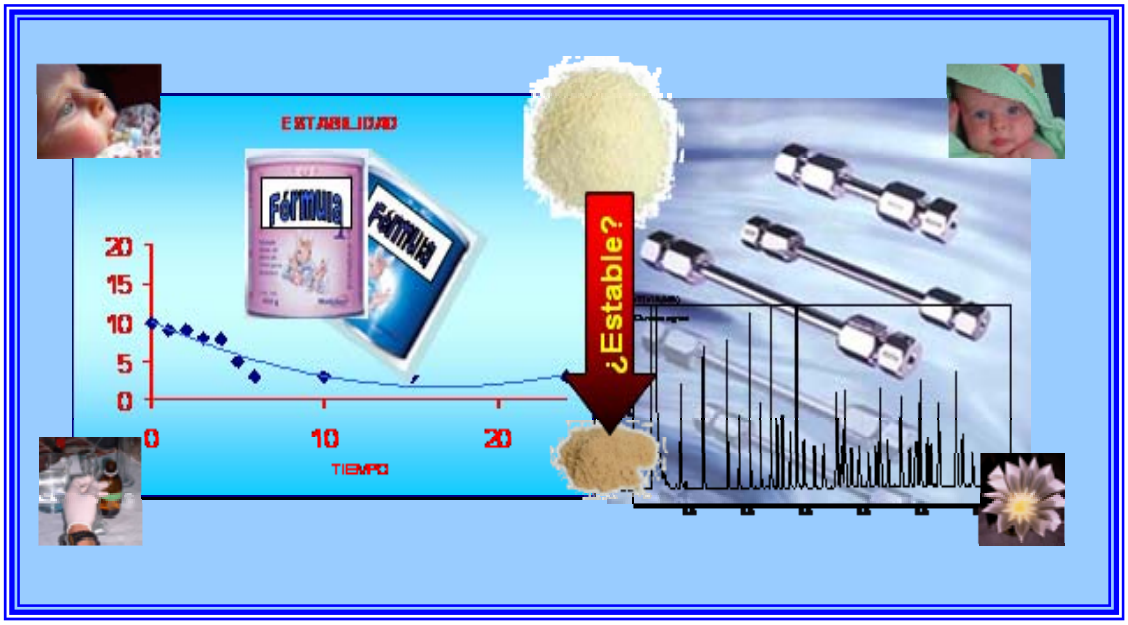
UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

DEPARTAMENT DE NUTRICIÓ I BROMATOLOGIA

Estudios de estabilidad en preparados de base láctea suplementados con diferentes fuentes de ácidos grasos poliinsaturados de cadena larga.

Jorge Luis Chávez-Servín, 2007



IV.- PUBLICACIONES

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PUBLICACIÓN 1

Análisis de mono- y disacáridos en formulas de base láctea por cromatografía líquida de alta eficacia con detección de índice de refracción.

Chávez-Servín, J. L., Castellote, A. I., & López-Sabater, M. C. (**2004**). Analysis of mono- and disaccharides in milk-based formulae by high-performance liquid chromatography with refractive index detection. *Journal of Chromatography A*, 1043(2), 211-215.

Se desarrolló y validó un método simple y reproducible para el análisis cualitativo y cuantitativo de mono- y disacáridos libres (fructosa, glucosa, galactosa, sacarosa, lactulosa y lactosa) en formulaciones de base láctea por cromatografía líquida de alta eficacia (HPLC) con detección de índice de refracción (RI). La fórmula se diluye con etanol- agua 1:1 (v/v), se eliminan las proteínas por precipitación (Carrez I y Carrez II) y adición de acetonitrilo, la solución se filtra por un cartucho activado Sep-Pak C_{18}, en donde quedan retenidos los compuestos no polares. Una alícuota es inyectada en el HPLC-RI. La cuantificación se realiza por rectas de calibrado con soluciones patrón. El método mostró buena linealidad con valores de r^2 superiores a 0.99. Los límites de detección (DL) en los azúcares estudiados siguiendo el orden de aparición antes mencionado fueron de 0.17, 0.13, 0.06, 0.16, 0.05 y 0.25 mg/ml respectivamente. Asimismo los límites de cuantificación (QL) fueron 0.27, 0.14, 0.20, 0.26, 0.22 y 0.38 mg/ml. Los coeficientes de variación (RSD) para la repetibilidad en la fructosa, sacarosa, lactulosa y lactosa fueron 0.78, 0.99, 2.91 y 0.46, y los RSD para la reproducibilidad fueron 4.8, 6.15, 7.04 y 2.49, siguiendo el mismo orden. Las recuperaciones en todos los azúcares estudiados se encontraron entre el 93% y el 113%. Este método resultó relativamente simple en términos de preparación de la muestra y obtención de resultados. Es útil para caracterizar y cuantificar los azúcares libres en las formulas estudiadas y se puede utilizar para análisis rutinarios a fin de observar tanto la evolución de éstos compuestos, así como una posible adulteración. El método mostró valores adecuados de precisión, recuperación y sensibilidad.

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Analysis of mono- and disaccharides in milk-based formulae by high-performance liquid chromatography with refractive index detection☆

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Abstract

A simple and reproducible method for the qualitative and quantitative analysis of free mono- and disaccharides (fructose, glucose, galactose, sucrose, lactulose and lactose) in milk-based formulae by high-performance liquid chromatography (HPLC) with refractive index (RI) detection was developed and validated. The method showed good linearity with determination coefficients exceeding 0.99. The limits of detection (DL) in these sugars were 0.17, 0.13, 0.06, 0.16, 0.05 and 0.25 mg/ml, respectively; and the limits of quantification (QL), 0.27, 0.24, 0.20, 0.26, 0.22 and 0.38 mg/ml. The relative standard deviations (R.S.D.s) for repeatability in fructose, sucrose, lactulose and lactose were 0.78, 0.99, 2.91 and 0.46 and the R.S.D.s for reproducibility were 4.8, 6.15, 7.04 and 2.49, respectively. Recoveries in all sugars were between 93 and 113%.

Keywords: Milk-based formulae; Food analysis; Saccharides

1. Introduction

The industry has deployed considerable technological resources to bringing the composition of infant formulae closer to that of human milk [1]. Besides, it has developed several milk-based formulae for adults, e.g. for pregnant women [2–6]. Milk-based formulae can be based on any appropriate blend of proteins, carbohydrates, fats, minerals and vitamins. Milk powders are usually free-flowing agglomerates formed by spray drying, which extend the shelf-life of dried milk from several days to 18–24 months [7,8].

One of the industry's main problems is to control the stability of milk-based formulae, because they contain a lot of components that may interact. Milk powders are especially sensitive to Maillard reaction, as they contain a relatively high concentration of lactose and proteins with a high lysine level, besides the high temperature applied during the manufacturing process and their storage for long periods of time [9]. During heat treatment, lactose undergoes the Lobry de Bruyn-Alberda van Eckenstein rearrangement, which gives rise initially to isomeric disaccharides, mainly lactulose. As lactulose is not known to occur naturally in milk and is only formed in heated dairy products [10–14], it is a good indicator of heat damage in milk products. Changes may occur during the formulae powders' long periods of storage, even under appropriate storage conditions, and may even be greater than those caused by heat treatment in the production process. The result is, an unacceptable product. Many milk-based formulae contain sugars besides lactose, the evolution of mono- and disaccharides needs to be evaluated. Observation of product stability will help determine whether there are any differences between the same formulae during storage time and the shelf-life.

Many techniques have been developed in order to evaluate sugar fraction during the Maillard reaction. One of the methods is spectrophotometric [15,16], also in some studies lactulose is identified by enzymatic method [17]. The major disadvantage of these consists in the difficulty to evaluate simultaneously different sugars.

Another method developed to evaluate damage in milk powders is the capillary electrophoresis [9], which consist in monitoring the β-lactoglobuline of the whey pro-

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tein fraction. In spite of the promising use, for preparation of sample the caseins need to be precipitated with HCl overnight at 4 °C this mean a lot of time in analysis sample. Yet another method is the high-pH anion-exchange separation with pulsed amperometric detection (AEC–PAD) for evaluating monosaccharides as glucose, fructose and disaccharides as lactulose, lactose, sucrose and maltose. Kaine and Wolnik [18] studied sugars in infant formulae by high pH AEC–PAD, Cataldi et al. [19] gave a comprehensive overview of analytical applications in food for carbohydrate analysis by high-pH AEC-PAD.

One of the methods commonly used in sugar analysis is the gas chromatography (GC). Troyano et al. [20,21] developed a GC method. With this is possible quantifying glucose, galactose, *myo*-inositol, lactulose, *N*-acetylglucosamine, *N*-acetylgalactosamine and other derivatives. GC has been used in the study of milk [20,22], dried skim milk [23], in model systems containing protein-bonded lactose [24] and in pasteurized milk [25]. Valero et al. [13] determined the intensity of the heat treatment in milk pasteurized for the amount of lactulose formed by GC of the trimethylsilyl derivatives of the free sugar, besides monosaccharides were determined. Also in UHT milk [26] and in milk permeate GC has been used [27]. In spite of GC is a sensitive method for sugar analysis, sample preparation is laborious. Besides in the CG procedure the anomeric composition of α- and β-anomers is obtained which mean more than one area peak for each compound. The procedure is tedious to be used routinely.

Finally, in many studies, HPLC is used for its accuracy, separation abilities and rapidity [28,29]. It appeared more then 20 years ago, but remains one of the most widely used techniques. HPLC with refractive index (RI) detection is a powerful technique for quantifying various types of carbohydrate compounds. HPLC–RI was used for determining sugars (glucose, fructose and sucrose) in apple juice [30], disaccharides in whey permeate (lactose, galactose and lactulose) [31], oligosaccharides (fructose, glucose, sucrose, maltose and lactose) in plain cereals, sugar coated cereals, canned fruits, canned vegetables, crackers cookies [32]. HPLC–RI has also been used for determining sugars (sucrose, glucose and fructose), in fruit and drink samples [33], sugars in meat products [34], oligosaccharides in lactose–sucrose systems for determining sucrose inversion by invertase [35] and in sugar casein systems [36]. Martins et al. [37] studied the kinetic modelling of amadori *N*-(1-deoxy-D-fructos-1-yl)-glycine (DPG; intermediate in the early stages of the Maillard reaction) pathways in aqueous model systems, the quantification of D-glucose and D-manose was made by HPLC using an ion-exchange column (ION-300), and sugars were detected by monitoring the refractive index.

Although difficulties of using eluent gradients and relatively poor sensitivity associated with refractometry, HPLC–RI appears to be an economical, simple and fast method for determination of sugars. The aims of this study were to design and to validate an easy HPLC–RI method that separates the free sugar fraction from components such as proteins and other macromolecules that could create interference in the system; and to analyze qualitative and quantitative free mono- and disaccharides in milk-based formulae.

2. Experimental

2.1. Reagents and standards

The chemicals used for sample preparation were of analytical reagent grade: HPLC-grade, SDS acetonitrile and methanol (Peypin, France), HPLC-grade, Panreac absolute ethanol, Carrez I and Carrez II reagents (Barcelona, Spain), deionised water purified through a Milli-Q system (Millipore, Bedford, MA, USA). The standard sugars (fructose, glucose, galactose, sucrose, lactulose and lactose) came from Sigma (St. Louis, MO, USA), were >99% pure and were stored in a vacuum desiccator, with silica gel as desiccant, until use.

2.2. Samples

The method can be applied to any kind of milk-based formula: infant formulae, formulae for pregnant women, etc. In this paper, the samples used were an experimental formula for pregnant women, which contained according to the label milk powder, animal fat, fructose, sucrose, minerals and artificial aroma, and an infant formula (58% carbohydrates), whose ingredients were whole milk powder, lactose, minerals and vitamins.

Both were obtained from a firm in Barcelona, Spain. The formulae were stored at room temperature (25 °C).

2.3. Instrument

The chromatographic analyses were carried out in a Shimadzu high-performance liquid chromatograph equipped with a LC-10AD double pump, a 7725 Rheodyne manual injector (Cotati, CA, USA) with a 20 μL loop, a RID-6A Shimadzu refractive index detector and a C-R6A chromatopac integrator. Chromatographic separation was achieved with a Tracer carbohydrates column (5 μm particle size; 250 mm × 4.6 mm i.d.), and an NH_2 precolumn (13 mm × 3 mm i.d.), both from Tracer (Teknokroma, Barcelona, Spain).

2.4. Sample preparation

Six hundred milligrams of milk-based formula was weighed and transferred to a 25 ml volumetric flask. The sample was dissolved in approximately 10 ml ethanol–water (1:1, v/v). It was placed in a 60 °C water bath and stirred for 25 min until it dissolved completely. After cooling at room temperature, 250 μl Carrez I solution (stirred 1 min)

and 250 μl Carrez II solution (stirred 1 min) were added. Five milliliters of acetonitrile (HPLC-grade) was added. These reagents were used to precipitate the protein and non-sugar fraction. The solution was made up to 25 ml with ethanol–water (1:1, v/v) in a volumetric flask, then was left for 1 or 2 h until complete formation and precipitation of protein clot. The resulting solution was filtered through filter paper and passed through a C_{18} Sep-Pak Plus cartridge Waters (Milford, MA, USA) previously conditioned with 10 ml of methanol (HPLC-grade) and 10 ml of Milli-Q water. This filtered extract was forced through a 0.45 μm nylon filter Tracer (Barcelona, Spain) and was injected into the HPLC system.

2.5. HPLC–RI conditions and quantification

Chromatographic separation was undertaken with an isocratic elution mobile phase of acetonitrile–water (75:25, v/v) and degassed before use. The flow-rate of this eluent was 1.8 ml/min and the volume of the sample injected was 20 μl (filling the loop completely). Column temperature was maintained at 25 °C. Peaks were identified by comparing retention times with sugar standards. The respective peak areas were used for the quantitative analysis. Calibration curves for each sugar were prepared at seven levels, from 0.5 to 10 mg/ml for fructose, glucose, galactose and sucrose; 2–15 mg/ml for lactose; and 0.25–3 mg/ml for lactulose, all dissolved in ethanol–water (1:1, v/v).

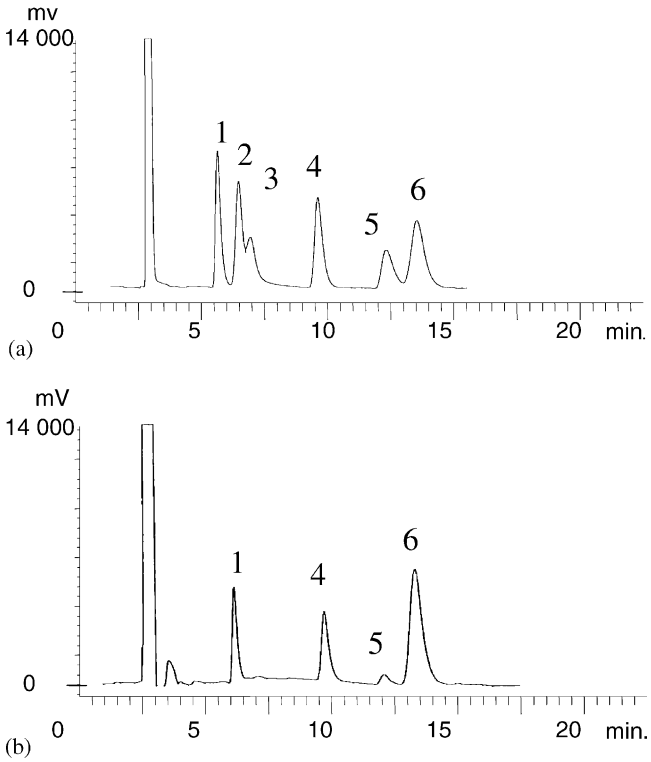

Fig. 1. Typical chromatogram of sugar analysis by the HPLC–RI method. See conditions in Section 2.5. Sugar peaks: 1, fructose; 2, glucose; 3, galactose; 4, sucrose; 5, lactulose; 6, lactose. (a) Saccharide standard content: 3 mg/ml fructose, glucose and galactose, respectively, 2 mg/ml sucrose, 1 mg/ml lactulose and 3 mg/ml lactose. (b) Formula for pregnant women sample, see contents in Table 3.

3. Results and discussion

HPLC–RI detection was used to determine fructose, glucose, galactose, sucrose, lactulose and lactose. Folks and Jordan [38] suggested as an appropriate mobile phase acetonitrile–water in the range 75:25 to 85:15. We experimented with 75:25, 80:20, 85:15 and 90:10 and found that with a 75:25 (v/v) the sugars eluted rapidly and the mobile phase provided better peak symmetry and acceptable separation peaks, except from glucose and galactose which were overlapped. Although this, glucose and galactose showed acceptable recoveries. Besides the analyzed samples not contain this sugars. Addition of galactose in milk-based formulae is not usual. Ferrerira et al. [1] determined sugars in infant formulae, follow-up milks and human milk, no galactose were founded in 50 samples studied.

The flow rate was 1.8 ml/min, with the following retention times: fructose, 5.8 min; glucose 6.8 min; galactose 7.4 min; sucrose 9.8 min; lactulose, 11.7 min and lactose 13.7 min (Fig. 1).

Ethanol is used to extract sugars in several analytical methods. We used a mixture of ethanol–water (50:50, v/v). The ethanol extracts contained high amounts of soluble non-sugar components. For this reason, reagents such as the Carrez solutions are needed to precipitate the compounds.

In previous analyses, when the samples were injected into the HPLC system, the mobile phase (acetonitrile 75%) precipitated the remains of the non-sugar component in spite of the addition of Carrez reagents. This caused, after injection into the HPLC system, a slight interference in the sugar peaks. For this reason, 5 ml of acetonitrile were added to the sample in order to completely precipitate all the substances which may interfere with the mobile phase (acetonitrile–water, 75:25, v/v) after HPLC injection.

In addition, Carrez solutions were reduced from 1 to 0.25 ml because the smaller volume is enough to precipitate and rid the solution sample of substances that might interfere with the sugar analysis. An excess of Carrez solution causes instability baseline after injection of several samples into the HPLC system, which interferes with the saccharide analysis.

3.1. Validation of proposed method

3.1.1. Linearity

Under the chromatographic conditions described above, a linear relationship between the concentrations of sugars (fructose, glucose, galactose, sucrose, lactulose and lactose) and RI was found. For all these sugars, the r^2 values were >0.99 at seven levels (Table 1).

Table 1
Linearity in sugars by RI detection

Compound	Range (mg/ml)	r^2[a]	Equation curve[b]
Fructose	0.5–10	0.998	$y = 563898x - 73672$
Glucose	0.5–10	0.998	$y = 514950x - 43672$
Galactose	0.5–10	0.997	$y = 413570x + 259.88$
Sucrose	0.5–10	0.999	$y = 570340x - 68877$
Lactulose	0.25–3	0.999	$y = 495929x - 10673$
Lactose	2–15	0.998	$y = 399859x - 66979$

[a] Determination coefficient.
[b] x: concentration (mg/ml); y: peak area.

Table 2
Detection (DL) and quantification limits (QL) in sugars by RI detection

Compound	DL (mg/ml)	QL (mg/ml)
Fructose	0.17	0.27
Glucose	0.13	0.24
Galactose	0.06	0.20
Sucrose	0.16	0.26
Lactulose	0.05	0.22
Lactose	0.25	0.38

3.1.2. Sensitivity

To check the sensitivity of this method both the detection limit (DL) and the quantification limit (QL) were studied according to the USP criteria [39]. DL and QL was determined by the chromatographic noise obtained for a blank of ethanol–water (1:1, v/v) through the method and injected under the HPLC conditions cited. The resulting standard deviation of areas was used to determine DL and QL. Results obtained showed acceptable sensitivity (Table 2).

3.1.3. Precision

To evaluate the repeatability of the method, six replicate determinations were carried out on the same day. For reproducibility, six determinations with the same reference (formula for pregnant women) sample on different days were done. The standard deviations and relative standard deviations (R.S.D.s) show good precision (Table 3) within the limits of acceptable variability in methods of analysis [40].

3.1.4. Recovery

The sugar standards fructose, sucrose and lactose which are the principal saccharides in the formula for pregnant women were added in a known mass at two levels in a previously analyzed formula. The trial was in duplicate and the samples injected in triplicate into the HPLC system (Table 4).

Table 3
Precision of the method

Compound	Repeatability ($n = 6$)			Reproducibility ($n = 6$)		
	Mean (g/100 g)	S.D.[a] (g/100 g)	R.S.D.[b] (%)	Mean (g/100 g)	S.D.[a] (g/100 g)	R.S.D.[b] (%)
Fructose	12.61	0.10	0.78	13.45	0.65	4.8
Sucrose	8.58	0.09	0.99	8.67	0.53	6.15
Lactulose	0.9	0.03	2.91	0.87	0.05	7.04
Lactose	16.39	0.08	0.46	16.04	0.40	2.49

Experimental formula for pregnant women. See chromatogram in Fig. 1.
[a] S.D.: standard deviation.
[b] R.S.D.: relative standard deviation.

Table 4
Results of the recovery of sugars

	Recovery (%)					
	Fructose	Glucose	Galactose	Sucrose	Lactulose	Lactose
Level 1	104 ± 4	110 ± 5	107 ± 4	99 ± 5	95 ± 4	110 ± 4
Level 2	108 ± 0.7	113 ± 5	103 ± 8	101 ± 1.8	95 ± 2.3	93 ± 3

Level 1 (mg/g): 27 of fructose, 6.25 of glucose, galactose and lactulose, respectively, 20 of sucrose and 40 of lactose. Level 2 (mg/g): 40 of fructose, 26 of glucose, galactose and lactulose respectively, 35 of sucrose and 70 of lactose. The results are expressed as mean values ± standard deviation ($n = 6$).

4. Conclusion

The results of sugar analysis in the experimental formula for pregnant women sample are given in Table 3, containing 13% of fructose, 9% of sucrose, 0.9% of lactulose and 16% of lactose. The infant formula sample analyzed only contain lactose (57.21 ± 0.2%). The establishment of thermal parameters, defined under specific temperature/time conditions, contributes to the classification of heat treated milks. These thermal parameters are mainly employed to identify and optimize processes, assess heat-loads and identify the degree of thermal damage. Lactulose was proposed by the International Dairy Federation [41] and the European Union [42] as parameter capable of differentiating between UHT milk and in-container sterilized milk. Both international bodies suggested 600 mg/L of lactulose as marker for distinguishing between the two milk types, so as to guarantee the quality of UHT milk and between 600 and 1400 mg/L for sterilized milk; experimental formula for pregnant women are in this last range. However, no limit to lactulose content in infant formulas and/or milk-based formulas has been established.

We developed a simple and reproducible HPLC–RI method to characterize and quantify the free sugar fraction. This method is suitable for routine analysis of mono- and disaccharides in milk-based formulae, in order to monitor the evolution of the compounds, and possible adulteration and stability in the sugar fraction of the formulae. The method provides acceptable precision, recovery and sensitivity.

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PUBLICACIÓN 2

Análisis de los furfurales potenciales y libres en formulas de base láctea por cromatografía líquida de alta eficacia. Evolución durante el almacenamiento.

Chávez-Servín, J. L., Castellote, A. I., & López-Sabater, M. C. (**2005**). Analysis of potential and free furfural compounds in milk-based formulae by high-performance liquid chromatography: Evolution during storage. *Journal of Chromatography A*, 1076(1-2), 133-140.

Se adaptó un método simple y reproducible por HPLC con detector de fotodiodos (DAD) para el análisis cualitativo y cuantitativo de los furfurales potenciales y libres (5-hidroximetil-2-furaldehído, HMF; 2-furaldehído, F; furilmetilcetona, FMC; y 5-metil-2-furaldehído, MF) en formulas de base láctea como indicadores de la extensión de la Reacción de Maillard avanzada. En resumen para determinar los compuestos furfurales potenciales, la fórmula se calienta con ácido oxálico a 100ºC y después se hace reaccionar con ácido tricloro-acético (TCA). Se separan los compuestos interferentes por centrifugación y el sobrenadante se filtra (0.45 μm) para ser inyectado en el HPLC. Para la determinación de los compuestos furfurales libres se realizó el mismo procedimiento, omitiendo el calentamiento a 100ºC. La determinación y cuantificación de los compuestos furfural se realizó mediante rectas de calibrado con soluciones patrón.

El método mostró buena linealidad con r^2 mayores a 0.999. Los límites de detección y cuantificación fueron aceptables para todos los furfurales estudiados. Los coeficientes de variación siguiendo el orden de mención de los furfurales, fueron: para la repetibilidad 1.05, 2.30, 1.66, 1.38, y para la reproducibilidad 2.68, 4.28, 3.51 y 3.32. Las recuperaciones estuvieron en el rango de 94.5 a 98.7%. En este trabajo se reporta además la evolución de los niveles de furfurales en una formula experimental para mujeres embarazadas (FPW), suplementada con aceite de pescado microencapsulado (MFO), almacenada a 25 y 37ºC desde su producción hasta 15 meses después.

HMF fue el principal compuesto detectado, seguido por F. Los niveles observados de furfurales fueron mayores a 37ºC que a 25ºC como era de esperarse, indicando que la temperatura de almacenamiento afecta la RM (a mayor temperatura, mayor RM). Los niveles encontrados antes del almacenamiento, para los furfurales libres fueron: HMF

379.80 ± 3.7 μg / 100 g y F 61.34 ± 4 μg / 100 g. Asimismo para los furfurales potenciales fueron: HMF 902.81 ± 10 μg / 100 g y F 128.40 ± 2.6 μg / 100 g.

El valor de los furfurales potenciales (HMF + F) en las formulas estudiadas antes de su almacenamiento (1031.21 μg / 100 g) fue mayor que los valores encontrados en preparados para lactantes de inicio (601.95 μg / 100 g) pero con una relación caseína/suero 40/60 y menor que los valores reportados en formulas de seguimiento (1362.03 μg / 100 g) con una relación caseína/suero 80/20 (misma relación que para la FPW estudiada). Esto puede ser explicado debido a que la reactividad del grupo ε-amino de la lisina es mayor para la caseína que para el suero.

En general los valores de furfurales fueron incrementando conforme incrementó el tiempo y la temperatura de almacenamiento, no obstante, los incrementos observados fueron de un modo irregular, probablemente debido al hecho de que los furfurales llegan a un estado de equilibrio entre su destrucción debido a los procesos oxidativos y a su formación de precursores.

No se encontraron ni FMC, ni MF a pesar de haber sobrecalentado la formula a 47ºC (durante 10, 30, 50, 100 y 120 días) para confirmar su formación durante el almacenamiento.

No existen límites legales en el contenido de furfurales, no obstante debido a que son indicadores de deterioro de las formulaciones de base láctea, se recomienda mantener los límites de estos compuestos tan bajo como sea posible.

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Analysis of potential and free furfural compounds in milk-based formulae by high-performance liquid chromatography☆ Evolution during storage

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Abstract

A simple and reproducible HPLC-diode array detection method for the qualitative and quantitative analysis of potential and free furfural compounds (5-hydroxymethyl-2-furaldehyde, HMF; 2-furaldehyde, F; 2-furyl methyl ketone, FMC; and 5-methyl-2-furaldehyde, MF) in milk-based formulae was developed and validated. The method showed good linearity with determination coefficients over 0.999. The limits of detection and quantification were acceptable for all furfurals. The relative standard deviations (RSDs) for repeatability and reproducibility were <4.28. Recoveries in all furfurals were between 94.5 and 98.7%. In addition, we report the evolution over shelf life of furfural compound levels in an experimental powder formula for pregnant women stored at 25 and 37 °C from production until 15 months.

Keywords: Furfural compounds; Milk-based formula powders; Maillard reaction; Storage

1. Introduction

The food industry has made several attempts to improve the quality and the nutrient content of milk-base products, and to develop products for specific stages of live (i.e. intrauterine, newborn, pregnancy and lactation [1]. Two of these products are the infant formulae (IFs) and the formulae for pregnant women (FPW), of which milk powder is one of the major constituents. One of the challenges of industry is to control the stability of these two kinds of product. Instability can occur because many factors make these powders susceptible to the Maillard reaction (MR), such as the reductor sugar content, lysine-rich proteins, high temperature applied during production and long storage times [2–6].

The reductor sugars and lysine are the main compounds involved in the initial states of the MR, and consequently a lactulosyl-lysine compound is produced [4,7–14]. In advanced states of MR undesirable compounds such as furfurals can be found [12,15,16]. These compounds can be useful indicators of food damage and can also be used to evaluate the extent of the MR [17,18].

Furfurals can be produced in two ways: via Amadori compounds (mostly ∈-*N*-deoxylactulosyl-L-lysine) from MR by enolization in acidic conditions, or through lactose isomerization [19,20], known as the Lobry De Bruyn-Alberda van Ekenstein transformation (L-A) and the subsequent degradation reactions [12,21]. Fig. 1 shows the schematic formation of furfural compounds from lactose and lysine.

To date, studies have focused on four furfural compounds in processed foods: HMF, 2-furaldehyde (F), 2-furyl methyl ketone (FMC) and 5-methyl-2-furfural (MF) [19,22–29].

Since the development of the Keeney and Basette method [28], a differentiation was made between free HMF and potential HMF. In this method, to determine the latter, the

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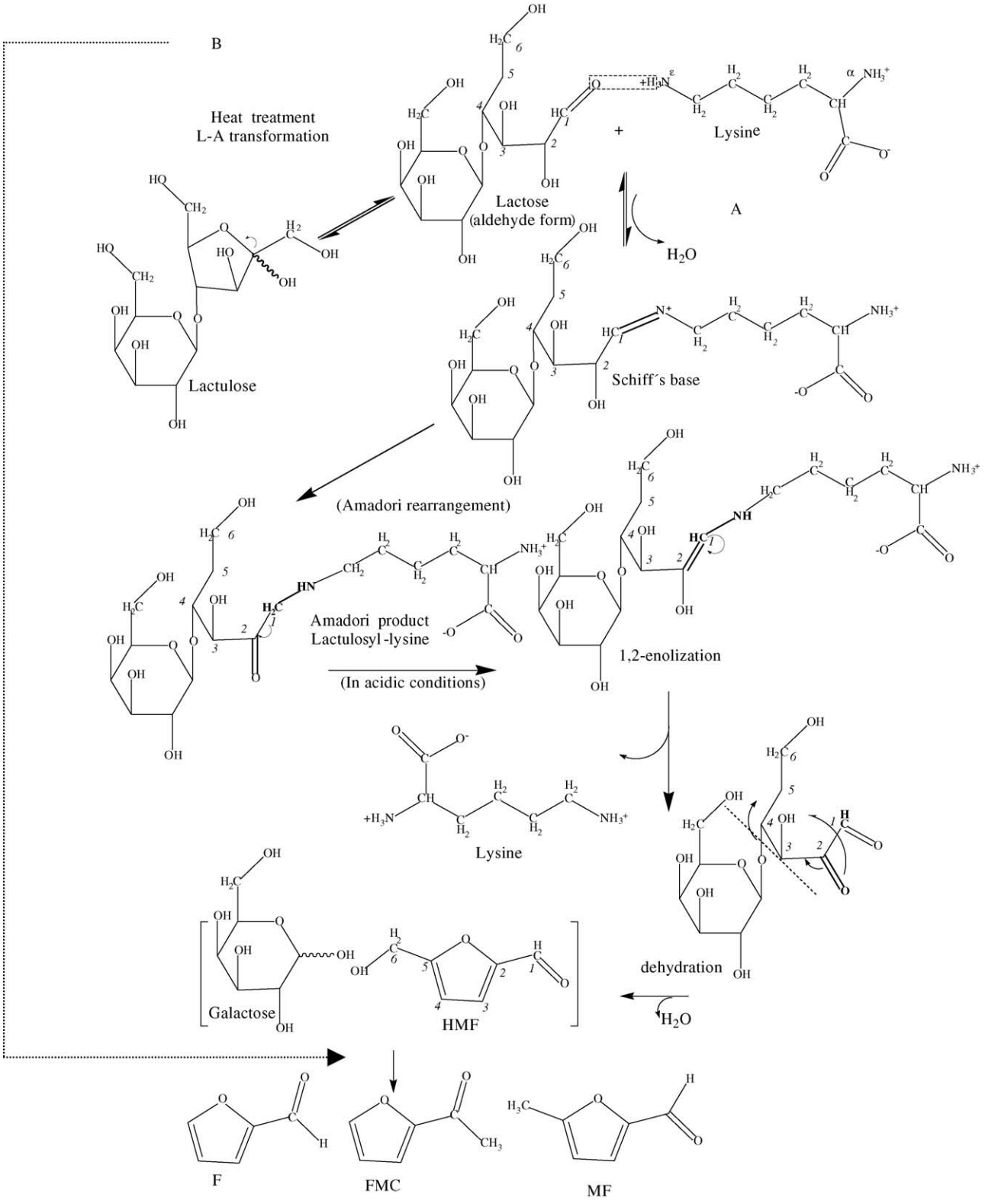

Fig. 1. Schematic presentation of furfural formation from amino ε-group of lysine and lactose in the Maillard reaction: (A) via Amadori compounds and (B) lactose isomerization (L-A). *Note*: Lactose isomerization to lactulose is not a MR process, but this reaction is important in the study of milk-based formulae.

heated-treated milk sample is reheated in 0.3 N oxalic acid at 100 °C to release HMF, because the formation of this furfural from the Amadori compound is induced under acidic conditions. Thus, potential HMF is the sum of the precursors of HMF (i.e. HMF bounded to protein; as Amadori products, HMF from reductor sugars, or novo HMF) and free HMF. Free HMF is determined by omitting the hydrolysis step.

Furfural compounds such as HMF can be measured by spectrophotometry with thiobarbituric acid (TBA). However, one disadvantage of this colorimetric method is that it is not specific for HMF detection because TBA lacks specificity for this compound. In addition, a strict control of time and temperature reaction is required because the reaction product measured colorimetrically is unstable [24]. This instability leads to highly variable results. At present, HPLC techniques can be used for accurate and reliable measurement of furfural compounds in several food products [16,21,26,29–33]. These techniques can determine furfural compounds specifically, and the formation of a colored derivative is not required because of the strong UV absorption of furfurals at approximately 280 nm.

Given that the formation of furfurals can be caused by many factors (temperature of heat treatment/time and composition of formulae), it is difficult to compare distinct formulae, and the amount of furfural compounds may differ. Nevertheless, a comparison of the evolution of furfural contents in the same formula during shelf life could be a useful indicator of changes caused by the MR.

The aims of this study were to validated an HPLC-diode array detection (DAD) method that separates furfurals from components such as proteins, fat and other interference macromolecules for the qualitative and quantitative analysis of potential and free furfural compounds (HMF, F, FMC and MF) in milk-based formula. We used this method and monitored the evolution of these compounds in an experimental FPW stored at 25 and 37 °C, during shelf life. In addition, this study aims at obtaining more information on furfural formation, formula stability, and the usefulness of furfural compound analysis to evaluate deterioration in this product.

2. Experimental

2.1. Reagents and standards

The chemicals used for sample preparation were of analytical reagent grade: acetonitrile HPLC-grade (SDS, Peypin, France), oxalic acid dihydrate >95.5% and trichloroacetic acid (TCA) >99.5% (Fluka, Buchs, Switzerland). Deionised water was purified through a Milli-Q system (Millipore, Bedford, MA, USA). Standards of 5-hydroxymethyl-2-furaldehyde (HMF), 2-furaldehyde (F), 2-furyl-methyl ketone (FMC) and 5-methyl-2-furaldehyde (MF) were >99% pure and were purchased from Fluka (Buchs, Switzerland).

2.2. Instrument

The HPLC system used consisted of a Hewlett-Packard HP 1050 series controller pump degassing device, a Waters 717 plus autosampler and a DAD HP 1040 M series II HPLC detection system. The HP 1090 Win Chemstation system was used for data acquisition.

Separation was performed on a Tracer ODS-2 C_{18} column (150 mm × 4.6 mm), with a 5 μm particle size (Teknokroma, Barcelona, Spain).

2.3. Chromatographic conditions

Separation was performed at 30 °C using a mixture of acetonitrile–water (4.5:95.5, v/v) as the mobile phase and a flow rate of 1 ml/min.

Detection was made at 284 nm for HMF, 277 nm for F, 274 nm for FMC and 293 nm for MF. The injection volume was 20 μl.

2.4. Samples

The method can be applied to any kind of milk-based formula (i.e. IFs, FPW, etc.). Here we tested an experimental FPW, which, according to the label, contained milk powder, animal fat, fructose, sucrose, minerals and artificial aroma (53.7% carbohydrate, 20.2% fat and 18.1% protein: casein/serum protein: 80/20), Vitamin A (2200 μg/100 g), Vitamin C (1800 mg/100 g), and minerals mg/100 g: Na (250), Ca (2000), P (1600), Mg (620); and a commercial IF (58% carbohydrate, 26% fat and 12% protein: casein/serum protein 40/60), whose ingredients were whole milk powder, lactose, minerals and vitamins. Both formulae were obtained from a firm in Barcelona, Spain.

2.5. Storage

To evaluate the evolution of furfurals only in FPW during shelf life, we kept the product in a storage chamber at 25 °C or 37 °C from production until 15 months.

2.6. Measurement of furfural compounds

Free and potential furfurals were measured by RP-HPLC-DAD, with a slight modification of the Albalá-Hurtado method [29]. Potential furfurals include free furfurals, furfurals bound to proteins (like Amadori products) and those formed from precursors. The procedure was as follows.

Potential furfurals: 2 g of formula powder was mixed with 10 ml of 0.2 N oxalic acid (freshly prepared) in a sealed tube covered with parafilm to prevent evaporation. The tube was heated in a water-bath system at 100 °C for exactly 25 min. It was then left to cool at room temperature and 3 ml of 40% (w/v) TCA solution was added. The mixture was stirred for 5 min. It was then centrifuged at 4000 rpm for 15 min. The supernatant phase was passed through a paper filter and col-

lected in a 25 ml volumetric flask. Ten milliliters of 4% (w/v) TCA was added to the solid residue. This was then mixed thoroughly for 10 min and then centrifuged again. The supernatant was filtered and added to the flask, and the solid phase was discarded. The solution was made up to 25 ml with 4% TCA in the volumetric flask. The mixture was then filtered through a 0.45 μm nylon filter before HPLC analysis.

Free furfurals: The sample was prepared as above but heating in the water-bath system was omitted.

Furfurals were identified by retention times and by their characteristic spectra. They were quantified by interpolation in a calibration curve in the range 0.05–2 μg/ml for F, FMC, MF and 0.05–5 μg/ml for HMF.

2.7. pH

The pH of the samples was measured in a pH meter micro-pH 2000 with a glass electrode (Crison Instruments, Barcelona, Spain). Following the manufacturer's instructions, the FPW was reconstituted with cold water (10–15 °C; 15 g in 200 ml) and after of the samples had reached room temperature the pH values were measured.

2.8. Statistical analysis

For statistical analysis, we used a one-way analysis of variance (ANOVA) and multiple comparisons using the Tukey HSD procedure for each furfural and temperature of storage, in order to detect differences in the FPW along storage time at 25 and 37 °C. The level of statistical significance was set at 5% for all analyses. We performed statistical analysis using the SPSS package for Windows version 11 (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Validation of proposed method

Before the method proposed here, first we used a Spherisorb ODS-2 C_{18} column 250 mm × 4.6 mm, 5 μm particle size [27,29]. A pool of standards for furfural compound (HMF, F, FMC, MF) was prepared and injected into the HPLC system. The time required for a single sample injection was about 30 min at room temperature. To observe a relationship between the column temperature and the time required for the detection of each furfural peak, in order to reduce the analysis time, we tested with 25, 30, 35, 50, 60, 70, 80 and 85 °C. Besides we want to know if the column temperature affect the quantity of furfural detected due to the lability of these compounds. We injected the pool of standards three times at each column temperature. A linear relation in each compound ($r^2 \geq 0.99$) was observed between the temperature of analysis and detection. In addition, the RSDs of the amount of each compound detected at all the temperatures tested were less than 0.81. No statistical differences were found between temperatures of analysis and furfural detection ($p > 0.05$). This observation indicates that the increases in column temperature did not affect the amount of compound. Nevertheless, when we prepared the extraction of furfural compounds from an IF and a FPW sample, the increase in temperature did not allow the reliable quantification of HMF because this compound eluted by joining to the matrix leftovers. We then used a shorter column, an ODS-2 C_{18} (4.6 mm × 150 mm), and repeated the analyses. The optimum temperature that allowed the separation of the furfural peaks was 30 °C. However, the HMF peak eluted by joining to a minimal residue, which interfered with the analysis of this compound. We examined the mobile phase using slight variations of water–acetonitrile (90:10, 93:7, 95.5:4.5 and 96:4), and observed that a 95.5:4.5 ratio gave the best result. The time required for each HPLC analysis was about 15 min.

3.1.1. Linearity

Under the chromatographic conditions tested, a linear relationship was verified in the range 0.05–2 μg/ml for F, FMC, MF and 0.05–5 μg/ml for HMF of standard solutions, by analysis of variance of the regression (r^2). For all these compounds, the r^2 values were >0.999 at seven levels. The concentration of HMF in the standard solution was higher than that of the other furfurals because HMF is the main furfural compound in milk-based formulae.

3.1.2. Sensitivity

To determine the sensitivity of the method, the detection limit (DL) and the quantification limit (QL) was studied following the USP criteria [34]. These two limits were determined by the chromatographic noise obtained by repeated analysis of a blank through the system, which was injected under the HPLC conditions described. This is the most common method used to estimate sensitivity in chromatographic procedures. The method showed acceptable sensitivity (Table 1).

3.1.3. Precision

Six replicate measurements were performed on the same day to evaluate repeatability. For reproducibility, eight determinations with the same formula were made on different days. FMC and MF (1 ppm) were added to each sample. The RSDs for HMF, F, FMC and MF were satisfactory according to Horwitz (Table 1) [35].

3.1.4. Recovery

Standards of HMF, F, FMC and MF were added (1 μg/ml of each) to milk-based formula that had been analyzed previously. The six replicate analyses showed acceptable recoveries (Table 1).

