Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 106 (2008) 299–309
Food Chemistry
www.elsevier.com/locate/foodchem

Vitamins A and E content in infant milk-based powdered formulae after opening the packet

Jorge L. Chávez-Servín, Ana I. Castellote, M. Carmen López-Sabater *

Dpt. de Nutrició i Bromatologia, Centre de Referència en Tecnologia dels Aliments (CeRTA), Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII s/n E-08028 Barcelona, Spain

Received 11 January 2007; received in revised form 25 March 2007; accepted 30 May 2007

Abstract

Vitamins A and E were determined by HPLC in 20 starting, milk-based powdered infant formulae from local markets. We traced the evolution of these compounds, once the packets had been opened, during 0, 30 and 70 days of storage at room temperature (≈25 °C; min. 23 °C, max. 25.5 °C). Immediately after opening the packets, vitamin A ranged from 0.55 to 0.94 mg RE/100 g (93.3–183 μg RE/100 kcal) and vitamin E from 6.58 to 27.8 mg α-TE/100 g (1.36–5.39 mg α-TE/100 kcal). All the samples had higher vitamins A and E contents than those declared on the label, vitamin A mean adequacy values: 134% ± 17, min. 98%, max. 162%, and vitamin E 185% ± 47, min. 101%, max. 286%, including values at 0, 30 and 70 days of storage.

All formulae covered the minimum limits for vitamins A and E established by the current Spanish and European legislations, even after 70 days of storage at room temperature.

Keywords: Retinols; Tocopherols; Infant formula powder; Storage; Stability

1. Introduction

In general, vitamin A refers to all-*trans*-retinol, which is the most active form of this vitamin, while vitamin E is a collective term for tocopherols (α, β, γ and δ) and tocotrienols (Blake, 2004; Blake, 2005). Vitamin A is essential for the maintenance of healthy vision, healthy skeletal and tooth development, cellular differentiation and proliferation, reproduction and integrity of the immune system (Olson, 1987; Olson, 1994; Spannaus-Martin, Cook, Tanumihardjo, Duitsman, & Olson, 1998; Tanumihardjo et al., 1990). The absorption of vitamin E occurs in the small intestine by a non-saturable, passive transport system that does not require carrier proteins but does require solubilisation in the form of micelles. The degree of absorption depends on the amount of fat absorbed. The efficiency of absorption of α-T decreases as intake increases. Between 50% and 70% of the vitamin E consumed (in an intake range of 0.4–1 mg) is absorbed (Farrell & Roberts, 1994), however, at pharmacological doses (i.e., 200 mg) absorption may decrease to less than 10%. The primary mode of action of vitamin E at the molecular level is not well understood, yet there is widespread agreement that the predominant physiologic function is its antioxidant activity. In cellular and subcellular membranes, vitamin E is in close proximity to phospholipid components (in particular PUFA), which are susceptible to peroxidation. Vitamin E protects these fatty acids by interfering with the free radical reactions that can result in cellular damage. Interest in vitamin E in infant nutrition began in the 1940s, when researchers demonstrated that erythrocytes were susceptible to hemolysis, in the presence of hydrogen peroxide, and that this effect was inhibited by vitamin E supplements (Bell, 1989). The significance of this effect became clear when formulae with high PUFA levels were given to preterm infants (Oski & Barness, 1967). The combination of high intakes of PUFA and low levels of vitamin E contributed to the development

* Corresponding author. Tel.: +34 93 4024512; fax: +34 93 4035931.
E-mail address: mclopez@ub.edu (M.C. López-Sabater).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.05.088

of hemolytic anemia, thrombocytosis, edema and reticulocytosis. These manifestations were later found to be prevented by supplementation with vitamin E. Given this interrelationship between vitamin E and the phospholipid components of cellular membranes, it is recognized that the requirement for α-tocopherol is proportional to the amount of PUFA consumed (Farrell & Roberts, 1994). Tocopherols (α, β, γ and δ) are naturally occurring lipid antioxidants which specifically inhibit the oxidation of polyunsaturated fatty acids (PUFA) such as linoleic (LA, C18:2, $n-6$), linolenic (ALA, C18:3, $n-3$), arachidonic (AA, C20:4, $n-6$), eicosapentaenoic (EPA, C20:5, $n-3$) and docosahexaenoic acids (DHA, C22:6, *n*-3). Infant formulae (IFs) contain tocopherols derived from the vegetable oils used as ingredients in their manufacture. The antioxidant activities of α-, β-, γ- and δ-tocopherols, those commonly found in vegetable oils, contribute as more biologically active components to the diet than any other tocopherol isomer, although α-tocopherol is less stable during manufacture and storage (Brigelius-FlohE & Traber, 1999).

Several studies on infant nutrition have addressed the effects of long-chain (LC)-PUFA on development and growth (Giovannini, Riva, & Agostoni, 1995; Lauritzen, Hansen, Jorgensen, & Michaelsen, 2001) and also their effects on the fetus and neonate (Crawford, 2000; Gibson, Neumann, & Makrides, 1996; Hornstra, 2000; Jeffrey, Weisinger, Neuringer, & Mitchell, 2001; Koletzko et al., 2001). Therefore, some IF manufacturers include LC-PUFA in formula composition. Tocopherols and retinol are added to IFs to improve vitamin content and to prevent lipid oxidation of these fatty acids during manufacture and storage, thereby helping to extend product shelf-life. Conventional bovine milk-based formula is usually manufactured using specific combinations of protein, fat, carbohydrate, vitamin and mineral components. The raw material mix is blended, pasteurised, homogenised, condensed and spray-dried or sterilised. The redistribution and interactions of components in the system occur during processing and storage (Guo, Hendricks, & Kindstedt, 1998). The factors that cause significant component interactions and redistributions in IFs, and their impact on biochemical and nutritional properties, such as lipid oxidation and vitamin losses, are not well understood. Therefore, fortification of IFs with the most stable vitamin esters, such as α-tocopherol acetate, retinol acetate or retinol palmitate, is required (Blake, 2005; Brigelius-FlohE & Traber, 1999; Parrish, 1980). These molecules are more stable than their isomers from vegetable oils and also less susceptible to oxidation. The European Communities Commission (CE, 1996) and the Spanish regulations (BOE, 1998) establish the respective limits of these vitamins, which can be added to meet nutritional requirements and to guarantee the stability of the product because of their antioxidant properties. It is hypothesised that the stability of vitamin homologues decreases quickly after packets of IFs have been opened, as a result of oxygen action and exposure to light. Oxygen and UV radiation induces lipid oxidation, therefore, once opened, milk products such as IFs are highly susceptible to oxidation at room temperature and, consequently, vitamins A and E are lost. In addition, light accelerates this process (Hardas, Danviriyakul, Foley, Nawar, & Chinachoti, 2002; Hardas, Danviriyakul, Foley, Nawar, & Chinachoti, 2000). No information is currently available about the stability of the isomers of vitamins A and E in IF powder, once the packet has been opened. Most methods used to analyse vitamin A involve a saponification step and reversed-phase (RP) columns. This implies that when α-tocopherol acetate is added to IFs it cannot be differentiated from the naturally occurring α-tocopherol. Furthermore, without a saponification step, quantification of the ester forms added, as well as the natural vitamins A and E homologues can be performed using a normal-phase (NP)-HPLC system (Chase & Long, 1998; Rodrigo, Alegria, Barbera, & Farre, 2002).

Here we used NP-HPLC-DAD to determine the content of α-tocopherol, α-tocopherol acetate, γ-tocopherol, δ-tocopherol as well as retinol palmitate and retinol acetate (from naturally occurring compounds of oils and from added vitamin), in 20 commercial powdered starting IFs from local markets. In addition, the stability of these compounds was monitored for 70 days at room temperature after opening the packets. The values obtained were compared with the European (CE, 1996) and Spanish (BOE, 1998) regulations. Finally, we comment on some points of interest regarding vitamins A and E contents in IFs.

