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High-performance liquid chromatographic determination of furfural compounds in infant formulas Changes during heat treatment and storage ☆

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Abstract

Furfural contents in adapted and follow-up infant formulas were measured by RP-HPLC. The evolution of furfural compound contents during storage (a year at 20 and 37 °C) was studied. 2-Furylmethylketone and 5-methyl-2-furaldehyde were not detectable in analysed samples. The differences in the furfural compounds at point zero between both infant formulas has to be ascribed to the differences in protein and iron contents. An increase in free 5-hydroxymethyl-2-furfuraldehyde (HMF), 2-furaldehyde (F) and HMF+F contents was observed in all samples, although the differences were not statistically significant. The storage temperature affected the total HMF content and the storage time affected the total HMF and F contents.

Keywords: Infant formulas; Milk; Food analysis; Furfurals; Aldehydes

1. Introduction

The interactions between infant formula components (proteins, fats, carbohydrates, vitamin and minerals) mainly affect carbohydrates and proteins (Maillard reaction, MR), but those involving proteins are especially important in products used in infant feeding because of the high protein requirements of infants. The fact that infant formulas have high lactose and lysine contents, that relatively high temperatures are applied during their manufacturing process and that their storage is quite long makes them highly sensitive to MR [1]. Moreover, the addition of vitamin A and iron contributes to this susceptibility [2].

Measurement of the losses of available lysine and/or of contents of undesirable compounds, such as furfurals, generated at advanced stages of MR is used to evaluate the intensity of the thermal treatment applied and/or the effects of storage [3]. When sugars are heated, furfural compounds can be formed by two possible pathways: in the first (MR) case, when amino groups are present from Amadori compounds by enolyzation in acidic conditions, and

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there is a subsequent dehydratation of 3-deoxyosones. The second pathway involves lactose isomerization known as Lobry De Bruyn–Alberda van Ekenstein transformation and the subsequent degradation reactions [4,5].

The most widely studied furfural compounds are: 2-furaldehyde (F) (which originates mainly from pentoses) and 5-hydroxymethyl-2-furfuraldehyde (HMF) (which is a product of hexose degradation). HMF contents have most often been measured using the spectrophotometric method proposed by Keeney and Basette [6]. The main disadvantage of this method lies in the lack of specificity of the reaction with thiobarbituric acid, so other carbonyl compounds intrinsic of milk or resulting from the MR can react with thiobarbituric acid. This fact is probably responsible for the overestimation of HMF contents in some reports that give unexpected HMF values in milk products. However, reversed-phase high-performance liquid chromatography (RP-HPLC) techniques are now available for accurate measurement of different furfural compounds in milk.

Several authors have measured the furfural compound contents of commercial milk in order to evaluate the effect of different thermal treatments [pasteurization, ultra-high temperature (UHT), sterilization] on HMF formation, using the HMF content as an indicator of the type of thermal treatment applied [7–15].

Reports on thermal treatment evaluation in infant formulas are less abundant and those available are difficult to compare, because furfural contents can be the result of many effects (thermal treatments and compositions) that do not always coincide in the analyzed formulas and that the authors do not evaluate individually. In some of the studies the quality of the raw matter (raw cow milk) is unknown, and in others the furfural compounds contents of formulas differing in their composition and in the thermal treatment applied are compared [16] or the comparison is carried out in formulas having the same casein/serum protein ratio, subjected to the same treatment but differing in sugar, vitamin and mineral contents [17]. Only a few authors have studied formulas subjected to the same thermal treatment but differing in their protein fraction [18].

The evolution of furfural compounds during storage have also been studied in milk [8,19,20] or in infant formulas [17,21–24]. In some of these studies the storage time taken into account was longer than the formula shelf life [24], while in others large amounts of caseins, vitamins and/or minerals were added to the formulas [25] or the storage temperatures were unknown [21,22]. Therefore, we have been unable to find studies evaluating furfural compound formation in real time and temperature storage conditions in powdered milk-based infant formulas that differ in their composition (protein), were manufactured using the same raw cow milk and have been subjected to the same thermal treatment.

The aim of our study was to measure by RP-HPLC the furfural compounds contents of an adapted and a follow-up infant formula manufactured using the same raw cow milk and thermal treatment, but differing in their protein composition, and to study and compare the evolution of furfural compounds during a year of storage at 20 and 37 °C.

2. Experimental

2.1. Material and methods

2.1.1. Samples

Samples of adapted and follow-up milk-based formulas were analyzed. Both were vacuum packed in commercial airtight 1 kg containers in an N_2–CO_2 (<3% O_2) modified atmosphere. For each type of formula enough packages from the same batch to carry out the storage study were sampled.

