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Effect of storage conditions on furosine formation in milk-cereal based baby foods

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Running title: Furosine in baby foods during storage

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Abstract

The effect of storage during 9 months at 25º, 30º and 37ºC on furosine formation in three milk-cereal based baby foods was studied to evaluate development of the Maillard reaction. Furosine was measured by HPLC-UV. Immediately after the manufacturing process, furosine contents were 310-340 mg/100 g protein and at the 9th storage month were 426-603 mg/100 g protein. Storage time and temperature have a significant increase ($p<0.05$) of furosine content during storage. Furosine contents were higher in sample containing honey than in those without honey. Interactions ($p<0.05$) between storage time and temperature or type of sample were found. A predictive model equation of the evolution of furosine during storage explaining 80% of the variability in furosine content was obtained. The blockage of lysine through storage calculated using the furosine and total lysine provided values ranged from 9.5 to 18.1 % for analysed baby foods.

Keywords: Storage, Maillard reaction, baby foods, furosine, lysine blockage.

1. Introduction

Baby foods have to provide nutrients in sufficient amounts to permit optimal development and growth and to prevent diseases. From the 5^{th} / 6^{th} month onwards, an exclusively milk based diet is gradually broadened with the introduction of other foods. Cereals in the form of paps prepared with milk are usually one of the first foods added in the diversification of the infant diet (Casado de Frías, Maluenda Carrillo & Casado, Sáenz, 1993; Martínez & Hernández, 1993). The European Commission (EC, 2006) has established guidelines on protein contents and requirements regarding the amino acid composition of cereal based baby foods.

At present, milk and cereal based ready-to-eat baby foods are available on the market. These products have a long shelf-life and can be consumed for up to one year after manufacture. Their composition, the thermal treatments applied in the manufacturing process and their shelf-life favour development of the Maillard reaction (MR), which reduces the quality of proteins, mainly due to a blockage of lysine and a decrease in digestibility (Meade, Reid & Gerrard, 2005).

To evaluate the early stages of MR, use can be made of the determination of furosine (ε-N-(furoylmethyl)-L-lysine), an artificial amino acid arising from acid hydrolysis of the Amadori compounds fructosyl-lysine, lactulosyl-lysine and maltulosyl-lysine produced by the reaction of ε-amino groups of lysine with glucose, lactose and maltose, respectively. Furosine content provides an estimation of blocked and therefore non-reactive lysine, and it is considered to be the most specific earliest indicator of MR (Guerra-Hernández, Corzo & Garcia-Villanova, 1999; Ferrer, Alegría, Farré, Abellán, Romero & Clemente, 2003).

Studies have been done on the effect of thermal treatment (Guerra-Hernández & Corzo, 1996; Guerra-Hernández et al., 1999) and storage (Ramírez- Jiménez, Guerra-

Hernández & García- Villanova, 2003; Rada-Mendoza, Sanz, Olano & Villamiel, 2004; Ramírez-Jiménez, García- Villanova & Guerra-Hernández, 2004a) on the furosine content of baby foods. However, only one of these studies included baby foods containing cereals and milk in their composition (Ramírez-Jiménez et al., 2004a).

Now many types of baby foods are found on the market, and their consumption has been rising (Mercasa, 2006). Ready-to-eat milk and cereal-based products are easy to use, and this characteristic will facilitate their diffusion. However, as mentioned their composition favours MR development. Therefore the purpose of the present study was to evaluate the influence of storage on the development of the earliest stages of MR by measuring the formation of furosine in three ready-to-eat milk and cereal- based baby foods.

2. Material and methods

2.1. Samples

Three different (A, B, C) ready-to-eat milk-cereal based baby foods commercially available in Spain and containing 88% skimmed milk and 8.8% alpha-amylase hydrolysed 8-cereals flour (wheat, corn, rice, oat, barley, rye, sorghum and millet) were analysed. All three had the same composition, except for 0.9% of honey in B, and 1.1% of fruit (banana, orange and apple) in C. The composition (g/ 100g) of the baby foods (A, B, C) was: proteins: 3.4, 3.5, 3.5; carbohydrates: 16.1, 15.7, 15.8, respectively, and lipids: 2.9, in all samples. The three products were obtained by the same manufacturing process that includes an indirect UHT thermal treatment. The milk used in the manufacturing process had also undergone a spray-drying treatment. Baby foods were packed in a commercial 250 mL Tetra Brik in nitrogen atmosphere and stored at three different temperatures (25º, 30º and 37ºC) for 9 months. They

were analysed by quadruplicate immediately after manufacture (zero time) and after 1, 2, 4, 5, 6, 7, 8 and 9 months of storage.

