

Effect of Ingredients on Non-enzymatic Browning, Nutritional Value and Furanic Compounds in Spanish Infant Formulas

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Abstract During their processing, the infant formulas (IFs) are subjected to different thermal processes, which affect their safety and nutritional value, being influenced by the quality and type of ingredients used. The objective of this study was to evaluate the effect of ingredients used in commercial powdered IFs on furosine, HMF, furfural, available lysine content and evaluation of possible toxic effect of furanic compounds. Principal components (PC) and cluster analysis (CA) were employed to investigate relationships among IFs and indicators. Two PC were obtained which explain 77.3% of the total variance, grouping the IFs in five clusters. Significant higher values of available lysine were obtained in IFs with whey milk or skimmed milk; likewise, furosine was obtained in IFs with lactose, whey milk, milk proteins, skimmed milk or partially hydrolyzed whey (PHW). Significant higher values of HMF and furfural were obtained in IFs with starch. The maximum free furanic compounds provided by IFs were of 1.7 mg/person/day. HMF content does not represent a risk to the babies' health. All IFs except one cover more than 90% of available lysine needs for a 3-month aged baby. PC and CA are useful to evaluate heat damage in IFs.

Keywords: *infant formulas, furosine, HMF, furfural, available lysine, toxic effect, principal components analysis*

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1. Introduction

Maillard reaction (MR) is one of the most important sources of heat-generated compounds [1]. The interactions between IFs components, mainly affect carbohydrates and proteins through of MR, but those involving proteins are especially important in products used in infant feeding because of the high protein requirements of infants [2]. The manufacture of IFs includes the component blending, homogenization, pasteurization, and spray-drying [2]. The fact that IFs 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 [3]. Detrimental effects of thermal processes are also relevant. The loss of thermo labile compounds such as vitamins, essential amino acids (lysine, tryptophan) and/or the formation of undesired tastes and off-flavours cause loss in the nutritional value and sensorial quality of heated foods [4,5,6]. Besides, protein and carbohydrate ingredients are also subjected to thermal processes for their production, so they can also present heat damage [7].

The extent of the MR in foods can be monitored with different chemical indexes. The main objective of such indicators are to define the nutritional status, organoleptic characteristics or even the possible toxicity of the foodstuff after the thermal process and storage, then obtaining food products of good quality and high nutritional value [8]. Furfural compounds (Hydroxymethylfurfural (HMF) and furfural) are a recognized indicator of the deterioration produced by excessive heating or storage in a wide range of carbohydrate-containing foods [7,9,10,11]. Furosine determination has also been used to study early stages of the MR during the heat treatment and storage of IFs [2,12,13]. Available lysine content is an indicator of both early and advanced MR phases [14], and several studies have been published on lysine loss due to the heat treatment and storage of IFs [2,13], and model systems [15]. The lysine, as one of the essential amino acids, is involved in this reaction, and bioavailability is lost [16].

Heat-generated compounds may create a risk to human health. For some years, adverse effects from neo-formed contaminants have been the subject to increased attention, particularly acrylamide, nitrosamines, heterocyclic amines, polycyclic aromatic hydrocarbons, furanic compounds (Furan, HMF) and advanced glycation end products

[17,18]. Furanic compounds arise from heat-treatment processing and contribute to the sensory properties of cooked foods, in general their presence is appreciated; however, due to its potential harmful effects on human health studies related with its formation, content in a variety of foods [19]. However, no relevance for humans concerning carcinogenic and genotoxic effects of HMF are available [20].

It is clear that the ingredients used in the IFs directly affect their quality; however, there are few studies where it has been established relationships between the presence or absence of ingredients and the different chemical indicators of MR. On the other hand, there are studies about heat damage by MR and nutritional value in powder IFs, but few where it is estimated the potential toxic risk for infants by furanic compounds in different types of commercial IFs. The purpose of this study was to find relationships between ingredients used and heat damage of IFs, in order to evaluate the nutritional value and possible toxic effect of different types of IFs from Spanish market.

2. Materials and Methods

2.1. Samples and Reagents

Thirteen commercial powdered IFs (eight adapted and five follow-up (hypoallergenic, and soybean-based)) were purchased in several local markets. The composition protein-sugar and ingredients used according to label are shown in the Table 1.

All chemicals used were of analytical grade. Methanol (HPLC grade), hydrochloric acid, acetonitrile and acetic acid glacial were obtained from Panreac (Barcelona, Spain). Sep-Pack cartridges (C₁₈) were purchased from Waters Millipore (Milford, MA, US). Trichloroacetic acid (TCA), N-ε-2, 4- dinitrophenyl-lysine (DNP-L-Lysine) HCl, fluoro-2, 4- dinitrobenzene (FDNB) were purchased from Sigma-Aldrich (Madrid, Spain). 5-(hidroxymethyl)-furfural and 2-furaldehyde were purchased from Merck (Darmstadt, Germany). Furosine was obtained from Neosystem Laboratories (Strasburgo, France).

