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MODIFICATION OF NUTRITIONAL VALUE DURING STORAGE OF INFANT FORMULAS ELABORATED WITH DIFFERENT INTACT AND PARTIALLY HYDROLYZED PROTEINS AND CARBOHYDRATES

MODIFICACIÓN DEL VALOR NUTRICIONAL DURANTE EL ALMACENAMIENTO DE FÓRMULAS INFANTILES ELABORADAS CON DIFERENTES PROTEÍNAS INTACTAS, PARCIALMENTE HIDROLIZADAS Y CARBOHIDRATOS

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ABSTRACT

Background: Human milk is considered the best source of nutrition for young infants. However, if mothers cannot provide adequate breast milk or if infants are premature or have a low birth weight, breast-feeding must often be replaced or complemented with infant formulas (IFs). The interactions between infant formula components (proteins, fats, carbohydrates, vitamin and minerals) mainly affect carbohydrates and proteins (Maillard reaction, MR), but those involving proteins are especially important in products used in infant feeding because of the high protein requirements of infants. On the other hand, fatty acids (FAs) are considered important in infant development. **Objectives:** The aim of the present work was to test the stability of IFs made with different ingredients, analyzing the available lysine losses (for protein stability) and the FAs content and the peroxide value (for fat stability) during stored at normal and adverse conditions and to propose a faster control of that stability. **Methods:** Available lysine analyzed by high-performance liquid chromatography (HPLC), lipid oxidation by titrimetric method and FAs profile by gas chromatography (GC) were determined in four types of IFs prepared with intact and partially hydrolyzed proteins and different carbohydrates (lactose or maltodextrins) during storage at 4, 20 and 30 ºC for 24 months at normal water activity (Aw=0.1-0.4), and at Aw of 0.65 at 20 and 30 ºC for 4 weeks. These IFs were prepared twice (IF_1 and IF_2) in different batches by a Spanish dietary product company. **Results:** At 30ºC, available lysine losses were 40-50% in all IFs analyzed. The behavior and percentage lysine loss between 1 and 4 weeks of storage at 30 °C with Aw=0.65 was similar to those obtained after 24 months of storage at 30 °C. No significant changes were observed in fatty acid profile during storage. Oxidation was only observed in opened packs and after 4 weeks/30 °C/Aw=0.65. **Conclusions:** The losses of available lysine increase to higher time and storage temperatures. The FAs shows a good stability for any storage condition; however peroxide values prove more sensitive than FAs changes for evaluating fat oxidation during the storage of IFs.

Keywords: Infant formulas, available lysine, milk protein, fatty acids, peroxides

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RESUMEN

Antecedentes: La leche materna es la mejor fuente nutricional para niños recién nacidos. Sin embargo, cuando las madres no pueden proveer leche, o si los niños son prematuros o tienen bajo peso al nacer, la lactancia debe ser reemplazada o complementada con fórmulas infantiles (IFs). La interacción entre los componentes de las IFs (proteínas, grasas, carbohidratos, vitaminas y minerales) principalmente afecta carbohidratos y proteínas (Reacción de Maillard, MR), pero los que implican proteínas son especialmente importantes en alimentación infantil debido a los altos requerimientos de proteína de los lactantes. Por otra parte, los ácidos grasos (FAs) son considerados importantes en el desarrollo de los niños. **Objetivos:** El objetivo fue evaluar la estabilidad de IFs elaboradas con diferentes ingredientes, analizando lisina útil (para estabilidad de proteínas), contenido de FAs y valor de peróxidos (para estabilidad de grasas) durante el almacenamiento bajo condiciones normales y adversas, y proponer un control más rápido de la estabilidad. **Metodos:** Los análisis de lisina útil por cromatografía liquida de alta resolución (HPLC), oxidación lipídica por el método de titulación y el perfil de FAs por cromatografía de gases (GC) fueron determinados en cuatro tipos de IFs preparadas con proteínas intactas y parcialmente hidrolizadas con diferentes carbohidratos (lactosa y maltodextrinas) durante el almacenamiento a 4, 20 y 30 ºC por 24 meses a actividad de agua normal (Aw=0,1-0,4), y a Aw de 0,65 a 20 y 30 ºC por 4 semanas. Estas IFs fueron preparadas dos veces (IF_1 y IF_2) en baches diferentes por una empresa Española de productos dietarios. **Resultados:** A 30ºC, las pérdidas de lisina útil fueron de 40-50% en todas las IFs analizadas. El comportamiento y porcentaje de pérdidas de lisina entre la 1 y 4 semana de conservación a 30 °C con Aw=0,65 fue similar a las obtenidas tras 24 de almacenamiento a 30 °C. No se observaron cambios significativos en el perfil de FAs durante el almacenamiento. Oxidación solo se observo en los envases abiertos y después de 4 semanas/30 °C/Aw=0.65. **Conclusiones:** Las pérdidas de lisina útil incrementan a mayor tiempo y temperatura de almacenamiento. Los FAs muestran una buena estabilidad para cualquiera de las condiciones de almacenamiento, sin embargo el valor de peróxidos evidencia mayor sensibilidad que los cambios en FAs para evaluar la oxidación de las grasas durante el almacenamiento de IFs.

Palabras clave: Formulas infantiles, lisina útil, proteína láctea, ácidos grasos, peróxidos

INTRODUCTION

Infant formulas (IFs) are designed as substitutes for maternal milk when breast feeding is not possible. Generally, they contain a source of protein such as milk protein, milk protein hydrolysate or soybean protein. Lactose, starch, maltodextrins, sucrose and triglycerides are used as energy sources (1). There are two types of formula for normal feeding, adapted formula suitable for infants under 4 months old and follow-up formula for those over 4–6 months old. IFs prepared with partially hydrolyzed proteins are also used in certain cases, e.g., to prevent or treat cow's milk allergies (2)

Production, handling and storage warrant greater attention during the manufacture of IFs compared with other food formulations, because they are more subject to reactions and interactions and their physical properties and nutrient availability can undergo major changes during storage. The storage temperature and relative humidity are two important factors in the control of physico-chemical properties and microbial quality of infant foods. Several types of physico-chemical damage can occur during storage, e.g., lactose crystallization, the Maillard reaction (MR), lipid peroxidation, caking, and interactions and reactions between proteins and polysaccharides, etc (1). The MR involves amino acids and reducing carbohydrates and can lead to loss of nutritional value (3). IFs containing reducing carbohydrates (lactose or dextrinomaltose) and proteins or free amino acids are prone to non-enzymatic browning as a result of the MR.

