Furosine and HMF determination in prebiotic-supplemented infant formula from Spanish market

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ABSTRACT

Evaluation of Maillard reaction (MR) in commercial prebiotic-supplemented and not supplemented infant formulas (IFs) was carried out through determination of furosine (2-furoylmethyl- ϵ -lysine) and hydroxymethyl furfural (HMF). Furosine was present in all studied IFs ranging from 94 to 1226 and 315 to 965 mg 100 g⁻¹ of protein, in samples without prebiotics and prebiotic-supplemented IFs respectively. HMF was found in all IFs in the range 62-510 μ g 100 g⁻¹ product. No statistical differences in HMF and furosine contents between prebiotic-supplemented and not supplemented IFs were observed. Storage of six representative IFs, with and without prebiotics, at room temperature during 15 months, did not produce changes in HMF content after 8 months of storage, while furosine content increased significantly throughout the storage time. The high amounts of furosine, found in some IFs may be attributed to excessive heat treatment during processing, inadequate storage of IFs or ingredients used in their manufacturing process. The use of furosine and HMF as thermal indicators allows to evaluate the quality of prebiotic-supplemented IFs which was similar to IFs without prebiotics. Finally, two supervised classification methods (Support Vector Machines and Random Forests) were applied to classify IFs according to protein and carbohydrate source and IF type.

Keywords: furosine; HMF; prebiotics; infant formula; storage; food analysis; Support Vector Machines; Random Forests

Chemical compounds studied in this article:

Furosine (PubChem CID: 92043346)

5-Hydroxymethylfurfural (PubChem CID: 237332)

1. Introduction

The food industry has made several attempts to develop an infant formula (IFs), that fulfil the nutritional needs of the new born or young infants. This is particularly important in instances where mothers cannot produce enough milk. In the last years IFs have been supplemented with prebiotic oligosaccharides as growth-promoting factors for bifidobacterias which are widely recognised as beneficial for the new-born health (Ben et al., 2008; Knol et al., 2005; Fanaro et al., 2005). IFs composition includes as major components carbohydrates (54-61 g 100 g⁻¹ of product) and proteins (11-15 g 100 g⁻¹ of product). Sugars such as corn syrups, lactose, sucrose or starch can be used to elaborate IFs. They can also be supplemented with prebiotics such as lactulose, galactooligosaccharides (GOS), fructooligosaccharides (FOS), mixtures GOS/FOS, polydextrose; isomaltooligosaccharides or xilooligosaccharides (Ackerman et al., 2017). Protein source can be varied although the most used is whey protein (Morales et al., 2004).

The manufacture of IFs includes different stages such as blending of ingredients, homogenisation, pasteurisation, spray-drying and storage that have a great influence in their final quality (Morales et al., 2004). Some of these processes, involving heat, reactions and /or interactions between constituents can give rise to a loss of nutritive value being this very important because IFs sometimes are the only source of infant nutrition during the first months of life. Maillard reaction (MR) is one of the main reactions causing deterioration of proteins during processing or storage of foods. Since IFs contain high concentrations of carbohydrates and proteins, MR plays an important role during elaboration from the point of view of losses of nutritive value.

MR is a very complex reaction that covers transformations that produces a large number of the so-called MR products (Kim, 2010). This reaction takes place during

processing of foods, between the carbonyl group of a reducing sugar and the free amino group of proteins, peptides or amino acids, therefore it plays an important role in IFs due to their carbohydrate and protein composition affecting adversely the protein quality by loss of the bioavailability of essential amino acids (Nasirpour et al., 2006). Loss of available lysine is the most negative nutritional consequence of the MR in IFs (Michalak et al., 2006). Storage of powdered IFs can also promote MR in different extension depending on environmental conditions (temperature and humidity) and product composition (Michalak et al., 2016).

Different compounds have been selected as indicators of different stages of MR being furosine (2-furoylmethyl- ϵ -lysine, 2-FM-Lys) one of the most used as an indicator of early stages of MR in processed foods. Furosine determination is an indirect measure of Amadori compounds which are formed in the first stages of MR and its determination provides information about early quality changes caused in treated or stored foods (Boitz and Mayer, 2016; Corzo-Martínez et al., 2012; Fratianni et al., 2017; Gökmen et al., 2008; Rios-Rios et al., 2018).