2.2. HMF and Furfural Determination

Furanic compounds were determined following a method described elsewhere [7], with some modifications. Approximately, 0.4 g of sample was clarified (with Carrez I and II) and the water fraction was filtered through 0.2-μm disk filter before injection. The HPLC equipment consisted of a Waters 600 controller (Waters Millipore) with a manual injector and Konic model 200 detector (Linear Instrument Corp, Reno Nevada, NV). The integrator program used was a Millennium chromatography manager (Waters Millipore). 50 μL of filtered solution were separated in a reversed-phase C₁₈ column (Nova-Pack 4 μm; 250 mm x 46 mm id., Cartridge, Waters, Milford, MA, US). The mobile phase was water-acetonitrile (95:5) and the flow rate was 1 ml/min. HMF and furfural were quantified using the external standard method. Duplicate analysis of duplicate samples was carried out (*n* = 4).

Table 1. Commercial Powdered IFs Composition According Label

Formula	% Protein	% Sugar
1 ^a	Whey milk, skimmed milk, milk proteins (9.5)	Lactose (57.9)
2 ^b	Whey milk, skimmed milk (12)	Lactose (62.1)
3 ^a	Whey milk, skimmed milk (12)	Lactose (54.5)
4 ^a	Milk proteins (10.4)	Lactose (55.5)
5 ^b	Milk proteins (11.6)	Lactose (40.2) Dextrinomaltose (13.2)
6 ^b	Skimmed milk (12.5)	Lactose (55.9)
7 ^a	Skimmed milk (12)	Lactose (43.4) Dextrinomaltose (14)
8 ^b	Skimmed milk (14.6)	Lactose (44.6) Dextrinomaltose (11.9)
9 ^{a*}	Partially hydrolyzed whey protein milk (11.5)	Lactose (40.4) Dextrinomaltose (17.3)
10 ^{a*}	Partially hydrolyzed whey protein milk (11.5)	Lactose (19.1) Dextrinomaltose (26) Starch (9.9)
11 ^{b*}	Partially hydrolyzed whey protein milk (12.1)	Lactose (20.1) Dextrinomaltose (21.6) Starch (12.6)
12 ^{a*}	Partially hydrolyzed whey protein milk (12.4)	Dextrinomaltose (33.7) Maltose (20.5)
13 ^a	Isolated soy protein (14.2)	Dextrinomaltose (52)

^a Adapted IF

^b Follow-up IF

* Hypoallergenic IF.

2.3. Furosine Determination

Furosine was determined following the method described by Resmini et al. [21] with some modifications [7]. The HPLC system consisted of a Perkin-Elmer model 250 pump (Norwalk, CT, USA) with a Waters 717 autosampler (Milford, MD, USA) and a Perkin-Elmer model 235 diode array detector (Norwalk, CT, USA). Data were collected with a 1020 software data system Perkin-Elmer. 50 μ L of filtered solution were separated using a C₈ furosine-dedicated column (250 mm x 4.6 mm, Alltech, DeerWeld, IL, USA). Calibration of the chromatographic system was made using the external standard method. Duplicate analyses of duplicate samples were carried out ($n = 4$).

2.4. Available Lysine Determination

ϵ -NDP-lysine was determined by HPLC following the method used for IFs by Contreras-Calderón et al. [2]. A sample containing approximately 4 mg of protein was derivatized by addition of FDNB. Hydrolysis of FDNB derivative was realized with HCl. The HPLC study was performed in a Perkin-Elmer 250 model with a Waters 717 automatic injector and Perkin-Elmer 235 UV diode array detector. The integrator-computer used here was a 1020 Perkin-Elmer Nelson model. 50 μ L of filtered solution were separated in a Nova pack reverse phase C₁₈ HPLC column (150 mm x 3.9 mm Waters) operating at room temperature. ϵ -DNP-lysine was determined by the external standard method. Duplicate analyses of duplicate samples were carried out ($n = 4$).

2.5. Statistical Analysis

Multivariate statistics (including PC analyses and CA) were employed to quantitatively investigate relationships among the 13 samples of IFs with respect to the 4 indicators; PC analyses variables were standardized before the analysis and principal components (PC) with eigenvalues greater than one were selected. CA was made through Ward method using square Euclidean distances.

One-way ANOVA was applied to the results; differences between clusters were established using LSD test with a level of significance of 95 %. Correlations among variables were assessed by means of the Pearson's correlation tests (level of significance of 95 %). The impact of ingredients used on IFs quality and chemical indicators was evaluated using multifactor ANOVAs, where factors were the ingredients in the IFs with two levels (present, absent). All the statistical analyses were performed in Statgraphics Centurion XVI®. Left censored data for HMF and furfural was assumed to be zero in the statistical analysis.

3. Results and Discussion

3.1. HMF and Furfural

HMF is an intermediate in the MR which is formed from the degradation of Amadori products during the intermediate stage of the MR if the heating conditions are appropriate [22]. HMF is considered a heat-induced marker for a wide range of carbohydrate containing foods such as milk [23]. HMF is also used for monitoring the heating process applied to cereal products such as baby cereals [24] and breakfast cereals [25]. But, significance of the measurement of HMF is different depending of the type of food being mainly used as marker of either adulteration or over-processing (i.e. milk), and marker of quality of ageing. In other foods, analysis of HMF is taken together with other furanic compounds such as furfural.