Raw material: both formulas were manufactured from raw cow milk (class A, means <400 000 somatic cells/ml and <100 000 cfu/ml).

Thermal treatments applied during processing: (1) Pasteurization (72 °C/15 s) of raw cow milk and milk whey; the latter was obtained by ultracentrifugation. (2) Concentration of the milk in a long-tube vertical, falling film evaporator combining three thermal effects (85, 66 and 58 °C) over a 5 min period. (3) Sterilization (high-temperature start time (HTST) 100 °C/22 s). (4) Spray-drying (air input 175–185 °C/air output 90–94 °C) of the mixture of concentrated milk, pasteurized whey and lipids, lactose and minerals.

The composition of the studied infant formulas as given on the label was:

(1) Adapted infant formula: proteins (casein/serum proteins, 40/60) 11.6%, carbohydrates (lactose) 55%, lipids 28%, iron 6 mg and vitamin A 450 μg.

(2) Follow-up infant formula: proteins (casein/serum proteins, 80/20) 16.%, carbohydrates 54% (lactose 32.4%+maltodextrine 21.6%), lipids 24% iron 8 mg and vitamin A 450 μg.

Storage: samples of both formulas were stored at 20 and 37 °C in a storage chamber (with<10% relative humidity and temperature controlled by a BJC heater with a Omron E5EW thermostat) for 12 months and analyzed just after manufacturing (at zero time) and after 3, 6, 9 and 12 months of storage. Samples were maintained in their airtight containers until analysis.

2.1.2. Apparatus

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of two LC-10AD pumps controlled by a CBM-10a, a Model 7725i manual injection valve (Rheodyne, Cotati, CA, USA) equipped with a 20 μl sample loop and an SPD-10AD UV–visible detector. Data were collected and analysed using the CLASS LC-10W/S software package.

Solvents and samples were filtered using a Millipore (Milford, MA, USA) system with 0.20 μm membrane filters (47 and 13 mm, respectively).

Block digestion system: Foss Tecator 2006 Digestor (Höganäs, Sweden). Distillation system: Kjeltec system 1026 distilling unit (Foss Tecator). Centrifuge: Jouan Model GT 422 (Saint Nazaire, France) equipped with a fixed-angle rotor, capable of centrifugation at 4000 *g*. pH meter: Crison Model GLP21 (Alella, Barcelona, Spain). Shaking bath: Selecta Digiterm 200 (Barcelona, Spain) −20 to 200±0.05 °C. Drying chamber: Heraeus Ut 6060 (Hanau, Germany).

2.1.3. Chemicals and materials

Acetonitrile 99.8% and methanol 99.8% HPLC quality were obtained from J.T. Baker (Deventer, The Netherlands); oxalic acid dihydrate 99.5% and trichloroacetic acid (TCA) 99.5%, HMF (5-hydroxymethyl-2-furaldehyde), F (2-furaldehyde), FMC (2-furylmethylketone) and MF (5-methyl-2-furaldehyde) from Fluka (Buchs, Switzerland).

All aqueous solutions were prepared with high-purity water produced with a Millipore system.

All reagents were of analytical-reagent grade unless the contrary is stated.

2.2. Procedures

2.2.1. Determination of free and total furfural compounds (HMF, F, FMC and MF)

Total and free furfurals of infant formulas were measured by RP-HPLC with UV detection, according to the method proposed by Albalá-Hurtado et al. [26]. Total furfurals include free furfurals, furfurals bound to proteins (as Amadori products) and furfurals formed from the precursors (or novo furfurals).

Sample preparation was based on the Van Boekel and Rehman [9] procedure.

(1) Total furfurals: 15 g of 15% (w/v) reconstituted infant formula was mixed with 5 ml of 0.15 *M* oxalic acid (freshly prepared daily) in a sealed tube to prevent evaporation. The tube was heated in a boiling water bath for exactly 25 min. After letting it cool at room temperature, 3 ml of a 40% (w/v) TCA solution was added, and the mixture was stirred (magnetic stirring plate) thoroughly for 5 min. It was then centrifuged at 2000 *g* for 15 min and two phases were obtained. The supernatant was collected and 10 ml of 4% (w/v) TCA was added to the solid residue, mixed thoroughly for 10 min and centrifuged at 2000 *g* for 15 min. The solid phase was discarded, and the two supernatants were combined. The volume was then measured, and the mixture was filtered through a 0.20 μm filter.

(2) Free furfurals: the sample was prepared as mentioned above for total furfurals but omitting the heating in the boiling water bath.