Water activity (A_w) and pH of the three baby foods were 1.00±0.01 and 6.59±0.05, respectively. A_w and pH did not change during storage period.

2.2. Chemicals and materials

HPLC-grade acetonitrile was obtained from J.T. Baker (Deventer, The Netherlands); high-purity water was supplied by the Milli-Q plus system from Millipore Corp. (Bedford, MA, USA); formic acid 98-100% was purchased from Merck (Darmstadt, Germany); sodium 1-heptane-sulphonate was from Sigma-Aldrich (St. Louis, MO, USA); furosine standard was purchased from Neosystem (Strasbourg, France). A standard solution of furosine 72 μg/mL in hydrochloric acid 0.1M was prepared and stored at -20ºC. Sep-Pak C_{18} plus cartridges were from Waters (Milford, MA, USA).

2.3. Apparatus

The HPLC system (Shimadzu Europe GmbH, Duisburg, Germany) consisted of two LC-10AD pumps controlled by a CBM-10 A (communications bus module), a model 7725i manual injection valve (Rheodyne, Cotati, CA, USA) equipped with a 20 μL sample loop and an SPD-10AV UV-visible detector. Data were collected and analysed using the CLASS LC-10 S/W software package. The column temperature was set with a model HIC-6A column heater manufactured by Shimadzu Europe GmbH (Duisburg, Germany).

A vacuum system consisting of a Speed Vac Plus AR SC110 centrifuge (Savant Instruments. Inc, Farmingdale, NY), a RCT60 trap (Jouan, St Herblain, France) and an RD-9 vacuum oil pump (Telstar, Terrassa, Spain) were used in the sample preparation step. A UT 6060 air-circulation drying oven (Heraus, Hanau, Germany) was used in sample hydrolysis.

A Pawkit water activity meter (Decagon Devices, Inc., Pullman, Washington, USA) calibrated with 6.0 M sodium chloride was used to measure the A_w of samples.

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

2.4. Sample preparation

An aliquot of sample containing 40-50 mg of protein (1.14-1.43 g) was weighed into a 10 mL Pyrex glass tube fitted with Teflon-lined screw caps. Eight mL of HCl 8N was added. After bubbling with nitrogen for 1 min, the closed tube was kept at 110ºC for 23 h. After hydrolysis the tubes were weighed and, if needed, a sufficient amount of 8N HCl was added to recover the weight prior to hydrolysis. The hydrolysate was filtered and collected in an opaque tube to protect it from light and then diluted with 3M HCl to obtain a protein content of 1-2 μg/μL (1 mL of hydrolysate and 4mL of 3M HCl). The solid-phase extraction prior to chromatographic analysis was performed as follows: 0.5 mL of hydrolysate was added to a pre-wetted (5 mL methanol and 10 mL H_2O) Sep-Pak C_{18} cartridge; the eluted liquid was discarded, and furosine was then eluted with 3 mL of 3N HCl. The sample was vacuum dried at 43ºC and then dissolved in 2 mL of mobile phase.

2.5. Chromatographic conditions

A Waters Spherisorb ODS2 column (250 x 4.6 mm, 5 μm) was used. The injection volume was 20 μL. Separations were carried out isocratically at 25ºC using a mobile phase consisting of 5 mM sodium 1-heptane-sulphonate with 20% acetonitrile and 0.2% formic acid at a flow rate of 0.8 mL/min. Four aliquots of each sample were prepared. Detection was carried out by UV at 280 nm.

Calibration curves (0.5, 1, 2, 4, 6 and 8 μg/mL furosine) were obtained by plotting absorbance, expressed in area units vs. μg/mL of furosine.

