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High-performance liquid chromatographic determination of furfural compounds in infant formulas during full shelf-life

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Abstract

In order to evaluate the extent of the Maillard reaction in adapted and follow-up infant formulas (IF), a study was made of the evolution of furfural compound (2-furaldehyde, 5-hydroxymethyl-2-furfuraldehyde, 2-furylmethylketone and 5-methyl-2-furaldehyde) in these products, along with their relation to available lysine during the shelf-life period (two years at 20 and 37 °C).

Total and free furfural contents were measured by RP-HPLC and UV detection, heating or not the sample in boiling water to free the furfurals bound to proteins and the furfurals formed from precursors. Only 2-furylmethylketone and 5-hydroxymethyl-2-furfuraldehyde were detected. Adapted and follow-up IF showed similar behaviour during shelf-life, with a significant increase in furfural compound contents at the end of the storage period that was more marked at 37 °C than at 20 °C. In both IF, a correlation was obtained between furfural contents and the cubic time variable, which explains the irregular increase of these compounds over time, and between available lysine and furfural compounds in the second storage year – indicating the Maillard reaction to be in an advanced stage.

Results obtained indicate that under storage conditions furfural content is a useful indicator of advanced Maillard reaction stages.
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Keywords: Furfural compounds; HPLC; Infant formulas; Maillard reaction; Storage

1. Introduction

Infant formulas (IF) which in Spain are marketed mainly in powdered form, combine a set of factors that makes them highly sensitive to Maillard Reactions (MR), namely the formula composition (high lactose and lysine contents), the relatively high temperatures applied during the manufacturing process, the packaging conditions, and storage for long periods of time (Palombo, Gertler, & Saguy, 1984; Van Mil & Jans, 1991). Moreover, IF are enriched with compounds (vitamin A, iron, and lactose) that increase their susceptibility to MR in comparison to cow milk (Caric, Gavaric, & Milanovic, 1984).

The contents of undesirable compounds such as furfurals generated at advanced stages of the Maillard reaction (Ferrer, Alegría, Farré, Abellán, & Romero, 1999) are used to evaluate in IF the intensity of the thermal treatment applied during manufacture and/or the effects of storage. Several studies have been carried out on the evolution of furfural compounds in milk (Fink & Kessler, 1986; Jiménez-Pérez, Corzo, Morales, Delgado, & Olano, 1992; Morales, Romero, & Jiménez-Pérez, 1997), IF (Albalá-Hurtado, Veciana-Nogués,

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Mariné-Font, & Vidal-Carou, 1998, 1999; Guerra-Hernández, León Gómez, García-Villanova, Corzo Sánchez, & Romera Gómez, 2002) and junior milks (Albalá-Hurtado et al., 1999). However, in these studies either the storage time taken into account was shorter than the IF shelf-life, as in the case of follow-up IF (Albalá-Hurtado et al., 1998), or the 5-hydroxymethyl-2-furfuraldehyde (HMF) contents in IF were assayed some time after manufacture without monitoring or indicating either storage time or storage temperature. As a result, the reported results are only isolated measures that do not allow follow-up of the changes throughout product storage or shelf-life (Angelini, Bellomonte, Carratù, & Sanzini, 1984; Pompei, Rossi, & Mare, 1987).

In a previous work (Ferrer, Alegría, Farré, Abellán, & Romero, 2002) the formation of furfural in adapted and follow-up IF as a consequence of the thermal treatment applied during manufacture and of storage at 20 and 37 °C for one year was studied. Increases in HMF and in free and total 2-furaldehyde (F) contents at both temperatures were observed.

The lack of reports in the literature taking into account the full shelf-life of the product led us to continue the study throughout the second storage year, using RP-HPLC to determine the contents of furfural compounds [F (2-furaldehyde), HMF, FMC (2-furylmethylketone) and MF (5-methyl-2-furaldehyde)] in adapted and follow-up milk-based IF stored at 20 and 37 °C, followed by an evaluation of furfural compound evolution in the mentioned IF during the entire shelf-life (two years).