3.2. pH analysis

Given that pH can enhance the formation of furfural compounds either by lactose isomerization (Lobry De Bruyn-Alberda van Ekenstein transformation, L-A) or by Amadori

Table 1
Validation method of furfural determination in milk-based formulae by HPLC-DAD

Analytical parameter	HMF	F	FMC	MF
Detection limit (μg/100 g)	32.07	0.32	5.66	18.58
Quantification limit (μg/100 g)	49.05	15.17	22.13	32.11
Repeatability (RSD, %)	1.05	2.30	1.66	1.38
Reproducibility (RSD, %)	2.68	4.28	3.51	3.32
Recovery (%, $n = 6$)	96.32 ± 2.58	96.24 ± 4.12	94.46 ± 3.32	98.71 ± 1.80

Table 2
Potential and Free HMF contents in stored formulae for pregnant women

Sample	Storage (months)	Potential HMF			Free HMF		
		μg/100 g	μg/100 ml	RSD (%)	μg/100 g	μg/100 ml	RSD (%)
FPW, 25 °C	0	902.81 ± 10^{i,1}	67.71 ± 0.7	1.13	379.80 ± 3.7^{i,1}	28.49 ± 0.2	0.98
	5	1040.83 ± 29 ^{j,3}	78.06 ± 2.1	2.80	392.75 ± 2.7^{i,1}	29.46 ± 0.2	0.70
	9	1142.84 ± 10 ^{k,2}	85.71 ± 0.7	0.87	443.47 ± 8.2^{j,3}	33.26 ± 0.6	1.86
	12	1426.10 ± 17 ^{l,5}	106.96 ± 1.3	1.20	356.73 ± 1.4^{i,1}	26.75 ± 0.1	0.42
	15	1562.30 ± 18 ^{m,4}	117.17 ± 1.3	1.15	315.34 ± 9.5^{k,4}	23.65 ± 0.7	3.04
FPW, 37 °C	0	902.81 ± 10 ^{a,1}	67.71 ± 0.7	1.13	379.80 ± 3.74^{a,1}	28.49 ± 0.2	0.98
	5	1199.31 ± 1.8^{b,2}	89.95 ± 0.1	0.16	547.78 ± 33.3^{b,2}	41.08 ± 2.5	6.08
	9	1180.66 ± 9.8^{b,2}	88.55 ± 0.7	0.83	558.19 ± 13.7^{b,2}	41.86 ± 1.1	2.46
	12	1521.12 ± 13^{c,4}	114.08 ± 1.1	0.92	319.75 ± 11.2^{c,4}	23.98 ± 0.8	3.52
	15	2618.66 ± 60^{d,6}	196.40 ± 4.5	2.32	1166.77 ± 65^{d,5}	87.51 ± 4.9	5.64

Values are expressed as mean ± standard deviation ($n = 4$). No coincidence in the superscript letters indicates a significant difference ($p < 0.05$) with the storage time of the same column. No coincidence in the superscript numbers indicates a significant difference ($p < 0.05$) with the temperature of storage.

compounds formation [26], the pH of the reconstituted FPW samples was measured each month of storage. No differences were observed in the evolution of pH values at 25 °C or 37 °C. The average pH over the 15 months of storage was 8.01 ± 0.15 and 8.11 ± 0.18, respectively. These values are slightly basic and could enhance the formation of Amadori compounds with subsequence formation of furfurals during determination.

3.3. Furfural contents

Heating at 100 °C not only released HMF but prolonged heating at this temperature also induced the formation of this compound. Therefore, the conditions of hydrolysis used in this method prevented HMF formation. These conditions were evaluated previously [29], and no furfural compounds were detected in raw milk samples. This observation implies that furfural content in milk-based formula depends on the heating process during manufacture and/or on changes caused by storage conditions.

The chromatograms of furfural in a standard solution, IF and FPW are given in Fig. 2. Potential and free furfural compounds (HMF, F and HMF + F), expressed as μg/100 g powder sample and μg/100 ml of reconstituted sample from FPW at 25 and 37 °C are reported in Tables 2–4.

Other studies refer to "total furfurals" instead of "potential furfurals". We believe that the term "total furfurals"

Table 3
Potential and free F contents in stored formulae for pregnant women

Sample	Storage (months)	Potential F			Free F		
		μg/100 g	μg/100 ml	RSD (%)	μg/100 g	μg/100 ml	RSD (%)
FPW, 25 °C	0	128.40 ± 2.6^{i,1}	9.63 ± 0.2	2.07	61.34 ± 4.0^{i,1}	4.60 ± 0.3	6.65
	5	216.61 ± 13^{j,3}	16.25 ± 1.1	6.15	63.78 ± 1.2^{i,1}	4.78 ± 0.1	1.91
	9	162.55 ± 7.7^{i,5}	12.19 ± 0.5	4.79	89.40 ± 0.4^{j,3}	6.70 ± 0.1	0.43
	12	249.84 ± 5.5^{j,2}	18.74 ± 0.4	2.21	84.95 ± 1.4^{j,3}	6.37 ± 0.1	1.65
	15	345.36 ± 5.6^{k,4}	25.90 ± 0.4	1.64	82.49 ± 2.3^{j,3}	6.19 ± 0.2	2.85
FPW, 37 °C	0	128.40 ± 2.6^{a,1}	9.63 ± 0.2	2.07	61.34 ± 4.0^{a,1}	4.60 ± 0.3	6.65
	5	274.87 ± 4.4^{b,2}	20.62 ± 0.3	1.63	123.12 ± 3.7^{b,2}	9.23 ± 0.2	3.06
	9	265.49 ± 4.7^{b,2}	19.91 ± 0.3	1.79	135.19 ± 6.6^{b,2}	10.14 ± 0.4	4.48
	12	328.06 ± 7.0^{c,4}	24.67 ± 0.5	2.13	106.68 ± 3.8^{c,4}	8.00 ± 0.3	3.62
	15	515.96 ± 13^{d,6}	38.70 ± 0.9	2.50	243.30 ± 6.4^{d,5}	18.25 ± 0.5	2.66

Values are expressed as mean ± standard deviation ($n = 4$). No coincidence in the superscript letters indicates a significant difference ($p < 0.05$) with the storage time of the same column. No coincidence in the superscript numbers indicates a significant difference ($p < 0.05$) with the temperature of storage.

Table 4
Potential and free HMF + F contents in stored formulae for pregnant women

Sample	Storage (months)	Potential HMF + F			Free HMF + F		
		μg/100 g sample	μg/100 ml sample	RSD (%)	μg/100 g sample	μg/100 ml sample	RSD (%)
FPW, 25 °C	0	$1031.21 \pm 12.8^{i,1}$	77.34 ± 0.9	1.24	$441.14 \pm 7.8^{i,1}$	33.09 ± 0.6	1.77
	5	$1257.44 \pm 42^{j,3}$	94.31 ± 3.1	3.38	$456.52 \pm 1.5^{i,1}$	34.24 ± 0.1	0.34
	9	$1305.39 \pm 2.2^{k,4}$	97.90 ± 0.2	0.17	$532.87 \pm 7.9^{j,3}$	39.96 ± 0.6	1.48
	12	$1675.93 \pm 22^{l,6}$	125.69 ± 1.7	1.35	$441.67 \pm 2.9^{i,1}$	33.13 ± 0.2	0.65
	15	$1907.68 \pm 23^{m,5}$	143.08 ± 1.7	1.21	$397.83 \pm 8.8^{i,1}$	29.84 ± 0.7	2.21
FPW, 37 °C	0	$1031.21 \pm 12.8^{a,1}$	77.34 ± 0.9	1.24	$441.14 \pm 7.8^{a,1}$	33.09 ± 0.6	1.77
	5	$1474.18 \pm 6.3^{b,2}$	110.56 ± 0.5	0.43	$670.90 \pm 37^{b,2}$	50.32 ± 2.7	5.52
	9	$1446.14 \pm 5.6^{b,2}$	108.46 ± 0.4	0.35	$693.38 \pm 7.7^{b,2}$	52.00 ± 0.6	1.11
	12	$1850.08 \pm 20^{c,5}$	138.76 ± 1.5	1.08	$426.43 \pm 9.6^{a,1}$	31.98 ± 0.7	2.25
	15	$3134.62 \pm 47^{d,7}$	235.00 ± 3.5	1.52	$1410.07 \pm 59^{d,4}$	105.75 ± 4.4	4.21

Values are expressed as mean ± standard deviation ($n = 4$). No coincidence in the superscript letters indicates a significant difference ($p < 0.05$) with the storage time of the same column. No coincidence in the superscript numbers indicates a significant difference ($p < 0.05$) with the temperature of storage.

can lead to confusion because it could be taken as the sum of the total furfurals present in a sample, for example HMF + F + FMC + MF, and not the potential of these compounds in terms of their precursors. Therefore, we refer to "potential furfurals" when referring to the sum of free furfurals, plus the furfurals bound to proteins such as Amadori products, furfural from reductor sugars and the furfurals from precursors.

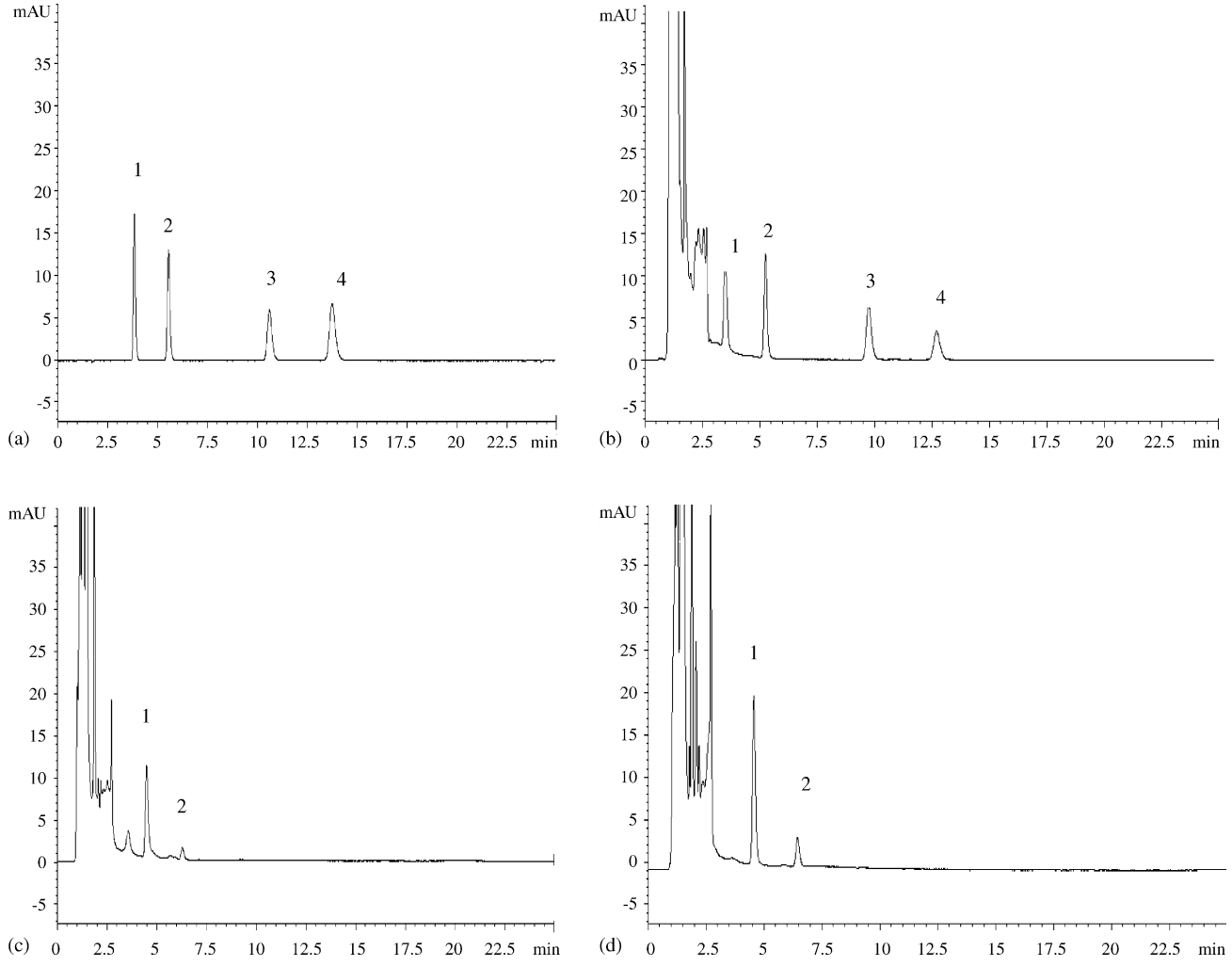

Fig. 2. Typical chromatograms of furfural determination by the HPLC-DAD method. See conditions in Section 2.3. Furfural peaks: 1, HMF; 2, F; 3, FMC; 4, MF. (a) Furfural standards; (b) recoveries; (c) infant formula and (d) formula for pregnant women.

It has been reported that the first furfural compound formed during the MR is HMF, and F, MF and FMC are products of the most advanced states of the reaction, or are formed by inter-conversion as a result of greater heating or longer storage periods [30]. To detect FMC and MF compounds and to corroborate their formation in milk-base formulae at advances stages of the MR, we stored IF and FPW at 47 °C, and then analyzed potential and free furfurals at 10, 30, 50, 100 and 120 days (data not show). Neither FMC nor MF was formed. This result is consistent with other studies that did not detect either of these compounds in IF or milk [26,27,29,30,36].

To our knowledge, no study has analyzed furfural compounds in FPW, which are specific formulae for adults. Since furfural formation is the result of many factors such as composition of formulae, thermal treatments during manufacturing and storage, etc., the comparisons of different milk-based formulae is complicated. However, comparison of furfural content in different milk-based formulae could be useful, in spite of these limitations.

The potential furfural value (HMF + F) found at point zero in the FPW was 1031.21 μg/100 g (Table 4). Other authors have reported, [26] 601.95 μg/100 g for an adapted IF (casein/serum 40/60) and 1362.03 μg/100 g for a follow-up formula (casein/serum 80/20). The FPW used in this study also had a casein/serum ratio of about 80/20. This could be explained because of the reactivity of the ε-amino group of lysine from casein is higher than that from serum as reported previously [12,26].

In general, the values of potential HMF in the FPW were slightly lower than those reported by Ferrer et al. [26] in follow-up formulae with the same casein/serum protein ratio (80/20), but distinct composition. Nevertheless, the values of potential and free F were higher in FPW than in the adapted formulae.

In addition, the values of potential and free HMF and F in the FPW were slightly lower than those reported in liquid and powdered IF by Albalá-Hurtado et al. [29], except in free HMF and F from liquid infant milk which have the lowest values, respectively.

At zero point, the level of potential HMF in the FPW was 67.71 μg/100 ml of reconstituted sample, which is similar to the concentration reported in several UHT milk samples [30].

3.4. Furfural evolution

In the FPW, distinct evolution of furfurals was observed with respect to storage at 25 and 37 °C. In general, HMF and F concentrations were greater with increased storage time, and this increment was higher in the FPW stored at 37 °C. This observation can be explained because storage at inadequate temperatures, such as at 37 °C, favor the MR. The sugar content of the FPW differed from that of the IFs. In addition to lactose, the FPW contained fructose and sucrose [37]. The reductor sugars such as lactose and fructose can favor the MR.

3.4.1. Potential furfurals

At the beginning of the study the potential HMF in the FPW was 902.81 μg/100 g, and after 15 months of storage at 25 °C increased to 1562.30 and to 2618.66 μg/100 g at 37 °C, respectively (Table 2). Similar results were observed for the potential F, whose initial content was 128.40 μg/100 g and at the end of 15 months of storage reached 345.36 and 515.96 μg/100 g at 25 and 37 °C, respectively. These results indicate that the storage temperature affects the MR; the greater the temperature, the faster the MR.

The potential HMF and F in the FPW increased with extended storage and higher temperatures. However, these increases were not regular (Tables 2 and 3). Other authors [26] have reported that the potential HMF and F contents vary in an irregular way with the storage time and temperature. These observations can be explained by the fact that HMF reaches a state of equilibrium between destruction by oxidation and formation from precursors [38].

3.4.2. Free furfurals

At zero point, free HMF in the FPW reached 28.49 μg/100 ml of reconstituted sample, which is slightly lower or higher than those reported for several UHT milk samples (in the range 7.40–65.22 μg/100 ml) [30].

The contents of free HMF in the FPW did not follow a regular pattern. At the beginning of the study, it was 379.80 μg/100 g, and at the end of 15 months of storage dropped to 325.34 and 1166.77 μg/100 g at 25 and 37 °C, respectively. Moreover, HMF concentrations fluctuated throughout storage (Table 2). Free F was 61.34 μg/100 g and after 15 months of storage reached 82.49 and 243 μg/100 g at 25 and 37 °C, respectively. Increasing values were observed during storage, and a decrease was detected only at 12 months of storage in samples at 25 and 37 °C.

Many studies have addressed the chemical changes that are produced by thermal process. The search for compounds induced by heating and the concentration of these as possible indicators of the heat treatment or product deterioration, such as furfural compounds, continues. The question whether or not HMF and F are really suitable indicators has not yet been satisfactorily answered.

4. Conclusions

The HPLC-DAD method used in this study is relatively simple and reproducible for measuring furfural compounds in milk-based formulae. It is suitable for routine analysis and shows acceptable precision, recovery and sensitivity.

HMF was the main furfural compound detected in the FPW, followed by F. Levels of furfurals were higher in FPW stored at 37 °C than at 25 °C. The levels found before storage were, for free HMF and F: 379.80 ± 3.7 and 61.34 ± 4 μg/100 g; and for potential HMF and F were 902.81 ± 10 and 128.40 ± 2.6 μg/100 g of sample, respec-

tively. No formation of FMC and MF were detected in any of the formulae. At present, there are no established limits for furfural compounds concentrations in milk-based formulae. In the case of IFs the recommendation is that the amount of unavailable lysine (or blocked), such as Amadori compounds, should be as low as possible [39]. This recommendation could be extended to other milk-based formulae.

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PUBLICACIÓN 3

Análisis simultáneo de vitaminas A y E en preparados para lactantes de base láctea por cromatografía líquida de alta eficacia en fase normal con detector de fotodiodos (NP-HPLC-DAD) utilizando una columna corta de resolución rápida.

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Se desarrolló y validó un método rápido, simple y reproducible por NP-HPLC-DAD para la determinación simultanea cualitativa y cuantitativa de Vitamina A (acetato de retinol y palmitato de retinol) y Vitamina E (acetato de α-tocoferol, α-tocoferol, γ-tocoferol, y δ-tocoferol) en preparados para lactantes de base láctea mediante una sola inyección. La preparación de la muestra fue basada en la precipitación de proteína como material interferente, y la extracción de las vitaminas con etanol, seguida de una re-extracción con hexano. El método de análisis cromatográfico se basó en el uso de una columna corta de resolución rápida (50 mm x 2.1 mm; 3 μm de tamaño de partícula) la que permite un menor uso de solvente, una baja presión en el sistema, una reducción de muestra en la fase estacionaria y provee de una alta sensibilidad en la detección de compuestos.

Asimismo varios tipos alternativos de preparación de muestra, basados en las más simples metodologías reportadas por otros autores, fueron llevados a cabo para comparar resultados, observándose ventajas en el método propuesto. Los valores de linealidad para los compuestos estudiados obtuvieron valores de r^2 superiores a 0.999. Los límites de detección expresados en μg / ml para el palmitato de retinol, acetato de retinol, acetato de α-tocoferol, α-tocoferol, γ-tocoferol, y δ-tocoferol fueron 0.3, 0.2, 8.8, 6.8, 5.3 y 4.9, y los límites de cuantificación 1.0, 0.7, 29.5, 22.6, 17.8 y 16.5, respectivamente. Los niveles de recuperación estuvieron comprendidos dentro del rango de 93% a 102.5%. Los coeficientes de variación para la repetibilidad fueron menores a 1.0% y para la reproducibilidad menores a 6.9%.

El método desarrollado mostró valores aceptables de precisión, recuperación y sensibilidad, y prueba ser muy simple para realizar análisis rutinarios, además de que se utiliza menor consumo de solventes

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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☆

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Abstract

A rapid, simple and reproducible normal-phase (NP) high-performance liquid chromatography (HPLC)–diode array detection (DAD) method for simultaneous qualitative and quantitative determination of Vitamin A (retinol acetate and retinol palmitate) and Vitamin E (α-tocopherol acetate, α-, γ- and δ-tocopherols) in milk-based infant formulae was developed and validated. The preparation sample was based on protein precipitation and vitamin extraction with ethanol, followed by re-extraction with hexane, while the chromatographic method was based on the use of a short narrow-bore column (50 mm × 2.1 mm; 3 μm particle size), which afforded less solvent consumption and higher mass sensitivity. The method showed acceptable values for precision, recovery and sensitivity, and proved very simple for routine analysis work.

Keywords: Vitamins A and E; Infant formula powder; Diode array detection; Short narrow-bore column

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 [1,2]. Tocopherols and retinol are added to infant formulae (IF) both to improve the vitamin content and to prevent lipid oxidation during manufacture and storage, which helps to extend product shelf life. Moreover, IF also contain tocopherols originating from the oils used in their manufacture. The antioxidant activities of α-, β-, γ- and δ-tocopherols, those commonly found in vegetable oils, contribute more biologically active components to the diet than any other tocopherol isomer, although α-tocopherol is less stable in many products during manufacture and storage [3]. Therefore, fortification of IF with the more stable vitamin esters, such as α-tocopherol acetate, retinol acetate, or retinol palmitate is necessary [1–4]. These molecules are more stable and less susceptible to oxidation. High-performance liquid chromatographic (HPLC) procedures have been of particular interest since initial reports of determination of Vitamin E in foods [4] have steadily risen. HPLC was first applied to the resolution of Vitamin E and other fat-soluble vitamins in 1971 by Schmit et al. [5]. Two reversed-phase packing materials, Permaphase ODS and Zipax HCP, were used to study the resolution of the fat-soluble vitamins, including α-tocopherol and α-tocopherol acetate. Permaphase ODS was a C_{18} column and Zipax HCP a hydrocarbon coating on Zipaz support. Van Niekerk [6] demonstrated the power of HPLC for vitamin analysis, and established important principles for the application of NP-HPLC to fat-soluble vitamins analysis: (1) as oils could be injected directly onto a silica column, no sample preparation other than dilution of the oil was required; (2) the positional isomers β- and γ-tocopherol could be resolved; (3) recovering added tocopherols to oils were high, approaching 100%. In addition, these procedures were "fast and easy", with wide applicability for the routine assay of fat-soluble vitamins in foods [7–10]. Detection of fat-soluble vitamins after HPLC resolution can be accomplished by UV (using diode array detection (DAD)), fluorescence (FLD), electrochemical (ED), or evaporative light scattering (ELSD)

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detection methods [11–15]. The most commonly used detector for Vitamins A and E analysis is FLD, which is considerably more sensitive and selective than UV, but less sensitive than ED.

NP-HPLC has been used to determine α-, β-, γ- and δ-tocopherols [16–19] and retinol in IF [20,21]. All of these procedures use FLD since FLD provides a sensitive and specific detection mode. For the simultaneous determination of Vitamins A and E, however, two different injections and mobile phases are necessary as FLD only works with one excitation wavelength (λ_{ex}) and one emission wavelength (λ_{em}). For Vitamin E determination, this means that it is necessary to adjust the FLD configuration to $\lambda_{ex} = 285$ nm, and $\lambda_{em} = 310$ nm; for Vitamin A determination, one must re-adjust the configuration to $\lambda_{ex} = 325$ nm, and $\lambda_{em} = 470$ nm. On the other hand, DAD can work with multiple UV wavelengths, which are traduced with more versatility, but which are less sensitive for the detection of analytes compared with FLD.

The recent introduction of shorter narrow-bore columns (i.e. 50 mm × 2.1 mm; 3 μm particle size) in place of traditional columns (250 mm and/or 150 mm × 4.6 mm; particle size 5 μm) offers several advantages, including less solvent consumption and higher mass sensitivity [22]. An increase in mass sensitivity can permit the use of DAD to simultaneously analyze Vitamins A and E in IF in the same injection once these fat-soluble vitamins have been extracted.

Depending on the sample matrix, extraction of fat-soluble vitamins is usually performed by direct solvent extraction or saponification. Most oils that contain high levels of Vitamin E can be diluted with hexane or by mobile phase and directly injected into NP columns. This straightforward approach works well unless one component has low solubility in the mobile phase, or if other interfering compounds exist, such as sometimes occurs in IF. In this case, more extensive cleanup procedures must be employed. Preparation of the fat-soluble vitamin fraction for injection into the column from 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 directly injected into a normal-phase column. Saponification converts the α-tocopherol acetate to α-tocopherol, and cannot be differentiated from the naturally occurring α-tocopherol [23]. In addition, saponification involves additional steps that are traduced when employed in increased-time analyses. Although there exist simplified methods involving saponification, it is reported that non-saponification of the total lipid dilution must be regarded as preferable to the saponification procedure as it offers higher accuracy [24]. Besides the elimination of the saponification step, this method permits the quantification of the added ester forms, as well as the natural Vitamins A and E homologues. The use of NP-HPLC makes fat removal unnecessary in IF, since it is known that the use of normal-phase silica allows the direct injection of oil; up to 2 mg per injection without influencing resolution, detection, or column life [16]. Finally, the stability of these fat-soluble vitamins is better both in the lipid matrix and with the ester forms of Vitamins A and E [21,23,24]. The solvent extraction method for fat-soluble vitamins used by Thompson and Hatina [16] has remained a standard for Vitamin E analysts for many years, although the extraction process is tedious. Moreover, the large solvent volumes required, and its attendant labor-intensiveness, renders this method less attractive. Solvents commonly used for fat-soluble vitamins extraction include chloroform–methanol (2:1), as employed in the Folch extraction method, acetone, diethyl ether, hexane, hexane–ethyl acetate, etc. [25].

The aim of this work was to develop and to validate an NP-HPLC–DAD method using a short narrow-bore column for simultaneously determining in a single injection α-tocopherol, α-tocopherol acetate, γ-tocopherol, δ-tocopherol, as well as retinol palmitate and retinol acetate, via a short, easy and straightforward direct-extraction method in IF.

2. Experimental

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), Standard of α-tocopherol acetate was obtained from Fluka (Buchs, Switzerland), and standards of α-, γ-, and δ-tocopherols and all-*trans*-retinol palmitate, and retinol acetate, were purchased from Sigma (St. Louis, MO, USA).

2.2. Instruments

We used a Hewlett-Packard liquid chromatographic system (Waldbronn, Germany) with an HP 1050 pump, an 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 mm × 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. Chromatographic conditions

Separation was performed at 30 °C using an isocratic mixture of 0.5% ethyl acetate in hexane as the mobile phase and a flow rate of 0.4 ml/min. Detection was at 284 nm for α-tocopherol acetate, 296 nm for α-, γ- and δ-tocopherols, and 326 nm for retinol acetate or palmitate. The injection volume was 20 μl. The total run time was 25 min between each routine injection.

2.4. Sample

A commercial powdered IF supplemented with α-tocopherol acetate and retinol acetate was provided from a firm in Barcelona just after manufacture. According to the label, the composition of the formulae was as follows: carbohydrate 58.3%, fat 26%, and protein 10.7%, Vitamin A 640 μg/100 g and Vitamin E 25 mg/100 g. The samples were maintained at 0–4 °C until subsequent analysis.

2.5. Sample preparations tested

2.5.1. Proposed method: Method I

Two grams of IF powdered sample were reconstituted with 8 ml of distillated water, which was then immersed in warm water (40 °C), and mixed until complete homogenization was achieved (5 min approximately). We subsequently used a vortex to complete the homogenization of the sample. One milliliter of reconstituted sample (20%, w/w) was transferred into a centrifuge tube. Three milliliters of absolute ethanol were added and shaken mechanically for 3 min, and then 1 ml of hexane was added, the whole shaken for another min. The sample was then left to stand for 5 min, after which, 3 ml of saturated NaCl was added to aid solvent separation, with the mixture then manually shaken by inversion. We centrifuged for 5 min at 3000 rpm at room temperature. The hexane phase was recovered, followed by:

(a) The hexane phase was directly filtered through a 0.22 μm nylon filter and collected in a 1 ml amber glass vial, 20 μl was then injected into the HPLC system.
(b) The hexane phase was transferred into a centrifuge tube and the remnant was added (1 ml of hexane) and then shaken for another minute. We centrifuged for another 5 min at 3000 rpm at room temperature. The two hexane phases were collected in the same tube and evaporated to dryness under nitrogen. The extract was reconstituted with 1 ml of hexane; we then proceeded as described in paragraph (a) above.

2.5.2. Other methods

2.5.2.1. Method II. Two hundred and fifty milligrams of IF powder was weighed in a centrifuge tube, with 4 ml of absolute ethanol then added and homogenized for 3 min, assisted by glass pearls inside the tube. One milliliter of hexane was then added and re-homogenized for an additional minute. We added 2 ml of water to aid solvent separation. We then centrifuged for 5 min at 3000 rpm. The hexanic phase was treated as described in Method I(a).

2.5.2.2. Method III. Method adapted from Rodrigo et al. [24]: 1 ml of reconstituted powdered IF (20%, w/w) was mixed with 5 ml of dichloromethane–methanol (2:1, v/v) and mechanically stirred for 3 min. The mixture was left to stand for 5 min, and then 1 ml of saturated NaCl solution was added to aid solvent separation. It was manually shaken by inversion (two or three times), and was then centrifuged at 2000 rpm for 10 min. The organic phase was recovered and dried under nitrogen, with the extract reconstituted with 1 ml of hexane; we then proceeded as described in Method I(a).

2.5.2.3. Method IV. The lipid fraction of the IF was first extracted with dichloromethane–methanol (2:1) following the modified Folch method as previously reported [26]. Once the fat had been extracted, we weighed 50 mg of it, dissolving it in 1 ml of hexane; we then proceeded as described in Method I(a).

3. Results and discussion

3.1. Optimization of chromatographic conditions for Vitamins A and E separation

We first tested the mobile phases of 2 and 4% isopropanol in hexane using a pool of standards (retinol acetate, retinol palmitate, α-, γ-, δ-tocopherols and α-tocopherol acetate). Since complete separation of the peaks was not possible, we lowered the quantity of isopropanol, yielding 1.0 and 0.5%, but without obtaining any satisfactory results. Tan and Brzuskiewicz [27] optimized solvents systems for amino-, and silica-normal-phase columns using mobile phases consisting of 99% hexane and 1% of a variety of polar modifiers such as isopropanol, *n*-butanol, tetrahydrofuran, and methylene chloride. Other authors also used ethyl acetate and isopropyl alcohol [20,24]. Abidi and Mounts [28] studied Vitamin E resolution in silica columns, demonstrating that the ability of mobile phases containing a weakly polar modifier, such as an ester (ethyl acetate) or a mono-functional ether (*tert*-butyl methyl ether), to resolve positional isomers was significantly greater than those mobile phases containing more polar modifiers. Therefore, we tested mobile phases containing 2, 1 and 0.5% ethyl acetate in hexane. With the latter, we encountered the best resolution and effective separation of chromatographic peaks, both in standards and in extracted samples. The optimum chromatographic conditions, as reported in Section 2.3, permitted the correct resolution of all studied analytes in less than 20 min. With normal-phase chromatography, the homologues of Vitamins A and E eluted in order of increasing polarity, as follows: retinol palmitate 1.7 min, retinol acetate 4.0 min, α-tocopherol acetate 5.0 min, α-tocopherol 5.6 min, γ-tocopherol 12.0 min, and δ-tocopherol 18.3 min (Fig. 1). The RSDs of repeatability retention times were less than 2%, and the RSDs reproducibility less than 5%.

3.2. Sample preparations tested

When using NP as in this study, most fat-soluble vitamins can be obtained with simple direct solvent extraction procedures. These procedures usually consist of the following sequence: addition of a protein denaturing solvent such as ethanol, methanol or isopropanol; addition of water or buffer to improve the extraction efficiency of the solvent; addition of the organic phase to extract the fat-soluble vitamins; centrifugation; and finally solvent evaporation if concentration of the analytes is required.

We developed and tested several procedures for extracting the above-mentioned fat-soluble vitamins based on the simplest methods currently found in the literature. Rodas et al. [26] developed a rapid extraction method for determining Vitamins A and E based on extraction with ethanol and re-extraction with hexane, similar to procedures reported by Huo et al. [29]. Rodrigo et al. [24] developed a simple method for tocopherols extraction using chloroform–methanol (2:1, v/v) based on the method of Folch et al. [30]. The optimum methods were described in Section 2.5. Based on the simplest methodology vis-a-vis the results

Table 1
Comparison of retinol acetate, α-tocopherol acetate and α-tocopherol contents obtained with the different preparation sample methods

Method ($n=6$)	Retinol acetate (μg/100 g)		α-Tocopherol acetate (mg/100 g)		α-Tocopherol (mg/100 g)	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Method I(a)	1030.1 ± 2.3	0.2	31.1 ± 0.4	1.4	5.5 ± 0.5	0.8
Method I(b)	1042.3 ± 15.1	2.4	29.9 ± 1.5	2.5	5.6 ± 0.1	2.2
Method II	718.2 ± 77.3	10.8	25.4 ± 2.4	9.3	4.2 ± 0.4	8.4
Method III	883.7 ± 48.4	5.5	24.6 ± 0.7	3.1	4.6 ± 0.3	5.7
Method IV	995.4 ± 11.4	1.1	30.5 ± 0.2	0.5	5.8 ± 0.0	0.7

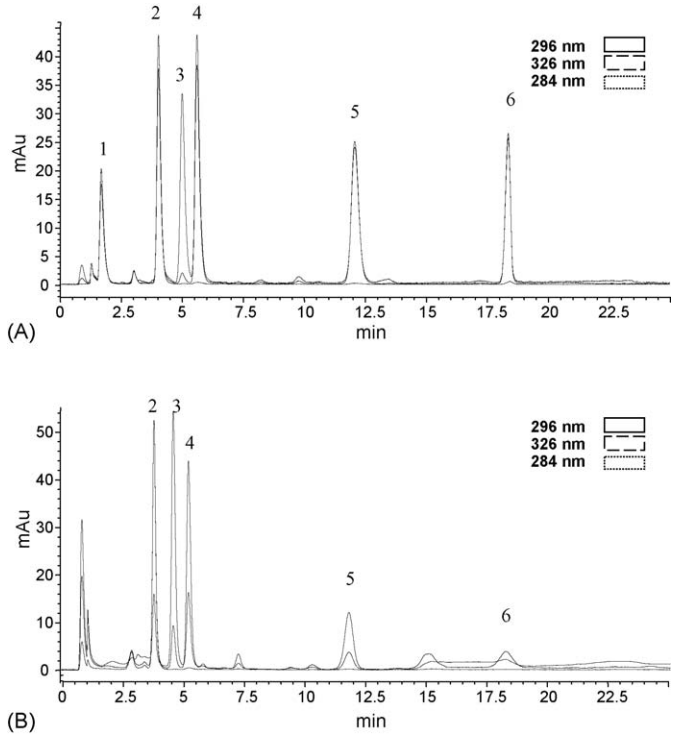

Fig. 1. Typical chromatogram of Vitamins A and E by HPLC–DAD in IF. See conditions in Section 2.3: (A) standards; (B) IF sample. Peaks: 1, retinol palmitate; 2, retinol acetate; 3, α-tocopherol acetate; 4, α-tocopherol; 5, γ-tocopherol; 6, δ-tocopherol.

obtained using the other methods, the most effective procedure proved to be Method I(a) (Table 1).

Method I(a) showed that a re-extraction step is not necessary to extract additional vitamins. Moreover, re-extraction followed by evaporation of the hexane phase and reconstitution results in increasing variability in the results (Table 1; Method I(b)).

Method II showed low recoveries of the studied vitamins, which was probably due to the fact that the addition of 2 ml of water was insufficient to achieve total separation of the hexane phase. For these reasons, we used 3 ml of NaCl saturated solution, as described in Method I. Moreover, we noted that when beginning this procedure with powder sample, the RSDs of several samples were greater that when 1 ml of reconstituted (20%, w/w) sample was used. One possible explanation is that the dispersion of the vitamin molecules into the powder sample is not perfectly homogenous; therefore, when multiple analyses of 250 mg powder sample are carried out, RSDs are greater when compared with 2 g (ratio 1:8) of powder sample for reconstituting the formula, thereby decreasing the variability of the results. Another explanation is that when the powder formula is submitted directly to absolute ethanol, it forms a clot, difficult homogenization, in spite of the glass pears added.

Although Method III showed better results than Method II (Table 1), the main inconvenience of the former, based on extraction with dichloromethane–methanol (2:1, v/v), was that recovery of the dichloromethane phase first required the removal of the methanol–water phase and the protein clot due to the dichloromethane phase was located at the bottom of the centrifuge tube. Finally, it was necessary to evaporate to dryness this organic phase and reconstitute the sample with hexane.

Method IV, based on a fat extraction process, used greater quantities of dichloromethane–methanol (2:1, v/v) and diethyl ether. Beginning with 30 g of IF powdered sample [26], it showed good results, similar to our proposed method (Table 1). Instead of large volumes of solvents for extraction and purification of the fat fraction, we included this method as it is commonly used in our laboratory to extract and analyze fat from IF.

Table 2
Linearity in studied Vitamins A and E by NP-HPLC–DAD method

Compound	Range (μg/ml)	λ detection (nm)	r^2[a]	Equation curve[b]
Retinol palmitate	0.2–5	326	0.9991	$y = 108.46x + 7.3056$
Retinol acetate	0.2–5	326	0.9999	$y = 169.54x - 0.7629$
α-Tocopherol acetate	2–100	284	0.9994	$y = 7.3294x + 0.2813$
α-Tocopherol	2–100	296	0.9993	$y = 13.994x - 23.418$
γ-Tocopherol	1–50	296	0.9994	$y = 18.438x - 19.171$
δ-Tocopherol	1–50	296	0.9993	$y = 13.517x - 14.280$

[a] Determination coefficient.
[b] x, concentration (μg/ml); y, peak area.

Table 3
Detection (DL) and quantification limits (QL) in studied Vitamins A and E by NP-HPLC–DAD method

Compound	DL (ng/ml)[a]	QL (ng/ml)[a]	DL (μg/ml)[b]	QL (μg/ml)[b]
Retinol palmitate	0.8	2.5	0.3	1.0
Retinol acetate	0.6	1.8	0.2	0.7
α-Tocopherol acetate	22.1	73.7	8.8	29.5
α-Tocopherol	16.9	56.5	6.8	22.6
γ-Tocopherol	13.3	44.5	5.3	17.8
δ-Tocopherol	12.4	41.2	4.9	16.5

[a] By apparatus.
[b] By sample.

3.3. Validation of the proposed method

3.3.1. Linearity

Under the chromatographic conditions tested, linear relationships of standard solutions were verified for the studied fat-soluble vitamins. In all cases, the analysis of variance of the regression r^2 values were > 0.999 at seven levels (Table 2).

3.3.2. Sensitivity

To determine the sensitivity of the method, the detection (DL) and the quantification (QL) limits were studied following the USP criteria [31]. These two limits were determined by the chromatographic noise obtained by repeated analysis of a hexane blank, which was injected under the HPLC conditions described previously. The DLs and QLs are shown in Table 3. Acceptable limits were observed for tocopherols, while excellent results were obtained for retinol acetate and palmitate due to the high UV absorbance of these last two compounds.

3.3.3. Precision

Six replicate determinations of Vitamins A and E in the same powdered IF were performed on the same day to evaluate repeatability. For reproducibility, six determinations with the same formula were made on different days. The RSD values for each vitamin were satisfactory, as described by Horwitz [32] (Table 4).

3.3.4. Recovery

For recovery, standards of retinol acetate, retinol palmitate, α-tocopherol acetate, and α-, γ-, δ-tocopherols were added to the IF samples at two levels: for tocopherols 5 and 10 μg; and for retinols 2 and 2.5 μg, respectively. Results are reported in Table 5 and show excellent recoveries.

Table 4
Precision of the proposed NP-HPLC–DAD method

Compound	Repeatability ($n = 4$)		Reproducibility ($n = 6$)	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Retinol acetate[a]	1030 ± 2.3	0.5	998.5 ± 61.9	6.2
α-Tocopherol acetate[b]	31 ± 0.4	1.0	29.7 ± 1.6	5.4
α-Tocopherol[b]	5.6 ± 0.0	0.8	5.5 ± 0.2	3.7
γ-Tocopherol[b]	1.4 ± 0.0	0.7	1.4 ± 0.1	6.9

[a] Expressed as μg/100 g of IF powder sample.
[b] Expressed as mg/100 g of IF powder sample.

Table 5
Recovery of the proposed NP-HPLC–DAD method

Compound	Recovery (%) $n = 4$ Level 1[a]	Recovery (%) $n = 4$ Level 2[b]
	Mean ± SD	Mean ± SD
Retinol palmitate	100.3 ± 6.3	98.5 ± 4.7
Retinol acetate	98.4 ± 1.2	99.4 ± 0.6
α-Tocopherol acetate	100.4 ± 0.6	95.8 ± 0.6
α-Tocopherol	97.9 ± 1.6	93.1 ± 4.6
γ-Tocopherol	96.6 ± 1.3	99.2 ± 4.4
δ-Tocopherol	102.5 ± 2.0	94.6 ± 4.1

[a] For retinols 2 μg and for tocopherols 5 μg of standards were added, respectively, to the IF.
[b] For retinols 2.5 μg and for tocopherols 10 μg of standards were added, respectively, to the IF.

The proposed method allows the simultaneous determination of retinol acetate or retinol palmitate, α-tocopherol acetate, and α-, γ-, δ-tocopherol in a single run using NP-HPLC–DAD with a short narrow-bore column. The main advantages include less solvent consumption, lower back pressure, reduction in stationary phase amount, and higher mass sensitivity [33]. In the narrow-bore column, the peaks eluted in much smaller volumes with less dispersion; therefore, detector signals are of greater intensity due to the higher concentrations in the detector flow cell. These characteristics permit the determination of compounds present in small amounts, such as fat-soluble vitamins in IF. The use of this short narrow-bore column allows the simultaneous analysis of Vitamins A and E using multiple UV wavelengths in DAD. Extraction of the above-mentioned fat-soluble vitamins and the NP-HPLC–DAD method are both very simple for routine use in laboratory analysis, with the latter demonstrating acceptable precision, recovery and sensitivity.

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PUBLICACIÓN 4

Evolución del contenido de mono- y disacáridos libres en fórmulas de base láctea en polvo durante el almacenamiento.

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El HPLC-RI se utilizó para estudiar la evolución del contenido de mono- y disacáridos durante la vida útil de dos tipos de formulaciones de base láctea en polvo suplementadas con distintos niveles de aceite de pescado (rico en LC-PUFA) microencapsulado (MFO). El primer tipo es una formula infantil y el segundo una formula experimental para mujeres embarazadas y/o en periodo de lactancia (FPW). Los resultados de la evolución se compararon con los que se obtuvieron de la realización de una evaluación sensorial. Las fórmulas se almacenaron a 25ºC, 37ºC (durante 12 meses) y 47ºC (durante 120 días). Al inicio del estudio, los valores de lactosa detectados en la formulación infantil fueron de 63 g/100g, y no se detectó lactulosa. En la FPW los siguientes azúcares fueron detectados: lactosa (25 g/100 g), fructosa (15.8 g/100 g), sacarosa (9.28 g/100 g) y lactulosa (0.9 g/100 g).