2. Materials and methods

2.1. Reagents and standards

The chemicals used for sample preparations were of analytical reagent grade. Hexane and ethyl acetate, both of HPLC-grade, were obtained from SDS (Peypin, France), absolute ethanol from Panreac (Barcelona, Spain), a standard of α-tocopherol acetate from Fluka (Buchs, Switzerland), and standards of α-, γ-, and δ-tocopherol and all-*trans*-retinol palmitate, and retinol acetate from Sigma (St. Louis, MO, USA).

2.2. Instruments

We used a Hewlett-Packard liquid chromatographic system (Waldbronn, Germany) with a HP 1050 pump, a HP-1040 M photodiode-array detector and a Waters 717 plus autosampler injector (Milford, MA, USA). We also used a pre-column guard cartridge (10 × 2 mm) and a pinnacle II silica short-narrow-bore column (50 mm × 2.1 mm i.d.), with a 3-μm particle size from Restek (Bellefonte PA, USA).

2.3. Samples

Twenty commercial milk-based powdered Ifs, of recognized brands, were purchased from local markets. We included only the "starting milks" or "first milks"; formu-

lated to meet the needs of healthy full-term infants, from 0–4 months of age. Table 1 indicates the general composition of each formula as described in the information provided on the packet.

2.4. Storage

All formulae were opened on the same day, approximately during the 5–9 months of their shelf-life. In addition, formulae were opened three times every day, each time the powder was stirred in the original package to maintain uniform exposure to environment and approximately 10 g of powder was discarded, simulating normal storage and preparation. We kept the formulae at room temperature (25 °C: min. 23 °C, max. 25.5 °C) and analyses were done at 0, 30 and 70 days, respectively. Once formulae had been properly stored as described above, analytical determinations were subsequently made.

2.5. Vitamins A and E determination

Tocopherols and retinol acetate or palmitate compounds in the formulae were measured by an NP-HPLC-DAD method, as we previously reported (Chávez-Servín, Castellote, & López-Sabater, 2006). Approximately 2 g of IF was reconstituted with 8 ml of distilled water. This mixture was then immersed in warm water (40 °C) and mixed until complete homogenisation was achieved (5 min approximately). We subsequently used a vortex to complete the homogenisation of the sample. We then transferred 1 ml of reconstituted sample (20%, w/w) into a centrifuge tube. Next, 3 ml of absolute ethanol was added and the tube was shaken mechanically for 3 min. We then added 1 ml of hexane and the tube was shaken for another minute. The sample was then left to stand for 5 min, after which, 3 ml of saturated NaCl was added to aid solvent separation. The mixture was then shaken manually by inversion. The tube was centrifuged for 5 min at 3000 rpm at room temperature. The hexane layer was recovered, filtered through a 0.22 μm nylon filter and injected into the HPLC system.

2.6. Statistical analysis

For statistical analysis, we used one-way analysis of variance (ANOVA), as well as multiple comparisons, using the

Table 1
Composition of infant milk-based formulae according to the information provided on labels

Formula	Lipid[a]	Protein[a]	Carbohydrate[a]	Main ingredients[b]
1	23.1	12.8	58.3	Skimmed milk, lactose, starch, palm olein, demineralized whey milk, colza, coconut and sunflower oils, soybean lecithin
2	24.0	12.5	58.6	Demineralized whey milk, skimmed milk, palm olein, maltodextrin, colza, palm and corn oils, soybean lecithin
3	24.0	12.5	58.6	Demineralized whey milk, palm olein, starch, skimmed milk, corn syrup, colza, coconut and corn oils
4	24.0	12.0	58.7	Demineralized whey milk, palm oil, skim milk, maltodextrins, vegetal oils (colza, sunflower)
5	24.5	11.0	59.7	Skimmed milk, vegetal fat matter (palm, coconut, colza and sunflower), demineralized whey milk, maltose, maltodextrins, soybean lecithin
6	27.5	13.9	52.4	Skimmed milk, lactose, glucose syrup, vegetal oils (palm, colza, corn and coconut)
7	28.0	11.6	56.0	Demineralized whey milk, vegetable oils, skimmed milk powder, lactose, soybean lecithin and monoglycerides of fatty acids
8	28.2	11.3	60.5	Whey powder, vegetable oils, skimmed milk, lactose, galacto-oligosaccharides, polyfructose, fish oil
9	24.4	11.7	58.6	Demineralized whey milk, milk powder (partially demineralized), vegetable oils (palm, colza, sunflower), lactose
10	27.0	11.5	55.0	Skim milk, fat milk, lactose, lactose, vegetable oils (palm, sunflower, colza) milk proteins.
11	25.4	12.1	50.0	Demineralized whey milk, vegetable oils (palm, coconut, soybean), lecithin
12	23.9	11.2	60.0	Milk protein, skimmed milk, whey protein milk, vegetable oils (palm, coconut, colza, sunflower), lactose, carob flour, glucose syrup
13	24.0	12.5	55.6	Demineralized whey milk, palm olein, starch, skimmed milk, corn syrup, colza, coconut and corn oils
14	29.0	11.0	56.0	Lactose, skim milk, palm oil, whey milk protein concentrate, coconut and soybean oils, vegetable oil rich in oleic acid, soybean lecithin
15	29.0	11.0	56.0	Lactose, skimmed milk, palm oil, whey milk protein concentrate, coconut and soybean oils, vegetable oil rich in oleic acid, soybean lecithin
16	26.4	11.5	56.4	Hydrolyzed whey milk protein minerals reduced, vegetable oils (palm olein, soybean, coconut, sunflower, high oleic acid), lactose, corn maltodextrin
17	26.0	11.5	57.7	Hydrolyzed whey milk protein minerals reduced, corn syrup, vegetable oils (palm olein, canola, coconut, sunflower, high oleic acid)
18	28.0	11.0	56.0	Skimmed milk, lactose, vegetal oils, fractionated milk protein (α-lactoalbumin), soybean lecithin, LC-PUFA (arachidonic and docosahexaenoic acid)
19	26.0	9.5	58.0	Skimmed milk, vegetable oils (palm, coconut, sunflower, soybean, high oleic acid), LC-PUFA (arachidonic and docosahexaenoic acid), lactose, maltodextrin, milk proteins, soybean lecithin
20	26.0	10.7	58.3	Lactose, vegetable oil, skimmed milk, maltodextrin, serum protein, egg phospholipids

[a] Expressed as g/100 g of powder.
[b] Ingredients are listed in the order in which they appear on the label.

Tukey HSD procedure for each IF and storage time (0, 30 and 70 days). We conducted statistical analysis using the SPSS package for Windows version 11 (SPSS, Chicago, IL, USA). The level of statistical significance was set at 5% for all analyses.

3. Results and discussion

Preparation of the fat-soluble vitamin fraction for injection into the column, for most food matrices, requires either saponification of the sample matrix or a concentrated lipid fraction or extraction of total lipids from the sample, which can then be injected directly into a NP column. Saponification converts α-tocopherol acetate to α-tocopherol, which cannot be differentiated from the naturally occurring α-tocopherol (Chase, Eitenmiller, & Long, 1997). Although several simplified methods involving saponification are available, non-saponification of the total lipid dilution is considered preferable to saponification as it offers higher accuracy (Rodrigo et al., 2002). Furthermore, the direct method permits the quantification of the ester forms added, as well as of the natural vitamins A and E homologues. Finally, the stability of these fat-soluble vitamins is improved both in the lipid matrix and with the ester forms of vitamins A and E (Chase et al., 1997; Rodrigo et al., 2002). The results of vitamins A and E contents in the studied formulae are reported in Tables 2 and 3, respectively. As a consequence of the heating treatments, IF undergoes chemical and biochemical changes that affect components, mainly proteins, carbohydrates and vitamins (Ferrer, Alegria, Courtois, & Farre, 2000). In the case of vitamins A and E, to compensate losses during manufacture and storage (before and after opening the packet), manufacturers generally add more vitamins A and E than that reported on the label (Tables 2 and 3). We found mean adequacy values for vitamin A of 134 ± 17%, min. 98% and max. 162%, relative to information on the labels, and for vitamin E, 185 ± 47%, min. 101% and max. 286%, respectively, taking into account the 0, 30 and 70 days of storage. According to the manufacturers' instructions, powdered IF must be used one month after being opened. Generally, formulae are consumed before this time. However, it is necessary to study the evolution of these vitamin homologues after this time. Here we studied evolution of these formulae until 70 days storage. Once opened, stored formulae were treated by simulating the normal use by consumers.