(3) RP-HPLC conditions: a Spherisorb ODS2 C_{18} 5 μm column (250×4.6 mm I.D.) was used. Separations were carried out isocratically at room temperature using a mixture of acetonitrile–water (5:95, v/v) at a flow-rate of 1 ml/min as the mobile phase. Detection in wavelength gradient at 284 nm for HMF and F, and 274 nm for FMC and at 293 nm for MF. The injection volume was 20 μl.

Furfurals were quantified by interpolation in a

Table 1
Furfural compounds determination in infant formulas: hydroxymethylfurfural (HMF), furfural (F), furylmethylcetone (FMC) and methylfurfural (MF)

Analytical parameter	HMF	F	FMC	MF
Intra-day precision $n=5$				
(μg/100 g formula)	83.0±3.7	31.7±4.0	90.2±6.6	86.2±6.6
(RSD, %)	4.4	12.7	7.3	7.7
Inter-day precision $n=9$				
(μg/100 g formula)	88.9±10.4	29.3±4.4	85.9±8.3	98.0±15.3
(RSD, %)	11.7	14.9	9.7	15.6
Recovery (%) $n=3$	101.0±3.5	93.1±3.6	113.7±3.1	112.7±27.0
Detection limit				
(μg/100 g formula)	6.7	13.3	13.3	13.3
(μg/ml assay)	0.005	0.01	0.01	0.01
Linearity	0.01–2 μg/ml assay 11–2222 μg/100 g formula			

n=Number of samples. RSD=Relative standard deviation.

calibration curve in the range of 0.01 to 2 μg/ml assay of HMF; F, FMC and MF.

The analytical parameters of the method are reported in Table 1.

2.2.2. Determination of total protein content

The Kjeldahl method (AOAC official method, 1980) was used to measure total nitrogen [27]. To convert the nitrogen values to protein, a factor of 6.25 was applied.

2.2.3. Determination of moisture

The water content of all the samples was measured by desiccation at 102±1 °C up to constant mass [28].

All analyses were carried out in triplicate.

2.3. Statistical analysis

Three- and two-factor analysis of variance (ANOVA) tests were applied to the free and total HMF, F and HMF+F contents in order to detect differences between the two studied infant formulas, the storage temperatures conditions (20 and 37 °C) and the storage period (0, 3, 6, 9, and 12 months).

In order to study the possible influence of storage period on free and total HMF, F and HMF+F contents, a simple regression was applied and different alternative models ($y=a+bx$, $1/y=a+bx$) were obtained.

3. Results and discussion

Free and total furfural compounds (HMF, F and HMF+F) contents, expressed as μg/100 g sample and mg/100 g protein, in stored adapted and follow-up infant formulas, are reported in Tables 2–4.

The structures of furfurals and the chromatograms corresponding to (a) standard solution, (b) adapted infant formula and (c) follow-up infant formula are included in Fig. 1.

The pH value and moisture content of the samples were measured at each sampling point because both factors can favor furfural formation either by lactose isomerization (Lobry De Bruyn–Alberda van Ekenstein transformation) or through Amadori compounds intermediates in the MR. No differences were found either in pH values or in moisture content among the different sampling periods and/or temperatures in the two types of infant formulas studied.

The mean pH and moisture values are: (1) adapted formula: pH 6.732±0.241 and moisture 2.613±0.458, ranging during the storage period from 6.435 to 7.047, and 1.978 to 3.158, respectively; (2) follow-up formula: pH. 6.754±0.216, and moisture

Table 2
Free and total HMF contents in stored infant formulas (mean±standard deviation)

