LAUANE NUNES

EVALUATION OF THE COMPOSITION AND DEVELOPMENT OF MAILLARD REACTION IN INFANT FORMULAS

Dissertation presented to the Federal University of Viçosa, as part of the requirements for the Graduate Program in Food Science and Technology, to obtain the title of *Magister Scientiae*.

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LIST OF ABBREVIATIONS

WHO - World Health Organization

Codex - *Codex Alimentarius Committee*

GOS - galactooligosaccharides

FOS - fructooligosaccharides

ARA - arachidonic acid

DHA - docosahexaenoic acid

HMF - 5-Hydroxymethylfurfural

F - 2-Furaldehyde

FMC - 2-furyl-methyl ketone

MF - 5-methyl-2-furaldehyde

a_w - water activity

MPC - milk protein concentrate

WMP - whole milk powder

SMP - skim milk powder

MRPs - Maillard reaction products

SMF - 5-sulfoxymethylfurfural

HAAs - heterocyclic aromatic amines

FDA - Food and Drug Administration

AGEs - advanced Glycation End Products

CML - carboxymethyllysine

DPPH - 1,1-diphenyl-2-picryl-hydrazyl

L* - luminosity

a* - intensity of red and green

b* - intensity of yellow and blue

ΔE - color difference

OPA - o-phthaldialdehyde

ABSTRACT

NUNES, Lauane, M.Sc., Universidade Federal de Viçosa, February, 2018. **Evaluation of the composition and development of Maillard reaction in infant formulas**. Adviser: Antônio Fernandes de Carvalho. Co-advisers: Ítalo Tuler Perrone and Pierre Schuck.

Infant formula has the function of replacing or complementing breast milk and its formulation is based on modified cow milk. Due to a large number of heat treatments, the formulas are subject to a series of reactions that cause a reduction in its quality, among them the Maillard reaction. In addition, it is important that these products provide sufficient quantities of all nutrients, therefore, a constant evaluation of the nutritional composition of products available in the market is necessary. Therefore, the objective of this work was evaluate the propagation of Maillard reaction in infant formulas and to investigate the centesimal composition of products under conditions of use over 60 days at 4 and 24 ºC. It was observed a decrease in lactose, lysine content and pH; increase in moisture, HMF content, water activity and color difference. Possibly, lactose is associated with some proteins and amino acids, such as lysine, in the initial phases of Maillard reaction, and with the prolongation of the storage, there is production of intermediates such as HMF, formic acid, water, among others, in such a way that the intermediate compounds polymerize forming the pigments known as melanoidins. However by decreasing the storage temperature, it was observed a decrease in the Maillard reaction rates, and consequently, lower nutritional losses. Regarding the centesimal composition, almost all the infant formulas analyzed were within the limits established. In addition, they does not pose health risks to the infant in relation to the level of HMF. However, studies are still needed to clarify the effect of Maillard reaction on this type of product, what the real harm to the infant and review the micronutrient values.

RESUMO

NUNES, Lauane, M.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Avaliação da composição e desenvolvimento da reação de Maillard em fórmulas infantis**. Orientador: Antônio Fernandes de Carvalho. Coorientadores: Ítalo Tuler Perrone e Pierre Schuck.

A fórmula infantil tem a função de substituir ou complementar o leite materno e sua formulação é baseada no leite de vaca modificado. Devido a uma grande quantidade de tratamentos térmicos, as fórmulas estão sujeitas a uma série de reações que causam redução de qualidade, entre elas a reação de Maillard. Além disso, é importante que estes produtos proporcionem quantidades suficientes de todos os nutrientes, por isso, é necessário uma avaliação constante da composição nutricional dos produtos disponíveis no mercado. Portanto, o objetivo deste trabalho foi avaliar a propagação da reação de Maillard em fórmulas infantis e investigar a composição centesimal de produtos em condições de uso ao longo de 60 dias a 4 e 24 ºC. Observou-se uma diminuição no teor de lactose, lisina e pH; aumento da umidade, teor de HMF, atividade da água e diferença de cor. Possivelmente, a lactose se associa a algumas proteínas e aminoácidos, como a lisina, nas fases iniciais da reação de Maillard e com o prolongamento do armazenamento, há produção de intermediários como HMF, ácido fórmico, água, entre outros, de tal maneira que os compostos intermediários polimerizam formando os pigmentos conhecidos como melanoidinas. No entanto, ao diminuir a temperatura de armazenamento, observou-se uma diminuição nas taxas de reação de Maillard e, consequentemente, menores perdas nutricionais. Quanto à composição centesimal, quase todas as fórmulas infantis analisadas estavam dentro dos limites estabelecidos. Além disso, elas não representam riscos para a saúde do lactente em relação ao nível de HMF. Porém, ainda são necessários estudos para esclarecer o efeito da reação de Maillard sobre esse tipo de produto, quais os danos reais para as crianças e avaliar os valores de micronutrientes.

CONTENTS

Scientific Context

The World Health Organization (WHO) recommends that breastfeeding be exclusive until the first 6 months of the child and complement to 2 years or more. However, in some situations, the mother cannot or does not wish to breastfeed, which makes it necessary to supplement or replace breast milk with infant formulas.

In Brazil, about 60 % of infants up to 6 months receive other foods than breast milk, such as water, teas, juices and mainly cow milk (Ministry of Health, 2009). However, infant feeding with bovine milk can be harmful to the development of the baby because it presents lipid, protein, lactose and mineral contents in imbalance with the physiological needs of the infant (Blanchard, Zhu, & Schuck, 2013).

According to the *Codex Alimentarius Committee* (Codex), infant formula is defined as a breast milk substitute that can by itself meet the nutritional needs of infants, from birth to the introduction of complementary feeding.

This product is especially predisposed to the initiation and propagation of the Maillard reaction due to their peculiar composition with high content in proteins and lactose besides the exposition to high temperatures during processing and prolonged storage period (Ferrer, Alegría, Farré, Abellán, & Romero, 2005; Perez-Locas & Yaylayan, 2010).

Considering the situation in which infant formulas are the only foods offered to infants during their first months of life, a constant assessment of the nutritional composition of products available on the market is important to recognize whether the requirements set by legislation are being met. In addition, the evaluation of the development of Maillard reaction under conditions of use is necessary to determine the impact of the products of this reaction on the infant health.

CHAPTER 1 - THE MAILLARD REACTION IN POWERED INFANT FORMULA

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Abstract

Infant formula has the function of replacing or supplementing breast milk and its formulation is based on cow milk and other ingredients as lactose, long-chain polyunsaturated fatty acids, vitamins and minerals. The manufacture of this food pass by dissolution of the dried ingredients in water or skimmed milk, pasteurization, addition of lipids and vitamins, homogenization, concentration in vacuum evaporator and drying in spray dryer. Due to a large number of thermal processes, the formulas are subject to a series of reactions that cause a reduction in its quality, among them the Maillard reaction. This phenomenon is the result of chemical reactions between a carbonyl group of the reducing sugar and a free amino group of the protein or amino acid. With prolongation of heating or storage, a wide variety of reactive compounds is formed, which can polymerize with protein residues, forming dark pigments or melanoidins. This reaction is affected by factors as pH, temperature, water activity, type of reducing sugar or the amine and presence of metals; and causing numerous consequences in infant formulas, such as, unavailability of amino acids and sugars; solubility loss and consequently increases the allergenicity of some proteins, and even impediment of metabolism and mineral

absorption. However, many studies are still needed to understand the consequences of this reaction in infant formulas in order to propose strategies to provide foods with high nutritional properties and safe to consumer.

Keywords: breast milk; cow milk; heat treatments; infants.

1.1. Introduction

The World Health Organization (2009) (WHO) recommends that breastfeeding be exclusive until the first 6 months of the child and supplement to 2 years or more. However, in some circumstances, mothers choose not to breastfeed or not to do it exclusively due to insufficient milk syndrome, breastfeeding failure, social factors (wage-earners mothers); when breastfeeding is medically contraindicated as some babies with metabolic errors or for premature and low-birth-weight infants. Under these and other conditions, it is necessary to substitute or supplement breastfeeding with infant formulas (Contreras-Calderón, Guerra-Hernández, & García-Villanova, 2009; Guo & Ahmad, 2014; Pereyra Gonzáles, Naranjo, Malec, & Vigo, 2003).

According to the *Codex Alimentarius Committee* (Codex), the main regulatory agency of infant formula guidelines at international level, this product is defined as a substitute for breast milk that can fulfill by itself the nutritional requirements of infants, from birth to the introduction of complementary feeding.

In the most of cases, the infant formulas are produced from cow milk with addition of other components such as lactose, long-chain polyunsaturated fatty acids, vitamins and minerals, in order to mimic the breast milk (Zou, Pande, & Akoh, 2016). All ingredients are mixed together and successive heating treatments (pasteurization, concentration, drying) are applied to guarantee their microbiological safety while, at the same time, promote undesirable reactions between the constituents (Blanchard et al., 2013; Happe & Gambelli, 2015; Schuck et al., 2016).

Infant formulas are especially predisposed to the initiation and propagation of the Maillard reaction due to their peculiar composition and exposition to high temperatures and prolonged storage period (Ferrer et al., 2005; Perez-Locas & Yaylayan, 2010). Due to the nutritional importance of this product and some negative effects of Maillard reaction on these, the objective of this review is to evaluate the consequences of the occurrence of the Maillard reaction in infant formulas.

1.2. Infant formulas

1.2.1. Composition of infant formulas

Since infant formulas are the main substitute or supplement of breast milk, the industry must be committed to providing safe and nutritionally adequate products to meet the infant's needs, since formulas are often the only source of nutrients during a significant period of rapid growth and development (Guo & Ahmad, 2014). Thus, it is very important that these products provide sufficient amounts of all nutrients, in appropriate ways, because the inclusion of unnecessary components or in inappropriate amounts may overload the physiological and metabolic functions of the infant (Francescato, Mosca, Agostoni, & Agosti, 2013).

Recommendations for nutrient limits are suggested in FAO & WHO (2007), in addition to other authors such as Blanchard; Zhu; Schuck (2013); Francescato et al. (2013) and Thompkinson; Kharb (2007). They are presented in Figure 1 together with the importance of the main nutrients.