3.1. Vitamin A content

Foods such as milk or IFs are most commonly fortified with vitamin A in the form of retinyl acetate (samples 1–4, 6, 9–11, 13, 16, 17 and 20) or retinyl palmitate (samples 5, 7, 8, 12, 14, 15, 18 and 19) because these molecules are more stable and less susceptible to oxidation than their respective isomers from vegetable oils. Human milk contains preformed vitamin A, mainly in the form of retinyl esters (i.e., retinyl palmitate and retinyl stearate) and carotenoids, which are vitamin A precursors (Canfield, Giuliano, Neilson, & Kelly, 1992; Canfield, Kaminsky, Taren, Shaw, & Sander, 2001; Khachik et al., 1997). The parent compound of the vitamin A group is all-*trans*-retinol. Vitamin A activity is expressed as international units (IU) or retinol equivalents (RE). Tables of food composition are based on equating 1 IU of vitamin A with 0.3 μg of all-*trans*-retinol or 0.6 μg all-*trans*-β-carotene. However, this 2 to 1 relationship does not accurately reflect the biological activity after ingestion. Consequently, the current convention is to express vitamin A activity as retinol equivalents (RE) with 1 RE equal to 1 μg all-*trans*-retinol, 6 μg all-*trans*-β-carotene and 12 μg of other provitamin A carotenoids, (Olson, 1989; Anonymous, 1998). Table 2 reports the values for retinyl acetate or palmitate and the respective values expressed as RE, in order to compare label contents, values in literature and those established by current European (CE, 1996) and Spanish (BOE, 1998) legislation, following the equivalence:

Retinol of 0.3 μg = 0.344 μg retinyl acetate = 0.55 μg retinyl palmitate or, what is the same, 1 μg retinol = 1.146 μg retinyl acetate = 1.832 μg retinyl palmitate.

European (CE, 1996) and Spanish (BOE, 1998) legislation establish the minimum limits for vitamin A in IFs at 60 μg RE/100 kcal. All our samples complied with this regulation, even after 70 days of storage after opening the packet (Table 2). The recorded values of vitamin A, immediately after opening were in the range 0.55–0.94 mg RE/100 g, or 93.3–183 μg RE/100 kcal, respectively.

Delgado-Zamarreno, Bustamante-Rangel, Garcia-Jimenez, Sanchez-Perez, and Carabias-Martinez (2006) reported values of vitamin A (as retinyl acetate) in the range 0.59–0.74 mg RE/100 g in four types of IFs. These values were also higher, between 113% and 120%, than those stated on their respective labels. Similarly, Albalá-Hurtado, Veciana-Nogues, Vidal-Carou, and Font (2001) reported values of vitamin A of 0.64–1.06 mg RE/100 g in the starting liquid, and 0.43–1.09 mg RE/100 g in the starting powdered IF. We observed a high variability in vitamin A content, but in general the values of this vitamin reported on the label were close to the values found (mean = 134% ± 17) in the samples. Other studies have reported values more than twice those declared on the labels in several IFs (Albalá-Hurtado, Veciana-Nogues, Vidal-Carou, & Marine-Font, 2000; Landen et al., 1985). Immediately after opening the packets, RE values of all samples were greater than those reported on the label. Toxicity from excessive intake is a concern, because vitamin A is stored in the body. Ingestion of very high doses of preformed vitamin A in humans causes many toxic manifestations, for example, headache, vomiting, anorexia, diplopia, alopecia, dryness of the mucus membranes, desquamation of the skin, bone abnormalities and liver damage (Snodgrass, 1992). European (CE, 1996) and Spanish (BOE, 1998) legislation establish the maximum limits for vitamin

Table 2
NP-HPLC-DAD analysis of vitamin A content of infant formulae during storage

Sample	Storage (days)	Retinyl acetate or palmitate (mg/100 g)	RE found (mg/100 g)	RE label (mg/100 g)	Losses (%)	Adequacy (%)	RE/100 kcal μg
1[I]	0	0.93 ± 0.03[a]	0.81 ± 0.03[a]	0.52	–	156	165 ± 5.46
	30	0.84 ± 0.03[b]	0.73 ± 0.03[b]		9.5	141	149 ± 6.48
	70	0.81 ± 0.01[b]	0.71 ± 0.01[b]		12.2	137	145 ± 2.36
2[I]	0	0.69 ± 0.03[a]	0.61 ± 0.03[a]	0.45	–	136	123 ± 5.29
	30	0.64 ± 0.01[b]	0.56 ± 0.01[b]		8.2	124	113 ± 1.68
	70	0.64 ± 0.01[b]	0.57 ± 0.01[b]		8.2	124	115 ± 2.88
3[I]	0	0.94 ± 0.04[a]	0.82 ± 0.04[a]	0.53	–	155	164 ± 8.25
	30	0.85 ± 0.04[b]	0.74 ± 0.04[b]		9.6	140	149 ± 7.70
	70	0.81 ± 0.02[b]	0.71 ± 0.02[b]		13.3	134	142 ± 4.14
4[I]	0	0.83 ± 0.02[a]	0.73 ± 0.02[a]	0.48	–	151	146 ± 3.44
	30	0.72 ± 0.01[b]	0.63 ± 0.01[b]		13.5	131	126 ± 1.63
	70	0.59 ± 0.01[c]	0.51 ± 0.01[c]		29.6	106	103 ± 0.86
5[II]	0	1.22 ± 0.03[a]	0.67 ± 0.02[a]	0.42	–	158	132 ± 3.06
	30	1.16 ± 0.06[a]	0.63 ± 0.03[a]		5	150	126 ± 6.90
	70	1.14 ± 0.01[a]	0.62 ± 0.01[b]		6.1	149	124 ± 1.06
6[I]	0	1.08 ± 0.03[a]	0.94 ± 0.03[a]	0.60	–	156	184 ± 5.44
	30	0.96 ± 0.04[b]	0.84 ± 0.04[b]		10.6	139	164 ± 6.91
	70	0.71 ± 0.06[c]	0.62 ± 0.06[c]		34.3	102	121 ± 1.65
7[II]	0	1.59 ± 0.04[a]	0.87 ± 0.02[a]	0.61	–	142	175 ± 4.85
	30	1.52 ± 0.09[a]	0.83 ± 0.05[a]		4.5	136	167 ± 5.13
	70	1.51 ± 0.10[a]	0.82 ± 0.09[a]		5.2	135	166 ± 8.80
8[II]	0	1.39 ± 0.01[a]	0.76 ± 0.01[a]	0.47	–	162	153 ± 0.81
	30	1.33 ± 0.06[a]	0.73 ± 0.04[a]		4.6	154	146 ± 7.20
	70	1.30 ± 0.04[b]	0.71 ± 0.02[b]		6.2	152	144 ± 4.33
9[I]	0	1.00 ± 0.02[a]	0.87 ± 0.02[a]	0.54	–	162	174 ± 4.98
	30	0.93 ± 0.03[a]	0.81 ± 0.03[a]		7.2	150	162 ± 5.45
	70	0.76 ± 0.09[b]	0.66 ± 0.09[b]		24.0	123	133 ± 8.25
10[I]	0	0.69 ± 0.04[a]	0.60 ± 0.04[a]	0.39	–	154	117 ± 8.04
	30	0.56 ± 0.05[b]	0.49 ± 0.04[b]		18.5	126	95.3 ± 7.39
	70	0.49 ± 0.04[b]	0.43 ± 0.04[b]		28.9	109	83.1 ± 7.39
11[I]	0	0.70 ± 0.03[a]	0.61 ± 0.03[a]	0.45	–	136	122 ± 5.42
	30	0.65 ± 0.01[a]	0.57 ± 0.01[a]		7.5	126	113 ± 1.06
	70	0.61 ± 0.06[b]	0.54 ± 0.06[b]		12.3	119	107 ± 11.20
12[II]	0	1.01 ± 0.03[a]	0.56 ± 0.02[a]	0.44	–	126	115 ± 4.20
	30	0.88 ± 0.06[a]	0.48 ± 0.03[a]		12.9	110	99.9 ± 6.91
	70	0.82 ± 0.10[b]	0.45 ± 0.06[b]		18.7	103	93.9 ± 7.55
13[I]	0	0.95 ± 0.03[a]	0.83 ± 0.03[a]	0.53	–	156	165 ± 6.63
	30	0.85 ± 0.01[b]	0.74 ± 0.01[b]		10.3	140	148 ± 1.50
	70	0.81 ± 0.01[b]	0.71 ± 0.01[b]		14.2	134	142 ± 1.98
14[II]	0	0.99 ± 0.06[a]	0.55 ± 0.05[a]	0.48	–	114	93.3 ± 8.93
	30	0.89 ± 0.05[a]	0.49 ± 0.03[a]		10.2	101	83.8 ± 5.13
	70	0.85 ± 0.02[b]	0.47 ± 0.01[b]		13.9	98	80.4 ± 2.47
15[II]	0	1.10 ± 0.05[a]	0.60 ± 0.03[a]	0.48	–	126	114 ± 5.80
	30	1.07 ± 0.05[a]	0.59 ± 0.03[a]		2.5	123	112 ± 5.37
	70	0.92 ± 0.04[b]	0.50 ± 0.02[b]		16.9	105	95.0 ± 4.20
16[I]	0	0.73 ± 0.01[a]	0.64 ± 0.01[a]	0.46	–	139	125 ± 0.79
	30	0.67 ± 0.01[b]	0.58 ± 0.01[b]		8.6	127	115 ± 0.46
	70	0.64 ± 0.02[c]	0.56 ± 0.02[c]		12.4	122	110 ± 3.73