Another indicator used to evaluate intermediate and advanced stages of MR in processed foods is hydroxymethylfurfural (HMF) (Albalá et al., 1997; Michalak et al., 2006). In acid media, HMF is also formed by degradation of hexoses and was one of the first compounds to be used as an indicator to evaluate the severity of heat treatment or length of storage in several foods as, milks (Guerra-Hernández et al., 2002b), orange juice, multifloral honey, breakfast cereals biscuits and jam (Teixidó et al., 2006). Depending on the procedure chosen to measure HMF it can be determined either free HMF or free HMF plus potential HMF derived from other browning intermediates (total HMF) (Morales et al., 1996).

A lot of data about the use of above mentioned indicators to assess the quality of liquid and powder IFs are available (Chávez-Servín et al., 2015; Contreras-Calderón et al., 2009; Ferrer et al., 2005; Guerra-Hernández et al., 2002a, 2002b; Michalak et al., 2006; Roux et al., 2016). However, in IFs supplemented with prebiotics, the studies are mainly focused on the influence of these carbohydrates on growth up and promotion of gut health of infants (Ackerman et al., 2017) or to know carbohydrate composition (Sabater et al., 2016). On the other hand, few studies have been done about the relationship among IFs and indicators (Contreras-Calderon et al. 2017) and the usefulness of quality parameters to classify samples.

Therefore, the aim of this study was to evaluate quality of IFs supplemented with prebiotics, compared to others without prebiotics, through thermal indices, furosine and free HMF determination, as well as the influence of storage of IFs (8 and 15 months) on MR. In addition, in order to study the relationships between the IFs studied grouped in different categories (starting and follow-up IFs, IFs containing proteins from different origin and different main carbohydrates) and quality parameters here determined, two supervised classification methods based on statistical learning theory have been applied: Support Vector Machines (SVM) and Random Forests (RF). They are promising methods applied on spectroscopic data of milk powder for brand identification and component analysis (Wu et al., 2008), and MS data for characterization and classification of cheese samples (Fabris et al., 2010).

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade. Hydrochloric acid, acetic acid and potassium chloride were obtained from Panreac (Barcelona, Spain). Sep-Pack cartridges

(C₁₈) were purchased from Waters Millipore (Milford, MA, USA). Ultrapure water quality (18.2 MΩ cm) with 1–5 ppb total organic carbon (TOC) and <0.001 EU mL⁻¹ pyrogen levels was produced in-house using a laboratory water purification Milli-Q Synthesis A10 system from Millipore (Billerica, MA, USA).

2.2. Samples

Different types of commercial powdered IFs (n=24) starting and follow-up formulas were purchased from local markets. Sixteen IFs containing prebiotics i.e. fructooligosaccharides (PFOS, n=3), galactooligosaccharides (PGOS, n=7), and mixtures of galactooligosaccharides and fructooligosaccharides (PGOS/FOS, n=6), and lactose (except PFOS3 sample) were studied. The other eight IFs were without prebiotics, four of them contained lactose and maltodextrins or starch (L) while the other four were lactose-free (LF) but contained maltodextrins (Sabater et al., 2016). Before analysis, IFs were stored at 4 °C and analysed before the sell-by date. Also, six representatives IFs were stored at room temperature for 15 months at room temperature 25 °C (20-33 °C) and humidity between 17 and 43%.

Also, for statistical analysis IFs were categorised according to three criteria i) proteins source: whey (n=14), milk (n=4), hydrolysed whey (n=4), and soy (n=2); ii) considering carbohydrates data of these IFs previously published (Sabater et al., 2016) we have considered samples with lactose as main carbohydrate (n=5), samples with lactose and maltodextrins (Mdx) (n=14) and with Mdx (n=5); and iii) by age: starting (n=11) and follow-up (n=13) IFs.