También se evaluaron los valores de pH. En las fórmulas estudiadas no se observaron cambios durante el almacenamiento a ninguna de las temperaturas evaluadas, con valores promedio de 6.9 para las formulas infantiles y de 7.1 para las FPW.

No se observó ninguna diferencia en la evolución de los azúcares en las fórmulas que contenían el suplemento MFO que en las que no lo contenían y fueron utilizadas como control, indicando que esta materia prima no afectó la evolución de azúcares durante su almacenamiento.

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Evolution of free mono- and di-saccharide content of milk-based formula powder during storage

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Abstract

HPLC-RI was used to monitor saccharide evolution during the shelf-life of two types of milk-based formulae powders supplemented with microencapsulated long-chain polyunsaturated fatty acids (LC-PUFA): infant formula and formula for pregnant and/or lactating women. In addition, these powders were subjected to sensory evaluation. The powders were stored at 25, 37 and 47 °C. Lactose was detected in the infant formula (63 g/100 g), while in the formula for pregnant women, the following were found: lactose (25 g/100 g), fructose (15.8 g/100 g), sucrose (9.28 g/100 g) and lactulose (0.9 g/100 g). Microencapsulated LC-PUFA supplementation of milk-based formula powders did not affect sugar changes.

Keywords: Milk-based formula powders; Saccharides; Storage; Sensory evaluation

1. Introduction

The food industry has made several attempts to improve the quality and the overall nutrient contents of formula-based milk, and to develop special products for specific stages of life (namely intrauterine, newborn, pregnancy and lactation). Two of these are the infant formulae (IF) and formula for pregnant and/or lactating women (FPW). Several studies on infant nutrition have addressed the effects of long-chain polyunsaturated fatty acids (LC-PUFA) on development and growth (Giovannini, Riva, & Agostoni, 1995; Lauritzen, Hansen, Jorgensen, & Michaelsen, 2001) and also their effects on pregnant and lactating women, the fetus and neonate (Hornstra, Al, van Houwelingen, & Foreman van Drongelen, 1995; Gibson, Neumann, & Makrides, 1996; Jensen, Maude, Anderson, & Heird, 2000; Hornstra, 2000; Makrides & Gibson, 2000; Al, van Houwelingen, & Hornstra, 2000; Koletzko et al., 2001).

Some IFs and FPWs include LC-PUFAs. Moreover, the addition of docosahexaenoic acid (DHA, C22:6 n-3) to IF results in improved neurofunctional responses in pre-term infants (Heird, Prager, & Anderson, 1997; Carlson & Neuringer, 1999; Crawford, 2000; Cunnane, Francescutti, Brenna, & Crawford, 2000; Jeffrey, Weisinger, Neuringer, & Mitchell, 2001).

Heating causes most of the chemical changes that occur during the manufacture of milk-based formulae. During heating, lactose undergoes the Lobry de Bruyn-Alberda van Ekenstein rearrangement, thereby initially producing isomeric disaccharides, mainly lactulose. Given that this compound does not occur naturally in milk, but is formed in heated dairy products, it is a good indicator of heat-induced damage during the manufacture of these products (Andrews, 1986; Olano et al.,

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1992; Friedman, 1996; Lopez-Fandino & Olano, 1999; Belloque, Villamiel, Lopez-Fandino, & Olano, 2001; Kockel, Allen, Hennigs, & Langrish, 2002). Formula milk powders are frequently stored for long periods before being consumed, and therefore changes in their composition may occur. The Maillard Reaction is generally slow at ambient temperatures in liquid systems, but in dried food it is fast. (Troyano, Olano, & Martinez-Castro, 1994).

Milk-based formulae vary in nutrient composition, depending on the type (IFs or FPW) and the manufacturer. Consequently, the speed of the Maillard Reaction and lactose isomerization may differ (Pereyra Gonzales, Naranjo, Malec, & Vigo, 2003).

The FPW and IF used in this study were supplemented with microencapsulated fish oil (MFO), a dry free-flowing powder that consisted of spherical particles made of marine oil, distributed in a food starch matrix, which is a source of DHA. This supplementation could affect the stability of formulae milk. To prevent oxidation and to increase the stability and durability of LC-PUFA, mainly DHA, MFO is coated with caseinate and sucrose. It is stabilized with ascorbyl palmitate, sodium ascorbate and tocopherols as antioxidants. The evolution of milk-based formulae during shelf-life should be monitored through analytical and sensorial methods. The evaluation of organoleptic properties is one of the most useful ways to study quality (Baker, 1983; Valero, Villamiel, Sanz, & Martinez-Castro, 2000). A sensorial analysis is useful for assessing the stability of the product and for determining differences in sensorial characteristics during storage (Valero, Villamiel, Miralles, Sanz, & Martinez-Castro, 2001).

No studies have addressed the evolution of lactose in milk-based powdered formulae when other sugars are added (namely fructose and sucrose), or whether these supplements affect the quality and sensory perception of the products during shelf-life.

Here we studied the evolution of mono-and di-saccharides in two kinds of milk-based formulae supplemented with microencapsulated fish oil. The results of evolution were then compared with those from a sensory analysis.

2. Materials and methods

2.1. Samples

The first formula was supplemented with MFO (SIF), containing 0.5 g/100 g of total fatty acids as DHA (C22:6 n-3) while the second, control infant formula (CIF), was not. These two formulae were packed in 400 g unbroken, laminated, foil bags and sealed under nitrogen. According to the label, the two formulae contained 58% of carbohydrates (lactose), 26% of fat and 12% of protein (casein/serum proteins, 40/60). Their main ingredients were skimmed milk powder, reduced minerals whey, lactose, minerals and vitamins.

In addition, an experimental FPW supplemented with MFO containing 20 g/100 g of total fatty acids as DHA, was analyzed. According to the label, this formula contained 53.7% of carbohydrates, 20.2% of fat and 18.1% of protein (casein/serum proteins, 80/20). The main ingredients of this formula were whole milk powder, animal fat, fructose, minerals, vitamins and artificial aroma. It was packed in a 15 g unbroken aluminium foil bag flushed with nitrogen. All formulae were obtained from a production plant immediately after manufacture.

2.2. Storage

For this study, the formulae were kept in a storage chamber equipped with a heater thermostat and were maintained at three temperatures (25, 37 and 47 °C) from production until 12 months of storage. Storage at 47 °C was for only 120 days after production.

2.3. Sensory analysis

External judges were previously selected on the basis of their performance in a Duo-Trio test, using IFs heated for 5 days at 47 °C and comparison with fresh IF. Fourteen judges were chosen and were trained for sensorial tests. In accordance with the manufacturer's instructions, the FPW was reconstituted with cold water (10–15 °C, 15 g in 200 ml) and the IF with warm water (37 °C, 15 g in 100 ml). The samples for both formulae were presented in white plastic cups (30 ml each).

Duo-trio and paired comparison tests were applied. The former, which used two samples (recently produced milk-based formula vs. stored formula), was conducted to determine whether storage affected sensorial quality. The paired comparison test was done to determine whether particular sensorial characteristics (better taste, better smell, and longer-lasting flavour) differed between the two samples. In this study, we accepted the option "no difference". For the statistical analysis we divided these scores evenly between the two samples (Meilgaard, Civille, & Carr, 1987). For each storage period analysis, each judge did three test on separate days. We conducted 42 sensory tests for each storage period analyzed (only at 25 °C) at 0, 5 and 9 months in FPW and 0, 6 and 9 months for SIF.

2.4. Analytical determinations

2.4.1. pH

The pH of the reconstituted samples was measured in a pH meter micro-pH 2000 with a glass electrode, (Crison Instruments, SA, Barcelona, Spain).

2.4.2. Instrument

The chromatographic analyses were carried out in a Shimadzu high-performance liquid chromatograph equipped with a LC-10AD double pump, a 7725 Rheodyne injector (Cotati, CA, USA) with a 20 μl loop, and a RID-6A Shimadzu refractive index detector. Chromatographic separation was performed in a Tracer carbohydrates column (5 μm particle size; 250 × 4.6 mm i.d.), and an NH_2 precolumn (13 mm × 3 mm i.d.), both from Tracer (Teknokroma, Barcelona, Spain).

2.4.3. Free mono-and di-saccharide analyses

HPLC-RI was used to determine free mono-and disaccharide contents (Chávez-Servín, Castellote-Bargallo, & Lopez-Sabater, 2004) as follows. The formulae were dissolved with a warm methanol–water mixture; Carrez-I and Carrez-II solutions and acetonitrile were then added. After precipitation of protein, the resulting solution was passed through filter paper and through a C18 Sep-Pak Plus cartridge from Waters (Milford, MA, USA). Finally, it was forced through a 0.45 μm nylon filter from Tracer (Barcelona, Spain). An aliquot was injected into the HPLC system. HPLC-RI analyses were performed in quadruplicate.

2.5. Statistical analysis

For the Duo-trio and the paired comparison tests, we used the χ^2-test and the paired *t*-test, respectively. For both sensorial tests, the two-sided hypotheses for each storage time against fresh formula were applied and a significance of $\alpha = 0.01$ (1%) (Meilgaard et al., 1987) was established. For the statistical analysis of mono- and di-saccharides, we used a one-way analysis of variance (ANOVA) for each sugar and formula, in order to detect differences in the formulae during the storage. Statistical analyses were performed using the SPSS package for Windows version 11 (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. pH analysis

The pH values of the formula samples were measured upon storage because this can favour either the sugar isomerization (Lobry De Bruyn-Alberda van Ekenstein transformation) or the formation of Amadori compounds (Pellegrino, Denoni, & Resmini, 1995).

In the CIF and SIF, no changes in pH values were detected from 0 to 12 months of storage (25 and 37 °C). The average pH of CIF and SIF (from 0 to 12 months) was 7.02 ± 0.17 and 6.93 ± 0.04, respectively. These values are common for IFs (Ferrer, Alegria, Farre, Abellan, & Romero, 2002; McSweeney, Mulvihill, & O'Callaghan, 2004).

The standard deviation of pH values in FPW, between storage at 25 and 37 °C, showed no variation. The pH averages were 7.02 ± 0.12 and 7.12 ± 0.18, respectively.

3.2. Sensory analysis

3.2.1. Duo-trio test

This test was chosen because is simple, and has the advantage that a reference sample is presented to panellists. No significant differences were detected in FPW at 0 vs. 5 or 0 vs. 9 months of storage at 25 °C (Table 1).

The panel did not distinguish between fresh SIF and that stored for 6 months at 25 °C. In addition, in 73.83% of the tests, the panel members distinguished between 0 and 9 months of storage. However, this did not represent a significant difference ($p > 0.01$) (Table 1).

3.2.2. Paired-comparison test

The paired comparison test was used to determine the way in which the taste, smell and flavour of SIF and FPW changed with storage time, compared with recently manufacturated products. This test is one of the simplest and most used sensory tests (Meilgaard et al., 1987). No significant differences were detected in "better taste", "better smell" or "longer lasting-flavour" between FPW at 0 months of storage and that stored for 5 and 9 months (Table 2). In addition, the sensory characteristics of the SIF were similar when comparing 0 vs. 5 and 0 vs. 9 months of storage at 25 °C (Table 2).

For both types of milk-based formulae, the characteristic "better smell" registered the highest values of "indifferent", indicating that the multiple chemical changes that occurred during storage are not reflected in odour compounds detectable by sensory analysis, at least until 9 months of storage (Table 2).

3.2.3. Mono- and di-saccharides

Table 3 shows the mono- and di-saccharide initial contents in the milk-based formulae. The intensity of the heat treatment in the production of IF powder was estimated by determining the amount of lactulose formed. This compound is a good indicator of heat damage in milk products. The International Dairy Federation (IDF) and the European Community (EC) Commission (Fox, 1997) proposed 600 mg/l of lactulose as a marker for distinguishing between UHT milk and from 600 to 1400 mg/l for sterilized milk; reconstituted formula for pregnant women falls within the latter. However, no limit has been established for lactulose content in IFs or milk-based formulae. No lactulose was detected in the CIF or SIF. Because of the mild thermal conditions in milk drying, lactulose formation is not significant (De Block et al., 2003). Moreover, no lactulose was formed during storage conditions at 25, 37 or 47 °C. This result indicates that the formation of

Table 1
Duo-trio test in formula for pregnant women (FPW) and in supplemented infant formula (SIF) stored at 25 °C

	Formula for pregnant women		Infant formula	
	FPW 0 months vs. FPW 5 months	FPW 0 months vs. FPW 9 months	SIF 0 months vs. SIF 6 months	SIF 0 months vs. SIF 9 months
Detected difference (%)	54.76	70.12	61.9	73.83
No detected difference (%)	45.23	29.88	38.1	26.19

The sensory assessment was made by 14 judges three times on different days. No significant difference was detected ($p > 0.01$).

Table 2
Paired comparison test (preference %) in formula for pregnant women (FPW) and supplemented infant formula (SIF) stored at 25 °C

Characteristics	FPW 0 months vs. FPW 5 months			FPW 0 months vs. FPW 9 months		
	0 months	5 months	Indifferent	0 months	9 months	Indifferent
Better flavour	26.19	57.14	16.67	14.29	65.71	20
Better smell	23.81	23.81	52.38	23.81	33.33	42.86
Longer-lasting flavour	21.43	42.86	35.71	30.95	50.00	19.05
	SIF 0 months vs. SIF 7 months			SIF 0 months vs. SIF 9 months		
	0 months	5 months	Indifferent	0 months	9 months	Indifferent
Better flavour	23.81	47.62	28.57	21.43	60.71	17.86
Better smell	21.43	21.43	57.14	10.71	32.14	57.14
Longer-lasting flavour	23.81	35.71	40.48	28.57	21.43	50

The sensory assessment was made by 14 judges three times on different days. No significant difference was detected ($p > 0.01$).

Table 3
Initial contents of mono and disaccharides in milk-based formulae and in the raw matter with microencapsulated DHA

Carbohydrate content (g/100 g powder)						
Sample	Lactose	Lactulose	Fructose	Sucrose	Glucose	Galactose
CIF[c]	65.0 ± 0.73	–[a]	–	–	–	–
SIF[d]	63.4 ± 0.92	–	–	tr[b]	–	–
FPW[e]	25.1 ± 0.74	0.9 ± 0.03	15.8 ± 0.07	9.28 ± 0.23	–	–
MFO[f] A	–	–	–	28.8 ± 0.11	–	–
MFO[f] B	–	–	–	28.8 ± 0.12	–	–

Values are expressed as means ± standard deviation of four determinations.
[a] = not detected.
[b] tr = traces below quantification limit.
[c] CIF, control infant formula.
[d] SIF, supplemented infant formula.
[e] FPW, formula for pregnant women.
[f] MFO raw matter.

this compound occurs only at elevated temperatures during heating, and not during storage. Nevertheless, in the case of FPW, an initial lactulose content of 0.9 g/100 g of powder was detected, which remained unchanged during the first 5 months of storage. After this time, the amount decreased to 0.39 g/100 g after 7 months and, after 9 months of storage at 25 °C lactulose was not detected (Fig. 1). Lactulose is much more difficult to reduce than lactose, (Hu, Kurth, Hsieh, & Krochta, 1996).

Neither glucose nor galactose was detected in formulae after production or during storage, which is consistent with other findings (Ferreira, Gomes, & Ferreira, 1998). In the IFs, lactose was the only sugar present; however, the SIF showed slight traces of sucrose, which were below quantification limits <0.20 mg/ml (Chávez-Servín et al., 2004).

The decrease in lactose concentration during the first period of storage in the IF and FPW is attributed to the combination of lactose with the ε-amino group of lysine, which leads to the formation of lactulosyl-lysine, (which remains bound to the protein), a key precursor in the formation of coloured products (Olano, Calvo, & Corzo, 1989; De Block et al., 2003). FPW stored at 25 °C showed a decrease in lactose from 25.1 g/100 g to 18.81 g/100 g, after 5 months of storage. In addition, after this time, no alterations in lactose concentrations were observed until 12 months (Fig. 1).

The concentration of lactose in CIF and SIF remained unaltered when these were stored at 25 and

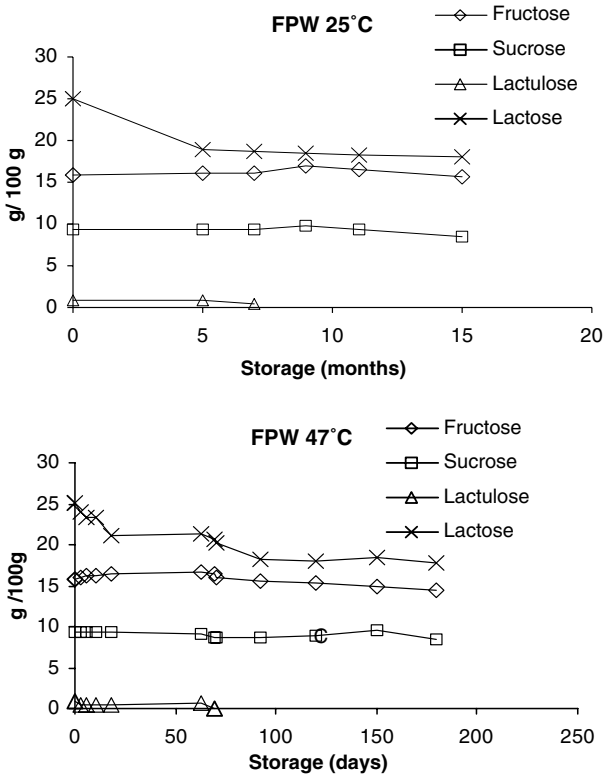

Fig. 1. Evolution of saccharides in formulae for pregnant and/or lactating women (FPW) stored at 25 and 47 °C.

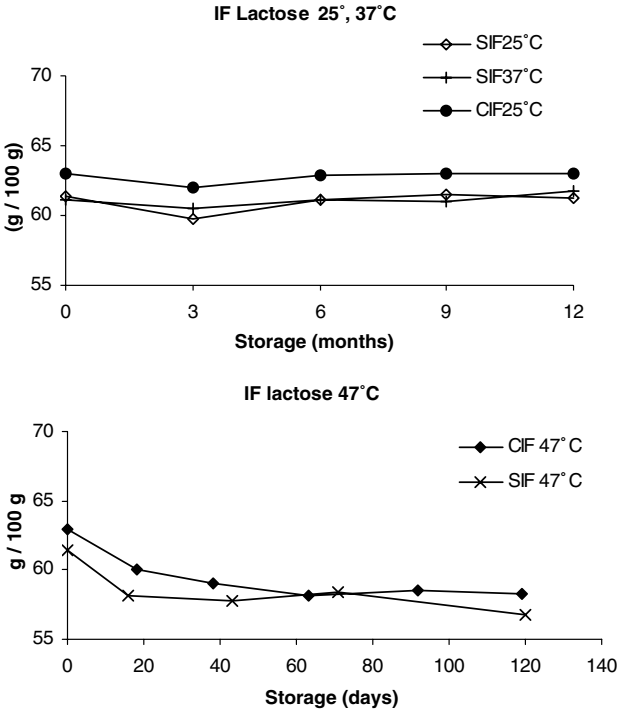

Fig. 2. Evolution of lactose in the infant formulae stored at 25, 37 and 47 °C. SIF, supplemented infant formula; CIF, control infant formula.

37 °C. However, at 47 °C, the concentration of this sugar decreased by about 4.6% at 18 days, 6% at 28 days and 7% from 63 until 120 days, compared with the initial content (Fig. 2).

No differences between the lactose evolution in CIF and SIF were observed, indicating that the addition of MFO did not affect the evolution of this sugar upon storage.

Sucrose forms part of the coated MFO. To observe the evolution of sucrose in this raw matter, the MFO was also stored at 47 °C. No changes were observed in the concentration of this sugar in two batches, (production separated by 1 year) after 120 days of storage (Fig. 3).

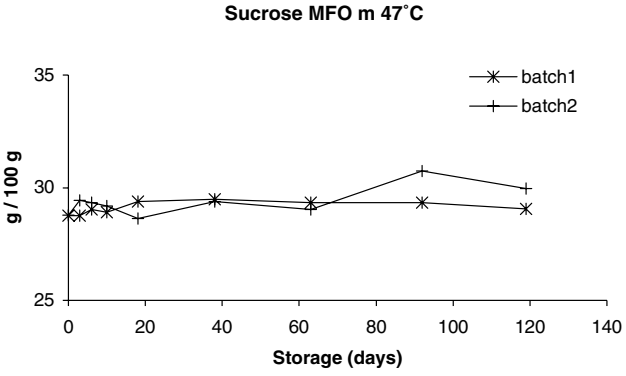

Fig. 3. Evolution of sucrose in the MFO raw matter in two batches stored at 47 °C.

Finally, fructose, which is listed in the contents label of FPW, is used as a sweetener. The initial content of this sugar after production in FPW was 15.82 g/100 g. The concentration of fructose did not show significant changes during storage at 25 °C or 47 °C. This observation is consistent with the results obtained from the sensory analysis.

3.2.4. Conclusions

The sugar evolution of IF and FPW was studied; not significant differences in sugar contents were found when products were stored at 25 or 37 °C.

On the basis of our findings, the addition of MFO to the milk-based formulas did not affect sugar evolution. Hardly any changes in sensorial quality were observed. The use of this MFO in the milk industry has considerable potential; however, more studies on the stability of supplemented products with this material are necessary, including more batches and further sensory analyses.

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PUBLICACIÓN 5

Evolución de los furfurales potenciales y libres en preparados para lactantes de base láctea durante el almacenamiento.

Chávez-Servín, J. L., Castellote, A. I., & López-Sabater, M. C. (**2006**). Evolution of potential and free furfural compounds in milk-based infant formula during storage. *Food Research International*, 39(5), 536-543.

Se evalúa la evolución de los furfurales potenciales y libres (5-hidroximetil-2-furaldehído, HMF; 2-furaldehído, F; furilmetilcetona, FMC; y 5-metil-2-furaldehído, MF) mediante el uso de RP-HPLC-DAD durante la vida útil de dos tipos de preparados para lactantes, una suplementada con ácidos grasos poliinsaturados de cadena larga (LC-PUFA) en la forma de aceite de pescado microencapsulado (MFO), y la otra no suplementada. Recién producidas, las fórmulas fueron almacenadas a 25ºC y 37ºC. Los contenidos iniciales de furfurales en la fórmula suplementada fueron: HMF potencial 485.88 μg/100 g, F potencial 167.13 μg/100 g, HMF libre 58.23 μg/100 g, y F libre no se detecto al inicio. En la fórmula no suplementada los valores encontrados fueron: HMF potencial 515.85 μg/100 g, F potencial 170.29 μg/100 g, HMF libre 84.92 μg/100 g, y F libre 1.19 μg/100 g. Estos valores están dentro del rango reportado por otros autores en diversas preparados para lactantes. En general, se observaron incrementos en los valores de furfurales conforme avanza el tiempo de almacenamiento, y los incrementos son más pronunciados en las fórmulas a 37ºC. Muy pocas diferencias en la evolución de los contenidos de furfurales se observaron entre la fórmula suplementada con respecto a la no suplementada. Los resultados sugieren que el uso del aceite de pescado microencapsulado utilizado como materia prima, no afectó la formación de furfurales. En ninguna de las fórmulas se encontró FMC ni MF.

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Evolution of potential and free furfural compounds in milk-based infant formula during storage

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Abstract

HPLC-DAD was used to monitor the evolution of potential and free furfural compounds (5-hydroxymethyl-2-furaldehyde, HMF; 2-furaldehyde, F; 2-furyl methyl ketone, FMC; and 5-methyl-2-furaldehyde, MF) over the shelf life of two types of infant powder formula: one supplemented with long-chain polyunsaturated fatty acids (LC-PUFA) in the form of microencapsulated fish oil (MFO), the other not supplemented with LC-PUFA. Following production, the formulae were stored at 25 and 37 °C. The initial furfural content in the supplemented formula was as follows: potential HMF (485.88 μg/100 g), potential F (167.13 μg/100 g), and free HMF (58.23 μg/100 g), while free F was not detected. In the unsupplemented formula, the following values were recorded: potential HMF (515.85 μg/100 g), potential F (170.29 μg/100 g), free HMF (84.92 μg/100 g), and free F (1.19 μg/100 g). In general, increased furfural content was observed during storage, an increase more pronounced in formula stored at 37 °C. Hardly any differences in furfural evolution during storage were observed between supplemented and unsupplemented infant formulae. The results suggest that the uses of MFO material for supplement formula not affected furfural formation. Neither FMC nor MF was formed in the formulae studied.

Keywords: Furfural compounds; Milk-based formula powders; Maillard reaction; Storage

1. Introduction

The food industry has continually striven to improve the quality and nutritional content of infant formulae (IF), attempting to bring the composition closer to that of human milk. This is particularly important in instances when breastfeeding is not an option available to a newborn (Ferreira, Gomes, & Ferreira, 1998). One such improvement consists in adding LC-PUFA to IF, since several studies on infant nutrition have addressed their beneficial effects on growth and development. Moreover, the addition of docosahexaenoic acid (DHA, C22:6$n-3$) to IF has demonstrated improved neurofunctional response in pre-term infants (Cunnane et al., 2000; Gibson, Neumann, & Makrides, 1996; Hornstra, Al, van Houwelingen, & Foreman van Drongelen, 1995; Rodriguez et al., 2003; Valenzuela & Nieto, 2001). One source of DHA is fish oil, though it remains very susceptible to oxidation due to high PUFA content. For this reason the food industry has developed microencapsulated fish oil (MFO); which contains 25% fish oil (approximately 5% EPA and 25% DHA), in the form of a dry free-flowing powder, consisting of spherical marine oil particles, distributed in a food starch matrix and coated with protein and carbohydrate to prevent oxidation and to increase the stability and durability of LC-PUFA, principally DHA. However, the protein–carbohydrate complex may react during manufacturing process or during storage, producing undesirable products. This microencapsulated oil is moreover stabilized with ascorbyl palmitate, sodium ascorbate, lecithin, and tocopherol (Hamilton, Kalu, McNeill, Padley, & Pierce, 1998). One challenge the industry faces is controlling the stability of this IF. Instability

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can result from many factors, including reducing sugar content, lysine-rich proteins, high temperatures applied during production, and long storage times, thereby rendering the formula susceptible to Maillard reaction (MR) (Agostoni et al., 1999; Baltes, 1982; Chávez-Servín, Castellote, & López-Sabater, 2004; Guo, Hendricks, & Kindstedt, 1998; Vanmil & Jans, 1991).

Reducing sugars and lysine are the main compounds involved in the initial states of MR, consequently producing a lactulosyl-lysine compound in which the lysine is unavailable, thereby affecting nutritional value. (Friedman, 1996; Naranjo, Malec, & Vigo, 1998; Pereyra Gonzales, Naranjo, Malec, & Vigo, 2003; Rehman, 2002; Rehman & Shah, 2003). In advanced states of MR, undesirable compounds as furfurals can be found (Ramirez-Jimenez, Garcia-Villanova, & Guerra-Hernandez, 2000; Rehman, Saeed, & Zafar, 2000; van Boekel, 1998). These compounds are formed during the thermal process (severity of temperature/time ratio) and/or storage. In addition, they can be useful indicators of food damage, as well as tools to evaluate the extent of MR (Guerra-Hernandez, Garcia-Villanova, & Montilla-Gomez, 1992; Lococo, Valentini, Novelli, & Ceccon, 1994).

To date, studies have focused on four furfural compounds in processed foods: 5-hydroxymethyl-2-furaldehyde (HMF), 2-furaldehyde (F), 2-furyl methyl ketone (FMC), and 5-methyl 2-furfural (MF) (Dinsmore & Nagy, 1974; Mijares, Park, Nelson, & Mciver, 1986; Pellegrino, Denoni, & Resmini, 1995). Since the development of the Keeney and Bassette method (1959), a distinction has been made between free HMF and potential HMF. To determine the latter, the milk sample is heated in 0.3 *N* oxalic acid at 100 °C in order to release HMF. Potential HMF is the sum of the HMF precursors (i.e., lactulosyl-lysine, 1-2 enolized product, etc.) and free HMF. Free HMF is determined by omitting the heating step. Given that the formation of furfurals can result from many factors (temperature of heat treatment/time and composition of formula), it is difficult to compare different formulae. Moreover, the amount of furfural compounds may vary from formula to formula. Nevertheless, an examination of furfural content evolution in a sample formula over the course of its shelf life could be a useful measure of change caused by MR.

The aim of the present study was to trace the evolution of furfural compounds (HMF, F, FMC and MF) in two types of formula via RP-HPLC-DAD monitoring, and study the effect of MFO supplementation, temperature and time of storage. Comparisons in furfural content were then made using storage time (0, 3, 6, 9 and 12 months), temperature of storage (25 and 37 °C), and MFO supplementation as variables (CIF vs. SIF).

2. Materials and methods

2.1. Samples

Two types of IF, differing only in microencapsulated LC-PUFA supplementation, were manufactured for this study by a firm in Barcelona, Spain. The first formula was supplemented (SIF) with MFO, using Dry n-3® 5:25 (BASF Health and Nutrition, Ballerup, Denmark), which contained in the final product 0.5 g/100 g of total fatty acids as DHA (C22:6*n* − 3), while the second, the control infant formula (CIF), was not. Formulae were packed in a commercial, unbroken, laminated and airtight, foil 1 kg bag and sealed under a nitrogen atmosphere (<2% O2). According to the label, the composition of the formulae was as follows: carbohydrate 58%, fat 26%, and protein 12% (casein/serum protein 40/60). The major ingredients were skimmed milk powder, reduced minerals whey, lactose, minerals, and vitamins. Sufficient formulae samples were obtained from the production plant immediately following manufacture.

2.2. Storage

To evaluate the evolution of potential and free furfurals over the 12-month shelf life, we kept the formulae at 25 and 37 °C from the time of production until 0, 3, 6, 9, and 12 months, respectively. Once formulae had been properly stored as described above, we kept it at −32 °C until analytical determinations were subsequently conducted.

2.3. Chemicals

The chemicals used for sample preparations were of analytical reagent grade: acetonitrile HPLC-grade (SDS, Peypin, France), oxalic acid dihydrate >95.5% and trichloroacetic acid (TCA) >99.5% (Fluka, Buchs, Switzerland). Deionized water was purified through a Milli-Q system (Millipore, Bedford, MA, USA). Standards of 5-hydroxymethyl-2-furaldehyde (HMF), 2-furaldehyde (F), 2-furyl-methyl ketone (FMC) and 5-methyl-2-furaldehyde (MF) were >99% pure, and were purchased from Fluka (Buchs, Switzerland).

2.4. Instrument

The HPLC system used consisted of a Hewlett Packard liquid chromatographic system (Waldbronn, Germany) with an HP 1050 pump system and a HP-1040 M photodiode-array detector and a Waters 717 plus autosampler injector (Milford, MA, USA). We used a Tracer ODS-2 C_{18} column (4.6 × 150 mm), with a 5 μm particle size (Teknokroma, Barcelona, Spain). Chromatographic separation was performed at 30 °C using a mixture of acetonitrile–water (4.5:95.5 v/v) for the mobile phase and a flow rate of 1 ml/min. Detection was made at 284 nm for HMF, 277 nm for F, 274 nm for FMC, and 293 nm for MF. The injection volume was 20 μl.

2.5. Analytical determinations

2.5.1. Measurement of furfural compounds

Free and potential furfurals were measured by RP-HPLC-DAD, a method previously validated (Chávez-Servín, Castellote, & López-Sabater, 2005). Potential furfurals

include free furfurals, furfurals bound to proteins (like Amadori products), and those formed from precursors. Furfurals were identified by their retention times, as well as by their characteristic spectra. They were quantified by interpolation in a calibration curve within a range of 0.05–5 μg/ml for HMF, and 0.05–2 μg/ml for F, FMC, and MF. Analyses were performed in quadruplicate.

2.5.2. pH

The pH of samples was measured using a pH meter micro-pH 2000 with a glass electrode (pH 0–12, 0–80 °C), and equipped with an automatic temperature compensator (Crison Instruments, SA, Barcelona, Spain). IF was reconstituted in warm water (40–45 °C; 4.7 g in 30 ml) according to the manufacturer's instructions. After a given sample had reached room temperature, the pH was determined.

2.5.3. Statistical analysis

For statistical analysis, we used one-way analysis of variance (ANOVA), as well as multiple comparisons, using the Tukey HSD procedure for each furfural, storage temperature (25 and 37 °C), and formula type (SIF or CIF). We conducted statistical analysis utilizing 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

3.1. pH analysis

The pH of reconstituted IF samples was measured during each month of storage since pH can enhance the formation of furfural compounds, either by lactose isomerization (Lobry De Bruyn-Alberda van Ekenstein transformation, L-A) or by Amadori compound formation; which are intermediates in the MR (Ferrer, Alegria, Farre, Abellan, & Romero, 2002). In the acid medium, dehydration of carbohydrates leads to the formation of HMF. Moreover, the MR can also take place, giving rise to Amadori compounds during the first steps of this reaction, and HMF as a consequence of further reactions (Rada-Mendoza, Sanz, Olano, & Villamiel, 2004). In the other hand, basic medium could cause Amadori compounds formation, with subsequence of furfurals formation. In the CIF and SIF groups, no differences were observed in the evolution of pH values at either 25 or 37 °C, with values remaining constant over 0–12 months of storage. The average pH in CIF and SIF over this 12-month period was 6.90 ± 0.15 (RSD 1.61%) and 6.92 ± 0.18 (RSD 1.16%), respectively. These are neutral pH values with no effect in furfural formation, and common for IF (Ferrer et al., 2002; McSweeney, Mulvihill, & O'Callaghan, 2004). These results show that furfural evolution was independent of pH changes, since pH values were constant along storage time at the two controlled temperatures while clear furfural evolution was observed.

3.2. Furfural content

In this study, we compare two formulae, both having the same composition and manufacturing process, and differing only in their LC-PUFA content by inclusion of MFO in one of the two: SIF. We compared our results with other studies on furfural content in IF and UHT milk, designating them as follows: Study A (Albala-Hurtado, Veciana-Nogues, Izquierdo-Pulido, & Vidal-Carou, 1997), Study B (Ferrer et al., 2002), Study C (Ferrer, Alegria, Courtois, & Farre, 2000), Study D (Albala-Hurtado, Veciana-Nogues, Marine-Font, & Vidal-Carou, 1998), Study E (Ferrer, Alegria, Farre, Abellan, & Romero, 2004), and Study F (Ferrer, Alegria, Farre, Abellan, & Romero, 2000).

Hydrolysis conditions implemented under this method prevented HMF formation since, as is well known, heating at 100 °C not only releases HMF, but prolonged heating at this temperature also induces its formation. These conditions were previously evaluated in Study A, with no furfural compounds detected in raw milk samples. This finding means that furfural content in IF depends on the heating process during manufacture, and/or on changes brought about by storage conditions.

A typical chromatogram of furfural compounds in a standard solution, as well as a chromatogram of furfural determination in IF, is shown in Fig. 1. Both potential and free furfural (HMF, F and HMF + F) content, expressed as μg/100 g of powder sample and μg/100 ml of reconstituted

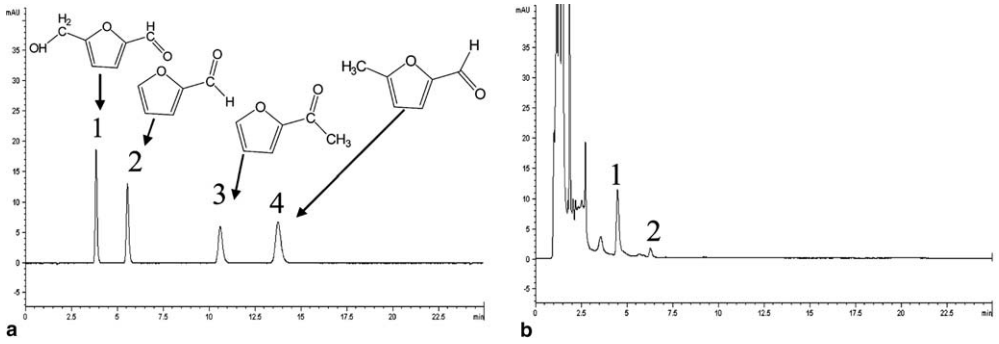

Fig. 1. Typical chromatograms of furfural determination by HPLC-DAD. See conditions in Section 2.3. Furfural peaks 1: HMF; 2: F; 3: FMC; and 4: MF. (a) Furfural standards; (b) infant formula.

sample from CIF and SIF stored at 25 and 37 °C, are recorded in Tables 1–3.

We noted that some studies refer to "total furfurals" instead of "potential furfurals". The term "total furfurals" can lead to some confusion since it could be taken as the sum of the total furfurals present in a sample (i.e., HMF + F + FMC + MF), and not as the potentially existent compounds in a sample, in terms of their precursors. Therefore, we have chosen to use the term "potential furfurals" when referring to the sum of compound precursors (i.e., furfurals bounded to protein, as occurs in Amadori products, furfurals from reducing sugars, or novo furfurals) plus free furfurals.

The potential furfural values (HMF + F) found at the zero points in the SIF and CIF were 653.01 and 686.13 μg/100 g, respectively, with no statistical difference ($p > 0.05$) between them. (Table 3). Study B reported values similar to our studied formulae: 601.95 μg/100 g for an adapted IF

Table 1
Potential and free hydroxymethyl-furaldehyde (HMF) content in stored infant formulas

Sample	Storage (months)	Potential HMF			Free HMF		
		μg/100 g	μg/100 ml	RSD (%)	μg/100 g	μg/100 ml	RSD (%)
SIF 25 °C	0	$485.88 \pm 4.90^{a\text{-}1\text{-}I}$	76.12 ± 0.77	1.01	$58.23 \pm 1.64^{a\text{-}1\text{-}I}$	9.12 ± 0.26	2.81
	3	$759.92 \pm 9.19^{b\text{-}2\text{-}II}$	119.05 ± 1.44	1.21	$67.46 \pm 0.13^{a\text{-}1\text{-}I}$	10.57 ± 0.02	0.2
	6	$879.58 \pm 8.35^{c\text{-}4\text{-}III}$	137.80 ± 1.31	0.95	$70.83 \pm 3.46^{a\text{-}1\text{-}I}$	11.10 ± 0.54	4.89
	9	$786.63 \pm 9.85^{b\text{-}2\text{-}II}$	123.24 ± 1.54	1.25	$70.28 \pm 4.65^{a\text{-}1\text{-}I}$	12.26 ± 0.73	5.94
	12	$726.37 \pm 19.70^{b\text{-}2\text{-}II}$	113.80 ± 3.09	2.71	$77.07 \pm 3.26^{a\text{-}1\text{-}I}$	12.07 ± 0.51	4.24
SIF 37 °C	0	$485.88 \pm 4.90^{i\text{-}1}$	76.12 ± 0.77	1.01	$58.23 \pm 1.64^{i\text{-}1}$	9.12 ± 0.26	2.81
	3	$1115.53 \pm 13.60^{j\text{-}3}$	174.77 ± 2.13	1.22	$63.49 \pm 1.80^{i\text{-}1}$	9.95 ± 0.28	2.84
	6	$1194.17 \pm 11.05^{k\text{-}5}$	187.09 ± 1.73	0.93	$82.06 \pm 2.98^{i\text{-}1}$	12.86 ± 0.47	3.63
	9	$1589.19 \pm 70.30^{l\text{-}6}$	248.97 ± 11	4.42	$109.58 \pm 0.25^{j\text{-}2}$	17.17 ± 0.04	0.23
	12	$1616.03 \pm 45.27^{l\text{-}6}$	253.18 ± 7.09	2.8	$121.83 \pm 0.55^{j\text{-}2}$	19.09 ± 0.09	0.45
CIF 25 °C	0	$515.85 \pm 16.06^{v\text{-}I}$	80.82 ± 2.52	3.11	$84.92 \pm 2.85^{k\text{-}I}$	16.30 ± 0.45	3.35
	3	$471.52 \pm 17.78^{v\text{-}I}$	73.87 ± 2.79	3.77	$101.25 \pm 3.96^{k\text{-}I}$	15.86 ± 0.62	3.91
	6	$533.66 \pm 17.15^{v\text{-}I}$	83.61 ± 2.69	3.21	$116.70 \pm 1.74^{k\text{-}II}$	18.28 ± 0.27	1.49
	9	$560.46 \pm 11.03^{v\text{-}I}$	87.81 ± 1.73	1.97	$122.46 \pm 9.90^{k\text{-}II}$	19.19 ± 1.55	8.08
	12	$784.36 \pm 8.11^{w\text{-}II}$	122.88 ± 1.27	1.03	$161.66 \pm 7.24^{l\text{-}III}$	25.33 ± 1.13	4.48

Values are expressed as the mean ± standard deviation ($n = 4$). Repetitions in superscripted characters within the same column indicate no significant differences ($p < 0.05$), according to the following: Letters, storage time (0–12 months); Arabic numbers, temperature of storage (25 vs. 37 °C); and Roman numbers, (SIF 25 vs. CIF 25 °C).