The analytical parameters of the method were previously estimated by Ferrer, Alegría, Courtois & Farré (2000).

2.6. Calculation of lysine blockage

The following formula: % blockage = (3.1 x furosine x 100) / (chromatographed lysine + 1.86 furosine) can be used to calculate the percentage of blocked lysine based on the furosine content, when in furosine determination hydrolysis is carried out with 6M HCl (Bujard & Finot, 1978, Finot, Deutsch & Bujard, 1981, Pizzoferrato, Manzi, Vivanti, Nicoletti, Corradini & Cogliandro, 1998). Furosine content varies depending on the concentration of HCl used in hydrolysis, as observed in baby cereals (Guerra-Hernández & Corzo, 1996) and in UHT-milk (Henle, Zehetner & Klostermeyer, 1995), and the application of the mentioned formula requires, in the present study, the use of a correction factor of 0.8 in application to the furosine contents (Evangelisti, Calcagno, Nardi & Zunin, 1999a).

Lysine contents were measured at zero time by RP-HPLC prior to derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and fluorescence detection (Bosch, Alegría & Farré, 2006). These lysine contents, together with the measured furosine contents, allowed calculation of the percentage of blocked lysine in the analysed baby foods.

2.7. Statistical analysis

A three factor ANOVA test (Statgraphics plus 3.1 statistical software package) was applied to the furosine contents in order to detect differences among the three types of milk-cereal based baby foods analysed, the storage temperature (25º, 30º and 37ºC) and the storage period (0, 1, 2, 4, 5, 6, 7, 8 and 9 months). In factors with more than two levels and exhibiting

a significant effect ($p<0.05$), a Tukey test was applied to identify those differing significantly from each other.

To study the effect of storage time, temperature and type of sample on furosine content, multiple regression was applied.

3. Results and discussion

3.1. Furosine content in baby foods

Furosine contents (expressed as mg/100 g of protein) in the three baby foods (A, B and C), stored for 9 months at 25º, 30º and 37ºC are reported in Table 1. Immediately after the manufacturing process, furosine contents ranged from 310 to 340 mg/100 g protein, and at the last month of storage from 426 to 603 mg/100 g protein.

Mean furosine contents in samples A, B and C, were 373, 418 and 371 mg/100 g protein, respectively. Furosine contents in sample B containing honey were significantly ($p<0.05$) higher than in samples A and C, without honey added. The three baby foods had similar protein contents, underwent the same thermal treatment, and contained milk of the same quality and in the same proportion. Thus, the higher furosine content in sample B could be ascribed to the higher monosaccharide content, which could increase susceptibility to the Maillard reaction. In sample B, honey contribution to fructose and glucose contents was of 3.5 and 3 g/kg baby food, respectively. This contribution has been estimated taking into account honey percentage (0.9%) and their glucose and fructose content (Souci, Fachmann & Kraut, 2000). The effect of honey has been previously reported in cereal based baby foods, although without milk protein, and containing cereal with or without soy (Guerra-Hernández et al., 1996, 1999). In sample C, fruits (apple, orange and banana) (1.1%) contributed monosaccharides similar to those of honey, though in lesser amounts (glucose 0.2-0.4 g/kg

baby food and fructose 0.3-0.6 g/kg baby food). This was also estimated taking into account the contribution of fruits (1.1%) and their glucose and fructose contents (Souci et al., 2000). The lesser contribution of fruits when compared to honey in terms of monosaccharide content in the studied baby foods could explain the lack of statistically significant differences between samples C and A, without added honey or fruits.

Comparison of the furosine values obtained in this study with those of previous reports is not easy, because data on furosine contents in baby foods are scarce. Moreover, the composition of the products included in this food category is highly varied, and even if they are cereal based they may or may not contain milk. In turn, different manufacturing processes are used, and the products can be marketed in powdered or in liquid form.