Temperature (°C)	Month	Free HMF		Total HMF	
		μg/100 g sample	mg/100 g proteins	mg/100 g proteins	μg/100 g sample
Adapted formulas					
20	0	97.06±16.19^{a-1}	0.77±0.13	570.07±17.86^{c-3}	4.52±0.14
	3	126.27±1.93^{a-1}	1.02±0.02	230.95±26.11^{e-3}	1.86±0.21
	6	113.41±19.76^{a-1}	0.93±0.16	537.30±9.13$^{c,d-3}$	4.43±0.08
	9	37.82±1.38^{a-1}	0.33±0.01	438.32±14.93$^{c,d-3}$	3.82±0.13
	12	123.26±1.31^{a-1}	1.08±0.01	234.8±13.64$^{d,e-3}$	2.05±0.03
37	0	97.06±16.19^{a-1}	0.77±0.13	570.07±17.86^{c-4}	4.52±0.14
	3	136.5±18.27^{a-1}	0.74±0.02	297.68±7.43^{e-4}	2.39±0.06
	6	176.91±0.51^{a-1}	1.49±0.01	658.04±37.8$^{c,d-4}$	5.56±0.32
	9	87.99±6.58^{a-1}	0.76±0.06	668.33±28.19$^{c,d-4}$	5.76±0.24
	12	214.54±6.68^{a-1}	1.95±0.06	396.6±13.9$^{d,e-4}$	3.47±0.12
Follow-up formulas					
20	0	474.03±50.88^{b-2}	3.06±0.33	1291.35±68.02^{f-5}	8.33±0.44
	3	443.27±28.33^{b-2}	2.91±0.19	702.67±12.01^{g-5}	4.61±0.08
	6	458.87±14.84^{b-2}	3.01±0.1	1137.60±52.81^{f-5}	7.46±0.35
	9	380.53±27.46^{b-2}	2.59±0.19	997.19±19.07$^{f,\ g-5}$	6.80±0.13
	12	533.29±10.16^{b-2}	3.8±0.07	674.04±20.48^{g-5}	4.81±0.15
37	0	474.03±50.88^{b-2}	3.06±0.33	1291.35±68.02^{f-6}	8.33±0.44
	3	505.5±12.97^{b-2}	3.33±0.09	810.17±39.27^{g-6}	5.34±0.26
	6	499.77±21.13^{b-2}	3.24±0.14	1435.04±14.28^{f-6}	9.30±0.09
	9	450.52±9.71^{b-2}	3.28±0.07	1158.56±54.69$^{f,\ g-6}$	8.44±0.40
	12	623.67±17.92^{b-2}	4.7±0.14	888.24±12.1^{g-6}	6.70±0.09

No coincidence in the superscript letters indicates significant differences ($P<0.05$) with the storage time of the same column.
No coincidence in the superscript numbers indicates significant differences ($P<0.05$) with the storage temperature of the same column.

2.653±0.313 ranging during the storage period from 6.435 to 7.000 and 2.389 to 3.374, respectively.

Free and total FMC and MF were not detectable in any of the analyzed samples.

In a previous study [18] no furfural compounds (HMF, F, FMC and MF) were detected in the raw cow milk used in the manufacture of the formulas studied here. Therefore, the HMF and F detected at the zero point are formed during the thermal treatment steps of the manufacturing process.

At point zero, the free and total HMF contents of the adapted formulas are, respectively, five and two times, lower than those obtained in the follow up formulas (Table 2). Both formulas were subjected to the same thermal treatments (pasteurization 72 °C, 15 s/sterilization 100 °C, 22 s/atomization: air input 175–185 °C, air output 90–94 °C) and in making them raw cow milk of the same quality was used. Therefore, the statistically significant differences ($P<0.05$) in the furfural compound content between the adapted and follow up formulas can be ascribed to the differences in their composition.

The analyzed infant formulas differed in the type of sugar but not in their contents. While the adapted formula only contained lactose, the follow-up formula had lactose and maltodextrine. The adapted and the follow-up formulas had the same lactose content during the thermal treatment steps, because it was at a later step that lactose or maltodextrine was added to the powdered milk base. The amount added was sufficient to reach a final total sugar content of 55 or 54% depending on the type of formula to be obtained, i.e., adapted or follow-up, respectively. According to that, only the lactose present in the milk base could be expected to give rise to furfural compound formation during the thermal treatment, and the contents at the zero point should be similar in both the adapted and follow-up formulas.

Several authors studied the effect of sugar type on lysine losses during the thermal treatment by using

Table 3
Free and total F contents in stored infant formulas (mean±standard deviation)