Loss of lysine availability and decrease of protein digestibility are the main nutritional consequences of the MR during the manufacture and storage of IFs (4, 5). This nutritional damage is of particular interest in IFs because they are frequently the only protein source during early infancy. The nutritional requirement for lysine of babies aged 0 to 6 months is around 107 mg/kg /day (6).

On the other hand, fatty acids (FAs) are considered important in infant development. The main FAs in human milk are palmitic (20%), oleic (38%) and linoleic (15%) (7). Unsatured FAs are highly susceptible to oxidation; lipid oxidation is the main cause of deterioration of lipid-containing IFs. The reaction between atmospheric oxygen and unsaturated lipids produces a wide range of hydroperoxides (8). Lipid oxidation has received much attention because of its undesirable implications for human health and its contribution to a decrease in the nutritional value of foods. Thus, peroxide values indicate the initial products of autoxidation and can only be used to study peroxide formation in the early stage of oxidation. During the course of oxidation, peroxide value may reach a peak and then decline (9).

There have been several studies on the lysine loss caused by heat treatment and storage of powered IFs (10-12) and on the influence of water activity during storage of powdered milk-like systems (13). However, there are no data on the effects of adverse storage conditions (temperature and Aw) that usually occur in the European Mediterranean areas, African, American, and Asian countries who consume these products or on comparisons between formulas made with different intact proteins and those made with protein hydrolysates prepared with different carbohydrates. Moreover, there are no comparative studies available on products with the same formulation but different batches of ingredients.

The purpose of this study was to determine the loss of available lysine and assess lipid oxidation by peroxide level and fatty acid profile during the storage of IFs prepared by industry with intact or hydrolyzed proteins under normal (with the original product water content) and under abnormal humidity conditions (water activity, Aw=0.65), using different ingredients (protein and carbohydrates) and using different batches of the same ingredients, to discover whether the IFs still complied with established requirements and European regulations after storage and to propose a faster control of that stability.

MATERIALS AND METHODS

Samples and reagents

The study was performed using four powdered IFs prepared with different carbohydrates (maltose-dextrin (dextrose equivalent (DE)=17), sucrose or lactose) and milk proteins (calcium caseinate (90% of protein), whey protein milk low in lactose (76% of protein), whey protein milk (64% of protein), and partially hydrolyzed (molecular weight of 8000–20,000 Da) whey protein milk low in lactose (82% of protein)). Two IFs had intact protein (A and B) and two had partially hydrolyzed whey protein (C and D) (Table 1). IFs A, C and D are adapted formulas and infant formula B is a follow-up formula. Each formula was prepared twice (IF_1 and IF_2) under the same conditions and their composition was given by the Spanish dietary product company (Table 1). Although the formulation was the same in both plants, the ingredients were from different batches. The infant formula D only was prepared once, with the same batch protein ingredient used in infant formula C2.

Ingredients were mixed with deionized water at <60 ºC under agitation to obtain 40% total solids (approximately 3 hours). Oil was added at 90-95 ºC for 22 sec. Spray-drying was performed at 215-230 ºC and the temperature of exit air was 85-95 ºC. There was a final amino acid addition stage in dry, partially hydrolyzed formulas; the added amino acids were L-Arginine, L-Phenylanaline, L-Histidine, L-Trytophan, and L Tyrosine. The final product was packed under nitrogen atmosphere in a 400g aluminium bottle.

Acetic acid, ethanol (HPLC grade), hexane, methanol, sodium thiosulfate, starch sulphuric acid and potassium iodide were obtained from Panreac (Barcelona, Spain). N-ε-2,4-DNP-L-lysine-HCl, butyl hydroxy anisol (BHA), chloroform, FAs methyl esters, sodium methylate, hexane, margaric acid (C:17) and derivative reagent 1-Fluoro-2,4-dinitrobenzene (FDNB) solution were purchased from Sigma-Aldrich (Madrid, Spain).

Table 1. Final composition of IFs.

Formulas	% Content	
A	Prepared 1	Prepared 2
Carbohydrates	54.9	
Sucrose	24.7	
Maltose-dextrin	30.2	
Proteins	11.33	11.56
Whey protein milk low in lactose	5.38	5.49
Calcium caseinate	5.90	6.10
Fat	27.7	
Linoleic acid	4.20	
Minerals	3.1	
Water content	3.3	
B	**Prepared 1**	**Prepared 2**
Carbohydrates	55.2	
Lactose	55.2	
Proteins	11.94	12.10
Whey protein milk	5.73	5.81
Calcium caseinate	6.21	6.29
Fat	27.3	
Linoleic acid	5.50	
Minerals	2.90	
Water content	3.00	
C	**Prepared 1**	**Prepared 2**
Carbohydrates	55.9	
Sucrose	8.4	
Maltose-dextrin	47.5	
Proteins	13.14	13.29
Partially hydrolyzed whey protein milk low in lactose	13.14	13.29
Fat	25.35	
Linoleic acid	4.10	
Minerals	2.90	
Water content	2.60	
D	**Prepared 1**	
Carbohydrates	55.90	
Sucrose	8.4	
Lactose	47.5	
Proteins	13.5	
Partially hydrolyzed whey protein milk low in lactose	13.5	
Fat	25.35	
Linoleic ccid	4.1	
Minerals	2.9	
Water content	2.6	

Storage conditions

Formulas, packed under nitrogen atmosphere in a 400g aluminum bottle were introduced into an oven at 20 or 30 ºC or cold chamber at 4 ºC and analyses were performed after 3, 6, 12, 15 and 24 months of storage (useful life of an IF). Storage at 4 ºC was not performed at 3 months. A different bottle was used for each storage time and temperature and was removed at the corresponding time. Another study was realized at Aw=0.65, samples of formula for each storage time and temperature were placed in open Petri dishes in desiccators with sodium nitrite saturate solution to obtain an Aw of 0.65 (14), which were then placed in an oven at 20 or 30 ºC during 1, 2, 3 and 4 weeks of storage. All IFs were tested at baseline (month 0 at room temperature). After the corresponding storage period, samples were kept at -20 ºC until their analysis.

