**JÚLIA D'ALMEIDA FRANCISQUINI**

# SCIENCE AND TECHNOLOGY APPLIED TO DEMAND OF CONCENTRATED AND DRIED DAIRY PRODUCTS

Thesis presented to Universidade Federal de Viçosa as part of the requirements for the Graduate Program in Food Science and Technology to obtain the title of *Doctor Scientiae.*

Advisor: Antônio Fernandes de Carvalho

Co-advisors: Ítalo Tuler Perrone
Rodrigo Stephani

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APPROVED: March 15th, 2021.

Assent:

Júlia d'Almeida Francisquini
Author

Antônio Fernandes De Carvalho
Advisor

I dedicate to the persons more important of my life my parents, Carolina, Guilherme and my girl, Helena.

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To my parents, sister and family who have always encouraged me to take new steps in my professional career and have always gone to any lengths to help me!

To my husband Guilherme, who has always been by my side at all times and has always supported me in all my decisions!

To my daughter Helena, for her I try to become a better person every day and I try to be the best professional I can be!

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# ABSTRACT

FRANCISQUINI, Júlia d'Almeida, D.Sc., Universidade Federal de Viçosa, March, 2021. **Science and technology applied to demand of concentrated and dried dairy products**. Advisor: Antônio Fernandes de Carvalho. Co-advisors: Ítalo Tuler Perrone and Rodrigo Stephani.

Dairy products exhibit quality from a nutritional point of view, pleasing the consuming public for their versatility and pleasant sensory characteristics. Within the world of dairy products, concentrated and dehydrated products stand out and within this line of research there are growing studies on the reduction of energy costs, product versus process interaction, development of new products to meet market demands and reduce environmental impacts by water and energy savings. Thus, the objective of this thesis was to meet three market demands: increased consumption and production of dairy products with zero lactose, increased consumption of instant dairy products and the study of instantaneousness, increased demand and concern with the nutritional quality of infant formulas. The hydrolysis of lactose in dulce de leche can affect the color, flavor, texture, intensity of the Maillard reaction and even some nutritional aspects. Thus, the first study studied the influence of different levels of lactose hydrolysis, the addition of sucrose and the initial pH in the development of the Maillard reaction. A process simulator with a multiple monitoring system was used to produce fifteen milk sweets. The Box-Behnken $3^3$ experimental design was applied with determination of lipids, proteins, ash, carbohydrates, water activity, dissolved solids, colorimetric and hydroxymethylfurfural analysis. In addition, the rehydration process is complex and occurs in four stages: wettability, sinking, dispersibility and solubility. Thus, the second work sought to analyze dispersibility and solubility by means of particle size distribution in order to establish a new rehydration index for whole powdered milk. The granulometric distribution and morphological characteristics of seven whole milk powder samples (from A to G) were analyzed. Finally, in some cases, mothers unable to breastfeed, need substitutes, nowadays, the substitutes that best mimic human milk are infant formulas. Different technological routes can be designed to produce infant formulas presenting the great challenge: the compromise between safe food and the damage caused by heat treatment. For this reason, the objective of the third work was to review the current scientific knowledge about how heat treatment affects milk macro and micronutrients, extrapolating the expected effects for infant formulas.

Keywords: Dulce de leche. Powdered milk. Infant formula. Whole milk.

# RESUMO

FRANCISQUINI, Júlia d'Almeida, D.Sc., Universidade Federal de Viçosa, março de 2021. **Ciência e tecnologia aplicada à demanda de produtos lácteos concentrados e desidratados**. Orientador: Antônio Fernandes de Carvalho. Coorientadores: Ítalo Tuler Perrone e Rodrigo Stephani.

Os produtos lácteos exibem qualidade do ponto de vista nutricional agradando o público consumidor por sua versatilidade e característica sensorial agradável. Dentro do mundo dos lácteos, destacam-se os produtos concentrados e desidratados e dentro desta linha de pesquisa crescem estudos da redução dos custos de energia, interação produto versus processos, desenvolvimento de novos produtos para atender às demandas de mercado e redução dos impactos ambientais por economia de água e energia. Assim, o objetivo desta tese foi atender à três demandas do mercado: aumento do consumo e da produção de lácteos com zero lactose, crescimento do consumo de produtos lácteos instantâneos e do estudo da instantaneidade, aumento da demanda e da preocupação com a qualidade nutricional de fórmulas infantis. A hidrólise da lactose em doce de leite pode afetar a cor, o sabor, a textura, intensidade da reação de Maillard e até mesmo alguns aspectos nutricionais. Desta forma, o primeiro trabalho estudou a influência de diferentes níveis da hidrólise da lactose, da adição de sacarose e do pH inicial no desenvolvimento da reação de Maillard. Um simulador de processo com sistema de monitoramento múltiplo foi usado para produzir quinze doces de leite. O delineamento experimental Box-Behnken $3^3$ foi aplicado com determinação de lipídios, proteínas, cinzas, carboidratos, atividade de água, sólidos dissolvidos, análise colorimétrica e hidroximetilfurfural. Além disto, o processo de reidratação é complexo e ocorre em quatro etapas: molhabilidade, afundamento, dispersibilidade e solubilidade. Assim, o segundo trabalho buscou analisar a dispersibilidade e a solubilidade por meio da distribuição granulométrica a fim de estabelecer um novo índice de reidratação para o leite em pó integral. Foram analisadas a distribuição granulométrica e as características morfológicas de sete amostras de leite em pó integral. Por fim, em alguns casos, mães impossibilitadas de amamentar, necessitam de substitutos, nos dias de hoje, os substitutos que melhor mimetizam o leite humano são as fórmulas infantis. Diferentes rotas tecnológicas podem ser projetadas para produzir fórmulas infantis apresentando o grande desafio:

o compromisso entre alimentos seguros e os danos causados pelo tratamento térmico. Por isto, o objetivo do terceiro trabalho foi revisar o conhecimento científico atual sobre como o tratamento térmico afeta os macro e micronutrientes do leite, extrapolando os efeitos esperados para as fórmulas infantis.

Palavras-chave: Doce de leite. Leite em pó. Fórmula infantil. Leite integral.

**SUMMARY**

# 1. GENERAL INTRODUCTION

The removal of water from milk is a way of preserving and offering it in new forms to the consumer. It leads to an increase in the shelf life, which limits the possibility of microorganism's growth. Concentrated and dried milks are classified as dairy products, resulting from evaporation, membrane separation and drying of milk by specific technological processes. Dulce de leche and milk powders are relevant products in concentrated and dried dairy products, and the main research topics are the reduction of energy costs, product versus process interaction, development of new products fitting market demands and reduction of environmental impacts by saving water and energy (De carvalho et al., 2013).

The global dairy market of lactose free products (45% to 50%) is shared by multinationals industries. The U.S global lactose free products market was at US$ 10,582.5 million at the end of 2017 and estimated in US$ 17,809.4 million in 2027 increasing 5,3% in 10 years (FMI, 2019). The demand for zero lactose products grows to meet the increase in the population that is really lactose intolerant or adept to functional lactose-free diets (Luthy et al., 2017). When a dulce de leche with hydrolyzed lactose is produced, there is an increase in the availability of glucose and galactose, which have higher solubility in water, sweeter in water, being directly fermented and immediately absorbed in the intestine, and may also assist in the control or even avoiding the appearance of lactose crystals noticeable on the palate. However, such hydrolysis can mainly affect the sensory characteristic of dulce de leche, increasing the difficulty to reach the product point in addition to resulting in different color (darker), texture (increased viscosity) and flavor (sweeter) characteristics of the traditional product in which lactose is intact. Such modifications have as main cause the intensification of the Maillard reaction and its products formation, for the greater concentration of reducing sugars (Sakkas et al., 2014).

Powdered dairy products, besides long storage periods, display physical and functional properties, are allowed to be used as ingredients in dairy industries (e.g. fermented milk, processed cheese, and ice cream) as well as in other food industries such as bakery, confectionery and meat. When used as ingredient, milk powder improves nutritional value of the final product while contributes to properties as color, texture, flavor, humidity, total solids, yields, emulsification, gelation, solubility, viscosity, foaming, and whipping (Sharma et al., 2012). The efficient rehydration of the powdered dairy product is essential so that the powder when added to water has a good wettability, penetrability, dispersivity, and solubility reaching the expected consumer characteristics (Richard et al., 2013). Tests to determine rehydration properties are usually highly dependent on the measurement technique and the classification scale, which in turn force industries to develop their own methods, adapted to specific applications or to meet clients' demands (Boiarkina et al., 2016). Works have been conducted with the aim at developing easier, more convenient and reproducible methods to evaluate the dissolution properties of dairy powders (Boiarkina et al., 2016).

Breast milk as the children's primary source of nutrition fulfils the babies' needs and can also provide immune protection. In some cases, when mothers are not able to breastfeed, an equivalent substitute is required. Nowadays, the best substitutes of the human breast milk are infant formulas (Euclydes, 2014). The infant formula represents about 70% of the world market for baby food and, in 2010, this percentage was around 40% (Talbot, 2015). The global baby food and infant formula market reached about US$ 48 billion by 2016 (Euromonitor International, 2016). Infant formulas are found in two readily consumable forms: liquid concentrated and powder form. The preparation of this food requires successive heat treatments, which can cause product problems. Both from the technological and sensorial point of view as well as from the nutritional aspect, which can lead to damages to the child's health. (Kliegman et al., 2014; Jiang and Guo, 2014). It is very important that these products provide sufficient quantity and quality of nutrients. Therefore, the food industry needs to gradually improve the elaboration techniques of infant formulas in order to be more similar to breast milk, thus reducing its negative consequences for the child's health (Euclydes, 2014).

In view of the above considerations, the thesis aimed to analyze and meet three current demands by three different papers:

- Higher number of individuals with lactose intolerance increasing the demand and consequent production of zero lactose products. **PAPER 1:** 5-Hydroxymethylfurfural formation and color change in lactose-hydrolyzed Dulce de leche.
- Growth of the consumer market for instant dairy products requiring mechanisms to assess such instantaneousness. **PAPER 2:** Particle size distribution applied to milk powder rehydration.

- Increased demand for infant formulas and increased concern for the nutritional quality of this product. **PAPER 3:** How the heat treatment affects the constituents of infant formulas: a review.

** The papers presented below are in the format requested by the different journals in which the works were published.

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## 2. PAPER 1

FRANCISQUINI, Júlia d'Almeida et al. 5-Hydroxymethylfurfural formation and color change in lactose-hydrolyzed Dulce de leche. **The Journal of dairy research**, v. 86, n. 4, p. 477-482, 2019.