(*continued on next page*)

Table 2 (*continued*)

Sample	Storage (days)	Retinyl acetate or palmitate (mg/100 g)	RE found (mg/100 g)	RE label (mg/100 g)	Losses (%)	Adequacy (%)	RE/100 kcal μg
17[I]	0	0.85 ± 0.02^{a}	0.74 ± 0.02^{a}	0.54	–	138	146 ± 3.98
	30	0.82 ± 0.05^{a}	0.71 ± 0.01^{a}		4	132	140 ± 0.94
	70	0.80 ± 0.03^{b}	0.70 ± 0.03^{a}		5.4	130	138 ± 5.52
18[II]	0	1.39 ± 0.05^{a}	0.76 ± 0.03^{a}	0.52	–	146	146 ± 5.95
	30	1.38 ± 0.06^{a}	0.75 ± 0.04^{a}		0.3	145	145 ± 7.28
	70	1.26 ± 0.08^{a}	0.69 ± 0.05^{a}		9.2	132	132 ± 8.71
19[II]	0	1.59 ± 0.04^{a}	0.87 ± 0.02^{a}	0.61	–	142	170 ± 4.76
	30	1.54 ± 0.03^{a}	0.84 ± 0.02^{a}		3.1	138	165 ± 3.20
	70	1.51 ± 0.05^{a}	0.83 ± 0.03^{a}		4.7	135	162 ± 5.86
20[I]	0	1.05 ± 0.01^{a}	0.92 ± 0.01^{a}	0.64	–	144	179 ± 2.17
	30	1.01 ± 0.04^{a}	0.88 ± 0.04^{a}		4	138	171 ± 7.20
	70	0.76 ± 0.01^{b}	0.66 ± 0.01^{b}		28.2	103	128 ± 1.58

Identical superscripted letters within the same column and formula indicate no significant differences ($p > 0.05$) comparing 0 vs. 30 vs. 70 days of storage.
[I] Retinyl acetate.
[II] Palmitate.

A in IF at 180 μg RE/100 kcal. All of our samples complied with this regulation. Only in IF 6 did we detect 184 RE/100 kcal immediately after opening the packet. However, this value decreased 10.6% after 30 days of storage and 34.3% after 70 days (121 RE/100 kcal). Moreover, in infants and young children, signs of toxicity usually appear only after daily intake greater than 6000 μg RE (20 000 IU) over a period of months or years (Bauernfeind, 1983; Brin & Bauernfeind, 1978), an amount which is much greater than the limit established by European legislation (CE, 1996), Spanish regulation (BOE, 1998) and the recommendation of the expert panel of the Life Sciences Research Office (LSRO) of the American Society for Nutritional Sciences concerning the "Assessment of Nutrient Requirements for Infant Formulas". This expert panel recommended a maximum vitamin A content of IF of 500 IU/100 kcal (150 μg/100 kcal). Although the 90th centile of the Food and Drug Administration (FDA) analyses of IFs is 570 IU/100 kcal, the upper centiles of the FDA values presumably reflect averages in vitamin A content above the current code of federal regulation (CFR) minimum of 250 μg/100 kcal. The expert panel concluded that an upper limit of 500 μg/100 kcal was more appropriate (Anonymous, 1998). The only known case of a vitamin A overdose was in an infant who died after being given about 27 000 mg RE/day (90 000 IU/day) for 11 days (Bush & Dahms, 1984).

IFs are generally well packed. Packaging (can, bag) of powdered formulae protects the product from light and oxygen, thereby preventing the alteration of components (Karatapanis, Badeka, Riganakos, Savvaidis, & Kontominas, 2006; Schroder, Scott, Bland, & Bishop, 1985; Vassila, Badeka, Kondyli, Savvaidis, & Kontominas, 2002). However, once opened, IFs are exposed to light and oxygen and therefore are more susceptible to oxidative reactions and vitamin losses. Our samples were all packed in cans (samples 1–7, 11–19) or bags (samples 8–10, 20), were protected from light and sunlight, had a controlled atmosphere, and contained approximately >0.2–0.3% of residual oxygen according to the manufacturers. We detected decreases in vitamin A content once the packets had been opened, however, these decreases were not significant in all the samples (Table 2). Only formulae 4, 6 and 16 showed significant losses ($p < 0.05$) at all the storage times (0 vs. 30 vs. 70 days), with losses after 70 days of storage of 29.6%, 34.3% and 12%, respectively (Table 2). These formulae were supplemented with retinyl acetate. In contrast, formulae 5, 7, 18 and 19 did not show significant losses ($p > 0.05$) after opening (0 vs. 30 vs. 70 days). They exhibited losses of only about 6.1%, 5.2%, 9.2% and 4.7%, respectively, after 70 days of storage (Table 2). Interestingly, these formulae were supplemented with retinyl palmitate. However, a comparison of IFs containing retinyl acetate versus those containing retinyl palmitate did not show differences ($p > 0.05$) in stability over 0, 15 and 70 days of storage. Therefore, the observation of no significant differences in formulae (5, 7, 18 and 19) was probably circumstantial. Environmental conditions directly affected the stability of vitamin A, once the IFs had been opened. Vitamin A was lost most as a result of exposure to oxygen and light. Most manufacturers added amounts above the declared levels in the labels. This practice is attributed to compensating for the potential losses of this vitamin during production and storage. After 70 days of storage, we recorded adequacy values close to 100% in some formulae, namely 106% (IF 6), 102% (IF 14), 105% (IF 15), and 103% (IF 20) (Table 2). These observations support that over-fortification of vitamin A with retinyl acetate or retinyl palmitate ensures the vitamin A content stated on the label.