Available lysine determination

ε-NDP-lysine was determined by HPLC, following the method used for IFs by Contreras-Calderón et al., 2009 (2). A sample containing approximately 4 mg of protein was derivatized by addition of FDNB solution in 3% v/v, ethanol. The FDNB derivative was hydrolyzed with HCl. HPLC was performed using a Perkin–Elmer Model 250 fitted with a Waters 717 automatic injector and a Perkin–Elmer 235 UV diode array detector, using a Model 1020 Perkin–Elmer Nelson integrator-computer. Fifty microliters of filtered solution were separated in a Novapack reverse-phase C_{18} HPLC column (4 μ, 150x3.9 mm, Waters, Milford, MA) operating at room temperature. ε-DNP-lysine was determined by the external standard method. The stock standard solution was 100 mg/ml of N-ε-2.4-DNP-L-lysine-HCl in methanol:water (1:4). Working standard solutions (2-10 mg/ml) were prepared by diluting stock standard solutions in 0.01 M sodium acetate (pH 5). A regression coefficient (r^2) of 0.9903 was obtained. The linear regression equation used was Y = 42082X + 2188, where Y is the peak area and X the ε- NDP-lysine concentration (mg/l). Duplicate analyses of duplicate samples were carried out (n = 4).

Fatty acid determination

Fat extraction was performed following the method described by Folch et al., 1957 (15). Two ml of IFs_1 (1.2 g/10 ml bi-distilled water) was added to 20 ml chloroform: methanol solution (2:1), 2 ml of BHA 1500 mg/l and 200 μl of internal standard C_{17} (20 mg/ml), shaking for 30 min in an amber Erlenmeyer flask with rubber stopper, then centrifuging the solution at 4500 rpm for 10 min to obtain three layers. The chloroform layer was evaporated under vacuum. Subsequently, 6 ml of sodium methylate was added and then boiled for 5 min. Following the method, 6 ml of a 3% (v/v) solution of sulphuric acid in methanol was added and then boiled for a

further 5 min. Finally, after 5 min of cooling, 4 ml of hexane was added, shaken gently, and water was added to bring the hexane layer to the neck of the flask. Finally, 2 μl were injected into a Perkin Elmer autosystem GC equipped with flame ionization detector (FID) (16) coupled to a Perkin-Elmer integrator. An SP-2330 capillary column (30 m x 0.75 mm) (Teknokroma, Barcelona, Spain) was used. Operating conditions were: injector 250 ºC, detector 300 ºC, oven at 50 ºC heated at 15 ºC/min to 190 ºC and held at 190 ºC for 5 min and then heated at 5 ºC/min to 230 ºC and held at 230 ºC for 5 min. Nitrogen at a flow rate of 1.43 ml/min was utilized as carrier gas. The split used was 1:35. Fatty acid methyl esters (FAMEs) were identified by comparison with relative retention times of standards. FAs concentrations were calculated according to the integrated peak area relative to the internal standard using the following correction factors for the individual FAMEs (two standard mixtures, each injected 3 times): 1.0527(C8), 0.989(C10), 0.913(C12), 0.958(C14), 0.908(C16), 0.947(C18), 0.914(C18:1), 0.997(C18:2), 1.13(C18:3), 1.015(C20), 1.061(C22). Range of FAMEs concentrations was 0.00094 - 0.015 mg/ml for C12, C16 and C18 and 0.0005-0.008 mg/ml for C6, C10, C14, C18:1, C18:2, C18:3, C20 and C22. Duplicate analyses of duplicate samples were carried out (n = 4).

Peroxide value

Fat extraction was performed following the method described by Folch et al., 1957 (15). Peroxide value was determined only in IFs_1 according to the peroxide value of oils and fats (17); 4 ml acetic acid-chloroform solution (3:1) was added to the fat obtained (0.25 g approximately) and dissolved by shaking. Subsequently, 1 ml of saturated potassium iodine solution was added, swirled for one min and stored in darkness for 5 min, followed by the addition of 10 ml of water. It was slowly titrated with 0.01 N sodium thiosulphate standard solution, vigorously shaking until the yellow color almost disappeared. Then, 0.5 ml of 1% starch solution was added and titration by vigorous shaking continued to release all iodine from the chloroform layer until the blue disappeared. Samples were analyzed in duplicate.

Statistical analysis

Statgraphics 5.1software was used for the statistical analysis. The two-Way ANOVA test was used to compare the storage times and the temperatures effect on available lysine (LSD test was used). The one-way ANOVA test was used to compare the storage times effect on FAs and peroxide value, and level of significance was set at 95%.

RESULTS

Available lysine determination

Precision of the entire available lysine procedure, including acid hydrolysis, sample preparation and RP-HPLC analysis, was evaluated using a commercial follow-up infant formula (n=8). The mean available lysine was 3.69 mg/100g of protein with a coefficient of variation of 4.6%. The detection limit (LOD) (three times the signal-to-noise ratio) was 7.25×10^{-4} mg/100g of protein, and the quantification limit (LOQ) (ten times the signal-to-noise ratio) was 2.42×10^{-3} g/100 mg of protein (2).

Normal storage

Available lysine contents during storage are shown in Table 2. During storage at normal conditions significant ($p < 0.05$) effects of time and temperature on lysine losses were observed in all IFs analyzed, except in the time for IFs B_2 and D.

Table 2. Available lysine content (mg/100 g of protein, dry weight), and percentage (%) of loss, during normal storage of IFs.