In baby cereals with an unknown honey percentage, furosine contents higher than those obtained in this study have been reported. Thus, greater furosine values have been described in the baby food with the composition (milk, rice and honey) most similar to that of the food analysed in the present study (Carratù, Boniglia, Filesi & Bellomonte, 1993). The lack of knowledge on the percentage contribution of the different ingredients to the product and on the manufacturing process does not allow us to explain this difference. Higher furosine contents in powdered baby cereals with honey, but without milk added (Guerra-Hernández et al., 1996, 1999), have been obtained. When compared to sample B (0.9% honey), differences mainly can be due to the manufacturing process.

In cereal-based baby foods containing fruits, furosine contents higher (360-1860 mg/100 g protein) than those found in sample C have been reported (Guerra-Hernández et al., 1996, 1999). Differences can be mainly ascribed to the thermal treatment. In only fruit based baby foods, lower furosine contents (40-180 mg/100 g protein) than those found here have been reported (Rada-Mendoza, Olano & Villamiel, 2002; Rada-Mendoza et al., 2004).

3.2. Evolution of furosine content through storage

The application of a three factors ANOVA (type of sample, temperature and time) showed statistically significant differences ($p<0.05$) in furosine content for all studied storage temperature, the mean values being 353, 394 and 415 mg/100 g protein at 25º, 30º and 37ºC, respectively.

No interaction was found between storage temperature and sample type, i.e., the evolution of furosine content with increasing temperature showed a similar behaviour in all three samples.

During storage, a gradual increase in furosine contents of the baby foods was observed, that varied over a broad range, from 37% in sample A (based on cereals and milk) at 25ºC, to 77% in sample B (with honey added) at 37ºC. Statistically significant differences ($p<0.05$) among furosine contents in samples stored for different periods of time are reported in Table 1.

Statistically significant ($p<0.05$) interactions between storage time-temperature and storage time-type of sample were found (Fig. 1): at higher temperature (37ºC), the formation of furosine during storage was faster than at lower temperatures (25 and 30ºC) (Fig. 1A), especially up to the fourth month. From this point onwards and up to the end of the storage period, the increase in furosine showed a similar behaviour at all three studied temperatures (25, 30 and 37ºC). The type of baby food (A, B and C) also affected the furosine increments (Fig 1B), the latter being faster in B, as mentioned above.

Taking into account the significant effect of storage time, storage temperature and type of sample (ANOVA) on furosine formation, multiple regression analysis was applied to quantify the effect of these factors. Considering that the type of sample is a qualitative variable, dummy variables were used in the analysis, i.e., two auxiliary variables were created (*sample 1 and sample 2)* (Jobson, 1991). The coefficient of variable *sample 1* measures what

sample B contents differ on average from those of sample A, taking the latter as reference. In the same way, the coefficient of the variable *sample 2* measures what sample C contents differ on average from those of sample A, taken as reference. Figure 2 show the observed versus predicted plots for furosine and the corresponding equation, that explains a high percentage of the variability found in the formation of furosine (R^2= 80%). The most important effect on furosine formation is ascribed to the interaction of *temperature*month* variables. The negative sign corresponding to the coefficient of the variable *month* does not mean a decrease in furosine content with increasing time. The effect of storage time is jointly described by the variable *month* and the interaction *temperature*month* in such a way that, in all cases, for a given temperature, furosine content increases with increasing storage time.

Data on the effects of storage time and temperature on the formation of furosine in baby foods are limited. Among them, mention may be made of a study on the effect of storage temperature and time in baby foods containing only fruits (Rada-Mendoza et al., 2004) or rice as the main ingredient (Ramírez-Jiménez et al., 2003), i.e., baby foods having matrices with compositions different to those studied here.

Moreover, to our knowledge, no studies on the interactions between time and temperature in relation to the formation of furosine or predictive models of their evolution during storage have been published.

The only report offering results that can be compared with those of the present study correspond to the formation of furosine in baby cereals with (40%) powdered milk stored under industrial conditions (32º and 55ºC for 1, 3, 6 or 12 months in air or nitrogen atmosphere) (Ramírez-Jiménez et al., 2004a). At zero time, samples had furosine contents greater than those found in our study. Increases in furosine contents close to those obtained in the present study in samples stored at 30ºC for 9 months have been reported for samples

containing milk stored at 32ºC for 12 months, although considering that in these samples milk content is the half than those of those analysed here their furosine contents are higher.