The performance of the method was evaluated in commercial follow-up infant formula, including the preparation and RP-HPLC analysis. Mean recovery values were 99.2% for HMF and 71.1% for furfural, and the relative standard deviations (RSD) were 2.42% and 1.23% for HMF and furfural respectively. The detection limit (LOD) (three times the signal-to-noise ratio) and quantification limit (LOQ) (ten times the signal-to-noise ratio) were 4.95×10^{-3} and 0,017 mg/100 g of protein for both HMF and furfural.

Table 2. Furosine, Available Lysine, HMF and Furfural Content in Commercial Powdered IFs.

Formula	HMF		Furfural		Furosine	Available lysine	
	mg/Kg	mg/100 g of protein	mg/Kg	mg/100 g of protein	mg/100 g of protein	mg/g	g/100 g of protein
1 ^a	ND	ND	ND	ND	936±29.8	4.20±0.09	4.42±0.10
2 ^b	ND	ND	ND	ND	1166±35.7	4.91±0.09	4.09±0.08
3 ^a	1.28±0.00	1.07±0.00	0.31±0.05	0.26±0.04	1392±31.2	4.80±0.41	4.00±0.34
4 ^a	5.01±0.13	4.82±0.13	0.08±0.01	0.08±0.01	796±16.1	4.57±0.02	4.39±0.02
5 ^b	3.45±0.03	2.97±0.03	0.12±0.00	0.10±0.00	865±25.7	4.52±0.10	3.74±0.08
6 ^b	5.17±0.33	4.14±0.26	0.08±0.00	0.06±0.00	757±16.6	4.85±0.06	4.18±0.05
7 ^a	2.08±0.07	1.73±0.06	0.19±0.01	0.16±0.01	830±21.3	5.33±0.02	4.44±0.01
8 ^b	4.52±0.05	3.10±0.03	0.14±0.00	0.10±0.00	608±20.7	6.26±0.00	4.29±0.00
9 ^{a*}	ND	ND	ND	ND	1459±10.8	3.93±0.25	3.41±0.22
10 ^{a*}	4.59±0.04	3.99±0.03	0.16±0.01	0.14±0.01	752±1.97	4.47±0.04	3.88±0.03
11 ^{b*}	14.2±0.50	11.7±0.41	0.62±0.04	0.51±0.03	859±10.1	5.32±0.08	4.26±0.06
12 ^{a*}	2.27±0.02	1.83±0.02	0.08±0.00	0.06±0.00	711±16.6	2.83±0.10	2.28±0.08
13 ^a	4.92±0.09	3.46±0.06	0.06±0.00	0.04±0.00	379±10.8	4.81±0.04	3.39±0.03

Values are expressed as the mean \pm standard deviation ($n=4$). ND not detected.

^a Adapted IF

^b Follow-up IF

* Hypoallergenic IF.

HMF and furfural values ranging between non-detected for IFs 1, 2, 9 and 11.7 and 0.51 mg/100 g of protein, respectively for IF 11 (Table 2). It is clear that the IF 11 has a higher thermal damage than other IFs. Because the ingredients used are similar to the other IFs, these high furanic compounds values may be due to a high heat treatment, long storage time, or that thermally damaged ingredients have been used. In IFs 1, 2 and 9 where no furanic compounds were detected they must have used good quality ingredients (low thermal damage); additionally, storage conditions and thermal treatments during manufacturing of IFs must not have been very drastic.

Comparing the results with those reported by other authors, it is observed that in general, the IFs of this study showed higher values of furanic compounds; thus, Albalá-Hurtado et al. [26] found that free HMF and furfural values between 10.3 and 23.5 $\mu\text{mol/L}$ (0.84 and 1.91 mg/100 g of protein), and 1.10 and 5.30 $\mu\text{mol/L}$ (0.09 and 0.43 mg/100 g of protein), respectively in powdered IFs stored during 9 months at a 20, 30 and 37°C. Ferrer et al. [27] found that HMF levels were 0.34 and 1.84 mg/100 g protein, and between not detected and 0.08 mg furfural/100 g of protein, respectively in adapted and follow-up powdered IFs. Chávez-Servín et al. [28] found that initial values of 0.85 mg HMF/Kg and 0.012 mg furfural/Kg during storage of powdered IFs not supplemented with long-chain polyunsaturated fatty acids, and 0.58 mg HMF/Kg in supplemented IFs, where no furfural was detected. The HMF values in IFs from this study were also higher to those obtained in a study conducted by Contreras-Calderón et al. [7], in protein ingredients used in the manufacture of IFs.

A significant ($p < 0.5$) positive correlation was observed between HMF and furfural ($r = 0.7826$), which is expected since it is considered that the formation of both is affected by the same factors. However, Albalá-Hurtado et al. [29], did not find correlation between the formation of HMF and furfural from their precursors in liquid IFs. The differences in these studies may be due to ingredients used in the IFs and the initial thermal damage of the ingredients.