Temperature (°C)	Month	Free F		Total F	
		μg/100 g sample	mg/100 g proteins	μg/100 g sample	mg/100 g proteins
Adapted formulas					
20	0	nd^{a-1}	nd	31.88±5.43^{c-3}	0.25±0.04
	3	18.19±0.51^{a-1}	0.15±0.01	16.79±1.33^{d-3}	0.14±0.01
	6	nd^{a-1}	nd	44.25±3.59$^{c,\ e-3}$	0.36±0.03
	9	17.51±1.80^{a-1}	0.15±0.02	50.23±3.93^{e-3}	0.44±0.03
	12	nd^{a-1}	nd	51.14±4.18^{e-3}	0.45±0.04
37	0	nd^{a-1}	nd	31.88±5.43^{c-3}	0.25±0.04
	3	17.59±0.53^{a-1}	0.12±0.05	19.14±2.26^{d-3}	0.15±0.02
	6	15.32±2.83^{a-1}	0.13±0.02	40.91±2.67$^{c,\ e-3}$	0.35±0.02
	9	23.78±1.68^{a-1}	0.20±0.01	56.78±6.82^{e-3}	0.49±0.06
	12	53.22±2.53^{a-1}	0.48±0.02	55.58±3.93^{e-3}	0.5±0.04
Follow-up formulas					
20	0	nd^{b-2}	nd	70.68±3.94^{g-4}	0.46±0.03
	3	15.28±3.69^{b-2}	0.10±0.02	31.03±1.28^{f-4}	0.20±0.01
	6	19.81±5.01^{b-2}	0.13±0.03	67.37±8.48^{g-4}	0.44±0.06
	9	66.89±4.28^{b-2}	0.46±0.03	76.08±7.40^{g-4}	0.52±0.05
	12	16.66±0.45^{b-2}	0.12±0.01	67.05±1.96^{g-4}	0.48±0.01
37	0	nd^{b-2}	nd	70.68±3.94^{g-4}	0.46±0.03
	3	20.23±2.68^{b-2}	0.13±0.02	31.34±4.53^{f-4}	0.21±0.03
	6	20.24±2.21^{b-2}	0.13±0.01	77.95±5.75^{g-4}	0.51±0.04
	9	20.86±0.57^{b-2}	0.15±0.01	79.23±7.30^{g-4}	0.58±0.05
	12	24.49±1.81^{b-2}	0.18±0.01	81.55±4.51^{g-4}	0.62±0.03

nd: Non detectable.
No coincidence in the superscript letters indicates significant differences ($P<0.05$) with the storage time of the same column.
No coincidence in the superscript numbers indicates significant differences ($P<0.05$) with the storage temperature of the same column.

Table 4
Free and total HMF+F contents (expressed as μg/100 g sample) in stored infant formulas

Temperature (°C)	Month	Adapted formulas		Follow-up formulas	
		Free HMF+F	Total HMF+F	Free HMF+F	Total HMF+F
20	0	110.36^{a-1}	601.95^{c-3}	487.33^{b-2}	1362.03^{e-5}
	3	144.46^{a-1}	247.74^{d-3}	458.55^{b-2}	733.70^{e-5}
	6	126.71^{a-1}	581.55^{c-3}	478.68^{b-2}	1204.97^{e-5}
	9	55.33^{a-1}	488.55^{c-3}	447.42^{b-2}	1073.27^{e-5}
	12	136.56^{a-1}	285.94$^{c,\ d-3}$	549.95^{b-2}	741.09^{e-5}
37	0	110.36^{a-1}	601.95^{c-4}	487.33^{b-2}	1362.03^{e-5}
	3	154.09^{a-1}	316.82^{d-4}	525.73^{b-2}	841.51^{e-5}
	6	192.23^{a-1}	698.95^{c-4}	520.01^{b-2}	1512.99^{e-5}
	9	111.77^{a-1}	725.11^{c-4}	471.38^{b-2}	1237.79^{e-5}
	12	267.76^{a-1}	452.18$^{c,\ d-4}$	648.16^{b-2}	969.79^{e-5}

No coincidence in the superscript letters indicates significant differences ($P<0.05$) with the storage time of the same column.
No coincidence in the superscript numbers indicates significant differences ($P<0.05$) with the storage temperature of the same column.