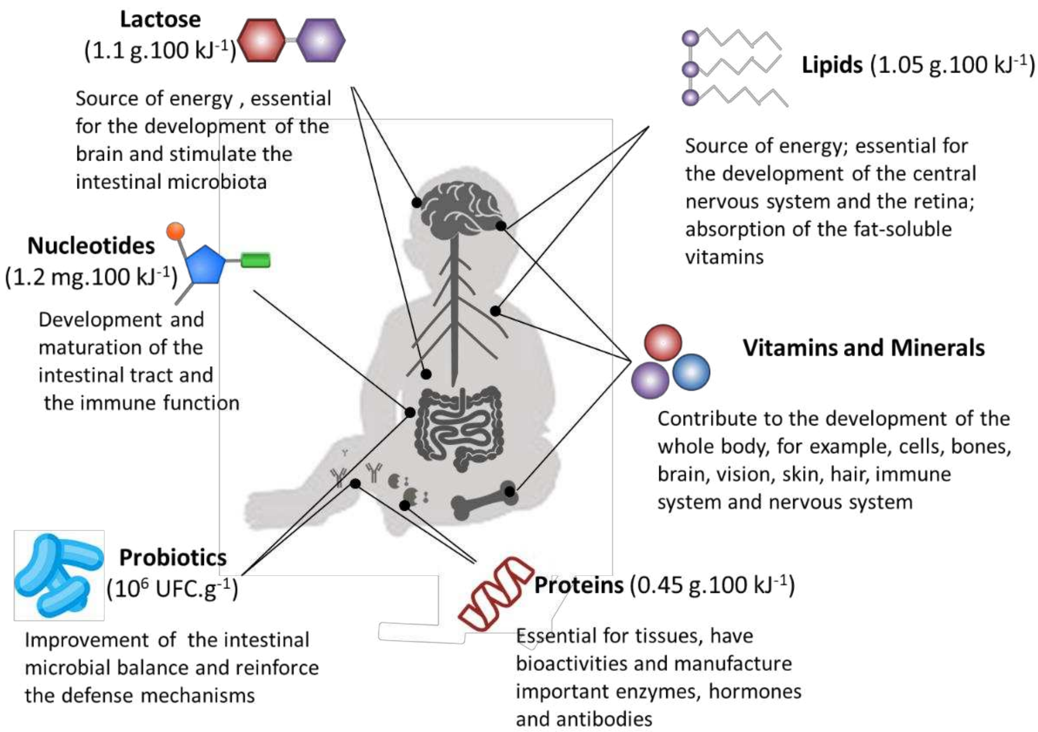

Figure 1 - The importance of the main nutrients and minimum recommendations for infants. Source: Blanchard et al., 2013; Thompkinson & Kharb, 2007; USDA & WIC, 2009b.

The main proteins used in infant formulas are cow milk protein, soy protein and goat milk protein isolates. Formulas based on cow milk should be enriched with some essential amino acids and present reduced protein content to 0.7 g.100 kJ^{-1} with a ratio of whey protein to casein of 60:40 (FAO & WHO, 2007).

Non-protein nitrogen is derived from a number of components such as free amino acids, peptides, creatine and creatinine, nucleic acids and nucleotides, urea, uric acid, ammonia, amino sugars, polyamines, carnitine, and other compounds (Geddes, Hassiotou, Wise, & Hartmann, 2016). A number of these components have important functions for the infant. For example, some studies suggest that nucleotides improve the development and maturation of the intestinal tract, as well as immune function (Alles, Scholtens, & Bindels, 2004; Guo & Ahmad, 2014).

Codex recommends a minimum total carbohydrate content in infant formulas of 2.2 g.100 kJ^{-1}. Carbohydrates in infant formula can theoretically contribute between 28 % and 56 % of energy. Fifty percent of the carbohydrates present in the formulas should be represented by lactose; however, glucose and fructose cannot be added due to effects on osmolarity, Maillard reaction and hereditary fructose intolerance (European Commission, 2003).

Infant formulas are reinforced in lactose in order to simulate the concentrations present in breast milk, so the minimum addition of 1.1 g.100 kJ^{-1} is required, with no maximum limit (European Commission, 2003). Lactose acts as a source of energy and it is essential for the development of the infant brain, and stimulate the development of the intestinal microbiota (Geddes et al., 2016; Thompkinson & Kharb, 2007).

Oligosaccharides like galactooligosaccharides (GOS), fructooligosaccharides (FOS) and inulin, are added for the purpose of mimicking the biological benefits of oligosaccharides present in breast milk (Blanchard et al., 2013).

Lipids provide about 50 % of a child daily caloric intake and their addition is allowed up to 1.4 g.100 kJ^{-1} (FAO & WHO, 2007; Geddes et al., 2016). The great majority of lipids (98 %) are in the form of triglycerides, the rest consisting of diglycerides, monoglycerides, free fatty acids, cholesterol and phospholipids (Andreas, Kampmann, & Mehring Le-Doare, 2015).

Long-chain polyunsaturated fatty acids are added in infant formulas since they are essential for the development of the central nervous system and the retina of the infant (Zou et al., 2016).

The content of trans fatty acids of formulas should be as low as possible. According to Codex, the maximum level of trans fatty acids should be 3 % of total fatty acids (Thompkinson & Kharb, 2007).

Seven elements (K, Na, Ca, Mg, P and Cl) are referred to as minerals and are present in mg.100 kJ^{-1}, while trace elements (Fe, Cu, Zn, Se, Mn and I) are present in µg or less than 100 kJ^{-1} (Montagne, van Dael, Skanderby, & Hugelshofer, 2009). These compounds should also be increased in infant formulas to approach breast milk and maintain optimal osmotic pressure for the infant (FAO & WHO, 2007).

Infant formulas provide the liposoluble and hydrosoluble vitamins, ensuring that the child is able to grow and develop normally without running the risk of developing an inadequate nutritional status (Thompkinson & Kharb, 2007). However, the absorption of liposoluble vitamins from infant formulas is related to the efficacy of lipid absorption of the formula, while the excess intake of hydrosoluble vitamins is easily eliminated from the body when compared to the first one (Montagne et al., 2009).

The most commonly studied and used species of probiotics today belong to genera *Lactobacillus*, *Bifidobacterium* and *Saccharomyces* (Bertelsen, Jensen, & Ringel-Kulka, 2016). These microorganisms, added in infant formulas, improve the intestinal microbial balance and reinforce the defense mechanisms, through antagonistic, competition and immunological effects (Thompkinson & Kharb, 2007).

Although it is a trend to add nucleotides, prebiotics, probiotics, arachidonic acid (ARA) and docosahexaenoic acid (DHA) in infant formulas, research are needed both for definition of daily use recommendations and to determine the health effect of such ingredients on the infant nutrition (Zou et al., 2016).

1.2.2. Production of powdered infant formula

Currently infant formulas are available in powder or concentrated liquid forms; however, due to advantages presented by the powered formula (lesser cost of production and transport, higher storage time at ambient temperature, practicality, among others), this one have better acceptance by consumers around the world.

The first dehydrated infant formulas were produced by the dry mixing process in which the powdered ingredients are purchased separately and mixed in large batches. However, cases of contamination with pathogens such as *Enterobacter sakazakii* and *Salmonella enterica* have questioned the microbiological safety of this process (Guo & Ahmad, 2014; Happe & Gambelli, 2015).

For this reason, the dry mixing has been substituted by the wet mixing process, shown in Figure 2, which consists in dissolve the dried ingredients in water or skimmed milk preheated, pasteurize and homogenize the mixture. The preparation is concentrated in a vacuum evaporator in order to reduce the energy costs associated to spray drying (Blanchard et al., 2013; Happe & Gambelli, 2015).

The spray drying of concentrated preparation is characterized by atomization of the liquid resulting in small droplets whose water molecules are evaporated by a flow of heated air. The hot air stream has temperature varying between 140 and 200 °C but, due to relatively brief exposure time of the particles to heating, their core temperature does not exceed 45 ºC (Mujumdar, Huang, & Chen, 2010; Silveira, Perrone, Rodrigues Júnior, & Carvalho, 2013).

The powder is collected after passing through a cyclone and packed in big bags or stored in silos while the quality control analysis are done (Jiang & Guo, 2014). Subsequently, it is normally canned in an aseptic packaging line under a modified atmosphere such as nitrogen, to prevent possible oxidation of the product (Happe & Gambelli, 2015).

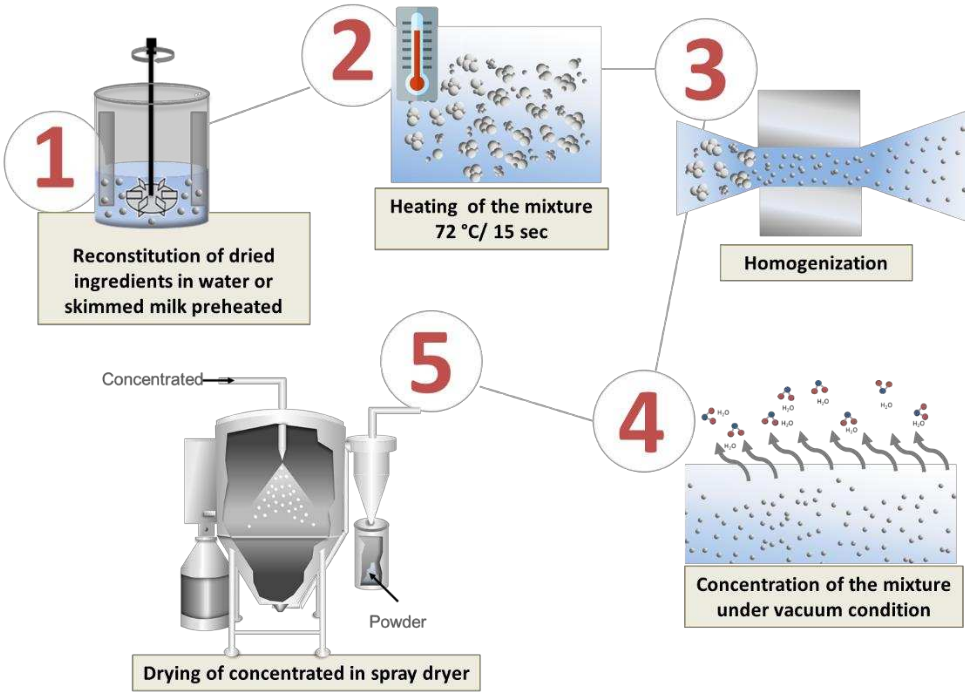

Figure 2 - Scheme of production of powdered infant formulas. Source: Blanchard et al., 2013; Happe & Gambelli, 2015; Mujumdar et al., 2010.

The product must be packed in suitable containers with the capacity to block the light; avoid transmission of water and O_2; also be resistant to impact and stacking damage; besides being stored in ideal conditions of humidity and temperature (Walsh, 2014). Even all points being served, the formulas are still subject to a series of reactions that cause a reduction in the quality of this product, such as the Maillard reaction.

1.3. Maillard reaction

The Maillard reaction is the result of chemical reactions between a carbonyl group of the reducing sugar and a free amino group of the protein or amino acid, forming a lactosyl-lysine, also known as Schiff's base (Martins & Van Boekel, 2005).

In the vast majority of infant formulas, lactose is the main reducing sugar present while the proteins are represent by casein and whey proteins (Andreas et al., 2015; European Commission, 2003). Due to high lactose (7.0-7.5 % w.v^{-1}) and protein (1.2-1.55 % w.v^{-1}) content, this kind of food when exposed to high temperatures is prone to initiate the Maillard reaction conducting to formation of Schiff's bases. As previously described, the infant formulas pass for successive heating treatments during its manufacture, which favor the initiation of Maillard reaction.

On the other hand, these bases are chemically unstable suffering additional isomerization, reaction known as Amadori rearrangement, leading to production of lactoulosyl-lysine (Amadori product) and loss of nutritional value due to blockage of the essential amino acids (Birlouez-Aragon et al., 2004; Martins & Van Boekel, 2005; Perez-Locas & Yaylayan, 2010). The Amadori rearrangement is driving under slight acid conditions which are reached thanks to accumulation of formic acid produced by parallel reactions between lactose molecules (Brands & van Boekel, 2001; Martins, Jongen, & van Boekel, 2000). With the prolongation of the heating or storage, the Amadori products are degraded under neutral or acid conditions (1,2 enolisation) to form a wide variety of furfurals compounds such as 5-Hydroxymethylfurfural (HMF), 2-Furaldehyde (F), 2-furyl-methyl ketone (FMC) and 5-methyl-2-furaldehyde (MF). As the infant formulas are slightly acidic or neutral, such compounds are preferably formed (Chávez-Servín, Castellote, & López-Sabater, 2006; Ferrer, Alegría, Farré, Abellán, & Romero, 2002).