Based on data reported in literature, it is not clear whether human exposure to HMF represents a potential health risk. Janzowski et al. [30] concluded that HMF does not pose a serious health risk, even though the highest concentrations in specific foods approach the biologically effective concentration range in cell systems. HMF was previously considered a quality indicator of thermally processed foods until its toxic properties were highlighted [31]. The Scientific Panel on food additives, flavorings, processing aids and materials in contact with foods estimated a dietary HMF intake of 1.6 mg/person per day based on an mTAMDI (modified Theoretical Added Maximum Daily Intake) – approach [32]. This estimate is far below the threshold of concern of 540 mg/person/day derived from a large database containing data on sub chronic and chronic animal studies [33]. Zaitzev et al. [34] suggested that a Tolerable Daily Intake (TDI) of 132 mg/person per day by applying a 40-fold margin of safety.

The currently available assessments of dietary intake remain far below this value. Chávez-Servín et al. [35] reported that the range of potential HMF consumed by an infant about 6 months old feeding only on formula was

estimated between 0.63 mg and 3.25 mg per day. In the present work, the maximum free HMF and furfural concentration was 14.8 mg/Kg for IF 11 (Table 2; HMF plus furfural). For a baby aged 6 months with an average weight of 8 kg [36], the consumption of IF is around 115.2 g/day (6 measures of 4.8 g per bottle for 4 bottles in 24 h, according to label recommendations); considering that 1 Kg of IF 11 has 14.8 mg HMF and furfural, 115.2 g of this IF will provide 1.7 mg/person of these compounds per day. This value is slightly higher than that established by EFSA [32] for the use of HMF as a food additive and is within the range set by Chávez-Servín et al. [35]. However, these values are far below the threshold of concern established by other authors [33,34]. It should be taken into account the HMF's genotoxicity potential, the importance of lowering HMF content in infants' milk-based formula and other heated foods, so the dietary exposure can be carefully re-evaluated, and it has also been recommended by Chávez-Servín et al. [35].

3.2. Furosine

The content of furosine (ϵ -N-2-furoylmethyl-L-lysine) present in foods is influenced by the kind of heat treatment and/or the storage time. Levels of furosine tend to decline after prolonged storage or after overheating to give rise to other compounds such as N ϵ -(carboxymethyl) lysine (CML) [37,38]. Furosine is the most specific and important indicator of the initial phase of the Maillard reaction. It is widely used in the analysis of cereal products, since lysine is the limiting amino acid of this product and, thus the presence of furosine is an important marker of protein biological value loss. The precision of the entire procedure, including acid hydrolysis, sample preparation, and RP-HPLC analysis, was evaluated for a commercial follow-up infant formula ($n = 8$) with a RSD of 3.4%. The detection limit (LOD) (three times the signal-to-noise ratio) was 0.105 mg/100 g of protein. The quantification limit (LOQ) (ten times the signal-to-noise ratio) was 0.35 mg/100 g of protein.

The furosine values had a wide range, between 379 mg/100 g of protein for IF 13 based on soy protein isolates and 1459 mg/100g of protein for IF 9 prepared with hydrolyzed whey proteins (Table 2). The average value of furosine obtained in the eight IFs made with intact dairy proteins (919 mg/100 g of protein) was similar to that obtained in the four IFs made with partially hydrolyzed milk protein (945 mg/100 g of protein). The mean value of furosine in adapted formulas was slightly superior to follow-up formulas (907 vs. 871 mg/100 g of protein), the major proportion of whey protein vs. casein used in the first formulas could justify this results [7].

The above values are similar to those found by other authors. Thus, in IFs made with intact proteins, it has been found values between 141 and 1551 mg/100 g of protein, and between 130 and 900 mg/100g of protein in IFs elaborated with hydrolyzed milk protein [12,13,39,40,41]. Baptista and Carvalho [42] found very low values in IFs, between 21.4 and 81.5 mg/100g of protein for a formulation without lactose and with partially hydrolyzed whey protein respectively. Contreras-Calderón et al. [2] found that similar values in IFs made with intact proteins; however, in IFs made with partially hydrolyzed proteins

(PHP) the values were lower (299 mg/100 g of protein) to those found in this study. The few data found in the literature on the analysis of IFs made with soy protein [39] are lower (approximately, 270 mg/100 g of protein) than those obtained in this study. High values of furosine in IFs with hydrolyzed protein would indicate the use of ingredients with high initial heat damage since furosine content in powdered hydrolyzed IFs is lower than in regular powdered formulas [40], as glycation in these IFs cannot be detected when is estimated by the furosine method [41]. In addition, hydrolyzed proteins typically have low levels of furosine [2].

The different formulations, the thermal damage of different ingredients and processing of IFs make that this indicator, unlike what happens in milk, can only be used to control the process of manufacturing and storage of the same formula.