An additional aim was to relate the obtained furfural compound contents to the values of available lysine determined in the same samples in a previous study (Ferrer et al., 2003), in order to evaluate the evolution and extent of MR in two IF types (adapted and follow-up) over the full shelf-life (two years) at two storage temperatures (20 and 37 °C).

2. Materials and methods

2.1. Samples

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

These adapted and follow-up IF differ in protein composition, but were manufactured with the same raw cow milk and subjected to the same thermal treatment. Details on the composition and manufacturing procedure were provided in a previous work (Ferrer et al., 2002).

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 heater (BJC Electrónica Josa S.A. Terrasa Barcelona, Spain) with an Omron E5EW thermostat) for 24 months. The samples were analyzed immediately after manufacture (at zero time), and again after 12 storage months in a previous work (Ferrer et al., 2002), and at 15, 18, 21 and 24 storage months in the present study. Samples were maintained in their airtight containers until analysis.

2.2. Procedure

The procedure applied and the analytical parameters of the method are reported in the previous work (Ferrer et al., 2002).

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 to room temperature, 3 ml of a 40% (w/v) TCA solution was added, and the mixture was stirred thoroughly for 5 min. It was then centrifuged at 2000*g* for 15 min. 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.

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

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, 274 nm for FMC, and 293 nm for MF was carried out. The injection volume was 20 μl.

Furfurals were quantified by interpolation in a calibration curve in the range of 0.01–2 μg/ml assay of HMF, F, FMC and MF. The quality of the method was evaluated through the values of the analytical parameters (Ferrer et al., 2002).

2.3. Statistical analysis

Two ANOVA, one of two factors (temperature and time) and another of three factors (temperature, time and formula type), and a Tukey's posteriori test were applied to the free and total HMF, F and HMF + F contents to detect statistically significant differences ($p < 0.05$) between the adapted and follow-up IF at the two storage temperatures (20 and 37 °C), during the sec-

ond year of storage (15, 18, 21 and 24 months), and over the full shelf-life (24 months).

In order to study the possible influence of storage time on free and total HMF, F and HMF + F contents, a simple regression analysis was applied. Different regression models were assayed, in which the free or total furfural content was the dependent variable (y), and available lysine content the independent variable (x). The Statgraphics 7.0 statistical package was used throughout.

3. Results and discussion

The contents (μg/100 g sample) of free and total HMF and F in adapted and follow-up IF during the second year of storage are reported in Table 1. The sum of HMF and F, free and total in the same formulas are given in Table 2. Neither free nor total FMC or MF were detected in any of the samples analyzed.

3.1. Comparison between adapted and follow-up IF

In similar way to the first storage year (Ferrer et al., 2002) the contents of free and total F, HMF and F + HMF recorded during the second year were higher in follow-up IF than in adapted IF (see Table 1) – the differences being statistically significant ($p < 0.05$) except for free F, the levels of which were either below or very near the detection limit. These differences between the two types of IF must be attributed to the differences in their casein/serum protein ratios and iron contents, considering that raw cow milk of the same quality was used in producing them, the same thermal treatment was applied to both formulas, and even the storage conditions (time and temperature) were the same for both IF (Ferrer et al., 2002).

3.2. Evolution of furfural compounds during shelf-life storage

In order to determine whether the furfural compounds display the same behavior throughout storage, the values obtained for the second storage year and those corresponding to the whole shelf-life were evaluated separately.

3.2.1. Influence of time

No free F was found in adapted IF, except at the 18th storage month at 37 °C – though the content was only slightly above the detection limit (18.97 μg/100 g vs. 13.3 μg/100 g). Free F was detected in follow-up IF only at the end of the storage period (21 and 24 storage months) (see Table 1).