If the infant formulas have basic pH, which varies according to the composition of these, Amadori products can also be degraded by other via (2,3 enolisation), forming reductones, and a variety of fission products, including acetol, pyruvaldehyde and diacetyl. These carbonyl compounds are highly reactive and participate in other reactions such as Strecker degradation, so they will react with amino acids to form aldehydes and aminoketones, that are important contributors to the aroma of foods, such as chocolate, coffee, tea, honey and bread (Martins et al., 2000; Nursten, 2005).

The products reductones, furfurals, aldehydes and other intermediate products react readily with amines to give “polymeric” high molecular mass, dark pigments called melanoidins (Perez-Locas & Yaylayan, 2010). Some positive and negative aspects about melanoidins will be discussed later.

1.3.1. Factors affecting reaction

A number of factors influences the speed of Maillard reaction, such as, when the pH is above the isoelectric point of the involved amino acid, usually alkaline conditions, they favor the reactivity of the amino group to the carbonyl group involved in the reaction, besides favoring the open chain form of the sugars. However, acidic environments, such as infant formulas, favor the hydrolysis of non-reactive sugars, such as sucrose, promoting amino-carbonyl interaction. In addition, pH affects the degradation pathway of Amadori products, that is, acidic to neutral pH favors formation of furfural, while under alkaline conditions, several products are formed, responsible for contributing to the organoleptic properties of the product, as explained above (Newton, Fairbanks, Golding, Andrewes, & Gerrard, 2012; John O’Brien, 2009; Perez-Locas & Yaylayan, 2010).

The rate of reaction is also influenced by temperature, increasing the reactivity between sugar and amino group (Martins & Van Boekel, 2005; van Boekel, 2001). Since the Q_{10} of the reaction is 3-4, the reaction rate triples or even quadruples at each 10 °C increase in temperature (McSweeney & Fox, 2009).

The water activity (a_w) is another important factor, since the condensation and dehydration reactions are part of the Maillard reaction pathways (Steele, 2004). In a dilute system (excess water), there is a decrease in the Maillard reaction rate, due to the dilution of the reagents, preventing the occurrence of many stages of this reaction. If water activity is reduced, the reactants may begin to lose essential mobility for the reaction resulting in a lower rate (Nursten, 2002; Pereyra Gonzales, Naranjo, Leiva, & Malec, 2010). Therefore, the browning rates reach a maximum around intermediate values of 0.5 to 0.8 (Cécile, Delphine, Emilie, Carole, & Thierry, 2016). However, an increase in the Maillard reaction in dry dairy products is often attributed to reduced water activity, due to the increased concentration of reactants as a result of drying (Newton et al., 2012; van Boekel & Brands, 1998).

The type of reducing sugar or the amine has a direct influence on the speed of the Maillard reaction. Pentoses are more reactive than hexoses, while reducing monosaccharides are more reactive than disaccharides or reducing polysaccharides. Non-reducing sugars, however, require the hydrolysis of the glycosidic bond to participate in the reaction. As in the vast majority of infant formulas, lactose is the main reducing sugar present, wich can positively influence reaction rates (Lingnert et al., 2002; Perez-Locas & Yaylayan, 2010). Lysine is one of the most reactive amino acids in this reaction, because beyond of the α-amino group in the terminal amino acid, it contains a side chain with a free ε-amino group that also participates of the reaction (Martins et al., 2000; Martins & Van Boekel, 2005). Although the sugars also reacts with arginine, methionine, tryptophan, and histidine (Mehta & Deeth, 2016).

The Maillard reaction can also be catalyzed by metals such as copper, iron, calcium and magnesium, which are present in the composition of infant formulas. The mechanisms of metal ions action on Maillard browning are still unclear but most of studies employed polyvalent transition metals suggesting an unspecified contribution of redox processes in the observed browning (Newton et al., 2012; Rizzi, 2008).

The lipid oxidation products can also be a source of carbonyls in later stages of the Maillard reaction, behaving similarly to carbohydrates. Particularly, the reaction of lipid oxidation products with amines, amino acids, and proteins has long been related to the browning observed in many foods during processing and storage (Newton et al., 2012; Zamora & Hidalgo, 2005).

1.3.2. Consequences in infant formulas

An important contribution of the Maillard reaction to most thermally processed foods is the browning during the processing of these products due to the formation of melanoidins, but this is undesirable, mainly in some dairy products (Bastos, Monaro, Siguemoto, & Séfora, 2012; Steele, 2004).

Another contribution is the formation of volatile compounds, mainly, aldehydes and aminoketones from the degradation of Strecker, that are important contributors to the aroma of some foods (Martins et al., 2000). The cooking flavors mainly affect processed dairy products such as milk powder, UHT milk, pasteurized milk and infant formulas (Cécile et al., 2016; Perez-Locas & Yaylayan, 2010).

Maillard reaction influences the texture of food via protein cross-linking during food processing which is one of the main causes of changes in milk powders during storage (Bastos et al., 2012). Le et al. (2013) observed a considerable amount of high-molecular-weight protein complexes that may be largely responsible for solubility loss in MPC 80 (milk protein concentrate), WMP (whole milk powder), and SMP (skim milk powder) via dicarbonyl compounds produced as advanced MRPs (Maillard reaction products), besides cross-links between molecules in casein micelles.

The degradation of the products of Amadori also forms acids, such as formic and acetic acid, responsible for the slight decrease in pH in stored dairy products (Brands & van Boekel, 2001; Martins et al., 2000).

One of the most obvious negative consequences of the Maillard reaction in food is the decrease of digestibility, destruction and/or biological inactivation of amino acids, especially the essentials (Martins et al., 2000; Teodorowicz, van Neerven, & Savelkoul, 2017). The quality and nutritional availability of proteins in dairy products is extremely relevant once they are often suppliers of essential amino acids (Perez-Locas & Yaylayan, 2010).

Furthermore, the thermal processing on food protein allergy decreases the protein solubility, promotes structural rearrangements and aggregation of proteins with sugar (Teodorowicz et al., 2017). An example is that allergenicity of β-lactoglobulin may be enhanced by the Maillard reaction, since higher degrees of glycation lead to a higher resistance to proteolysis, because the protein cleavage sites are masked (Nursten, 2002; John O'Brien, 2009; Teodorowicz et al., 2017).

Melanoidins have metal-chelating properties, may be undesirable when there complexing nutritionally essential metals (Ca, Mg, Cu, Zn, Fe) and subsequent impediment of metabolism and mineral absorption (Cécile et al., 2016; Perez-Locas & Yaylayan, 2010). The milk processing conditions decrease the solubility of calcium resulting in significantly lower values of apparent calcium absorption and retention (Seiquer, Delgado-Andrade, Haro, & Navarro, 2010). Other authors also observed lower iron and zinc bioavailability in bottle-sterilized formula compared to the reconstituted powder infant formula (Sarria & Vaquero, 2001, 2004).

1.3.3. Products of Maillard reaction potentially harmful

HMF is one of the intermediate compounds formed during the Maillard reaction. Generally, it could be used as marker of the extent of the thermal treatment applied or inadequate storage conditions (Morales, 2009). There are reports of a possible mutagenic, genotoxic and carcinogenic effect for HMF, as well as its metabolites, mainly 5-sulfoxymethylfurfural (SMF), which can be metabolized *in vivo*. The interaction of this reactive intermediate with cellular nucleophiles (i.e., DNA, RNA, and proteins) may result in structural damage (Arribas-Lorenzo & Morales, 2010; Capuano & Fogliano, 2011). Special attention has been given to the HMF content in infant formulas or baby foods in general (Morales, 2009).

Acrylamide has been classified as a probable carcinogen and it is reported in carbohydrate-rich foods processed at a relatively high temperature. This compound is formed mainly from the thermal degradation of free asparagine in the presence of sugars in the Maillard reaction (Lingnert et al., 2002; Mottram, Low, & Elmore, 2006), however; its levels in dairy products are relatively low (<100–1000 $\mu g.kg^{-1}$) (Petersen & Tran, 2005). The mean content of acrylamide in the baby foods ranged from 2 to 516 $\mu g.kg^{-1}$ determined by Mojska; Gielecińska; Stoś (2012).

There is one group of non-volatile compounds, called heterocyclic aromatic amines (HAAs), that are highly mutagenic and have been found in protein-rich cooked foods subjected to high temperatures (Mottram et al., 2006). The Maillard reaction contributes generating crucial intermediates including aldehydes, pyridines and pyrazines through the Strecker degradation reaction (Perez-Locas & Yaylayan, 2010), which have been shown to be tumor inducers in long-term animal studies. There is also a possible carcinogenic effect of HAAs for humans genetically susceptible and/or moderately to highly exposed (Nursten, 2005). The presence of such mutagens has not yet reported in dairy products (John O'Brien, 2009).

In early 2004, the US Food and Drug Administration (FDA) expressed concern at the presence of furan, a possible human carcinogen, in a number of heat-treated foods (FDA, 2008). Maillard reaction products such as furfural and furoic acid have been proposed as furan precursors but their levels in heated dairy products is considerably low (generally <20 $\mu g.kg^{-1}$ in infant formulas and evaporated milks) (John O'Brien, 2009).

1.3.4. Advanced Glycation End Products (AGEs)

Maillard reaction occurs in biological systems and the final products are referred as AGEs. A large number of AGEs have been identified *in vivo* including carboxymethyllysine (CML), pentosidine and pyrraline and dietary MRPs contribute to the accumulation of AGES in the body (Bastos & Gugliucci, 2015; van Rooijen et al., 2013).

AGEs can cause tissue damage from changes in the structure and conformation of the proteins; inter- or intramolecular cross-linking; formation of free radicals and inflammation due to the interaction of these compounds with the specific receptors (Delgado-Andrade, 2014; Nicoloff, Baydanoff, Petrova, & Christova, 2002).

As a result, excess AGEs can accumulate in the tissues seems to contribute to the development of disorders such as diabetes, weight gain, cardiovascular and neurodegenerative diseases, *i.e.,* Alzheimer, Parkinson and schizophrenia (Byun et al., 2017; Kouidrat et al., 2015; Losso, 2016; Robert & Labat-Robert, 2006; Uribarri et al., 2015; Wautier, Guillausseau, & Wautier, 2017). In addition, they may contribute to the development of arthritis, loss of bone mass and promotion of disorders in the function and/or structure of DNA and RNA molecules (Bastos et al., 2012; Delgado-Andrade, 2014; Losso, 2016; Turner, 2017; van Rooijen et al., 2013).

Younger children have greater gastrointestinal/epithelial permeability and this has been suggested to contribute to the risk of atopic disease. The immaturity of the immune system might also make infants more susceptible to environmental/epigenetic influences (Smith, Masilamani, Li, & Sampson, 2016).

Effects of these compounds can be avoided or regulated by renal AGEs elimination and antioxidant systems, but this depends on dietary intake, presence of pathologies as well as the amount and type of compound ingested (Bastos et al., 2012; Teodorowicz et al., 2017; van Rooijen et al., 2013).