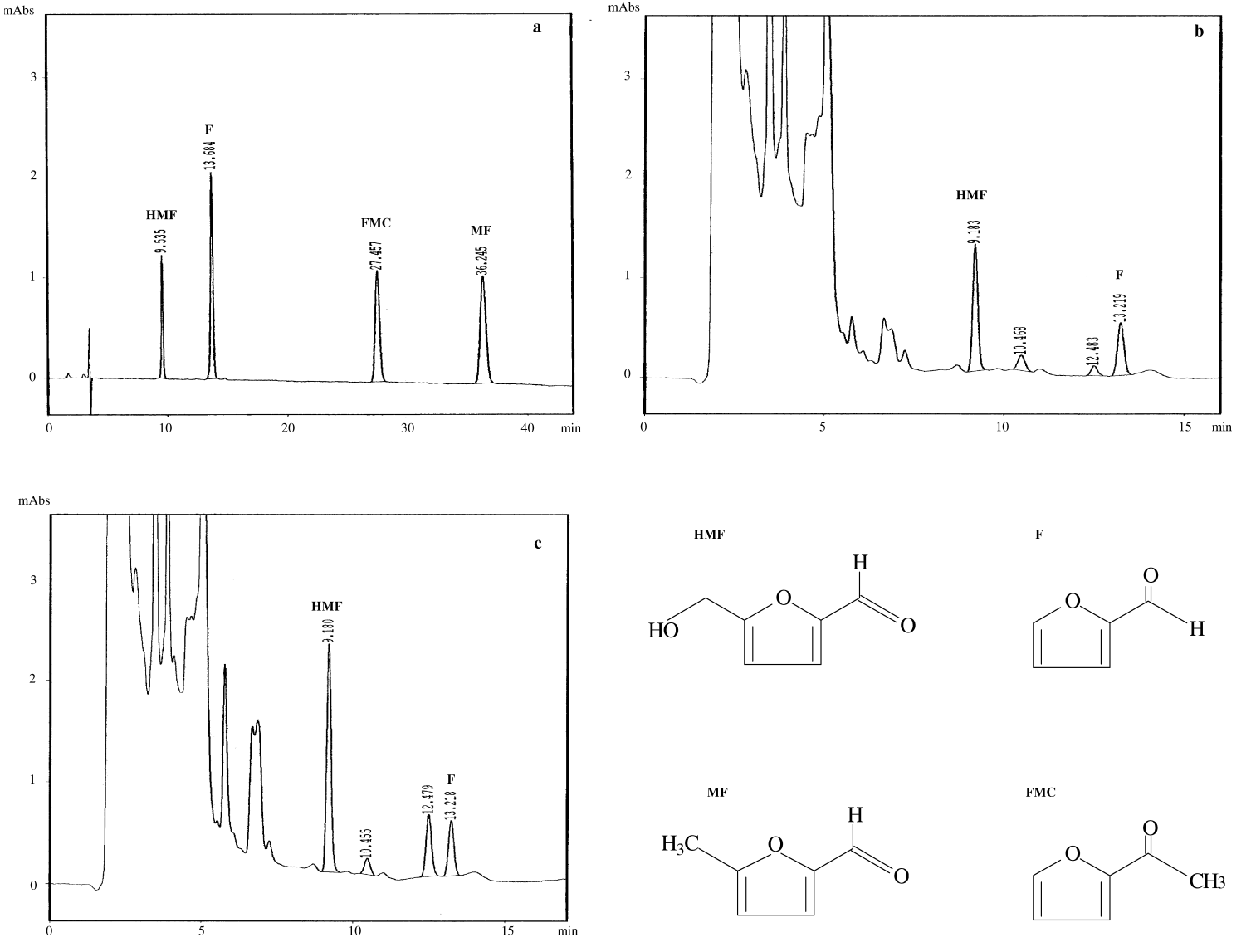

Fig. 1. The structures of furfurals and the chromatograms corresponding to (a) standard, (b) adapted infant formula and (c) follow-up infant formula.

Table 5
HMF contents (μmol/l) in milk and infant formulas reported by different authors

Sample	Free HMF	Total HMF	Ref.
Adapted infant formulas			
Pasteurized	0.03	0.3	[16][a]
UHT	0.07–0.75	1.49–4.20	
Sterilized	0.50–0.82	2.65–4.50	
Sterilized and atomized	0.043–0.098	0.174–0.300	[18][c]
	nd–0.013[b]	0.019–0.037[b]	
Follow-up infant formulas			
Sterilized and atomized	0.338–0.481	0.645–0.913	[18][c]
Sterilized	0.014–0.021[b]	0.029–0.046[b]	
	0.6–1.6	9.0–12.2	[17]
	0.2–0.6[b]	1.4–2.3[b]	
Spray-drying	10.3–23.5	15.0–34.7	
	1.0–5.3[b]	1.8–6.8[b]	
UHT	nd	3.0–8.8	[24]
	nd[b]	1.2–3.2[b]	
Junior milk			
UHT	1.0–2.2	13.2–20.5	[24]
	0.3–1.2[b]	2.3–3.4[b]	
Other infant milks			
–	0.3–2.3	2.2–34.3	[21][c,d]
–	–	1.2–6.1	[22][c]
–	–	21.0–43.9	[25]
Cow's milk			
Pasteurized	–	0.5–4.9	[7]
	–	1.11	[12]
		2.1[d]	
	nd	0.95–2.14	[13]
	–	1.5–2.5	[14]
UHT	–	3.1–16.8	[7]
		5.7–28.4	[2][d]
	–	6.0–8.5	[8]
	–	6.5	[9]
	–	5.0–20.0	[10]
	–	4.56–12.01	[11]
	–	5.16	[12]
		8.1[d]	
	0.29–0.60	3.46–6.47	[13]
	–	5.6–17.0	[14]
	nd–65.22	8.73–66.44	[15][e]
Sterilized	–	11.7–24.1	[7][d]
	–	21.1–21.7	[8][d]
	–	12.43	[12]
		17.1[d]	
	1.34–2.50	15.52–21.38	[13]
	–	22.0	[14]
Evaporated	–	79.4–116.0	[8][d]