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**Research Article**

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Color; dairy; evaporation; lactose-free; Maillard reaction

**Author for correspondence:**
Ítalo Tuler Perrone,
Email: italotulerperrone@gmail.com

### 5-Hydroxymethylfurfural formation and color change in lactose-hydrolyzed Dulce de leche

Júlia d'Almeida Francisquini[1], Júlia Rocha[1], Evandro Martins[1], Rodrigo Stephani[2], Paulo Henrique Fonseca da Silva[3], Isis Rodrigues Toledo Renhe[4], Ítalo Tuler Perrone[5] and Antônio Fernandes de Carvalho[1]

[1]Department of Food Technology, Federal University of Viçosa (UFV), 36570-900, Viçosa, MG, Brazil; [2]Department of Chemistry, Federal University of Juiz de Fora (UFJF), 36036-330, Juiz de Fora, MG, Brazil; [3]Department of Nutrition, Federal University of Juiz de Fora (UFJF), 36036-330, Juiz de Fora, MG, Brazil; [4]Candido Tostes Dairy Institute (EPAMIG) – Rua Tenente Luís de Freitas, 116, Santa Terezinha, 36045-560, Juiz de Fora, MG, Brazil and [5]Department of pharmaceutical sciences, Federal University of Juiz de Fora (UFJF), Campus Universitário – Rua José Lourenço Kelmer, s/n – São Pedro, 36036-330, Juiz de Fora, MG, Brazil.

**Abstract**

The work described in this Research Communication concerns the production of Dulce de leche (DL), that is a traditional product from South America obtained by concentration. Maillard reaction (MR) products are mainly responsible for the formation of color and flavor in this product. Lactose-hydrolyzed products have been developed to supply consumer demand, but this hydrolysis may affect the flavor, color, taste, texture and even some nutritional aspects of the product. We studied the influence of different levels of lactose-hydrolysis, sucrose addition and initial pH on the development of MR, appraised by the determination of 5-hydroxymethylfurfural (HMF). A process simulator with multi-monitoring system was used to produce 15 DL. Box-Behnken $3^3$ experimental design was applied for the three factors: pH, lactose-hydrolysis level and sucrose concentration. Lipids, protein, ashes, carbohydrates, water activity, dissolved solids, colorimetric analysis and HMF (free and total) are among the physicochemical attributes and MR indicators analyzed in this work. The products showed significant differences in composition but all the values were in agreement with the literature. Moreover, higher levels of lactose hydrolysis and higher pH presented a direct relation with the development of MR, observed by an increase in coloration (lower luminosity) and more formation of HMF, both free and total. The present study expands the knowledge about DL spread made of lactose-hydrolyzed milk, allowing the food industries to produce a lactose free DL with nutritional and sensory characteristics closer to the traditional product.

About 70% of the worldwide adult population presents some level of lactose intolerance, with higher incidence in individuals of South America, Africa and Asia (Lule *et al.*, 2015). It is estimated that 50% of dairy products manufactured by multinational dairy companies are lactose free (Lule *et al.*, 2015), although not all are marketed as such. Among the industrial technologies, lactose reduction by the action of β-galactosidase (lactase) enzyme has been the most applied method for dairy production. This enzyme hydrolyzes the lactose into glucose and galactose whose concentrations increase in the final product. The methodology can be applied to various dairy products, including the concentrated ones such as Dulce de leche (DL: Rodriguez *et al.*, 2016).

DL is typically produced by countries in South America (Sabione *et al.*, 1984) and has the potential to be commercialized on the international market. Considering its high amount of lactose, approximately 10.43% w/w (Perrone *et al.*, 2011), the use of lactase enzyme is a promising solution to the formation of lactose crystals on the product (Sabione *et al.*, 1984) and, at the same time, to allow its consumption by people with intolerance to this carbohydrate.

DL is produced by milk concentration at considerably high temperatures (Brazil, 1997). Hence, the hydrolyzed product is prone to develop Maillard reaction (MR) products because of the higher availability of reducing carbohydrates (galactose and glucose). Besides compromising nutritional aspects, excessive MR may alter the sensory characteristics of DL because of more intense color or sandiness texture (Walstra *et al.*, 2006). Therefore, ways to monitor and control the development of MR during low lactose DL production must be considered.

The kinetics of MR product formation is influenced by factors as pH, type and amount of amine/reducing sugar, and temperature of heating treatment (Martins *et al.*, 2001). By changing these factors it is possible to control the intensity of the reaction and, consequently, the excess accumulation of dark compounds (melanoidins) that contribute to the non-conform color of the product (Francisquini *et al.*, 2017).

Therefore, the aim of this study was to monitor the development of MR in lactose hydrolyzed DL through evaluation of 5-hydroxymethylfurfural (HMF) concentration. The effects of



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# 5-Hydroxymethylfurfural formation and color change in lactose-hydrolyzed dulce de leche

Júlia d'Almeida Francisquini[1], Júlia Rocha[1], Evandro Martins[1], Rodrigo Stephani[2], Paulo Henrique Fonseca da Silva[3], Isis Rodrigues Toledo Renhe[4], Ítalo Tuler Perrone[5]* and Antônio Fernandes de Carvalho[1]

[1]Department of Food Technology – Federal University of Viçosa (UFV), 36570-900, Viçosa, MG, Brazil.juliafrancisquininutri@gmail.com; evandrombi@yahoo.com.br; afc1800@yahoo.com.
[2]Department of Chemistry – Federal University of Juiz de Fora (UFJF), 36036-330, Juiz de Fora, MG, Brazil.rochajulia13@gmail.com; rodrigostephani@gmail.com;
[3]Department of Nutrition – Federal University of Juiz de Fora (UFJF), 36036-330, Juiz de Fora, MG, Brazil. paulohenrique.ufjf@gmail.com.
[4]Candido Tostes Dairy Institute (EPAMIG) – Rua Tenente Luís de Freitas, 116, Santa Terezinha, 36045-560, Juiz de Fora, MG, Brazil. isis@epamig.br
[5]Department of pharmaceutical sciences - Federal University of Juiz de Fora (UFJF), Campus Universitário - Rua José Lourenço Kelmer, s/n - São Pedro, 36036-330, Juiz de Fora, MG, Brazil. italotulerperrone@gmail.com

Short title: **5-Hydroxymethylfurfural and color in dulce de leche**

*Correspondence: Ítalo Tuler Perrone
Department of pharmaceutical sciences
Federal University of Juiz de Fora
Campus Universitário
José Lourenço Kelmer, s/n, São Pedro
36036-330
Juiz de Fora, MG, Brazil.
+553221023893
*italotulerperrone@gmail.com*

## Summary

This Research Paper is about Dulce de leche (DL) that is a traditional product from South America obtained by concentration. Maillard reaction (MR) is mainly responsible for the formation of color and flavor on this product. Lactose-hydrolyzed products have been developed to supply a consumers demand but this hydrolysis may affect the flavor, color, taste, texture and even some nutritional aspects of the product. The aim of this work was to study the influence of different levels of lactose-hydrolysis, sucrose addition and initial pH on the development of MR, appraised by the determination of 5-hydroxymethylfurfural (HMF). A process simulator with multi-monitoring system was used to produce the fifteen DL. Box-Behnken $3^3$ experimental design was applied forthe three factors: pH,lactose-hydrolysis level and sucrose concentration. Lipids, protein, ashes, carbohydrates, water activity, dissolved solids, colorimetric analysis and HMF (free and total) are among the physicochemical attributes and MR indicators analyzed in this work. The products showed significant differences in composition but all the values were in agreement with the literature. Moreover, higher levels of lactose hydrolysis and higher pH presented a direct relation with the development of MR, observed by an increase in coloration (lower luminosity) and more formation of HMF, both free and total. The present study expands the knowledge about DL spread made of lactose-hydrolyzed milk, allowing the food industries to produce a lactose free DL with nutritional and sensory characteristics closer to the traditional product.

**Key-words:** Lactose-free; Maillard reaction; dairy; color; evaporation.

**Introduction**

About 70% of worldwide adult population presents some level of lactose intolerance, with higher incidence in individuals of South America, Africa and Asia (Lule et al., 2016). Global dairy marketestimates 50% of products manufactured by multinational companies to be lactose free (Lule et al., 2016). Among the industrial technologies, lactose reduction by the action of β-galactosidase (lactase) enzyme has been the most applied method for dairy production. This enzyme hydrolyzes the lactose into glucose and galactose whose concentrations increase in the final product. The methodology can be applied to various dairy products, including the concentrated ones as dulce de leche (Rodriguez et al., 2016).

DL is typically produced by countries in South America (Sabione et al., 1984) and has potential to be commercialized on international market. Considering its high amount of lactose, approximately 10.43% w/w (Perrone et al., 2011), the use of lactase enzyme is a promising alternative to the formation of lactose crystals on the product (Sabione et al., 1984) and, at the same time, to allow its consumption by people with certain degree of intolerance to this carbohydrate.

DL is produced by milk concentration at considerable high temperatures (Brazil, 1997). Hence, the hydrolyzed product is keen to develop Maillard reaction because of the higher availability of reducing carbohydrates (galactose e glucose). Besides compromising nutritional aspects, excessive MR may sensory alter the dulce the leche because of more intense color or sandiness texture (Walstra et al., 2006). Ergo, ways to monitor and control the development or Maillard reaction during low lactose DL production must be consider.

The kinetics of MR products formations is influenced by factors as pH, type and amount of amine/reducing sugar, and temperature of heating treatment (Martins et al., 2001). Changing this factors is possible to control the intensity of the reaction and, consequently, the excess accumulation of dark compounds (melanoidins) that contribute to the non-conform color of the product (Francisquini et al., 2017).

Therefore, the aim of this study is to monitor the development of Maillard reaction in lactose hydrolyzed DL through evaluation of 5-hydroxymethylfurfural (HMF) concentration. The effects of sucrose concentration, pH and level of hydrolysis on HMF content and the products color were also assessed. Two hypotheses guided this work. The first was: the HMF amount in DL is dependent of the percentage of added sucrose, pH of milk and lactose hydrolysis level. The second was: the intensity of dulce de leche color cannot be explained only by HMF amount formed during evaporation.

**Material & methods**

Brazilian legislation (1997) allows a maximum 30 kg of sucrose per 100 L of milk for dulce de leche production. Therefore, concentrations of 15, 20 or 25% $w \times v^{-1}$ of sucrose were studied. To determine the effect of the variables lactose hydrolysis, pH and sucrose content on HMF formation in dulce de leche, a factorial Box-Behnken $3^3$ design with 15 trials was defined (Table 1).

DL spread was produced from pasteurized whole milk previously adjusted for pH and lactose content, then added of sucrose according to the methodology described by Perrone et al. (2011). The ingredients (milk and sugar) were mixed and concentrated by evaporation in a process simulator Thermomix® TM5 (Vorwerk, Wuppertal, Germany) coupled with a load cell (Ramuza IDR 7.500, Santana de Parnaíba, Brazil) of 1g precision and a temperature sensor PT-100. The process was continued until concentration of 68 ± 2 °Brix was measured by digital refractometer (AR200, Reichert Technologies Analytical Instruments, New York). The warm products were packaged in 200g plastic containers and storage at room temperature until analysis.