Time (months)	Formula A_1						Formula A_2					
	4ºC	%	20ºC	%	30ºC	%	4ºC	%	20ºC	%	30ºC	%
0	4993±133^{a}	0	4993±133^{a}	0	4993±133^{a}	0	5870±41.0^{a}	0	5870±41.0^{a}	0	5870±41.0^{a}	0
3	-		4777±21.0^{b1}	4	4642±10.0^{b2}	7	-		4977±20.0^{b1}	15	4940±124^{b1}	16
6	4822±2.00^{b1}	3	4334±10.0^{c2}	13	4435±7.00^{c3}	11	4998±165^{b1}	15	4813±31.0^{c1}	18	4764±93.0^{b1}	19
15	4308±82.0^{c1}	14	4282±41.0^{c1}	14	4032±93.0^{d2}	19	4854±21.0^{b1}	17	4650±10.0^{d2}	21	4143±155^{c3}	29
24	-		-		2533±93.0^{e}	49	-		-		2817±0.00^{d}	52
Time (months)	**Formula B_1**						**Formula B_2**					
	4ºC	**%**	**20ºC**	**%**	**30ºC**	**%**	**4ºC**	**%**	**20ºC**	**%**	**30ºC**	**%**
0	6438±183^{a}	0	6438±183^{a}	0	6438±183^{a}	0	5951±30.0^{a}	0	5951±30.0^{a}	0	5951±30.0^{a}	0
3	-		5630±143^{b1}	13	5369±61.0^{b2}	17	-		5387±409^{b1}	9	5267±901^{a1}	11
6	5272±143^{b1}	18	5047±41.0^{c2}	22	4857±195^{c2}	25	5090±162^{b1}	14	4937±429^{b2}	17	4612±225^{a2}	23
15	4761±82.0^{c1}	26	4852±7.00^{d1}	25	4437±51.0^{d2}	31	4614±193^{c1}	22	4344±82.0^{c1}	27	4038±7.00^{b2}	32
24	-		-		3730±133^{e2}	42	-		-		3238±338^{c}	46
Time (months)	**Formula C_1**						**Formula C_2**					
	4ºC	**%**	**20ºC**	**%**	**30ºC**	**%**	**4ºC**	**%**	**20ºC**	**%**	**30ºC**	**%**
0	5694±235^{a}	0	5694±235^{a}	0	5694±235^{a}	0	5307±92.0^{a}	0	5307±92.0^{a}	0	5307±92.0^{a}	0
3	-		5491±7.00^{a1}	4	5307±10.0^{b2}	7	-		4597±153^{b1}	13	4568±113^{b1}	14
6	5568±9.00^{a1}	2	5501±21.0^{a2}	3	5100±9.00^{c3}	10	4174±31.0^{b1}	21	4045±92.0^{c1}	24	4024±110^{c1}	24
15	4366±7.00^{b1}	23	4306±93.0^{b1}	24	4198±1.00^{d2}	26	4225±41.0^{b1}	20	3984±102^{c2}	25	3727±62.0^{d3}	30
24	-		-	-	3389±218^{e}	40	-		-	-	2977±21.0^{e}	44
Time (months)	**-**						**Formula D**					
	-	**-**	**-**	**-**	**-**	**-**	**4**	**%**	**20**	**%**	**30**	**%**
0	-	-	-	-	-	-	5295±173^{a}	0	5295±173^{a}	0	5295±173^{a}	0
3	-	-	-	-	-	-	-		5252±92.0^{a1}	1	5331±41.0^{a1}	0
6	-	-	-	-	-	-	5206±6.00^{a1}	2	5283±31.0^{a1}	0	5321±21.0^{a1}	0
15	-	-	-	-	-	-	5196±71.0^{a1}	2	5008±41.0^{b2}	5	5104±62.0^{b2}	4
24	-	-	-	-	-	-	-		-		3354±238^{c}	37

n = 4. Different letters indicate significant differences between storage times (columns) and different numbers between temperatures (rows).

Formulas with intact proteins

Formula A. For the IFs A, showed significant losses ($p < 0.05$) respect to the control sample (0 months of storage), from the third month for all temperatures tested, and were higher at elevated temperature and storage time. Available lysine losses were between 3% after 6 months at 4ºC for IF A_1 and 52% after 24 months at 30 ºC for IF A_2.

Formula B. Losses respects to the control sample (0 months of storage) in IFs B were significant ($p < 0.05$) after 3 months for all temperatures tested, except in IF B_2 at 30°C. Available lysine loss after 24 months at 30 ºC ranged between 42 and 46% for IF B_1 and IF B_2 respectively (Table 2).

Formulas with partially hydrolyzed proteins

Formulas C and D. Losses in available lysine respect to the control sample (0 months of storage) in the IFs C samples were significant ($p < 0.05$) from 3 months in all IFs, except in IF C_1 at 4 and 20°C. Available lysine losses were between 20 and 30% after 15 month for the three temperatures and between 40 and 44% after 24 months at 30 ºC for IF C_1 and IF C_2 respectively (Table 2).

IF D (with lactose), with the same protein ingredient as IF C_2, and prepared only once, showed a significant losses ($p < 0.05$) only after 15 months of storage at 20 and 30 °C, with a loss of available lysine at the end of the storage of around 37% (Table 2).

Storage at Aw = 0.65

The available lysine contents during storage at 20 and 30 °C are shown in Table 3. Losses in available lysine in all IFs were significant ($p < 0.05$) at the two temperatures tested from the 1 week respect to the control sample (0 weeks of storage). The effect of time and temperature on lysine losses was significant ($p < 0.05$) in all IFs.

Formulas with intact proteins

Formulas A and B. Available lysine losses from formulas A and B after 1 and 4 weeks of storage were statistically significant ($p < 0.05$) at the two temperatures tested (20 and 30 °C) (Table 3). At 30 °C, losses ranged from 42 to 62% after 4 weeks. After 4 weeks of storage at 20 °C, losses of lysine were almost the same as at 30 °C after 1 week for all prepared product (A and B), except for IF B_1 where losses were lower at 20 °C. For the same storage times, significant losses ($p < 0.05$) were observed between 20 and 30 °C except in IF B_2 after 4 weeks.