2.3. Infant formula characterisation

All IFs were reconstituted in water at 10% (w/v) and the pH values were measured using a Mettler Toledo Five Easy Plus pH Meter. The dry matter (DM) content was determined gravimetrically by drying the samples until constant weight according to the AOAC method 950.01 (AOAC, 1990a). Water activity (a_w) measurement was carried out in an AW Sprint TH-500 instrument (Novasina, Pfäffikon, Switzerland). Saturated aqueous solutions of LiCl, MgCl₂, Mg(NO₃)₂, NaCl, BaCl₂ and K₂Cr₂O₇ were used to calibrate the sensor unit. The Kjeldahl method was used to determine total nitrogen (TN) using 6.25 as conversion factor (TN x 6.25) following the AOAC method 920.165 (AOAC, 1990b). All determinations were carried out in duplicate and data were expressed as mean $\pm$ standard deviation (SD).

2.4. Determination of furosine

Furosine was determined by ion-pair RP-HPLC according the method of Resmini et al. (1990). Before analysis, samples (0.250 mg) were hydrolysed with 4 mL of 8 N HCl at 110 °C for 24 h under inert conditions. The hydrolysate was filtered through Whatman No. 40 filter paper and 0.5 mL of filtrate was applied to a Sep-Pak C₁₈ cartridge (Waters) previously activated with methanol and water. Furosine was eluted with 3 mL of 3 N HCl and 50 μ L were injected into the chromatograph. RP-HPLC analysis was carried out in a C₈ column (250 mm x 4.6 mm, 5 μ m particle size) (Alltech furosine-dedicated, Nicholasville, KY) thermostated at 34 °C, using a linear binary gradient at a flow rate of 1.2 mL/min. Mobile phase consisted of solvent A, 0.4% acetic acid, and solvent B, 0.34% KCl in phase A. The elution program was as follows: 100% A from 0 to 8 min, 100% B from 8 to 21 min, and 100% A from 21 to 25 min. Detection was performed using a variable wavelength UV detector set at 280 nm

(Agilent Technologies 1260 Infinity LC System – 1260 RID, Böblingen, Germany). Acquisition and processing of data were achieved with Agilent ChemStation Rev. C.01.05 software.

Calibration was performed by the external standard method using commercial furosine (Neosystem Laboratories, Strasbourg, France) as standard. All samples were analysed in duplicate, and data shown are the average values expressed as mg of furosine per 100 g of protein $\pm$ standard deviation (SD). The linearity of the method was evaluated with furosine standard in a concentration range of 0.023 to 6.990 mg L⁻¹ and the obtained calibration curve was $y=2.9378x+0.0286$ with a R² of 0.9995. Furthermore, limit of detection (0.018 mg L⁻¹) and quantification (0.060 mg L⁻¹) were established after the injection and analysis of the most diluted standard of furosine based on a signal/noise ratio of 3:1 and 10:1, respectively (Teixidó, Santos, Puignou & Galceran, 2006).

2.5. Determination of hydroxymethyl furfural (HMF)

The analysis of HMF was carried out by HPLC using a ACE-5 C₁₈ column (ACE, UK) (250 mm x 4.6 mm, 5 μ m) thermostated at 25 °C and a linear gradient from methanol:water (5:95) to methanol:water (80:20) in 6 min, isocratic elution was then continued for 6 min and, finally, initial conditions were reestablished in 1 min and held for 10 min. The flow rate was 1 mL min⁻¹ and injection volume 50 μ L. The UV detector was set at 283 nm (Rada-Mendoza et al., 2004).

Before chromatographic analysis, fat and protein interferences were removed from IFs by precipitation using Carrez reagents. IFs (400 mg) were gently mixed with 3 mL water and equal volumes (0.4 mL) of Carrez I (7.2% w/v K₄Fe(CN)₆·3H₂O in water) and Carrez II (14% w/v ZnSO₄·7H₂O in water) in a 10 mL volumetric flask, added 2 mL

of methanol and diluted to volume with water. Then, supernatant was collected, centrifuged at 10,000g for 5 min at room temperature and passed through a filter of 0.45 μm (Waters) and then injected. Quantification was carried out employing the external standard method using a commercial standard of HMF (Sigma, St. Louis, MO, USA). Data were the mean values of duplicates expressed as $\text{mg } 100 \text{ g}^{-1}$ of product. The linearity of the method was evaluated with HMF standard in a concentration range of 0.4 to 400.0 $\mu\text{g L}^{-1}$ (equivalent to 1 at 1000 $\mu\text{g } 100 \text{ g}^{-1}$ of product) and the obtained calibration curve was $y = 3.51x + 1.7836$, $R^2 = 0.9998$. Furthermore, limit of detection (0.3 $\mu\text{g L}^{-1}$, 0.75 $\mu\text{g } 100 \text{ g}^{-1}$ of product) and quantification (0.7 $\mu\text{g L}^{-1}$, 1.75 $\mu\text{g } 100 \text{ g}^{-1}$ of product).