3.3. Lysine blockage through storage via the furosine method

Furosine content has been used to estimate the percentage of blocked lysine in samples similar to those used in this study, such as different types of milk subjected to varied thermal treatments (Bujard & Finot, 1978; Finot, Deutsch & Bujard, 1981; Evangelisti et al., 1999a) and during storage (Hurrell, Finot & Ford, 1983), and also in cereal and milk products (Erbersdobler & Hupe, 1991; Pizzoferrato et al., 1998; Evangelisti, Calcagno, Zunin & Nardi, 1999b).

In just manufactured (zero time) baby foods, lysine contents were found to be 76.2±1.4, 73.8±2.4 and 67.6±3.6 mg/g protein in samples A, B and C, respectively (Bosch et al., 2006). The percentages of blockage were: a) in just manufactured samples 9.5, 10.7 and 10.7% in A, B and C, respectively; and b) after 9 months of storage 12.8-15.7, 16.0-18.1 and 15.2-16.4% in A, B and C, respectively. The results obtained show that lysine blockage is important not only during the baby food manufacturing process (mean value of 10.3%), but also during the storage period (mean value of 6.4%).

The percentages of lysine blockage obtained in this study are in agreement with the 12% of lysine blockage in non-stored, ready-to-eat rice-milk based baby food reported by Pizzoferrato et al. (1998).

In baby cereals containing 40% of milk during 12 months of storage at 32ºC, a percentage lysine loss of 8% was reported (Ramírez-Jiménez, García-Villanova & Guerra-Hernández, 2004b). The percentage loss is comparable to that found in our study, taking into account that the storage time was only 9 months. Storage of the same baby cereals at 25ºC and A_w=0.65 for 1 month originated lysine losses of 13%, i.e., much higher than those

observed in our study, though an A_w=0.65 is optimal for MR development, given that MR shows its maximum at A_w=0.55-0.75 (Cheftel & Cheftel, 1999), while the milk-cereal based baby foods analysed in this study had an Aw= 1.00, as reported in the Samples section.

Although honey addition to milk-based cereal baby foods improves their organoleptic properties (odour and taste) results obtained in this study show that the presence of honey can favour the first steps of MR, decreasing nutritional value (basically through lysine losses). This effect can be relevant in the type of samples studied, because these foods are intended for a population group with high nutritional needs, namely infants in the early stages of life, and the selection of food varieties in young infants is quite low which requires a high quality of the single items.

The results obtained show a clear increase in furosine content during milk-cereal based baby food storage, and also demonstrate the effect of a high storage temperature (37ºC). Although storage is usually carried out at lower temperatures than those applied during the manufacturing process, it exerts a deleterious effect on available lysine contents, resulting in a mean percentage of blocked lysine (6.4%) lower than that produced by the manufacturing process (10.3%), but nevertheless important from the nutritional point of view. Thus, the effect of storage time also must be taken into account, and it is important for manufacturers, stock facilities and stores to control the storage conditions (temperature and time) in order to prevent losses in the nutritional value of baby foods.