1.3.5. Benefits of Maillard reaction in infant formula

Some works have evaluated the possible antioxidant, antihypertensive, prebiotic and antimicrobial activity of MRPs, mainly as melanoidins (Bastos et al., 2012; Delgado-Andrade, 2014; Monente et al., 2015; Patrignani, Rinaldi, & Lupano, 2016). A higher antioxidant activity could be obtained with the intermediate and especially the final stage Maillard reaction in a system of milk proteins and reducing sugars, the same results were obtained for milk powder, sweetened condensed milk and "dulce de leche" (Yáñez, Gagneten, Leiva, & Malec, 2018). Pre-heated milk protein and sugar mixtures effectively prevent lipid oxidation of dairy beverages probably due to the Maillard reaction products provided at the initial stage of sterilization (Giroux, Houde, & Britten, 2010). Other works performed by Oh et al. 2014, 2016 also observed antioxidant activity of Maillard reaction products in a system constituted by milk proteins and sugars.

This antioxidant effect has been associated to intermediate reductone compounds that could break the radical chain by donation of a hydrogen atom. Others Maillard reaction products, as melanoidins, have also been reported to be powerful scavengers of reactive oxygen species, peroxyl and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) (Lingnert et al., 2002; Vhangani & Wyk, 2016).

Some of the dietary AGEs may accumulate in body tissues or the MRPs that are not absorbed can be metabolized by intestinal microbes, giving prebiotic function (van Rooijen et al., 2013).

1.3.6. Inhibition of Maillard reaction in infant formula

Many sulphur compounds are inhibitors of the Maillard reaction in milk systems by making carbonyl compounds unavailable for further reactions and browning (John O'Brien, 2009).

The concentration of oxygen may influence both the overall rate of browning and the formation of low molecular weight oxidation products. The replacement of air by nitrogen is efficient in reducing the rate of this and other reactions of deterioration, this conservation strategy has already been used by industry (John O'Brien, 2009).

The replacement of a carbohydrate by another less reactive in infant formula would be a strategy (Naranjo, Pereyra Gonzales, Leiva, & Malec, 2013). Another one being researched is the optimization of microwave heating of a model of infant formula showing that it is possible to produce it with reduction of formed MRPs and deterioration of the added spores (Laguerre et al., 2011; Schuck et al., 2016).

Moreover, several authors as Evangelisti et al. (1999); Mendoza; Olano; Villamiel (2005); Messia; Candigliota; Marconi (2007); and Tossavainen; Kallioinen (2007) recommend that storage of milks at ≤ 4 ºC, especially the lactose-hydrolyzed, should be compulsory in order to limit protein damage. In addition, maintain the moistures below 30 % $w.w^{-1}$ to limit the mobility of molecules, in dairy powders (Lingnert et al., 2002).

1.4. Conclusions

Despite the importance of the formulas for infant nutrition, there are few studies in this area, especially studies that clarify the effect of Maillard reaction on this type of product and what the real harm to the infant. Understanding the chemical, nutritional and toxicological consequences of browning reactions and related transformations, in infant formulas, can help create strategies for the production of safer and healthier products.

CHAPTER 2 - COMPOSITIONAL CHARACTERIZATION OF COMMERCIAL INFANT FORMULAS

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Abstract

Infant formula has the function of replacing or supplementing breast milk thanks to a composition strategically calculated to provide the essential nutrients for the healthy development of the child. However, a constant evaluation of the nutritional composition of products available in the market is necessary in order to guarantee compliance with the requirements established by legislation. For this, the formulas were evaluated with respect to the composition, under conditions of use, along the storage to 4 ºC and 24 ºC for 60 days. In general, the values of moisture, ash, lipids, protein content, lactose and water activity presented within the limits established by the legislation, although one of the brands has a lower protein content than the ideal. It was observed a decrease in lactose and increase in moisture content of the formulas stored at room temperature; but the conservation of infant formulas under refrigeration reduces the gain in water and the rate of decrease of lactose, after 60 days of opening.

Keywords: centesimal composition; infants; breast milk; lactose.

2.1. Introduction

The World Health Organization (2009) (WHO) recommends that breastfeeding be exclusive until the first 6 months of the child and complement up to 2 years or more. However infants are often fed with products such as water, teas, juices and especially cow milk (Ministry of Health, 2009). In several countries, such as Australia, United States, England, China, less than 17 % of newborns were exclusively breastfed for up to six months (Brownell et al., 2015; de Jager et al., 2015; Mc Andrew, 2012; Yi, Yim, & Chow, 2016). In situations which the mother cannot or does not want to breastfeed, it is recommended to substitute or supplement breast milk with infant formulas (M Guo & Ahmad, 2014; Pereyra Gonzáles et al., 2003).

This product is available on the market in powder or liquid form (concentrated or ready for consumption) (USDA & WIC, 2009a) and most of them are prepared from cow milk added with carbohydrates, mainly lactose; long-chain polyunsaturated fatty acids; vitamins and minerals (European Commission, 2003; Schuck et al., 2016; Thompkinson & Kharb, 2007; Zou et al., 2016). There are also various types of preparations for specific medical purposes, as example, lactose-free formulas, soy-based, hypoallergenic and anti-regurgitation.

The composition of cow milk is modified to resemble the composition of breast milk to meet the nutritional needs of infants (Blanchard et al., 2013). It is should be remember that the inclusion of unnecessary or in inappropriate amounts of components, may overload the physiological and metabolic functions of the infant (Francescato et al., 2013). To this end, the *Codex Alimentarius Committee* (Codex) establishes the limit of the nutrients necessary in the composition of the infant formulas, in such a way that they must comply with the provisions from the legal point of view.

Infant formula represents about 70 % of the global infant food market, and in 2010 this percentage was around 40 % and sales will continue to grow rapidly, demonstrating the importance of this product in infant feeding (Happe & Gambelli, 2015).

Considering the significance of infant formulas, especially when replacing breast milk, a constant assessment of the composition is important to recognize whether the requirements set by legislation are being met, and also to ensure the nutrients needed by the child. The objective of this work was to evaluate the composition of infant formulas and to verify their adequacy regarding the norms in force during the period of use.

2.2. Material and Methods

2.2.1. Materials

Six commercial infant formulas (first age) of different lots from two different brands (A and B) were acquired in the local market. The formulas presented between 2 and 4 months of shelf life at the time of acquisition, and the composition of the label given by the manufacturer is shown in the Table 1.

Table 1 - Composition of the infant formulas analyzed, given by the manufacturer

Nutrients	**Brand A**	**Brand B**
Energetic value (kcal. 100g^{-1})	484	505
Lactose (g.100g^{-1})	53	55
Ashes (g.100g^{-1})	1.62	1.53
Lipids (g.100g^{-1})	26	28
Proteins (g.100g^{-1})	9.8	9.3

Sulfuric acid for milk-analysis (92.25 %; Micro-Química; Brazil), isoamyl alcohol (P.A.; Neon; Brazil), sulfuric acid (> 95.0 %, Alphatech; Brazil), boric acid (> 99.5 %, Vetec; Brazil), methyl red and methylene blue (P.A.; Cromato Produtos Químicos; Brazil), hydrochloric acid (P.A.; Vetec; Brazil), sodium hydroxide (P.A.; Vetec; Brazil), digesting mixture of potassium sulfate and copper sulfate (P.A.; Cromato Produtos Químicos; Brazil) were used for analysis of centesimal composition.

2.2.2. Storage and simulation of the use conditions of infant formulas

Infant formulas of the branches A and B were opened and storage at 4 or 24 ºC during 60 days. The simulation of domestic use was carried out by daily homogenization of infant formulas with a spoon. The temperatures and relative humidity of storage were measured daily with HigroFlex 5 thermohygrometer (Rotronic; Bassersdorf, Switzerland).

According to the manufacturer's recommendations, the product should be consumed within 30 days, but considering situations of longer use, such as complementary feeding, then it is important to evaluate the composition of these in a period of at least 60 days. In addition, several authors recommended that storage of milks, especially the lactose-hydrolysed, at ≤ 4 ºC should be compulsory in order to limit protein damage from the Maillard reaction (Mendoza et al., 2005; Messia et al., 2007; Tossavainen & Kallioinen, 2007).

2.2.3. Analytical determinations

Infant formulas in dried form were analyzed for moisture, by the gravimetric technique. About 5 g of the sample was heated to 105 °C and cooled in a desiccator to constant weight. The percentage of moisture is given by the loss of mass of the sample divided by the weight of this, multiplied by 100 (AOAC, 1990).

The ash content was also verified by the gravimetric method. About 5 g of sample was weighed, left in the oven at 105 °C for 2 hours and incinerated in a muffle at 550 °C for 3 hours. The residue was cooled in a desiccator and weighed. The ash percentage is given by the amount of ash, divided by the weight of the sample, multiplied by 100 (AOAC, 1990)

The lipid content was determined in a special butyrometer for powdered milk. 2.5 g of sample were transferred to butyrometer with 5 mL of distilled water at 40-50 °C, a further 5 mL of water was added and the sample was dissolved. Subsequently, 10 mL of sulfuric acid for milk analysis and 1 mL of isoamyl alcohol were transferred. It was centrifuged at 1200 rpm for 5 minutes, stored in a water bath at 65 °C for 2 to 3 minutes. The value obtained in the scale corresponds directly to the percentage of lipids (AOAC, 1990).

Total protein was evaluated by the Kjeldahl method which 1 g of sample was transferred to Kjedahl tube, 2 g of the catalytic mixture and 5 mL of sulfuric acid were added in the digester block with the plate at 450 °C until the material is clear. After cooling, the sample was inserted into the distillation system, neutralized with 40 % ($w.v^{-1}$) sodium hydroxide, heated until evaporation and condensation of all the nitrogen present in the sample in Erlenmeyer with 4 % ($w.v^{-1}$) boric acid and 3 drops of mixed indicator. The mixture was then titrated with 0.1 N hydrochloric acid solution. The percentage of protein was given by the multiplication of the volume spent in the titration, correction factor and concentration of the hydrochloric acid solution, equivalent gram of nitrogen, factor of general conversion of the nitrogen in protein, divided by the weight of the sample, multiplied by 100 (AOAC, 1990).

For the formulas analyzed, the carbohydrate content includes only lactose; GOS and FOS are counted separately. For this, lactose was quantified by enzymatic kit Lactose/D-glucose of R-Biopharm AG according the instruction of manufacturer.

The a_w was determined at 25 ° C by using an Aqualab (Decagon 3TE, USA).

All physical analysis were carried out in triplicate.

2.2.4. Statistical analysis

The results were evaluated by analysis of variance (ANOVA) and t-Student test for comparison of means for a significance level of 5 %, using the statistical program Assistat version 7.7 beta.

2.3. Results and Discussion

The composition of the infant formulas was evaluated along the open storage under refrigeration, temperature at 3.8 ± 1.8 ºC, relative humidity of 16.6 ± 4.8 %; and ambient temperatures at 24.4 ± 1.3 °C with relative humidity of 62.5 ± 5.0 %, for 60 days.

The minimum and maximum limits established for infant formulas are presented in the form of quantity per 100 kJ or 100 kcal by legislation. However, to facilitate the interpretation of the data, the values were adapted for both brands analyzed in the form of quantity per 100 g, since each brand presents a different energetic value, as presented in Table 2.