Water activity ($a_w$) was analyzed by AquaLab (4 ATE model, Meter Group Inc., São Paulo). The colorimetric analyses were determined by Hunter Lab colorimeter (Color Quest XE, Reston, USA). All the analyses were conducted in duplicate.

Moisture analysis was conducted by drying 1.5 g of DL at 105°C to an oven (model 404D, Nova Ética, São Paulo). Fat content was analyzed using Gerber method. Protein was analyzed trough micro Kjeldahl method using 1 mL of sample solution (20% $m \times v^{-1}$) of DL. Ashes content was obtained after incineration of 1 g of sample in furnace at 550°C (LF0612 model, Jung, Florida). Sample composition analysis followed AOAC International official methods. All the analyses were done in duplicate.

DL samples were collected throughout the production on times 0, 20, 40 and 80 min to monitor total and free HMF formation. All analyses were conducted in duplicate according to methodology of Keeney and Bassette (1959). HMF concentration was obtained from the analytical curve.

The results were analyzed using Minitab® 17.3.1, Excel, 2011 and MATLAB 7.10 software. More detailed information's are described in the supplementary material.

**Results and discussion**

Analyses of the products (Table 2) confirmed that all formulations meet the legislation standards for DL physical-chemical and compositional criteria (Brasil, 1997; Perrone et al. 2011) despite variations on lactose hydrolysis degree, sucrose content and pH (Silva et al., 2015). Therefore, it is possible to infer that the process simulator used on this study is an efficient laboratorial tool to simulate industrial processes of DL production on open pan evaporator.

Products 1, 2, 3 and 4 (Tables 2), without lactose reduction, can be considered traditional formulations applied on the production of DL. Hence, for the purpose of this study, these formulations will be considered as control for HMF concentration and color formation against lactose hydrolyzed products.

*Hydroxymethyl furfural analysis throughout production time*

The formulations showed a HMF formation potential of 16,478 ± 7,838 $\mu mol \times g^{-1}$. The differences can be explained by variations on the raw material. However, the dulce de leches showed lower free HMF values than total HMF, which suggests that heating was not enough to induce the maximum formation of this compound (Tabela 1).

Considering control samples (Free HMF = 55.3 $\mu mol \times g^{-1}$), samples with lactose hydrolysis >99 % (Free HMF = 110.4 $\mu mol \times g^{-1}$) and 50 % hydrolysis (Free HMF = 86.1 $\mu mol \times g^{-1}$) presented respectively means 2.0 and 1.5 times bigger than control samples. This result strongly suggests that lactose hydrolysis has big influence on HMF formation in dulce de leche.

Other authors also observed that lactose hydrolyzed dairy products tend to present higher HMF formation and consequently higher concentration of MR products (Jansson et al., 2014).

The increase in sucrose concentration did not necessarily result in higher rates of HMF accumulation and its contribution to HMF formation was no apparent. Sucrose is a non-reducing sugar and does not directly take part in Maillard reaction with the formation of HMF (Newton et al., 2012).

As observed for % of sucrose, the pH influence on HMF formation was variable. The pH affects the intensity of Maillard reaction with a maximum velocity at the alkaline range of pH 9 and 10 (Francisquini et al., 2017; Rodriguez et al., 2016). Milk pH is within this ideal range so, when heat treated, this food becomes very susceptible to the increase of MR concentration and your products formation (Van Boekel, 1998).

An acidity reducer is added during dulce the leche manufacturing with the aim of reducing or avoiding precipitation, besides help the development of sensorial characteristics of the food (Perrone, 2011). Therefore, this ingredient influences pH increase, resulting in darker coloration of DL. According to Francisquini et al. (2018), free HMF index tends to increase in dulce de leches with higher concentration of sodium bicarbonate (acidity reducer).

Specifically in this study, the pH variation (6.85 to 7.05) was limited in order to avoid casein micelles precipitation during production of dulce de leche, hence the influence of this parameter was not well established as in other studies.

Analysis of each parameter separate indicated that % of lactose hydrolysis has a central role on the rate of HMF accumulation on dulce de leche. However, the role of variables as % of sucrose and pH was not well enlightening. On the other hand, it is feasible to consider that these parameters synergistic take part on the HMF formation which justifies an analysis of the simultaneous contributions.

*HMF relation versus lactose hydrolysis, sucrose concentration, and pH*

With the aim of evaluating the synergistic effect of the variables, contour curves were created keeping at least one of the parameters constants (Figure 1). The major HMF accumulation is observed in dulces with higher lactose hydrolysis and lower amount of sucrose (Figure 1A); higher degree of hydrolysis and pH slightly alkaline (Figure 1B); and low amount of sucrose and pH alkaline (Figure 1C). The analysis of variables combined two by two allows inferring that the variables take part to HMF formation in higher or lower levels.

From the Box-Behnken $3^3$ factorial design, it is possible to identify that the parameters do not independently act on the HMF formation and the contribution of each variable can be predicted by equation 2 at level of 95% confidence.

Eq 2. $$\text{Free HMF} = -36303 - 2.56\times H - 45.9\times S + 10546\times pH - 0.00126\times H^2 - 0.026\times S^2 - 766\times pH^2 - 0.0640\times H\times S + 0.650\times H\times pH + 7.10\times S\times pH$$

Where: Free HMF = HMF concentration expressed as μmol×$g^{-1}$; H = % hydrolysis; S = % sucrose; pH = potential of hydrogen ion activity

*Relation colorimetric analysis and HMF*

According to lightness values, samples can be grouped as light (L>40) or dark ($L \leq 40$) (Figure 1). Dulces with hydrolyzed lactose tend to be darker than the traditional ones, which can be partly explained by a higher accumulation of HMF and final products of Maillard reaction (melanoidins).

Therefore, HMF seems to influence the dark coloration on the dulces, while pH is decisive in luminosity definition.

This happens because the Maillard reaction begins with a condensation of a reducing sugar with an amino group of an aminoacid, peptide or protein. The increase of pH promotes a nucleophilic attack, which is necessary to start the condensation, and consequently promoting a bigger stimulus to the start the Maillard reaction. On the other hand, the decrease in pH decreases the nucleophilic attack, stopping the condensation and the consequent beginning of MR (Newton, 2012; Walstra et. al., 2006). Therefore, high pH increases Maillard reaction and its products formation (HMF), resulting in darker foods.

*Relation of HMF, physical chemical and compositional attributes*

From Principal Component Analysis (PCA) it is possible to verify that samples without lactose hydrolysis (1, 7, 3 and 5) formed one group. Products with 50% and 100% of lactose hydrolysis (4, 8, 9, 10, 13, 14 and 15) were grouped far from the ones with 0% of hydrolysis. Characteristics of physical-chemistry, composition and HMF formation were similar between samples in a group. Ergo, it is clear that lactose hydrolysis presented bigger influence on the results found for the analyzed attributes when compared to sucrose concentration and pH.

## Conclusions

Free HMF is a compound indicator of Maillard reaction and can be related to dulce de leche color. Despite the formation of this indicator be influenced by processing time, sucrose content, pH of milk and by lactose hydrolysis level, this work demonstrated that the final color of dulce de leche cannot be only explained by higher free HMF concentration on the product.

The pH was a central factor on the definition of the intensity of luminosity on the product, which implies that this sensorial attribute can be controlled by adjusting the initial pH of the formulation. From this observation, it is possible to bring free-lactose products sensory closer to traditional and well established products in the market.

**Acknowledgment**: This work was supported by Research Foundation of Minas Gerais State (FAPEMIG), Brazilian National Council for Scientific and Technological Development (CNPq) and Coordination for the Improvement of Higher Education Personnel (CAPES).

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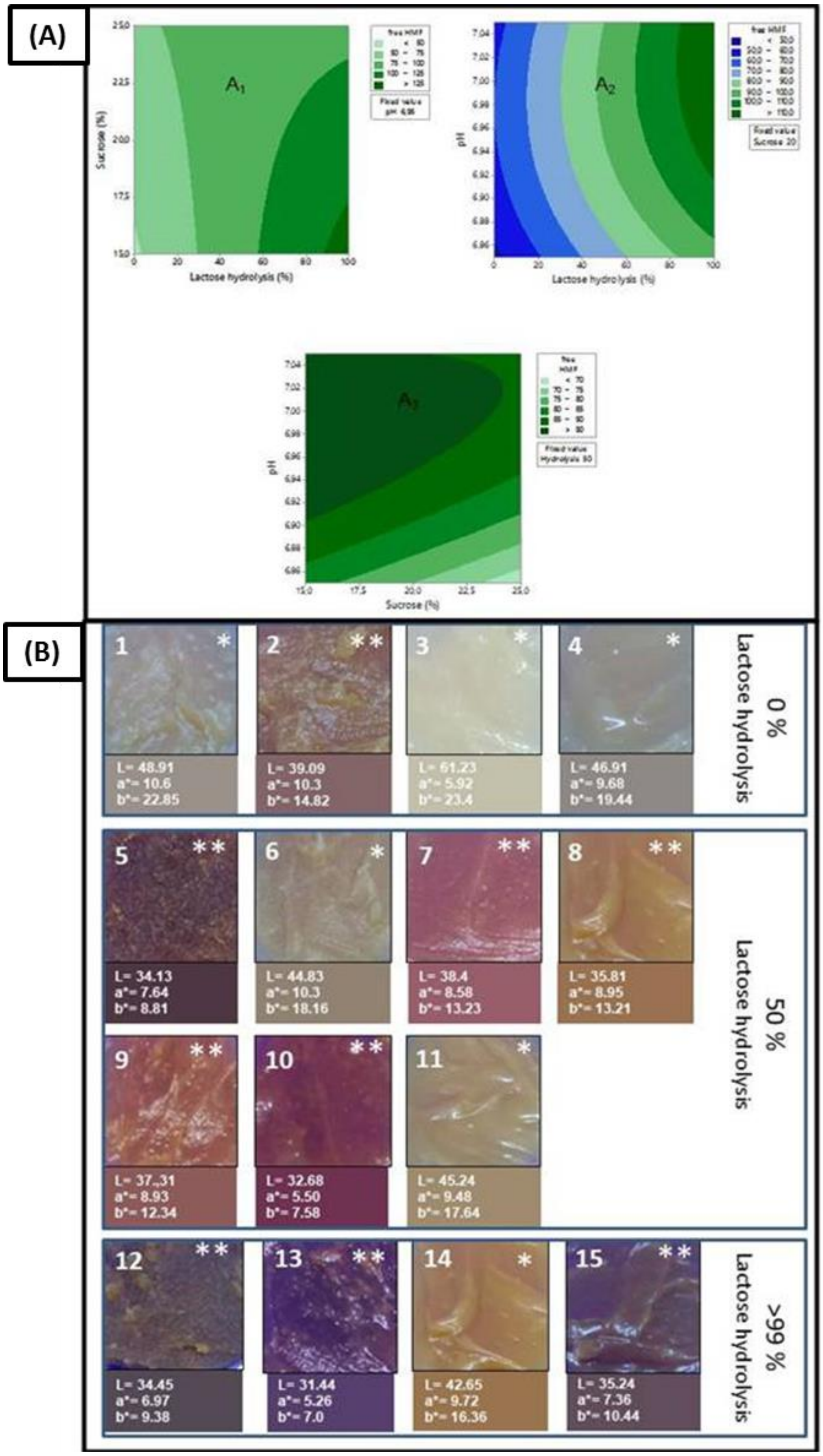
**Figure legends:**

**Figure 1:**

**(A)** Contour graph of free HMF formed during dulce de leche production: ($A_1$) constant pH at level 0 (6.95); ($A_2$) sucrose content constant at level 0 (20%); ($A_3$) lactose hydrolysis constant at level 0 (50%). **(B) V**isual appearance and color analysis of dulce de leche: Lightness (L), red/green coordinate ($a^*$) and yellow/blue coordinate ($b^*$) of the samples with different levels of lactose hydrolysis.* Light samples ($L > 40$); ** Dark samples ($L \leq 40$).