A significant ($p < 0.05$) negative correlation was observed between HMF and furosine ($r = -0.3889$), which suggests a progress of the MR, since at high temperatures (spray drying), the Amadori product may be further degraded by different pathways during the advanced stage of the MR [43]. However, it is the IF 13 with isolated soy protein which had the lowest furosine and moderate HMF content. Contreras-Calderón et al. [7] noted that soy proteins analyzed did not contain furanic compounds and furosine values were low, so it is possible that the moderate values of HMF may come from maltodextrins [44] present in the formulation and not to the MR during processing. In the case of the IF 13, furosine would be the thermal damage indicator since the MR is in the early stages.

No significant correlation ($p > 0.05$) was found between furfural and furosine which would indicate that in these samples Amadori compounds (measured as furosine) are not furfural precursors since furfurals are both the result of Amadori compounds from the MR or of lactose isomerization [45], or still furfural already present in sugar ingredients [44].

3.3. Available Lysine

The precision of the method was evaluated in a commercial follow-up infant formula ($n = 8$), with a RSD of 4.6%. The detection limit (LOD) (three times the signal-to-noise ratio) for adapted infant formula was 7.25×10^{-4} g/100 g of protein. The quantification limit (LOQ) (ten times the signal-to-noise ratio) was 2.42×10^{-3} g/100 g of protein [2].

Available lysine values were between 2.28 and 4.44 g/100g of protein (Table 2), respectively for IF 12 made with hydrolyzed whey protein and IF 7 made with skim milk. The 62% of IFs presented values above 4.00 g/100 g of protein, being mainly the IFs with PHP and soy protein isolate the ones showing lower values compared with IFs elaborated with intact proteins. Contreras-Calderón et al. [7] observed that the same trend in this type of proteins. Pereyra González et al. [46] also found that available lysine contents in soy based IFs were significantly lower than those found in milk protein based formulas.

The nutritional requirements of lysine for babies aged 0 to 6 months are around 107 mg/kg/day [47]. For a baby aged 3 months with an average weight of 6.2 kg [36], the formula consumption is around 150 g/day (7 measures of

4.3 g per bottle for 5 bottles in 24 h, according to label recommendations), and the need for lysine is approximately 663.4 mg/day. Only the IF 12 does not cover the needs of children since it provides 425 mg lysine/day. The other IFs provide more than 90% of DRI's, between 590 and 800 mg lysine/day, for IFs 9 and 7, respectively. However, Contreras-Calderón et al. [48] found that adapted IFs exceed the minimum estimated need of infants, which can be attributed to the IFs in the present study or the protein ingredients used had higher heat damage as is observed by the low available lysine (Table 2). Given these results, it is important that the preparation of bottles and daily consumption of IFs are made based on the content of available lysine and not on the total lysine of the protein.

The nutritional requirements of lysine for babies aged 7 to 12 months are around 89 mg/kg/day [47]. For a baby aged 12 months with an average weight of 10.2 kg [36], consumption of formula is around 115.2 g/day (8 measures of 4.8 g per bottle for 3 bottles in 24 h, according to label recommendations), and the need for lysine is approximately 907.8 mg/day. Only IF 8 covers the minimum requirements of lysine since it provides 939 mg lysine/day. The other IFs provide between 678 and 798 mg lysine/day for IFs 5 and 11, respectively, which makes sense because for a child of 12 months IFs are not the only food. These results differ from those found by Contreras-Calderón et al. [48], where the follow-up IFs covered the needs of infants. Similar to the observed in adapted IFs, the follow-up IFs of this study or protein ingredients used, could also have high heat damage.

The data found in the literature are highly variable; thus, Ferrer et al. [27] in adapted and follow-up IFs found values of 6.67 and 6.61 g/100g of protein, respectively. Pereyra-González et al. [46], in different commercial powder IFs, made with soy protein isolates, casein, milk, whey fortified milks and cow's milk, found values between 3.3-5.2; 4.13-7.0; 6.2-8.11; 5.4-7.8 and 8.04 g/100 g of protein, respectively. Ferrer et al. [12], analyzed an adapted and follow-up IFs elaborated with milk protein, finding initial available lysine values of 8.43 and 7.9 g/100 g of protein, respectively. Chávez-Servín et al. [49], during store of powder IFs based on milk proteins and lactose, found initial available lysine values ranged between 6.01 and 6.04 g/100 g of protein. Contreras-Calderón et al. [2], in powdered IFs found values between 4.88 and 5.88 g/100g of protein. Finally, Contreras-Calderón et al. [48], in similar IFs found values between 4.99 and 6.44 g/100 g of protein. In general, the values reported in literature are much higher than those obtained in the present work, which may be due to a high heat treatment, long storage time, or that ingredients thermally damaged have been used.

Additionally, no significant correlation ($p > 0.05$) was found between furosine and lysine; this is surprising if we regard that in milk products, the MR occurs between lactose and lysine, leading a significant decrease in lysine bioavailability, through the formation of the Amadori compound [50]. Similar results were reported by Contreras-Calderón et al. [7], in protein ingredients, although they found a larger amount of available lysine associated with a larger amount of furosine. On the other hand, Contreras-Calderón et al. [2] found that lysine losses

were inversely proportional to increases in furosine during processing of IFs. These contradictory results can be explained because it is not easy to compare different types of IFs, as there are studies confirming that the specific formulation, processing conditions applied for IFs and the initial damage of the original ingredients are closely related with an increased risk of protein damage by glycation and oxidation reactions [2,51].