Just after opening, the infant formulas had their composition analyzed and the results are summarized in Table 3. The composition is in agreement with that presented on the label of the infant formulas analyzed (Table 1), only the ashes presented relatively higher than the mineral content, since this attribute is related to the inorganic residue obtained from the burning of the organic matter, generating CO_2 and oxides of nitrogen, besides the mineral residues that are in the form of oxides, sulfates, phosphates, silicates and chlorides (Marshall, 2010).

Table 2 - Amount of nutrients recommended for infant formulas

Nutrients	**BRAND**			
	A		**B**	
	Minimum	**Maximum**	**Minimum**	**Maximum**
Ashes (g.100g^{-1})	1.02	3.22	1.07	3.37
Lipids (g.100g^{-1})	21.26	28.35	22.26	29.68
Proteins (g.100g^{-1})	9.11	14.18	9.54	14.84
Carbohydrates (g.100g^{-1})*	44.55	66.83	46.64	69.96

*Necessarily, fifty percent of the carbohydrates present in the formula may be represented by lactose. Source: adapted from FAO & WHO, 2007.

During 60 days of opening with daily mixing of infant formulas, there was no significant difference in the ash, lipid and protein attributes in both brands independently of storage temperatures (Table 3), since in low water activities, such as the formulas analyzed, there are low rates of reactions in food (Fennema, Damodaran, & Parkin, 2010). However, a significant increase in the values of moisture (40 %) and water activity (43 %) and a decrease of lactose (7 %) content was found (Table 3) for formulas stored at 24 °C, up to 60 days.

Table 3 - Composition of commercial infant formulas

Analysis	BRAND A			BRAND B		
	0 DAY	60 DAYS		0 DAY	60 DAYS	
	24 ºC	4 ºC	24 ºC	24 ºC	4 ºC	24 ºC
Lactose ($g.100g^{-1}$)	52.99 ± 0.14^{bcd}	51.06 ± 0.25^{ef}	49.64 ± 0.27^{g}	54.64 ± 0.28^{a}	52.83 ± 0.03^{bcd}	51.04 ± 1.02^{ef}
Ashes ($g.100g^{-1}$)	2.46 ± 0.06^{a}	2.49 ± 0.08^{a}	2.54 ± 0.06^{a}	2.47 ± 0.10^{a}	2.46 ± 0.10^{a}	2.48 ± 0.11^{a}
Lipids ($g.100g^{-1}$)	25.74 ± 0.18^{b}	25.56 ± 0.11^{b}	25.60 ± 0.14^{b}	27.65 ± 0.13^{a}	27.70 ± 0.18^{a}	27.53 ± 0.15^{a}
Proteins ($g.100g^{-1}$)	9.60 ± 0.18^{a}	9.70 ± 0.05^{a}	9.76 ± 0.03^{a}	9.22 ± 0.10^{b}	9.20 ± 0.06^{b}	9.25 ± 0.01^{b}
Moisture ($g.100g^{-1}$)	2.33 ± 0.07^{d}	3.08 ± 0.35^{ab}	3.25 ± 0.29^{a}	2.18 ± 0.24^{d}	2.88 ± 0.27^{abc}	3.09 ± 0.16^{ab}
Water activity	0.25 ± 0.02^{de}	0.31 ± 0.01^{bc}	0.35 ± 0.01^{a}	0.22 ± 0.01^{f}	0.32 ± 0.01^{bc}	0.33 ± 0.01^{ab}

Means followed by equal letters in the same line do not differ at the 5 % level of significance.

According to the manufacturers, lactose is the only carbohydrate reducer added to food, which can be associated with some proteins, in the initial phases of Maillard reaction (Martins & Van Boekel, 2005). With the prolongation of the storage, occurs a series of reactions, forming several compounds, being able to have release of molecules of water that can significantly alter the a_w and moisture of infant formulas over time (Pereyra Gonzales et al., 2010). Furthermore, after opening, water molecules can be absorbed by dairy powder leading to additional gain of moisture. In its turn, the increasing in the water content can boost the Maillard reaction kinetics accelerating the nutritional losses and the browning process, which consists of the polymerization of intermediate compounds forming melanoidins (Perez-Locas & Yaylayan, 2010; Steele, 2004).

However by decreasing both temperature and ambient humidity, it is expected a reduction in the Maillard reaction rates. A zero-order reaction is observed for the reaction, for instance in milk systems (Claeys, Van Loey, & Hendrickx, 2003; van Boekel, 2001), then it is expected that rates occur at a slower speed, at 4 °C. With this 2 times reduction in storage temperature value, the reaction rate is expected to decrease from 9 to 16 times, since the Q_{10} of the reaction is 3-4 (McSweeney & Fox, 2009).

The conservation of infant formulas under refrigeration reduces both rates of formation of Maillard reaction intermediates and the gain in water, observed by the lower increase in moisture (32 %) and water activity (34 %). The lactose molecules continue to be consumed (3.5 %), but at a lower rate when compared with the infant formulas kept at ambient temperature.

The attributes ashes, lipids and lactose presented values between the limits established by the legislation, up to 60 days, at both storage temperatures, as shown in Table 2, but for proteins, only the products of brand A presented conformity, those of brand B showed relatively low values, from the moment the formulas were opened, since on the label of those products a protein concentration (9.3 $g.100g^{-1}$) was declared lower than required by the legislation. Carneiro; Carvalho; Pires (2016) determined levels of lipids, carbohydrates and proteins in commercial infant formulas with values within the limits required, which also corroborate with the results presented in this study. Contreras-Calderón; Guerra-Hernández; García-Villanova, (2009) also observed similar values to those determined in this work, for the previous attributes. Sabater et al. (2016) determined similar values of carbohydrates, while Kelly et al. (2016) found similar values to lipids.

There is no regulation on moisture in infant formulas, however, according to the Technical Regulation on Identity and Quality of Milk Powder (Ministry of Agriculture Supply and Agrarian, 1996), the maximum moisture content for this product should be 3.5 % ($w.w^{-1}$). However, for FAO & WHO (2011) the maximum moisture content for powdered milk may be up to 5 % ($w.w^{-1}$). Based on these values, the evaluated products are in compliance with the moisture, during 60 days, at both storage temperatures.

By regarding water activity, the recommendation of the specialized literature is around 0.20 and the products evaluated presented values relatively close to the ideal, in time 0 (Schuck, Anne, & Jeantet, 2012). However, significant increases in these values occurs in 60 days in both storage temperatures. Thus, the increase in water and moisture activity may favor the initiation of degrading reactions, such as the Maillard reaction, for example (Fennema et al., 2010). Kelly et al. (2016) and Tham; Yeoh; Zhou (2017) found similar values for moisture and water activity in infant formulas when opened.

2.4. Conclusions

Based on the results obtained, it is inferred that in general the infant formulas presented nutrient values analyzed within the limits established by the legislation, although one of the brands has lower protein content than the ideal.

It was observed a decrease in lactose and increase in moisture content of the formulas stored at room temperature. Under refrigeration, there was reduction in water gain and rate of decrease of lactose, after 60 days of opening.

Despite these compositional variations over time, based only in the attributes evaluated here, the formulas can be consumed during 60 days instead the 30 days proposed by manufactures. However, to this observation be kept, futures experiments should be carried out in order to revise the micronutrient values during the period of use after the expiration date.

Finally, to confirm the hypothesis that the reduction of the lactose content is related to the Maillard reaction, future investigations must be carried out.

CHAPTER 3 - EVALUATION OF MAILLARD REACTION IN INFANT FORMULAS AFTER OPENING

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Abstract

The infant formula is a substitute or complement of breast milk and it is subject to Maillard reaction due to its composition, which may be initiated during the heat treatment steps and prolonged in storage. It was evaluate the propagation of Maillard reaction in infant formulas under conditions of use by physico-chemical analysis, along the storage to 4 ºC and 24 ºC for 60 days. It was observed increase of HMF content, change of color, decrease in lactose, lysine and pH and increasing in water activity and moisture of the formulas stored at room temperature. The infant formulas stored at refrigeration temperature showed lower nutritional losses, as well as reduced HMF. The level of HMF found in infant formulas does not create risks for the infant, and it is recommended to keep the product under refrigeration during the storage of the formula in the period of consumption.

Keywords: 5-hydroxymethylfurfural; breast milk; nutritional losses; storage.

3.1. Introduction

Exclusive breastfeeding is indicated for infants from birth to 6 months and supplement to 2 years or more, due to balanced nutritional composition and immunological protection provided by human milk. Infants fed with breast milk show better development and lowers risk of disease throughout life (Olson & Hayward, 2017; Pereyra Gonzáles et al., 2003); but despite these advantages, the breastfeeding can be interrupted or not conducted exclusively in cases of baby blues, insufficient milk syndrome, social factors (wage-earners mothers), medical recommendation in babies with metabolic disturbances, among others (Contreras-Calderón et al., 2009; M Guo & Ahmad, 2014; Pereyra Gonzáles et al., 2003). Under these circumstances, infant formulas are many times the only food offered to children and, for this reason; they should be safe besides providing all necessary nutrients to their development.

The most of commercial infant formulas are produced from combination between the bovine milk and others components as lactose, long-chain polyunsaturated fatty acids vitamins and minerals (Zou et al., 2016). In several process of production of these foods, all ingredients are mixture, solubilized in water, heat treated to eliminate pathogenic bacteria, dehydrated in spray dryer and packaged under aseptic conditions. Despite the heat treatment is extremely necessary to guarantee the microbiological safety of product, it can generate nutritional losses and affects techno-functional properties of infant formulas such as the rehydration degree, color modification and the formation of volatile compounds (Birlouez-Aragon et al., 2004; Perez-Locas & Yaylayan, 2010).

As infant formulas are fortified with vitamins, minerals such as iron and lactose, they are subjected to suffering Maillard reaction during the steps of heat treatments, drying and storage (Ferrer et al., 2005; Perez-Locas & Yaylayan, 2010). This reaction is the result of the interaction between a carbonyl group of the reducing sugar and a free amino group of the protein or amino acid, forming the Schiff's base (Birlouez-Aragon et al., 2004; Martins et al., 2000; Nursten, 2005). These bases are chemically unstable and successive reactions promote the formation of a wide variety of heterocyclic, carboxylic and aliphatic compounds such as dicarbonyls, reductones, Strecker degradation products and furfural compounds, such as HMF (Ferrer et al., 2002; Martins et al., 2000; Perez-Locas & Yaylayan, 2010).

Particular attention has be paid to HMF because it is an intermediate compounds of the Maillard reaction commonly used as an indicator of the severity of heat treatments and also of the length and conditions of storage of some foods (Chávez-Servín, De La Torre Carbot, García-Gasca, Castellote, & López-Sabater, 2015; Ferrer et al., 2005; Guerra-Hernández, Leon, Corzo, García-Villanova, & Romera, 2002). Furthermore, studies have suggested that HMF has a possible mutagenic, genotoxic and carcinogenic effect and, for adults, its recommended daily intake must not exceed 2.5 mg by body mass in kilograms (equivalent to 20 $\mu mol.kg^{-1}.day^{-1}$) (Kowalski, Lukasiewicz, Duda-Chodak, & Zięć, 2013; Morales, 2009; Rosatella, Simeonov, Frade, & Afonso, 2011).

Briefly, in excess, the Maillard reaction promotes the significant unavailability of essential amino acids as lysine and reducing sugar as lactose, besides produces toxic and dark compounds as HMF and melanoidins, respectively (Mottram et al., 2006; Pereyra Gonzáles et al., 2003; Perez-Locas & Yaylayan, 2010).