2.7. Statistical analysis

ANOVA tests and Tukey's test for $p < 0.05$ were applied to data of furosine and HMF contents in order to detect differences between the storage period (0, 8 and 15 months) and between the groups of IFs studied: IFs with and without prebiotics, starting and follow-up IFs, IFs containing proteins from different origin and different main carbohydrates. Box and Whisker Plot was used to summarise the data for furosine and HMF contents. In addition, Pearson correlation coefficient was calculated for all variables.

Multivariate data analysis was employed to investigate relationships among the 24 IFs with respect to quality parameters: generals (pH, a_w , protein content), Maillard reaction (MR) indicators (furosine and HMF) and carbohydrate contents, prebiotic and reducing sugars (considering the sum of monosaccharides, lactose, maltose and prebiotics) determined previously in our laboratory (Sabater et al., 2016). Two

supervised classification methods were used, Support Vector Machines (SVM) and Random Forests (RF). All variables were scaled and centred before the analysis. SVM classification algorithms try to find patterns in empirical with regard to label classes (protein and carbohydrate source and IF type in this work). For SVM classification a radial basis function kernel was chosen.

In RF, a multitude of decision trees are constructed, outputting the different classes. Each node is split using the best among a subset of predictors randomly chosen. This method classifies very effectively compared to many others, including discriminant analysis, SVM and neural networks, and is robust against overfitting (Breiman, 2001).

These classification methods were validated by 10-fold cross-validation and tested with new samples. All statistical analyses were performed on R (R Core Team). SVM classification was performed with e1071 SVM package (Meyer et al., 2017). RF classification was performed with random Forest package (Liaw and Wiener, 2002).

3. Results and discussion

Data on physic-chemical characteristics of analysed infant formulas (IFs) are shown in Table 1; as it can be observed, DM content and a_w were within the range of 94.5 to 99.2 and 0.07 to 0.28, respectively. In general, the composition of IFs was as expected for this type of products. Values of pH and protein content were in the range 6.67 - 7.23 and 8.31 to 16.0% (w/w), respectively. These values were fairly close to those found by Morales et al., (2004) and Chavez-Servín et (2015) in powdered infant formula.

3.1. Evaluation of Maillard reaction in commercial IFs

The content of furosine in commercial IFs is shown in Fig. 1. Furosine was detected in all studied IFs in variable amounts ranging from 94 to 1226 mg 100 g⁻¹ protein, in samples without prebiotics and from 315 to 965 mg of furosine 100 g⁻¹ of protein in prebiotic-supplemented IFs. No statistically significant differences between the presence and absence of prebiotics were found. Morales et al., (2004) also obtained high values of furosine (271-1050 mg 100 g⁻¹ protein) in Spanish commercial powdered IFs without prebiotics. Lactose containing IFs (L) showed the low levels except L3, which presented 1226 mg of furosine 100 g⁻¹ of protein; this fact could be attributed to the presence of whey protein hydrolysate which have high levels of free amino acids to participate in the Maillard reaction (MR). Contreras-Calderón et al. (2008) found high values of furosine (644 – 1435 mg 100 g⁻¹ of protein) in whey proteins with different lactose contents used as ingredients in IFs, which presented high available lysine content. Taken into account prebiotic-enriched IFs, those containing galactooligosaccharides (PGOS) showed the highest amount of furosine i.e. 965 mg 100 g⁻¹ of protein, while among fructooligosaccharide (FOS) enriched formulas, PFOS3 had the highest content (897 mg 100 g⁻¹ of protein).

As is well known, the final quality of IFs depends on formulation and processing design being both influential factors in the evolution of MR (Contreras-Calderón et al., 2009). Therefore, sample PGOS5, with similar formulation to that of PGOS2, presented higher furosine level, which could be attributed to a higher temperature conditions during processing or storage.