Similar results were obtained in the two IF, both on considering only the second year of storage and the full shelf-life. Total F, free and total HMF, and free and total HMF + F contents increased significantly ($p < 0.05$) during the full shelf-life period (see Tables 1 and 2). In turn, between the 15th and 24th storage months an over-

Table 1
Free and total HMF and F contents (mean ± standard deviation, expressed as μg/100 g sample) in stored infant formulas

T (°C)	Month	HMF		F	
		Free	Total	Free	Total
Adapted formulas					
20	15	$92 \pm 6^{a\text{-}1}$	$499 \pm 5^{c\text{-}5}$	$nd^{e\text{-}9}$	$36 \pm 0.1^{f\text{-}10}$
	18	$148 \pm 15^{a\text{-}1}$	$392 \pm 7^{c\text{-}5}$	$nd^{e\text{-}9}$	$28 \pm 1^{g\text{-}10}$
	21	$62 \pm 8^{a\text{-}1}$	$481 \pm 12^{c\text{-}5}$	$nd^{e\text{-}9}$	$19 \pm 2^{h\text{-}10}$
	24	$211 \pm 37^{b\text{-}1}$	$1089 \pm 41^{d\text{-}5}$	$nd^{e\text{-}9}$	$86 \pm 1^{i\text{-}10}$
37	15	$187 \pm 1^{a\text{-}2}$	$857 \pm 24^{c\text{-}6}$	$nd^{e\text{-}9}$	$38 \pm 2^{f\text{-}11}$
	18	$224 \pm 30^{a\text{-}2}$	$645 \pm 6^{c\text{-}6}$	$19 \pm 5^{e\text{-}9}$	$33 \pm 1^{g\text{-}11}$
	21	$174 \pm 10^{a\text{-}2}$	$893 \pm 16^{c\text{-}6}$	$nd^{e\text{-}9}$	$24 \pm 2^{h\text{-}11}$
	24	$342 \pm 18^{b\text{-}2}$	$1738 \pm 57^{d\text{-}6}$	$nd^{e\text{-}9}$	$87 \pm 4^{i\text{-}11}$
Follow-up formulas					
20	15	$475 \pm 30^{j\text{-}3}$	$1114 \pm 41^{l\text{-}7}$	$nd^{e\text{-}9}$	$55 \pm 4^{n\text{-}12}$
	18	$425 \pm 17^{j\text{-}3}$	$866 \pm 61^{l\text{-}7}$	$nd^{e\text{-}9}$	$40 \pm 4^{n\text{-}12}$
	21	$477 \pm 2^{j\text{-}3}$	$1314 \pm 138^{l\text{-}7}$	$nd^{e\text{-}9}$	$70 \pm 11^{n\text{-}12}$
	24	$1042 \pm 26^{k\text{-}3}$	$2102 \pm 5^{m\text{-}7}$	$33 \pm 0.001^{e\text{-}9}$	$115 \pm 7^{o\text{-}12}$
37	15	$539 \pm 42^{j\text{-}4}$	$1649 \pm 156^{l\text{-}8}$	$nd^{e\text{-}9}$	$90 \pm 1^{n\text{-}13}$
	18	$523 \pm 22^{j\text{-}4}$	$1273 \pm 47^{l\text{-}8}$	$nd^{e\text{-}9}$	$56 \pm 4^{n\text{-}13}$
	21	$612 \pm 16^{j\text{-}4}$	$1367 \pm 44^{l\text{-}8}$	$41 \pm 2^{e\text{-}9}$	$68 \pm 11^{n\text{-}13}$
	24	$1133 \pm 132^{k\text{-}4}$	$2909 \pm 18^{m\text{-}8}$	$32 \pm 3^{e\text{-}9}$	$133 \pm 16^{o\text{-}13}$

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

Table 2
Free and total HMF + F contents (expressed as μg/100 g sample) in stored infant formulas