Formulas with partially hydrolyzed proteins

Formulas C and D. At both 20 and 30 °C, there were significant lysine losses ($p < 0.05$) from these IFs, prepared with hydrolyzed proteins, at 1 week and from 1 to 4 weeks of storage, except for formula C_2 and D at 30 °C between 1 and 4 weeks (Table 3). After 4 weeks at 30 °C, losses were 42% and 34% for IF C_1 and C_2 respectively, lower than those found from the IFs prepared with intact proteins (42 to 62%). After 4 weeks of storage at 20 °C, available lysine losses were almost the same as those at 30 °C after 1 week for both preparations (1 and 2) of formulas C and D. For the same storage times, significant losses ($p < 0.05$) were observed between 20 and 30 °C except in IFs C_2 and D after 4 weeks.

Table 3. Available lysine content (mg/100 g of protein, data expressed in dry weight) and percentage (%) of loss during storage at Aw 0.65 of IFs.

Time (weeks)	A_1				A_2			
	20°C	**%**	**30°C**	**%**	**20°C**	**%**	**30°C**	**%**
0	4933 ± 133^{a}	0	4933 ± 133^{a}	0	5870±41.0a	0	5870 ± 41.0^{a}	0
1	3091 ± 42.0^{b1}	38	2604 ± 2.00^{b2}	48	4813 ± 21.0^{b1}	18	3895 ± 32.0^{b2}	34
4	2640 ± 31.0^{c1}	47	1903 ± 11.0^{c2}	62	3837 ± 42.0^{c1}	35	2541 ± 53.0^{c2}	57
Time (weeks)	**B_1**				**B_2**			
	20°C	**%**	**30°C**	**%**	**20°C**	**%**	**30°C**	**%**
0	6438 ± 183^{a}	0	6438 ± 183^{a}	0	5951 ± 30.0^{a}	0	5951 ± 30.0^{a}	0
1	4642 ± 42.0^{b1}	28	3833 ± 41.0^{b2}	40	4391 ± 51.0^{b1}	26	3542 ± 10.0^{b2}	40
4	4313 ± 63.0^{c1}	33	3065 ± 0.00^{c2}	52	3523 ± 41.0^{c1}	41	3461 ± 31.0^{c2}	42
Time (weeks)	**C_1**				**C_2**			
	20°C	**%**	**30°C**	**%**	**20°C**	**%**	**30°C**	**%**
0	5694 ± 235^{a}	0	5694 ± 235^{a}	0	5307 ± 92.0^{a}	0	5307 ± 92.0^{a}	0
1	4412 ± 95.0^{b1}	23	3740 ± 54.0^{b2}	34	4590 ± 63.0^{b1}	14	3715 ± 43.0^{b2}	30
4	3549 ± 42.0^{c1}	38	3286 ± 33.0^{c2}	42	3431 ± 32.0^{c1}	35	3491 ± 21.0^{b1}	34
Time (weeks)	**-**				**D**			
	-	**-**	**-**	**-**	**20°C**	**%**	**30°C**	**%**
0	-	-	-	-	5295 ± 173^{a}	0	5295 ± 173^{a}	0
1	-	-	-	-	4159 ± 32.0^{b1}	21	3309 ± 42.0^{b2}	38
4	-	-	-	-	3306 ± 21.0^{c1}	38	$3308 \pm 5\ .00^{b1}$	38

n = 4. Different letters indicate significant differences between storage times (columns) and different numbers between temperatures (rows).

Fatty acid determination

The total Fatty acid content in IFs during storage ranged between 18.2 and 23.4 g/100 g of sample, being the oleic acid (C18:1 cis9) the major fatty acid present in the IFs (Table 4). No significant ($p < 0.05$) changes were observed in fatty acid profile during storage.

Peroxide value

Table 5 shows the behavior of peroxide values, expressed in meq O_2/kg, during storage of IFs. No oxidation was observed after 6 months of storage at 30 °C or after 15 months at 20 °C in closed retail packs (Table 5).

No oxidation was observed after 1 week of storage in opened pack at 30 °C/Aw=0.65%. The significant increases in the peroxide values were significant ($p < 0.05$) from 12 months in opened pack at 30 °C in all IFs and after 30°C/4week Aw=0.65% in IFs A and B respect to the control sample (0 months and 0 weeks of storage respectively). Peroxide values ranged from 10.6 to 12.6 meq O_2/kg after 15 months in opened pack at 30 °C and from 0 to 4.89 meq O_2/kg after 4 weeks in opened pack at 30 °C/Aw=0.65.

Table 4. Fatty acid content in IFs during storage (g/100 g of sample).