3.4. Principal Components Analysis (PCA) and Cluster Analysis (CA)

In this study, two PC were obtained which explain 77.3% of total variance. Fig. 1 showed that the biplot (PC_2 vs. PC_1) of IFs samples and variables. PC_1 explained 48.6% of the total variance, which has a positive influence of HMF, furfural, and in lesser extent, a negative influence of furosine and a positive influence of lysine. PC_2 explained 28.7 % of the variance; it is mainly characterized by a positive contribution of lysine and furosine (Figure 1). PC_1 could be seen as an indicator of intermediate stages of MR, as it takes into account the positive contribution of furanic compounds and the negative contribution of

furosine; lysine also exhibited a positive contribution probably due to the fact it is released during the intermediate stages of MR in the 1-2 and 2-3 enolisation pathways, and lysine is finally incorporated into melanoidins polymers just at final stages of MR [52]. On the other hand, PC_2 could be seen as an indicator of initial stages of MR. That is, PC_2 is higher with higher lysine contents which is due to the fact that lysine is the most reactive amino acid concerning the MR; additionally, PC_2 is higher with higher furosine contents; this is useful as an estimation of the advance of initial stages of MR.

CA was performed based on the PCA. Figure 2 showed the distribution of samples inside the clusters according to PC_1 and PC_2 , in which five clusters were formed. Cluster 3 is formed by samples 4, 5, 6, 7, 8 and 10; this cluster is located close to coordinate origin, which means that these samples show average for furosine (768 mg/100 g protein), HMF (3.46 mg/100 g protein), furfural (0.11 mg/100 g protein) and lysine (4.15 g/100 g protein) nearby to the global average (885, 2.98, 0.12 mg/100 g protein for furosine, HMF and furfural, respectively and 3.91 g/100 g protein for lysine). It implies that IF sin cluster 3 exhibited intermediate heat damage.

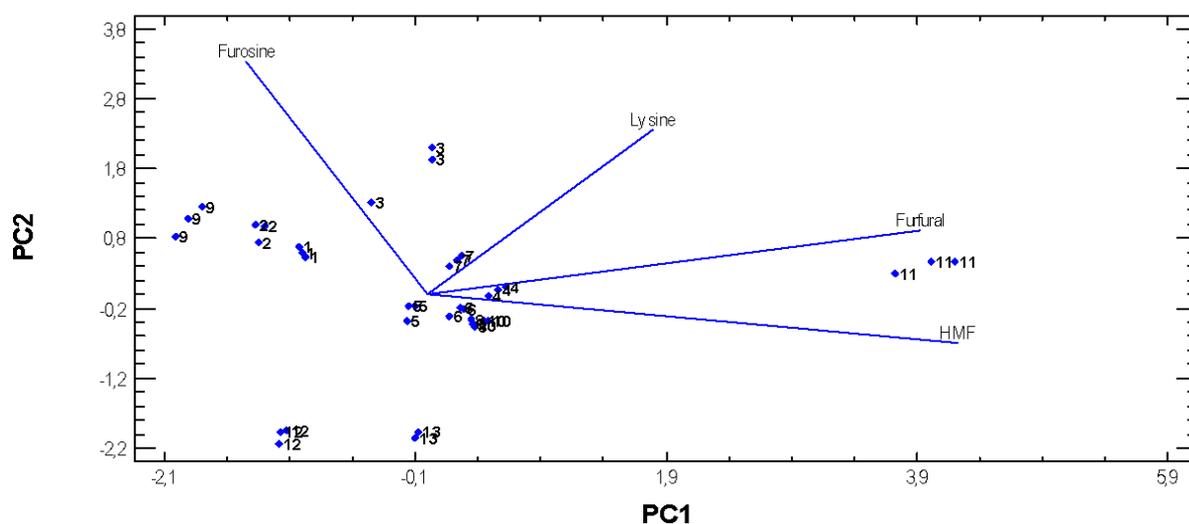


Figure 1. Principal component analysis (PCA) of the IFs according to the determinations of heat damage indicators