**Figure 1:**


(A)
A1
Sucrose (%)
Lactose hydrolysis (%)
free HMF
Fixed value
A2
pH
Lactose hydrolysis (%)
free HMF
Fixed value
Sucrose 20
A3
pH
Sucrose (%)
free HMF
Fixed value
Hydrolysis 50
(B)
1 *
L= 48.91
a*= 10.6
b*= 22.85
2 **
L= 39.09
a*= 10.3
b*= 14.82
3 *
L= 61.23
a*= 5.92
b*= 23.4
4 *
L= 46.91
a*= 9.68
b*= 19.44
0 % Lactose hydrolysis
5 **
L= 34.13
a*= 7.64
b*= 8.81
6 *
L= 44.83
a*= 10.3
b*= 18.16
7 **
L= 38.4
a*= 8.58
b*= 13.23
8 **
L= 35.81
a*= 8.95
b*= 13.21
9 **
L= 37.,31
a*= 8.93
b*= 12.34
10 **
L= 32.68
a*= 5.50
b*= 7.58
11 *
L= 45.24
a*= 9.48
b*= 17.64
50 % Lactose hydrolysis
12 **
L= 34.45
a*= 6.97
b*= 9.38
13 **
L= 31.44
a*= 5.26
b*= 7.0
14 *
L= 42.65
a*= 9.72
b*= 16.36
15 **
L= 35.24
a*= 7.36
b*= 10.44
>99 % Lactose hydrolysis

**Tables legends:**

**Table 1:** Box-Behnken $3^3$ design matrix for dulce de leche.

**Table 2:** Statistical analysis of Dulce de leche composition and free HMF just after production.

**Table 1:**

| Trials | Hydrolysis (level) | Hydrolysis (%) | Sucrose (level) | Sucrose (%) | pH (level) | Ph |
|---|---|---|---|---|---|---|
| **1** | -1 | 0 | -1 | 15 | 0 | 6.95 |
| **5** | | | 0 | 20 | +1 | 7.05 |
| **7** | | | 0 | 20 | -1 | 6.85 |
| **3** | | | +1 | 25 | 0 | 6.95 |
| **9** | 0 | 50 | -1 | 15 | +1 | 7.05 |
| **11** | | | -1 | 15 | -1 | 6.85 |
| **13** | | | 0 | 20 | 0 | 6.95 |
| **14** | | | 0 | 20 | 0 | 6.95 |
| **15** | | | 0 | 20 | 0 | 6.95 |
| **10** | | | +1 | 25 | +1 | 7.05 |
| **12** | | | +1 | 25 | -1 | 6.85 |
| **2** | +1 | >99,9 | -1 | 15 | 0 | 6.95 |
| **6** | | | 0 | 20 | +1 | 7.05 |
| **8** | | | 0 | 20 | -1 | 6.85 |
| **4** | | | +1 | 25 | 0 | 6.95 |

**Table 2:**

| Product | $X_1$ (% of lactose hydrolysis) | $X_2$ (% of sucrose) | $X_3$ (pH) | Dissolved solids (°Brix) | $a_w$ | Moisture (g/100g) | Protein (g/100g) | Lipids (g/100g) | Ashes (g/100g) | Carbohydrates* (g/100g) | Free HMF (μmol.g$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Reference values** | | | | 66 a 68 | 0.83 – 0.89 | Max.30.0 | Min. 5.0 | 6.0 to 9.0 | Max. 2.0 | Max. 30 kg.100L$^{-1}$ of milk | - |
| **1** | 0 | 15 | 6.95 | 66.5±0.2$^{e}$ | 0.849±0.001$^{a}$ | 28.1±0.2$^{a}$ | 8.4±0.4$^{abc}$ | 8.5±0.0$^{a}$ | 2.2±0.1$^{a}$ | 52.8±0.5$^{b}$ | 47.6±1.9$^{a}$ |
| **5** | | 20 | 7.05 | 72.5±0.1$^{a}$ | 0.825±0.000$^{bcd}$ | 24.6±1.1$^{a}$ | 6.4±0.1$^{abc}$ | 6.5±0.0$^{bc}$ | 2.0±0.0$^{b}$ | 64.2±0.0$^{a}$ | 59.5±1.3$^{ab}$ |
| **7** | | | 6.85 | 72.2±0.5$^{a}$ | 0.831±0.003$^{b}$ | 31.3±7.3$^{a}$ | 6.6±0.5$^{abc}$ | 6.8±0.4$^{bc}$ | 1.8±0.0$^{cd}$ | 53.6±6.5$^{b}$ | 53.9±5.9$^{a}$ |
| **3** | | 25 | 6.95 | 69.4±0.0$^{bcd}$ | 0.829±0.002$^{bc}$ | 24.3±0.7$^{a}$ | 6.8±1.4$^{abc}$ | 7.0±0.0$^{abc}$ | 1.6±0.0$^{f}$ | 60.4±0.8$^{ab}$ | 60.2±3.4$^{ab}$ |
| **9** | 50 | 15 | 7.05 | 70.3±0.2$^{b}$ | 0.796±0.004$^{g}$ | 24.5±0.7$^{a}$ | 5.5±0.6$^{bc}$ | 7.0±0.0$^{abc}$ | 2.3±0.0$^{a}$ | 53.8±0.1$^{b}$ | 85.6±1.3$^{cd}$ |
| **11** | | | 6.85 | 68.8±0.1$^{d}$ | 0.817±0.001$^{de}$ | 26.4±0.1$^{a}$ | 8.8±0.7$^{ab}$ | 6.0±0.0$^{c}$ | 2.0±0.0$^{b}$ | 56.7±0.9$^{ab}$ | 74.6±0.0$^{ce}$ |
| **13** | | 20 | | 70±0.1$^{bc}$ | 0.806±0.005$^{fg}$ | 26.4±0.4$^{a}$ | 8.6±1.6$^{abc}$ | 7.0±0.0$^{bcd}$ | 1.9±0.0$^{c}$ | 56.1±1.2$^{b}$ | 88.7±1.9$^{d}$ |
| **14** | | | | 69.7±0.4$^{bcd}$ | 0.809±0.002$^{ef}$ | 26.9±0.7$^{a}$ | 9.0±0.8$^{a}$ | 6.0±1.4$^{abc}$ | 1.8±0.0$^{cd}$ | 56.3±0.2$^{b}$ | 90.7±2.2$^{d}$ |
| **15** | | | 6.95 | 69.1±0.0$^{bcd}$ | 0.816±0.002$^{def}$ | 27.7±0.7$^{a}$ | 6.1±0.4$^{abc}$ | 6.0±0.0$^{ab}$ | 1.8±0.0$^{de}$ | 54.5±0.6$^{b}$ | 96.2±0.0$^{d}$ |
| **10** | | 25 | 7.05 | 68.7±0.0$^{d}$ | 0.834±0.000$^{b}$ | 27.9±1.3$^{a}$ | 6.9±1.7$^{abc}$ | 5.5±0.0$^{c}$ | 1.7±0.0$^{ef}$ | 57.9±0.4$^{ab}$ | 96.2±3.1$^{d}$ |
| **12** | | | 6.85 | 66.3±0.0$^{e}$ | 0.836±0.001$^{b}$ | 31.5±0.4$^{a}$ | 6.7±0.6$^{abc}$ | 5.5±0.0$^{c}$ | 1.4±0.0$^{g}$ | 54.9±0.2$^{b}$ | 71.0±1.9$^{be}$ |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **2** | 99 | 15 | 6.95 | 72.4±0.8$^{a}$ | 0.770±0.000$^{h}$ | 24.2±0.1$^{a}$ | 7.9±0.9$^{abc}$ | 8.0±0.0$^{ab}$ | 2.3±0.0$^{a}$ | 60.1±0.0$^{ab}$ | 144.5±0.0$^{f}$ |
| **6** | | 20 | 7.05 | 68.9±0.4$^{cd}$ | 0.825±0.000$^{bcd}$ | 28±1.4$^{a}$ | 5.3±0.1$^{c}$ | 6.8±0.4$^{bc}$ | 1.8±0.0$^{cd}$ | 58.0±1.9$^{ab}$ | 111.3±8.1$^{g}$ |
| **8** | | | 6.85 | 68.8±0.2$^{d}$ | 0.809±0.002$^{ef}$ | 26.9±1.5$^{a}$ | 5.4±0.2$^{c}$ | 5.8±0.4$^{c}$ | 1.7±0.0$^{de}$ | 55.6±2.1$^{b}$ | 92.7±3.1$^{d}$ |
| **4** | | 25 | 6.95 | 69.4±0.2$^{bcd}$ | 0.819±0.002$^{cde}$ | 26.3±1.7$^{a}$ | 5.7±0.1$^{bc}$ | 6.0±0.0$^{c}$ | 1.6±0.0$^{f}$ | 60.5±1.6$^{ab}$ | 93.1±1.9$^{d}$ |
| **Means** | - | - | - | 69.5±1.8 | 0.818±0.018 | 27.0±2.7 | 6.9±1.4 | 6.6±0.9 | 1.9±0.3 | 57.0±3.4 | 84,4±24,4 |
| **Minimum value** | - | - | - | 66.3 | 0.770 | 23.8 | 5.1 | 5.0 | 1.4 | 49.0 | 46,2 |
| **Maximum value** | - | - | - | 72.9 | 0.850 | 36.4 | 9.7 | 8.5 | 2.3 | 64.2 | 144,5 |
| **Standard deviation of the mean** | - | - | - | 0.3 | 0.003 | 0.5 | 0.3 | 0.4 | 0.0 | 1.9 | 3,2 |

*calculated values

**Limits established by legislation (Brazil, 1997) and/or literature (Perrone *et al.,* 2011)

***For free HMF there are no reference values.

# 3. SUPPLEMENTARY FILE OF PAPER 1

## 5-Hydroxymethylfurfural formation and color change in lactose-hydrolyzed dulce de leche

Júlia d'Almeida Francisquini[1], Júlia Rocha[1], Evandro Martins[1], Rodrigo Stephani[2], Paulo Henrique Fonseca da Silva[3], Isis Rodrigues Toledo Renhe[4], Ítalo Tuler Perrone[5]* and Antônio Fernandes de Carvalho[1]

### Introduction

European companies operations on this market allow Occident Europe to be considered the biggest producer of lactose free milk and dairy products. According to Future Market Insights report (APEJ, 2018), North America market for dairy products with lactose free claim was worth US$ 10,582.5 million by the end of 2017 with growing projection to became US$ 17,809.4 million by the end of 2027. Therefore, the partial or total hydrolysis of lactose have been adopted by food industries as consequence of an increased number of consumers with lactose intolerance or that adopted a lactose free diet.