Fatty Acids	A1			
	Baseline	**30°C/15 months**	**30°C/1week/Aw0.65%**	**30°C/4week/Aw0.65%**
C8	0.63±0.00	0.62±0.01	0.64±0.05	0.64±0.09
C10	0.52±0.03	0.48±0.01	0.48±0.05	0.48±0.05
C12	3.22±0.06	3.22±0.05	3.20±0.51	3.23±0.17
C14	1.33±0.01	1.33±0.02	1.38±0.16	1.44±0.01
C16	1.86±0.01	1.75±0.01	1.75±0.06	1.84±0.06
C18:0	0.80±0.03	0.73±0.03	0.73±0.10	0.80±0.31
C18:1	9.73±0.02	8.98±0.32	8.99±0.12	9.86±0.40
C18:2	4.35±0.04	3.91±0.09	3.92±0.15	4.22±0.31
C18:3	0.43±0.02	0.37±0.01	0.42±0.03	0.43±0.03
C20	0.07±0.06	0.07±0.01	0.07±0.01	0.08±0.00
C22	0.15±0.03	0.14±0.02	0.13±0.03	0.16±0.02
Total	23.1±0.30	21.6±0.60	21.7±1.30	23.2±1.40
Fatty Acids	**B1**			
	Baseline	**30°C/15 months**	**30°C/1week/Aw0.65%**	**30°C/4week/Aw0.65%**
C8	0.47±0.02	0.43±0.07	0.57±0.01	0.58±0.10
C10	0.34±0.01	0.41±0.01	0.42±0.00	0.40±0.06
C12	2.62±0.03	2.91±0.16	2.94±0.01	2.91±0.33
C14	1.29±0.06	1.32±0.07	1.32±0.01	1.29±0.13
C16	1.84±0.07	1.90±0.02	1.80±0.05	1.76±0.08
C18:0	0.86±0.03	0.84±0.01	0.83±0.05	0.81±0.01
C18:1	9.40±0.64	10.3±0.07	9.50±0.52	9.27±0.15
C18:2	4.37±0.29	4.56±0.09	4.45±0.23	4.25±0.08
C18:3	0.46±0.02	0.43±0.02	0.44±0.03	0.44±0.01
C20	0.11±0.06	0.09±0.00	0.07±0.01	0.09±0.00
C22	0.19±0.01	0.19±0.00	0.18±0.02	0.17±0.01
Total	22.0±1.20	23.4±0.50	22.5±1.00	22.0±0.90
Fatty Acids	**C1**			
	Baseline	**30°C/15 months**	**30°C/1week/Aw0.65%**	**30°C/4week/Aw0.65%**
C8	2.01±0.07	2.26±0.10	2.43±0.25	2.41±0.21
C10	1.99±0.12	2.01±0.26	2.04±0.24	2.02±0.02
C12	1.85±0.21	2.18±0.17	2.18±0.30	2.20±0.01
C14	0.64±0.29	0.74±0.23	0.91±0.12	0.92±0.03
C16	1.21±0.09	1.41±0.08	1.38±0.02	1.42±0.13
C18:0	0.54±0.10	0.59±0.09	0.55±0.09	0.55±0.07
C18:1	5.87±0.36	6.60±0.19	6.04±0.37	6.21±0.56
C18:2	3.54±0.31	3.97±0.43	3.89±0.25	3.99±0.32
C18:3	0.37±0.02	0.41±0.06	0.42±0.02	0.43±0.03
C20	0.07±0.01	0.07±0.01	0.06±0.01	0.06±0.00
C22	0.11±0.10	0.12±0.03	0.11±0.01	0.09±0.00
Total	18.2±1.70	20.3±1.70	20.1±1.70	20.3±1.40

n = 4

Table 5. Peroxide value in IFs during storage (meq O_2/kg).

	A_1	B_1	C_1
Pto 0	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
30ºC/6 month (closed pack)	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
30ºC/12 month (opened pack)	8.67 ± 0.20^b	5.96 ± 0.10^b	6.38 ± 0.30^b
30ºC/15 month (opened pack)	12.1 ± 0.1^c	10.6 ± 0.2^c	12.6 ± 0.10^c
30ºC/1week Aw 0.65%	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
30ºC/4week Aw 0.65%	4.89 ± 0.10^d	4.23 ± 0.30^d	0.00 ± 0.00^a

n = 2. Different letters indicate significant differences between storage times.

DISCUSSION

Available lysine determination

Normal storage

Formulas with intact proteins

Formula A. The results (Table 2) show that the temperature and storage time had a significant effect on lysine losses. Differences in lysine losses between the two formulas (A_1 and A_2) derived from differences in the batches of proteins used, since their treatments were identical. It was also observed during the processing (2) that the initial content of available lysine differed between the two formulas. The linear inverse correlation between storage time and available lysine content was > 0.93 (r^2) and statistically significant ($p < 0.05$) at 30 ºC storage temperature for both formulas (Table 6).

Formula B. The linear inverse correlation obtained between storage time and available lysine content was > 0.92 (r^2) for formula B_2 at the three temperatures (Table 6). The correlation was statistically significant ($p < 0.05$) for both B formulas at 30 ºC but only for IF B_2 at 20 ºC. No significant differences in IF B_2 after 3 month at 30°C may be due to the high standard deviation of this sample (Table 2).

Table 6. Inverse linear correlations (r^2) between normal storage time and available lysine.

Temperature (ºC)	Time (months)	Formulas						
		A_1	A_2	B_1	B_2	C_1	C_2	D
4	15	0.973	0.764	0.891	0.924	0.899	0.606	0.730
20	15	0.734	0.614	0.732	0.929★	0.927★	0.670	0.867
30	24	0.934★	0.949★	0.859★	0.939★	0.998★	0.895★	0.756

★ $p < 0.05$

4 ºC. n = 3

20 ºC. n = 4

30 ºC. n = 5

For both types of formula made with complete proteins (A and B), losses were similar after 24 months of storage at 30 °C. These formulas were prepared with different types of carbohydrate (maltodextrins versus lactose), and with different kinds of whey protein. These data are consistent with lysine losses measured during the manufacture of both formulas (2). While there was always loss of lysine during storage at the temperatures studied, losses were smallest at 4 ºC, and it may be desirable to keep the formulas routinely under refrigeration. With long-term (15 months) storage, losses were similar at 4 and 20 °C. The latter temperature should certainly not be exceeded during storage, because changes are much higher at 30 °C.

Guerra-Hernández et al., 2002 (12) reported available lysine losses of 8.9% in IF elaborated with milk protein and lactose and stored for 3 months at

20 °C and of 88.3% in IF stored for 3 months at 55 °C. The IF stored at 20 °C showed slightly lower losses than those observed in the present study. Ferrer et al., 2003 (4) stored adapted and follow-up IFs elaborated with milk protein and lactose at 20 and 37 °C for 24 months. Initial available lysine values were 8430 and 7900 mg/100 g of protein respectively, and they found storage losses for adapted IF of 20% at 20 °C and 24% at 37 °C and for follow-up IF of 48% and 49% at 20 and 37 °C, respectively. In the present study, initial levels of available lysine in the IFs were lower, but the losses were similar. Chávez-Servín et al., 2008a (18) store powder IF based on milk proteins and lactose at 20 and 37 °C for 12 months. The initial available lysine values were ranged between 6010 and 6040 mg/100 g of protein, similar to obtained in the present study and an average value accepted for human milk (6.6 g/100 g of protein) (19), however these authors only obtain losses when the storage is at 37 °C. In dried milk, El and Kavas, 1997 (20) found lysine losses of around 17% when skim milk powder was stored for 18 months at room temperature (20 – 30 °C). These same authors found that the available lysine of milk powders decreases during the drying process and storage (6 months) around 14 and 15% respectively.