T (°C)	Month	Adapted formulas		Follow-up formulas	
		Free	Total	Free	Total
20	15	$105^{a\text{-}1}$	$535^{c\text{-}3}$	$488^{e\text{-}5}$	$1169^{g\text{-}7}$
	18	$161^{a\text{-}1}$	$420^{c\text{-}3}$	$438^{e\text{-}5}$	$906^{g\text{-}7}$
	21	$75^{a\text{-}1}$	$500^{c\text{-}3}$	$491^{e\text{-}5}$	$1383^{g\text{-}7}$
	24	$224^{b\text{-}1}$	$1175^{d\text{-}3}$	$1075^{f\text{-}5}$	$2217^{h\text{-}7}$
37	15	$200^{a\text{-}2}$	$895^{c\text{-}4}$	$552^{e\text{-}6}$	$1739^{g\text{-}8}$
	18	$237^{a\text{-}2}$	$678^{c\text{-}4}$	$536^{e\text{-}6}$	$1329^{g\text{-}8}$
	21	$193^{a\text{-}2}$	$917^{c\text{-}4}$	$653^{e\text{-}6}$	$1436^{g\text{-}8}$
	24	$355^{b\text{-}2}$	$1825^{d\text{-}4}$	$1165^{f\text{-}6}$	$3042^{h\text{-}8}$

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

all increase in furfural compound contents was also observed – though the increase was not constant and included falls as well as rises.

We applied simple regression analysis between F + HMF content dependent variable and storage time independent variable (x, x^2 and x^3), and assayed the different alternative models suggested by the graphic plot of points. In each case, the model yielding the highest explained square sum (SC) was chosen (r^2 = explained SC/total SC).

The best models obtained for free and total HMF + F during the second storage year and full shelf-life of the analyzed IF are included in Table 3. Significant simple regression models were also obtained between some free and total F or HMF contents and time, the models and variability percentages being similar to those mentioned in Table 3. They are not included because they yielded no additional information on the evolution of furfural compound contents.

A positive correlation was obtained between furfural contents and the cubic time variable (x^3), explaining the irregular increase observed throughout IF storage.

Table 3
Simple regression analysis – best models between free and total F + HMF contents (y) and storage time (x)

	Second year of storage	Shelf-life
Adapted IF		
Free F + HMF	–	$y = 8.46\times10^{-3}x^3 + 132$, $r^2 = 29$; $p = 0.02$
Total F + HMF	$y = 0.079x^3 + 228$, $r^2 = 52$; $p = 0.04$	$y = 0.058x^3 + 440$, $r^2 = 53$; $p = 0.0006$
Follow-up IF		
Free F + HMF	$y = 0.058x^3 + 204$, $r^2 = 75$; $p = 0.006$	$y = 0.034x^3 + 449$, $r^2 = 62$; $p = 0.0001$
Total F + HMF	$y = 0.121x^3 + 675$, $r^2 = 55$; $p = 0.03$	$y = 0.086x^3 + 1014$, $r^2 = 53$; $p = 0.0006$

r^2 is the percentage of variability explained by the model (explained square sum/total square sum). p is the significance level.

These results are explained by the equilibrium between the oxidation-mediated destruction of HMF and its formation from precursors (Morales et al., 1997).

The models between the two variables obtained during IF shelf-life are basically attributable to the evolution of furfural contents during the second storage year. In the first storage year the significant correlations between furfural contents were scarce – as has been reported previously (Ferrer et al., 2002).

3.2.2. Influence of temperature

The application of a two-factor ANOVA test to total F, free and total HMF, and free and total HMF + F contents in IF showed statistically significant differences ($p < 0.05$) between the two temperatures studied, the furfural compound contents being higher at 37 °C than at 20 °C both during the second year of storage and for the full shelf-life – except for the free F contents. The latter observation is reasonable, since as has been commented above the free F content was either non-detectable or close to the detection limit. As storage temperature increases, higher HMF levels can be expected in relation to storage time (Morales et al., 1997).

Two chromatograms corresponding to an adapted IF stored for 15 months (20 and 37 °C), and showing the differences in total furfural content according to storage temperature, are shown in Fig. 1. Comparison of the results obtained during the second storage year with those corresponding to the first year (Ferrer et al., 2002) shows differences in the evolution of furfural contents in the two periods.