Composition, physical chemistry characteristics, colorimetric analysis and heat treatment indicators are important parameters of characterization, classification and processing monitoring applied during manufacture. And, according to Van Boekel (1998), 5-hydroxymethylfurfural or HMF concentration direct increases with the intensity of heat treatment, so it is consider a good indicator for MR in dairy products. Besides, this indicator can be easily detected by spectrophotometric technics (Francisquini et al., 2018), which make it easy the continuous assessment even by less specialized personnel.

### Material & methods

*Lactose-hydrolysis in milk*

Pasteurized whole milk was added of 0.2% $w.w^{-1}$ of lactase (Maxilact-DSM®) and kept at 34 ±1°C for 24 h, according to Fialho et al. (2017). The hydrolysis was conducted until ~100% lactose concentration reduction as measured by Lactozym® Pure Lacto Monitor™ (Novozymes/Dinamarca) following manufacturer instructions.

Pasteurized whole milk with 50% of lactose reduction was obtained by mixture of equal amounts of regular and lactose free milk.

Food industry adopts the total or partial hydrolysis of lactose on dairy products manufacture (Luthy et al., 2017), hence 50 and 100% reduction were studied on this work. Pasteurized whole milk (0% lactose hydrolysis) was used as control.

*Adjustment of milk pH*

The pH of whole milk with or without lactose hydrolysis was measured with pH meter (PG 180 - Gehaka) and adjustments were done with sodium bicarbonate (Farmax).

To avoid technological problems as protein precipitation during production, milk initial pH must be around 7 (Perrone et al., 2007; Fennema, 2010). For this work, milk pH was adjusted to 6.85, 6.95 or 7.05.

*Dulce de leche production*

Brazilian legislation (1997) allows a maximum 30 kg of sucrose per 100 L of milk for dulce de leche production. Therefore, concentrations of 15, 20 or 25% $w.v^{-1}$ of sucrose were studied.

The warm products were packaged in 200g plastic containers and storage at room temperature until analysis.

To determine the effect of the variables lactose hydrolysis, pH and sucrose content on HMF formation in dulce de leche, a factorial Box-Behnken $3^3$ design with 15 trials was defined (Table 1).

**Table 1:** Box-Behnken $3^3$ design matrix for dulce de leche.

| Trials | Hydrolysis (level) | Hydrolysis (%) | Sucrose (level) | Sucrose (%) | pH (level) | pH |
|---|---|---|---|---|---|---|
| **1** | -1 | 0 | -1 | 15 | 0 | 6.95 |
| **5** | | | 0 | 20 | +1 | 7.05 |
| **7** | | | 0 | 20 | -1 | 6.85 |
| **3** | | | +1 | 25 | 0 | 6.95 |
| **9** | 0 | 50 | -1 | 15 | +1 | 7.05 |
| **11** | | | -1 | 15 | -1 | 6.85 |
| **13** | | | 0 | 20 | 0 | 6.95 |
| **14** | | | 0 | 20 | 0 | 6.95 |
| **15** | | | 0 | 20 | 0 | 6.95 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **10** | | | +1 | 25 | +1 | 7.05 |
| **12** | | | +1 | 25 | -1 | 6.85 |
| **2** | +1 | >99,9 | -1 | 15 | 0 | 6.95 |
| **6** | | | 0 | 20 | +1 | 7.05 |
| **8** | | | 0 | 20 | -1 | 6.85 |
| **4** | | | +1 | 25 | 0 | 6.95 |

*Water activity and colorimetric analysis*

The colorimetric analyses were determined through direct reading of system reflectance of coordinates L* (lightness), a* (red/green coordinate) and b* (yellow/blue coordenate), using CIELAB color scale with Illuminant D65 and standard observer function of 10° (Nachtigall *et al*. 2009).

*Analysis of moisture, lipids, protein, ashes and carbohydrates*

In moisture analysis the sample was weighted on a dish with sand and took to an oven. The samples were periodically removed from the oven, put on a desiccator to cool down and weigh. The process was repeated until constant weigh.

Fat analysis was performed in a milk butyrometer with scale range from 0 to 8% (Original, São Paulo), 11 mL of dulce de leche solution (20 % $m.v^{-1}$) was added replacing milk. After mixing with the other reagents, the butyrometer was centrifuged for 5 min at 200xg (ThermoScientific™ Heraeus™ Biofuge™ Stratos™ ThermoFisherScientific, EUA) and transferred to boiling bath at 65± 2ºC for 10 min before direct reading on the butyrometer scale. The result was obtained after multiplication by dilution factor (5x).

The carbohydrates were obtained by difference from the other determined attributes.

*Analysis of Maillard reaction indicators*

Free HMF was analyzed without dilution on times 0 and 20 min, while 4 g of dulce were diluted in 20 g of distilled water before analysis on times 40 and 80 min. Total HMF was analyzed after dilution of 0.25 g of dulce de leche in 100 g of water.

For free HMF, 5 mL of sample/solution was transferred to a tube and added 5 mL of oxalic acid 0.3 $mol.L^{-1}$(> 99.5 % PA; Dinâmica) and 5 mL of trichloroacetic acid 40% w/v (TCA > 99 % PA; Vetec). After filtration in paper filter (Quanty – J. Prolab, 15 cm, 8 µm), 4 mL of permeated was transferred to a tube, added 1 mL of thiobarbituric acid 0.05 mol.L$^{-1}$ (> 97.5 % PA; Merck) and then heated at 40°C for 30 min. Afterwards, samples were read in UV/Visible spectrophotometer (model Evolution 60S, ThermoScientific®, Madison) at 443 nm. For total HMF, the sample and the oxalic acid were kept in boiling water for 60 min, cooled down and then added the of TCA. From this point, the analysis followed the same protocol of free HMF.

HMF concentration (µmol.kg$^{-1}$) was obtained from the analytical curve (Figure 1):

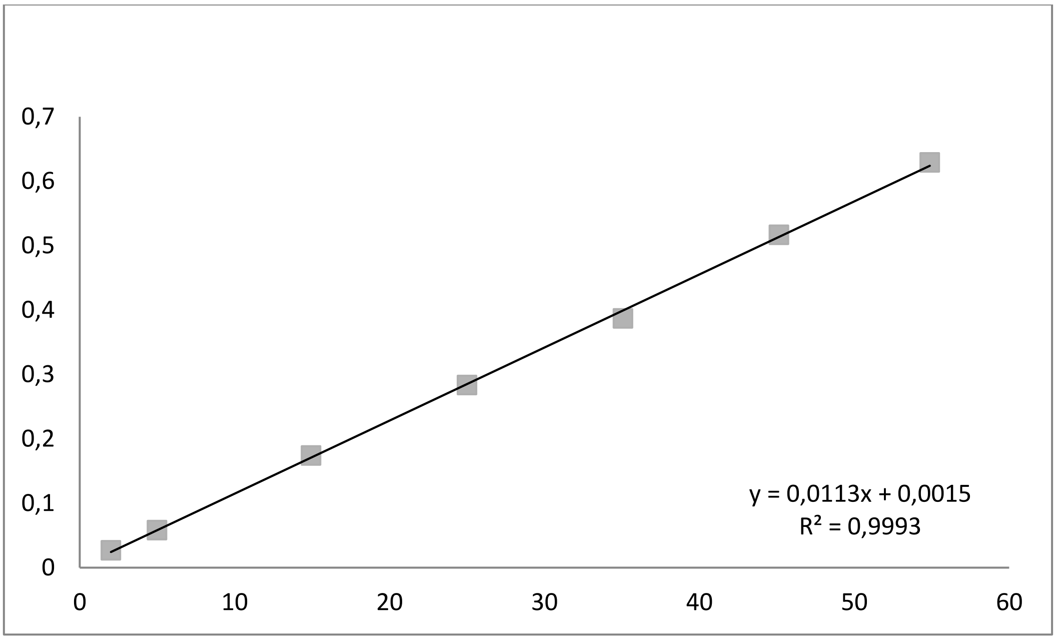



**Figure 1:** Standard curve for obtaining the concentration of 5-hydroxymethylfurfural (µmol.kg$^{-1}$). In which y represents the absorvance; x represents HMF content; and $R^2$ is the coefficient of correlation.

*Statistical analysis*

MATLAB 7.10 software was used for Principal Components Analysis (PCA). The data were normalized using SNV (Standard Normal Variate) pre-processing, which normalizes the data using the weighted average. In the model constructed by PCA, two main components (CPs) were chosen, with about 98% of the total variance captured. The choice of CPs was based on the graph of eigenvalues versus number of principal components.

**Results and discussion**

Dulce de leche production with or without lactose hydrolysis on manufacture scale is produced in open pan evaporators with steam jacket. Considering the necessity to precisely control product's temperature to study the development of Maillard reaction, a process simulator was used to better adjust heating rate and agitation.

*Hydroxymethylfurfural analysis throughout production time*

During dulce de leche production, the ingredients mixture (milk + sucrose) was kept at controlled temperature of 110 ± 15 °C until concentration achieved 62 °Brix. Production time took between 80 and 100 min depending on formulation.

Periodically analyses during the 80 min production length for the different formulations (Figure 1) were used to evaluate the HMF accumulation rate. Free HMF versus processing time curves were built from groups of similar samples with same degree of lactose hydrolysis (Figure 1A), percentage of sucrose (Figure 1 B) or pH (Figure 1C).

The relation between Free HMF concentration and processing time can be adjusted by quadratic equations as shown by Figure 1. Free HMF accumulation rate at 80 minutes increased within the increase of the degree of lactose hydrolysis, with values of 0.32; 0.58 e 0.67 $\mu mol.g^{-1}.min^{-1}$ for 0, 50 e >99% of hydrolysis, respectively (Figure 1A).