The kinetic of Maillard reaction is primarily influenced by temperature, humidity, pH, type of reducing sugar or the amine, light exposition, oxygen, and thus the conditions of processing and storage are crucial to define the intensity of propagation of this reaction in infant formulas (Martins et al., 2000; Newton et al., 2012; John O'Brien, 2009).

Considering the importance of this food to child nutrition, the objective of this work was evaluate the propagation of Maillard reaction in infant formulas under conditions of use by evaluating indicators of reaction: HMF, lysine and lactose content.

3.2. Materials and methods

3.2.1. Materials and Methods

Six commercial infant formulas (first age) of different lots and from two different brands (A and B) were acquired in the local market. The formulas presented between 2 and 4 months of shelf life at the time of acquisition.

Oxalic acid (> 99.5 % PA; Fmaia; Brazil), trichloroacetic acid (TCA > 99 % PA; Vetec; Brazil), thiobarbituric acid (> 97.5 % PA; Êxodo Científica, Brazil), sodium dodecylsulfate (> 99 %; Bio-Rad; Japan), o-phthaldialdehyde (≥ 99 %; Sigma-Aldrich; China), ethyl alcohol (95 % PA; Cap-Lab; Brazil), sodium tetraborate (> 99 % PA; Vetec; Brazil), 2-mercaptoethanol (> 99 %; Sigma-Aldrich; Germany), casein from bovine milk technical grade (Sigma-Aldrich; United States) and standard of HMF was > 99 % pure, purchased from Sigma-Aldrich (Brazil), were used to determine the content of HMF, lactose and lysine in the infant formula samples.

3.2.2. Storage and simulation of the conditions of use of infant formulas

Infant formulas of the branches A and B were opened and storage to 4 and 24 ºC during 60 days. The simulation of domestic use was carried out by daily homogenization of infant formulas with a spoon. The temperatures and the relative humidity of storage were measured daily with HigroFlex 5 thermohygrometer (Rotronic; Bassersdorf, Switzerland).

3.2.3. Analytical determinations

3.2.3.1. Physical analysis

Infant formulas in dried form were analyzed for moisture, by the gravimetric technique, evaluating the weight loss of the material submitted to the temperature of 105 ºC in oven (AOAC, 1990). The a_w was determined at 25 ° C by Aqualab (Decagon 3TE, USA).

The color of powders was measured by using the color scale CIELAB, with coordinates "L*" (luminosity), "a*" (intensity of red and green) and "b*" (intensity of yellow and blue), illuminant D65 and observation angle of 10°, in colorimeter Colorquest XE (Hunter Lab, Reston, USA). The results were expressed as total color difference (ΔE), according to the equation $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$.

All physical analysis were carried out in triplicate.

3.2.3.2. Chemical analysis

pH measurement

The infant formulas were reconstituted according to the instructions of manufacturers and the pH was determined by using a pH meter digital Gehaka PG1800 (São Paulo, Brazil).

Evaluation of the indicators of Maillard reaction

The determination of HMF was made by spectrophotometric analysis in acidified medium according to Keeney & Bassette (1959).

Five milliliters of infant formulas diluted 6 times, 5 mL of oxalic acid 0.3 $mol.L^{-1}$ and 5 mL of trichloroacetic acid 40 % ($w.v^{-1}$) were filtered on filter paper (Whatman GR 40 150 mm). 4 mL of the filtrate and 1 mL of thiobarbituric acid 0.5 $mol.L^{-1}$ were added in a capped test tube and placed in a water bath at 40 °C for 30 minutes. After cooling to room temperature, reading was done in a UV-visible spectrophotometer (Global Trade Technology; UV-5100; China) at 443 nm.

The evaluation of the lysine content was performed by spectrophotometric analysis with o-phthaldialdehyde (OPA). The methodology was adapted according to Goodno et al. (1981) and Vigo et al. (1992). The infant formula was dissolved in sodium dodecyl sulfate solution at 10 % (w.v^{-1}), in such an amount that it contained 50 µg of protein, and 50 µL was added to 2 mL of OPA reagent. The absorbance was measured at 340 nm and plotted against a standard curve of purified casein dissolved in 0.1 M sodium tetraborate buffer solution at pH 9.0. The interference of free amino groups of amino acids, small peptides and amines was verified in the supernatant of samples dissolved in sodium tetraborate buffer solution at pH 9.0 subsequently treated with 10 % (w.v^{-1}) trichloroacetic acid solution, this interference being considered as negligible.

The lactose was quantified by enzymatic kit Lactose/D-glucose of R-Biopharm AG according the instruction of manufacturer.

All chemical analysis were carried out in triplicate.

3.2.4. Statistical analysis

The results were evaluated by analysis of variance (ANOVA) and t-Student test for comparison of means for a significance level of 5 %, using the statistical program Assistat version 7.7 beta.

3.3. Results and discussion

3.3.1. Development of Maillard reaction in infant formulas at ambient temperatures

The formation of Maillard reaction products is induced by heating treatments during the production of infant formulas (Birlouez-Aragon et al., 2004; Chávez-Servín et al., 2015; Mehta & Deeth, 2016). As previously described, in excess, HMF shows toxigenic and mutagenic properties that can be harmful to the health of the newborn and infant in the long term, justifying its quantification in this kind of food.

Infant formulas provided from two different brands (A and B) were evaluated for the HMF content just after the opening and, an amount of approximately 120 $\mu mol.L^{-1}$ was found in both of them (Figure 3A). However, depending of the use conditions, it is expected to increase or decrease the rate of HMF formation over time (Albalá-Hurtado et al., 1998; Chávez-Servín et al., 2006; Ferrer et al., 2005; Guerra-Hernández et al., 2002).

To investigate this hypothesis, the formulas A and B were first evaluated under domestic use conditions including temperature at 24.4 ± 1.3 ºC, relative humidity of 62.5 ± 5.0 % and daily mix of the formulas with a spoon. After opening, the manufacturers advise consumption during 30 days; however, the experiment were conducted up to 60 days to simulate an abusive condition imposed by consumer.

After 30 days of opening, the HMF content significantly increased of approximately 10 % for both brands (Figure 3A). Until the sixtieth day, the HMF index increased to 14 % only to brand B while the brand A kept constant (Figure 3A). These results are corroborated by other authors that verified an increasing between 20 and 30 % of HMF during 90 days of storage (Albalá-Hurtado et al., 1998; Ferrer et al., 2000, 2002; Guerra-Hernández et al., 2002).

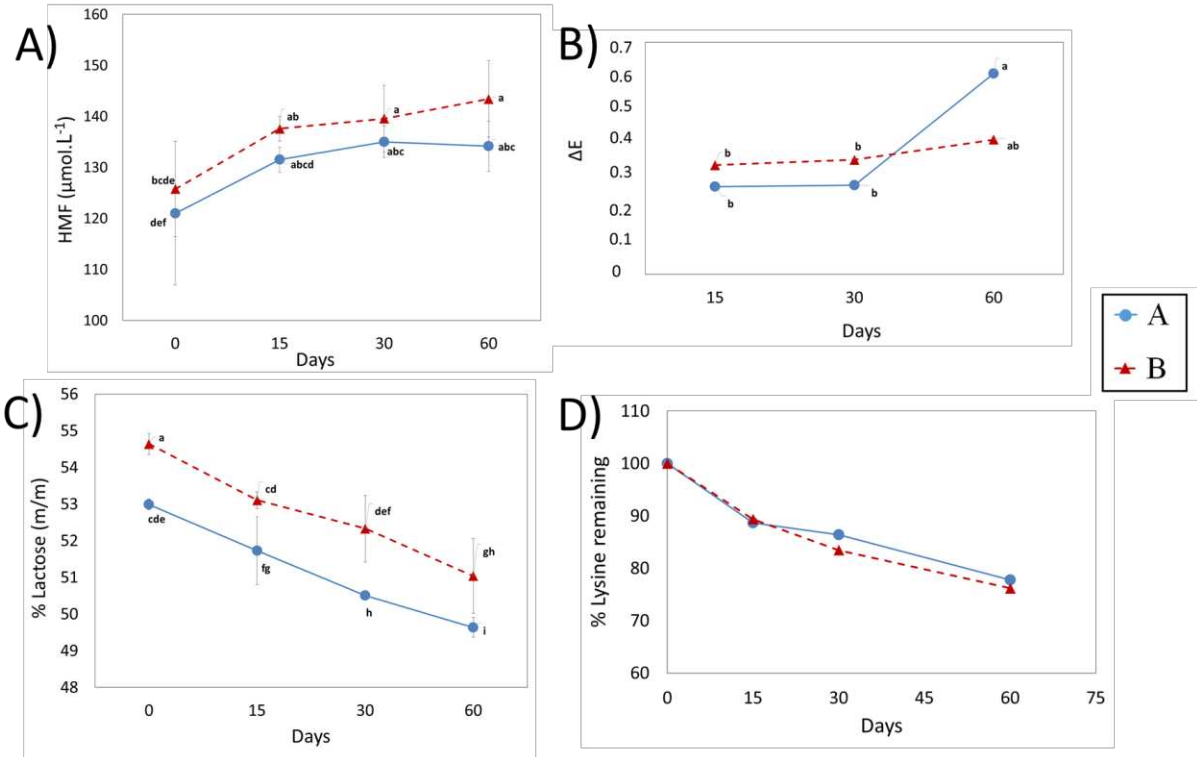

Figure 3 - HMF (A), color variation (B), lactose (C) and lysine remaining (D) in infant formulas stored at 24 ºC. Means followed by equal letters in the same line do not differ at the 5 % level of significance.

Intermediate compounds as HMF, polymerizes throughout the Maillard reaction forming complex brown pigments (melanoidins) responsible by undesirable browning of the infant formulas. Thus, the concentration of HMF is strongly correlated with changes in milk's color (Cais-Sokolinska, Pikul, & Dankow, 2004).

The products from A-brand showed color variation after 60 days of opening, even though it is not visually noticeable, while no significant variation was observed for formula from B-brand (Figure 3B). Others researchers also found color variation in infant formulas and, in some cases, reports demonstrate an increasing in color variation after 15 days of storage (Albalá-Hurtado et al., 1998; Guerra-Hernández et al., 2002; Perez-Locas & Yaylayan, 2010).

The accumulation of HMF formed by the Maillard reaction results in a decreasing in the content of other components as lactose and lysine, precursors of reaction. According to the manufacturers, lactose is the only carbohydrate reducer added to food, which can be associated with lysine molecules in the initial phases of Maillard reaction. Studies carried out by McSweeney & Fox (2009) also suggest that lysine blockage due to Maillard reaction is more intense in infant formulas with high proportion of lactose-protein.

Lactose is a very important component in infant nutrition, once it stimulates the development of the intestinal microbiota, acts as a source of energy and it is related to ideal development of the infant brain (Blanchard et al., 2013). In the same direction, lysine is essential for the fatty acids metabolism, reduction of cholesterol levels and absorption of calcium in bones and connective tissues. Deficient diets in lysine cause kidney stones, nausea, loss of appetite, fatigue, slow growth, anemia, and other physiological disorders (Mehta & Deeth, 2016).

After 15 days of opening, significant reductions in the lactose and lysine contents were found for infant formulas of both brands (Figure 3C and 3D). Lactose reduced from 54 to 50 $g.g^{-1}$ of formula after 60 days of storage, corresponding to a loss of 7 %, while lysine reduced up to 24 % in the same period (Figure 3C and 3D).