Table 3
Analysis of vitamin E content of infant formulae during storage

Sample	Storage (days)	α-T (mg/100 g)	γ-T (mg/100 g)	δ-T (mg/100 g)	α-TAc (mg/100 g)	α-TE Found (mg/100 g)	α-TE Label (mg/100 g)	Losses (%)	Adequacy (%)	α-TE μg/ 100 kcal
1	0	3.97 ± 0.17^{a}	1.38 ± 0.19^{a}	–	7.86 ± 0.35^{a}	9.75 ± 0.44^{a}	4	–	244	1.98 ± 0.09
	30	3.77 ± 0.17^{a}	1.39 ± 0.06^{a}	–	7.67 ± 0.30^{a}	9.42 ± 0.35^{a}		8.1	235	1.91 ± 0.07
	70	3.77 ± 0.10^{a}	1.36 ± 0.03^{a}	–	7.67 ± 0.21^{a}	9.41 ± 0.24^{a}		13.6	235	1.91 ± 0.05
2	0	3.67 ± 0.12^{a}	3.05 ± 0.05^{a}	–	14.7 ± 0.37^{a}	14.5 ± 0.29^{a}	6.63	–	219	2.92 ± 0.06
	30	3.57 ± 0.05^{a}	2.95 ± 0.05^{a}	–	14.4 ± 0.39^{a}	14.3 ± 0.32^{a}		2.9	215	2.87 ± 0.06
	70	3.58 ± 0.13^{a}	2.92 ± 0.06^{a}	–	14.4 ± 0.34^{a}	14.2 ± 0.37^{a}		3.7	214	2.86 ± 0.07
3	0	2.82 ± 0.13^{a}	2.24 ± 0.08^{a}	–	7.53 ± 0.18^{a}	8.54 ± 0.23^{a}	4.02	–	212	1.71 ± 0.05
	30	2.80 ± 0.15^{a}	2.27 ± 0.05^{a}	–	7.51 ± 0.43^{a}	8.51 ± 0.33^{a}		5.4	211	1.70 ± 0.07
	70	2.64 ± 0.09^{a}	2.12 ± 0.02^{b}	–	7.47 ± 0.34^{a}	8.30 ± 0.28^{a}		6.6	206	1.66 ± 0.06
4	0	2.67 ± 0.04^{a}	1.84 ± 0.07^{a}	1.18 ± 0.05^{a}	10.4 ± 0.28^{a}	10.4 ± 0.24^{a}	6	–	174	2.10 ± 0.05
	30	1.47 ± 0.06^{b}	1.33 ± 0.03^{b}	0.78 ± 0.03^{b}	10.4 ± 0.22^{a}	9.06 ± 0.21^{b}		2.6	151	1.82 ± 0.04
	70	1.03 ± 0.03^{c}	0.86 ± 0.05^{c}	0.37 ± 0.00^{c}	10.6 ± 0.09^{a}	8.62 ± 0.08^{c}		5.3	144	1.73 ± 0.02
5	0	4.64 ± 0.09^{a}	2.83 ± 0.05^{a}	–	6.85 ± 0.24^{a}	10.0 ± 0.19^{a}	5.2	–	192	1.99 ± 0.04
	30	4.64 ± 0.31^{a}	2.77 ± 0.06^{a}	–	6.71 ± 0.12^{a}	9.90 ± 0.29^{a}		8.1	190	1.97 ± 0.06
	70	4.08 ± 0.08^{b}	2.66 ± 0.14^{a}	–	6.58 ± 0.17^{a}	9.22 ± 0.11^{b}		9.5	177	1.83 ± 0.02
6	0	5.36 ± 0.25^{a}	4.06 ± 0.52^{a}	–	7.93 ± 0.09^{a}	11.7 ± 0.22^{a}	4.1	–	286	2.29 ± 0.04
	30	5.26 ± 0.23^{a}	3.38 ± 0.13^{a}	–	7.72 ± 1.03^{a}	11.4 ± 0.93^{a}		2.1	276	2.21 ± 0.18
	70	4.65 ± 0.44^{b}	3.26 ± 0.35^{b}	–	7.34 ± 0.28^{a}	10.9 ± 0.95^{b}		2.2	255	2.04 ± 0.11
7	0	6.52 ± 0.15^{a}	6.09 ± 0.15^{a}	1.98 ± 0.10^{a}	8.12 ± 0.64^{a}	13.6 ± 0.59^{a}	5.73	–	238	2.75 ± 0.12
	30	6.08 ± 0.18^{a}	5.89 ± 0.20^{a}	2.08 ± 0.05^{a}	8.04 ± 0.26^{a}	13.1 ± 0.31^{a}		8.1	229	2.64 ± 0.06
	70	5.74 ± 0.49^{b}	5.73 ± 0.45^{a}	2.15 ± 0.13^{a}	7.95 ± 0.49^{a}	12.7 ± 0.85^{a}		17.7	221	2.55 ± 0.17
8	0	9.02 ± 0.23^{a}	2.90 ± 0.08^{a}	–	6.12 ± 0.41^{a}	13.9 ± 0.52^{a}	9.62	–	144	2.80 ± 0.10
	30	7.99 ± 0.27^{b}	2.61 ± 0.04^{b}	–	5.97 ± 0.14^{a}	12.7 ± 0.24^{b}		1.3	132	2.56 ± 0.05
	70	7.91 ± 0.28^{b}	2.52 ± 0.09^{b}	–	5.85 ± 0.57^{a}	12.5 ± 0.18^{b}		2.6	130	2.52 ± 0.04
9	0	5.19 ± 0.15^{a}	1.80 ± 0.07^{a}	3.04 ± 0.05^{a}	2.50 ± 0.22^{a}	7.61 ± 0.29^{a}	3	–	254	1.52 ± 0.06
	30	4.91 ± 0.17^{a}	1.68 ± 0.05^{b}	2.88 ± 0.13^{a}	2.08 ± 0.07^{b}	6.99 ± 0.22^{b}		8.1	233	1.40 ± 0.04
	70	4.55 ± 0.38^{b}	1.57 ± 0.04^{c}	2.76 ± 0.02^{b}	2.04 ± 0.02^{b}	6.57 ± 0.39^{b}		13.6	219	1.31 ± 0.08
10	0	7.48 ± 0.51^{a}	1.04 ± 0.09^{a}	2.58 ± 0.25^{a}	–	7.95 ± 0.55^{a}	6.2	–	128	1.54 ± 0.11
	30	7.26 ± 0.40^{a}	1.05 ± 0.09^{a}	2.51 ± 0.36^{a}	–	7.72 ± 0.45^{a}		2.9	124	1.50 ± 0.09
	70	7.19 ± 0.60^{a}	1.08 ± 0.07^{a}	2.48 ± 0.18^{a}	–	7.66 ± 0.63^{a}		3.7	123	1.49 ± 0.12
11	0	2.91 ± 0.21^{a}	5.43 ± 0.26^{a}	2.85 ± 0.40^{a}	15.0 ± 0.86^{a}	14.8 ± 0.84^{a}	13	–	114	2.96 ± 0.17
	30	2.37 ± 0.13^{b}	4.97 ± 0.22^{b}	2.80 ± 0.40^{a}	14.8 ± 0.23^{a}	14.0 ± 0.28^{a}		5.4	108	2.80 ± 0.06
	70	2.34 ± 0.14^{b}	4.74 ± 0.14^{b}	2.81 ± 0.19^{a}	14.7 ± 0.23^{a}	13.8 ± 0.10^{a}		6.6	106	2.76 ± 0.02

(*continued on next page*)

Table 3 (*continued*)