Considering samples with the same percentage of sucrose (Figure 1B), HMF accumulation rate in 80 minutes of process was 0.40; 0.57 and 0.53 $\mu mol.g^{-1}.min^{-1}$ for treatments with 15, 20 and 25% of sucrose. Using the same approach for samples at the same pH (Figure 1C), HMF accumulation rate was 0.58; 0.43 and 0.58 $\mu mol.g^{-1}.min^{-1}$ for pH 6.85; 6.95 and 7.05, respectively.

The rate of change in HMF concentration in the samples were influenced to a greater or lesser extent by the % of lactose hydrolysis, % of sucrose and pH of the formulations (Figure 1). The major rate of variation on HMF accumulation (0.32 to 0.67 $\mu mol.g^{-1}.min^{-1}$) was observed for % of lactose hydrolysis, which suggests that the presence of glucose and galactose promote Maillard reaction propagation.

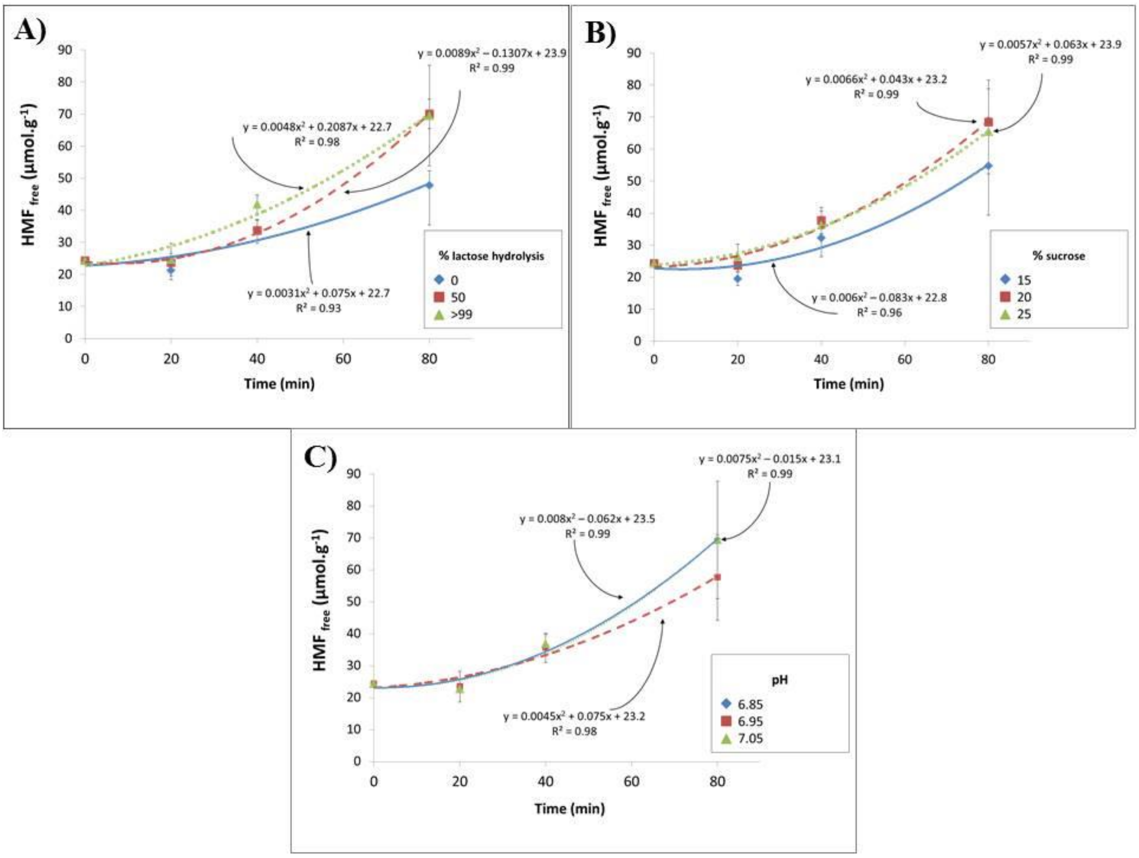



**Figure 2:** Kinetics of Free HMF accumulation during processing of different dulce de leche formulations. Lactose hydrolysis effect (A); sucrose percentage (B) and pH (C).

Maillard reaction starts with the condensation of a reducing sugar with the amino group of aminoacids, which is more likely to happen at higher pH (pH>9) (Fennema, 2010). Ergo, slightly alkaline dairy products are expected to present a tendency to accumulate more HMF.

*Relation colorimetric analysis and HMF*

Formulation at pH 6.85 and 7.05 were respectively light and dark, despite the level of hydrolysis or sucrose concentration (Table 2). Products at pH 6.95 presented different colors as function of the level of lactose hydrolysis: 0% lactose hydrolysis (light color); 50 or >99% lactose hydrolysis (dark color) (Table 3). Sucrose concentration did not present any influence on the samples classification as dark or light.

**Table 2:** Color of formulations at pH 6.85 or 7.05.

| Trials | Hydrolysis(%) | Sucrose (%) | pH | Color |
|---|---|---|---|---|
| **2** | 0 | 20 | 7.05 | Dark |
| **3** | | 20 | 6.85 | Light |
| **5** | 50 | 15 | 7.05 | Dark |
| **6** | | 15 | 6.85 | Light |
| **10** | | 25 | 7.05 | Dark |
| **11** | | 25 | 6.85 | Light |
| **13** | >99 | 20 | 7.05 | Dark |
| **14** | | | 6.85 | Light |

**Table 3:** Color of formulations  at pH 6.95.

| Trials | Hydrolysis (%) | Sucrose (%) | pH | Color |
|---|---|---|---|---|
| **1** | 0 | 15 | 6.95 | Light |
| **4** | 0 | 25 | | Light |
| **8** | 50 | 20 | | Dark |
| **12** | >99 | 15 | | Dark |
| **15** | >99 | 25 | | Dark |

Dulces with lower luminosity show a tendency of higher HMF concentration, despite poor correlation of a direct relation between these two factors (Figure 3).

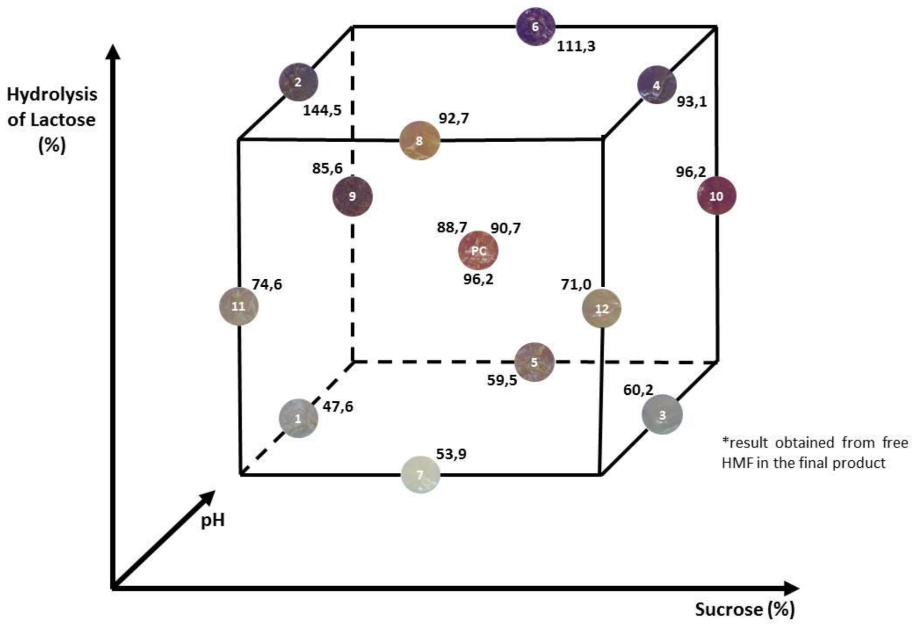



**Figure 3:** Schematic representation of the three levels used on the Box-Behnken design.

*Relation of HMF, physical chemical and compositional attributes*

The Box-Behnken desing used on this work is a rotation class or approximately rotation class, corresponding to a second order design and being based on an incomplete factorial design of three levels. These three levels can be graphically represented to help understand the obtained results (Ferreira *et al*. 2007). Figure 3 shows the graph that represents this study.

From Figure 3, it is possible to observe the products arrangement according to the parameters: % of sucrose, % of lactose hydrolysis and pH, in addition to the specific coloration of each product and its respective free HMF index. This graph confirms the previous presented results and shows the clear influence of the different parameters used on this design on coloration and free HMF index.

Figure 4 shows the Principal Component Analysis (PCA), which was elaborated to graphically show the observations, to facilitate the visualization and to evaluate the similarities and differences between the attributes analyzed in the present work.

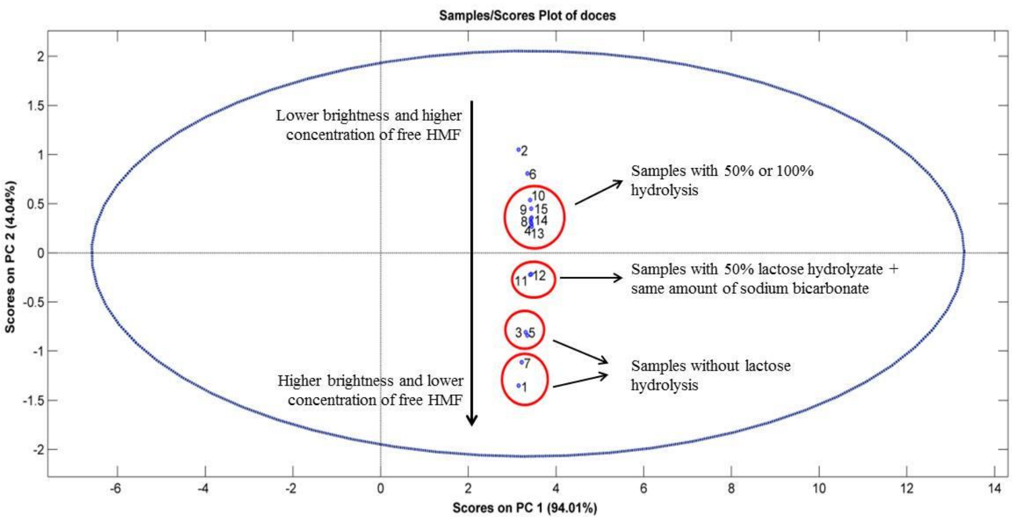



**Figure 4:** Graphic of scores PC1 versus PC2 of composition characterization data and free HMF formation at the times 0, 20, 40, 60, 80 and final minutes of evaporation (soluble solids content, water activity, moisture content, protein, lipid, ashes, carbohydrate, luminosity, free HMF 0, free HMF 20, free HMF 40, free HMF 80, free HMF final).