The literature review carried out indicates that to date there have been no reports on MR changes during full shelf-life storage. The results obtained indicate that the evolution of furfural compound contents varies according to the storage period considered. It is therefore important to consider the entire shelf-life when either globally or individually evaluating and analyzing all changes taking place in the MR.

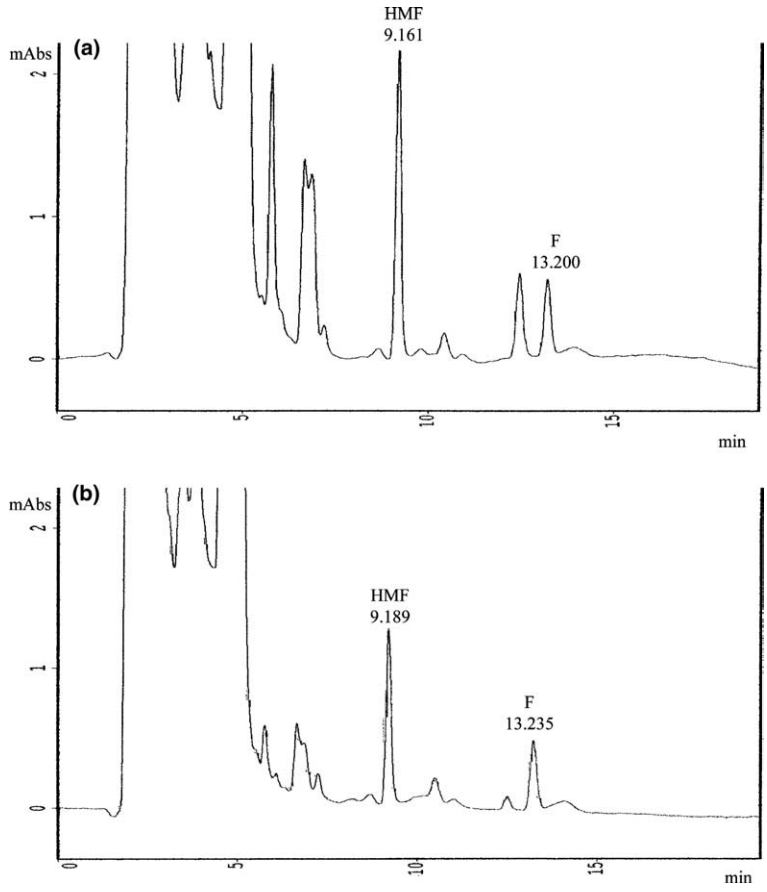

Fig. 1. Chromatograms corresponding to the total furfural compounds in adapted infant formulas stored for 15 months at 37 °C (a) and 20 °C (b).

3.3. Comparisons with the literature

The furfural compound (F and HMF) contents in sterilized follow-up IF stored for nine months at 20, 30 and 37 °C, reported by Albalá-Hurtado et al. (1998, 1999), were comparable to those obtained in this study for adapted IF. In addition, the results presented by these authors for spray-dried IF and junior milks stored for the aforementioned periods of time and under the same conditions, were comparable to those obtained here for follow-up IF. Albalá-Hurtado et al. (1998, 1999) and Guerra-Hernández et al. (2002) reported an increase in free and total furfural compound contents with storage time and – as in this study – the values corresponding to the higher storage temperature were greater than those corresponding to lower temperatures. However, it should be pointed out that while the values reported by Albalá-Hurtado et al. (1998) are similar, in the present study the storage period was longer (24 vs. 9 months). Moreover, the contents found were higher than those reported by Albalá-Hurtado et al. (1998, 1999) for follow-up UHT IF, by Ferrer, Alegría, Farré, Abellán, and Romero (2000) for powdered adapted and follow-up IF, and by Guerra-Hernández et al. (2002) for liquid infant milk – though the storage periods in these studies were nine, six and three months, respectively. In these studies (Albalá-Hurtado et al., 1998; Ferrer et al., 2000; Guerra-Hernández et al., 2002), an increase in furfural compound contents with time and temperature was reported, in coincidence with the present study.