Sample	Storage (days)	α-T (mg/100 g)	γ-T (mg/100 g)	δ-T (mg/100 g)	α-TAc (mg/100 g)	α-TE Found (mg/100 g)	α-TE Label (mg/100 g)	Losses (%)	Adequacy (%)	α-TE μg/ 100 kcal
12	0	6.19 ± 0.13^{a}	1.98 ± 0.06^{a}	–	–	6.58 ± 0.14^{a}	5.1	–	129	1.36 ± 0.03
	30	6.02 ± 0.48^{a}	1.96 ± 0.04^{a}	–	–	6.41 ± 0.49^{a}		2.6	125	1.32 ± 0.10
	70	5.85 ± 0.19^{a}	1.92 ± 0.14^{a}	–	–	6.23 ± 0.21^{a}		5.3	122	1.29 ± 0.04
13	0	3.61 ± 0.34^{a}	3.39 ± 0.05^{a}	1.63 ± 0.04^{a}	7.51 ± 0.17^{a}	9.70 ± 0.35^{a}	4.2	–	231	1.94 ± 0.07
	30	3.30 ± 0.10^{a}	3.37 ± 0.09^{a}	1.54 ± 0.04^{a}	6.84 ± 0.29^{b}	8.92 ± 0.19^{b}		8.1	212	1.78 ± 0.04
	70	3.19 ± 0.06^{b}	3.28 ± 0.05^{a}	1.47 ± 0.09^{b}	6.83 ± 0.35^{b}	8.78 ± 0.22^{b}		9.5	209	1.76 ± 0.04
14	0	4.01 ± 0.57^{a}	4.22 ± 0.63^{a}	1.36 ± 0.05^{a}	12.1 ± 1.56^{a}	13.5 ± 1.25^{a}	6.3	–	214	2.31 ± 0.02
	30	3.86 ± 0.22^{a}	3.93 ± 0.18^{a}	1.23 ± 0.06^{b}	12.1 ± 0.71^{a}	13.2 ± 0.50^{a}		2.1	210	2.26 ± 0.09
	70	3.81 ± 0.27^{a}	4.00 ± 0.22^{a}	1.51 ± 0.07^{c}	12.0 ± 1.42^{a}	13.2 ± 1.01^{a}		2.2	209	2.26 ± 0.17
15	0	2.79 ± 0.11^{a}	3.85 ± 0.09^{a}	1.21 ± 0.04^{a}	14.7 ± 5.90^{a}	14.0 ± 4.18^{a}	6.3	–	222	2.65 ± 0.79
	30	2.15 ± 0.02^{b}	3.60 ± 0.03^{b}	1.00 ± 0.09^{b}	14.1 ± 3.24^{a}	12.8 ± 2.26^{a}		8.1	204	2.43 ± 0.43
	70	2.08 ± 0.02^{b}	3.38 ± 0.05^{c}	0.69 ± 0.06^{c}	12.4 ± 0.90^{a}	11.5 ± 0.64^{a}		17.7	182	2.18 ± 0.12
16	0	2.67 ± 0.04^{a}	4.29 ± 0.02^{a}	1.54 ± 0.03^{a}	11.6 ± 0.08^{a}	11.8 ± 0.09^{a}	6.8	–	173	2.31 ± 0.02
	30	2.56 ± 0.03^{b}	4.20 ± 0.03^{a}	1.52 ± 0.10^{a}	11.5 ± 0.17^{a}	11.6 ± 0.15^{a}		1.3	171	2.28 ± 0.03
	70	2.55 ± 0.03^{b}	3.99 ± 0.14^{b}	1.47 ± 0.06^{a}	11.4 ± 0.80^{a}	11.5 ± 0.57^{a}		2.6	169	2.26 ± 0.11
17	0	3.72 ± 0.08^{a}	1.63 ± 0.07^{a}	1.36 ± 0.06^{a}	6.94 ± 0.19^{a}	9.03 ± 0.15^{a}	4.08	–	222	1.77 ± 0.03
	30	3.58 ± 0.02^{a}	1.55 ± 0.02^{a}	1.36 ± 0.15^{a}	6.54 ± 0.13^{b}	8.61 ± 0.11^{b}		4.7	211	1.69 ± 0.02
	70	3.60 ± 0.09^{a}	1.36 ± 0.08^{b}	1.34 ± 0.17^{a}	6.51 ± 0.24^{b}	8.57 ± 0.15^{b}		5.2	209	1.68 ± 0.03
18	0	4.26 ± 0.06^{a}	4.89 ± 0.12^{a}	2.09 ± 0.05^{a}	6.07 ± 0.10^{a}	9.69 ± 0.17^{a}	5.8	–	167	1.86 ± 0.03
	30	3.83 ± 0.07^{b}	4.57 ± 0.09^{b}	2.05 ± 0.07^{a}	60.3 ± 0.19^{a}	9.18 ± 0.17^{b}		5.3	158	1.76 ± 0.03
	70	3.59 ± 0.02^{c}	4.31 ± 0.06^{c}	2.01 ± 0.08^{a}	5.89 ± 0.08^{a}	8.78 ± 0.07^{c}		9.5	151	1.69 ± 0.01
19	0	6.47 ± 0.21^{a}	4.90 ± 0.06^{a}	1.21 ± 0.09^{a}	6.46 ± 0.53^{a}	12.1 ± 0.57^{a}	6.1	–	198	2.38 ± 0.11
	30	6.08 ± 0.25^{a}	4.69 ± 0.25^{a}	1.16 ± 0.13^{a}	6.27 ± 0.11^{a}	11.5 ± 0.31^{a}		4.7	189	2.26 ± 0.06
	70	5.74 ± 0.24^{b}	4.55 ± 0.12^{b}	1.08 ± 0.05^{a}	6.07 ± 0.29^{a}	11.0 ± 0.31^{b}		9.0	180	2.16 ± 0.06
20	0	6.02 ± 0.31^{a}	1.50 ± 0.03^{a}	–	30.6 ± 2.16^{a}	27.8 ± 1.79^{a}	25	–	111	5.39 ± 0.35
	30	5.82 ± 0.10^{a}	1.44 ± 0.03^{a}	–	28.9 ± 0.88^{a}	26.3 ± 0.60^{a}		5.2	105	5.11 ± 0.12
	70	5.58 ± 0.04^{b}	1.30 ± 0.13^{b}	–	27.6 ± 1.12^{b}	25.2 ± 0.81^{b}		9.4	101	4.89 ± 0.16

Identical superscripted letters within the same column and formula indicate no significant differences ($p > 0.05$) comparing 0 vs. 30 vs. 70 days of storage.

3.2. Vitamin E content

As reviewed by Farrell and Roberts (1994), the standard for comparison is dl-α-tocopherol, which is defined to be 1.49 IU/mg or 1 tocopherol equivalent (TE). The activities of other isomers relative to the α isomer are dl-α-tocopheryl acetate = about 70% (0.7 TE/mg), β = 40% or 0.4 TE/mg, γ = 10–30% or 0.1–0.3 TE/mg, and δ = about 1% or 0.1 TE/mg. The relevance of these activities to IFs is in the relative concentrations of these isomers in the oils used in their manufacture. For example, corn and soy are major sources of vitamin E in the American diet. In both cases, there is a much higher proportion of the γ (about 60 mg/100 g) than in the α form (slightly more than 10 mg/100 g) (Farrell & Roberts, 1994). Table 3 shows the isomer content in our samples. We also summarised these values and they were expressed as α-TE, following the formula:

$$\begin{aligned}&\alpha - \text{TE mg/100 g}\\&\quad = \alpha - \text{T mg/100 g} * (1) + \gamma - \text{T mg/100 g} * (0.2)\\&\qquad + \delta - \text{T mg/100 g} * (0.1) + \alpha - \text{TAc mg/100 g}(0.7).\end{aligned}$$

IFs contain tocopherols derived from the vegetable oils used as ingredients and from the specific addition of α-T or α-TAc during their manufacture. Because of its stability when exposed to air and light, α-TAc is the most frequently used form of the vitamin (Miquel, Alegria, Barbera, Farre, & Clemente, 2004), and thus provides a stable systemic vitamin E source. Our results confirm this, as all the samples had α-TAc additions, with the exception of 10 and 12.