At last, the significant differences in furosine content within the three groups of IFs (i.e considering source of protein, main carbohydrates and starting/follow up types)

were studied using two-way ANOVA. Significant differences depending on source of protein were observed, especially between IFs with soy protein and IFs with whey hydrolysed protein, confirmed by a pairwise test (Fig. 1b); however, no other differences were found (Fig. 1c; 1d).

In general, the furosine values determined in IFs were in the range found by Contreras-Calderon et al. (2009), Contreras-Calderon et al. (2017), Fenaille et al. (2006) and Ferrer et al. (2003), Martysiak-Zurowska and Stolyhwo (2007) in conventional, hypoallergenic and soybean-based IFs. However, other studies reported lower furosine contents in this type of products (Michalak et al., 2006). Therefore it is necessary to control the heat treatment applied to IFs because high furosine values can indicate advanced degradation of proteins resulting in a considerable decrease of lysine in IFs and therefore decreasing of nutritive value (Contreras-Calderon et al., 2017).

The content of HMF in all analysed IFs is shown in Fig. 3. Free HMF values ranged from 62 to 510 $\mu\text{g } 100 \text{ g}^{-1}$ of product, and only statistically significant differences between PGOS/FOS IFs and conventional (L) and PGOS IFs were found. On the other hand, there were no significant differences between IFs containing: proteins from different origin (Fig. 2b), different main carbohydrate (Fig. 2c), nor starting and follow-up IFs (Fig. 2d).

These results agree with those obtained by Chavez-Servin et al. (2006) in milk-based IFs and Contreras-Calderon et al., (2017) in adapted, follow-up, hypoallergenic and soybean-based IFs. Nevertheless, free HMF contents were lower than those reported in several studies for powdered IFs (Albalá-Hurtado et al., 1997; Guerra-Hernández et al., 2002a; Michalak et al., 2006; Kocadagli et al., 2014). Differences in the HMF levels could be attributable to IFs composition, thermal treatments during manufacturing and storage.

3.2. Progress of Maillard reaction in IFs during storage

The influence of IFs storage (8 and 15 months), at room temperature, on MR progress has also been studied in some representative samples; the results are depicted in Fig. 4. Furosine content increased significantly ($p < 0.05$) in lactose-containing IF L4 and lactose-free IF LF4 (with soy protein) at the end of the storage period, these samples have a low initial level of furosine. In contrast, it can be observed that furosine levels found in two samples (LF1, PFOS3) were lower in the 15th month of storage. These results suggest that furosine may suffer degradation, since other compounds from more advanced MR stages are formed (Ferrer et al., 2003). Ferrer et al. (2003) Gliguém et al. (2005) and Guerra-Hernandez et al. (2002a) studied the evolution of MR products throughout the shelf-life storage period and observed an increase of furosine content which was statistically significant. On the other hand, no changes during storage were found for the HMF, with the exception of FOS-enriched formula PFOS3 where HMF content increased, according with the furosine decrease after 15 months. Chavez-Servin et al. (2006) found no increase in free HMF in milk-based IFs after 12 months of storage. However, these results differ from those reported by Chavez-Servin et al. (2005), who studied the evolution over shelf life of HMF levels in milk-based formulae from production until 15 months. They found a significant increase of free HMF after 15th months, and in another study (Chavez-Servin et al., 2015) determined slight increases of furfural contents in powdered IFs after 70 days of storage at room temperature. Ferrer et al. (2005) also reported an increase in the HMF content of milk-based IFs at the end of the storage during 24 months at 20 °C.

3.4. Multivariate data analysis

In order to obtain more information on MR development in IFs here studied, the relationships between furosine and HMF and the different parameters determined in this paper (pH, a_w and protein content) as well as those published by Sabater et al. (2016) (reducing sugars and prebiotic contents), calculating the Pearson correlation coefficient, have been studied. The results have shown a strong correlation between HMF and prebiotic contents (correlation coefficient 0.49) (Fig. 4 b) increasing HMF with prebiotic content. However, a great dispersion of furosine values were observed (Fig. 4 a) and not significant correlation with prebiotic content (correlation coefficient 0.21) was obtained.