Table 2
Potential and free furaldehyde (F) content in stored infant formulas

Sample	Storage (months)	Potential F			Free F		
		μg/100 g	μg/100 ml	RSD (%)	μg/100 g	μg/100 ml	RSD (%)
SIF 25 °C	0	$167.13 \pm 4.98^{a\text{-}1\text{-}I}$	26.18 ± 0.78	2.98	$ND^{a\text{-}1\text{-}I}$	–	–
	3	$214.53 \pm 8.54^{b\text{-}2\text{-}II}$	33.61 ± 1.34	3.98	$27.50 \pm 2.49^{b\text{-}2\text{-}II}$	4.31 ± 0.39	9.05
	6	$234.68 \pm 5.93^{b\text{-}2\text{-}II}$	36.77 ± 0.93	2.53	$35.62 \pm 1.57^{b\text{-}2\text{-}II}$	5.58 ± 0.25	4.41
	9	$192.71 \pm 6.91^{b\text{-}3\text{-}III}$	30.19 ± 1.08	3.59	$56.14 \pm 5.49^{c\text{-}5\text{-}III}$	8.80 ± 0.86	9.77
	12	$186.40 \pm 17.3^{b\text{-}3\text{-}III}$	29.20 ± 2.70	9.26	$ND^{a\text{-}1\text{-}I}$	–	–
SIF 37 °C	0	$167.13 \pm 4.98^{i\text{-}1}$	26.18 ± 0.78	2.98	$ND^{i\text{-}1}$	–	–
	3	$232.06 \pm 5.39^{j\text{-}2}$	36.36 ± 0.84	2.33	$10.54 \pm 2.38^{j\text{-}3}$	1.65 ± 0.37	22.6
	6	$199.74 \pm 11.27^{k\text{-}2}$	31.29 ± 1.76	5.64	$15.45 \pm 2.72^{j\text{-}3}$	2.42 ± 0.43	17.6
	9	$201.77 \pm 9.68^{k\text{-}3}$	31.61 ± 1.52	4.8	$27.09 \pm 1^{k\text{-}6}$	4.24 ± 0.16	3.7
	12	$198.22 \pm 7.97^{k\text{-}3}$	31.05 ± 1.25	4.02	$17.64 \pm 2.06^{j\text{-}3}$	2.76 ± 0.32	11.7
CIF 25 °C	0	$170.29 \pm 7.44^{v\text{-}I}$	26.68 ± 1.17	4.37	$1.19 \pm 2.38^{v\text{-}I}$	0.19 ± 0.37	3.8
	3	$147.17 \pm 6.66^{v\text{-}I}$	23.06 ± 1.04	4.53	$ND^{v\text{-}I}$	–	–
	6	$153.86 \pm 10.92^{v\text{-}I}$	24.10 ± 1.71	7.1	$9.70 \pm 0.82^{w\text{-}IV}$	1.52 ± 0.13	8.48
	9	$156.09 \pm 3.11^{v\text{-}I}$	24.45 ± 0.49	2.1	$11.15 \pm 0.52^{w\text{-}IV}$	1.75 ± 0.08	4.63
	12	$212.21 \pm 18.9^{w\text{-}II}$	33.25 ± 2.96	8.92	$14 \pm 0.35^{w\text{-}IV}$	2.19 ± 0.05	2.48

Values are expressed as the mean ± standard deviation ($n = 4$). Repetitions in superscripted characters within the same column indicate no significant differences ($p < 0.05$), according to the following: Letters, storage time (0–12 months); Arabic numbers, temperature of storage (25 vs. 37 °C); and Roman numbers, (SIF 25 vs. CIF 25 °C).

Table 3
Potential and free hydroxymethyl-furaldehyde + furaldehyde (HMF + F) content in stored infant formulas

Sample	Storage (months)	Potential HMF + F			Free HMF + F		
		μg/100 g	μg/100 ml	RSD (%)	μg/100 g sample	μg/100 ml	RSD (%)
SIF 25 °C	0	$653.01 \pm 3.75^{a\text{-}1\text{-}I}$	102.30 ± 0.59	0.57	$58.23 \pm 1.64^{a\text{-}1\text{-}I}$	9.12 ± 0.26	2.81
	3	$974.45 \pm 8.54^{b\text{-}2\text{-}II}$	152.66 ± 1.34	0.88	$94.96 \pm 2.61^{a\text{-}1\text{-}I}$	14.88 ± 0.41	2.76
	6	$1114.25 \pm 14^{c\text{-}4\text{-}III}$	174.57 ± 2.24	1.28	$106.45 \pm 4.05^{b\text{-}2\text{-}II}$	16.68 ± 0.63	3.80
	9	$979.34 \pm 12^{b\text{-}5\text{-}IV}$	153.43 ± 1.95	1.27	$134.42 \pm 2.10^{b\text{-}2\text{-}II}$	21.06 ± 0.33	1.57
	12	$912.77 \pm 2.4^{b\text{-}5\text{-}IV}$	143.03 ± 0.38	0.27	$77.07 \pm 3.26^{a\text{-}1\text{-}I}$	12.07 ± 0.51	4.23
SIF 37 °C	0	$653.01 \pm 3.75^{i\text{-}1}$	102.30 ± 0.59	0.57	$58.23 \pm 1.64^{i\text{-}1}$	9.12 ± 0.26	2.81
	3	$1347.59 \pm 17.8^{j\text{-}3}$	211.12 ± 2.78	1.32	$74.03 \pm 0.58^{i\text{-}1}$	11.60 ± 0.09	0.78
	6	$1393.91 \pm 21.64^{j\text{-}6}$	218.38 ± 3.39	1.55	$97.51 \pm 3.41^{j\text{-}2}$	15.28 ± 0.53	3.50
	9	$1790.96 \pm 80^{k\text{-}7}$	280.58 ± 12.5	4.47	$136.66 \pm 0.75^{j\text{-}2}$	21.41 ± 0.12	0.55
	12	$1814.24 \pm 39.3^{k\text{-}7}$	284.23 ± 6.16	2.17	$139.47 \pm 1.53^{j\text{-}2}$	21.85 ± 0.24	1.10
CIF 25 °C	0	$686.13 \pm 17.88^{v\text{-}I}$	107.49 ± 2.80	2.61	$86.10 \pm 3.57^{v\text{-}I}$	13.49 ± 0.56	4.14
	3	$618.68 \pm 24.36^{v\text{-}I}$	96.93 ± 3.82	3.94	$101.25 \pm 3.96^{v\text{-}I0}$	15.86 ± 0.62	3.91
	6	$687.52 \pm 27.78^{v\text{-}I}$	107.71 ± 4.35	4.04	$126.40 \pm 1.05^{v\text{-}II}$	19.80 ± 0.16	0.83
	9	$716.54 \pm 10.08^{v\text{-}I}$	112.26 ± 1.58	1.41	$133.61 \pm 10.3^{w\text{-}II}$	20.93 ± 1.61	7.68
	12	$996.56 \pm 24.09^{w\text{-}II}$	156.13 ± 3.77	2.42	$175.66 \pm 7.30^{x\text{-}III}$	27.52 ± 1.14	4.16

Values are expressed as the mean ± standard deviation ($n = 4$). Repetitions in superscripted characters within the same column indicate no significant differences ($p < 0.05$), according to the following: Letters, storage time (0–12 months); Arabic numbers, temperature of storage (25 vs. 37 °C); and Roman numbers, (SIF 25 vs. CIF 25 °C).

(casein/serum 40/60). In general, the values of potential HMF in the SIF and CIF were slightly higher than those reported in Study B for adapted formulae with the same casein/serum protein ratio (40/60), but of distinct composition. Nevertheless, the values of free HMF were both lower in the SIF and similar in CIF, when making comparisons with the same study. In the case of potential F, the values found at the zero points in SIF and CIF were 167.13 μg/100 g and 170.29 μg/100 g, respectively. Lower values of potential F were reported in Study B (31.88 μg/100 g). In fresh SIF, no free F was detected, while in the CIF only 1.19 μg/100 g was observed, a value near the detection limit (Chávez-Servín et al., 2005).

In addition, the values of potential and free HMF in the CIF and SIF were similar to those reported in liquid and powdered IF in Study A, except for the free HMF in powdered infant milk, which exhibited the highest values. With regard to potential and free F, similar values were found when comparing the CIF and SIF with those values reported in Study A for both liquid and powdered infant milk. The only exception was in the case of potential F, which in the studied samples was higher in both SIF and CIF.

At the zero points, the levels of potential HMF in the SIF and CIF were 76.12 and 80.82 μg/100 ml of reconstituted sample, respectively, concentrations slightly higher than those reported in Study C for several UHT milk samples.

These results confirm that exist differences in the furfural content reported by other authors, mainly due to formula composition, thermal treatments during manufacturing process and storage. However, in spite of these differences, comparison with other authors in different formulae are necessary for determine the usefulness of furfurals as suitable indicators of food damage. In general, the values of furfurals founded are similar when are compared with infant formulas with the same casein/serum (60:40) content.

Likewise is more useful to contrast the potential furfurals (HMF + F) than free furfurals, due to potential furfurals involve not only the furfurals present in the sample, but also their precursors, which provide a better information of the extension of MR and the food damage. Concerning free furfural comparisons in different samples is more complicated due to their behavior during the storage as it is shown next.

3.3. Furfural evolution

For evaluate furfural compounds evolution, we compare the variables as follows: For MFO supplementation (SIF 25 °C vs. CIF 25 °C), for storage temperature (SIF 25 °C vs. SIF 37 °C), and for storage time, we compare the values respect to 0, 3, 6, 9 and 12 months, respectively (Tables 1–3). We note that values of CIF 37 °C were omitted to avoid overload information since the effect of temperature were already studied in SIF. In the SIF, a distinct furfural evolution was observed with respect to storage temperatures of 25 and 37 °C. In general, HMF and F concentrations were greater with increased storage time, an increment greater in the SIF stored at 37 °C. These observations are explained by the fact that storage under inadequate temperatures, such as 37 °C, tends to favor MR. However, evolution did not follow regular patters, as shows next.

3.3.1. Potential furfurals

At the beginning of the present study, the potential HMF in the SIF and CIF was 485.88 and 515.85 μg/100 g,

respectively. Following 12 months of storage at 25 °C, these values increased to 726.37 and to 784.36 μg/100 g, respectively, while the SIF stored at 37 °C exhibited a value of 1616.03 μg/100 g (Table 1). Similar results were observed for potential F, whose initial content in SIF and CIF was 167.13 and 170.29 μg/100 g, respectively, while at the end of 12 months storage 186.40 and 212.21 μg/100 g, respectively. Following 12 months of storage at 37 °C, SIF exhibited a content of 198.22 μg/100 g. In general, the potential HMF and F in both of the studied IF groups (CIF and SIF) increased over extended storage and higher temperatures, consistent with Studies A and D. These results indicate that storage temperature affects MR; indeed, the higher the temperature, the faster the rate of MR. We noted, however, that the evolution of potential furfurals, principally the potential F, in the SIF and CIF groups did not follow a clear pattern upon storage (Tables 1–3). Studies B and E reported that potential HMF and F content vary in an irregular manner over changes in storage time and temperature. These findings can be explained by the fact that HMF reaches a state of equilibrium between destruction by oxidation and formation from precursors (Morales, Romero, & Jimenez-Perez, 1997). We noted that the values of potential HMF, in the SIF at 37 °C showed a constant increase while the SIF 25 °C and CIF 25 °C showed increases and decreases (Table 1). This means that the storage at 37 °C increases the MR producing a major precursors of HMF, being greater their production than their destruction in comparison with formulae at 25 °C.

3.3.2. Free furfurals

In fresh IF, free HMF in the SIF and CIF was 9.12 and 16.30 μg/100 ml of reconstituted sample, respectively, values similar or lower than those reported for several UHT milk samples (within the range 7.40–65.22 μg/100 ml) in Study C. The contents of free HMF in the SIF and CIF increased as storage time lengthened or temperatures increased. At the beginning of the present study, values of 58.23 μg/100 g for SIF and 84.92 μg/100 g for CIF were recorded, while by the end of 12 months storage at 25 °C, these had increased to 77.07 and 161.66 μg/100 g, respectively. Following 12 months of storage at 37 °C, we measured 121.83 μg/100 g for SIF (Table 1). Free F at the zero time point in SIF was not detected, while in CIF, free F was present at a barely detectable level: 1.19 μg/100 g. After 12 months storage at 25 °C, free F was not detected in the SIF, while in the CIF it measured at 14 μg/100 g and in SIF at 37 °C 17.64 μg/100 g (Table 2). In neither formula did free F follow a regular pattern, instead exhibiting both increases and decreases observed upon storage.

Studies have reported that the first furfural compound formed during MR is HMF, with F. The MF and FMC either being the by-products of the reaction's most advanced states, or forming by inter-conversion as a result of higher temperatures or longer storage periods (van Boekel, 1998). Study A reported that FMC and MF, along with HMF and F, were present when IF was heated to extreme temperatures. To detect FMC and MF compounds, as well as to corroborate their formation in IF during advanced stages of MR, we maintained the IF at 47 °C, analyzing furfurals at 10, 30, 50, 100, 120, and 200 days (data not show). Neither FMC nor MF formed, a result consistent with Studies B, C, D, E, and F, which detected none of these compounds in either IF or milk. On the basis of these findings and the available literature, the identification of FMC and MF in infant formulae is not deemed essential since only one previous report demonstrated their presence, and then only when the IF had been heated to 100 °C over 3 h, conditions quite uncommon in the manufacturing process. Moreover, analysis time in the HPLC would be reduced by half (see Fig. 1).

The evolution of furfurals in SIF and in CIF was similar, with hardly any differences observed between them. This result is consistent with a previous study (Chávez-Servín et al., in press) in which sugar stability of MFO material (used in the formulation of SIF) was evaluated, and no recorded changes were observed after 120 days of storage at 47 °C.

4. Conclusions

We identified furfural compounds in the CIF and SFI by RP-HPLC-DAD analysis. HMF was the principal furfural compound detected, followed by F. Furfural levels were higher in IF stored at 37 °C than at 25 °C. Furfural levels increased as storage time lengthened. Neither FMC nor MF formation was detected in any of the formulae. In agree with other authors, as in this study, is concluded that high storage temperatures, as 37 °C, increase furfural levels, and it seems that this variable is more important than storage time at room temperature for furfurals development. With regard to the uses of MFO, not significant differences were observed in furfurals, comparing CIF and SIF. On the basis of these findings, we believe that supplementing infant formulae with MFO has no effect on furfural formation or evolution. The use of MFO material in the infant formulation has considerable potential. However, more studies on the stability of these supplemented products are necessary. Studies on the chemical changes produced by both the thermal and storage processes must continue. The search for compounds induced by heating, as well as the concentration of these compounds as possible indicators of heat treatment or product deterioration, remains ongoing, the most prominent example being furfural compounds, principally HMF, as intermediaries in the Maillard Reaction. The question of whether or not HMF and F are really suitable indicators has yet to be satisfactorily answered, due to their irregular pattern, showing both increases and decreases upon storage. At present, there are no established limits for furfural compound concentrations in IF. The general recommendation is that the amount of furfurals should remain as low as possible (Bremer et al., 1987), since they are related indicators of degeneration in food products.

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PUBLICACIÓN 6

Evolución de los contenidos de lisina disponible y lactosa en preparados para lactantes en polvo suplementadas con aceite de pescado microencapsulado durante el almacenamiento.

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En este trabajo se muestra la evolución de los contenidos de lisina disponible y lactosa, así como del contenido de hidroperóxidos, como indicadores de calidad y estabilidad en dos tipos de fórmulas para lactantes de base láctea en polvo almacenados a 25ºC y37ºC durante 12 meses. El primer tipo de fórmula fue suplementada con aceite de pescado microencapsulado, que consiste en pequeñas partículas de polvo en una matriz recubierta de caseinato y sacarosa para prevenir la oxidación de los PUFA del aceite de pescado. El segundo tipo de fórmula no fue suplementada y se uso como referencia. La determinación de lisina disponible fue realizada por un método HPLC en fase reversa con un detector DAD, utilizando la técnica de FDNB como agente derivatizante, que reacciona con la lisina disponible y forma un complejo Nε-DNP-lys que es extraído y determinado en el cromatógrafo. El microencapsulado utilizado como materia prima, podría no ser estable durante el almacenamiento y afectar negativamente la calidad de la fórmula, básicamente de dos maneras: por la oxidación de los PUFA, los cuales podrían posteriormente reaccionar con la lisina, bloqueándola o haciéndola no biodisponible, o, por hidrólisis de la sacarosa, produciendo azúcares reductores e incrementando la reacción de Maillard.

Solamente pequeños cambios fueron observados en la evolución de lisina disponible y lactosa durante las condiciones de almacenamiento. Se observó pérdida de lisina sólo después de los 9 y 12 meses de almacenamiento a 37ºC, típico de la reacción de Maillard en fórmulas para lactantes en polvo. Cuando se compararon los dos tipos de fórmulas, se observa que la adición o uso del aceite de pescado microencapsulado no afectó negativamente ninguno de los parámetros estudiados, indicando por lo tanto una estabilidad aceptable en las condiciones experimentales estudiadas.

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Original Article

Evolution of available lysine and lactose contents in supplemented microencapsulated fish oil infant formula powder during storage

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Summary In this work we report the evolution of available lysine, lactose and lipid hydroperoxide contents as indicators of food quality and stability of two types of infant formula powder stored at 25° and 37 °C for 12 months. The first type was supplemented with microencapsulated fish oil (MFO), made of little powder particles in a food starch-coated matrix of caseinate and saccharose to prevent the oxidation of polyunsaturated fatty acid (PUFA). The second type was not supplemented and was used for reference. MFO might not be stable during storage and affect negatively the quality of the formula in two ways: by oxidation of PUFA, which may also easily react with lysine upon oxidation, blocking lysine, or by hydrolysis of saccharose, producing reducing sugars and increasing the Maillard reaction. Slight changes were observed in lysine and lactose evolution. By comparing the two formulae, this study shows that the MFO material did not negatively affect the studied parameters, indicating therefore acceptable stability in the conditions tested.

Keywords Available lysine, infant formula powder, lactose, microencapsulated fish oil, storage.

Introduction

Human milk is the optimum food for neonates; it generally covers all their nutritional requirements. However, breastfeeding is not always possible and must sometimes be complemented with or substituted by infant formula (IF). Given that IF are often the only food source that the infant receives, they must provide all the nutrients required and guarantee the stability of their contents throughout shelf life. The food industry has therefore made several attempts to improve the composition of formulae in order to increase its resemblance to human milk and to guarantee safety. One of these improvements includes supplementation with long-chain polyunsaturated fatty acids (LC-PUFA), as several studies on infant nutrition have reported the beneficial effects of these fatty acids on growth and development (Cunnane *et al*., 2000; Lauritzen *et al*., 2001; Innis, 2003). Nowadays there are a lot of IF supplemented with LC-PUFA. A number of highly unsaturated dietary lipid sources are currently available, such as egg yolk lipids, fish oils and oils synthesised from fungal (*Mortierella alpine*) and algal (*Crypthenocodium cohnii*) organisms that synthesise oils rich in arachidonic acid and docosahexaenoic acid, respectively. Since LC-PUFA are highly susceptible to the oxidation process, controlling the stability of these supplemented IF's is necessary. The fish oil matter is generally coated with protein and carbohydrate in the form of microencapsulated fish oil (MFO) to prevent oxidation and to increase the fish oil's stability and durability of the infant formulation. Powder IF combine a set of factors that makes them highly sensitive to the Maillard reaction (MR), namely reducing sugar content, lysine-rich proteins, high temperature applied during manufacture and long storage time (Ferreira *et al*., 1998; Ferrer *et al*., 2005). Likewise, high PUFA content may increase oxidation, which is the main cause of deterioration of lipid-containing IF. Reaction of atmospheric oxygen with unsaturated lipids produces a wide range of hydroperoxides. The MR's most significant nutritional effect is the loss of protein quality brought about during manufacture and storage of IF. Reducing carbohydrates react with free amino acid side-chains from proteins, mainly the ϵ-amino group of lysine, and produces (via formation of a Schiff's base and the Amadori rearrangement) lactuloselysine, bound to the protein structure. The formation of this so-called Amadori product results in non-bioavailability of this amino acid (blocked lysine) for nutritional purposes (Evangelisti *et al*., 1994; Friedman, 1996; Van Boekel,

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1998; Rehman, 2002; Rehman & Shah, 2003; Ramirez-Jimenez *et al*., 2004; Chávez-Servín *et al*., 2005) (Fig. 1); lactuloselysine products cannot be digested by proteolytic enzymes (Finot *et al*., 1976; Hernandez & Alvarezcoque, 1992). PUFA may also easily react with lysine upon oxidation, blocking lysine. Lysine is an essential amino acid, which is generally used as an indicator of the biological value of food protein product (Mauron & Mottu, 1958; Naranjo *et al*., 1998). Lysine can be measured in two ways: total lysine content and available lysine content. The former is usually determined after acid hydrolysis; in the case of the MR, however, this measurement does not reflect the amount of available lysine or lysine that is unavailable for nutritional purposes. The analysis of available lysine previously requires a derivative reagent, generally 1-fluoro-2,4-dinitrobenzene (FDNB), which interacts with the ϵ-amino group from lysine. The bound lysine does not react with this reagent and consequently the available lysine (or reactive lysine) can be determined (Carpenter, 1960; Hurrell *et al*., 1983).

Here we track the evolution of available lysine, lactose and hydroperoxides' content in two types of infant formulae powder: one supplemented with MFO and one not supplemented (control), during 12 months of storage at different temperatures (25º and 37 ºC). Afterwards, we compared these results with previous research and European Union (EU) legislation.

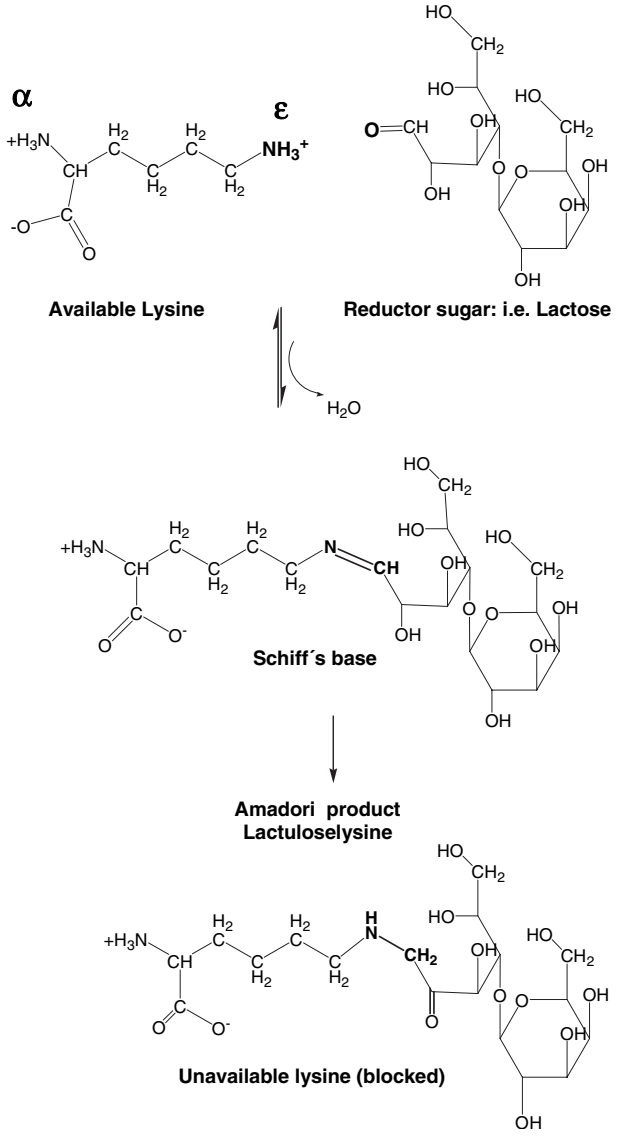

Figure 1 Schematic presentation of initial Maillard reaction from amino ϵ-group of available lysine with a reducing sugar (lactose) in milk-based infant formulae.

Materials and methods

Materials

The chemicals used for sample preparation were of analytical reagent grade: methanol high-performance liquid chromatographic (HPLC)-grade (SDS, Peypin, France), ethanol absolute, acetic glacial acid and hydrochloric acid came from Panreac (Barcelona, Spain), and sodium acetate was purchased from Probus (Barcelona, Spain). Deionised water was purified through a Milli-Q system (Millipore, Bedford, MA, USA). The derivative reagent FDNB was obtained from Fluka (Buchs, Switzerland). Standards of *N*ϵ-2,4-dinitro-phenyl-L-lysine hydrochloride, *N*α-acetyl-L-lysine and lactose (> 99% pure) were purchased from Sigma (St. Louis, MO, USA).

Instrumentation and chromatographic conditions

For available lysine determination we used a Hewlett Packard liquid chromatographic system (Waldbronn, Germany) with an HP 1050 pump system, an HP-1040 M photodiode-array detector and a Waters 717 plus autosampler injector (Milford, MA, USA), and an ODS-2 C_{18} column (4.6 × 150 mm), with a 5-μm particle size, and a precolumn (13 mm × 3 mm i.d.), both made by Tracer (Teknokroma, Barcelona, Spain). Separation was performed at 30 ºC using a mixture of 35% methanol and 65% 0.01 M sodium acetate solution (adjusted to pH 4.5 with acetic glacial acid, and passed through a 0.45-μm nylon filter) as the mobile phase and a flow rate of 1 mL min^{-1}. Detection was at 362 nm, and the injection volume was 20 μL. *N*ϵ-DNP-Lys was completely separated in 7 min. After 8 min of injection, a clean programme was executed, which consisted of increasing the methanol proportion from 35% to 95% during 5 min, and then returning to the initial propor-

tion (35%) of methanol and re-equilibrating for 7 min. *N*ϵ-DNP-Lys was determined by the external standard method and expressed as lysine.

The chromatographic analyses of lactose were carried out on a Shimadzu HPLC equipped with a LC-10AD double pump, a 7725 Rheodyne injector (Cotati, CA, USA) with a 20-μL loop, and a RID-6A Shimadzu refractive index detector. Chromatographic separation was performed on a Tracer carbohydrates column (5-μm particle size; 250 × 4.6 mm i.d.), and an NH_2 precolumn (13 mm × 3 mm i.d.), both made by Tracer (Teknokroma).

Samples

The two types of IF powder samples were produced at a pilot scale by a company in Barcelona, Spain, using the same basal composition and processing conditions. The MFO used as raw material for the manufacturing process was the Dry *n*-3® 5:25, obtained from BASF Health and Nutrition (Ballerup, Denmark). The first type was supplemented (SIF) with LC-PUFA by adding Dry *n*-3® 5:25 to the final powder, until obtaining 0.5 g 100 g^{-1} of total fatty acids as DHA (C22:6*n*-3). The second type, the control infant formula (CIF), was not supplemented. The formulae were packed in airtight 400-g aluminium foil bags flushed with nitrogen in a modified atmosphere ($< 2\%$ O_2). Formula composition is reported in Table 1. The initial moisture content determined gravimetrically was 3.2%.

Table 1 Composition of the studied formulae according to the label information

	Mean values 100 g^{-1}
Energetic value (kJ $kcal^{-1}$)	2152/514
Protein (g)	12
Carbohydrate (g)	58
Fat (g)	26
Sodium (mg)	175
Potassium (mg)	535
Chloride (mg)	290
Calcium (mg)	420
Phosphorus (mg)	230
Magnesium (mg)	42
Iron (mg)	6
Zinc (mg)	4
Copper (μg)	300
Iodine (μg)	70
Selenium (μg)	11
Vitamin A (μg)	640
Vitamin D (μg)	10
Vitamin E (mg)	25
Vitamin K (μg)	42
Vitamin B1 (μg)	520
Vitamin B2 (μg)	620
Vitamin B6 (μg)	825
Vitamin B12 (μg)	2
Vitamin C (mg)	60

Storage conditions

To evaluate the evolution of selected parameters during the shelf life of IF, we kept the product at 25º or 37 ºC from production until 0, 3, 6, 9 and 12 months, respectively. Once the storage periods tested were completed, peroxide value (PV) was immediately determined. The samples were then kept at –32 °C until available lysine and lactose were analytically determined for a period no longer than 3 weeks.

Measurement of available lysine

*N*ϵ-NDP-Lys was determined by HPLC, adapting the method proposed by Albalá-Hurtado *et al*. (1997) to our laboratory: 250 mg of powder sample was placed in a 100-mL Erlenmeyer flask. We next added 10 mL of 8% (w/v) sodium bicarbonate solution and the powder was dissolved by gently swirling. Fifteen millilitres of derivative reagent (FDNB 3% in ethanol) was added to the flask, which was then sealed with a stopper and shaken for 2 h on a magnetic plate. After the lysine derivative reaction, ethanol was evaporated in a water bath at 95 ºC for approximately 15 min, until no effervescence was produced when shaken. Then, 40 mL of 8 M HCl was added into the flask. The Erlenmeyer flask was connected to a reflux tube and was immersed in a silicone bath at 160 ºC for 2 h 30 min. After hydrolysis, the solution was filtered through a Whatman paper number 541 while still hot, and the resultant solution was collected in a 250-mL volumetric flask. The flask and the residues were washed thoroughly with distilled water until the total filtrate reached 250 mL. The filtrate was shaken thoroughly to prevent the formation of a dinitrophenol precipitate. Fifteen microlitres was transferred to a 50-mL volumetric flask and the pH was adjusted to 5.0 with a 2 M NaOH solution. We then added 10 mL of HPLC-grade methanol and made up the volume to 50 mL with Milli-Q water. Before injection, each sample was filtered through a 0.45-μm nylon filter. All analyses were carried out in quadruplicate. *N*ϵ-DNP-Lys was identified by its retention time and characteristic spectra. It was quantified by interpolation in a calibration curve, expressed as lysine, in the range of 0.05–7 μg mL^{-1}.

Determination of lactose

Saccharide contents were determined by the HPLC-RI method exactly as we reported previously (Chávez-Servín *et al*., 2004).

Determination of peroxide value

Lipid hydroperoxides were determined by an iodometric method described in the Regulation EEC/2568/91 of the European Union Commission (1991).

pH values

The pH of the samples was measured in a pH meter micro-pH 2000 with a glass electrode (pH 0–12, 0–80 ºC) equipped with an automatic temperature compensator (Crison Instruments, S.A., Barcelona, Spain). Following the manufacturer's instructions, the IF was reconstituted in warm water (40–45 ºC; 4.7 g in 30 mL). The pH was determined after samples had reached room temperature.

Protein content

Nitrogen content was determined by the Kjeldahl method (IDF: 20, 1962) using a 1007 Tecator digestor system DS-6 and a Tecator Kjeltec 1002 distillation unit (Tecator AB, Höganas, Sweden). The factor 6.38 for dairy products was used to calculate the protein content.

Statistical analysis

For statistical analysis, we used a one-way analysis of variance (ANOVA), as well as multiple comparisons, using the Tukey HSD procedure for content of: available lysine, lactose and hydroperoxides, with storage temperature (25 and 37 ºC) and formula type (SIF or CIF). We performed 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.

Results and discussion

Analytical parameters of available lysine method

Under the chromatographic conditions tested, a linear relationship of standard solutions was verified for *N*ϵ-DNP-Lys, expressed as only-lysine, in the range of 0.05–7 μg mL^{-1}, by analysis of variance of the regression (r^2;). The r^2 value was >0.999 at seven levels and the equation curve was $y = 48.9x + 0.7602$. Six replicate measurements were performed on the same day to evaluate repeatability (RSD 2.45%). For reproducibility, we carried out eight determinations with the same formula on different days (RSD 6.53%). The RSD values for available lysine were satisfactory, as described by Horwitz (1982). To determine the sensitivity of the method, the detection (DL) and the quantification (QL) limits were studied following the United States Pharmacopeia (USP) criteria (1989). The DL of available lysine was 0.53 mg 100 g^{-1} and the QL 2.78 mg 100 g^{-1} of powder sample, respectively. Recovery by using Nα-acetyl-lysine was 94.18% ± 3.87% (RSD 4.11%).

pH analysis

As high pH values can enhance the loss of available lysine through the formation of Amadori compounds, the pH of the reconstituted IF sample was measured at each month of storage. No differences in the evolution of pH values at 25º or 37 ºC were observed in the CIF or SIF. The pH values remained constant from 0 to 12 months of storage. The average pH in CIF and SIF over the 12-month period was 6.91 ± 0.14 (RSD 1.60%) and 6.92 ± 0.17 (RSD 1.20%), respectively. These values are common for IF (Ferrer *et al.*, 2003; McSweeney *et al.*, 2004).

Available lysine contents

The total protein content in CIF and SIF was identical, with a recorded value of 12.31% ± 0.08% (RSD 0.65%). A typical chromatogram of *N*ϵ-DNP-Lys in a standard solution as well as a chromatogram of an IF sample after the method is shown in Fig. 2. The initial available lysine content in our SIF and CIF before storage was 6.01 and 6.04 g 100 g^{-1} of protein, respectively (Table 2). These values are within the range of available lysine contents reported in a previous study (Pereyra Gonzales *et al.*, 2003) on commercial IF powder based on milk and milk enriched with whey (5.38–8.11 g 100 g^{-1} protein). In this cited study the average available lysine content in formulae based on milk proteins was 6.67 ± 0.95 g 100 g^{-1} protein, which is a similar amount to the mean value accepted for human milk (6.6 g 100 g^{-1}) (WHO, 2000; Pereyra Gonzales *et al.*, 2003).

Available lysine contents in our IF were slightly lower than those reported in adapted (6.67 g 100 g^{-1} protein) and follow-up (6.61 g 100 g^{-1} protein) IF (Ferrer *et al.*, 2000). Likewise, our values were slightly higher than those reported by Albalá-Hurtado *et al.* (1997) in powdered infant milk (5.97 g 100 g^{-1} protein) and liquid infant milk (5.02 g 100 g^{-1} protein). Our results are also comparable with a previous study in liquid and powdered IF (5.7 and 6.5–6.7 g 100 g^{-1} protein, respectively) (Tomarelli *et al.*, 1985), and for liquid infant formulae (4.81–7 g 100 g^{-1} protein) (Pompei *et al.*, 1988).

Evolution of available lysine

After production, formulae were stored at 25º and 37 ºC. After 12 months of storage at 25 ºC the SIF showed (5.87 ± 0.20) g 100 g^{-1} protein, which represents a loss of 2.32% of available lysine compared with

Table 2 Available lysine content in stored infant formulae

Storage		Available lysine[z]			
Sample	Months	g 100 g^{-1} protein	g 100 g^{-1} powder	mg 100 mL^{-1} [x]	Losses (%)
Supplemented infant formula 25 ºC	0	6.01 ± 0.15 $^{a-1-I}$	0.740 ± 0.02	115.94 ± 2.84	–
	3	5.87 ± 0.12 $^{a-1-I}$	0.723 ± 0.01	113.28 ± 2.34	2.30
	6	6.00 ± 0.39 $^{a-1-I}$	0.739 ± 0.05	115.77 ± 7.56	0.15
	9	5.85 ± 0.08 $^{a-1-I}$	0.720 ± 0.01	112.87 ± 1.58	2.64
	12	5.87 ± 0.20 $^{a-1-I}$	0.723 ± 0.02	113.25 ± 3.80	2.32
Supplemented infant formula 37 ºC	0	6.01 ± 0.15 $^{h-1}$	0.740 ± 0.02	115.94 ± 2.84	–
	3	5.97 ± 0.11 $^{h-1}$	0.735 ± 0.01	115.12 ± 2.15	0.71
	6	5.84 ± 0.21 $^{h-1}$	0.719 ± 0.03	112.60 ± 4.13	2.88
	9	4.95 ± 0.17 $^{h-2}$	0.609 ± 0.02	95.39 ± 3.28	17.72
	12	4.49 ± 0.24 $^{h-2}$	0.553 ± 0.03	86.62 ± 4.65	25.29
Control infant formula 25 ºC	0	6.04 ± 0.08 $^{m-I}$	0.744 ± 0.01	116.51 ± 1.56	–
	3	5.98 ± 0.12 $^{m-I}$	0.737 ± 0.01	115.42 ± 2.29	0.94
	6	5.56 ± 0.30 $^{m-I}$	0.684 ± 0.04	107.22 ± 5.86	7.98
	9	5.47 ± 0.17 $^{m-I}$	0.673 ± 0.02	105.47 ± 3.24	9.48
	12	5.61 ± 0.14 $^{m-I}$	0.690 ± 0.02	108.15 ± 2.76	7.18
Control infant formula 47 ºC	122 days	1.38 ± 0.22	0.17 ± 0.03	26.71 ± 4.19	77.08
	148 days	0.66 ± 0.04	0.08 ± 0.01	12.67 ± 0.74	89.13

[z]Values are expressed as mean ± standard deviation of four determinations. No coincidence in the superscript characters in the same column indicates a significant difference ($P < 0.05$).
[x]Reconstituted formula at 14% following manufacturer's instructions.
Letters, storage time (0–12 months); Arabic numerals, temperature of storage (25º vs. 37 ºC); Roman numerals, SIF 25º vs. CIF 25 °C.

fresh formula. Similarly, at the end of 12 months of storage at 25 ºC, the CIF showed (5.61 ± 0.14) g 100 g^{-1} protein, which represents a loss of 7.18% of available lysine compared with recently produced formula. Nevertheless, statistical analyses showed no difference ($P > 0.05$) between formula analysed immediately after production and formula stored for 3, 6, 9 and 12 months, respectively (Table 2). This result is consistent with a previous study (Albala-Hurtado *et al.*, 1998) in which two types of IF (liquid and powder) were monitored over 9 months of storage at 20º, 30º and 37 ºC. In this case, no changes in available lysine content were detected at any of these temperatures. We noted that when our SIF was stored at 37 ºC for the first 6 months, no significant changes in available lysine content were observed. However, in the 9- and 12-month storage, significant ($P < 0.05$) reductions were recorded: for the 9-month period from 6.01 to 4.95 (17.72% loss) and for the 12-month period from 6.01 to 4.49 g 100 g^{-1} protein (25.29% loss) (Table 2).

In two previous studies, the first in powdered milk stored at 20 ºC for 3 years (Vanmil & Jans, 1991), and the second in an IF stored for 1 year at 20 ºC (King *et al.*, 1991), no changes in available lysine were detected. Nevertheless, the first study found a 10% decrease in available lysine in samples stored at 35 ºC, and the second study a 21% reduction in lysine after 1 year of storage at 37 ºC. This latter study is consistent with our results: the values detected in our SIF samples stored at 25º and 37 ºC showed no statistical differences ($P > 0.05$). Only after 9 and 12 months of storage at 37 ºC was the loss of available lysine significant ($P < 0.05$). Ferrer *et al.* (2003) tracked the evolution of available lysine in adapted and follow-up milk-based IF over 2 years of storage at 20º and 37 ºC, and a significant decrease was reported at these two temperatures. After 24 months of storage, loss of available lysine in the adapted formulae reached 19.73% and 23.82% at 20º and 37 ºC, and in the follow-up formulae 47.59% and 48.69%, respectively, compared with the respective formula immediately after manufacture. Despite statistical differences ($P < 0.05$), only minor differences between 20º and 37 ºC were observed and no obvious effect of storage temperature on loss of available lysine was detected. To corroborate the effect of temperature on lysine loss, we stored the CIF at 47 ºC for 122 and 148 days, and observed significant losses of 77% and 89%, respectively, confirming temperature effect (Table 2).

The MR in milk-based formulae occurs between a reducing sugar (namely lactose) and the ϵ-amino group of lysine, forming lactuloselysine products in the initial steps of this reaction, and furfurals in subsequent steps. In a previous study on furfural evolution in the same IF we reported increases in furfural compounds after storage, which were higher in formula stored at 37 ºC than at 25 ºC (Chávez-Servín *et al.*, 2006a). It has been proposed that available lysine loss can be estimated

from HMF values (Renner, 1988); however, we observed increasing HMF values throughout storage and no correlated losses in available lysine contents. Because no relationship was found between furfural compound increases and insignificant losses in available lysine evolution upon storage, this indicates that the increase in furfural compounds arose from the initial precursors (lactuloselysine products), which are the result of the initial steps of MR, formed during the manufacturing process. This shows that the main blockage of lysine occurs during the manufacture of formulae rather than during storage.

Lactose content

To determine the lactose content, we carried out RP-HPLC-RI determination method of mono- and disaccharides (Chávez-Servín *et al.*, 2004). In the samples analysed lactose was the only sugar present, with initial contents of 62.4 g 100 g^{-1} of powder and 62.5 g 100 g^{-1} of powder in SIF and CIF, respectively. However, traces of saccharose below quantification limits < 0.20 mg mL^{-1} was also detected in SIF. This is because of the SIF being supplemented with the MFO Dry *n*-3® in the form of dry free-flowing powder particles, which according to the technical bulletin of this material, contains fish oil microencapsulated in a food starch-coated matrix of caseinate and saccharose to prevent the oxidation of PUFA.

Table 3 shows the evolution of the hydroperoxide levels through storage. The PV is a usual indicator for the determination of primary oxidation products. In the CIF and SIF the initial PV before storage were 0.55 and 0.85 meq O_2 kg^{-1}, respectively. In the CIF stored at 25 ºC only slight increases were recorded. PV after 12 months of storage was 0.83 meq O_2 kg^{-1}. In the SIF stored at 25 ºC also slight increases were observed, showing after 12 months of storage 2.02 meq O_2 kg^{-1}. Furthermore in the SIF stored at 37 ºC the PV after 12 months of storage was 4.28 meq O_2 kg^{-1}. In the SIF stored at 25º and 37 ºC, hydroperoxide levels increased slowly, being 2.4 and 5.0 times greater than the initial values, while in the CIF stored at 25 ºC after 12 months the level was only 1.50 times greater than the initial value. However, the limiting PV specified by the codex alimentarius (CODEX stan 210, 1991) for refined oils is 10 meq O_2 kg^{-1}. The encapsulated fish oil used for SIF was expected to be less reactive because of the protection of the oil as mentioned before, showing that these results are satisfactory (Table 3).

Saccharose (from MFO) is not a reducing sugar, however during the manufacturing process it would be hydrolysed to glucose and fructose, which are reducing sugars and could increase the MR. However, this phenomenon was not observed. Furthermore, the quantity of saccharose detected in the formulae was insignificant. We also studied previously the sugar stability in the raw material MFO Dry *n*-3®, submitting this to 47 ºC for 120 days. No changes were observed in sugar fraction in two batches (production separated by 1 year) (Chávez-Servín *et al.*, 2006b). Table 4 shows the lactose contents over 12 months of storage. In the formulae stored at 25 ºC (CIF and SIF) no significant changes were observed, the concentration of lactose remained constant. However, in the SIF stored at 37 ºC the values of lactose fell to 57.8 and 54.4 g 100 g^{-1} of powder, respectively, in the 9 and 12 months of storage. These results are correlated with the lysine evolution (Table 2).

The European Commission (96/4/EC) (1996) establishes that the basic composition of IF, reconstituted following the manufacturer's instructions, must have an energetic value for each essential and semi-essential amino acid equal to the content in the human milk, in the case of lysine, 122 mg 100 $kcal^{-1}$ (available lysine).

Table 3 Hydroperoxides content in stored infant formulae

	Peroxide values (meq O_2 kg^{-1}) [z]		
Storage (months)	**Supplemented infant formula 25 ºC**	**Supplemented infant formula 37 ºC**	**Control infant formula 25 ºC**
0	0.85 ± 0.03 $^{a\text{-}I\text{–}1}$	0.85 ± 0.03 $^{a\text{-}I}$	0.55 ± 0.05 $^{a\text{–}1}$
3	1.12 ± 0.05 $^{a\text{-}I\text{–}2}$	1.22 ± 0.10 $^{a\text{-}I}$	0.63 ± 0.10 $^{a\text{–}1}$
6	1.48 ± 0.08 $^{a\text{-}I\text{–}2}$	2.36 ± 0.07 $^{b\text{-}II}$	0.76 ± 0.11 $^{a\text{–}1}$
9	1.87 ± 0.09 $^{a\text{-}I\text{–}2}$	2.84 ± 0.11 $^{b\text{-}II}$	0.82 ± 0.12 $^{a\text{–}1}$
12	2.02 ± 0.10 $^{a\text{-}I\text{–}2}$	4.28 ± 0.15 $^{c\text{-}III}$	0.83 ± 0.08 $^{a\text{–}1}$

[z]Values are expressed as mean ± standard deviation of three determinations. No coincidence in the superscript characters indicates a significant difference ($P < 0.05$).
Letters, storage time (0–12 months); Roman numerals, temperature of storage (25º vs. 37 ºC); Arabic numerals, SIF 25º vs. CIF 25 ºC.