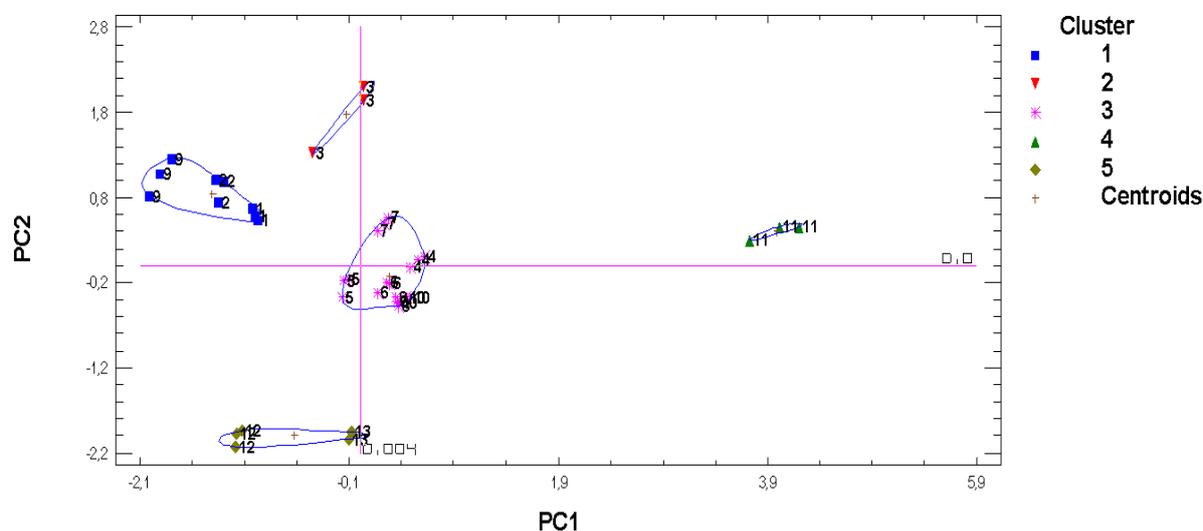


Figure 2. Clusters formed from IFs samples according to PC_1 and PC_2

Cluster 1, formed by samples 1, 2 and 9 exhibited the lower value in PC₁ and slightly high value in PC₂, which presented average close to the global average for lysine (3.97 g/100 g protein), higher average for furosine (1186 mg/100 g protein) and lower average for HMF and furfural (no detected) than the global average. These results suggest that samples in cluster 1 did not react in greater extent until intermediate stages of MR, but with a high advance in initial stages. This could indicate that samples in cluster 1 were manufacture with good quality raw material (with minimal heat damage) and the thermal processing was not too strong, as MR just advanced until initial stages.

Cluster 2 represents sample 3 which has the higher value in PC₂ and intermediate value in PC₁ with values close to the average for lysine (4.00 g/100 g protein), high average than global average for furfural (0.26 mg/100 g protein) and furosine (1392 mg/100 g protein) and lower average than global for HMF (1.07 mg/100 g protein). The high furfural contents could be due to isomerization of lactose through LA (Lobry de Bruyn- Alberda Van Ekenstein) transformation to lactulose and its posterior degradation to HMF which subsequently could react to form furfural [10]. The high furosine content indicates that important heat damage was induced during the manufacturing process or storage, which can promote the lactose isomerization, as was established by Pereyra González et al. [46] who found significantly higher lactulose concentrations in whey-enriched IFs compared to others milk based IF's. Therefore, probably this sample has high initial heat damage and it is starting the intermediate stages of MR.

Cluster 4 represents sample 11 which has the higher value for PC₁ and an intermediate value for PC₂ with values close to the global average for furosine (858 mg/100 g protein) and lysine (4.26 g/100 g protein), and higher average for HMF (11.70 mg/100 g protein) and furfural (0.51 mg/100 g protein) than global average. In

fact, sample 11 showed the highest values for HMF and furfural. Sample 11 could be manufactured with damaged ingredients or it could be subjected to an intense heat treatment during processing or storage. However, the formation of furfural and HMF could have been carried out not only by MR, but also by other mechanisms already mention, as the lysine content was not further decreased in comparison with other samples. Based on the results, it could be said that this IF exhibited the largest thermal damage.

Cluster 5 is formed by samples 12 and 13 which has the lower value for PC₂ and intermediate value for PC₁ with lower average values for lysine (2.83 g/100 g protein), furosine (545 mg/100 g protein) and furfural (0.05 mg/100 g protein) than global average, and values close to global average for HMF (2.64 mg/100 g protein). The low available lysine content could be due to these IFs are elaborate with PHP and soy protein isolate, which showed lower values than IFs elaborated with intact proteins [7,46], and it does not reflect a high thermal damage, as can be confirmed by the low values of furosine and furfural. The intermediate values for HMF might be from maltodextrins [44], so these samples exhibit low heat damage.

Ingredients (Table 1) were used to try to explain the belonging of the samples to the clusters. It was found that cluster 5 is formed by one adapted hyper allergenic IF and the only IF with isolated soy protein, both the only samples without lactose; samples in clusters 3, 4 and 5 do not have whey milk and cluster 3 is formed by samples without partially hydrolyzed whey, except for sample 10. However, it was not possible to find a general relation between ingredients used in the IFs and the heat damage indicators that explained the clustering of the samples, maybe because there are other important underlying variables as the possible interactions between ingredients, the impact of manufacturing process of each IF, the quality of the raw material, etc., which were unknown.