The second step was to apply two classification methods to study the relationship between three previously established categories of IFs according to i) protein source, ii) carbohydrate source and iii) starting/follow up type, and the set of quality parameters studied, generals (pH, a_w , protein), related with their carbohydrate composition (reducing sugars and prebiotic content) (Sabater et al. 2016), and with MR indicators furosine and HMF. The supervised models, trained with 70% IFs (including stored samples during 8 months) and tested with 30% IFs, were applied. As can be seen using a SVM classification method high classification rate was obtained (Fig. 5): a and b) protein source training, validation and test rates, 95.0, 92.5 and 90.0% (cost value 2.0, gamma value 0.5), c and d) carbohydrate source training, validation and test rates, 100, 90.0 and 90.0% (cost value 1.0, gamma value 0.5) and e and f) starting/follow up IF training, validation and test rates, 100, 92.5 and 90.0% (cost value 2.0, gamma value 0.5). Cost parameter indicates how much the misclassification of samples should be avoided during the SVM application. A large cost gives high variance and low bias

because the cost of misclassification is highly penalized. A small cost gives higher bias and lower variance. Gamma parameter defines how far the influence of a single training example reaches, with low values meaning a high influence. Therefore, a small gamma value gives low bias and high variance while a large gamma value gives higher bias and low variance. Therefore, it can be said that good classifications are obtained. With regard to variable importance, furosine and reducing sugars content showed the highest coefficients (0.41 and 0.27, respectively) when IFs were classified according to their protein source. When IFs were classified according to their carbohydrate source, reducing sugars showed the highest coefficient, 0.60. Finally, when IFs were classified according to their type, furosine and protein content showed the highest coefficients (0.34 and 0.29, respectively). In all three cases, the models were able to accurately predict new samples.

On the other hand, to corroborate the good fit classification reached when SVM was used, RF was also applied to classify IFs, and similar results were obtained. According to their protein source, the training, validation and test rates were 90, 89 and 80%. This RF classification model was built with 150 trees and 1 variable tried at each split. With regard to variable importance in this classification, prebiotic and reducing sugars content showed the highest Mean Decrease Accuracy (10.0 and 9.1, respectively) while reducing sugars and furosine content showed the highest Mean Decrease Gini (3.8 and 3.6, respectively). These two parameters represent the loss of accuracy and the mean decrease in node purity when a variable is removed. pH, protein and reducing sugars content were the most influent variables to classify IFs containing amino acids (coefficients -1.0, -1.7, 1., respectively). pH and reducing sugars content were the most influent variables to classify milk IFs (coefficients 8.2 and 6.3, respectively). Furosine and prebiotic content were the most influent variables to classify soy IFs (coefficients

7.0 and 5.3, respectively). HMF and prebiotic content were the most influent variables to classify IFs containing whey (coefficients 7.5 and 7.5, respectively). Protein content was the most influent variable to classify IFs containing whey hydrolysate (coefficient 5.3).

When RF were applied to classify IFs according to their carbohydrate source, the training, validation and test rates were 95, 92 and 90%. This RF classification model was built with 100 trees and 1 variable tried at each split. With regard to variable importance in this classification, reducing sugars content showed the highest Mean Decrease Accuracy and Mean Decrease Gini (8.9 and 6.5, respectively). Protein and a_w were the most influent variables to classify lactose IFs (coefficients 5.5 and 5.2, respectively). Reducing sugars and HMF content were the most influent variables to classify IFs containing lactose and maltodextrins (coefficients 6.1 and 4.0, respectively). Reducing sugars and HMF content were the most influent variables to classify IFs containing maltodextrins (coefficients 9.3 and 5.1, respectively).

At last, when RF were applied to classify IFs according to starting/follow up, the training, validation and test rates were 97.5, 92 and 85%. This RF classification model was built with 100 trees and 3 variables tried at each split. With regard to variable importance in this classification, furosine and reducing sugars content showed the highest Mean Decrease Accuracy (9.4 and 8.1, respectively) and highest Mean Decrease Gini (5.7 and 5.3, respectively). Reducing sugars and furosine content were the most influent variables to classify IFs by infant ages being the coefficients 8.2 and 7.8, for starting and 6.6 and 7.3 for follow up IFs.