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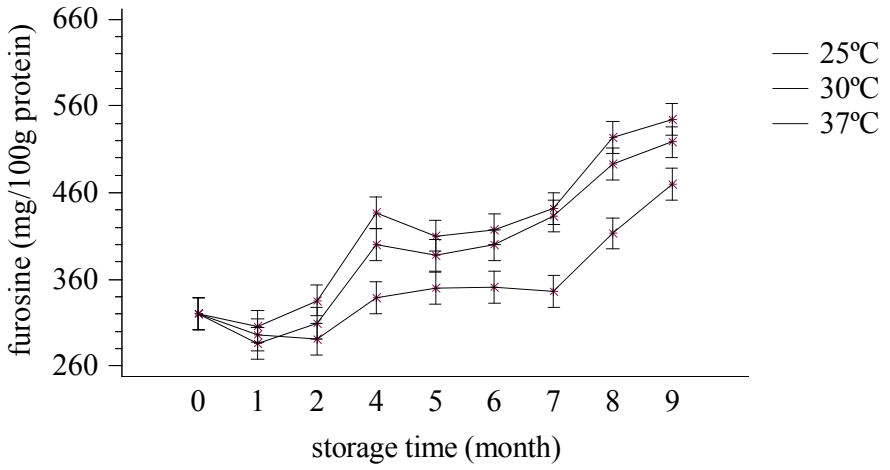
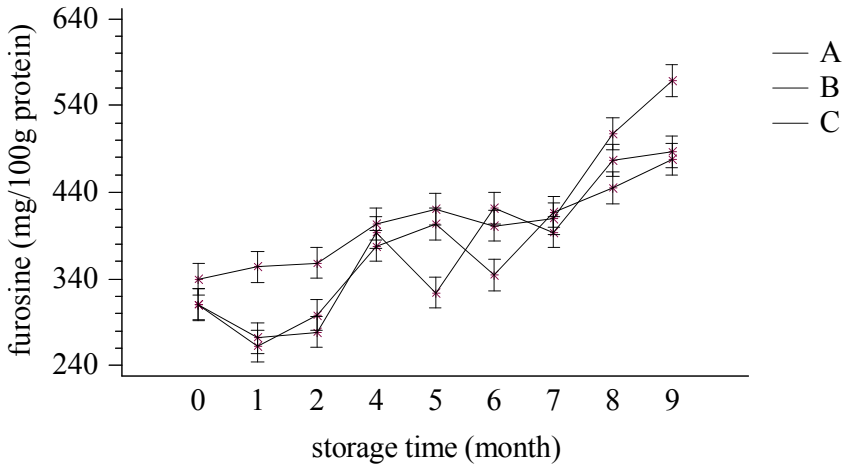
Figure(s)

Fig. 1. Furosine interactions: storage time-temperature (25º, 30º and 37ºC) (A) and storage time-type of sample (A, B and C) (B).

A



B

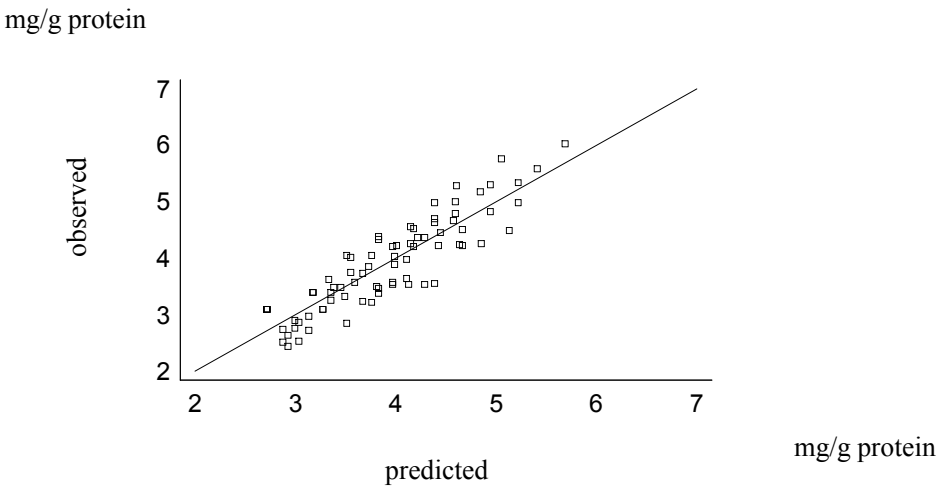


A: milk-cereal based baby foods

B: milk-cereal based baby foods with 0.9% honey

C: milk-cereal based baby foods with 1.1% fruits

Fig. 2. Observed versus predicted values for furosine and obtained regression equation of the model.



Furosine = 2.72 + 0.01temperature.month – 0.09month + 0.45sample 1 - 0.02sample2
(R^2=80%)

Table(s)

Table 1. Furosine contents[1] (mg/ 100 g of protein) of the three (A, B, C) milk-cereal based baby foods analyzed.