Table 3. Results from Multifactor ANOVAs Relating Ingredients of IFs and Heat Damage Indicators

Ingredient	Present	Furosine	HMF	Furfural	Lysine
		Mean	Mean	Mean	Mean
Lactose	No	545.4*	2.65	0.05	2.84*
	Yes	947.3*	3.05	0.13	4.10*
Dextrinomaltose	No	1010	2.01	0.08	4.22
	Yes	808.0	3.60	0.14	3.71
Starch	No	900.1*	2.10*	0.08*	3.88*
	Yes	805.4*	7.84*	0.32*	4.07*
Whey milk	No	801.7*	3.77*	0.13	3.83*
	Yes	1165*	0.36*	0.09	4.17*
Skimmed milk	No	831.7*	4.11	0.13	3.62*
	Yes	948.2*	1.67	0.10	4.24*
Milk proteins	No	855.5*	2.25	0.10	4.06*
	Yes	911.2*	3.62	0.13	3.77*
Partially hydrolyzed whey	No	858.9*	2.37*	0.09	4.10*
	Yes	945.4*	4.38*	0.18	3.46*
Isolated soy protein	No	927.7	2.95	0.12	3.95*
	Yes	379.3	3.46	0.04	3.39*

Mean with asterisk (*) showed statistically significant differences ($p < 0.05$) between the two level of a same factor.

Thus, four multifactor ANOVA's were made in order to find the relation of ingredients in the IFs and the heat damage indicators. Eight ingredients were selected: lactose, dextrinomaltose, starch, whey milk, skimmed milk, milk proteins, partially hydrolyzed whey (PHW) and isolated soy protein (ISP). All factors with two levels: presence or absence of the ingredient in the IF (Table 3).

Available lysine showed significant ($p < 0.05$) higher values in IFs with lactose, starch, whey milk or skimmed milk and absence of PHW, milk proteins or ISP. This is expected because the whey milk contained higher lysine levels than PHW and ISP [7]. It is difficult to explain how the lactose favored high lysine content since both the high lactose content and the supplementation with whey proteins promote MR in IFs [51]. However, in this study all IFs have lactose, except two IFs elaborated with PHW and ISP, proteins with low available lysine content [7], so in this case the high lysine content in IFs with lactose is because these IFs contain proteins with higher lysine content. Pereyra Gonzáles et al. [46] also found higher available lysine contents in IFs based on skim milk than in IFs based on whole milk and caseins. The starch (a non-reducing polysaccharide) reduces the MR, favoring the conservation of lysine. Explain available lysine content is very difficult because not only depends on the thermal damage but the type and amount of protein used.

Samples with lactose, whey milk, milk proteins, skimmed milk or PHW and absence of starch, showed significant ($p < 0.05$) higher values of furosine. These results were expected, because it is known that the most dominant among the MR products in milk is certainly lactulosyllysine, the Amadori product of lactose and lysine side chains of the milk proteins [53]. However, the higher levels of furosine can be expected in IFs with whey protein respect to other IFs [54], and also whey protein can already be highly damaged [7]. On the other hand, Fenaille et al. [40] and Contreras-Calderón et al. [2] found lower values of furosine in IFs with PHW, so as already it is mentioned above the PHW used in these IFs may have high initial heat damage. Likewise, the starch does not favor the MR.

HMF showed significant ($p < 0.05$) higher values in IFs with starch or PHW and absence of whey milk. These results could be explained by the HMF present in the starch because during its production process is subjected to various thermal processes that can hydrolyze the starch to reducing sugars which can participate in MR and caramelization [55]. On the other hand, the only two IFs with starch are made with PHW, so we believe that this is the reason why statistical analysis relates this protein with a high content of HMF, but this is not necessarily related to the heat damage of the IF or protein. Contreras-Calderón et al. [7] did not found HMF in protein hydrolyzed. These same authors found HMF only in whey proteins, so it is not expected these results since whey proteins usually have higher heat damage than other proteins, which confirm that the high HMF values are due to presence of starch, as the IFs with whey proteins have not this carbohydrate.

Furfural was significant ($p < 0.05$) higher in samples with starch in comparison with the samples which did not have this ingredient in their composition. As already mentioned above, the starch may be an important source of furanic compounds, indicating an initial damage of ingredient but not of the IFs during processing or storage.

4. Conclusions

MR indicators are intrinsically linked as they represent complex relations of reactants in the different MR pathways which depend on the nature of the reactants and the conditions of the heat treatment applied to the food matrix. These complex relations are difficult to follow or explain if just one variable is considered. Statistical analysis is a useful tool to help to interpret results of multiple variables. The main advantage of multivariate analysis is to simplify the interpretation of multiple variables results regarding to a common phenomenon. In the present study, it was possible to reduce the number of variables using PC analysis to see more easily the relation between response variables and it was also possible to group samples with similar heat damage and that way made some hypothesis about the quality of the ingredients used and the manufacture process. There are a variety of formulas with different degrees of thermal damage and biological value in the market. Available lysine values were low however all IFs except one covered more than 90% of needs for a baby aged 3 months. Multivariate statistical analysis proved to be a useful tool in the interpretation of the relation of the MR indicators and their connection with the quality of ingredients used and the manufacture process of IFs. PCA explained the 77.3 % of the total variance with two PC, reducing the number of variables from four to two, which facilitated the CA of samples. CA created 5 clusters and efficiently separated samples according to their heat damage. Finally, HMF content does not represent a risk to health of babies.

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Statement of Competing Interests

The authors have no competing interests.

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