RF reinforce results from SVM. In general, SVM classification performance was slightly better than RF classification. Although, in both cases, these results indicate that

it is possible to classify the IFs according to the protein or carbohydrate source and starting/follow up type considering the set of quality parameters determined in this study, which could be applied on IFs from unknown origin for its classification considering the high prediction rates of new samples.

4. Conclusion

The results here obtained show thermal damage of proteins of different types of IFs through measurement of furosine (2-FM-Lys) and HMF. Although great variability in furosine and HMF contents was found in the analysed commercial IFs, no significant differences were found in furosine content taking into account factors as presence of prebiotics, main carbohydrates presents or starting or follow-up IFs. With respect to HMF content, scarce differences between IFs with and without prebiotics were observed. In addition, storage of IFs at room temperature did not produce important changes in the furosine and HMF contents. Therefore, the high levels of furosine detected in some IFs may be attributed to excessive heat treatment during processing, elevated temperature during storage or presence of high proportion of furosine in some of the ingredients used in IFs elaboration. The use of furosine and HMF as indicators for thermal damage, allows establishing quality of prebiotic-supplemented IFs which is similar to IFs without added prebiotics. Finally, the utility of the set of quality parameters here determined to classify the IFs using the appropriate statistical tools (SVM and RF) has been established. IFs were classified according to the protein or carbohydrate source and starting/follow up type using these supervised models which could be applied on samples from unknown origin.

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Fig. 1. Furosine content found in infant formulas (IFs): **(a)** with lactose (L, n=4), lactose-free (LF, n=4) and prebiotic-supplemented (with fructooligosaccharides, PFOS n=3; with galactooligosaccharides, PGOS n= 7 and PGOS/FOS n=6); **(b)** according to their protein source: amino acids (n=2), milk (n=4), soy (n=2), whey (n=14) and whey hydrolysate (n=2); **(c)** according to their carbohydrate source: lactose (n=5), maltodextrins (Mdx, n=5), lactose + Mdx (n=14); and **(d)** by age, follow-up (n=13) and starting (n=11). (Fig. 1b, ^{a,b} Statistically significant differences ($p < 0.05$) between IFs).

Fig. 2. Hydroxymethyl furfural (HMF) amounts found in infant formulas (IFs) **(a)** with lactose (L, n=4), lactose-free (LF, n=4)) and prebiotic-supplemented (with fructooligosaccharides, PFOS n=3; with galactooligosaccharides, PGOS n= 7 and PGOS/FOS n=6). **(b)** HMF levels found in IFs according to their protein source: amino acids (n=2), milk (n=4), soy (n=2), whey (n=14) and whey hydrolysate (n=2). **(c)** HMF levels found in IFs according to their carbohydrate source: lactose (n=5), maltodextrins (Mdx, n=5) and lactose + Mdx (n=14) and. **(d)** HMF levels found in IFs according infant age: follow-up (n=13) and starting (n=11). (Fig. 2a, ^{a,b} Statistically significant differences ($p < 0.05$) between IFs).

Fig. 3. Evolution of furosine content (mg 100 g⁻¹ protein) during storage (8 and 15 months) at room temperature of six IFs: with lactose (L), lactose-free (LF) and prebiotic-supplemented IFs (PFOS and PGOS)..^{a,b,c} Statistically significant differences of furosine content during storage ($p < 0.05$).

Fig. 4. Relationships between furosine and HMF and prebiotic content in studied IFs.

Fig. 5. Relationships between furosine and HMF and prebiotic content in studied IFs according to their protein and carbohydrate source and starting/follow-up type.

Table 1. Dry matter (DM), water activity (a_w), pH and protein determined in the infant formula under study (n = 2, data shown as mean $\pm$ SD).