From the comparison of the results obtained with individual furfural compound contents reported by other authors, it is interesting to point out that the values corresponding to follow-up IF are similar to those mentioned by Park and Hong (1991) in IF enriched with iron and vitamin A; equal to or lower than those obtained by Pompei et al. (1987) in powdered IF analyzed a year before the expiry date and in liquid formulas at different time periods; and lower than the values reported by Angelini et al. (1984) for different formulas stored under unstated conditions of time and temperature. The recorded furfural compound contents are higher than those reported by Rossi and Pompei (1991) in pasteurized, UHT and sterilized adapted IF, though their free and total HMF contents were measured immediately after manufacture.

3.4. Relationship between different MR indicators

A simple regression analysis was carried out between available lysine content (an indicator of all MR stages), determined in a previous work (Ferrer et al., 2003), and

free and total F + HMF (indicators of advanced MR stages), with the purpose of assessing MR evolution during the shelf-life of IF.

A significant relationship ($p < 0.05$) between available lysine and free and total F + HMF contents was obtained during the second storage year. For total F + HMF contents, this followed a multiplicative model in adapted IF ($y = 423.55x^{-4.30}$; $p = 0.009$) and follow-up IF ($y = 1340.01x^{-1.55}$; $p = 0.001$). Both models are significant and are able to explain 71% and 84% of the variability of available lysine contents in adapted and follow-up IF, respectively. For free F + HMF contents, significant reciprocal models [($1/y = 9.12 \times 10^{-4}x + 0.98$; $p = 0.05$) and ($1/y = 8.96 \times 10^{-4}x + 0.52$; $p = 0.0002$)] were obtained for adapted and follow-up IF, respectively. These models can explain 49% and 91% of the variability of available lysine contents in adapted and follow-up IF, respectively.

No significant correlation ($p < 0.05$) was obtained between furfural compound contents and available lysine contents during the first year of storage.

When the two storage years (shelf-life of IF) were considered as a whole, a statistically significant ($p < 0.05$) correlation was obtained between available lysine and free and total F + HMF in both IF (adapted and follow-up) – though the correlation was lower than in the first case (second storage year). The following models were obtained between available lysine contents and:

- Total F + HMF contents: adapted IF $y = -3669.12x + 3941.98$; $p = 0.003$; follow-up IF $y = -2077.78x + 3435.12$; $p = 0.007$. Both models were significant and can explain 44% and 38% of the variability of available lysine contents in adapted and follow-up IF, respectively.
- Free F + HMF contents: adapted IF $1/y = 8.06 \times 10^{-4}x + 0.99$; $p = 0.002$; follow-up IF $1/y = 9.18 \times 10^{-4}x + 0.49$; $p = 0.000$. Both models were significant and can explain 44% and 85% of the variability of available lysine contents in infant and follow-up IF, respectively.

To summarize, the best correlations between free and total furfural compound contents correspond to the second storage year – no correlations being found during the first storage year. Moreover, the correlations corresponding to the full shelf-life period were lower than those obtained for the second year.

4. Conclusions

Relevant formation of furfural compounds occurs during the second storage year of IF, indicating progression from initial to advanced MR stages. Furfurals therefore constitute a useful indicator of advanced MR stages under IF storage conditions.

Adapted and follow-up IF display similar behavior during the second year of storage and throughout shelf-life, with a more-or-less significant increase in furfural compound contents at the end of the storage period – the latter being more accentuated at 37 °C than at 20 °C.

The observed correlation between free and total furfural contents and time indicates different stages in MR (initial and advanced) at the first and second storage year, respectively. Thus, the effect of time on furfural content is not continuous, and consequently the individual models corresponding to the second storage year or to the full shelf-life period would not be useful for predicting furfural compound behavior during storage.

As a general conclusion, the results obtained point to the need for strict control of the IF storage conditions (temperature and time), even recommending a turnover of stocks in periods lower than one year or as low as possible, in order to ensure the greatest nutritional quality of products of this kind.

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