	A			B			C		
Month	25ºC	30ºC	37ºC	25ºC	30ºC	37ºC	25ºC	30ºC	37ºC
0		310±20^{a}			340±18^{a}			311±11^{a}	
1	274±6^{b}	265±6^{b}	276±3^{b}	364±5^{b}	348±12^{b}	350±16^{b}	251±8^{b}	245±9^{b}	291±12^{b}
2	253±15^{a,b}	273±12^{a,b}	311±4^{a,b}	333±13^{a,b}	357±25^{a,b}	386±16^{a,b}	287±4^{a,b}	298±15^{a,b}	311±18^{a,b}
4	340±15^{c,d}	402±14^{c,d}	439±5^{c,d}	351±7^{c,d}	423±14^{c,d}	438±21^{c,d}	325±19^{c,d}	376±23^{c,d}	433±19^{c,d}
5	285±14^{c}	322±18^{c}	365±3^{c}	358±19^{c}	436±12^{c}	468±17^{c}	406±17^{c}	405±18^{c}	398±23^{c}
6	373±20^{c,d}	422±29^{c,d}	471±20^{c,d}	355±25^{c,d}	423±24^{c,d}	427±13^{c,d}	325±18^{c,d}	354±19^{c,d}	356±13^{c,d}
7	337±21^{d}	421±16^{d}	423±11^{d}	355±6^{d}	424±26^{d}	450±13^{d}	347±20^{d}	453±16^{d}	452±5^{d}
8	403±13^{e}	498±14^{e}	530±27^{e}	446±24^{e}	517±20^{e}	559±16^{e}	390±30^{e}	463±11^{e}	482±13^{e}
9	426±10^{f}	500±7^{f}	533±35^{f}	528±30^{f}	575±21^{f}	603±40^{f}	456±12^{f}	480±18^{f}	498±12^{f}

[1] Mean ± standard deviation (n=4); A: milk-cereal based baby foods; B: milk-cereal based baby foods with 0.9% honey; C: milk-cereal based baby foods with 1.1% fruits

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

Reviewer 1

Page 8, line 11 says: "Mean furosine contents in samples A, B and C were 373, 418 and 371 mg/100g protein...". However the values of the furosine show at the table 1 at 0 month are 310.3, 339.8 and 310.8 mg/100g protein. Are they talking about the same samples? Please, clarify!

The mentioned mean furosine contents refer to the mean furosine contents calculated for each type of sample (A, B and C), independently of the other considered factors (temperature and time). To check the effect of the factor sample on the value of furosine an analysis of variance (ANOVA) was applied, and to do this the means of experimental furosine contents corresponding to each one of the samples (A, B and C) were calculated, being these the reported values that did not correspond to the furosine contents at 0 months.

Page 10, line 4. Values of 353, 394 and 415 mg/100g. Where they do come from? Are they final values? Initial values? Please, clarify!

The mentioned furosine contents are the means of the values corresponding to the different temperatures and were estimated to study the effect of temperature on furosine content, independently of the other considered factors (type of sample and time).

Reviewer 2

Specific observations/recommendations

Table 1. Please check the correctness of informed number of significant decimal places on each value. For example I would inform 274 +/- 6, instead of 274.2 +/- 6.4

The number of significant decimal places has been revised and table 1 corrected accordingly.

Please, discuss if acid composition of honey may have play a role in enhancing furosine loss.

Since pH of the three analysed baby foods was 6.59±0.05 and it did not change during storage period (page 5, lines 3-4), we consider that acid composition of honey had no influence on furosine formation during storage.

Figure 1. Please, indicate if confidence intervals are included behind of and hidden by figure symbols.

Confidence intervals were not included in the figures of furosine interactions, but according to reviewer's comment, confidence intervals have been added in the revised version of the manuscript.

From Figure 1 It seems that temperature dependence for furosine formation is not as dependent of temperature as color or HMF formation. Could the authors calculate, or estimate rate constants and activation energies and compare them to those values reported for other deteriorative changes in milk products (usually 100 -120 KJ/mol)? Please, discuss why the temperature dependence is quite low in this system.

Rate constants and activation energies (Ea) have been calculated, as follows:

Simple regression analyses of the values of the furosine and storage time were carried out for the three temperatures studied. The obtained correlation coefficients are reported in Table I. To evaluate the data zero-order kinetics was used, as described by the equation:

$$C = Co + k.t$$

Where C is the value of furosine for a fixed storage time, temperature and type of sample; Co is the initial value of furosine; k is the reaction rate constant; and t is the storage time (months). The rate constants (k), obtained from the slope of the fitted straight lines, are reported in Table I, the high/ important standard deviations have to be noted.