Just during processing of infant formulas, lysine losses between 10 and 35 % can be expected (Contreras-Calderón et al., 2009) and an additional reduction (12–19 %) occurs during 6 months at ambient temperature (Ferrer et al., 2000). Birlouez-Aragon et al. (2004) found statistically significant losses of available lysine (about 30 %) in the studied infant formulas with respect to raw cow milk as a consequence of the thermal treatments.

Together with lactose and lysine reductions, a decreasing in 0.3 units of pH was observed in the infant formulas after 60 days of storage (Figure 4A), which is in accordance with other authors that also observed a reduction between 0.3 and 0.4 units of pH (Albalá-Hurtado et al., 1998; Brands & van Boekel, 2001; Liu, Li, Kong, Li, & Xia, 2014). The slight pH reduction is also related to Maillard reaction once the degradation of the Amadori products conduct to formation of formic acid during storage of product (Brands & van Boekel, 2001; Martins et al., 2000).

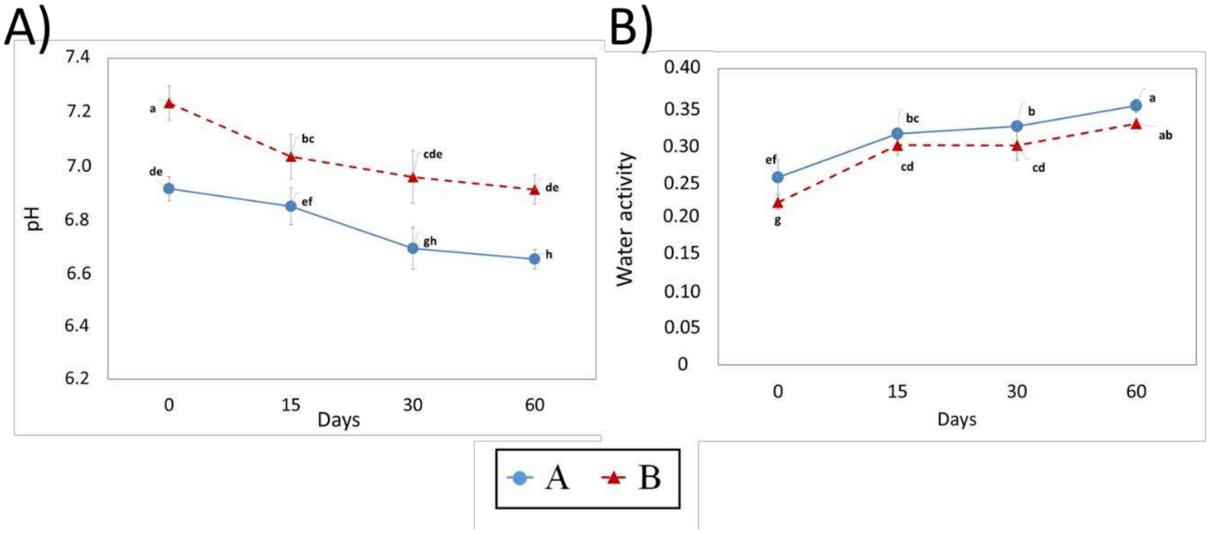

Figure 4 - pH (A) and water activity (B) in infant formulas stored at 24 ºC. Means followed by equal letters in the same line do not differ at the 5 % level of significance.

Besides the nutritional losses and browning, the Maillard reaction releases water molecules that can significantly alter the a_w and moisture of infant formulas over time (Pereyra Gonzales et al., 2010). Even with a minimal increase in moisture content, water is known to have a strong plasticizing effect that reduces the glass transition temperature of powder inducing degradative changes in the product, such as stickiness, caking, collapse and acceleration of nonenzymatic browning (Nasirpour et al., 2007).

Furthermore, after opening, water molecules can be absorbed by dairy powder leading to additional gain of moisture. In its turn, the increasing in the water content can boost the Maillard reaction kinetics accelerating the nutritional losses and the browning process (Steele, 2004). Several authors report losses of lysine in a_w of 0.44 (whey powder); 0.3-0.6 (skim milk powder) and 0.3-0.7 (whey protein concentrate) (Malec, Pereyra Gonzales, Naranjo, & Vigo, 2002).

The browning rates begin to increase when the a_w approaches a value between 0.2 and 0.3; reaching a maximum in values between 0.5 and 0.8 (Lingnert et al., 2002; Perez-Locas & Yaylayan, 2010). In this work, the water activity of the products ranged from 0.22 to 0.35 (Figure 4B) and an increase in humidity of 39.2 and 41.7 % (results not shown) for brand A and B, respectively, was also found. Therefore, the water activity and relative humidity of the environment favors the reaction rate, which helps to explain the pronounced accumulation of HMF over time for both brands (Figure 3A).

3.3.2. Development of Maillard reaction in infant formulas under refrigeration

In the previous section, the deleterious effects of Maillard reaction on the infant formulas at ambient temperatures were demonstrated; however by decreasing both temperature and ambient humidity, it is expected a reduction in the reaction rates. In other words, the nutritional losses and browning can be softened just by keeping of infant formulas under refrigeration. To investigate it, infant formulas from A and B brands were preserved at 3.8 ± 1.8 °C with relative humidity of 16.6 ± 4.8 % during 60 days also simulating conditions of use.

Contrary to observed under ambient temperature, the levels of HMF reduced to ~100 $\mu mol.L^{-1}$ for both brands after 60 days of storage (Figure 5A), however, the lactose and lysine contents have kept reducing on average 3.5 and 8.5 %, respectively (Figure 5B and 5C).

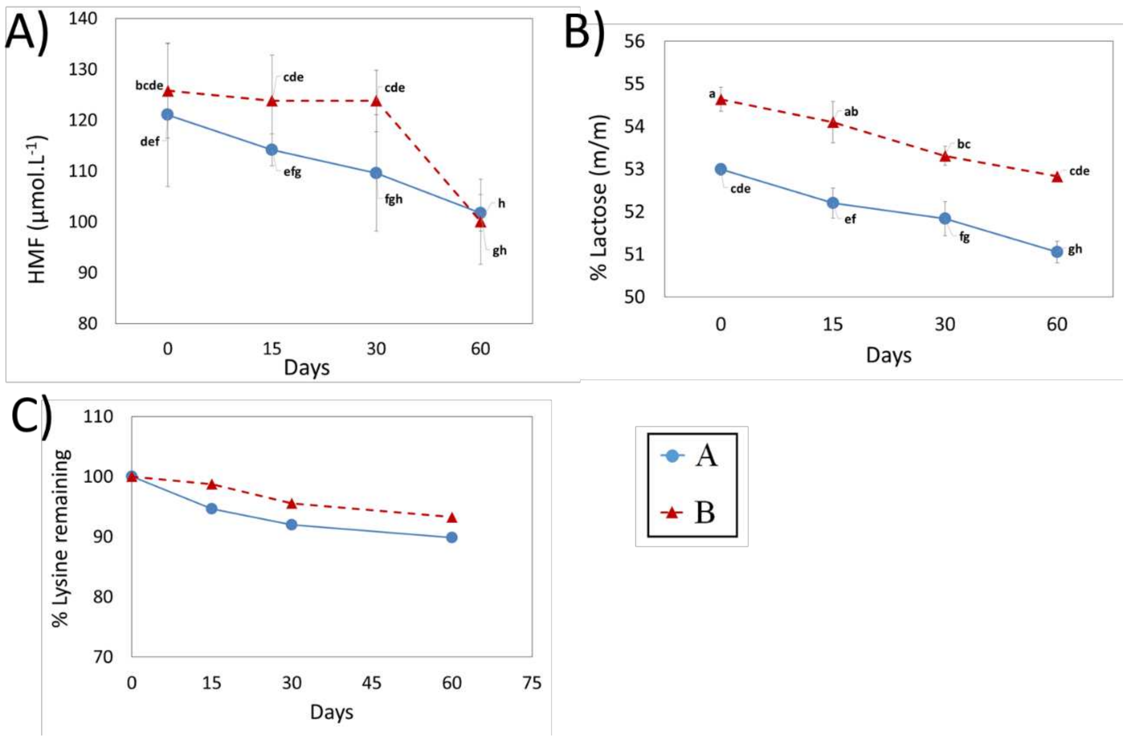

Figure 5 - HMF (A), lactose (B) and lysine remaining (C) in infant formulas stored at 4 ºC. Means followed by equal letters in the same line do not differ at the 5 % level of significance.

This results can be justified by the fact that during Maillard reaction the HMF molecules are simultaneously formed and degraded via oxidation or other transformations as decarboxylation, hydration and reduction, for example (Arribas-Lorenzo & Morales, 2010; Chávez-Servín, Castellote, & López-Sabater, 2005; Jimenez-Perez, Corzo, Morales, Delgado, & Olano, 1992). Therefore under low temperatures, the rate of HMF formation is probably lesser than the degradation rate resulting in the diminution this intermediate over time (Figure 5A).

On the other hand, the lactose and lysine molecules continue to be consumed to form HMF, even in lower rate, when compared with the infant formulas kept at ambient temperature (Figure 5B and 5C). Morales, Romero, & Jiménez-Pérez (1997) reported small losses of HMF at 6 °C in infant formulas. Naranjo, Pereyra Gonzales, Leiva, & Malec (2013) demonstrated that by reducing the storage temperature of lactose-hydrolyzed skim milk powder from 20 to 4 °C the losses of lysine was drastically reduced from 55 to 3 %.

No color variation in the refrigerated infant formula was observed, since there is no formation of melanoidins in sufficient contents to modify this parameter; while the pH followed similar behavior showed at ambient temperature (Figure 6A). However, powders kept under ~ 4 °C showed lower gain in a_w (Figure 6B) and moisture which was 32 % for both brands after 60 days.

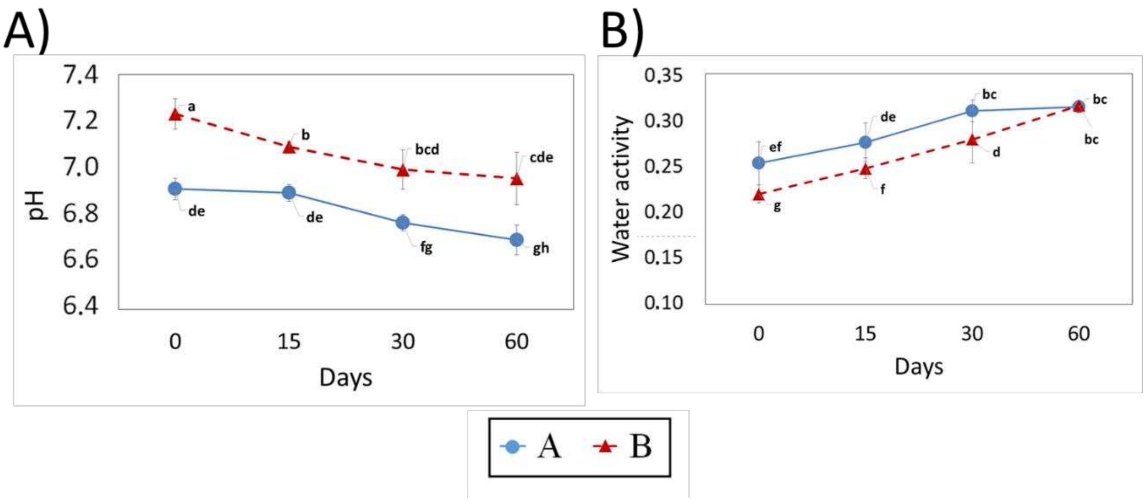

Figure 6 - pH (A) and water activity (B) in infant formulas stored at 4 ºC. Means followed by equal letters in the same line do not differ at the 5 % level of significance.