[a] mg/l.
[b] *F* values.
[c] mg/100 g sample.
[d] Colorimetric tiobarbituric acid (TBA) method.
[e] μg/100 ml.

model solutions [29] and milk [30]. As far as we know, no studies focusing only on the effect of the type of sugar in furfural formation have been reported, but Albalá-Hurtado et al. [24] obtained higher HMF and F contents in liquid infant formulas containing lactose and maltodextrine than in those having only lactose. However, the formulas compared had undergone different thermal treatments (sterilization and UHT, respectively), and this contributes to the differences mentioned by the authors.

In view of the above and given that the formulas studied also differ in their protein and mineral contents, the observed differences in the furfural compound contents at point zero probably cannot be ascribed to the differences in sugar contents.

The casein/serum protein ratios of the analyzed formulas differed: 40/60 and 80/20 in adapted and follow-up formulas, respectively. In the case of milk, the reacting amino groups are mainly the lysine residues in milk proteins (given that the content of free amino acids in milk is quite low), and it seems that the reactivity of lysine residues from casein is higher than that from serum proteins [4].

Our results agree with those reported by Caric et al. [2], indicating an increase in the furfural content of sterilized milk when the casein/serum protein ratio increases. In contrast, Albalá-Hurtado et al. [24] reported a high furfural content with a low casein/serum protein ratio in liquid infant formulas. However, in this case the formulas compared had undergone different thermal treatments (sterilization and UHT treatment in formulas with low and high casein content, respectively). On the other hand, Morales et al. [29] using the same temperature/time conditions obtained a higher furfural formation in the model system lactose/serum proteins than in the (model system) lactose/casein.

Finally, some authors have pointed out that cow milk [2] or formulas [25] enriched with iron and vitamin A had higher HMF contents than the non supplemented ones. Our formulas differ only in their iron content (6.0 and 8.0 mg in adapted and follow-up formulas, respectively).

In conclusion, the differences in the HMF and F contents between the studied adapted and follow-up formulas detected at the zero point must be ascribed to the differences in their casein/serum protein ratios and in iron content, given that in the manufacture of

both formulas raw cow milk of the same quality was used, the same thermal treatment was applied, and both have similar sugar/carbohydrate contents.

3.1. HMF and F contents

Studies on furfurals in cow milk subjected to different thermal treatments focus mainly on total HMF determination, while in infant formulas some authors have evaluated the HMF and F formation (Table 5).

The free and total HMF contents obtained in the adapted formula are comparable to the values reported by Rossi and Pompei [16] for pasteurized adapted infant formulas and lower than those given by the same authors for UHT, sterilized and sterilized–atomized infant formulas [16].

Albalá-Hurtado et al. [17,24] measured free and total F and HMF in different types of follow-up infant formulas (powdered, liquid) and in junior milks. The free and total values reported for the powdered formulas, respectively, are higher or slightly higher than ours. For the rest of formulas the reported contents are similar to ours, except for free HMF and total F which are lower and higher, respectively, than ours.

The free and total furfural (HMF and F) contents of the adapted and follow-up formulas analyzed are slightly higher than those obtained by us in a previous study [18].

The values reported by other authors are higher than ours, but often the type of formula and the thermal treatments applied are unknown and/or the method applied was spectrophotometric, which as mentioned before, overestimates the values [21,22,25].

The total HMF contents in pasteurized and UHT cow milk reported by some authors [7,9,10,12–14] are comparable to those obtained in our adapted formula. The values reported on UHT thermal treatments or more severe ones, like sterilization or evaporation [2,7,8,10,12–15] are higher than those of our adapted formula and similar to or higher than the values corresponding to the follow-up formula. The differences between our adapted and follow-up formulas agree with the results of Caric et al. [2], indicating higher total HMF contents in UHT milk when sodium caseinate was added to samples.

3.2. Influence of temperature and time of storage on furfural compounds content

The free and total HMF and F contents and their sum (HMF+F, free and total) in samples stored at 20 and 37 °C for different periods of time during the 1 year storage are reported in Tables 2–4.