Code	Dry matter (%)	a_w	pH	% Protein (N x 6.25)	Protein source*	Carbohydrates**
Infant formula without prebiotics						
<i>Conventional</i>						
L1 (S)	95.4 $\pm$ 0.5	0.15	6.74	9.6 $\pm$ 0.2	Whey protein	Lactose
L2 (S)	99.2 $\pm$ 0.0	0.11	6.93	10.1 $\pm$ 0.0	Whey protein	Lactose, mdx ¹
L3 (F)	96.1 $\pm$ 0.0	0.12	6.83	12.1 $\pm$ 0.0	Whey protein hydrolysate	Lactose, starch
L4 (F)	98.3 $\pm$ 0.0	0.14	6.70	16.0 $\pm$ 0.1	Milk protein	Lactose, mdx
<i>Without lactose</i>						
LF 1(S)	96.8 $\pm$ 0.0	0.13	6.67	10.4 $\pm$ 0.2	Milk protein	Mdx
LF 2(F)	97.9 $\pm$ 0.2	0.07	6.74	11.1 $\pm$ 0.1	Milk protein	Mdx
LF3 (S)	98.9 $\pm$ 0.0	0.13	7.12	12.1 $\pm$ 0.5	Soy protein	Mdx
LF4 (S)	97.3 $\pm$ 0.1	0.11	6.83	12.1 $\pm$ 0.3	Soy protein	Mdx
Infant formula with prebiotics						
<i>With fructooligosaccharides</i>						
PFOS1 (S)	96.8 $\pm$ 0.0	0.21	6.89	9.8 $\pm$ 0.0	Whey protein	Lactose, FOS ² /inulin
PFOS2 (F)	96.6 $\pm$ 0.2	0.17	6.80	12.8 $\pm$ 0.0	Whey protein	Lactose, FOS/inulin
PFOS3 (S)	97.7 $\pm$ 0.4	0.11	6.96	11.6 $\pm$ 0.4	Whey protein	Without lactose, FOS
<i>With galactooligosaccharides</i>						
PGOS1 (S)	98.0 $\pm$ 0.1	0.10	7.04	10.6 $\pm$ 0.0	Whey protein	Lactose, GOS ³
PGOS2 (F)	97.8 $\pm$ 0.2	0.16	7.22	10.4 $\pm$ 0.1	Whey protein	Lactose, GOS, mdx
PGOS3 (F)	96.5 $\pm$ 0.2	0.13	7.00	10.3 $\pm$ 0.5	Whey protein	Lactose, GOS, mdx
PGOS4 (S)	98.5 $\pm$ 0.4	0.12	6.92	10.5 $\pm$ 0.2	Whey protein	Lactose, GOS, mdx
PGOS5 (F)	94.5 $\pm$ 0.6	0.13	6.83	12.1 $\pm$ 0.2	Whey protein	Lactose, GOS, mdx
PGOS6 (S)	96.7 $\pm$ 0.4	0.15	7.13	10.7 $\pm$ 0.1	Milk protein	Lactose, GOS, mdx
PGOS7 (S)	96.6 $\pm$ 0.4	0.13	7.16	10.5 $\pm$ 0.0	Whey protein, amino acids	Lactose, GOS, mdx
<i>With GOS and FOS (All samples were follow-up)</i>						
PGOS/FOS1	96.5 $\pm$ 0.2	0.20	6.91	9.2 $\pm$ 0.0	Whey protein hydrolysate	Lactose, GOS/FOS
PGOS/FOS2	98.5 $\pm$ 0.1	0.14	7.12	9.6 $\pm$ 0.1	Whey protein	Lactose, GOS/FOS, mdx
PGOS/FOS3	95.0 $\pm$ 0.5	0.28	6.80	8.3 $\pm$ 0.3	Whey protein	Lactose, GOS/FOS, mdx
PGOS/FOS4	95.6 $\pm$ 0.8	0.24	6.68	9.3 $\pm$ 0.2	Whey protein	Lactose, GOS/FOS, mdx
PGOS/FOS5	98.4 $\pm$ 0.2	0.11	6.99	11.2 $\pm$ 0.3	Amino acids	Lactose, GOS/FOS, mdx
PGOS/FOS6	97.5 $\pm$ 0.8	0.14	7.23	12.3 $\pm$ 0.2	Whey protein	Lactose, GOS/FOS, mdx

* Data on the label; ** Sabater et al.,(2016) . ¹mdx, maltodextrins; ²FOS, fructooligosaccharides ³GOS, galactooligosaccharides. S: Starting IFs. F: Follow-up IF