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**Declarations of interest:** none

**Contributors**: All authors participated materially in the research and / or preparation of the article and all authors approved the final article.

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## 4. PAPER 2

FRANCISQUINI, Júlia d'Almeida et al. PARTICLE SIZE DISTRIBUTION APPLIED TO MILK POWDER REHYDRATION. **Química Nova**, v. 43, n. 2, p. 226-230, 2020.



PARTICLE SIZE DISTRIBUTION APPLIED TO MILK POWDER REHYDRATION

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Luiz Fernando Cappa De Oliveira
Federal University of Juiz de Fora

# PARTICLE SIZE DISTRIBUTION APPLIED TO MILK POWDER REHYDRATION

**Júlia d'Almeida Francisquini[a], Evandro Martins[a], Isis Rodrigues Toledo Renhe[b], Luiz Fernando Cappa de Oliveira[c*], Rodrigo Stephani[c], Ítalo Tuler Perrone[d], Antônio Fernandes de Carvalho[a]**

[a]Department of Food Technology – Federal University of Viçosa (UFV), Avenida Peter Henry Rolfs, s/n - Campus Universitário, 36570-900, Viçosa, MG, Brazil.
[b]CandidoTostes Dairy Institute (EPAMIG) – Street Tenente Luís de Freitas, 116, Santa Terezinha, 36045-560, Juiz de For a, MG, Brazil.
[c]Department of Chemistry – Federal University of Juiz de Fora (UFJF), Campus Universitário - Rua José Lourenço Kelmer, s/n - São Pedro, 36036-330, Juiz de Fora, MG, Brazil.
[d]Department of pharmaceutical sciences - Federal University of Juiz de Fora (UFJF), Campus Universitário - Rua José Lourenço Kelmer, s/n - São Pedro, 36036-330, Juiz de Fora, MG, Brazil.

[*]e-mail: luiz.oliveira@ufjf.edu.br
PARTICLE SIZE DISTRIBUTION APPLIED TO MILK POWDER REHYDRATION

Besides increasing shelf life of fluid milk, milk powder has physical and functional properties that allow it to be used as ingredient. The rehydration process is complex and happens in four steps: wettability, sinkability, dispersibility and solubility. Works have been conducted aiming at the development of an easier, more convenient and reproducible method to evaluate the dissolution properties of dairy powders. Therefore, the aim of this work was to evaluate dispersibility and solubility through particle size distribution in order to establish a new rehydration index for whole milk powder. The particle size distribution and morphological characteristics of seven samples of milk powder (from A to G) were analyzed. Samples F and C differed from the others which formed a similar group. Principal component analysis divided the samples into three different groups, allowing the indication of an efficient rehydration index to determine the powders dispersability.

Key words: Dairy products; Laser diffraction; Microscopy; Dispersibility; Solubility.

## INTRODUCTION

Milk is very important for diet because of its nutritional value, which may build up immune protection and aid in the prevention of some illnesses. However, the nutrients and high humidity make it very perishable. Hence, dairy products as milk powder became an alternative to increase shelf live (OLORUNNISOMO, 2008). Besides long storage periods, milk powder displays physical and functional properties that allow it to be used as ingredient in dairy industries (e.g. fermented milk, processed cheese, and ice cream) as well as in other food industries such bakery, confectionery and meat. When used as ingredient, milk powder improves nutritional value of final product while contributes to properties as color, texture, flavor, humidity, total solids, yields, emulsification, gelation, solubility, viscosity, foaming, and whipping (ALVAREZ et al., 2005; BARBUT, 2010; SHARMA, JANA, 2012).

The rehydration process in water is complex and can be described in four stages or inherent properties of the powder. The first is wettability, the powder ability to absorb water on its surface and to penetrate the surface of water; second comes the sinkability, which is based on the powder capacity to immerse in water after getting wet; dispersibility comes next and represents the aggregates separation with release of individual particles; in the end comes solubility, a characteristic related to the speed of dissolution and total solubility which is also related to the quantity of material dissolved in a saturated solution (SHARMA, JANA, 2012; GAVA, 1977; RICHARD et al., 2013).

Brazilian law sets dispersibility for instant milk powder (%w/w) as 85% for the whole product and 90 % for the skimmed and the partially skimmed milk powder (BRASIL, 1997). The International Dairy Federation (IDF) sets the ISO 17758 | IDF 087 methodology as reference for dispersibility determination, which is by definition the percentage by mass of the dry matter of the samples that can be dispersed in a defined amount of water. Water content of the sample must be previously analyzed for this determination. However, tests to determine rehydration properties are usually highly dependent on the measurement technique and the classification scale, which in turn force industries to develop their own methods, adapted to specific applications or to meet clients demands (BOIARKINA et al., 2017). Works have been conducted with the aim of developing easier, more convenient and reproducible methods to evaluate the dissolution properties of dairy powders (BOIARKINA et al., 2017; LEE et al., 2014).

It is well known that these properties are influenced by composition and chemistry of particles surface, as well as particle size distribution and density of the particles (BOIARKINA et al., 2017). Ergo, analysis of particle size distribution has been frequently used to characterize rehydrated milk powder (MIMOUNI et al., 2010; TORRES et al., 2017).

In this way, this work intended to evaluate dispersibility and solubility through particle size distribution in order to establish a new rehydration index for whole milk powder.

## EXPERIMENTAL PART

Seven samples of whole milk powder from different brands were analyzed (A, B, C D, E, F, G codification).The powders morphological analyses were conduct through images of scanning electron microscopy - SEM (x500) (Hitachi TM 3000, Hitachi Ltd., Tokyo, Japan). Particle size distribution was analyzed by laser diffraction (Beckman Coulter LS 13 320, Miami, FL, EUA) coupled to the aqueous liquid module (Beckman Coulter, Miami, FL, EUA).

Powders form samples, without rehydration, were slowly added to the analysis module, filled with room temperature water at 25 °C, until reading signal levels of 50 % + or – 5 % were achieved on the photodetectors PIDS (Polarization Intensity Differential Scattering System). Data were collected on the region of 0.04 to 2.000 μm every 90 seconds over 5 different times (1.5; 3.0; 4.5; 6.0; and 7.5 minutes).

Results were calculated with refractive indices of 1.332 for the dispersant (water) and 1.57 for the casein micelles, and 1.47 for the fat globules aiming at the observation of total solubility (MIMOUNI et al., 2009; MICHALSKI, BRIARD, MICHEL, 2001). Data were represented by the percentage of occupied volume by the particles as a function of their size. Statistical analyses were conducted with the equipment software.

## RESULTS AND DISCUSSION

Figure 1 shows the morphological analysis images for the different brands of powders. According to the morphological results, it is possible to observe that sample F presented lower amounts of fine particles, big individual particles and probably the occlusion of air was bigger during spray drying, resulting in different particle structure and agglomeration. In contrast, sample C presented a pattern of higher amount of broken particles. The remaining samples showed similar characteristics of agglomeration and presence of fine particles.

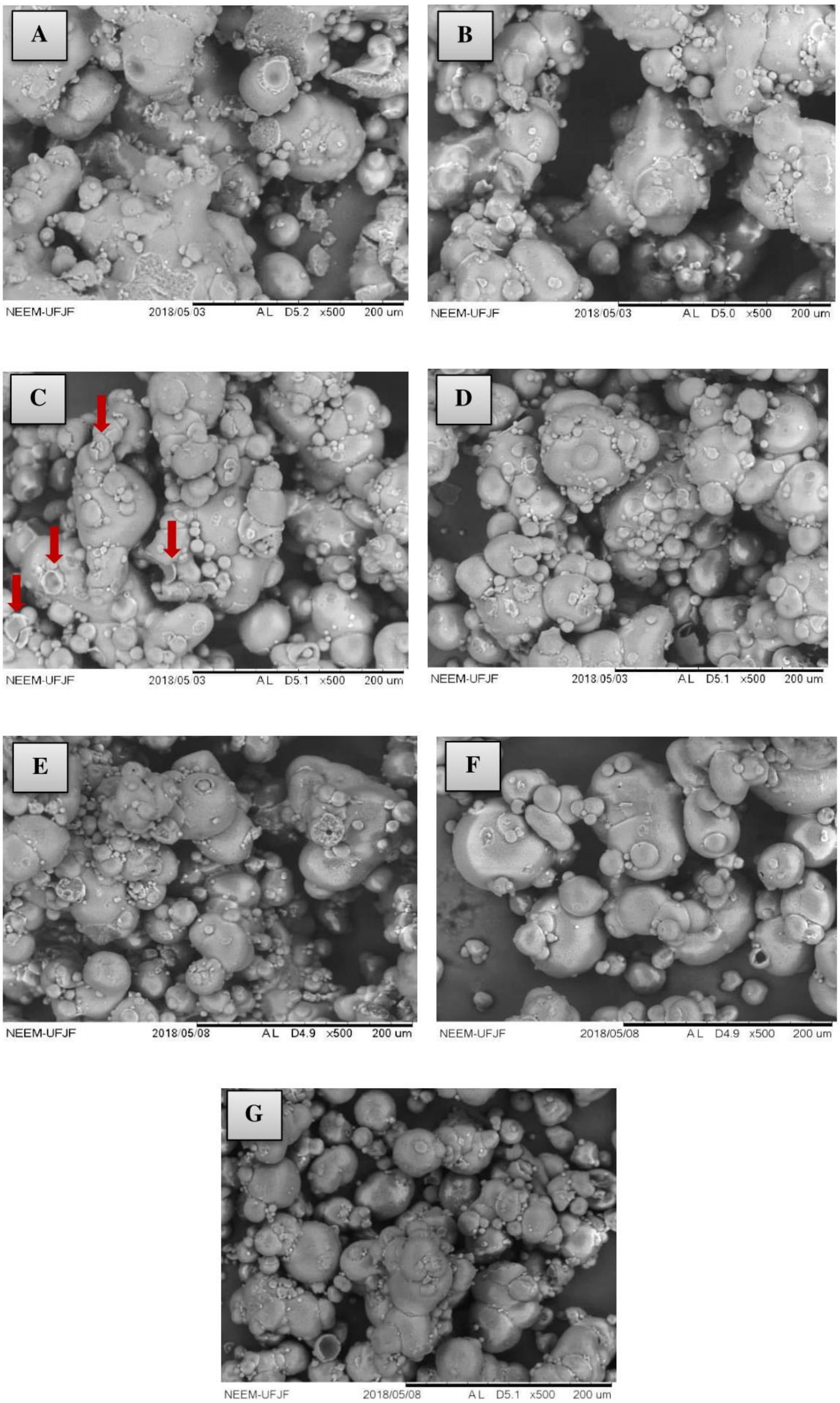


***Figure 1.*** *SEM images (x500) of samples*

The properties of the powders can be determined by the type of agglomeration which is affected by the process used to return the fines to the system (TAMIME, 2009). If the fines are added close to the atomizer, the high humidity leads to more compacted agglomerates with a structure that resembles an onion. On the contrary, if the distance from the fines introduction to the atomizer is increased, the agglomerates are less compacted and called raspberry or grapes structures in descending order of compaction.