Table 4 Lactose content in stored infant formulae

	Lactose (g 100 g^{-1} powder) [z]		
Storage (months)	**Supplemented infant formula 25 ºC**	**Supplemented infant formula 37 ºC**	**Control infant formula 25 ºC**
0	62.35 ± 2.52 $^{a\text{-}I\text{–}1}$	62.47 ± 1.32 $^{a\text{-}I}$	63.25 ± 1.15 $^{a\text{–}1}$
3	61.82 ± 2.57 $^{a\text{-}I\text{–}1}$	62.15 ± 2.11 $^{a\text{-}I}$	62.27 ± 2.01 $^{a\text{–}1}$
6	62.09 ± 1.42 $^{a\text{-}I\text{–}1}$	61.05 ± 2.04 $^{a\text{-}I}$	62.55 ± 1.72 $^{a\text{–}1}$
9	61.91 ± 2.04 $^{a\text{-}I\text{–}1}$	57.83 ± 2.17 $^{a\text{-}I}$	62.15 ± 2.05 $^{a\text{–}1}$
12	61.37 ± 2.15 $^{a\text{-}I\text{–}1}$	54.36 ± 2.14 $^{b\text{-}II}$	61.92 ± 2.15 $^{a\text{–}1}$

[z]Values are expressed as mean ± standard deviation of four determinations. No coincidence in the superscript characters indicates a significant difference ($P < 0.05$).
Letters, storage time (0–12 months); Roman numerals, temperature of storage (25º vs. 37 ºC); Arabic numerals, SIF 25º vs. CIF 25 ºC.

The two types of formulae studied complied with this regulation. Only in SIF stored at 37 ºC for 9 and 12 months did the levels of available lysine fell below this value (118.48 and 107.58 mg 100 $kcal^{-1}$, respectively). These levels represent lysine losses of about 17.72% and 25.29% compared with the initial content in fresh formulae (Table 2). However, storage at 37 ºC is uncommon. It should be noted that the average customers of IF would not consider the importance of high temperature effect on these food products. Also, certain customers looking for bargains may bulk-buy IF products to stock-up. Therefore, to ensure that the nutritive value is not lost, storage temperature must be controlled. At present, there is no legal maximum content of blocked lysine; however, the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) recommends that the amount of blocked lysine for preterm infants should be kept to a minimum (Bremer *et al.*, 1987). The evolution of available lysine in SIF and CIF throughout storage was similar, with hardly any differences observed, evidencing the benefits of this expensive technology. However, more studies on the stability of the MFO-supplemented formulae are recommended.

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PUBLICACIÓN 7

Contenido de vitaminas A y E en preparados para lactantes de base láctea en polvo, después de abierto el envase.

Chávez-Servín, J. L., Castellote, A. I., & López-Sabater, M. C. (**2007)** Vitamin A and E content in infant milk-based powdered formulae after opening the packet. *Food Chemistry,* in press.

Los contenidos de vitaminas A (acetato de retinol y palmitato de retinol) y Vitamina E (acetato de α-tocoferol, α-tocoferol, γ-tocoferol, y δ-tocoferol); provenientes tanto de los aceites (utilizados en la fabricación) que los contienen en forma natural, así como los añadidos en la industria, fueron determinados por un método en fase normal HPLC con detector DAD en 20 preparados para lactantes de inicio, compradas en locales comerciales.

La evolución de estos componentes fue seguida una vez que se abrieron los envases, a los cero, treinta y setenta días de almacenamiento a temperatura ambiente (aproximadamente 25ºC, máxima 25.5ºC y mínima 23ºC). Inmediatamente después de abrir los envases los contenidos de vitamina A se encontraban entre el rango de 0.55 a 0.99 mg RE/100 g (93.32 a 183 μg RE/100 kcal) y los de vitamina E entre 6.58 a 27.77 mg α-TE/100 g (1.36 a 5.39 mg α-TE/100 kcal). Todas las fórmulas estudiadas presentaron contenidos de vitaminas A y E superiores a los declarados en la etiqueta: El promedio del valor de adecuación de la vitamina A fue 134% ± 17, mínimo 98% y máximo 162%, mientras que el promedio de los valores de adecuación del contenido de vitamina E respecto al etiquetado fue 185% ± 47, mínimo 101% y máximo 286%, incluyendo los valores a tiempo cero, treinta y setenta días de almacenamiento. Todas las fórmulas estudiadas cubren los límites de niveles mínimos establecidos por la legislación española y europea, inclusive después de los 70 días de almacenamiento después de abierto el envase. En este trabajo, además se comentan algunos puntos de interés, relativos al contenido de estas vitaminas en los preparados para lactantes.

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

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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

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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-

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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.

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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 A in IF at 180 μg RE/100 kcal. All of our samples complied

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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

(*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
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
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.

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" (1998). 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-

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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*)

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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.

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ortification of vitamin A with retinyl acetate or retinyl palmitate ensures the vitamin A content stated on the label.

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 (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.

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Compuestos volátiles por cromatografía de gases de espacio en cabeza y perfil de ácidos grasos en preparados para lactantes comerciales de base láctea. Evolución después de abierto el envase

Chávez-Servín, J. L., Castellote, A. I., & López-Sabater, M. C. (**2007)** Volatile compounds and fattty acid profiles in cimmercial milk-based infant formulae by static headspace has chromatography. Evolution after opening the packet. *Food Chemistry,* in revision.

En este trabajo se utilizó el método de cromatografía de gases de espacio en cabeza para determinar los principales compuestos volátiles en 20 preparados para lactantes de base láctea en polvo. Asimismo, la evolución de los compuestos volátiles y el contenido de ácidos grasos en éstos una vez abiertos los envases fueron determinados durante el almacenamiento a 25ºC durante 70 días. El método utilizado no pretende realizar una investigación detallada de la caracterización de los perfiles aromáticos de cada una de las marcas comerciales de las fórmulas, determina cualitativa y cuantitativamente propanal, pentanal y hexanal como los principales compuestos volátiles presentes en las fórmulas en polvo. Estos aldehídos son compuestos secundarios de la oxidación lipídica y por lo tanto son indicadores de deterioro. Pocos cambios fueron observados en los contenidos de ácidos grasos durante el almacenamiento, pero perdidas significativas fueron encontradas en el C18:2 n-6 y el C18:3 n-3 de algunas fórmulas, mientras que los compuestos volátiles estudiados incrementaron a lo largo del almacenamiento en todas las fórmulas. De los 3 compuestos volátiles estudiados, el hexanal mostró mayores incrementos durante el almacenamiento en los envases abiertos de las fórmulas y por lo tanto, éste puede utilizarse como un indicador potencial para evaluar la estabilidad oxidativa en los preparados para lactantes. Correlaciones significativas ($p<0.05$) fueron encontradas entre el contenido de hexanal a los 70 días y la disminución del C18:2 n-6, así como del contenido de propanal a los 70 días y la disminución del PUFA n-3, específicamente de la reducción del C18:3 n-3. Los resultados muestran que la generación de los compuestos volátiles estudiados no se relacionaron directamente con la oxidación de los ácidos grasos con un número mayor de instauraciones, pues no se observaron cambios aparentes en el contenido de AA y DHA en las fórmulas que los contenían, después de 70 días de abiertos los envases. Más estudios en la formación de compuestos volátiles en los preparados para lactantes son necesarios.

Volatile compounds and fatty acid profiles in commercial milk-based infant formulae by static headspace gas chromatography. Evolution after opening the packet.

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Abstract

The evolution of volatile contents (propanal, pentanal and hexanal) and fatty acid profiles was examined in 20 infant formulae (IF) milk powders during storage at 25ºC for 70 days after opening the packets. Few changes were observed in the fatty acid content during storage, but significant losses were found in C18:2 n-6 and C18:3 n-3 of some formulae. All the three volatiles increased during storage in all formulae, confirming oxidative stability decreases once packets were opened. Significant correlation ($p<0.05$) was detected between hexanal content and oxidation of n-6 PUFA, specifically C18:2 n-6 losses, and between propanal content and oxidation of n-3 PUFA, specifically from C18:3 n-3 losses.

***Keywords*:** Infant formula powders; Volatile compounds; Propanal; Pentanal; Hexanal; Fatty acids

1. Introduction

Lipid oxidation causes quality deterioration during manufacture and storage of lipid-containing foods. In the peroxidation of unsaturated fatty acids, lipid hydroperoxides are formed during the propagation phase. These primary compounds are unstable and rapidly decompose in the presence of trace elements to give a range of new free-radicals and other non-radical compounds, including alkoxyl and alkyl radicals, aldehydes, ketones and a range of carboxyl compounds that form a complex mixture of secondary lipid oxidation products, which spoil infant formula (IF) milk powders. Traditionally, the peroxide value (POV) has been used to determine primary lipid oxidation products, and the thiobarbituric acid (TBA) assay for secondary oxidation products. Several protocols have been described for the determination of POV in milk products (Newstead & Headifen, 1981; FIL-IDF, 1991). However, with complex foods such as IFs, a previous lipid extraction is required for POV measurement and this may introduce error and increase analysis time (Perkins, 1984). In contrast, the TBA test can be applied directly to the sample. However, the

appropriateness of the TBA assay, especially when applied to milk and milk products, has been questioned (Ward, 1985). Researchers then focused their efforts on new technologies and methods that were simple, fast, reliable and sensitive for the evaluation of food lipid damage in less time and with the minimal sample treatment and/or sample alteration. Direct injection of fat into the heated injection port of a gas chromatograph and quantification of volatile substances originating from the thermal breakdown of lipid peroxides was a widely used approach for measuring rancidity in fatty foods (Dupuy, Fore & Goldblat, 1973). This idea was later modified for application to automatic samplers for static or dynamic HS-GC methods for the determination of volatile compounds produced by oxidation (Snyder, Frankel, Selke & Warner, 1988; Ulberth & Roubicek, 1993). Many studies have reported the development of off-flavours in milk at a given storage time, usually at the end of the product's shelf-life (Cormier, Raymond, Champagne & Morin, 1991; Vallejo-Cordoba & Nakai, 1994; Contarini & Pavolo, 2002).The deterioration of IF powder results in off-flavours, mainly caused by volatiles. Volatile compounds such as hexanal and pentanal have been associated with the development of undesirable flavours and are proposed as potential markers of fresh milk quality (Kim & Morr, 1996; Contarini et al., 2002; Toso, Procida & Stefanon, 2002; Marsili & Miller, 2003; Karatapanis, Badeka, Riganakos, Savvaidis & Kontominas, 2006). Concentrations of saturated aldehydes and hydrocarbons correlate well with sensory flavour (Hall & Andersson, 1985; Hall, Andersson, Lingnert & Olofsson, 1985). The so-called "cardboard-like" off-flavour is frequently associated with dehydrated milk products. This effect is highly correlated ($r = 0.89$) with the headspace concentration of hexanal (Hall et al., 1985). In stored UHT milk, concentrations of pentanal and hexanal are also related to the development of off-flavour (Rerkrai, Jeon & Bassette, 1987). Several sampling techniques are currently available for the isolation and measurement of volatile compounds, such as gas chromatography (GC)-mass spectrometry-based electronic nose, solid-phase microextraction (SPM), GC/mass spectrometry (GC/MS), vacuum distillation, simultaneous steam distillation and extraction GC, static headspace (SHS), dynamic HS/ purge and trap GC, and direct thermal desorption (Jung, Yoon, Lee & Min, 1998; Kolb, 1999; Marsili, 1999a;

Marsili, 1999b; Hardas, Danviriyakul, Foley, Nawar & Chinachoti, 2002; Cruwys, Dinsdale, Hawkes & Hawkes, 2002; Contarini et al., 2002; Fenaille, Visani, Fumeaux, Milo & Guy, 2003). The SHS technique requires minimal sample treatment and reduces artifactual volatile compound formation. The static head space gas chromatography (SHS-GC) method reported by Romeu-Nadal et al. (2004) for IF analysis is an easier, faster and more reliable method to determine the main volatile compounds in these samples.

Many milk-based IF powders are supplemented with polyunsaturated fatty acids such as arachidonic acid (C20:4, n-6) (AA) and docosahexaenoic acid (C22:6, n-3) (DHA); which are more susceptible to oxidation than linoleic acid (C18:2, n-6) (LA), and may produce undesirable flavours and odours. However, LA is the main polyunsaturated fatty acid in IFs (Ulberth & Roubicek, 1995). The content of hexanal, which is a major breakdown product of LA oxidation (Frankel, 1993), has been used to follow the course of lipid oxidation and off-flavour development in foods (Dupuy et al., 1977). Pentanal and hexanal are the specific volatile oxidation products of n-6 PUFA and propanal of n-3 PUFA (Romeu-Nadal, Castellote & Lopez-Sabater, 2004). In spite of literature related to volatile content in milk (liquid and powdered), information about these compounds in IFs is scarce. Van Ruth et al. (2005) studied the volatile profiles of 13 IFs by proton transfer reaction-mass spectrometry (PTR-MS). Fenaille et al. (2006) measured the levels of secondary lipid oxidation products (malondialdehyde and hexanal) in relation to the processing conditions of IF, pasteurized and UHT milk samples.

When stored, IFs are usually protected from light and maintained at room temperature. However, because of the long storage life of these powders (usually 2 years), PUFAs can be oxidized, giving rise to a loss of nutritive value and to the generation of volatile compounds from peroxides. UV light induces lipid oxidation, therefore milk products such as IFs, once opened, are highly susceptible to lipid oxidation at room temperature, and light accelerates this process (Hardas, Danviriyakul, Foley, Nawar & Chinachoti, 2000). There is a lack of information on the concentration and evolution of volatile compounds in commercial IFs. According to the

manufacturers' instructions, once opened IFs should be used within a month. Generally, they are consumed before this time. However, when IFs are used for complementary feeds, the product could be stored longer. Therefore it would be of interest to study the evolution of volatiles not only during the one-month's life once opened, but also after this time, extending the analysis, for example, until 70 days after opening the packets, a period greater than double the established time for formula consumption.

Here we measured and analysed the quantity of propanal, pentanal and hexanal in several brands of milk-based IF, as potential indicators of lipid oxidation and consequently formula decomposition. For this purpose we used SHS-GC, a simple and sensitive method developed in our laboratory (Romeu-Nadal et al., 2004). In addition, we evaluated oxidative stability of IFs by examining the evolution of the contents of these volatiles and the fatty acid profiles during the 70 days after opening the packets. It is hypothesized that oxidative stability decreases quickly once packets are opened as a result of product exposure to the action of oxygen and light, and that IFs with major contents of LC-PUFAs generate more volatiles due to their major susceptibility to oxidation. Correlations between fatty acid losses and volatiles increased were done.

2. Materials and methods

2.1. Samples

Twenty commercial milk-based powdered IFs of recognized brands were purchased from several markets. Table 1 indicates the general composition of the studied formulae, as stated on the product label.

2.2. Storage

All IFs were opened on the same day; approximately in the 5-9 month of their shelf-lives. In addition, IFs were opened three times every day thereafter; each time the powder was stirred in the original packet to maintain uniform exposure to environment and two scoops of powder were discarded, thereby simulating normal storage and preparation. We kept the IFs at room temperature (25ºC: min 23, max 25.5), and the contents were analysed at 0, 15, 30, 50 and 70 days, respectively. The volatile compound content was determined on-line. We placed aliquots of approximately 15 g in amber glass flasks, sealed them under nitrogen and kept them at -80ºC until fatty acid measurements.

2.3. Chemicals

Deionised water was purified through a Milli-Q system (Millipore, Bedford, MA, USA). Standards of propanal (propionaldehyde, 97% of pure), pentanal (valeraldehyde, 97% of pure) and butyl acetate (99% of pure), as volatile internal standard (IS), were purchased from Aldrich (Steinheim, Germany), hexanal (98% of pure), Supelco™ 37 component fatty acid methyl esters mix, tridecanoic acid (C13:0), used as the fatty acid internal standard (IS), were purchased from Sigma (St. Louis, MO, USA). Boron trifluoride in methanol (20% w/v), n-hexane, sodium chloride and anhydrous sodium sulphate were from Merck (Darmstadt, Germany), sodium methylate was obtained from Fluka (Buchs, Switzerland) and dry methanol from Panreac (Barcelona, Spain).

2.4. Instruments

For the analysis of volatiles, we used a Shimadzu gas chromatograph system model GC-14 A, coupled to a flame ionization detector and a split-splitless injector (Shimadzu, Kyoto, Japan). We used a supelcowax™-10 fused silica capillary column (30m x 0.25 mm x 0.25 μm film thickness) from Supelco (Bellafonte, PA, USA). The aldehydes were separated isothermally at 75ºC. The

injector and detector temperatures were 185 and 200ºC, respectively; with a split ratio of 1:20, helium was used as a carrier gas at a linear velocity of 20.39 cm/sec. Data acquisition was performed in an HP GC chemstation software for Windows (Hewlett-Packard).

For the fatty acid analysis, we used a Shimadzu GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Shimadzu AOC-20i autoinjector. The fatty acid methyl esters (FAMEs) were separated on a fast capillary column (10 m × 0.10 mm i.d.), coated with a SGE-BPX70 cross-linked stationary phase (70% cyanopropyl polysilphenylene-siloxane, 0.20 μm film thickness) from SGE (SGE Europe Ltd., United Kingdom).

2.5. Analytical determinations

2.5.1. Measurement of volatile compounds

Volatile compounds in the IFs were measured by static headspace gas chromatography with a flame ionization detector (HS-GC-FID), following Romeu-Nadal et al. (2004). Approximately 500 mg of IF powder was weighed into a 10 ml headspace vial, then 2.5 ml of Milli-Q water and 0.5 ml of butyl acetate as IS (containing 1 μg/ml) were added. The vials were sealed with silicone rubber PTFE caps. Samples were homogenized by a 1 min vortex. Samples were equilibrated at 60ºC for 15 min in a 2t® vial heater model VH 6200 from Tracer (Teknokroma, Barcelona, Spain), then 500 μl of headspace volume was measured by a static headspace sampler MHS 123. Sampling time was 30 seconds. The gas volume sample was then injected in the GC system. Propanal, pentanal and hexanal were identified by comparison of retention times with those of the standards and were quantified by interpolation in a calibration curve within a range of 0.03 to 0.70 μg/ml. Analyses were performed in quadruplicate.

2.5.2. Fatty acid analysis

Fatty acid profiles were analyzed by fast GC after derivatization to FAMEs. IF samples were prepared as described López-López et al. (2002), using sodium methylate and boron trifluoride in

methanol, thereby obtaining the FAMEs in n-hexane for subsequent injection into the gas chromatograph. Fast GC analysis was carried out following Bondia-Pons (2004). Operating conditions were as follows: the split–splitless injector was used in split mode with a split ratio of 1:100. The injection volume of the sample was 1 μL. The injector and detector temperatures were kept at 250 °C and 270 °C respectively. The temperature programme was as follows: initial temperature 60ºC, increasing at 50ºC/min until 175ºC (holding 1 min), then, increasing at 20ºC/min until 250ºC (holding 0.5 min). Data acquisitions and processing were performed with the Shimadzu Chemstation software for GC systems. Analyses were carried out in triplicate.

2.6.Statistical Analysis

For statistical analysis, we used one-way analysis of variance (ANOVA), as well as multiple comparisons, using the Tukey HSD procedure for each volatile, storage time and formula brand. Pearson's correlations were done between content of volatile compounds and fatty acids. Data on volatiles and fatty acids were subjected to the principal component analysis (PCA). Statistical analysis was performed using the SPSS package for Windows version 12 (SPSS, Chicago, IL, USA). The level of statistical significance was set at 5% for all analyses.

3. Results and discussion

3.1. Volatile content

In our 20 IF samples, which differed in brand and type, the only volatiles found were propanal, pentanal and hexanal (Fig. 1). The composition data of the major components of the IFs, such as lipid, protein and carbohydrate (Table 1), were subjected to a PCA (Fig. 2a). As expected, this analysis showed that the global composition of the IF samples was very similar. Only formulae 6 and 11 showed slight differences, which were the result of lower carbohydrate content, 52.4 and 50%, respectively, compared with the remaining samples. However, the volatile contents of the

brands differed markedly (Tables 2-4); a fact that is not surprising, considering that the studied IFs came from different manufactures and contain different raw matters. In newly opened (time 0) formulae 2, 6, 9, 12, 13, 17 and 20, we did not detect propanal (Table 2), while the remaining IFs registered values between 0.420 – 1.717 mg/kg. However, at 70 days of storage, all IFs showed between 0.319 and 3.450 mg/Kg of propanal. This compound is a product of the oxidation of n-3 polyunsaturated fatty acids. In spite of the variability of the results, a clear increase was appreciated.

Pentanal was not detected in seven (2, 4, 6, 9, 12, 13 and 20) of the twenty formulae at time 0, and in one (IF 20) of the 20 after 15 days: thereafter it increased like propanal (Table 3). In the remaining 13 newly opened IFs, pentanal content ranged from 0.250 to 1.719 mg/kg and increased constantly during storage to 0.025 – 4.739 mg/kg after 70 days.

Finally, hexanal content, which was not found in four IFs (4, 9, 12 and 13) at time 0, (Table 4) ranged from 0.026 to 5.109 mg/kg. However, hexanal content increased markedly during storage, to 0.345 mg/kg – 14.671 mg/kg at 70 days. The increase in these three volatiles is consistent with the findings of other studies, e.g. Karatapanis et al., (2006), which evaluated the changes in volatiles of whole pasteurized milk during storage. These authors did not report the concentrations of these compounds but showed only increasing/decreasing trends for each compound, which they related to storage under fluorescent light for 7 days. Ulberth et al. (1995) monitored oxidative deterioration of milk powder by HS-GC and reported hexanal (from 10 μg/kg to 106 μg/kg) as the main volatile during storage at room temperature under direct light for 130 days at 30ºC. They also reported headspace volatiles in a milk-based IF stored in air at 40ºC, with the major compounds, in order of amount, being hexanal, heptanal and pentanal. Kim and Morr (1996) used dynamic HS to monitor volatile compounds in commercial fluid milk stored in Pyrex test tubes and exposed to fluorescent light. In their study the major volatiles were hexanal, pentanal, dimethyl disulfide, 2-butanone and 2-propanol. Earlier reports (Cadwallader & Howard, 1997; Cladman, Scheffer, Goodrich & Griffiths, 1998) also show that the most common volatile compounds in light-oxidized milk were pentanal and hexanal. Although the profile of volatiles in IFs differ, these two compounds were

detected in our IF samples. The concentrations of the volatiles measured after 70 days of storage were submitted to PCA (Fig. 2c). Hexanal, pentanal and propanal were used as variables. The two extracted components explained 95.93% of the variance (cumulative percentage). Component 1 explained 82.42% of the variance, and in the graph (Fig. 2c) one group of samples is located near to the x axis on the left side, with values of the y axis close to zero. The first extracted group (IFs 1, 2, 3, 6, 8, 9, 12, 13, 15, 17 and 20) showed lower concentrations of volatiles in comparison with the remaining IFs (5, 7, 10, 11, 14, 16, 18 and 19). This graph shows that IF 4 is separate from the two groups as a result of exhibiting the highest concentration of propanal (11.03 mg/Kg). IF 4 showed a significant reduction ($p<0.05$) of n-3 PUFA comparing time 0 vs. 70 days (Table 5), and propanal formation is consistent with this observation.

Pentanal and hexanal are the specific volatile oxidation products of n-6 PUFA. In all formulae the volatile content increased, especially hexanal, which is a more sensitive indicator of lipid oxidation. Gaafar (1991) determined volatiles in UHT milk stored for 24 weeks at room temperature using direct static HS-GC method and reported hexanal levels up to 100 μg/kg. In another study on unspecified milk-based products purchased from supermarkets, hexanal values of 240 – 290 μg/kg were recorded by dynamic HS-GC (Park & Goins, 1992). Also, in fresh whole milk powder, thin-layer chromatographic analysis of derivatised carbonyl components detected an increase in hexanal from 90 μg/kg to 1190 μg/kg for a storage period of 16 months at 37ºC in air (Boon, Keen & Walker, 1976). Reported hexanal concentrations in stored milk-products vary considerably, depending among other factors, on the analytical technique used, raw material, manufacturing conditions, formula composition, metal ion content, packaging materials as well as time and storage conditions (Bassette & Jeon, 1983). Therefore the use of the same analytical technique for volatile measurements in several IF types, is a good starting point for future comparisons of these compounds.

Flavour strongly affects the infant acceptance of IF (Mennella & Beauchamp, 1998; Mennella, Griffin & Beauchamp, 2004). IFs are generally well packed. Packaging (can, bag) directly prevents

the development of light-induced off-flavour and lipid oxidation by protecting the product from both light and oxygen (Schroder, Scott, Bland & Bishop, 1985; Vassila, Badeka, Kondyli, Savvaidis & Kontominas, 2002; Karatapanis et al., 2006). We assumed that once IF packets are opened, the powder is exposed to light and oxygen and therefore is more susceptible to oxidative reactions. This hypothesis was confirmed by the constant production of propanal, pentanal and, more markedly, hexanal, in the formulae. This observation corroborates the contribution of oxygen and oxidation reactions to the development of volatiles in IFs. Although the differences in volatile increases were not always statistically significant, they may nevertheless have been sufficient to generate sufficient off-flavours to render the product unacceptable.

The formation of light-activated flavour in milk and dairy products is due to photosensitized oxidation of lipids and amino acid sulphur groups. Light, oxygen and substrate are required for this reaction (Foote, 1968; Foote, 1991; Kim et al., 1996). The development of light-activated flavour depends on the wavelength and intensity of the light, exposure time, product temperature and the light transmission properties of the container. The IFs we tested were packed in cans or bags and were therefore protected from light and sunlight and had a controlled atmosphere, containing approximately >0.2% of residual oxygen.

To show the overall behaviour of the volatiles found, we plotted a graph which presents the average of volatiles detected in the IFs along storage. Although great variability in this content was detected, the graph is an easy way to show the overall evolution of these compounds, independently of brand and composition of formulae (Fig 3). We determined the POV in ten IF samples through storage and related these values to headspace hexanal content. Low correlations were found when lineal data (r^2= 0.467) and logarithmically transformed data (r^2= 0.667) were used. Ulberth et al. (1995) reported a highly significant relationship between hexanal content and the POV (r^2 = 0.931) when these values where logarithmically transformed. This finding can be attributed to the fact that they used a steam-distillation HS-GC procedure. In this technique, not only is the free aldehyde content of the headspace gas above the sample determined, but also the aldehyde content resulting

from the thermally induced breakdown of lipid peroxides. Therefore, a high relationship between hexanal content and POV was found. The method used in our study is presented as a rapid way to evaluate the extent of oxidation of IFs by determining free aldehyde content.

3.2. Fatty acid profiles

Fatty acid profiles were determined at times 0, 15, 30, 50 and 70 days after opening IF packets. The concentrations recorded were submitted to a PCA (Fig. 2b), using as data variables: MUFA, PUFA, LC-PUFA, n-3 PUFA, n-6 PUFA, n-6 LC-PUFA and SFA contents. The two extracted components explained 71.05% of the variance (cumulative percentage). The IFs showed similar compositions, except IFs 4, 8, and 12, and 11, 15 and 16 respectively in the graph (Fig. 2b). IFs could also be differentiated on the basis of whether they were supplemented with AA and DHA. Due to the low differences between fatty acids over time, we report only the initial fatty acid profiles (Table 5). Unexpectedly, only two of fatty acids studied exhibited statistical significant decreases at the end of the study, the first being the C18:2 n-6 in nine formulae: IF5 (from 16.48% to 16.38%), IF7 (from 16.79% to 16.42%), IF11 (from 16.89% to 16.75%), IF12 (from 11.20% to 10.96%), IF14 (from 18.59% to 18.41%), IF15 (from 18.34% to 18.26%), IF16 (from 21.14% to 21.06%), IF19 (from 17.48% to 17.32%) and IF20 (from 16.29% to 15.98%), in average 0.18% ± 0.10 of decreases in this fatty acid. The other was the C18:3 n-3 in three formulae: IF4 (from 1.72% to 1.58%), IF7 (from 1.38% to 1.31%) and IF12 (from 1.72% to 1.57%).

PUFAs, in particular, are easily attacked by free radicals that react with their double bonds, thereby yielding several products such as short-chain aldehydes. The major susceptibility of fatty acids to oxidation is thought to be directly dependent on their degree of unsaturation, and thus DHA, EPA and AA, which are the most unsaturated FAs in IFs, would be more susceptible to oxidation. The major saturated fatty acid (SFA) in our IFs was C16:0, in the range 17.62-36.15%, the major monounsaturated fatty acid (MUFA) was C18:1 n-9 in the range 26.81-44.11%, and the major LC-PUFA was C18:2 n-6 in the range 10.96-21.14%. The powders that simultaneously

showed the highest concentrations of propanal, pentanal and hexanal were IFs 4, 7, 10, 14, 16 and 18 (Tables 2-4). On the basis of this observation, we expected that all these IFs were supplemented with LC-PUFA. However, this was not the case for IFs 4, 10, 14 and 16. Hexanal and pentanal arose as a result of the oxidation of n-6 PUFA. The samples with mayor n-6 PUFA content were IF 3 (17.14%), hexanal 0.527 mg/Kg; IF 7 (17.23%), hexanal 10.66 mg/Kg; IF 9 (17.14%), hexanal 6.33 mg/Kg; IF 13 (17.15%), hexanal 0.68 mg/Kg; IF 14 (18.69%), hexanal 12.84 mg/Kg; IF 15 (19.02%), hexanal 3.31 mg/Kg; IF 16 (21.27%), hexanal 16.38 mg/Kg; and IF 19 (17.88%), hexanal 10.08 mg/Kg. Therefore, no clear relation between n-6 PUFA content and hexanal increase was observed. In the same way, propanal came from n-3 PUFA. The IFs with major n-3 PUFA content were IF 4 (1.76%), propanal 11.03 mg/Kg; IF 6 (1.74%), propanal 2.01 mg/Kg; IF 8 (2.14%), propanal 1.47 mg/Kg; IF 12 (1.76%), propanal 0.33 mg/Kg; IF 15 (2.22), propanal 1.12 mg/Kg; IF 16 (1.92%), propanal 2.94 mg/Kg; and IF 17 (1.80%), propanal 1.42 mg/Kg. In the same manner, no clear relation between n-3 PUFA and propanal increase was observed. The content of volatile compounds after 70 days of storage and MUFA, SFA, n-3 PUFA and n-6 PUFA contents were used as variables for the PCA. The two extracted components explained 69.19% of the variance (cumulative percentage). IFs 4, 11 and 16 were observed to be separated from the rest (Fig. 2d). IF 4, due to the high content of propanal (as stated above), pentanal and hexanal, while formulae 11 and 16 exhibit the highest content o hexanal and n-6 PUFA. These last two formulae showed a significant reduction in n-6 PUFA content as a result of the reduction of LA content during storage (Table 5).

In general, marked increases in volatile evolution with no obvious change in fatty acid profiles were observed. However, we noted a significant reduction ($p<0.05$) of C18:2, n-6 (the major n-6 PUFA in IFs) in IFs 5, 7, 11, 12, 14, 16, 19 and 20. A positive relationship between propanal content and the difference from initial minus final value of n-3 PUFA was observed (Table 6), indicating that the increase in propanal is directly related to the oxidation of n-3 PUFA, specifically from C18:3, n-3. Also a significant positive correlation between the difference from the initial to

final C18:2, n-6 and hexanal content was observed. This finding indicates that the hexanal content derives mostly from the degradation of C18:2, n-6 fatty acid.

4. Conclusions

Significant losses were found in C18:2 n-6 and C18:3 n-3 of some formulae; however, no significant decreases were observed in the others, including the C20:4 n-6 and C22:6 n-3. Contrary to expectations, the observed stability of the fatty acid profiles in the studied formulae 70 days after opening the packets, [even in the cases that were found significant losses], is noteworthy due to the very small differences between a given fatty acid at the beginning and the end of this study. To gain better information on volatile formation and evolution in IFs, the same basal IF composition should be used, varying only in the fatty acid content. Hexanal could be used as a rapid indicator of formula oxidation. This aldehyde is proposed as a potential marker to evaluate freshness and overall oxidation quality in IFs.

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Table 1
Composition of infant milk-based formulae as stated on product label

Formula	Lipid[a]	Protein[a]	Carbohydrate[a]	Main ingredients[b]
1	23.1	12.8	58.3	Skim milk, lactose, starch, palm olein, demineralised whey milk, colza, coconut and sunflower oils, soybean lecithin.
2	24.0	12.5	58.6	Demineralised whey milk, skim milk, palm olein, maltodextrin, colza, palm and corn oils, soybean lecithin.
3	24.0	12.5	58.6	Demineralised whey milk, palm olein, starch, skim milk, corn syrup, colza, coconut and corn oils.
4	24.0	12.0	58.7	Demineralised whey milk, palm oil, skim milk, maltodextrins, vegetal oils (colza, sunflower).
5	24.5	11.0	59.7	Skim milk, vegetal fat matters (palm, coconut, colza and sunflower), demineralised whey milk, maltose, maltodextrins, soybean lecithin.
6	27.5	13.9	52.4	Skim milk, lactose, glucose syrup, vegetal oils (palm, colza, corn and coconut).
7	28.0	11.6	56.0	Demineralised 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	Demineralised whey milk, milk powder (partially demineralised), 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	Demineralised whey milk, vegetable oils (palm, coconut, soybean), lecithin.
12	23.9	11.2	60.0	Milk protein, skim milk, whey protein milk, vegetable oils (palm, coconut, colza, sunflower), lactose, carob flour, glucose syrup.
13	24.0	12.5	55.6	Demineralised whey milk, palm olein, starch, skim 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, skim 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	Skim milk, lactose, vegetal oils, fractionated milk protein (α-lactoalbumin), soybean lecithin, LC-PUFA (arachidonic and docosahexaenoic acid).
19	26.0	9.5	58.0	Skim 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, skim milk, maltodextrin, serum protein, egg phospholipids.

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

Table 2
Evolution of propanal in infant milk-based formulae once opened and storage at 25ºC

	Storage (days)				
	0	15	30	50	70
Formula	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
1	0.608 ± 0.002^{a}	0.766 ± 0.039^{b}	0.971 ± 0.002^{c}	1.031 ± 0.025^{c}	1.439 ± 0.105^{d}
2	ND^{a}	ND^{a}	ND^{a}	ND^{a}	1.124 ± 0.173^{b}
3	0.428 ± 0.006^{a}	0.429 ± 0.023^{a}	0.518 ± 0.018^{a}	0.516 ± 0.005^{a}	0.500 ± 0.002^{a}
4	0.665 ± 0.004^{a}	3.927 ± 0.022^{b}	8.7 ± 0.061^{c}	9.683 ± 0.009^{d}	11.031 ± 0.395^{e}
5	1.163 ± 0.029^{a}	1.714 ± 0.045^{b}	2.666 ± 0.154^{c}	2.520 ± 0.291^{c}	3.242 ± 0.040^{d}
6	ND^{a}	0.886 ± 0.122^{b}	1.139 ± 0.115^{c}	1.565 ± 0.105^{d}	2.009 ± 0.219^{e}
7	1.717 ± 0.154^{a}	1.982 ± 0.078^{a}	2.584 ± 0.234^{b}	2.348 ± 0.258^{b}	2.646 ± 0.377^{b}
8	0.420 ± 0.015^{a}	0.667 ± 0.005^{b}	0.779 ± 0.009^{b}	0.802 ± 0.011^{c}	1.468 ± 0.126^{d}
9	ND^{a}	ND^{a}	ND^{a}	ND^{a}	0.719 ± 0.061^{b}
10	ND^{a}	1.647 ± 0.004^{a}	2.085 ± 0.055^{a}	2.247 ± 0.215^{b}	3.450 ± 0.106^{c}
11	0.746 ± 0.018^{a}	1.161 ± 0.036^{b}	1.241 ± 0.005^{b}	1.547 ± 0.085^{b}	1.934 ± 0.196^{c}
12	ND^{a}	ND^{a}	ND^{a}	0.322 ± 0.001^{b}	0.333 ±0.006 ^{b}
13	ND^{a}	ND^{a}	0.590 ± 0.009^{b}	0.321 ± 0.006^{b}	0.319 ± 0.011^{b}
14	0.852 ± 0.023^{a}	1.342 ± 0.022^{b}	1.818 ± 0.199^{c}	2.044 ± 0.448^{c}	2.199 ± 0.404^{c}
15	0.700 ± 0.049^{a}	0.696 ± 0.005^{a}	0.893 ± 0.034^{a}	1.024 ± 0.078^{b}	1.122 ± 0.083^{b}
16	1.247 ± 0.027^{a}	2.897 ± 0.039^{b}	2.848 ± 0.225^{b}	2.965 ± 0.127^{b}	2.944 ± 0.005^{b}
17	ND^{a}	ND^{a}	ND^{a}	0.329 ± 0.001^{b}	1.424 ± 0.045^{c}
18	1.787 ± 0.123^{a}	2.107 ± 0.086^{a}	2.500 ± 0.099^{b}	2.875 ± 0.064^{b}	2.883 ± 0.263^{b}
19	0.669 ± 0.001^{a}	1.447 ± 0.046^{b}	1.539 ± 0.086^{b}	1.955 ± 0.137^{c}	1.874 ± 0.094^{c}
20	ND^{a}	ND^{a}	0.285 ± 0.010^{b}	0.320 ± 0.001^{b}	0.321 ± 0.004^{b}

Values are expressed in mg/ Kg of powder as the mean ± standard deviation (n = 4). Repetitions in superscripted characters within the same file indicate no significant differences ($p<0.05$).

Table 3
Evolution of pentanal in infant milk-based formulae once opened and storage at 25ºC

	Storage (days)				
	0	15	30	50	70
Formula	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
1	0.167 ± 0.006^{a}	0.613 ± 0.013^{b}	0.777 ± 0.010^{b}	0.729 ± 0.018^{b}	1.247 ± 0.096^{c}
2	ND^{a}	0.382 ± 0.013^{b}	0.438 ± 0.042^{b}	0.489 ± 0.026^{b}	0.684 ± 0.069^{c}
3	0.250 ± 0.012^{a}	0.331 ± 0.001^{a}	0.414 ± 0.007^{a}	0.438 ± 0.019^{a}	0.405 ± 0.013^{a}
4	ND^{a}	2.175 ± 0.131^{b}	3.994 ± 0.142^{c}	4.509 ± 0.480^{d}	4.739 ± 0.547^{d}
5	1.165 ± 0.046^{a}	1.729 ± 0.018^{b}	2.157 ± 0.382^{c}	2.337 ± 0.880^{c}	2.502 ± 0.372^{c}
6	ND^{a}	1.314 ± 0.025^{b}	1.026 ± 0.088^{b}	1.543 ± 0.040^{b}	1.434 ± 0.278^{b}
7	1.719 ± 0.088^{a}	1.757 ± 0.021^{a}	1.981 ± 0.128^{a}	2.336 ± 0.026^{a}	2.270 ± 0.236^{a}
8	0.444 ± 0.028^{a}	0.615 ± 0.023^{a}	0.805 ± 0.030^{b}	0.855 ± 0.050^{b}	0.910 ± 0.085^{b}
9	ND^{a}	0.413 ± 0.010^{b}	0.437 ± 0.010^{b}	0.498 ± 0.001^{b}	0.509 ± 0.027^{b}
10	0.436 ± 0.006^{a}	0.452 ± 0.013^{a}	2.558 ± 0.241^{b}	3.109 ± 0.566^{c}	3.292 ± 0.644^{c}
11	0.559 ± 0.020^{a}	2.467 ± 0.039^{b}	4.065 ± 0.121^{c}	1.778 ± 0.172^{b}	1.678 ± 0.258^{b}
12	ND^{a}	0.301 ± 0.023^{b}	0.378 ± 0.026^{b}	0.452 ± 0.041^{b}	0.443 ± 0.018^{b}
13	ND^{a}	0.346 ± 0.022^{b}	0.528 ± 0.065^{b}	0.526 ± 0.058^{b}	0.519 ± 0.069^{b}
14	1.618 ± 0.030^{a}	3.315 ± 0.090^{b}	3.495 ± 0.010^{b}	4.041 ± 0.472^{c}	4.686 ± 0.121^{d}
15	0.477 ± 0.003^{a}	0.985 ± 0.002^{b}	1.123 ± 0.211^{b}	0.946 ± 0.176^{b}	1.257 ± 0.193^{b}
16	1.564 ± 4.478^{a}	4.478 ± 0.449^{b}	4.533 ± 0.449^{b}	3.916 ± 0.072^{b}	4.689 ± 0.315^{b}
17	0.337 ± 0.011^{a}	0.644 ± 0.006^{b}	0.576 ± 0.033^{b}	0.697 ± 0.007^{b}	0.954 ± 0.172^{b}
18	1.695 ± 0.021^{a}	2.297 ± 0.252^{b}	2.906 ± 0.481^{b}	2.797 ± 0.212^{b}	3.047 ± 0.501^{c}
19	0.296 ± 0.010^{a}	1.061 ± 0.032^{b}	1.469 ± 0.216^{b}	1.685 ± 0.124^{c}	1.539 ± 0.003^{c}
20	ND^{a}	ND^{a}	0.022 ± 0.001^{b}	0.025 ± 0.001^{b}	0.025 ± 0.001^{b}

Values are expressed in mg/ Kg of powder as the mean ± standard deviation (n = 4). Repetitions in superscripted characters within the same file indicate no significant differences ($p<0.05$).