3.2.1. Free furfural compounds

After 12 months of storage an increase in the free HMF, F and HMF+F contents is observed in samples stored at 20 and 37 °C with respect to the contents of these furfural compounds at the zero point (with the exception of the free F contents in the adapted formula stored at 20 °C). Although the differences are not statistically significant ($P<0.05$), the results corroborate those obtained in a previous study [18].

A simple regression analysis was applied and two significant models ($P<0.05$) were obtained for the follow-up infant formula:

(a) Between the free F content, dependent variable (y), and the storage time, independent variable (x), $1/y=-0.0025x+0.0680$ ($r=-0.63$).

(b) Between the free HMF+F content the dependent variable (y), and the cubic storage time, independent variable (x^3), $y=0.057x^3+476.417$ ($r=0.670$).

Both models are significant and can explain 40.03% and 44.86% of the variability in the free F and free HMF+F content, respectively.

Albalá-Hurtado et al. [17] studied the free HMF and F formation in spray-dried powdered and sterilized liquid follow-up infant formulas stored for 9 months at 20, 30 and 37 °C. The levels of free HMF and F and the length of storage were statistically correlated, except for free F in UHT-follow-up formula. The authors observed an increase in HMF and F contents in all samples during the sample storage, and the powdered formulas showed higher contents than the liquid samples. In the powdered sample differences between the storage at 37 °C and storage at 20 and 30 °C were detected, while in the liquid formula the temperature effect was observed only in free F contents. A statistically significant correlation between temperature of storage in free HMF and F was found (except for free HMF in sterilized follow-up formulas).

3.2.2. Total furfural compounds

Samples stored at 20 °C have lower mean total HMF (402.3 and 960.6 μg/100 g sample for adapted and follow-up infant formulas, respectively) and F (38.9 and 62.4 μg/100 g sample for adapted and follow-up infant formulas, respectively) contents than those stored at 37 °C (total HMF 518.1 and 1116.7 μg/100 g sample for adapted and follow-up infant formulas, respectively, and total F 40.9 and 68.2 μg/100 g sample for adapted and follow-up infant formulas, respectively). However, the application of a two-factor ANOVA test to the results showed that the storage temperature (20 and 37 °C) affected the total HMF contents, while no statistically significant differences were found in the total F contents. Statistically significant differences were obtained for total HMF+F contents in adapted infant formula but not in follow-up infant formula. In a previous study we did not detect any temperature effect on the HMF and F contents in adapted and follow-up infant formulas stored for 6 months at 20 and 37 °C [18]. HMF is an intermediate product of the MR and its relationship with the main inducing factor, i.e., temperature, can be explained. As the storage temperature increases, higher HMF levels can be expected in response to storage time [19].

The application of a two-way ANOVA test to total HMF and F contents in both infant formulas revealed statistically significant differences between the storage times studied, except for total HMF+F in the follow-up infant formula. The total HMF and F contents of both formulas varied in an irregular way with the storage time. However, the application of a simple regression analysis gave a significant relationship between the total F contents and the storage time in adapted infant formulas that follows a linear model ($y=2.62x+24.16$; $r=0.81$); $r^2=65.64$.

In the same way Rossi and Pompei [23] observed an irregular variation with the storage time in the HMF content of liquid infant formulas stored for 531 days at 4, 20 and 38 °C. These results can be explained by the fact that HMF is known to be in a state of equilibrium between its destruction by oxidation and formation from precursors [19].

However, other authors [17] reported an increase in total furfural compounds in liquid and powdered follow-up formulas during 9 months of storage at 20, 30 and 37 °C. And in agreement with our results, the production of total HMF and F compounds after the same time elapse was statistically higher in samples stored at 37 °C than in those stored at 20 or 30 °C.

In a later study carried out in the same temperature and storage times conditions (9 months at 20. 30 and 37 °C), in liquid UHT and sterilized follow-up and UHT junior milk, the same authors [24] observed that the levels of total furfural compounds and the length of storage were statistically correlated, except for the total HMF in liquid sterilized milk formulas stored at 20 °C. Excluding the total F in liquid sterilized milk formulas, a statistically significant correlation between temperature of storage and total furfural compounds was also found. Moreover, the total HMF and F production was greater when samples were stored at 37

°C than at 30 °C and 20 °C.

As in our study, several authors have observed that at high storage temperatures high total HMF levels could be expected in response to storage time in different types (UHT, pasteurized, sterilized) of cow's milk. To be precise, an increase in HMF contents during storage at 35, 40 and 50 °C has been reported [2,8,19,20]. However, no significant variations in total HMF content were found under refrigeration or at room temperatures of storage (4 and 20 °C [8], 20 °C [19]), although the latter reported small losses at 6 °C.

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