The values of α-TE found in our samples, immediately after opening the packets, were in the range of 6.58–27.8 mg α-TE/100 g or 1.36–5.39 mg α-TE/100 kcal. Using RP-HPLC, Albalá-Hurtado et al. (2001) reported ranges of vitamin E, in starting liquid milk, from 8.9 to 24.4 mg α-TE/100 g and in starting powdered milk from 6.66 to 22.2 mg α-TE/100 g. Delgado-Zamarreno et al. (2006) studied tocopherols in several IFs using pressurised liquid extraction and liquid chromatography with amperometric detection, obtaining values of vitamin E of 6.63 mg α-TE/100 g in a starter hypoallergenic IF and 14.5 mg α-TE/100 g in a starter adapted protein IF. Miquel et al. (2004) reported values of vitamin E by NP-HPLC of 2.20, 1.90, 1.61 and 1.78 mg α-TE/100 kcal in four IF; the first two supplemented with α-T and the last two with α-TAc, respectively, immediately after opening packets. We also observed high variability in vitamin E contents, the mean value was 185% with respect to the composition stated on the labels. Some formulae (IF 1-3, 6, 7, 9, 13, 14, 15 and 17) had more than twice the declared values, which is consistent with other reports (Albalá-Hurtado et al., 2001; Landen et al., 1985). None of our samples had lower vitamin E contents than the values given on the label, even 70 days after opening. The adequacy value of formula 20 at 70 days is 101% (Table 3). However, this formula had the highest label value of α-TE (25 mg/100 g).

Human milk has been reported to contain 3.0–5.6 mg/L (0.45–0.8 mg/100 kcal) of vitamin E (Barbas & Herrera, 1998; Bohm et al., 1997; Chappell, Francis, & Clandinin, 1985; Romeu-Nadal, Morera-Pons, Castellote, & Lopez-Sabater, 2006). European legislation (CE, 1996) establishes a minimum vitamin E content in IF of 0.5 mg α-TE/g of PUFA, but not less than 0.5 mg α-TE/100 kcal. This requirement is based on the mean minus one standard deviation value for vitamin E in human milk and the absence of data to justify a change in current legislation. A maximum level is not specified for vitamin E in IF. Evidence for potential vitamin E toxicity in human term infants comes from extrapolation of data from preterm infants. Several studies report an association between intravenous administration of a racemic mixture of α-tocopherol acetate and hepatic and renal failure, leading to the death of 38 preterm infants and serious illness in many others. Also, hemorrhagic complications, increased risk of sepsis, necrotizing enterocolitis, and increased incidence of retinal haemorrhages have been described (Anonymous, 1998). Although there are no direct toxicity studies in term infants, it is unlikely that they are more susceptible to adverse effects than preterm infants. Bell (1989) proposed that the upper limit for vitamin E in IFs be set at 10 mg/100 kcal, which coincides with the level of vitamin E in human milk. Also, according to the American Society for Nutritional Sciences, relative to the assessment of Nutrient requirements for infant formulas, a maximum vitamin E content of 5 mg α-TE/g of PUFA in IF, based on the 90th centile of the FDA analyses of IF, is recommended. All our samples presented levels inferior to these recommended limits. The expert panel concluded that this maximum content of vitamin E is below the intake levels that would result in toxicity, as interpreted from the review of animal data, adult toxicology and reports of adverse effect in preterm infants (Anonymous, 1998).

In general, we observed that vitamin E was stable once the packet had been opened. No statistical changes ($p > 0.05$) were found in the IFs stored at 0, 30 and 70 days respectively, only in formulae 4 and 18 were significant differences detected in α-TE when comparing 0 vs. 30 vs. 70 days of storage ($p < 0.05$). These differences were basically due to the loss of natural tocopherols (namely α, γ, and δ-T). In addition, the stability of the α-TAc in the IF samples was confirmed (Table 3).

In summary, a comparison of our results on vitamin A and E contents in IFs with those declared by the manufacturers shows that most of the samples had higher fat-soluble vitamin contents than those declared. This observation can be attributed to manufacturers wishing to ensure that at the end of the shelf-life of the formula, vitamins A and E contents are at least as high as the label states. Vitamin A levels were between 1.4 and 3 times higher than the minimum level recommended by both Spanish (BOE, 1998) and European (CE, 1996) legislation for IFs. Vitamin E levels were between 2.6 and 10.8 times higher than the minimum level recommended by Spanish (BOE, 1998) and European (CE, 1996)

legislation for IFs. In general, vitamin E showed better stability than vitamin A, no significant changes were recorded in vitamin E content in most of the samples during storage at 0, 30 and 70 days, at room temperature. Similarly, vitamin A contents during storage and once the packet had been opened, at 0, 30 and 70 days showed only a slight decrease, being significant ($p < 0.05$) in 15% of the samples.

The over-fortification with α-TAc and retinyl acetate or palmitate to the IFs assures vitamins A and E content as stated in the label, for up to 70 days after opening the packet. Besides, these molecules can act as antioxidants during the shelf-life of the product. However, further studies are needed to confirm whether over-fortification in different IFs are really necessary, and at which levels.

Acknowledgements

The authors thank Robin Rycroft for correcting the English manuscript. Special thanks go to CONACYT (Mexico) for supporting J.L.C.-S.

References

Albalá-Hurtado, S., Veciana-Nogues, M. T., Vidal-Carou, M. C., & Font, A. M. (2001). Concentration of selected vitamins in infant milks available in Spain. *Journal of Food Science, 66*(8), 1191–1194.

Albalá-Hurtado, S., Veciana-Nogues, M. T., Vidal-Carou, M. C., & Marine-Font, A. (2000). Stability of vitamins A, E, and B complex in infant milks claimed to have equal final composition in liquid and powdered form. *Journal of Food Science, 65*(6), 1052–1055.

Anonymous (1998). Vitamins. *The Journal of Nutrition, 128*(11S), S2182.

Barbas, C., & Herrera, E. (1998). Lipid composition and vitamin E content in human colostrum and mature milk. *Journal of Physiology and Biochemistry, 54*(3), 167–173.

Bauernfeind, J. C. (1983). Vitamin-A – Technology and applications. *World Review of Nutrition and Dietetics, 41*, 110–199.

Bell, E. F. (1989). Upper limit of vitamin E in infant formulas. *Journal of Nutrition, 119*(Suppl. 12), 1829–1831.

Blake, C. J. (2004). Committee on food nutrition. *Journal of AOAC International, 87*(1), 285–286.

Blake, C. J. (2005). Committee on food nutrition – Fat-soluble vitamins. *Journal of AOAC International, 88*(1), 325–329.

BOE., (1998). Real Decreto 72/1998, de 23 de enero, por el que se aprueba la Reglamentación técnico-sanitaria específica de los preparados para lactantes y preparados de continuación. BOE núm 30 R.D. 72 de 04/02/1998, 3772–3780.

Bohm, V., Peiker, G., Starker, A., Weske, E., Schaarmann, G., Schubert, R., et al. (1997). The contents of vitamins B-1, B-2, A, and E as well as beta-carotene in transitional human milk and comparative examinations with maternal blood and cord blood. *Zeitschrift fur Ernahrungswissenschaft, 36*(3), 214–219.

Brigelius-FlohE, R., & Traber, M. G. (1999). Vitamin E: Function and metabolism. *Faseb Journal, 13*(10), 1145–1155.

Brin, M., & Bauernfeind, J. C. (1978). Nutrition needs of elderly .4. Vitamin needs of elderly. *Postgraduate Medicine, 63*(3), 155–163.

Bush, M. E., & Dahms, B. B. (1984). Fatal hypervitaminosis-A in A neonate. *Archives of Pathology and Laboratory Medicine, 108*(10), 838–842.

Canfield, L. M., Giuliano, A. R., Neilson, E. M., & Kelly, B. E. (1992). Quantitation of retinol in mature breast-milk by HPLC. *Faseb Journal, 6*(4), A1497.

Canfield, L. M., Kaminsky, R. G., Taren, D. L., Shaw, E., & Sander, J. K. (2001). Red palm oil in the maternal diet increases provitamin A carotenoids in breastmilk and serum of the mother–infant dyad. *European Journal of Nutrition, 40*(1), 30–38.

CE., (1996). Directiva 96/4/CE de la Comisión, de 16 de febrero de 1996, por la que se modifica la directiva 91/321/CEE relativa a los preparados para lactantes y preparados de continuación. DOUE n. 49 de 28/02/1996, 12–16.