The nutritional requirements of lysine for babies aged 0 to 6 months are around 107 mg/kg/day (6). A baby of 1-2 weeks weighing 3.3 kg therefore requires 353 mg of lysine/day, and the daily consumption of IF is 77.4 g (3 measures of 4.3 g per bottle for 6 bottles in 24 h according to label recommendations). For a baby aged 3 months weighing 5 kg, consumption of formula is around 150 g/day (7 measures of 4.3 g per bottle for 5 bottles in 24 h), and the need for lysine is approximately 535 mg/day. Lysine contents of IF A_1 and IF A_2 were 5000 and 5870 mg/100g of protein, respectively, exceeding the minimum estimated needs of infants, who require IF to provide a minimum of around 3200 mg of lysine per 100 g protein. This was the case for all storage times and temperatures, except for 24 months at 30 °C (Table 2). Recommended dietary allowances of lysine for infants aged 7 to 12 months are around 89 mg/kg/day (6). For a baby aged 12 months weighing 9 kg, consumption of formula is around 125 g/day (5 measures of 8.33 g per bottle for 3 bottles in 24 h according to label recommendations), and the need for lysine is approximately 801 mg/day. Lysine contents of IF B_1 and IF B_2 were 6440 and 5950 mg/100g of protein, respectively, more than covering the needs of infants, who require IF to provide a minimum of around 5300 mg of lysine per 100 g protein. Likewise, the stored IF B samples would cover infant needs up to 3 months of storage at all three temperatures tested (Table 2). However when children use follow–up formulas must consume other foods that can provide lysine. At present, there is no legal maximum content of blocked lysine; however, the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) recommend that the amount of blocked lysine for preterm infants should be kept to a minimum (21).

Formulas with partially hydrolyzed proteins

Formulas C and D. The loss of available lysine with time was nearly linear ($r^2 = 0.90$), and was significant ($p < 0.05$) for IF C_1 at 20 and 30 °C and for IF C_2 at 30 °C (Table 6). No significant differences in IF C_1 after 3 month at 4 and 30°C may be due to the high standard deviation of the control sample (0 months of storage) (Table 2).

In our study, the reduction of lysine in IF D was not significantly time-dependent (Table 6). At 30 °C lysine losses were similar in IF D, containing lactose, to those in IF C_2 with maltodextrins, as observed during their processing (2).

Available lysine losses after storage for 24 months at 30 °C were slightly lower in IFs prepared with hydrolyzed proteins (C and D) than in those prepared with intact proteins (A and B), probably more amino acids are available to react in IFs made with partially hydrolyzed proteins (22), proportionally reducing the amount of lysine that reacts. Similar lysine losses were observed during long-term IF storage at 4 and 20 °C, suggesting that product refrigeration is unlikely to be beneficial in these cases. These similar losses are due to the advance of MR which decreases the amount of reactants (sugars and amino acids) until the reaction is stabilized.

Storage at Aw = 0.65

It is important to study the stability of these formulas under the non-optimal storage conditions that can be found in some geographic areas, e.g., on the Mediterranean coast, where relative humidity is high and summer temperatures exceed 30 °C. Additionally, it is also useful to provide the industry with a tool for evaluating possible lysine losses after shorter periods of storage.

Formulas with intact proteins

Formula A and B.

At 30 °C and Aw=0.65, only 4 weeks were needed to achieve the same available lysine losses as those obtained after 24 months of storage at normal Aw. The time-dependency of available lysine on storage time was non-significant, probably due to the small data numbers.

Malec et al., 2002 (13) found losses of around 60% in a lactose-casein model system stored at 37 °C for 800 hours (around 1 month) with Aw=0.69. These losses were similar to those from IF A (prepared with sucrose and maltose-dextrin) and higher than those from IF B (prepared with lactose) after 4 weeks of storage at 30 °C/Aw = 0.65% (Table 4). Gen Pen and Melton, 2007 (23) found losses of around 4% in a lactose-sodium caseinate dry model system heated at 60 °C for 96 hours with relative humidity (RH) between 65 - 75%.

Formulas with partially hydrolyzed proteins

Formulas C and D.

The IFs prepared with hydrolyzates and stored at 30 °C and Aw=0.65 only required between 1 and 4 weeks to achieve the same loss as that obtained after 24 months of storage with normal Aw. IFs C_2 and D, with the same proteins but different carbohydrate (maltodextrins vs. lactose) had a similar initial content of lysine and loss during storage. No major difference in available lysine loss was observed between weeks 1 and 4, with all formulas showing the highest losses during the first week (30 - 48%), when humidity reached maximum values (around 9% in some products) before stabilizing (unpublished data).

The results show a clear dependence of time and temperature of storage on the content of available lysine in IFs. High temperature and long-term storage periods increase the loss of available lysine.

Fatty acid determination

The major fatty acid present in the IFs in quantitative terms was oleic acid (C18:1 cis9) with around 42% of total FAME in formulas elaborated with intact protein and 32% in formulas elaborated with hydrolyzed protein. Likewise the oleic acid content reported for human milk is the range of 24-40% (24). The level of saturated fatty acids (SFA) in IFs was around 36% in formulas elaborated with intact protein and 46% in formulas elaborated with hydrolyzed protein (also within the range found in human milk of 35-50%). The content in medium-chain FAs (MCFA C10:0-C14:0) was around 20% in IFs elaborated with intact protein and around 25% in formulas elaborated with hydrolyzed protein and these contents were much higher than that in human milk, especially due to the high content of C12:0 (approximately 12% in all IFs vs. 3-8% in human milk). Linoleic acid (LA C18:2 cis9-cis12) was the most abundant fatty acid of the PUFA fraction, with values of approximately 4% (91% of PUFA), which are lower than those present in human milk (8-18%) but within the range established by European regulations (25) (between 1.53 and 6.15) (Table 7). The content of alpha linolenic acid (ALA) (C18:3 cis9-cis12-cis15) account for approximately 0.4% and within that established by European regulations (25) (> 0.25) (Table 7). The LA/ALA ratio of the studied formulas range from 9.5-10.1, which falls almost with the ratio in human milk and that established by European regulations (25) (between 5 and 15) (Table 7). The FAs content were similar to reports by other authors for IFs (26-29).

Table 7. Comparison between recommendations for the fatty acid composition of IFs and values obtained.