Table 4
Evolution of hexanal in infant milk-based formulae once opened and storage at 25ºC

	Storage (days)				
	0	15	30	50	70
Formula	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
1	0.701 ± 0.026^{a}	1.086 ± 0.027^{b}	1.516 ± 0.005^{c}	1.769 ± 0.034^{d}	2.661 ± 0.076^{e}
2	0.026 ± 0.002^{a}	0.088 ± 0.005^{a}	0.129 ± 0.011^{b}	0.443 ± 0.021^{c}	0.345 ± 0.009^{c}
3	0.157 ± 0.008^{a}	0.330 ± 0.018^{a}	0.431 ± 0.007^{b}	0.437 ± 0.006^{b}	0.527 ± 0.045^{b}
4	ND^{a}	0.561 ± 0.004^{b}	4.526 ± 0.012^{c}	10.746 ± 0.497^{d}	14.671 ± 1.294^{e}
5	3.958 ± 0.178^{a}	5.512 ± 0.070^{b}	7.168 ± 0.248^{c}	7.218 ± 0.088^{c}	9.611 ± 0.211^{d}
6	0.783 ± 0.021^{a}	1.961 ± 0.177^{b}	2.318 ± 0.099^{c}	3.489 ± 0.236^{d}	3.909 ± 0.208^{d}
7	6.228 ± 0.558^{a}	9.444 ± 0.247^{b}	8.745 ± 0.996^{b}	10.918 ± 1.228^{b}	10.656 ± 1.417^{b}
8	0.511 ± 0.007^{a}	0.747 ± 0.024^{a}	0.950 ± 0.033^{b}	0.913 ± 0.107^{b}	0.953 ± 0.053^{b}
9	ND^{a}	0.343 ± 0.002^{b}	0.403 ± 0.008^{b}	0.529 ± 0.054^{b}	6.331 ± 0.213^{c}
10	0.494 ± 0.001^{a}	4.443 ± 0.376^{b}	6.667 ± 0.530^{c}	8.678 ± 0.490^{d}	13.814 ± 0.453^{e}
11	1.028 ± 0.012^{a}	1.938 ± 0.354^{b}	2.771 ± 0.323^{c}	3.945 ± 0.010^{d}	14.428 ± 0.681^{e}
12	ND^{a}	0.249 ± 0.007^{b}	0.116 ± 0.003^{b}	0.412 ± 0.035^{b}	0.612 ± 0.003^{c}
13	ND^{a}	0.259 ± 0.034^{b}	0.486 ± 0.061^{c}	0.513 ± 0.024^{c}	0.686 ± 0.003^{c}
14	3.546 ± 0.270^{a}	7.458 ± 0.120^{b}	7.337 ± 0.591^{b}	10.433 ± 0.496^{c}	12.844 ± 0.103^{c}
15	0.312 ± 0.011^{a}	1.723 ± 0.153^{b}	1.904 ± 0.230^{b}	2.333 ± 0.134^{c}	3.308 ± 0.297^{d}
16	4.091 ± 0.117^{a}	13.179 ± 0.24^{b}	12.452 ± 0.41^{b}	13.454 ± 0.403^{b}	16.382 ± 0.411^{b}
17	0.250 ± 0.019^{a}	0.991 ± 0.055^{b}	1.102 ± 0.168^{b}	1.261 ± 0.170^{c}	1.343 ± 0.059^{c}
18	5.109 ± 0.097^{a}	6.915 ± 0.774^{b}	8.823 ± 0.447^{c}	10.653 ± 0.475^{c}	11.787 ± 0.499^{c}
19	0.860 ± 0.045^{a}	4.986 ± 0.251^{b}	6.159 ± 0.316^{c}	8.564 ± 0.036^{d}	10.078 ± 0.052^{e}
20	0.665 ± 0.036^{a}	0.819 ± 0.051^{a}	0.852 ± 0.108^{a}	1.065 ± 0.048^{b}	1.095 ± 0.021^{b}

Values are expressed in mg/ Kg of powder as the mean ± standard deviation (n = 4). Repetitions in superscripted characters within the same file indicate no significant differences ($p<0.05$).

Table 5 Fatty acid profiles of the studied infant milk-based formulae immediately after opening the packet (% wt/wt)

	IF1	IF2	IF3	IF4	IF5	IF6	IF7	IF8	IF9	IF10
Fatty acids:	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd
C4:0	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	0.05 ± 0.01	Tr ± 0.00	Tr ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.59 ± 0.01
C6:0	0.05 ± 0.00	0.05 ± 0.00	0.12 ± 0.00	0.04 ± 0.00	0.13 ± 0.00	0.10 ± 0.00	0.12 ± 0.00	0.13 ± 0.03	0.03 ± 0.00	0.52 ± 0.01
C8:0	0.53 ± 0.01	0.52 ± 0.01	1.32 ± 0.03	0.06 ± 0.00	1.66 ± 0.03	1.27 ± 0.01	1.36 ± 0.01	1.67 ± 0.00	0.04 ± 0.00	0.44 ± 0.02
C10:0	0.59 ± 0.01	0.58 ± 0.01	1.15 ± 0.03	0.12 ± 0.04	1.40 ± 0.01	1.08 ± 0.00	1.12 ± 0.00	1.37 ± 0.00	0.10 ± 0.00	0.97 ± 0.01
C12:0	7.63 ± 0.22	7.56 ± 0.07	9.13 ± 0.18	0.43 ± 0.08	11.14 ± 0.13	8.99 ± 0.04	9.19 ± 0.01	11.04 ± 0.03	0.28 ± 0.00	1.33 ± 0.02
C14:0	3.08 ± 0.07	3.16 ± 0.19	4.06 ± 0.07	1.25 ± 0.14	4.71 ± 0.05	3.87 ± 0.03	3.99 ± 0.01	4.57 ± 0.01	0.94 ± 0.01	4.38 ± 0.03
C14:1	0.16 ± 0.03	0.15 ± 0.03	0.14 ± 0.02	0.11 ± 0.02	0.18 ± 0.01	0.10 ± 0.01	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	0.41 ± 0.00
C15:0	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.09 ± 0.02	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.07 ± 0.01	0.08 ± 0.01	0.41 ± 0.00
C16:0	26.30 ± 0.01	26.31 ± 0.06	23.44 ± 0.09	36.03 ± 0.19	25.20 ± 0.13	24.50 ± 0.03	21.77 ± 0.01	17.62 ± 0.07	30.72 ± 0.11	31.15 ± 0.04
C16:1.n-7	0.18 ± 0.01	0.18 ± 0.00	0.25 ± 0.03	0.34 ± 0.08	0.20 ± 0.05	0.20 ± 0.01	0.20 ± 0.00	0.27 ± 0.03	0.26 ± 0.04	0.92 ± 0.06
C17:0	0.09 ± 0.00	0.09 ± 0.00	0.11 ± 0.02	0.14 ± 0.03	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.02	0.11 ± 0.03	0.13 ± 0.05	0.29 ± 0.00
C17:1	0.06 ± 0.01	0.06 ± 0.00	0.08 ± 0.00	0.09 ± 0.02	0.07 ± 0.00	0.06 ± 0.01	0.05 ± 0.00	0.07 ± 0.00	0.07 ± 0.03	0.13 ± 0.01
C18:0	3.91 ± 0.06	3.89 ± 0.03	3.35 ± 0.04	3.55 ± 0.13	3.26 ± 0.01	2.92 ± 0.03	3.98 ± 0.00	3.16 ± 0.02	3.54 ± 0.15	5.97 ± 0.02
C18:1.n-9	40.16 ± 0.27	40.17 ± 0.09	37.66 ± 0.19	43.93 ± 0.86	33.24 ± 0.14	40.00 ± 0.09	38.42 ± 0.03	43.44 ± 0.11	43.50 ± 0.06	37.10 ± 0.06
C18:2.n-6	14.78 ± 0.05	14.75 ± 0.05	16.98 ± 0.05	11.20 ± 0.34	*16.48 ± 0.04	14.07 ± 0.05	*16.79 ± 0.03	12.92 ± 0.01	17.76 ± 0.13	12.93 ± 0.01
C18:3.n-6	0.04 ± 0.00	0.04 ± 0.00	0.13 ± 0.00	0.04 ± 0.01	0.05 ± 0.00	0.10 ± 0.00	0.07 ± 0.00	0.15 ± 0.01	0.09 ± 0.00	0.05 ± 0.00
C18:3.n-3	1.60 ± 0.02	1.60 ± 0.01	1.24 ± 0.01	*1.72 ± 0.04	1.34 ± 0.01	1.71 ± 0.01	*1.38 ± 0.00	1.82 ± 0.01	1.32 ± 0.02	1.44 ± 0.01
C20:0	0.33 ± 0.01	0.34 ± 0.02	0.29 ± 0.01	0.30 ± 0.04	0.25 ± 0.01	0.30 ± 0.01	0.26 ± 0.00	0.29 ± 0.01	0.34 ± 0.01	0.26 ± 0.01
C20:1.n-9	0.27 ± 0.01	0.27 ± 0.00	0.28 ± 0.00	0.28 ± 0.02	0.24 ± 0.01	0.35 ± 0.01	0.15 ± 0.01	0.38 ± 0.00	0.31 ± 0.00	0.29 ± 0.01
C20:2.n-6	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.06 ± 0.00
C20:3.n-6	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	0.03 ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00
C20:4.n-6	ND ± 0.00	ND ± 0.00	ND ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.31 ± 0.00	0.08 ± 0.00	ND ± 0.00	0.04 ± 0.00
C22:0	0.11 ± 0.01	0.11 ± 0.00	0.13 ± 0.01	0.08 ± 0.01	0.15 ± 0.01	0.10 ± 0.01	0.29 ± 0.00	0.26 ± 0.00	0.19 ± 0.00	0.14 ± 0.00
C20:5.n-3	ND ± 0.00	ND ± 0.00	ND ± 0.00	ND ± 0.00	ND ± 0.00	ND ± 0.00	0.05 ± 0.01	0.04 ± 0.00	ND ± 0.00	ND ± 0.00
C24:0	0.09 ± 0.01	0.09 ± 0.00	0.08 ± 0.00	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.14 ± 0.00	0.13 ± 0.00	0.11 ± 0.00	0.07 ± 0.00
C22:6.n-3	ND ± 0.00	ND ± 0.00	ND ± 0.00	ND ± 0.00	ND ± 0.00	ND ± 0.00	0.16 ± 0.00	0.29 ± 0.00	ND ± 0.00	ND ± 0.00
(1) SFA	42.73 ± 0.24	42.75 ± 0.10	43.22 ± 0.16	42.20 ± 0.30	48.11 ± 0.09	43.35 ± 0.04	42.36 ± 0.01	40.46 ± 0.07	36.52 ± 0.11	46.53 ± 0.02
(2)MUFA	40.82 ± 0.30	40.83 ± 0.07	38.40 ± 0.20	44.75 ± 0.80	33.93 ± 0.10	40.71 ± 0.10	38.82 ± 0.03	44.21 ± 0.08	44.24 ± 0.05	38.91 ± 0.02
(3) PUFA	16.45 ± 0.07	16.42 ± 0.05	18.38 ± 0.05	13.05 ± 0.50	17.96 ± 0.04	15.94 ± 0.06	18.82 ± 0.02	15.33 ± 0.01	19.24 ± 0.15	14.57 ± 0.00
n-3 PUFA	1.60 ± 0.02	1.60 ± 0.01	1.24 ± 0.01	*1.76 ± 0.05	1.39 ± 0.01	1.74 ± 0.01	*1.59 ± 0.01	2.14 ± 0.01	1.36 ± 0.01	1.49 ± 0.01
n-6 PUFA	14.85 ± 0.05	14.82 ± 0.05	17.14 ± 0.05	11.29 ± 0.36	*16.57 ± 0.04	14.20 ± 0.05	*17.23 ± 0.03	13.18 ± 0.01	17.88 ± 0.14	13.08 ± 0.01
n-3 LC-PUFA	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	0.05 ± 0.00	0.03 ± 0.00	0.21 ± 0.01	0.32 ± 0.00	0.04 ± 0.01	0.04 ± 0.00
n-6 LC-PUFA	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.06 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.37 ± 0.00	0.12 ± 0.01	0.03 ± 0.01	0.10 ± 0.00

Values are the mean of three measurements. * = significant difference ($p<0.05$) between immediately after opening the packet (T0) vs. 70 days after opening the packet. (1) Saturated fatty acids, (2) monounsaturated fatty acids, (3) polyunsaturated fatty acids; (1+2+3= 100% of the fatty acids studied). ND = not detected. Tr = Traces <0.01%

Table 5 (continue)

	IF11	IF12	IF13	IF14	IF15	IF16	IF17	IF18	IF19	IF20
Fatty acids:	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd
C4:0	Tr ± 0.00	0.05 ± 0.01	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	0.05 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	Tr ± 0.00	Tr ± 0.00
C6:0	0.17 ± 0.00	0.04 ± 0.00	0.12 ± 0.00	0.11 ± 0.00	0.11 ± 0.00	0.13 ± 0.01	0.12 ± 0.00	0.13 ± 0.00	0.10 ± 0.01	0.06 ± 0.00
C8:0	2.00 ± 0.01	0.06 ± 0.00	1.35 ± 0.04	1.36 ± 0.02	1.34 ± 0.02	1.37 ± 0.03	1.24 ± 0.01	1.36 ± 0.02	1.40 ± 0.06	0.49 ± 0.01
C10:0	1.61 ± 0.01	0.12 ± 0.04	1.18 ± 0.04	1.08 ± 0.01	1.08 ± 0.00	1.13 ± 0.00	1.06 ± 0.01	1.13 ± 0.00	1.20 ± 0.04	0.54 ± 0.01
C12:0	13.31 ± 0.10	0.43 ± 0.08	9.33 ± 0.39	9.00 ± 0.07	9.24 ± 0.03	9.00 ± 0.01	8.35 ± 0.05	9.04 ± 0.03	9.76 ± 0.29	6.68 ± 0.09
C14:0	5.55 ± 0.07	1.25 ± 0.14	4.12 ± 0.16	3.94 ± 0.04	4.15 ± 0.00	4.07 ± 0.01	3.78 ± 0.02	4.09 ± 0.04	4.12 ± 0.09	2.78 ± 0.05
C14:1	Tr ± 0.00	0.11 ± 0.02	0.14 ± 0.03	Tr ± 0.00	Tr ± 0.00	0.04 ± 0.00	Tr ± 0.00	Tr ± 0.00	0.15 ± 0.01	0.16 ± 0.01
C15:0	0.04 ± 0.00	0.09 ± 0.02	0.05 ± 0.00	0.06 ± 0.00	0.12 ± 0.07	0.12 ± 0.00	0.11 ± 0.00	0.16 ± 0.04	0.03 ± 0.00	0.04 ± 0.00
C16:0	27.06 ± 0.10	36.03 ± 0.19	23.29 ± 0.06	22.47 ± 0.04	22.58 ± 0.04	23.71 ± 0.05	25.47 ± 0.04	21.88 ± 0.05	21.74 ± 0.02	25.52 ± 0.78
C16:1.n-7	0.31 ± 0.01	0.34 ± 0.08	0.26 ± 0.03	0.36 ± 0.08	0.33 ± 0.10	0.35 ± 0.02	0.38 ± 0.13	0.38 ± 0.06	0.12 ± 0.02	0.32 ± 0.04
C17:0	0.14 ± 0.01	0.14 ± 0.03	0.10 ± 0.01	0.19 ± 0.12	0.23 ± 0.01	0.10 ± 0.00	0.14 ± 0.08	0.24 ± 0.04	0.06 ± 0.01	0.09 ± 0.01
C17:1	0.08 ± 0.00	0.09 ± 0.02	0.08 ± 0.00	0.13 ± 0.01	0.13 ± 0.07	0.11 ± 0.04	0.11 ± 0.06	0.19 ± 0.04	0.04 ± 0.00	0.03 ± 0.00
C18:0	3.88 ± 0.12	3.55 ± 0.13	3.36 ± 0.05	3.83 ± 0.15	3.73 ± 0.07	3.91 ± 0.02	3.60 ± 0.06	4.11 ± 0.03	3.67 ± 0.07	3.82 ± 0.20
C18:1.n-9	26.81 ± 0.03	43.93 ± 0.86	37.49 ± 0.51	36.35 ± 0.14	34.85 ± 0.08	32.05 ± 0.03	37.48 ± 0.16	37.94 ± 0.08	37.55 ± 0.42	40.93 ± 0.43
C18:2.n-6	*16.89 ± 0.02	*11.20 ± 0.04	16.98 ± 0.02	*18.59 ± 0.08	*18.34 ± 0.15	*21.14 ± 0.04	15.11 ± 0.11	16.48 ± 0.10	*17.48 ± 0.04	*16.29 ± 0.03
C18:3.n-6	0.08 ± 0.00	0.04 ± 0.01	0.13 ± 0.00	0.07 ± 0.01	0.10 ± 0.01	0.09 ± 0.00	0.12 ± 0.01	0.06 ± 0.00	0.03 ± 0.00	0.08 ± 0.00
C18:3.n-3	1.48 ± 0.02	*1.72 ± 0.04	1.23 ± 0.00	1.50 ± 0.02	1.90 ± 0.01	1.86 ± 0.01	1.57 ± 0.01	1.40 ± 0.01	1.19 ± 0.01	1.00 ± 0.02
C20:0	0.23 ± 0.01	0.30 ± 0.04	0.28 ± 0.04	0.27 ± 0.01	0.30 ± 0.01	0.27 ± 0.02	0.29 ± 0.00	0.25 ± 0.02	0.24 ± 0.01	0.26 ± 0.01
C20:1.n-9	0.13 ± 0.00	0.28 ± 0.02	0.27 ± 0.01	0.17 ± 0.00	0.28 ± 0.00	0.16 ± 0.00	0.37 ± 0.00	0.17 ± 0.00	0.13 ± 0.00	0.27 ± 0.00
C20:2.n-6	Tr ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.00	0.03 ± 0.00	Tr ± 0.00	Tr ± 0.00	0.02 ± 0.00	0.03 ± 0.00
C20:3.n-6	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	0.03 ± 0.00	0.01 ± 0.00
C20:4.n-6	ND ± 0.00	0.01 ± 0.00	ND ± 0.00	ND ± 0.00	0.54 ± 0.00	ND ± 0.00	0.21 ± 0.00	0.32 ± 0.00	0.31 ± 0.00	0.13 ± 0.00
C22:0	0.11 ± 0.00	0.08 ± 0.01	0.12 ± 0.01	0.22 ± 0.00	0.18 ± 0.00	0.15 ± 0.00	0.16 ± 0.00	0.29 ± 0.00	0.27 ± 0.01	0.25 ± 0.00
C20:5.n-3	ND ± 0.00	ND ± 0.00	ND ± 0.00	ND ± 0.00	0.05 ± 0.00	ND ± 0.00	0.04 ± 0.00	0.07 ± 0.00	0.04 ± 0.01	0.02 ± 0.00
C24:0	0.07 ± 0.00	0.06 ± 0.01	0.08 ± 0.01	0.11 ± 0.00	0.12 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.12 ± 0.04	0.12 ± 0.01	0.11 ± 0.00
C22:6.n-3	ND ± 0.00	ND ± 0.00	ND ± 0.00	ND ± 0.00	0.27 ± 0.00	ND ± 0.00	0.19 ± 0.00	0.16 ± 0.01	0.17 ± 0.00	0.07 ± 0.00
(1) SFA	54.17 ± 0.02	42.20 ± 0.30	43.39 ± 0.48	42.64 ± 0.18	43.18 ± 0.09	44.11 ± 0.03	44.43 ± 0.11	42.83 ± 0.08	42.72 ± 0.39	40.65 ± 0.67
(2)MUFA	27.33 ± 0.02	44.75 ± 0.80	38.23 ± 0.48	37.12 ± 0.07	35.58 ± 0.10	32.70 ± 0.01	38.33 ± 0.03	38.69 ± 0.02	38.00 ± 0.43	41.70 ± 0.42
(3) PUFA	18.50 ± 0.01	13.05 ± 0.50	18.38 ± 0.02	20.24 ± 0.12	21.24 ± 0.16	23.19 ± 0.04	17.24 ± 0.12	18.48 ± 0.10	19.28 ± 0.06	17.65 ± 0.25
n-3 PUFA	1.53 ± 0.02	*1.76 ± 0.05	1.23 ± 0.00	1.55 ± 0.02	2.22 ± 0.00	1.92 ± 0.00	1.80 ± 0.01	1.63 ± 0.01	1.40 ± 0.02	1.09 ± 0.02
n-6 PUFA	*16.97 ± 0.02	*11.29 ± 0.06	17.15 ± 0.02	*18.69 ± 0.10	*19.02 ± 0.16	*21.27 ± 0.04	15.44 ± 0.11	16.85 ± 0.10	*17.88 ± 0.04	*16.56 ± 0.04
n-3 LC-PUFA	0.05 ± 0.00	0.04 ± 0.01	Tr ± 0.00	0.05 ± 0.01	0.31 ± 0.00	0.06 ± 0.00	0.23 ± 0.00	0.23 ± 0.01	0.21 ± 0.01	0.09 ± 0.00
n-6 LC-PUFA	Tr ± 0.00	0.06 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.58 ± 0.00	0.03 ± 0.00	0.21 ± 0.00	0.32 ± 0.00	0.36 ± 0.00	0.18 ± 0.00

Values are the mean of three measurements. * = significant difference ($p<0.05$) between immediately after opening the packet (T0) vs. 70 days after opening the packet. (1) Saturated fatty acids, (2) monounsaturated fatty acids, (3) polyunsaturated fatty acids; (1+2+3= 100% of the fatty acids studied). ND = not detected. Tr = Traces <0.01%

Table 6
Pearson's correlation coefficients between selected fatty acids and volatile contents after storage
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Fatty acids a-b[d]	Hexanal	Pentanal	Propanal
C17:1	0.001	-0.006	-0.031
C18:0	0.069	0.135	0.153
C18:1 n-9	0.022	-0.055	-0.130
C18:2 n-6	*0.499	0.185	0.305
C18:3 n-6	0.021	0.149	0.253
C18:3 n-3	0.127	0.235	*0.544
C20:0	0.039	0.265	0.115
C20:1 n-9	0.018	0.247	0.116
C20:4 n-6	0.042	0.105	0.024
C20:5 n-3	-0.129	-0.012	-0.025
C22:6 n-3	-0.070	0.030	-0.009
SFA	0.140	0.286	0.207
MUFA	-0.257	-0.395	-0.349
PUFA	0.221	0.201	0.382
n-3 PUFA	0.145	0.267	*0.543
n-6 PUFA	0.406	0.178	0.310
n-3 LC-PUFA	-0.129	-0.003	-0.040
n-6 LC-PUFA	-0.022	-0.076	-0.100

* Significant Pearson's correlation at level $p<0.05$. [d] = difference resulting from initial fatty acid content (a) minus final value of fatty acid content (b).

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Análisis de los contenidos de vitaminas A, E y C, hierro y selenio en preparados para lactantes de base láctea en polvo durante su vida útil.

Chávez-Servín, J. L., Castellote, A. I., & López-Sabater, M. C. (**2007)** Analysis of vitamins A, E and C, iron and selenium contents in infant milk-based powdered formula during full shelf-life. *Food Chemistry,* in revision.

La estabilidad de las vitaminas A, E y C, así como de los contenidos de hierro y selenio fueron determinadas durante 18 meses de almacenamiento a 25ºC y 40ºC de dos tipos de preparados para lactantes de base láctea en polvo suplementadas con ácidos grasos poliinsaturados de cadena larga (LC-PUFA). El primer tipo (IF-A) fue suplementada con vitamina A como acetato de retinol, y el segundo tipo (IF-B) como palmitato de retinol. Ambos tipos de formula fueron también suplementadas con vitamina E como acetato de α-tocoferol y vitamina C como ácido ascórbico. Las dos fórmulas estudiadas presentaron mayores contenidos de vitamina A (140 y 139%), vitamina E (109 y 198%) y vitamina C (167 y 118%) en relación al etiquetado respectivo. Asimismo presentaron valores más bajos de hierro (65 y 65.25%) y selenio (72.90 y 79.44%) de acuerdo con la respectiva etiqueta del producto. Como se esperaba, todas las vitaminas estudiadas mostraron descensos a lo largo del almacenamiento, siendo más pronunciados en las fórmulas almacenadas a 40ºC. Las pérdidas de vitamina A a 40ºC después de 18 meses de almacenamiento fueron del 27.5% en la IF-A y 29% en la IF-B, mientras que las pérdidas de la vitamina E en las mismas condiciones fueron 23.1% y 28.1%, y las de vitamina C fueron 28.4% y 48.6% respectivamente. Todas estas pérdidas justifican la sobre-fortificación de éstas vitaminas en los preparados para lactantes suplementadas con LC-PUFA. En cuanto a los contenidos de hierro y selenio, éstos permanecieron sin cambios a través del almacenamiento.

Analysis of vitamins A, E and C, iron and selenium contents in infant milk-based powdered formula during full shelf-life

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Abstract

The stability of vitamins A, E and C, and the iron and selenium content were determined in two types of long chain-polyunsaturated fatty acid (LC-PUFA) supplemented milk-based powdered infant formulas (IF) during an 18-month storage period at 25ºC and 40ºC. The first type (IF-A) was supplemented with vitamin A as retinol acetate. The second type (IF-B) was supplemented with vitamin A as retinol palmitate. Both types were also supplemented with vitamin E as α-tocopherol acetate and with vitamin C as ascorbic acid. The two formulas studied had higher vitamin A (140 and 139%), vitamin E (109 and 198%) and vitamin C (167 and 118%), but lower iron (65 and 65.25%) and selenium (72.90 and 79.44%) than those amounts declared on the label. As expected, all the studied vitamins showed decreases during storage, and these decreases were higher in formulas stored at 40ºC. The losses of vitamin A at 40ºC after 18 months of storage were 27.5% in IF-A and 29% in IF-B, while vitamin E losses in the same conditions were 23.1% and 28.1%, and vitamin C losses in the same conditions were 28.4 and 48.6%. All these losses justify the over-fortification of the aforementioned vitamins in these LC-PUFA supplemented IFs. Iron and selenium content remained unchanged throughout storage.

***Keywords*:** Infant Formula Powder; Retinols; Tocopherols; Ascorbic acid; Iron; Selenium; Storage

1. Introduction

Human milk is the ideal food for newborns. It provides all nutrient needs, such as protein, carbohydrates and lipids. Human milk contains micronutrients, namely vitamins and minerals, which are essential during the first month of a baby's life. 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), and vitamin C (L-ascorbic acid) is a water soluble vitamin. Vitamin A is essential for the maintenance of healthy vision, healthy skeletal and tooth development, cellular differentiation, proliferation and reproduction, and

integrity of the immune system (Olson, 1987; Olson, 1994; Spannaus-Martin, Cook, Tanumihardjo, Duitsman & Olson, 1998; Tanumihardjo et al., 1990). The predominant physiological function of vitamin E is its antioxidant activity. Vitamin E protects the fatty acids by interfering with the free radical reactions that can result in cellular damage. Finally, vitamin C functions in the body as a cofactor for critical enzyme systems and as a reducing agent (Levine et al., 1996). On the other hand, iron-containing compounds serve many essential biologic functions, including oxygen transport and storage (hemoglobin and myoglobin) and the generation of cellular energy as ATP via oxidative metabolism involving the iron-containing cytochrome enzymes (Yip, 1994). Finally, the biochemical importance and the essential nature of selenium are related to its presence in a number of functionally active proteins and as an essential component of glutathione peroxidase that functions to reduce lipid peroxidation, and its prooxidant role in products with low water content as IF (Driskell, Giraud, Drewel & Davy, 2006; Kim, Lora, Giraud & Driskell, 2006).

Currently, it is suggested that IF should contain long chain polyunsaturated fatty acids (LC-PUFA), similar to that of human milk. Therefore, some IF manufacturers include LC-PUFA in formula composition. Fatty acid unsaturation increases the rate of oxidation and, although the amount of LC-PUFA in supplemented formulas are low, these needs substantial antioxidant protection (Gonzalez-Corbella, Tortras-Biosca, Castellote-Bargallo & Lopez-Sabater, 1999). Tocopherols are naturally occurring lipid antioxidants that 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) and docosahexaenoic (DHA, C22:6, n-3) acids. Tocopherols, retinol and ascorbic acid are added to IFs both to improve vitamin content and to prevent lipid oxidation during manufacture and storage, thereby helping to extend product shelf life. 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 less susceptible to oxidation. The European Communities Commission (2006/141/CE) establish the respective limits of vitamins A, E and C, which can be

added to meet nutritional requirements and to guarantee the stability of the product because of their antioxidant properties. It is usual for manufacturers to add more quantity of vitamins (A, E and C) to the IFs than the indicated on the formulas' label, for compensate losses during manufacture and storage. However, the real content of those vitamins after manufacture and storage at different temperatures needs to be checked to ensure correct intake and the accuracy of the label statements. It exists lack of information about the stability of the isomers of vitamin A and E, vitamin C, iron and selenium in LC-PUFA supplemented IF powder.

The main aims of this work were: first, to survey the content of vitamin A (as retinol palmitate or retinol acetate), vitamin E (as α-tocopherol, α-tocopherol acetate, γ–tocopherol and δ-tocopherol), vitamin C (as ascorbic acid), and the iron and selenium content in two types of LC-PUFA supplemented infant milk-based powdered formulas in relation to the label statements; second, to evaluate the stability of the aforementioned micronutrients during the full shelf-life of the product in different storage conditions (25ºC and 40ºC); and, third, to study the compliance of those vitamin and mineral contents with the recently published European legislation (2006/141/CE).

2. Experimental

2.1. Reagents and Standards

The chemicals used for sample preparations were of analytical reagent grade. Hexane, methanol and ethyl acetate, all of HPLC-grade, were obtained from SDS (Peypin, France), absolute ethanol and HPLC-grade acetic acid from Panreac (Barcelona, Spain), meta-phosphoric acid, and standard of α-tocopherol acetate from Fluka (Buchs, Switzerland), standards of α–, γ–, and δ-tocopherol, all-trans-retinol palmitate, and retinol acetate from Sigma (St. Louis, MO, USA). The ascorbic acid standard was obtained from Merck (Darmstadt, Germany)

2.2. Instruments

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

For vitamin C determination, an HP liquid chromatographic system (Waldbronn, Germany) with HP 1050 pump, Waters 717 plus autosampler injector (Milford, MA, USA) and a UV-vis detector, SPD-10 AV VP (Shimadzu, Kyoto, Japan) was used. The analytical column used was a Tracer excel 120 ODSB (250 x 4.0 mm I.D., 5 μm particle size) protected with a guard cartridge (Tracer, C_{18}, 5 μm), both from Tracer (Tecknokroma, Barcelona, Spain).

2.3. Samples

The two types of LC-PUFA supplemented IF powder samples were obtained from a pilot scale food plant. The first type (IF-A) was supplemented with vitamin A (640 μg of retinol equivalents [RE]/100g) in the form of retinol acetate, and the second type (IF-B) was supplemented with vitamin A (606 μg RE/100g) in the form of retinol palmitate. Both formulas were also supplemented with vitamin E as α-tocopherol acetate, IF-A containing 25 mg α-tocopherol equivalents [TE]/100g while IF-B containing 6.1 mg α-TE /100g and vitamin C (IF-A: 60 mg/100g, IF-B: 68 mg/100g) as ascorbic acid. The formulas were packed in airtight containers flushed with nitrogen, modified atmosphere N_2/CO_2 (<2% O_2). Formula composition is reported in Table 1.

2.4. Storage

To evaluate the evolution of selected vitamins and minerals during the shelf life of IFs, the product was kept at 25 ºC or 40 ºC from production until 0, 1, 3, 6, 9, 12, 15 and 18 months

respectively. 25ºC constitutes the usual ambient temperature in markets and food stores, while 40ºC is a temperature which can be reached under extreme conditions in stores without air conditioning in the summer. Once the storage periods tested were completed, analytical determinations were subsequently conducted.

2.5. Vitamin A, E and C determinations

Tocopherols (α-tocopherol, α-tocopherol acetate, γ–tocopherol and δ-tocopherol) and retinol acetate or palmitate compounds in the formulas were measured according to the previously reported HPLC method (Chávez-Servín, Castellote & López-Sabater, 2006). Vitamin C content in the formulas were measured according to the HPLC method reported by Romeu-Nadal et al. (2006). All analyses were carried out in quadruplicate.

2.6. Iron and Selenium determinations

Iron and selenium analyses were determined by graphite furnace atomic absorption spectrometry as reported in Aleixo et al. (2003)

2.7. Statistical Analysis

For statistical analysis, we used one-way analysis of variance (ANOVA), as well as multiple comparisons, using the Tukey HSD procedure for each IF and storage time. We conducted statistical analysis using the SPSS package for Windows version 12 (SPSS, Chicago, IL, USA). The level of statistical significance was set at 5% for all analyses.

Results and discussion

3.1. Vitamin A content

Vitamin A activity is expressed 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, (Anonymous, 1998; Olson, 1989). Table 2 reports the values for retinol acetate or palmitate and the respective values expressed as RE, following the equivalence:

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

IFs are most commonly fortified with vitamin A in the form of retinol acetate (as IF-A) or retinol palmitate (as IF-B) because these molecules are more stable and less susceptible to oxidation than their respective isomers from vegetable oils. At the beginning of the study, in the IF-A (time 0), a value of 0.90 mg RE/100 g was found, while in the label is expressed to contain 0.64 mg RE/100 g; therefore, this represents an adequacy value of 140%. Also, in the IF-B (time 0) a value of 0.85 mg RE/100 g was found, which means an adequacy value of 139% according to the label statements (Table 2). However, a constant decrease of vitamin A (as retinol acetate and retinol palmitate) during storage was observed in both formulas. On the one hand, in the formulas stored at 25ºC, after 18 months of storage, IF-A recorded a value of 0.73 mg RE/100 g, which represents losses of 18% with respect to the initial content, and an adequacy of 114.5% according to the label. IF-B in the same conditions recorded a value of 0.67 mg RE/100 g, which represents losses of about 21% with respect the same formula at beginning of the study. On the other hand, when the formulas were stored at 40ºC, losses of vitamin A were higher than expected. IF-A showed values of 0.65 mg RE/100 g which represents losses of about 27.5% and a label adequacy of 102%, while IF-B showed values of 0.60 mg RE/100 g, showing losses of 29% and a label adequacy of 99%. In both formulas, vitamin A losses in the form of retinol acetate (IF-A) and retinol palmitate (IF-B) were observed in a similar way.

Overfortification of vitamin A is regulated due to possible toxicity from excessive intake because vitamin A is stored in the body. European legislation (2006/141/CE) establishes the limits for vitamin A in IF, the minimum being 60 μg RE / 100 Kcal and the maximum 180 μg RE / 100 Kcal. The studied formulas are between these limits even after 18 month of storage at 40ºC (Table 2). At the beginning of the study, IF-A and IF-B contained higher amounts (2.9 times and 2.77 times, respectively) than this minimum level. On the other hand IF-A showed 174.26 μg RE/100 Kcal and IF-B 166.55μg RE/100 Kcal in agreement with this upper limit.

Delgado-Zamarreño et al. (2006) reported values of vitamin A (as retinol acetate) in the range of 0.59 to 0.74 mg RE / 100 g in four types of IFs. These values were between 113% and 120% of adequacy respect to the labels' information. Similarly, Albalá-Hurtado et al. (2001) reported values of vitamin A of 0.64 to 1.06 mg RE / 100 g in starting liquid, and 0.43 to 1.09 mg RE / 100 g in starting powdered IF, so the reported values on the label were close to the values found (mean 134% ± 17) in 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).

3.2. Vitamin E content

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-tocopherol acetate = about 70% (0.7 TE / mg), β = 40% or 0.4 TE / mg, γ = 10 to 30% or 0.1 to 0.3 TE / mg, and δ = about 1% or 0.1 TE / mg. The vitamin E content is expressed as α-TE, following the formula:

α-TE mg / 100 g = α-T mg / 100 g * (1) + γ-T mg / 100 g * (0.2) + δ-T mg / 100 g * (0.1) + α-TAc mg / 100 g (0.7).

The studied formulas were supplemented with α-tocopherol acetate at two levels, IF-A (25 mg α-TE/100 g) and IF-B (6.1 mg α-TE/100 g). However IFs also contain tocopherols derived from the vegetable oils used for formula manufacturing. In both formulas, at the beginning of the study (time 0), similar quantities of α-tocopherol were observed (IF-A: 6.18 mg/100g and IF-B: 6.47 mg/100 g), as well as γ-tocopherol (IF-A: 1.62 mg/100g and IF-B: 4.90 mg/100 g), and δ-tocopherol only in IF-B (1.21 mg/100 g)was detected (Table 3). The initial value of α-TAc in IF-A was 29.75 mg/100 g, and at the end of the study, was 24.19 mg/100 g (18.7% of losses) at 25ºC and 19 mg/100 g (35.3% of losses) at 40ºC. In IF-B, a value of 6.46 mg α-TAc/100 g was recorded at the beginning of the study (time 0). After 18 month of storage at 25ºC was 5.86 mg α-TAc /100 g (9.3% of losses) and at 40ºC was 5.12 mg α-TAc /100 g (20.7% of losses) (Table 3).

Finally, according to the label, IF-A contains 25 mg α-TE/100 g, the value found being analytically 27.3 mg α-TE/100 g, which represents an adequacy value of 109.3%. However, after 18 months of storage the recorded values were 22.4 mg α-TE/100 g at 25ºC (which represent 89.7% of the stated label) and 21 mg α-TE/100 g at 40ºC (adequacy of 84%).

According to the label statement, the IF-B content is 6.1 mg α-TE/100 g, and the initial recorded value (time 0) was 12.09 mg α-TE/100 g, which represents an adequacy value of 198%. After 18 months of storage at 25 and 40ºC, recorded values were 10.5 and 8.7 mg α-TE/100 g, which represents adequacy values of 173 and 142% with respect to label information.

In formula IF-A, the over-fortification to ensure the content of the label information was not enough; however, the averages of the common contents of α-TE according to the labels in the most commercial IFs is about 5.9 α-TE/100 g (in the range 3-13 α-TE). IF-A claims a higher vitamin E content (25 mg α-TE/100 g) in comparison with the rest of common commercial IFs. IF-B showed values closer to twice the declared values (Table 3), which is consistent with other reports (Albalá-Hurtado, Veciana-Nogues, Vidal-Carou & Font, 2001; Landen et al., 1985). Albalá-Hurtado et al. (2001) reported ranges of vitamin E in starting liquid milk from 8.9 to 24.44 mg α-TE /100 g and in

starting powdered milk from 6.66 to 22.22 mg α-TE/100 g, saponificating the samples and analyzing by RP-HPLC. Delgado-Zamarreño et al. (2006) studied tocopherols in several IFs, using pressurized liquid extraction and liquid chromatography with amperometric detection and 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 IFs; the first two supplemented with α-T and the last two with α-TAc, immediately after opening packets, the mean adequacy value being 185% with respect to the composition stated on the labels. Human milk has been reported to contain between 3.0 and 5.6 mg/L (0.45 to 0.8 mg / 100 Kcal) of vitamin E (Barbas & Herrera, 1998; Bohm et al., 1997; Chappell, Francis & Clandinin, 1985). European legislation (2006/141/CE) establishes a minimum vitamin E content in IF of 0.5 mg α-TE/ g of PUFA, and never less than 0.5 mg α-TE / 100 Kcal. IF-A and IF-B have 10.62- and 4.76 times higher the minimum recommended level (Table 3). Previously no maximum limit was specified for vitamin E (96/4/CE). Nowadays (2006/141/CE, 2006) a maximum level of 5 mg α-TE / 100 Kcal is currently specified for vitamin E. IF-A presented slightly higher levels (1.06 times) than this maximum limit, and it must be adapted to the new regulation.

3.3. Vitamin C content

At the beginning of the study, in IF-A (time 0), a value of 100.40 mg/100 g of vitamin C was found, while the label stated the value to be 60 mg/100g, which represents an adequacy of 167.33%. In IF-B (time 0), a value of 80.48 mg/100 g of vitamin C was found, which means an adequacy value of 118.35% according to the composition expressed in the label information (Table 4). Following storage, constant decreases were observed in the conditions tested. For formulas stored at 25ºC after 18 months of storage, IF-A recorded a value of 79.94 mg/100 g, which represents a loss of 20.38% with respect to the initial content (adequacy to label 133.23%). In the same conditions, IF-B recorded a value of 52.46 mg/100 g of vitamin C, which means a loss of 34.82%. In this case

this value is below the label statement (adequacy of only 77.15%). IF-B showed values lower than the referred label value of 68 mg/100 g after the 9-month of storage at 25ºC. When formulas were stored at 40ºC, greater losses were observed. IF-A after 18 months of storage a value of 71.88 mg/100 g of vitamin C was recorded, which represent losses of 28.41%. However this value complies with the label statements (adequacy of 119.80%). In the same conditions in IF-B, a value of 41.33 mg/100 g of vitamin C was recorded after 18 months of storage, which represents losses of 48.65% of the vitamin C with respect to the initial content (adequacy 60.78%). After 9 months of storage at 40ºC, IF-B showed levels lower than the referred value in the product's label (Table 4). In the IF-B, overfortification of vitamin C was not enough to ensure the correctness of the label statement after 9 months of storage at 25ºC nor 40ºC.

The values found in the two formulas are in agreement with those reported by Behrens and Madere (1989) in powdered IF in the range 46.4-86.3 mg/100 g. Also, the values found are in agreement with those reported by Martin et al. (1987) in IF manufactured in the United States (15.3 ± 3.2 mg/ 100 Kcal in milk-based). Our values are slightly higher than the values reported by Esteve et al. (1995) on milk-based and soy-based infant formula with the mean of 49.28 mg/100 g and 45.27 mg/100 g, respectively, and those reported by Fontannaz et al. (2006), who found values of ascorbic acid in IF in the range 39.5-62.2 mg/100 g.

The vitamin C content of mature milk varies widely from 30 to 100 mg/L (4.5 to 15 mg/100 Kcal) (Bank, Kirksey, West & Giacoia, 1985; Bates, Prentice, Prentice & Whitehead, 1982; Byerley & Kirksey, 1985; Salmenpera, 1984; Sneed, Zane & Thomas, 1981) and decreases during the course of lactation. The current European legislation (2006/141/CE, 2006) establish a minimum of 10 mg/100 Kcal and a maximum of 30 mg/100 Kcal. In all tested conditions the studied formulas complied with this new regulation, with the exception of IF-B at 40ºC after 15 months of storage (Table 4).

3.4. Iron contents

The iron levels in IF-A in all studied conditions were between 3.51-3.95 mg/100 g (0.68-0.77 mg/100 Kcal), which represents an adequacy from 58.50-65.83% (Table 5) with respect to label information in which is specified a content of 6 mg/100 g. Values of iron in IF-B were between 3.98-4.59 mg/100 g (0.78-0.90 mg/ 100 Kcal), which represents adequacy values from 65.25-75.25% with respect to label statement. In both studied formula values of iron were lower than the values reported in labels. The found values are slightly lower than values reported by other authors in IF from Nigeria milk-based powder (8.49 ± 1.21 mg/l), United Kingdom milk-based liquid first and follow-on formulas (11.30 ± 2.26 mg/l), United States of America milk-based powder formulas (9.30 ± 0.46 mg/l) and soy-based powder formula (9.14 ± 0.29 mg/l) (Ikem, Nwankwoala, Odueyungbo, Nyavor & Egiebor, 2002).