Chappell, J. E., Francis, T., & Clandinin, M. T. (1985). Vitamin-A and vitamin-E content of human-milk at early stages of lactation. *Early Human Development, 11*(2), 157–167.

Chase, G. W., Eitenmiller, R. R., & Long, A. R. (1997). Liquid chromatographic analysis of all-rac-alpha-tocopheryl acetate, tocopherols, and retinyl palmitate in SRM 1846. *Journal of Liquid Chromatography and Related Technologies, 20*(20), 3317–3327.

Chase, G. W., & Long, A. R. (1998). Liquid chromatographic method for analysis of all-rac-alpha-tocopheryl acetate and retinyl palmitate in milk-based infant formula using matrix solid-phase dispersion. *Journal of AOAC International, 81*(3), 582–586.

Chávez-Servín, J. L., Castellote, A. I., & López-Sabater, M. C. (2006). Simultaneous analysis of Vitamins A and E in infant milk-based formulae by normal-phase high-performance liquid chromatography-diode array detection using a short narrow-bore column. *Journal of Chromatography A, 1122*, 138–143.

Crawford, M. (2000). Placental delivery of arachidonic and docosahexaenoic acids: Implications for the lipid nutrition of preterm infants. *The American Journal Of Clinical Nutrition, 71*(1), 275S–284S.

Delgado-Zamarreno, M. M., Bustamante-Rangel, M., Garcia-Jimenez, M., Sanchez-Perez, A., & Carabias-Martinez, R. (2006). Off-line coupling of pressurized liquid extraction and LC/ED for the determination of retinyl acetate and tocopherols in infant formulas. *Talanta, 70*(5), 1094–1099.

Farrell, P. M., & Roberts, R. J. (1994). Vitamin E. In M. Shils, J. A. Olson, & M. Shike (Eds.), *Modern Nutrition in Health and Disease* (8th ed., pp. 326–341). Philadelphia: Lea and Febiger.

Ferrer, E., Alegria, A., Courtois, G., & Farre, R. (2000). High-performance liquid chromatographic determination of Maillard compounds in store-brand and name-brand ultra-high-temperature-treated cows' milk. *Journal of Chromatography A, 881*(1–2), 599–606.

Gibson, R. A., Neumann, M. A., & Makrides, M. (1996). Effect of dietary docosahexaenoic acid on brain composition and neural function in term infants. *Lipids, 31*, S177–S181.

Giovannini, M., Riva, E., & Agostoni, C. (1995). Fatty-acids in pediatric nutrition. *Pediatric Clinics of North America, 42*(4), 861–877.

Guo, M. R., Hendricks, G. M., & Kindstedt, P. S. (1998). Component distribution and interactions in powdered infant formula. *International Dairy Journal, 8*(4), 333–339.

Hardas, N., Danviriyakul, S., Foley, J. L., Nawar, W. W., & Chinachoti, P. (2000). Accelerated stability studies of microencapsulated anhydrous milk fat. *Lebensmittel-Wissenschaft und-Technologie, 33*(7), 506–513.

Hardas, N., Danviriyakul, S., Foley, J. L., Nawar, W. W., & Chinachoti, P. (2002). Effect of relative humidity on the oxidative and physical stability of encapsulated milk fat. *Journal of the American Oil Chemists Society, 79*(2), 151–158.

Hornstra, G. (2000). Essential fatty acids in mothers and their neonates. *The American Journal Of Clinical Nutrition, 71*(5), 1262S–1269S.

Jeffrey, B. G., Weisinger, H. S., Neuringer, M., & Mitchell, D. C. (2001). The role of docosahexaenoic acid in retinal function. *Lipids, 36*(9), 859–871.

Karatapanis, A. E., Badeka, A. V., Riganakos, K. A., Savvaidis, I. N., & Kontominas, M. G. (2006). Changes in flavour volatiles of whole pasteurized milk as affected by packaging material and storage time. *International Dairy Journal, 16*(7), 750–761.

Khachik, F., Spangler, C. J., Smith, J. C., Canfield, L. M., Steck, A., & Pfander, H. (1997). Identification, quantification, and relative concentrations of carotenoids and their metabolites in human milk and serum. *Analytical Chemistry, 69*(10), 1873–1881.

Koletzko, B., Agostoni, C., Carlson, S. E., Clandinin, T., Hornstra, G., Neuringer, M., et al. (2001). Long chain polyunsaturated fatty acids (LC-PUFA) and perinatal development. *Acta Paediatrica, 90*(4), 460–464.

Landen, W. O., Hines, D. M., Hamill, T. W., Martin, J. I., Young, E. R., Eitenmiller, R. R., et al. (1985). Vitamin-A and vitamin-e content of infant formulas produced in the United States. *Journal of the Association of Official Analytical Chemists, 68*(3), 509–511.

Lauritzen, L., Hansen, H. S., Jorgensen, M. H., & Michaelsen, K. F. (2001). The essentiality of long chain *n*-3 fatty acids in relation to development and function of the brain and retina. *Progress in Lipid Research, 40*(1–2), 1–94.

Miquel, E., Alegria, A., Barbera, R., Farre, R., & Clemente, G. (2004). Stability of tocopherols in adapted milk-based infant formulas during storage. *International Dairy Journal, 14*(11), 1003–1011.

Olson, J. A. (1987). Recommended dietary intakes (rdi) of vitamin-A in humans. *American Journal of Clinical Nutrition, 45*(4), 704–716.

Olson, J. A. (1989). Upper limits of vitamin A in infant formulas, with some comments on vitamin K. *Journal of Nutrition, 119*(12), 1820–1824.

Olson, J. A. (1994). Vitamins – The tortuous path from needs to fantasies. *Journal of Nutrition, 124*(9), S1771–S1776.

Oski, F. A., & Barness, L. A. (1967). Vitamin E deficiency: A previously unrecognized cause of hemolytic anemia in the premature infant. *Journal of Pediatrics, 70*(2), 211–220.

Parrish, D. B. (1980). Determination of vitamin E in foods – A review. *Crc Critical Reviews in Food Science and Nutrition, 13*(2), 161–187.

Rodrigo, N., Alegria, A., Barbera, R., & Farre, R. (2002). High-performance liquid chromatographic determination of tocopherols in infant formulas. *Journal of Chromatography A, 947*(1), 97–102.

Romeu-Nadal, M., Morera-Pons, S., Castellote, A. I., & Lopez-Sabater, M. C. (2006). Determination of gamma- and alpha-tocopherols in human milk by a direct high-performance liquid chromatographic method with UV–vis detection and comparison with evaporative light scattering detection. *Journal of Chromatography A, 1114*(1), 132–137.

Schroder, M. J. A., Scott, K. J., Bland, M. A., & Bishop, D. R. (1985). Flavor and vitamin stability in pasteurized milk in polyethylene-coated cartons and in polyethylene bottles. *Journal of the Society of Dairy Technology, 38*(2), 48–52.

Snodgrass, S. R. (1992). Vitamin neurotoxicity. *Molecular Neurobiology, 6*(1), 41–73.

Spannaus-Martin, D.J., Cook, L.R., Tanumihardjo, S.A., Duitsman, P.K., & Olson, J.A., (1998). Vitamin A and vitamin E statuses of preschool children of socioeconomically disadvantaged families living in the midwestern United States (Vol. 51, p. 864, 1997). *European Journal of Clinical Nutrition*, *52*(4), 310.

Tanumihardjo, S. A., Muhilal Yuniar, Y., Permaesih, D., Sulaiman, Z., Karyadi, D., & Olson, J. A. (1990). Vitamin-A status in preschool-age Indonesian children as assessed by the modified relative-dose-response assay. *American Journal of Clinical Nutrition, 52*(6), 1068–1072.

Vassila, E., Badeka, A., Kondyli, E., Savvaidis, I., & Kontominas, M. G. (2002). Chemical and microbiological changes in fluid milk as affected by packaging conditions. *International Dairy Journal, 12*(9), 715–722.