The temperature dependence of the reaction rate constants was modeled with the Arrhenius equation:

$$k = k_o . e^{-Ea/RT}$$

Where k is the reaction rate constant; k_o is the pre-exponential factor; Ea is the activation energy (kcal/mol); R is the universal gas constant (1.987 cal/mol.K); and T is the temperature in K. On plotting the negative logarithm of the rate constants ($-\ln k$) vs the reciprocal of absolute temperature (see Fig. I), a slope (Ea/R) was obtained and used to calculate the activation energies (Ea) in furosine formation. The obtained Ea and R^2 values (%) are reported in Table II. R^2 values indicate a moderately strong relationship between the variables. It could be possible that the model used for predicting Ea did not gave reliable values and the studied system presented two different Ea values, that is, an Ea for a determined range temperature and another one for another range, but in this work, it was not possible to calculate both Ea because storage has been carried out at three temperatures. Then, though the kinetics study would be of interest it is not possible to carry it out.

In any case, activation energies are higher for advanced than for early stages of MR (Morales FJ, Van Boekel MAJS (1998) Int Dairy J 8:907-915). As activation energy measures the sensitivity of the reaction to changes in temperature, the formation of furosine will be less sensitive to temperature changes than the formation of advanced or final MR products. Therefore, furosine could be formed at low temperatures, and a temperature increase would have a low effect on its formation. Moreover, since MR is favored in milk-cereal infant foods, initial products form rapidly, and furosine - an indicator of the early stages of the reaction - is soon detected, and it will not be necessary a temperature increase to detect furosine.

Table I. Coefficient correlations (*r*) and rate constants (*k*) of zero-order Maillard reactions for furosine formation in infant foods

Infant food	TºC	*r*	*k* (month^{-1})
A	25	0.813	0.516±0.140
	30	0.904	0.877±0.157
	37	0.917	0.949±0.155
B	25	0.710	0.505±0.189
	30	0.919	0.795±0.128
	37	0.922	0.902±0.143
C	25	0.810	0.568±0.155
	30	0.918	0.829±0.135
	37	0.901	0.784±0.143

A: milk-cereal based infant foods

B: milk-cereal based infant foods with 0.9% honey

C: milk-cereal based infant foods with 1.1% fruits

Table II. Activation energies (Ea) (Kcal/mol) and R^2 values (%) obtained by the application of Arrhenius equation for furosine formation in infant foods.

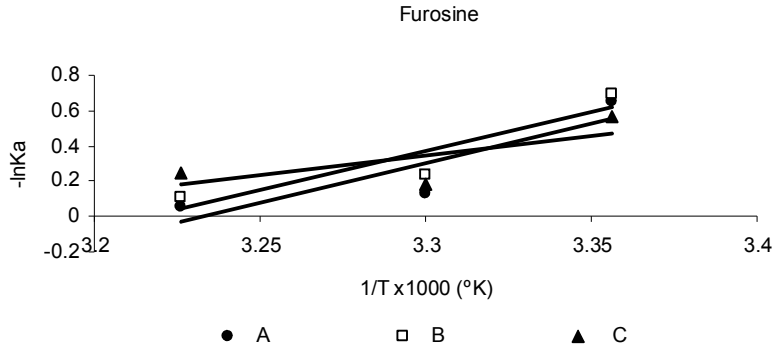
Infant food	Ea	R^2 values (%)
A	8.83	78.34
B	8.70	85.20
C	4.46	54.48

A: milk-cereal based infant foods

B: milk-cereal based infant foods with 0.9% honey

C: milk-cereal based infant foods with 1.1% fruits

Figure I. Arrhenius plots of infant foods A and B stored at three different temperatures for furosine.



This information was available when we wrote the manuscript, but in our opinion it was no appropriate to include it, because the results for the three temperatures showed a high variability, as show the high standard deviation values for k (table I), and so the calculation of activation energies was no robust (table II).