Human milk iron content is highest in early lactation, diminishes over the course of about five months, and gradually rises thereafter (Casey, Smith & Zhang, 1995). The iron content of human milk does not appear to be affected by maternal iron status (Lonnerdal, 1986). The reported mean values for mature human milk range from 0.2 to 0.8 mg/L (0.03 to 0.12 mg/100 kcal) (Anderson, 1992; Feeley, Eitenmiller, Jones & Barnhart, 1983; Hirai et al., 1990; Lonnerdal & Hernell, 1994). However, the ability to make recommendations about the appropriate iron content of infant formulas is limited by several factors including, among others: a) the lack of an ideal standard. Unlike other nutrients, human milk cannot be used as the standard for establishing minimum iron levels for infant formulas, primarily because of evidence to indicate that exclusively breast-fed infants are at risk of iron deficiency anemia after 6 months of life, b) the bioavailability of iron from various sources including breast milk and various formulas is not known at all, c) concerns about the impact of iron on gastrointestinal function and health of the infant, d) the potential nutrient interactions make estimation of appropriate iron content of infant formulas difficult, e) the safety of "low-iron" formulas, and f) the risks associated with excessive iron intake.

The difficulty in making recommendations about iron content in infant formulas is due in part to the incomplete understanding of iron status in infants, particularly breast-fed infants. An incongruity exists between the concentrations of iron in breast milk and the infant's ability to maintain adequate iron status at least through the first six months of life. Some commercially produced "iron-fortified" formulas contain approximately 12 mg/L of iron (1.8 mg/100 kcal). Formula products labeled as "low-iron" contain between 1.3 and 4.7 mg/L (0.2 and 0.7 mg/100 kcal) of iron. The justification for low-iron products is that their use will alleviate gastrointestinal problems (mainly constipation) presumed to be associated with the iron content of conventional formulas. These perceptions have been tested empirically in a number of studies (Bradley, Hillman, Sherman, Leedy & Cordano, 1993; Hyams et al., 1995; Nelson, Ziegler, Copeland, Edwards & Fomon, 1988; Oski, 1980). Also, iron has been reputed to interact with the absorption of several other minerals including copper, zinc, and manganese (Solomons, 1986). On the other hand, a number of deleterious effects have been attributed to excessive iron intakes. For example, iron acts as a catalyst for free radical generating reactions; consequently, an excess of free iron through the enhancement of cellular oxidative reactions such as lipid peroxidation can lead to oxidative damage (Halliwell, 1994). The past Commission of the European Communities (96/4/CE, 1996) specified a minimum iron level as 0.5 mg/100 kcal and a maximum iron level of 1.5 mg/100 kcal, a value which was recently modified (2006/141/CE, 2006) to limits of 0.3 mg/ 100 Kcal and 1.3 mg/ 100 Kcal, respectively. Analysis showed that iron levels remain unchanged at the conditions tested during storage, and these levels complied with the current European legislation. It would be interesting to analyze Fe(II) and Fe(III) during storage due to the oxidant activity of iron, in order to increase understanding of oxidation reactions during storage of powder IF.

3.5. Selenium contents

Table 6 shows the selenium found in the analysed samples. Analysis showed that selenium levels remain unchanged throughout storage in the conditions tested. The selenium levels in IF-A in all tested conditions were between 6.90-7.90 μg/ 100 g (1.34-1.53 μg/ 100 Kcal), which represents adequacy values from 64.49-73.83% with respect to the label, where a content of 10.7 μg/ 100 g is specified. On the other hand, values of IF-B were between 6.80-7.55 μg/ 100 g (1.34-1.48 μg/ 100 kcal), and these are numbers which represent adequacy values from 75.56-83.89% with respect to label statements. Both types of formulas showed lower levels of selenium than the labels state. IF-A average content was 10.00 ± 0.48 μg/l and IF-B was 9.67 ± 0.27 μg/l. Using liquid chromatography hydride generation atomic fluorescence spectrometry, Viñas et al. (2005) reported selenium in starting milk, follow-on milk, and soy-based IF in the range of 4.5-11.2 μg/100 g. Lower values based on flow injection hydride atomic absorption spectrometry were reported in Spanish milk-based IF in the range 1.8-5.0 μg/l. Using inductively coupled plasma atomic emission spectrometry with hydride generator, Navarro-Blasco and Alvarez-Galindo (2004) found values in Spanish IF from 2.9 to 17.3 μg/l. L'Abbé et al. (1996) used a diamino-napthalene fluorimetric method and reported selenium in Canadian IF, finding values from 3 to 31 μg/l in unsupplemented IF and 16 to 35 μg/l in supplemented IF. Foster and Sumar (1996) determined selenium in IF available in the UK by hydride generation atomic absorption spectrometry and reported figures that ranged from 3.4 to 9.3 μg/100 g for bovine casein and whey-based powdered IF, 2.3-9.3 μg/100 g for preterm powdered formulas and 2.7-4.9 μg/100 g for hospital administered low birth weight formulas. Unfortified commercially available infant formulas marketed in the United States have been reported to contain 2.2 to 9.5 μg/L or 0.33 to 1.4 μg of selenium/100 kcal (Daniels, Gibson & Simmer, 1997; Johnson, Smith, Chan & Moyermileur, 1993; Smith, Chen & Thomas, 1995; Smith, Picciano & Milner, 1982). Van Dael and Barclay (2006) reported on selenium levels in IF by using continuous flow hydride generation atomic spectrometry in several samples of infant and follow-on

formulas from France, Germany, Spain, Netherlands, Brazil, Mexico, USA, China, India, Australia and Africa. The endogenous selenium levels found varied between 3.4 and 13.6 μg/l.

Although the data on the bioavailability of selenium from various milks fed to healthy term infants are limited, there are sufficient data to deduce that differences do exist and that they are related to the source and form of selenium in the diet (Daniels, 1996; Lonnerdal, 1985). An additional consideration is the potential for interactions between selenium and other nutrients including protein, iron, magnesium, vitamin B6, and ascorbic acid (Jimenez, Planells, Aranda, SanchezVinas & Llopis, 1997; Lonnerdal et al., 1994; Yin et al., 1991). The practical implications of these potential interactions with selenium for the establishment of recommendations for the selenium content of infant formulas are unclear at this time. However, it appears that fortification of infant formula at selenium concentrations comparable to those found in human milk will result in a selenium status equivalent to that of breast-fed infants nursed by selenium-adequate mothers.

The Commission of the European Communities (2006/141/CE, 2006) have recently specified a minimum and maximum of 1 and 9 μg/100 kcal for selenium in infant formula. The values found in the samples we studied are in agreement with this legislation.

Losses of vitamins A, E and C during storage were observed in both LC-PUFA supplemented infant formulations. In spite of the stability of the α-TAc in comparison with the tocopherol isomers (namely, α-T, γ-T and δ-T), losses of α-TAc were observed in IF-A, during storage. Vitamin C showed the greatest losses-through-storage of the studied vitamins. The iron and selenium values found remained unchanged during storage and were lower than the label statements in both formula types and should therefore be adapted. The results justify the finding that over-fortification in these IFs with vitamin A, E and C is necessary to ensure that those vitamins meet the content stated on the labels. Further studies are required in other supplemented IFs.

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Table 1

Composition of the studied formulas according to the information on the label

	Mean values per 100 g	
	IF-A	IF-B
Energetic value ((KJ / kcal)	2038 / 487	2132 / 509
Protein (g)	10	9.5
Carbohydrate (g)	53.3	58
Lactose (g)	46.1	41
Fat (g)	26	26
Arachidonic acid (mg)	47	91
Docosahexaenoic acid (mg)	25	53
Sodium (mg)	175	129
Potassium (mg)	540	401
Chloride (mg)	330	341
Calcium (mg)	420	348
Phosphorus (mg)	230	174
Magnesium (mg)	42	45
Iron (mg)	6	6.1
Zinc (mg)	6	4.5
Copper (μg)	300	273
Iodine (μg)	100	53
Selenium (μg)	10.7	9
Vitamin A (μg RE))	640	606
Vitamin D (μg)	10.3	8.3
Vitamin E (mg α-TE)	25	6.1
Vitamin K (μg)	42	38
Vitamin B1 (μg)	520	568
Vitamin B2 (μg)	620	908
Vitamin B6 (μg)	825	379
Vitamin B12 (μg)	2	1.3
Vitamin C (mg)	60	68
Niacin (mg)	6	8.3
Folic acid (μg)	42	68
Pantothenic acid (mg)	3.2	3
Biotin (μg)	16	15

Table 2. Analysis of vitamin A content in infant formulas during storage

Sample	Storage (months)		Retinyl acetate[I] or palmitate[II] (mg/100g)	RE Found (mg/100g)	Label (mg/100g)	Losses (%)	Adequacy (%)	μg RE/100Kcal
IF-A[I]	0		1.03 ± 0.03[a]	0.90 ± 0.03[a]	0.64	-	140.2	174.26 ± 5.10
25ºC	1		1.00 ± 0.01[a]	0.87 ± 0.01[a]		2.8	136.3	169.41 ± 2.04
	3		0.99 ± 0.03[a]	0.86 ± 0.02[a]		3.9	134.8	167.51 ± 4.65
	6		0.94 ± 0.01[b]	0.82 ± 0.01[b]		8.5	128.2	159.37 ± 2.41
	9		0.85 ± 0.04[c]	0.74 ± 0.04[c]		17.7	115.4	143.36 ± 7.42
	12		0.85 ± 0.04[c]	0.74 ± 0.03[c]		17.5	115.7	143.76 ± 6.78
	15		0.85 ±0.01[c]	0.74 ± 0.00[c]		17.8	115.2	143.19 ± 0.97
	18		0.84 ± 0.00[c]	0.73 ± 0.00[c]		18.3	114.5	142.29 ± 0.37
IF-A[I]	0		1.03 ± 0.03[a]	0.90 ± 0.03[a]	0.64	-	140.2	174.26 ± 5.10
40ºC	1		0.99 ± 0.04[a]	0.86 ± 0.04[a]		3.8	134.9	167.62 ± 7.59
	3		0.97 ± 0.03[a]	0.85 ± 0.02[a]		5.8	132.1	164.14 ± 4.44
	6		0.82 ± 0.02[b]	0.71 ± 0.02[b]		20.7	111.3	138.27 ± 3.68
	9		0.78 ± 0.02[c]	0.68 ± 0.02[c]		24.2	106.3	132.08 ± 3.72
	12		0.77 ± 0.01[c]	0.67 ± 0.01[c]		24.9	105.3	130.86 ± 2.06
	15		0.77 ± 0.02[c]	0.67 ± 0.02[c]		25.5	104.5	129.82 ± 3.26
	18		0.75 ± 0.02[c]	0.65 ± 0.01[d]		27.5	101.7	126.39± 2.63
IF-B[II]	0		1.55 ± 0.04[a]	0.85 ± 0.02[a]	0.61	-	139.0	166.55 ± 4.30
25ºC	1		1.37 ± 0.03[b]	0.75 ± 0.02[b]		11.9	122.4	146.73 ± 3.30
	3		1.36 ± 0.05[b]	0.74 ± 0.02[b]		12.7	121.3	145.43 ± 4.87
	6		1.35 ± 0.01[b]	0.74 ± 0.01[b]		13.3	120.5	144.41 ± 1.33
	9		1.25 ± 0.09[c]	0.68 ± 0.05[c]		19.3	112.2	134.46 ± 9.18
	12		1.25 ± 0.08[c]	0.68 ± 0.05[c]		19.8	115.5	133.58 ± 8.85
	15		1.24 ± 0.03[c]	0.68 ± 0.02[c]		20.1	111.0	133.041 ± 3.24
	18		1.23 ± 0.03[c]	0.67 ± 0.01[c]		21.0	109.8	131.62 ± 2.94
IF-B[II]	0		1.55 ± 0.04[a]	0.85 ± 0.02[a]	0.61	-	139.0	166.55 ± 4.30
40ºC	1		1.37 ± 0.02[b]	0.75 ±0.01[b]		12.0	122.3	146.63 ± 1.95
	3		1.35 ± 0.06[b]	0.74 ± 0.03[b]		13.3	120.5	144.43 ± 5.90
	6		1.28 ± 0.07[b]	0.70 ± 0.04[b]		17.9	114.1	136.80 ± 7.09
	9		1.24 ± 0.07[c]	0.68 ± 0.04[c]		20.1	111.0	133.04 ± 7.83
	12		1.22 ± 0.05[c]	0.66 ± 0.03[c]		21.7	108.8	130.39 ± 5.73
	15		1.18 ± 0.03[c]	0.64 ± 0.01[c]		24.0	105.6	126.58 ± 2.85
	18		1.10 ± 0.03[d]	0.60 ± 0.02[d]		29.0	98.7	118.29 ± 3.37

Values are expressed as means ± standard deviation of four determinations. Repetitions in superscripted letters within the same column and in formula indicate no significant differences ($p > 0.05$).

Table 3 Analysis of vitamin E content in different infant formulas during storage by NP-HPLC-DAD

Sample	Storage (days)	α-T (mg/100g)	γ-T (mg/100g)	δ-T (mg/100g)	α-TAc (mg/100g)	α-TE Found (mg/100g)	α-TE Label (mg/100g)	Losses (%)	Adequacy (%)	α-TE mg/100Kcal
IF-A[I]	0	6.18 ± 0.29[a]	1.62 ± 0.11[a]	-	29.75 ± 1.28[a]	27.33 ± 0.99[a]	25	-	109.3	5.31 ± 0.19
25ºC	1	5.88 ± 0.08[a]	1.48 ± 0.02[b]	-	29.07 ± 0.92[a]	26.53 ± 0.72[a]		2.9	106.1	5.15 ± 0.14
	3	6.02 ± 0.15[a]	1.48 ± 0.03[b]	-	29.12 ± 0.88[a]	26.69 ± 0.77[a]		2.3	106.8	5.18 ± 0.15
	6	5.89 ± 0.09[a]	1.42 ± 0.03[b]	-	28.36 ± 0.50[a]	26.03 ± 0.42[a]		4.8	104.1	5.05 ± 0.08
	9	5.34 ± 0.27[b]	1.29 ± 0.07[c]	-	25.89 ± 0.87[b]	27.32 ± 0.85[a]		13.2	94.9	4.61 ± 0.16
	12	5.35 ± 0.25[b]	1.30 ± 0.07[c]	-	25.96 ± 0.85[b]	23.78 ± 0.80[b]		13.0	95.1	4.62 ± 0.16
	15	5.27 ± 0.13[b]	0.82 ± 0.00[d]	-	25.71 ± 0.14[b]	23.43 ± 0.11[b]		14.3	93.7	4.55 ± 0.02
	18	5.35 ± 0.48[b]	0.74 ± 0.02[e]	-	24.19 ± 0.61[b]	22.43 ± 0.56[b]		17.9	89.7	4.35 ± 0.11
IF-A[I]	0	6.18 ± 0.29[a]	1.62 ± 0.11[a]	-	29.75 ± 1.28[a]	27.33 ± 0.99[a]	25	-	109.3	5.31 ± 0.19
40ºC	1	5.95 ± 0.17[a]	1.59 ± 0.03[a]	-	29.06 ± 1.77[a]	26.62 ± 1.47[a]		2.6	106.5	5.17 ± 0.29
	3	6.02 ± 0.15[a]	1.58 ± 0.06[a]	-	28.47 ± 1.43[a]	26.26 ± 0.78[a]		3.9	105.1	5.10 ± 0.15
	6	5.83 ± 0.11[b]	1.40 ± 0.08[b]	-	27.77 ± 1.40[b]	25.54 ± 1.43[a]		6.5	102.2	4.96 ± 0.28
	9	5.09 ± 0.14[c]	1.29 ± 0.02[c]	-	24.09 ± 1.76[c]	22.20 ± 1.14[b]		18.8	88.8	4.31 ± 0.22
	12	5.04 ± 0.20[c]	1.23 ± 0.03[d]	-	23.87 ± 1.74[c]	22.00 ± 1.16[b]		19.5	88.0	4.27 ± 0.23
	15	5.20 ± 0.09[c]	0.81 ± 0.04[e]	-	24.03 ± 1.61[c]	22.19 ± 1.06[b]		18.8	88.7	4.31 ± 0.21
	18	4.78 ± 0.06[d]	0.71 ± 0.02[f]	-	19.25 ± 1.04[d]	21.01 ± 0.69[b]		23.1	84.0	4.08 ± 0.13
IF-B[II]	0	6.47 ± 0.21[a]	4.90 ± 0.06[a]	1.21 ± 0.03[a]	6.46 ± 0.53[a]	12.09 ± 0.57[a]	6.1	-	198.2	2.38 ± 0.11
25ºC	1	6.06 ± 0.05[b]	4.56 ± 0.06[b]	1.15 ± 0.04[b]	6.20 ± 0.53[a]	11.44 ± 0.40[a]		5.4	187.6	2.25 ± 0.08
	3	5.96 ± 0.05[b]	4.65 ± 0.08[b]	1.08 ± 0.05[c]	5.89 ± 0.08[a]	11.12 ± 0.10[b]		8.1	182.2	2.18 ± 0.02
	6	5.93 ± 0.10[b]	4.64 ± 0.09[b]	0.71 ± 0.16[d]	5.96 ± 0.15[a]	11.10 ± 0.21[b]		8.2	182.0	2.18 ± 0.04
	9	5.61 ± 0.14[c]	4.48 ± 0.03[c]	-	5.84 ± 0.21[a]	10.59 ± 0.29[b]		12.4	173.7	2.08 ± 0.06
	12	5.58 ± 0.10[c]	4.45 ± 0.03[c]	-	5.80 ± 0.15[a]	10.52 ± 0.22[b]		13.0	172.5	2.07 ± 0.04
	15	5.60 ± 0.11[c]	4.57 ± 0.07[c]	-	5.92 ± 0.26[a]	10.66 ± 0.52[b]		11.8	174.8	2.09 ± 0.10
	18	5.55 ±0.11[c]	4.50 ± 0.13[c]	-	5.86 ± 0.23[a]	10.55 ± 0.56[b]		12.8	172.9	2.07 ± 0.11
IF-B[II]	0	6.47 ± 0.21[a]	4.90 ± 0.06[a]	1.21± 0.09[a]	6.46 ± 0.53[a]	12.09 ± 0.57[a]	6.1	-	198.2	2.38 ± 0.11
40ºC	1	5.97 ± 0.09[b]	4.51 ± 0.12[b]	0.99 ± 0.07[b]	6.22 ± 0.30[a]	11.33 ± 0.29[a]		6.3	185.7	2.23 ± 0.06
	3	5.93 ± 0.12[b]	4.56 ± 0.19[b]	-	5.97 ± 0.36[a]	11.02 ± 0.26[b]		8.8	180.7	2.17 ± 0.09
	6	5.60 ± 0.18[c]	4.54 ± 0.17[b]	-	5.85 ± 0.45[a]	10.60 ± 0.18[b]		12.3	173.8	2.08 ± 0.04
	9	5.03 ± 0.10[d]	4.51 ± 0.11[b]	-	5.57 ± 0.42[a]	9.83 ± 0.78[b]		18.7	161.2	1.93 ± 0.15
	12	4.92 ± 0.12[d]	4.29 ± 0.07[c]	-	5.46 ± 0.33[b]	9.60 ± 0.33[c]		20.6	157.4	1.89 ± 0.08
	15	4.63 ± 0.03[e]	4.06 ± 0.09[d]	-	5.39 ± 0.49[b]	9.22 ± 0.12[c]		23.7	151.2	1.81 ± 0.06
	18	4.42 ± 0.10[f]	3.49 ± 0.07[e]	-	5.12 ± 0.36[b]	8.70 ± 0.21[d]		28.1	142.6	1.71 ± 0.04

Values are expressed as means ± standard deviation of four determinations. Repetitions in superscripted letters within the same column and formula indicate no significant differences ($p > 0.05$).

Table 4. Analysis of vitamin C content in infant formulas during storage

Sample	Storage (months)		Vitamin C[x] (mg/100g)	Label (mg/100g)	Losses (%)	Adequacy (%)	mg /100Kcal
IF-A	0		100.40 ± 3.51[a]	60	-	167.33	19.50 ± 0.53
25ºC	1		99.09 ± 2.19[a]		1.30	165.15	19.24 ± 0.21
	3		98.00 ± 3.51[a]		2.39	163.33	19.03 ± 0.52
	6		95.25 ± 2.01[b]		5.13	158.75	18.50 ± 0.21
	9		88.26 ± 2.57[c]		12.09	147.10	17.14 ± 0.25
	12		85.30 ± 3.52[c]		15.04	142.17	16.56 ± 0.51
	15		80.06 ± 2.31[d]		20.26	133.43	15.55 ± 0.23
	18		79.94 ± 3.47[d]		20.38	133.23	15.52 ± 0.35
IF-A	0		100.40 ± 3.51[a]	60	-	167.33	19.50 ± 0.53
40ºC	1		93.80 ± 5.54[b]		6.57	156.33	18.21 ± 0.22
	3		91.72 ± 3.17[b]		8.65	152.87	17.81 ± 0.31
	6		87.86 ± 3.71[c]		12.49	146.43	17.06 ± 0.33
	9		83.03 ± 4.01[c]		17.30	138.38	16.12 ± 0.41
	12		80.01 ± 2.21[c]		20.31	133.35	15.54 ± 0.33
	15		75.05 ± 2.71[d]		25.25	125.08	14.57 ± 0.32
	18		71.88 ± 2.22[e]		28.41	119.80	13.96 ± 0.20
IF-B	0		80.48 ± 2.52[a]	68	-	118.35	15.81 ± 0.25
25ºC	1		78.78 ± 2.01[a]		2.11	115.85	15.48 ± 0.21
	3		76.48 ± 3.11[a]		4.97	112.47	15.03 ± 0.34
	6		75.11 ± 2.19[b]		6.67	110.46	14.76 ± 0.22
	9		63.32 ± 1.99[c]		21.32	93.12	12.44 ± 0.21
	12		60.52 ± 2.56[c]		24.80	89.00	11.89 ± 0.25
	15		56.22 ± 1.57[d]		30.14	82.68	11.05 ± 0.19
	18		52.46 ± 2.02[e]		34.82	77.15	10.31 ± 0.20
IF-B	0		80.48 ± 2.52[a]	68	-	118.35	15.81 ± 0.25
40ºC	1		75.26 ± 2.03[b]		6.49	110.68	14.79 ± 0.22
	3		73.11 ± 2.15[b]		9.16	107.51	14.36 ± 0.22
	6		70.49 ± 1.81[c]		12.41	103.66	13.85 ± 0.26
	9		60.94 ± 2.75[d]		24.28	89.62	11.97 ± 2.27
	12		53.52 ± 1.97[e]		33.50	78.71	10.51 ± 0.18
	15		45.81 ± 2.05[f]		43.08	67.37	9.00 ± 0.11
	18		41.33 ± 2.37[g]		48.65	60.78	8.12 ± 0.25

[x] Values are expressed as means ± standard deviation of four determinations. Vitamin C is referred to as ascorbic acid. Repetitions in superscripted letters within the same column and formula indicate no significant differences ($p > 0.05$).

Table 5. Analysis of iron content in infant formulas during storage

Sample	Storage (months)		Iron (mg/100g)	Iron (mg/L)	Iron (mg/100 Kcal)	Adequacy (%)
IF-A	0		3.90 ± 0.26[a]	5,27	0.76	65.00
25ºC	1		3.82 ± 0.26[a]	5,16	0.74	63.67
	3		3.75 ± 0.27[a]	5,06	0.73	62.50
	6		3.54 ± 0.11[a]	4,78	0.69	59.00
	9		3.52 ± 0.25[a]	4,75	0.68	58.67
	12		3.79 ± 0.13[a]	5,12	0.74	63.17
	15		3.72 ± 0.16[a]	5,02	0.72	62.00
	18		3.80 ± 0.32[a]	5,13	0.74	63.33
IF-A	0		3.90 ± 0.26[a]	5,27	0.76	65.00
40ºC	1		3.84 ± 0.05[a]	5,18	0.75	64.00
	3		3.61 ± 0.13[a]	4,87	0.70	60.17
	6		3.58 ± 0.21[a]	4,83	0.70	59.67
	9		3.65 ± 0.22[a]	4,93	0.71	60.83
	12		3.95 ± 0.36[a]	5,33	0.77	65.83
	15		3.51 ± 0.42[a]	4,74	0.68	58.50
	18		3.89 ± 0.23[a]	5,25	0.76	64.83
IF-B	0		3.98 ± 0.14[a]	5,25	0.78	65.25
25ºC	1		4.00 ± 0.35[a]	5,28	0.79	65.57
	3		4.24 ± 0.33[a]	5,60	0.83	69.51
	6		4.22 ± 0.19[a]	5,57	0.83	69.18
	9		4.47 ± 0.57[a]	5,90	0.88	73.28
	12		4.37 ± 0.41[a]	5,77	0.86	71.64
	15		4.35 ± 0.41[a]	5,74	0.85	71.31
	18		4.21 ± 0.28[a]	5,56	0.83	69.02
IF-B	0		3.98 ± 0.14[a]	5,25	0.78	65.25
40ºC	1		4.24 ± 0.16[a]	5,60	0.83	69.51
	3		4.56 ± 0.12[a]	6,02	0.90	74.75
	6		4.50 ± 0.31[a]	5,94	0.88	73.77
	9		4.42 ± 0.12[a]	5,83	0.87	72.46
	12		4.48 ± 0.22[a]	5,91	0.88	73.44
	15		4.59 ± 0.59[a]	6,06	0.90	75.25
	18		4.55 ± 0.32[a]	6,01	0.89	74.59

Values are expressed as means ± standard deviation of three determinations. Repetitions in superscripted letters within the same column and formula indicate no significant differences ($p > 0.05$).

Table 6. Analysis of selenium content in infant formulas during storage

Sample	Storage (months)		Selenium (μg/100g)	Selenium (μg/L)	Selenium (μg/100 Kcal)	Adequacy (%)
IF-A	0		7.80 ± 0.46[a]	10,53	1.51	72.90
25ºC	1		7.64 ± 0.32[a]	10,31	1.48	71.40
	3		6.90 ± 0.33[a]	9,32	1.34	64.49
	6		7.08 ± 0.42[a]	9,56	1.37	66.17
	9		7.04 ± 0.33[a]	9,50	1.37	65.79
	12		7.58 ± 0.45[a]	10,23	1.47	70.84
	15		7.44 ± 0.35[a]	10,04	1.44	69.53
	18		7.60 ± 0.37[a]	10,26	1.48	71.03
IF-A	0		7.80 ± 0.46[a]	10,53	1.51	72.90
40ºC	1		7.68 ± 0.32 [a]	10,37	1.49	71.78
	3		7.22 ± 0.34[a]	9,75	1.40	67.48
	6		7.16 ± 0.45[a]	9,67	1.39	66.92
	9		6.90 ± 0.33[a]	9,32	1.34	64.49
	12		7.90 ± 0.28[a]	10,67	1.53	73.83
	15		7.02 ± 0.38[a]	9,48	1.36	65.61
	18		7.78 ± 0.42[a]	10,50	1.51	72.71
IF-B	0		7.15 ± 0.27[a]	9,44	1.40	79.44
25ºC	1		6.80 ± 0.17[a]	8,98	1.34	75.56
	3		7.38 ± 0.35[a]	9,74	1.45	81.98
	6		7.17 ± 0.28[a]	9,47	1.41	79.71
	9		7.52 ± 0.41[a]	9,93	1.48	83.56
	12		7.43 ± 0.37[a]	9,81	1.46	82.54
	15		7.40 ± 0.32[a]	9,76	1.45	82.17
	18		7.16 ± 0.35[a]	9,45	1.41	79.52
IF-B	0		7.15 ± 0.27[a]	9,44	1.40	79.44
40ºC	1		7.21 ± 0.18[a]	9,51	1.42	80.09
	3		7.35 ± 0.31[a]	9,70	1.44	81.67
	6		7.52 ± 0.25[a]	9,93	1.48	83.56
	9		7.45 ± 0.32[a]	9,83	1.46	82.78
	12		7.55 ± 0.41[a]	9,97	1.48	83.89
	15		7.45 ± 0.35[a]	9,83	1.46	82.78
	18		7.52 ± 0.25[a]	9,93	1.48	83.56

Values are expressed as means ± standard deviation of three determinations. Repetitions in superscripted letters within the same column and formula indicate no significant differences ($p > 0.05$).

PUBLICACIÓN 10

Estudio de la estabilidad durante el almacenamiento de formulas infantiles suplementadas con ácidos grasos poliinsaturados de cadena larga, provenientes de aceites sintetizados por microorganismos unicelulares o de fosfolípidos de huevo.

Chávez-Servín, J. L., Castellote, A. I., & López-Sabater, M. C. (**2007)** Stability during storage of a single cell oils- and an egg yolk LC-PUFA-supplemented infant formula. *Food Chemistry,* in revision.

Actualmente se recomienda que la composición de los preparados para lactantes debe ser lo más similar posible a la leche materna, con referencia en particular a los ácidos grasos poliinsaturados de cadena larga (LC-PUFA), específicamente el ácido araquidónico (AA) y el ácido docosahexaenóico (DHA), debido a que éstos proveen de beneficios funcionales y bioquímicos al recién nacido. No obstante los LC-PUFA son muy susceptibles a la oxidación, por lo que la estabilidad y contenido de dichas fórmulas debe ser controlado. En este estudio fue evaluada la estabilidad durante el almacenamiento a 25 y 40ºC de dos tipos de preparados para lactantes de base láctea en polvo suplementadas con LC-PUFA proveniente de diferentes fuentes. La primera (IF-EPL) suplementada con fosfolípidos de huevo (EPL) y la segunda (IF-SCO) con aceites sintetizados por microorganismos unicelulares (SCO) en forma de triacilglicéridos. Los siguientes parámetros fueron evaluados: índice de peróxidos, contenido de compuestos volátiles (propanal, pentanal y hexanal) y perfil de ácidos grasos como indicadores de la estabilidad oxidativa. Asimismo se evaluó el contenido de furfurales potenciales y furfurales libres (5-hidroximetil-2-furaldehído y 2-furaldehído) como indicadores de la reacción de Maillard. Además se realizó una evaluación sensorial en los dos tipos de fórmulas almacenadas a 25ºC. Los parámetros estudiados mostraron una estabilidad lipídica aceptable en los dos tipos de fórmulas en polvo, observándose mejores resultados en la IF-EPL. Pérdidas significativas ($p<0.05$) del ácido linoleico fueron encontradas al final del estudio en ambos tipos de fórmula, no obstante, no se encontraron disminuciones significativas en ningún otro ácido graso, incluyendo el AA y el DHA. En lo que se refiere al contenido de compuestos furfural, principalmente el HMF potencial, éste incrementa de una manera similar en los dos tipos de fórmula

indicando la reacción de Maillard típica de este tipo de productos durante su almacenamiento.

Stability during storage of a single cell oils- and an egg yolk LC-PUFA-supplemented infant formula

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Abstract

Nowadays the medical community recommends that infant formula mimic breast milk as much as possible, particularly in regards to long-chain polyunsaturated fatty acids (LC-PUFA). Specifically, these include arachidonic acid (AA) and docosahexaenoic acid (DHA), since both provide biochemical and functional benefits to newborns. However LC-PUFAs are very susceptible to oxidation and formulae must be carefully controlled. In this study, the stability of two types of LC-PUFA-supplemented milk-based powdered infant formulas was evaluated over the course of 18 months storage at 25 and 40ºC. The first (IF-EPL) was supplemented with LC-PUFA in the form of egg phospholipids (EPL) the second (IF-SCO) in the form of triglycerides synthesized by single cell oils (SCO). The following parameters were monitored: peroxide values, volatiles content (propanal, pentanal and hexanal), fatty acid profiles, and potential and free furfural content (5-hydroxymethyl-2-furaldehyde and 2-furaldehyde). In addition, these formulae were subjected to sensory evaluations. The studied parameters revealed acceptable lipid stability in both types of formula powders, with better results occurring in IF-EPL. At the end of the study period, significant deficits ($p<0.05$) in linoleic acid were noted in both formulae. However, no significant decreases were observed in the other acids, including AA and DHA. In regards to furfural content, both formulae exhibited a similar increase, indicative of the typical Maillard reaction characteristic of products stored for extended periods.

***Keywords*:** LC-PUFA supplementation; infant formula; stability; storage**.**

1. Introduction

The food industry has continually striven to improve the quality and nutritional content of infant formulae (IF), attempting to bring its composition closer to that of human milk. One such improvement consists of adding LC-PUFA to IF, since several

78 studies on infant nutrition have demonstrated their beneficial effects vis-à-vis growth
79 and development. Increasing evidence suggests that docosahexaenoic acid (DHA, C22:6
80 n-3) and arachidonic acid (AA, C20:4 n-6) are of particular importance, as these fatty
81 acids constitute the large majority of fatty acids found in brain tissue. Moreover, they
82 are also present in human milk, although are absent from cow's milk (Obrien &
83 Sampson, 1965). Furthermore, the addition of docosahexaenoic acid (DHA, C22:6 n-3)
84 to IF has been shown to improve neurofunctional response in preterm infants (Hornstra,
85 Al, van Houwelingen & Foreman van Drongelen, 1995; Gibson, Neumann & Makrides,
86 1996; Cunnane, Francescutti, Brenna & Crawford, 2000; Valenzuela & Nieto, 2001;
87 Rodriguez et al., 2003).

88 PUFAs above C_{18} cannot be synthesized by higher plants in any significant amounts
89 due to a lack of the requisite enzymes. Traditional sources of AA are animal livers and
90 egg yolks, and for DHA fish oils (Ward & Singh, 2005). The growing interest in PUFA
91 in relation with their importance in health and dietary requirements has focused
92 attention on identifying suitable sources of these compounds. Isolation of highly
93 efficient microorganisms has led to the development of new technologies for PUFA
94 production as an alternative to agricultural and animal processes. The production of
95 microbial oils, referred to as single cell oils (SCOs), is now an economic reality. SCOs
96 are now produced by various microorganisms as commercial sources of arachidonic
97 acid (AA) and DHA. One of the most representatives microorganisms for the
98 production of AA is *Mortierella alpina* (Ratledge, 2004), while for DHA, it is
99 *Crythecodinium Cohnii* (Ward et al., 2005). These oils are increasingly used as dietary
100 supplements in infant formulation. Although DHA has long been known to be a major
101 fatty acid in fish oils, it always occurs in tandem with eicosapentaenoic acid (EPA,
102 C20:5 n-3), which has been contraindicated for inclusion in infant diets since it affects
103 the uptake of DHA (Craig-Schmidt & Huang, 1998), which is crucial for neural

development. However, fish oils often possess objectionable aftertastes and/or odours, as well as variations in oil quality. In addition, their satisfactory utilization requires the removal of cholesterol and small amounts of potentially toxic impurities (e.g., pollutants including dioxins, PCBs, and heavy metals such as mercury) (Certik & Shimizu, 1999; Ratledge, 2004).

On the other hand, LC-PUFAs are very sensitive to oxidative reactions, since fatty acid unsaturation increases the rate of oxidation. Hence, supplemented LC-PUFA formulae are very sensitive to oxidation, despite their low fatty acid content (Gonzalez-Corbella, Tortras-Biosca, Castellote-Bargallo & Lopez-Sabater, 1999). Lipid oxidation is well recognized as a major cause of quality deterioration during the processing and storage of milk-based formulae. In the peroxidation of unsaturated fatty acids, lipid hydroperoxides form during the propagation phase. These primary compounds are unstable and rapidly decompose, giving rise to a range of new free-radicals and other non-radical compounds, including alkoxyl and alkyl radicals, aldehydes, ketones, as well as to a variety of carboxyl compounds that form a complex mixture of secondary lipid oxidation products. Volatile compounds such as hexanal and pentanal have been associated with the development of undesirable flavours and have been proposed as potential markers of fresh product quality (Kim & Morr, 1996; Contarini & Pavolo, 2002; Toso, Procida & Stefanon, 2002; Marsili & Miller, 2003; Karatapanis, Badeka, Riganakos, Savvaidis & Kontominas, 2006).

Arachidonic acid (C20:4, n-6) (AA) and docosahexaenoic acid (C22:6, n-3) (DHA) are more susceptible to oxidation than linoleic acid (C18:2, n-6) (LA). However, LA is the main polyunsaturated fatty acid in IFs (Ulberth & Roubicek, 1995). The content of hexanal, which is a major breakdown product of LA oxidation (Frankel, 1993), has been used to follow the course of lipid oxidation in foods (Dupuy et al., 1977). Pentanal and hexanal are the specific volatile oxidation products of n-6 PUFA; propanal of n-3 PUFA

(Romeu-Nadal, Castellote & Lopez-Sabater, 2004). PUFAs can be oxidized, resulting not only in a loss of nutritive value, but also in the generation of the aforementioned volatile compounds.

Another key target for modification in formulae is the protein entity; in particular, the well-known Maillard reaction (MR), which is responsible for decreasing protein digestibility and nutritional supply (Fenaille et al., 2006). Reducing carbohydrates present in infant formula brings about a reaction with free amino acids, mainly lysine, giving rise to lactuloselysine (van Boekel, 1998; Ramirez-Jimenez, Garcia-Villanova & Guerra-Hernandez, 2004; Chávez-Servín, Castellote & López-Sabater, 2005). PUFAs may also easily react with lysine upon oxidation, blocking lysine and increasing the MR. In advanced MR states, undesirable compounds such as furfurals can be found (van Boekel, 1998; Rehman, Saeed & Zafar, 2000; Ramirez-Jimenez, Garcia-Villanova & Guerra-Hernandez, 2000). These can serve as useful indicators of food damage, as well as tools for evaluating the severity of the Maillard reaction (Guerra-Hernandez, Garcia-Villanova & Montilla-Gomez, 1992; Lococo, Valentini, Novelli & Ceccon, 1994).

There is currently scant information regarding product stability and sensory evaluation during the full shelf life of SCO- and egg yolk LC-PUFA-supplemented infant formula. In this study, two types of LC-PUFA supplemented infant formula were examined: one containing egg yolk phospholipids (IF-EPL) and the other with triacylglycerides synthesized by SCO (IF-SCO). The following parameters were monitored over an 18-month storage period at 25 and 40ºC: the peroxide values, the evolution of the fatty acid profiles and the contents of propanal, pentanal and hexanal, as indicators of lipid oxidation. The evolution of potential and free furfural compounds (5-hydroxymethyl-2-furaldehyde: HMF; 2-furaldehyde: F), as an indicator of food deterioration via a Maillard reaction was also evaluated. These results were then

compared with those based on sensory analysis. Comparisons of the studied parameters were then made with storage time and temperature used as variables.

2. Materials and methods

2.1. Samples

Two types of LC-PUFA-supplemented IF were obtained from the production plant immediately following manufacture. The first formula (IF-EPL) was supplemented with egg yolk phospholipids [Ovotin®], the final product containing 47 mg/100 g of AA and 25 mg/100 g of DHA (C22:6 n-3). The second formula (IF-SCO) was supplemented with SCO [DHASCO® (DHA) from the alga *Crypthecodinium cohnii*, and ARASCO® (AA) [from Mortierella alpina] in the form of triacylglyceride, with the final powder containing 91 mg/100 g of AA and 53 mg/100 g of DHA. The formulae were packed in airtight containers flushed with nitrogen, providing a modified atmosphere of N_2/CO_2 (<2% O_2). The formula composition is detailed in Table 1.

2.2.Storage

To evaluate the evolution of selected parameters during the shelf life of IFs, the product was kept either at room temperature (25ºC: min 23, max 25.5) or at 40 ºC from production until 0, 1, 3, 6, 9, 12, 15, and 18 months. 25ºC constitutes the typical ambient temperature found in markets and food stores, while 40ºC can occur under extreme summer time conditions in stores lacking air conditioning. Once the data had been compiled, analytical determinations were subsequently conducted. The peroxide values and volatile compound content were determined online. We placed a sufficient number of aliquots (approximately 15 g each) in amber glass flasks. These were sealed under nitrogen and kept at -80ºC until the remaining analyses were carried out. For sensory analysis, the original package was maintained intact; when the required storage

182 periods had ended, products were kept at -80ºC until the sensory evaluation could be
183 conducted.
184
185 *2.3.Chemicals*
186 The chemicals used for sample preparations were of analytical reagent grade:
187 acetonitrile HPLC-grade (SDS, Peypin, France). Acetic glacial acid, hydrochloric acid
188 and dry methanol were obtained from Panreac (Barcelona, Spain). Deionized water was
189 purified through a Milli-Q system (Millipore, Bedford, MA, USA). Oxalic acid
190 dihydrate >95.5%, trichloracetic acid (TCA) >99.5%, 5-hydroxymethyl-2-furaldehyde
191 (HMF), 2-furaldehyde (F), 2-furyl-methyl ketone (FMC), 5-methyl-2-furaldehyde (MF)
192 and sodium methylate were purchased from Fluka (Buchs, Switzerland). Propanal
193 (propionaldehyde, 97% of pure), pentanal (valeraldehyde, 97% of pure), and butyl
194 acetate (99% of pure) were purchased from Aldrich (Steinheim, Germany). Hexanal
195 (98% of pure), Supelco™ 37 component fatty acid methyl esters mix, and tridecanoic
196 acid (C13:0), which used as a fatty acid internal standard (IS), were purchased from
197 Sigma (St. Louis, MO, USA). Boron trifluoride in methanol (20% w/v), n-hexane,
198 sodium chloride, and anhydrous sodium sulphate were from obtained from Merck
199 (Darmstadt, Germany),
200
201 *2.4. Instruments*
202 *2.4.1 Fatty acid determination*
203 For fatty acid analysis, we used a Shimadzu GC-2010 gas chromatograph (Shimadzu,
204 Kyoto, Japan) equipped with a flame ionization detector and a Shimadzu AOC-20i
205 autoinjector. The fatty acid methyl esters (FAMEs) were separated on a fast capillary
206 column (10 m × 0.10 mm i.d.) coated with an SGE-BPX70 cross-linked stationary

207 phase (70% cyanopropyl polysilphenylene-siloxane, 0.20 μm film thickness) from SGE
208 (SGE Europe Ltd., United Kingdom).
209
210 *2.4.2 Volatiles determination*
211 For volatiles analysis, we used a Shimadzu gas chromatograph system model GC-14
212 A, coupled to a flame ionization detector and a split-splitless injector (Shimadzu, Kyoto,
213 Japan). We also used a supelcowax™-10 fused silica capillary column (30m x 0.25 mm
214 x 0.25 μm film thickness) from Supelco (Bellafonte, PA, USA). The aldehydes were
215 separated isothermally at 75ºC. The injector and detector temperatures were 185 and
216 200ºC, respectively; with a split ratio of 1:20, helium was used as a carrier gas at a
217 linear velocity of 20.39 cm/sec. Data acquisition was performed using HP GC
218 Chemstation software for Windows (Hewlett-Packard).
219
220 *2.4.3 Furfural determination*
221 A Hewlett Packard liquid chromatographic system (Waldbronn, Germany) with an
222 HP 1050 pump system, a HP-1040 M photodiode-array detector, and a Waters 717 plus
223 autosampler injector (Milford, MA, USA) was used for furfural determination. We used
224 a Tracer ODS-2 C18 column (4.6 x 150 mm) with a 5 μm particle size (Teknokroma,
225 Barcelona, Spain). Chromatographic separation was performed at 30ºC using a mixture
226 of acetonitrile-water (4.5:95.5 v/v) for the mobile phase and a flow rate of 1 ml/min.
227 The injection volume was 20 μl.
228
229 *Analytical determinations*
230 *2.5.1. Fatty acid analysis*
231 Fatty acid profiles were analyzed by fast GC following their derivatization to
232 FAMEs. IF samples were prepared as described López-López et al. (2002), using

sodium methylate and boron trifluoride in methanol, thereby obtaining the FAMEs in n-hexane for subsequent injection into the gas chromatograph. Fast GC analysis was carried out following Bondia-Pons (2004). Operating conditions were as follows: the split–splitless injector was used in split mode with a ratio of 1:100. The sample injection volume was 1 μL. The injector and detector temperatures were kept at 250 °C and 270 °C, respectively. The temperature programme was as follows: initial temperature 60ºC, increasing 50ºC/min until reaching 175ºC, which was held 1 min; then, increased again 20ºC/min until reaching 250ºC, where it was held for 0.5 min. Data acquisitions and processing were performed using the Shimadzu Chemstation software for GC systems. Analyses were carried out in triplicate.