3.3.3. Maillard reaction in infant formula

Based on ours founds and reports described by literature, a hypothetical mechanism of Maillard reaction propagation in infant formulas will be proposed (Figure 7).

In infant formulas in which lactose is the only reducing sugar present, it becomes necessary in the initial stages of the reaction (Figure 7, step I). Therefore, lactose can reacts with proteins and amino acids as lysine, arginine, methionine, tryptophan, and histidine, conducting the formation of Schiff's bases (Martins & Van Boekel, 2005; Mehta & Deeth, 2016). Between the possible amino acids involved in the reaction, special attention is done to lysine once it is more reactive because its side chain containing a free ε-amino group (Figure 7, step I) (Martins et al., 2000). Note that during the link between the lactose and lysine, one molecule of water is released which can alter the a_w and moisture of powder.

Due to unstable nature of Schiff's bases, additional isomerization (Amadori rearrangement) occurs in an Amadori product (lactulosyl-lysine), under slightly acidic conditions (Figure 7, step II); at this stage the amino acid is nutritionally unavailable and its digestibility is also reduced (Nursten, 2005; Perez-Locas & Yaylayan, 2010).

The initial stages are favored in conditions of high temperatures (40 to 70 °C), elevated humidity (30 to 70 %), acidic or neutral pH, intermediate water activity (0.5 to 0.8), amino acids with high reactivity and reducing sugars, besides the presence of metallic ions and/or light (Newton et al., 2012; Nursten, 2002; Perez-Locas & Yaylayan, 2010).

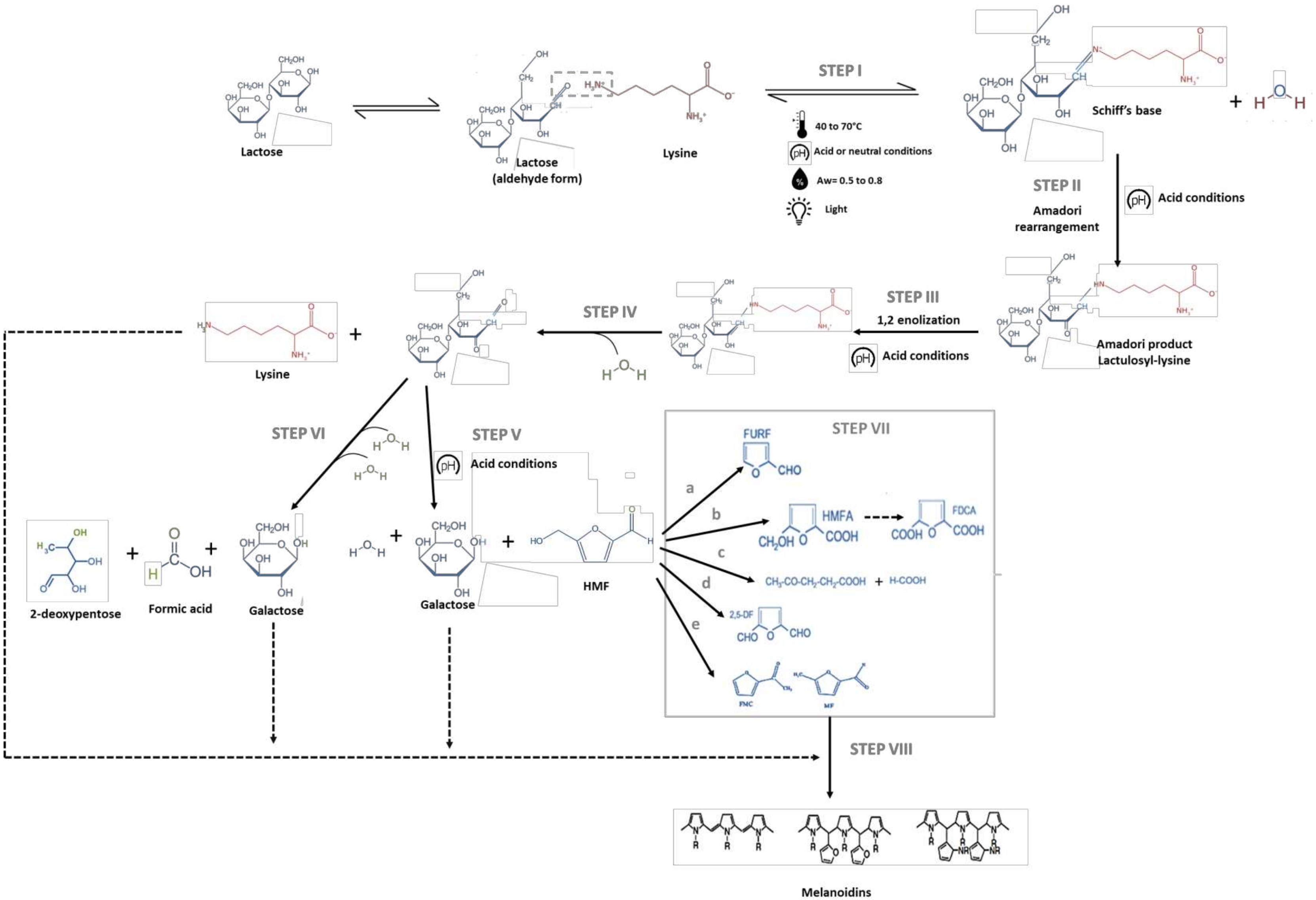

Figure 7 - Schematic presentation of Maillard reaction in infant formulas. Source: adapted from Arribas-Lorenzo & Morales, 2010; Brands & van Boekel, 2001; Chávez-Servín et al., 2005; Perez-Locas & Yaylayan, 2010.

As the storage temperature of infant formulas may influence the reaction rate, there was greater loss of lysine and lactose in infant formulas stored at room temperature than at refrigeration temperatures. Hence, several authors recommended that storage of milks at ≤ 4 ºC, especially the lactose-hydrolyzed, should be compulsory in order to limit protein damage (Mendoza et al., 2005; Messia et al., 2007; Tossavainen & Kallioinen, 2007).

With the prolongation of the heating or storage, the Amadori product undergoes an isomerization reaction called 1,2-enolization in which the ketone group of carbon 2 is broken down and a new double bond is formed between carbons 1 and 2 (Figure 7, step III). The Amadori product in its isomerized form is hydrolyzed, releasing lysine and a molecule (step IV) which is degraded (Figure 7, step V) under acid or neutral conditions, forming furfurals compounds such as HMF and a molecule of galactose (Martins et al., 2000). On here, another molecule of water is released which can alter the a_w and moisture of powder. The furfurals may be related to DNA damage, formation of growth inhibitors, in addition to cytotoxic, genotoxic, mutagenic and carcinogenic activities. They can be produced by the Maillard reaction and lactose isomerization, known as the Lobry De Bruyn-Alberda van Ekenstein transformation (via not shown) (Chávez-Servín et al., 2005).

The degradation of the products of Amadori also forms formic acid (Figure 7, step VI), responsible for the slight decrease in pH in stored dairy products. Berg & van Boekel (1994) reported formic acid as a main degradation reaction product for the Maillard reaction of lactose.

Once the initial steps are dependent of environmental temperature, the conservation of infant formulas under refrigeration reduces both rates of formation of Maillard reaction intermediates and the gain in water (Figure 5A and Figure 6B). A zero-order reaction is observed for the formation of HMF, for instance in milk systems (Claeys et al., 2003; van Boekel, 2001), then it is expected that rates occur at a slower speed at 4 °C. With this 2 times reduction in storage temperature value, the reaction rate is expected to decrease from 9 to 16 times, since the Q_{10} of the reaction is 3-4 (McSweeney & Fox, 2009).

In addition, the fact that in the refrigeration temperature a substantial decrease in HMF has been attributed by other authors to the possibility that part of the HMF thus generated is lost through decarboxylation (Figure 7, step VIIa) or other transformations as oxidation (Figure 7, step VIIb) as hydration (Figure 7, stepVIIc), reduction (Figure 7, stepVIId) and other reactions to form intermediates such as 2-Furaldehyde (F), 2-furyl-methyl ketone (FMC) and 5-methyl-2-furaldehyde (MF) (Figure 7, stepVIIe) (Arribas-Lorenzo & Morales, 2010).

Finally, the compounds originated in the step VII are cyclic and highly reactive which can polymerize with carbohydrates as galactose and amino acid as lysine, resulting in stable compounds and culminating in the molecular weight increase of dark pigments, known as melanoidins (Figure 7, step VIII), responsible for the undesirable color changes in some dairy products (Perez-Locas & Yaylayan, 2010). For the formulas stored under refrigeration, there were no significant changes in color, which is explained by the lower rate of reactions from steps I to VI and VIII.

3.4. Conclusions

For infants, a recommendation of HMF daily intake was not yet proposed by literature although of harms that this compound to health. Taking account that one infant of 6 months with 7.5 Kg intakes approximately 1 L of formula by day, in our worst case

(B-brand after 60 days; Figure 3A), the daily consummation of HMF is 19 μmol. Considering similar the recommended maximum consumption of HMF for infants and adults (20 $\mu mol.kg^{-1}.day^{-1}$), the infant can intake until 150 μmol of HMF by day.

Based on these considerations, it is possible predict that infant formulas with 60 days of storage does not pose health risks in relation to the level of HMF, although the nutritional losses of other components not analyzed can limit the extent of product validity.

Under refrigeration, the infant formulas showed lower losses of lactose and lysine besides maintaining the initial coloration even after 60 days. Thus, it is recommended to keep infant formula under refrigeration to avoid physico-chemical changes and improve the product from a nutritional point of view.

In future works, it is aimed to study sensible components of infant formulas such as vitamins and fatty acids in order to determine the best storage conditions and the expiration date of product under use conditions.

Final considerations

In the infant formulas analyzed, the Maillard reaction possibly happened through the association between lactose and some proteins/aminoacids, such as lysine, in the initial phases of reaction, observed by decreasing levels of these. With the prolongation of the storage, there is production of HMF, formic acid responsible by pH reducing; release of water molecules with water activity and moisture increasing and synthesis of dark polymers as melanoidins.

However, by decreasing the temperature, it was observed a decrease in the Maillard reaction rates and nutritional losses. In addition, it was possible to conclude that infant formulas with 60 days of storage does not pose health risks in relation to the level of HMF. Regarding the composition, almost all the infant formulas analyzed in this study were within the limits established by the legislation. Then, based on the analyzes made, the infant formulas can be consumed up to 60 days.

Despite the importance of the formulas for infant nutrition there are few studies, especially studies that clarify the effect of Maillard reaction on this type of product and what the real harm to the infant.

It is also necessary to revise the micronutrient values, due to their nutritional importance, during the period of use, to assess whether there is any unavailability or loss of any of these. The requirements of trace elements are critical during infancy and early childhood due to the high growth rate of children, and insufficient intake of these can lead to deficiencies that can impair the functioning of the body.

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