FAs	Recommendations*	Values obtained
Lauric and myristic acids	<20% of total FAs	13.7-19.7
Linoleic acid	1.53-6.15 g/100 g of sample	3.54-4.37
α- linolenic acid	>0.25 g/100 g of sample	0.37-0.46
Linoleic/ α- linolenic	5-15	9.5-10.1

* European Union: Commission Directive 2006/141/EC on infant formulae and follow-on formulae and amending Directive 1999/21/EC (25).

No significant changes ($p < 0.05$) in FAs composition were observed during the storage of any of the three IFs under study. The results obtained by other authors are contradictors, Roumeu-Nadal et al., 2007 (27) found significant losses in supplemented formulas with LC-PUFA stored at 25 ° C for 15 months but not in unsupplemented formulas, as happens in our case. However, Rodriguez-Alcala et al., 2007 (30) found significant oleic and linolenic acid losses in adapted and follow-up formulas stored at room

temperature for 4 years. Chavez-Servin et al., 2009 (8) found significant linoleic losses in formulas with LC-PUFA supplemented stored at 25 and 40 °C for 18 months, but did not find significant losses for the same acid in the 55% of commercial formulas stored in open package at 25°C for 70 days (31). Manglano et al., (2005) (32) also found greatest significant loss of linoleic acid from storage month 15 onwards in IFs at 22 and 37 °C. Neither, Kus, Aued-Pimentel, & Mancini-Filho (2011b) (33) found significant losses in PUFA in 20% commercial formulas analyzed after 8 months of storage at room temperature. Rufian-Henares et al., 2005 (16) studied the FAs profile during storage of liquid enteral formula prepared with similar ingredients, and found statistically significant ($p < 0.01$) losses in oleic (C18:1), linoleic (C18:2) and linoleic (C18:3) acids after all time periods assayed (1-4 weeks at 32 and 55 °C) but there were no losses in the other FAs. A different behavior was observed during long-term storage (36 weeks): at 20 and 30 °C, there were statistically significant ($p < 0.01$) losses in all of the FAs analyzed, whereas at 4 °C, significant losses were only observed in the unsaturated FAs. The greater stability of infant versus enteral formulas may be due to their different states (powder vs. liquid), that is a different heat treatment: pasteurization vs sterilization. These results show that the analysis of FAs profiles can be used to establish the composition of three IFs but not to know the influence of storage conditions. On the other hand, this analysis can lacks of the sensitivity necessary for evaluating the stability lipid in the IFs.

Peroxide value

Peroxide values were higher in formulas elaborated with intact proteins (IF A_1 and IF B_1) than formula elaborated with hydrolyzed protein (IF C1). The content of unsaturated FAs in formulas A and B was higher than obtained in formula C (64 vs 54%). The peroxide value is a good indicator of the quality of fat. Freshly refined fats should have hydroperoxide levels of less than 1 meq O_2/kg (34). The limiting peroxide value specified by Joint FAO/WHO in 1989 (35) standards for refined oil is 10 meq O_2/kg. Roumeu-Nadal, Chávez-Servín, Castellote, Rivero, and López-Sabater (2007) (27) studied the oxidation stability of the lipid fraction in milk powder formulas and reported initial values of 0.52, 0.85 and 0.98 meq O_2/kg, respectively, for non-supplemented formula (NSF) and supplemented formulas (SFA and SFB) with different levels of n-3 and n-6 LC-PUFA. After 15 months of storage at 25 °C, NSF showed a hydroperoxide level of only 1.48 meq O_2/kg. In contrast, these levels increased slowly in SFA at 25 and 37 °C to be 4.5 and 10.5 fold higher than initial values, respectively. The hydroperoxide value of formulas without supplementary PUFA was very low or zero when stored at room temperature (20 – 25 °C) and in closed retail packs, similar results to those obtained by us. These same authors (Chávez-Servín, Castellote, Martin, Chifré, & López-Sabater, 2009) (8) studied the stability during storage of two IFs supplemented with PUFA stored at 25° and 40 °C for 18 months, the initial peroxide values ranged between 0.34 and 1.26 meq O_2/kg and reached values between 48.56 and 54.97 meq O2/kg after 18 months of storage at 40°C. In these IFs the oxidation occurred before storage, different to observed in the IFs of the present study where peroxides were not observed at the beginning of storage. Manglano et al., (2005) (32) also found no oxidation in all newly manufactured IFs with values between 1.3 and 65.1 meq O2/kg in IFs stored at 22 and 37 °C during 17 months. This same authors found significant differences ($p = 0.000$) with respect to storage time, though not depending on the storage temperature.

The peroxide values in the IFs under study were within permitted values for refined oils except for IFs stored at 30 °C for 15 months in opened packs. No oxidation was observed in formulas in closed retail packs, and oxidation was only observed in opened packs after > 6 months of storage at 30 °C. These small changes were not observed in the FAs determination probably because this analysis lacked the necessary sensitivity for evaluating lipid stability in IFs. Therefore peroxide values can serve as indicators of lipid oxidation and food damage.

CONCLUSIONS

Our study suggests that the losses of available lysine increased with higher storage temperatures up to 37 - 52%, according to the IF in question, after storage at 30 °C for 24 months. Similar losses are obtained when using maltodextrin instead of lactose as a carbohydrate. Percentage losses between 1 and 4 weeks of storage at 30 °C with a water activity of 0.65 were similar to those after 24 months of storage at 30 °C at normal water activity. This

may offer a useful method for rapidly estimating possible lysine losses in long-term storage. The rate of available lysine loss was higher in IFs prepared with intact protein than in those prepared with hydrolyzed proteins, which may be because other amino acids besides lysine take part in the MR. The lysine requirements of infants could be met by IFs stored at 4, 20 and 30 °C for 15 months but not by those stored at 30 °C for 24 months. The FAs values obtained were within ranges established by European regulations. FAs composition showed no significant changes during storage for any time period at any temperature or Aw. Peroxide values proved more sensitive than FAs changes for evaluating fat oxidation during the storage of IFs.

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CONFLICT OF INTERESTS

Authors have declared no conflict of interests.

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