2.5.2. Measurement of volatile compounds

Volatile IF compounds were measured by static headspace gas chromatography with a flame ionization detector (HS-GC-FID), exactly as reported by Romeu-Nadal et al. (2004). Propanal, pentanal, and hexanal were identified by comparing retention times with those of the standards. Levels were then quantified by interpolation using a calibration curve within a range of 0.03 to 0.70 μg/ml. Analyses were performed in quadruplicate.

2.5.3. Measurement of peroxide values

Lipid hydroperoxides were determined using an iodometric method described in Regulation EEC/2568/ 91 published by the European Union Commission (1991)

2.5.4. Measurement of furfural compounds

Free and potential furfurals were measured by RP-HPLC-DAD, exactly as we have reported previously (Chávez-Servín et al., 2005). Furfurals were identified by their

retention times, as well as by their characteristic spectra. They were quantified by interpolation using a calibration curve within a range of 0.05 to 5 μg/ml for HMF, and 0.05 to 2 μg/ml for F, FMC, and MF. Analyses were performed in quadruplicate.

2.5.5. pH

The pH of samples was measured using a pH meter micro-pH 2000 with a glass electrode (pH 0-12, 0-80ºC), and equipped with an automatic temperature compensator (Crison Instruments, S.A, Barcelona, Spain). IF was reconstituted in warm water according to the manufacturer's instructions. After a given sample had reached room temperature, pH was determined.

2.5.6. Sensory analysis

External judges were selected beforehand and trained for the sensorial tests. A Duo-Trio and Pairs Comparison tests were applied accordingly, as previously reported (Chávez-Servín, Romeu-Nadal, Castellote & López-Sabater, 2006). A Pairs Comparison test was carried out to determine particular sensorial characteristics (better flavour, better smell, more rancid flavour and longer lasting-flavour) evaluated the two formulas throughout storage. For this test, we accepted the option "indifferent". In addition, for the statistical analysis we divided the scores between the two compared samples (Meilgaard, Civille & Carr, 1987). A Duo-Trio test was used to determine not only if sensory differences (undetected or detected) existed in the same type of formula, but also whether these parameters changed over time. For sensory analysis, the formulae were reconstituted in accordance with the manufacturer’s instructions. Only with the formulae stored at 25ºC did we carry out both tests. For each storage period analysis, we conducted 80 sensory tests. Comparisons were run in accordance with the following

284 storage times: For IF-EPL (T0 [fresh formula] *vs.* 12 months, and T0 *vs.* 18 months) and
285 for IF-SCO (T0 vs. 6 months, and T0 *vs.* 12 months).
286
287 *2.6. Statistical Analysis*
288 For statistical analysis, we carried out one-way analysis of variance (ANOVA) and
289 multiple comparisons tests, using the Tukey HSD procedure for each studied parameter
290 in order to detect differences in the formulae during storage Statistical analysis was
291 conducted using the SPSS package for Windows version 12 (SPSS, Chicago, IL, USA).
292 The level of statistical significance was set at 5% for all analyses. For the Duo-Trio and
293 the Pairs Comparison tests, we used the x^2-test and the paired *t*-test, respectively. For
294 both sensorial tests, two-sided hypotheses for each storage time *vs.* the fresh formula
295 (T0) were applied and a significance of $\alpha = 0.01$ (1%) (Meilgaard et al., 1987) was
296 established.
297
298 **3. Results and discussion**
299 *3.1. pH analysis*
300 As it can serve as a marker of product stability, the pH of reconstituted IF samples
301 was measured during each month of storage. Moreover, it can enhance the formation of
302 furfural compounds, either by lactose isomerization (Lobry De Bruyn-Alberda van
303 Ekenstein transformation, L-A) or by Amadori compound formation (Ferrer, Alegria,
304 Farre, Abellan & Romero, 2002). No differences in the evolution of pH values at 25 or
305 40ºC were observed in either formula type; in fact, the pH values remained constant
306 from 0 to 18 months of storage. The average pH for IF-EPL over the 18-month period
307 was 6.79 ± 0.05 (RSD 0.72%) while for IF-SCO it was 7.39 ± 0.05 (RSD 0.61%),
308 values typically found in IF (Ferrer et al., 2002; McSweeney, Mulvihill & O'Callaghan,
309 2004).

310

311 *3.2.Fatty acid profiles*

312 Fatty acid profiles were determined at the following time points and temperatures: 0,
313 1, 3, 6, 9, 12, 15 and 18 months, both at 25 and at 40ºC. However, due to the low
314 differences founded between fatty acids over time, we have included only those fatty
315 acid profiles determined at the starting point (T0), after 12 months of storage, and after
316 18 months of storage (Table 2). Unexpectedly, only in linoleic acid (C18:2, n-6) did we
317 detect any statistical difference ($p<0.05$) at the end of its shelf life; this was also true in
318 IF-EPL after 18 months of storage at 40ºC (from 16.29% ± 0.23 to 15.43% ± 0.31) and
319 in IF-SCO after 18 months of storage at 25ºC (from 17.48% ± 0.04 to 16.75% ± 0.10)
320 and at 40ºC (from 17.48% ± 0.04 to 16.71% ± 0.09). The quantity of saturated fatty acid
321 in IF-EPL was 40.65% while in IF-SCO it was 42.72%. Monounsaturated fatty acid
322 measured 41.70% in IF-EPL and 38.00% in IF-SCO; polyunsaturated fatty acid in IF-
323 EPL was 17.65% while in IF-SCO it totalled 19.28%. In addition, n-3 PUFA in IF-EPL
324 measured 1.09% and 1.40% in IF-SCO; n-6 PUFA totalled 16.56% in IF-EPL and
325 17.88% in IF-SCO. PUFAs, in particular, are easily attacked by free radicals that react
326 with their double bonds, thereby yielding several products such as short-chain
327 aldehydes. It is believed that the fatty acids' susceptibility to oxidation is largely
328 dependent upon their degree of unsaturation. Thus DHA and AA, the most unsaturated
329 fatty acids in the studied formulae, would be more susceptible to oxidation than any
330 others. However, contrary to expectations, DHA and AA levels remained constant
331 throughout storage. As stated above, only linoleic acid (the major n-6 PUFA in the
332 studied formulae) exhibited decreases (Table 2). The observed stability of the fatty acid
333 profiles in both formulae under the tested conditions, even in the case of C18:2 n-6, is
334 noteworthy due to the very small differences between the fatty acids at the beginning
335 and end of this study. These results show that the analysis of fatty acid profiles is useful

for determining the exact composition of an infant formula without the influence of storage conditions (e.g., time and temperature). On the other hand, this analysis lacked the necessary sensitivity for evaluating lipid stability in the formulae.

3.3. Peroxide values and volatile compounds content

To determine the effects of storage temperature on LC-PUFA supplemented formulae (25 and 40ºC), as well as of storage time on the oxidative stability of the powder formulae, we measured hydroperoxides and volatile compounds, as primary and secondary oxidation products, respectively. The peroxide value is a good indicator of fat quality. The limiting peroxide value specified for refined oil is 10 meq O_2/ Kg (Romeu-Nadal, Chávez-Servín, Castellote, Rivero & López-Sabater, 2006). At the beginning of this study, a value of 0.34 meq O_2/ Kg was recorded for the IF-EPL, which increase to 6.83 meq O_2/ Kg following 18 months of storage at 25ºC, and to 48.56 meq O_2/ Kg after 18 –months of storage at 40ºC. For IF-SCO, a value of 1.26 meq O_2/ Kg was recorded at the outset of our study, while values of 13.33 and 54.97 meq O_2/ Kg were recorded at the study's end for 25 and 40ºC, respectively. For both formulae, peroxide values increased in similar fashion during storage, with greater increases occurring at 40ºC. Although IF-SCO exhibited greater peroxide values than IF-EPL, its initial value (1.26 meq O_2/ Kg) indicates that oxidation occurred before storage. As peroxide values have proven to be sensitive indicators of increased primary oxidation products, they can serve as equally useful indicators of lipid oxidation and food damage. On the other hand, the only volatiles found were propanal, pentanal, and hexanal. At the beginning of the study, hexanal was detected only in IF-EPL (0.508 mg/Kg), while propanal (0.814 mg/kg), pentanal (0.460 mg/Kg), and hexanal (1.527 mg/Kg) were found in IF-SCO. These results are in agreement with the initial peroxide value, which was greater in IF-SCO. In both of the formula types stored at 25ºC, increased volatiles were observed

362 over time, with hexanal exhibiting the greatest increase (Table 3). At the end of the
363 study, the hexanal increased 2.44 times in IF-EPL (1.24 mg/Kg) and 2.61 times in IF-
364 SCO (3.98 mg/Kg). In the formulae stored at 40ºC, we encountered surprising results.
365 While we expected only volatiles to have increased (in formulae stored at 25ºC), these
366 compounds exhibited irregular behaviours (Table 3). This finding may be explained by
367 the high volatility of the studied compounds which, when formulae were stored at 40ºC,
368 were probably lost before analysis. Another possible explanation is that these secondary
369 oxidation products rapidly decompose, consequently reacting with other compounds in
370 the powder sample. In a previous study (Garcia-Llatas, Lagarda, Romero, Abellan &
371 Farre, 2006) on the determination of hexanal and pentane in infant formula, the authors
372 reported that hexanal contents were lower in samples stored for seven months *vs.* four.
373 The authors suggest that this decrease could stem from the reaction of hexanal with
374 other sample components, such as the Maillard reaction products. Ulberth et al. (1995)
375 monitored the oxidative deterioration of milk powder by HS-GC and reported hexanal
376 (from 10 μg/kg to 106 μg/kg) to be the main volatile present during the storage at room
377 temperature (30ºC) under direct light for 130 days . They also reported headspace
378 volatiles in a milk-based IF stored in air at 40ºC, with the major compounds, ranked by
379 amount, being hexanal, heptanal, and pentanal. Kim and Morr (1996) used dynamic HS
380 to monitor volatile compounds in commercial fluid milk stored in Pyrex test tubes and
381 exposed to fluorescent light. In their study, the major volatiles were hexanal, pentanal,
382 dimethyl disulfide, 2-butanone, and 2-propanol. Earlier reports (Cadwallader &
383 Howard, 1997; Cladman, Scheffer, Goodrich & Griffiths, 1998) also showed that the
384 most common volatile compounds in light-oxidized milk were pentanal and hexanal.
385 Although the profile of volatiles in IFs differ, pentanal and hexanal, the specific volatile
386 oxidation products of n-6 PUFA, were detected in our IF samples. In all formulae, the
387 volatiles content increased, especially hexanal, which is a more sensitive indicator of

388 lipid oxidation. Using a direct static HS-GC method, Gaafar (1991) determined volatiles
389 in UHT milk stored for 24 weeks at room temperature, reporting hexanal levels up to
390 100 μg/kg. In another study on unspecified milk-based products purchased from
391 supermarkets, hexanal values of 240 – 290 μg/kg were recorded by dynamic HS-GC
392 (Park & Goins, 1992). In addition, thin-layer chromatographic analysis of derivatized
393 carbonyl components from fresh whole milk powder detected an increase in hexanal
394 from 90 μg/kg to 1190 μg/kg during a storage period of 16 months at 37ºC in air (Boon,
395 Keen & Walker, 1976). The volatiles examined in this study are related to flavour,
396 which strongly affects infant acceptance of IF (Mennella & Beauchamp, 1998;
397 Mennella, Griffin & Beauchamp, 2004).
398
399 *3.4. Sensory analysis*
400 *3.4.1 Duo-trio test*
401 This test was chosen not only because it is simple, but also because a reference
402 sample is presented to panellists. No significant difference ($p<0.01$) was detected in IF-
403 EPL when compared with fresh formula (T0) *vs.* formula stored for 12 months.
404 However, when comparisons were made between T0 *vs.* formula stored for 18 months,
405 a sensory difference was detected in 90.79% of the tests (Table 4). In the case of IF-
406 SCO, when comparisons between T0 formula stored for 6 months were made, no
407 sensory differences were detected. However, when the same comparison was made
408 between T0 and formula stored for 12 months, a significant difference ($p>0.01$) was
409 detected (Table 4). The reason we carried out comparisons for both formula types (IF-
410 EPL and IF-SCO) during different months was due to the fact that at the beginning of
411 study, we had first conducted a duo-trio test comparing T0 *vs.* formula stored for 12
412 months. Since no significant differences had been detected in IF-SCO, we decided not
413 to compare T0 vs. T18 (in IF-SCO); instead, we compared T0 vs. formula stored for 6

414 months. The sensory results revealed IF-EPL to have better stability than IF-SCO
415 during storage, which is consistent with our earlier results pertaining to peroxide values
416 and volatiles content, in which IF-SCO exhibited signs of oxidation even prior to
417 storage.
418
419 *3.4.2 Paired-comparison test*
420 A paired comparison test was used to determine the way in which the taste, smell,
421 and flavour of the studied formulae changed during storage, compared with recently
422 manufactured formula (T0). This sensory test is one of the simplest and most widely
423 used now available (Meilgaard et al., 1987). In regards to IF-EPL, no significant
424 differences were observed in terms of "better flavour", "better smell", "more rancid
425 flavour" or "longer lasting flavour" when comparing T0 *vs.* formula stored for 12
426 months. When we compared T0 *vs.* formula stored for 18 months, however, the former
427 was rated as having better flavour and better smell in 90.79% and 77.68% of the tests,
428 respectively. In addition, formula stored for 18 months was rated as having a more
429 rancid flavour in 77.63% of the tests (Table 5). On the other hand, when IF-SCO was
430 compared (T0 *vs.* formula stored for 6 months), the former was rated as having better
431 flavour in 63.75% of the tests. Finally, more differences were detected when comparing
432 T0 *vs.* formula stored for 12 months; i.e., the former was rated as having better flavour
433 and smell, the latter more rancid flavour (Table 5). These results are in agreement with
434 the analytical parameters outlined above, in which IF-EPL was found to maintain
435 greater stability than IF-SCO.
436
437 *3.5. Furfural content*
438 It has been reported that the initial furfural compounds formed during the Maillard
439 reaction consist of HMF, and F, methyl furaldehyde (MF), and furyl-methyl ketone

(FMC). Indeed, these stem from the most advanced states of the reaction, or are formed by inter-conversion as a result of greater heating or longer storage periods (van Boekel, 1998; Ferrer, Alegria, Courtois & Farre, 2000). In this study, only HMF and F were detected; they are described in Table 6. Other studies detected neither MF nor FMC (Morales, Romero & Jimenez-Perez, 1992; Albalá-Hurtado, Veciana-Nogues, Izquierdo-Pulido & Vidal-Carou, 1997; Ferrer, Alegria, Farre, Abellan & Romero, 2000; Ferrer et al., 2002). At the beginning of the present study, similar quantities of HMF were detected: in IF-EPL, 566.66 μg/100 g of potential HMF; and in IF-SCO, 607.88 μg/100 g. After 18 months of storage at 25ºC, the following values were recorded: for IF-EPL 660.26 μg/100 g for IF-EPL and 998.03 μg/100 g for IF-SCO. In similar fashion, after 18 months of storage at 40ºC, the recorded values proved higher: 1378.36 μg/100 g for IF-EPL and 1305.75 μg/100 g for IF-SCO. In regards to potential F and free furfurals (free HMF and free F), no clear behaviour was observed (Table 6). In other studies (Ferrer et al., 2002; Ferrer, Alegria, Farre, Abellan & Romero, 2005), F and free furfurals reportedly varied in an irregular manner across changes in storage time and temperature. These findings can be explained by the fact that furfurals reach a state of equilibrium between destruction effected by oxidation and by precursors (Morales, Romero & Jimenez-Perez, 1997). In both formula types, furfural values were similar. Evolution of these compounds at 25 and 40ºC was found to vary. In general, potential HMF increased with storage time; in fact, it was incrementally higher when formulae were stored at 40ºC. This is due to inadequate storage conditions (e.g., 40ºC), which favors the Maillard reaction. Of the studied furfurals, potential HMF was the most sensitive indicator, exhibiting a clear evolution during storage and proving to be an effective indicator of the Maillard reaction. The values recorded for both formula types were similar to those reported in other studies (Albalá-Hurtado et al., 1997; Albalá-Hurtado, Veciana-Nogues, Marine-Font & Vidal-Carou, 1998; Ferrer et al.,

466 2000; Ferrer et al., 2002; Chávez-Servín et al., 2005; Chávez-Servín, Castellote &
467 López-Sabater, 2006). There are currently no established limits for furfural
468 concentration in infant formulae. Due to the fact that these compounds have been
469 associated with the Maillard reaction, and consequently with formula deterioration,
470 most studies recommend that the amount of potential HMF be kept as low as possible
471 (Bremer et al., 1987).
472
473 Depending upon the raw material used, supplementation can increase the retail price
474 of formula from 5-25%. As infant formula production is very competitive, this added
475 premium may restrict a particular formula's competitiveness. The search for low cost
476 materials for LC-PUFA supplementation, such as SCO sources, remains ongoing. With
477 regard to the studied formulae, the chosen parameters exhibited acceptable lipid stability
478 in both formula types, albeit with somewhat better results in IF-EPL than in IF-SCO. By
479 the end of the study, significant C18:2 n-6 losses were noted in both formula types.
480 However, no significant decreases were observed in the others, including AA and DHA.
481 Contrary to expectations, the observed stability of the fatty acid profiles is noteworthy
482 due to the very small differences found between a given fatty acid at the beginning and
483 the end of the study period, even in the case of C18:2 n-6 loses. In regards to furfural
484 content, specifically potential HMF, this increased in similar fashion in both formulae,
485 indicating the presence of the ubiquitous Maillard Reaction typically encountered in this
486 type of product during storage.
487
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489
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666
667

668 Table 1. Composition of the studied formulae according to the label information

	Mean values per 100 g	
	IF-EPL	IF-SCO
Energetic value (KJ)	2038	2132
(Kcal)	487	509
Carbohydrate (g)	53.3	58
Lactose (g)	46.1	41
Protein (g)	10	9.5
Fat (g)	26	26
Arachidonic acid (mg)	47	91
Docosahexaenoic acid (mg)	25	53
Vitamin A (μg RE))	640	606
Vitamin D (μg)	10.3	8.3
Vitamin E (mg α-TE)	25	6.1
Vitamin K (μg)	42	38
Vitamin B1 (μg)	520	568
Vitamin B2 (μg)	620	908
Vitamin B6 (μg)	825	379
Vitamin B12 (μg)	2	1.3
Vitamin C (mg)	60	68

669
670
671

672 Table 2. Fatty acid profiles of infant milk-based formulae during storage (% wt/wt)

	IF-EPL					IF-SCO				
	T0	**12m 25ºC**	**12m 40ºC**	**18m 25ºC**	**18m 40ºC**	**T0**	**12m 25ºC**	**12m 40ºC**	**18m 25ºC**	**18m 40ºC**
Fatty acids:	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd
C4:0	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00	Tr ± 0.00
C6:0	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.10 ± 0.01	0.10 ± 0.00	0.12 ± 0.00	0.11 ± 0.01	0.11 ± 0.00
C8:0	0.49 ± 0.01	0.50 ± 0.01	0.52 ± 0.01	0.49 ± 0.01	0.51 ± 0.03	1.40 ± 0.06	1.38 ± 0.02	1.50 ± 0.07	1.35 ± 0.03	1.35 ± 0.01
C10:0	0.54 ± 0.01	0.55 ± 0.01	0.56 ± 0.01	0.50 ± 0.01	0.52 ± 0.04	1.20 ± 0.04	1.18 ± 0.01	1.25 ± 0.06	1.10 ± 0.00	1.10 ± 0.00
C12:0	6.68 ± 0.09	6.73 ± 0.09	6.75 ± 0.08	6.52 ± 0.12	6.72 ± 0.46	9.76 ± 0.29	9.56 ± 0.10	10.19 ± 0.62	9.37 ± 0.04	9.38 ± 0.04
C14:0	2.78 ± 0.05	2.78 ± 0.02	2.79 ± 0.02	2.69 ± 0.05	2.78 ± 0.19	4.12 ± 0.09	4.03 ± 0.03	4.28 ± 0.21	3.97 ± 0.02	3.98 ± 0.01
C14:1	0.16 ± 0.01	0.14 ± 0.03	0.15 ± 0.01	0.13 ± 0.02	0.15 ± 0.02	0.15 ± 0.01	0.12 ± 0.01	0.11 ± 0.00	0.14 ± 0.01	0.12 ± 0.01
C15:0	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
C16:0	25.52 ± 0.78	25.29 ± 1.38	25.33 ± 1.14	25.34 ± 1.33	25.99 ± 0.42	21.74 ± 0.02	21.75 ± 0.01	21.71 ± 0.15	21.81 ± 0.13	21.85 ± 0.02
C16:1.n-7	0.32 ± 0.04	0.31 ± 0.01	0.33 ± 0.00	0.35 ± 0.01	0.35 ± 0.08	0.12 ± 0.02	0.13 ± 0.00	0.14 ± 0.00	0.14 ± 0.00	0.14 ± 0.01
C17:0	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.22	0.06 ± 0.01	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
C17:1	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
C18:0	3.82 ± 0.20	3.87 ± 0.12	3.96 ± 0.19	3.79 ± 0.07	3.88 ± 0.27	3.67 ± 0.07	3.74 ± 0.02	3.69 ± 0.07	3.89 ± 0.05	3.90 ± 0.05
C18:1.n-9	40.93 ± 0.43	41.12 ± 0.92	40.92 ± 0.86	41.44 ± 0.75	41.04 ± 0.38	37.55 ± 0.42	37.91 ± 0.14	36.90 ± 0.71	38.59 ± 0.23	38.66 ± 0.08
C18:2.n-6	16.29 ± 0.23	16.18 ± 0.44	16.16 ± 0.38	16.20 ± 0.29	*15.43 ± 0.31	17.48 ± 0.04	17.40 ± 0.06	17.43 ± 0.12	*16.75 ± 0.10	*16.71 ± 0.09
C18:3.n-6	0.08 ± 0.00	0.08 ± 0.00	0.09 ± 0.00	0.09 ± 0.01	0.09 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
C18:3.n-3	1.00 ± 0.02	0.99 ± 0.02	1.00 ± 0.03	1.01 ± 0.02	1.05 ± 0.07	1.19 ± 0.01	1.17 ± 0.01	1.19 ± 0.01	1.18 ± 0.03	1.21 ± 0.01
C20:0	0.26 ± 0.01	0.28 ± 0.02	0.29 ± 0.01	0.28 ± 0.02	0.32 ± 0.01	0.24 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.27 ± 0.01	0.23 ± 0.02
C20:1.n-9	0.27 ± 0.00	0.27 ± 0.00	0.27 ± 0.01	0.28 ± 0.01	0.29 ± 0.02	0.13 ± 0.00	0.14 ± 0.00	0.13 ± 0.01	0.14 ± 0.00	0.12 ± 0.01
C20:2.n-6	0.03 ± 0.00	0.04 ± 0.02	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C20:3.n-6	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
C20:4.n-6	0.13 ± 0.00	0.13 ± 0.00	0.14 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	0.31 ± 0.00	0.31 ± 0.00	0.31 ± 0.01	0.32 ± 0.00	0.31 ± 0.00
C22:0	0.25 ± 0.00	0.27 ± 0.02	0.27 ± 0.01	0.26 ± 0.00	0.27 ± 0.02	0.27 ± 0.01	0.29 ± 0.01	0.26 ± 0.01	0.31 ± 0.00	0.27 ± 0.01
C20:5.n-3	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.01
C22:6.n-3	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.17 ± 0.00	0.17 ± 0.00	0.18 ± 0.00	0.17 ± 0.01	0.17 ± 0.00
(1) SFA	40.65 ± 0.67	40.59 ± 1.41	40.79 ± 1.26	40.17 ± 1.07	41.29 ± 1.27	42.72 ± 0.39	42.47 ± 0.10	43.43 ± 0.81	42.42 ± 0.24	42.39 ± 0.02
(2)MUFA	41.70 ± 0.42	41.87 ± 0.96	41.70 ± 0.86	42.25 ± 0.75	41.88 ± 0.30	38.00 ± 0.43	38.35 ± 0.14	37.34 ± 0.71	39.04 ± 0.23	39.08 ± 0.47
(3) PUFA	17.65 ± 0.25	17.54 ± 0.46	17.52 ± 0.41	17.58 ± 0.31	16.83 ± 1.04	19.28 ± 0.06	19.18 ± 0.05	19.24 ± 0.11	18.54 ± 0.46	18.53 ± 0.09
n-3 PUFA	1.09 ± 0.02	1.09 ± 0.02	1.10 ± 0.03	1.11 ± 0.02	1.14 ± 0.06	1.40 ± 0.02	1.39 ± 0.01	1.41 ± 0.01	1.40 ± 0.04	1.43 ± 0.01
n-6 PUFA	16.56 ± 0.24	16.45 ± 0.44	16.42 ± 0.38	16.47 ± 0.30	*15.69 ± 0.32	17.88 ± 0.04	17.80 ± 0.06	17.82 ± 0.11	*17.14 ± 0.10	*17.10 ± 0.09
n-3 LC-PUFA	0.09 ± 0.00	0.09 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.01	0.21 ± 0.01	0.22 ± 0.00	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.00
n-6 LC-PUFA	0.18 ± 0.00	0.18 ± 0.01	0.17 ± 0.00	0.17 ± 0.00	0.17 ± 0.01	0.36 ± 0.00	0.36 ± 0.00	0.36 ± 0.01	0.36 ± 0.00	0.36 ± 0.00

673 Values represent the mean of three measurements. * = significant difference (p<0,05) with respect to T0 of the same formula. (1) Saturated fatty acids,
674 (2) monounsaturated fatty acids, (3) polyunsaturated fatty acids; (1+2+3= 100% of the fatty acids studied). m = months, T0 = before storage, Tr =
675 Traces <0.01%

676 Table 3. Peroxide values and volatiles during storage
677

Sample	Storage (months)	Peroxide value (meq O_2/Kg)	Propanal (mg/Kg)	Pentanal (mg/Kg)	Hexanal (mg/Kg)
IF-EPL	0	0.34	-	-	0.508 ± 0.040
25ºC	1	0.99	-	0.236 ± 0.028	0.473 ± 0.152
	3	1.11	-	0.306 ± 0.022	0.561 ± 0.027
	6	2.99	0.525 ± 0.06	0.380 ± 0.080	0.739 ± 0.053
	9	4.54	0.634 ± 0.03	0.383 ± 0.090	1.040 ± 0.112
	12	5.52	0.639 ± 0.03	0.385 ± 0.050	1.093 ± 0.019
	15	5.78	0.677 ± 0.03	0.543 ± 0.020	1.120 ± 0.060
	18	6.83	0.693 ± 0.03	0.776 ± 0.010	1.241 ± 0.053
IF-EPL	0	0.34	-	-	0.508 ± 0.040
40ºC	1	1.79	-	-	0.194 ± 0.039
	3	2.33	-	0.233 ± 0.120	0.226 ± 0.082
	6	4.52	0.424 ± 0.072	0.380 ± 0.017	0.443 ± 0.041
	9	5.28	-	-	0.436 ± 0.039
	12	6.53	-	-	0.445 ± 0.013
	15	9.21	-	-	0.391 ± 0.062
	18	48.56	1.558 ± 0.118	1.630 ± 0.045	7.312 ± 0.222
IF-SCO	0	1.26	0.814 ± 0.110	0.460 ± 0.13	1.527 ± 0.450
25ºC	1	2.18	0.905 ± 0.048	0.569 ± 0.07	1.778 ± 0.195
	3	2.48	1.068 ± 0.096	0.831 ± 0.04	2.947 ± 0.280
	6	2.55	1.225 ± 0.019	0.879 ± 0.00	3.148 ± 0.101
	9	5.69	1.598 ± 0.176	1.217 ± 0.08	3.639 ± 0.186
	12	10.08	1.663 ± 0.178	1.311 ± 0.11	3.686 ± 0.076
	15	11.43	0.828 ± 0.050	1.659 ± 0.27	3.690 ± 0.320
	18	13.33	1.296 ± 0.034	1.070 ± 0.13	3.987 ± 0.164
IF-SCO	0	1.26	0.814 ± 0.110	0.460 ± 0.13	1.527 ± 0.450
40ºC	1	4.03	0.808 ± 0.171	0.470 ± 0.17	1.511 ± 0.337
	3	3.89	0.976 ± 0.380	0.709 ± 0.22	1.656 ± 0.419
	6	4.77	0.521 ± 0.029	0.293 ± 0.07	0.359 ± 0.022
	9	7.22	-	-	0.306 ± 0.067
	12	11.49	-	-	0.537 ± 0.187
	15	12.45	-	0.766 ± 0.01	0.638 ± 0.020
	18	54.97	-	0.541 ± 0.01	0.403 ± 0.077

678 Values are expressed as the mean ± standard deviation of three determinations.

679
680 Table 4. Duo-Trio test in infant formulae stored at 25°C.
681

	IF-EPL		IF-SCO	
Comparison	T0 vs. 12 months	T0 vs. 18 months	T0 vs. 6 months	T0 vs. 12 months
Detected difference (%)	40.28	90.79*	45.00	68.42*
No detected difference (%)	59.72	9.21	55.00	31.58

682 Results of 80 tests. * = significant difference ($p>0.01$). T0 = formula before storage.

683 Table 5. Paired comparison test (preference %) in infant formulae stored at 25°C.
684

Characteristics	IF-EPL (T0 *vs.* 12 months)			IF-EPL (T0 *vs.* 18 months)		
	0 months	12 months	no differences	0 months	18 months	no differences
Better flavour	41.67	34.74	23.61	90.79*	3.95	5.26
Better smell	18.06	26.39	55.56	73.68*	3.95	22.37
More rancid flavour	25.00	19.44	55.56	3.95	77.63*	18.42
Longer lasting flavour	34.72	25.00	40.28	13.16	42.11	44.74
	IF-SCO (T0 *vs.* 6 months)			IF-SCO (T0 *vs.* 12 months)		
	0 months	6 months	indifferent	0 months	12 months	Indifferent
Better flavour	63.75*	22.50	13.75	78.95*	15.79	5.26
Better smell	12.50	43.75	43.75	59.21*	7.89	32.89
More rancid flavour	12.50	47.50	40.00	9.21	63.16*	27.63
Longer lasting-flavour	31.25	16.25	52.50	43.42	21.05	35.53

685 Results of 80 tests. * = significant difference ($p>0.01$). T0 = formula before storage
686

Table 6. Potential furfural content in stored infant formulae

Sample	Storage (months)	Potential HMF (μg/100 g)	Potential F (μg/100 g)	Potential HMF + F (μg/100 g)	Free HMF (μg/100 g)	Free F (μg/100 g)	Free HMF + F (μg/100 g)
IF-EPL	0	566.66 ± 19.43 $^{a-1-I}$	92.51 ± 07.21 $^{a-1-I}$	659.16 ± 17.30 $^{a-1-I}$	127.18 ± 02.13 $^{a-1-I}$	- $^{a-1-I}$	127.18 ± 02.13 $^{a-1-I}$
25ºC	1	673.73 ± 29.53 $^{a-1-I}$	113.84 ± 07.50 $^{a-2-I}$	787.57 ± 29.30 $^{a-1-I}$	167.31 ± 07.76 $^{a-1-II}$	- $^{a-1-I}$	167.31 ± 07.76 $^{a-1-I}$
	3	761.76 ± 20.56 $^{b-2-II}$	131.10 ± 06.80 $^{b-2-II}$	892.86 ± 17.90 $^{b-2-II}$	196.72 ± 21.06 $^{b-2-III}$	7.41 ± 2.35 $^{a-2-II}$	204.1 ± 19.8 $^{b-2-II}$
	6	751.18 ± 08.13 $^{b-2-II}$	108.04 ± 03.29 $^{a-2-I}$	859.23 ± 06.20 $^{b-2- II}$	152.47 ± 09.24 $^{a-2-I}$	- $^{a-1-I}$	152.47 ± 09.24 $^{a-2-I}$
	9	649.11 ± 45.76 $^{a-2-I}$	116.54 ± 14.67 $^{a-2-I}$	765.6 ± 58.07 $^{a-1-I}$	118.75 ± 02.16 $^{a-1-I}$	- $^{a-1-I}$	118.75 ± 02.16 $^{a-1-I}$
	12	645.76 ± 07.08 $^{a-2-I}$	74.72 ± 20.23 $^{a-2-I}$	720.49 ± 28.05 $^{a-1-I}$	134.46 ± 22.97 $^{a-1-I}$	- $^{a-1-I}$	134.46 ± 22.97 $^{a-2-I}$
	15	650.94 ± 18.98 $^{a-2-I}$	84.10 ± 10.86 $^{a-2-I}$	735.01 ± 29.03 $^{a-1-I}$	135.78 ± 05.49 $^{a-1-I}$	7.21 ± 2.23 $^{a-2-I}$	142.99 ± 07.23 $^{a-2-I}$
	18	660.26 ± 34.64 $^{a-2-II}$	82.94 ± 05.09 $^{a-2-I}$	743.20 ± 35.05 $^{a-1-I}$	158.55 ± 08.33 $^{a-1-I}$	5.25 ± 0.09 $^{a-2-I}$	163.80 ± 08.40 $^{a-2-II}$
IF-EPL	0	566.66 ± 19.43 $^{a-1-I}$	92.51 ± 07.21 $^{a-1-II}$	659.16 ± 17.30 $^{a-1-I}$	127.18 ± 02.13 $^{a-1-I}$	- $^{a-1-I}$	127.18 ± 02.13 $^{a-1-I}$
40ºC	1	649.03 ± 47.69 $^{a-1-I}$	127.18 ± 14.50 $^{b-2-II}$	776.21 ± 52.60 $^{a-1-II}$	127.29 ± 07.66 $^{a-1-I}$	20.21 ± 8.62 $^{b-2-II}$	147.5 ± 02.3 $^{a-1-II}$
	3	779.03 ± 73.28 $^{b-2-II}$	120.26 ± 19.50 $^{a-2-III}$	889.29 ± 84.50 $^{b-2-III}$	131.34 ± 17.22 $^{a-3-I}$	16.34 ± 5.69 $^{b-2-II}$	147.7 ± 16.2 $^{a-1-II}$
	6	904.64 ± 77.65 $^{c-3-III}$	104.94 ± 07.06 $^{a-2-III}$	1009.57 ± 81.70 $^{b-2-III}$	170.59 ± 05.98 $^{b-2-II}$	11.73 ± 4.10 $^{b-2-II}$	182.3 ± 08.8 $^{b-2-III}$
	9	1119.22 ± 67.50 $^{d-4-IV}$	122.94 ± 17.61 $^{a-2-I}$	1242.16 ± 57.21 $^{c-3-IV}$	161.57 ± 18.45 $^{a-2-III}$	10.44 ± 7.24 $^{a-3-I}$	172.0 ± 17.6 $^{b-2-III}$
	12	1255.45 ± 57.88 $^{e-5-V}$	86.41 ± 10.06 $^{a-2-I}$	1341.85 ± 59.01 $^{c-3-IV}$	161.24 ± 28.39 $^{a-1-III}$	13.77 ± 7.19 $^{b-2-II}$	175.0 ± 34.5 $^{b-2-III}$
	15	1338.92 ± 76.16 $^{e-5-V}$	122.37 ± 06.76 $^{a-2-I}$	1461.29 ± 74.12 $^{c-3-V}$	164.48 ± 29.79 $^{a-1-III}$	- $^{a-1-I}$	199.72 ± 33.46 $^{b-2-III}$
	18	1378.36 ± 89.10 $^{e-5-V}$	87.28 ± 10.87 $^{a-2-I}$	1465.64 ± 94.35 $^{c-3-V}$	163.00 ± 28.98 $^{a-1-III}$	13.99 ± 7.10 $^{b-2-II}$	191.84 ± 22.50 $^{b-2-III}$
IF-SCO	0	607.88 ± 34.48 $^{a-1-I}$	78.20 ± 06.24 $^{a-1-I}$	686.09 ± 36.10 $^{a-1-I}$	123.73 ± 12.40 $^{a-1-I}$	- $^{a-1-I}$	123.73 ± 12.40 $^{a-1-I}$
25ºC	1	630.34 ± 30.72 $^{a-1-I}$	150.06 ± 06.22 $^{b-2-II}$	780.41 ± 33.01 $^{a-2-I}$	187.01 ± 19.60 $^{b-2-II}$	13.35 ± 2.33 $^{b-2-II}$	200.40 ± 17.5 $^{b-2-II}$
	3	741.99 ± 7.04 $^{b-2-II}$	132.61 ± 12.91 $^{b-2-II}$	874.60 ± 18.41 $^{b-3-II}$	167.86 ± 6.69 $^{b-2-III}$	15.97 ± 7.90 $^{b-2-II}$	183.80 ± 05.3 $^{b-2-II}$
	6	782.92 ± 26.31 $^{b-2-II}$	106.08 ± 03.70 $^{a-1-I}$	889.00 ± 26.30 $^{b-3-II}$	196.85 ± 23.05 $^{b-2-II}$	11.86 ± 3.26 $^{b-2-II}$	208.70 ± 25.5 $^{b-2-II}$
	9	788.17 ± 28.56 $^{b-2-II}$	109.53 ± 13.01 $^{a-1-I}$	897.70 ± 18.30 $^{b-3-II}$	137.30 ± 08.11 $^{a-2-I}$	12.35 ± 4.43 $^{b-2-I}$	149.60 ± 06.6 $^{a-2-I}$
	12	792.51 ± 24.17 $^{b-2-II}$	109.31 ± 21.45 $^{a-1-II}$	901.82 ± 38.80 $^{b-3-II}$	175.52 ± 42.58 $^{a-2-I}$	5.39 ± 0.51 $^{a-1-I}$	180.90 ± 43.1 $^{b-2-II}$
	15	810.61 ± 30.60 $^{b-2-II}$	111.45 ± 19.77 $^{a-1-II}$	922.06 ± 33.90 $^{b-3-II}$	193.71 ± 33.18 $^{b-2-II}$	6.01 ± 0.54 $^{a-1-I}$	199.72 ± 33.46 $^{b-2-II}$
	18	998.03 ± 46.52 $^{c-5-III}$	121.63 ± 03.71 $^{a-2-II}$	1119.65 ± 43.62 $^{b-6-III}$	186.62 ± 23.04 $^{b-2-II}$	5.22 ± 1.25 $^{a-1-I}$	191.84 ± 22.50 $^{b-2-II}$
IF-SCO	0	607.88 ± 34.48 $^{a-1-I}$	78.20 ± 06.24 $^{a-1-I}$	686.09 ± 36.10 $^{a-1-I}$	123.73 ± 12.40 $^{a-1-I}$	- $^{a-1-I}$	123.73 ± 12.40 $^{a-1-I}$
40ºC	1	774.42 ±	100.62 ±	875.04 ±	162.90 ±	8.08 ±	171.01 ±

	11.42 $^{b-2-II}$	13.34 $^{a-1-II}$	24.10 $^{b-2-II}$	07.08 $^{a-2-I}$	0.56 $^{a-2-II}$	06.5 $^{b-2-II}$
3	807.51 ± 33.42 $^{b-2-II}$	111.59 ± 09.48 $^{b-3-III}$	919.10 ± 40.30 $^{b-3-III}$	160.93 ± 10.55 $^{a-2-I}$	10.17 ± 0.57 $^{a-2-II}$	171.10 ± 10.0 $^{b-2-II}$
6	1074.29 ± 14.43 $^{c-3-IV}$	113.78 ± 08.90 $^{b-3-III}$	1188.10 ± 08.80 $^{c-4-IV}$	174.81 ± 10.54 $^{b-2-II}$	- $^{a-1-I}$	174.810 ± 01.54 $^{b-2-III}$
9	1177.98 ± 18.57 $^{c-3-IV}$	98.37 ± 10.01 $^{a-1-I}$	1276.34 ± 27.50 $^{c-4-IV}$	176.61 ± 07.24 $^{b-2-III}$	- $^{a-1-I}$	176.610 ± 07.24 $^{b-2-III}$
12	1194.62 ± 28.42 $^{c-3-V}$	95.36 ± 12.21 $^{a-1-I}$	1289.98± 36.20 $^{c-4-IV}$	203.77 ± 07.59 $^{b-2-III}$	- $^{a-1-I}$	203.77 ± 07.59 $^{b-2-III}$
15	1299.37 ± 57.00 $^{d-4-V}$	89.40 ± 08.15 $^{a-1-I}$	1388.77 ± 50.52 $^{d-5-V}$	170.27 ± 12.52 $^{b-2-III}$	- $^{a-1-I}$	170.27 ± 12.52 $^{b-2-III}$
18	1305.75 ± 43.93 $^{d-4-V}$	77.38 ± 23.44 $^{a-1-I}$	1383.14 ± 43.41 $^{d-5-v}$	164.01 ± 24.40 $^{a-2-III}$	- $^{a-1-I}$	164.01 ± 24.40 $^{b-2-III}$

Values are expressed as the mean ± standard deviation (n=4). Repetitions in superscripted characters within the same column indicate no significant differences ($p<0.05$), according to the following parameters: Letters, storage time (0-18 months); Arabic numerals, storage temperature (25 *vs.* 40ºC); and Roman numerals, (IF-EPL *vs.* IF-SCO).