Particle size distribution was analyzed to understand the rehydration process and the results are shown in Figure 2.

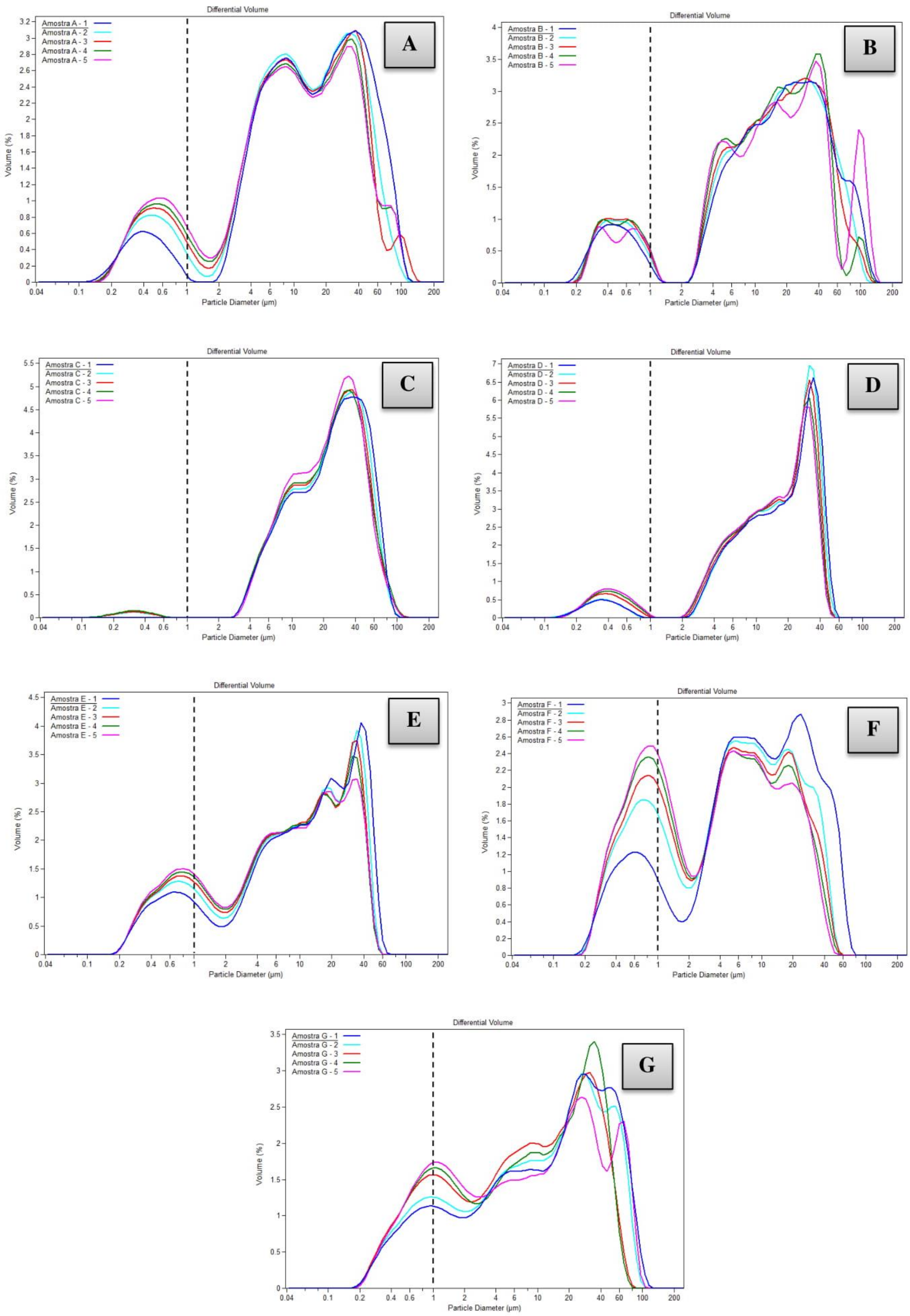


***Figure 2.*** *Particle size distribution over time (1.5; 3.0; 4.5; 6.0 e 7.5 minutes)*

Each sample showed a particular pattern for particle size distribution but, once again, samples F and C presented a distinguished behavior among the other samples. There was also some similarity of samples A and B with sample F, while samples D, E and G had a pattern more like sample C.

The proposed methodology considers that the ideal condition for particles distribution is in the region under 1µm, the nanometric region of caseins. This could be observed with sample F, which also displayed an increase in particles at this region with the course of analysis time. On the contrary, sample C particles stayed above 1µm, which can be considered the region in which fat and other non-hydrated particles can be found. The caseins may occupy the region above 1 µm when participating in the formation of fat globules after homogenization for milk powder production (VIGNOLLES et al., 2007).

Therefore, statistical analyses of particle size distribution were conducted to confirm the differences among the analyzed samples, as shown in Table 1.

**Table 1.** Particle size distribution of milk powder samples on the last reading (7.5 minutes)

| Sample | $d_{10}$ (µm) | $d_{90}$ (µm) | $d_{100}$ (µm) | <1µm (% volume) |
|---|---|---|---|---|
| A | 0.66 | 44.64 | 133.70 | 13.90 |
| B | 0.86 | 71.09 | 161.20 | 11.10 |
| C | 7.02 | 50.84 | 133.70 | 0.00 |
| D | 2.25 | 33.59 | 57.77 | 9.81 |
| E | 0.59 | 33.17 | 69.61 | 18.20 |
| F | 0.49 | 21.61 | 57.77 | 27.70 |
| G | 0.69 | 54.66 | 121.80 | 16.40 |
| Means | 1.79 | 44.23 | 105.08 | 13.87 |

Note: $d_{10}$, $d_{90}$ and $d_{100}$ represent 10 %, 90 % and 100 %, respectively, of the cumulative particle size distribution.

Statistical analysis confirmed the differences for samples F and C while the others showed more similar pattern and values among each other. Chemometric analysis was performed with the goal of classifying the samples according to their properties and the results can be seen in Figure 3.

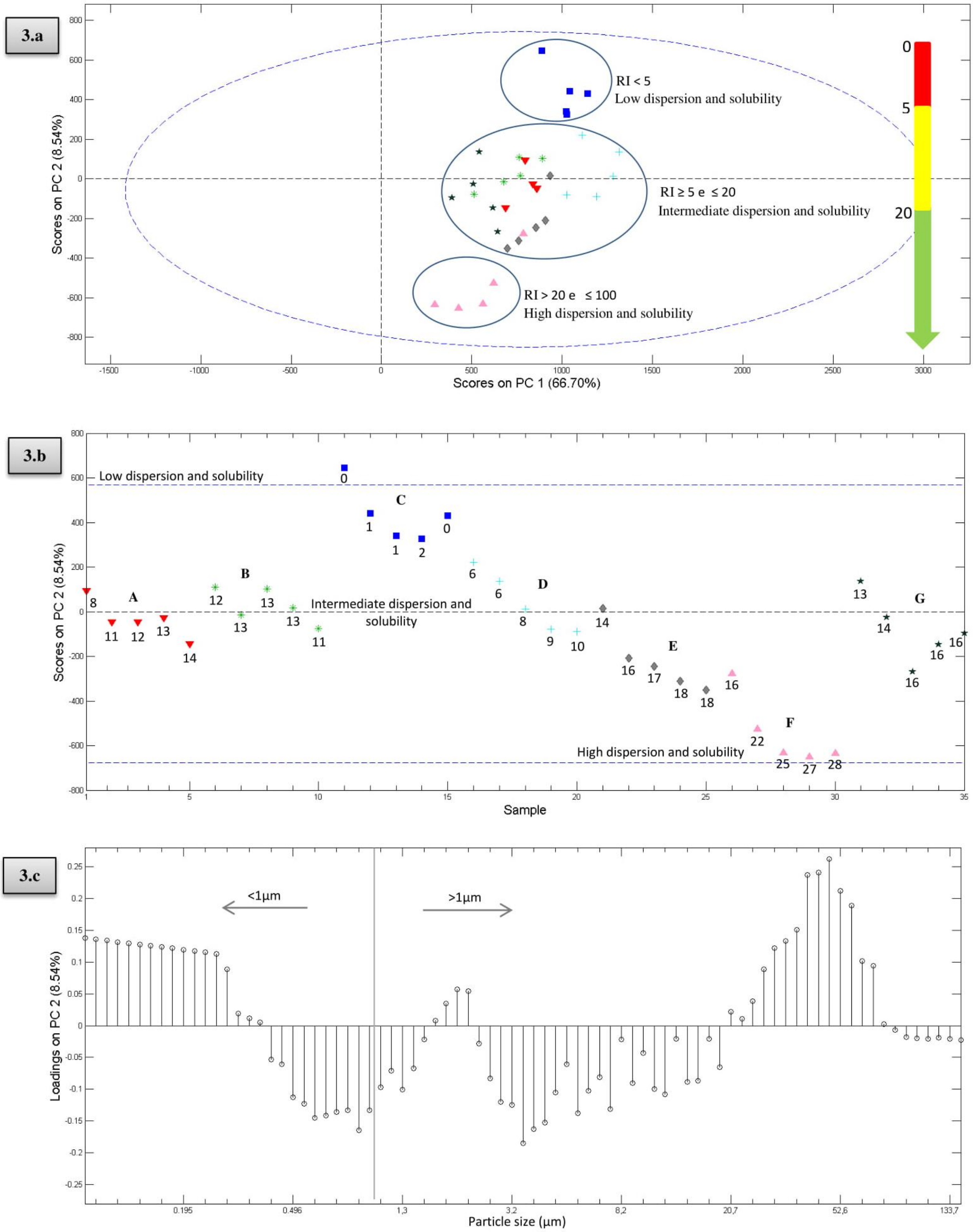


***Figure 3.*** *(a) Graphic of scores PC1 versus PC2; (b) graphic of samples versus scores PC2 (the numbers displayed in the graphic are referent to the rehydration indexes); and (c) graphic of loading PC2. Graphics represent the particle size analysis of seven milk powder samples A ( ▼ ), B ( ✳ ), C ( ■ ), D ( + ), E ( ◆ ), F ( ▲ ) and G ( ★ )*

Figure 3.a shows an 66.7 % of accumulation of responses in PC1 with a small range of variation inversely to the accumulation of responses in PC2 (8.54 %), in which there is a bigger range of variation. Because of this bigger variation and to avoid the agglomeration of results, Figure 3.b shows the samples division with their respective rehydration indexes and possible classification in low, intermediate and high properties of dispersion and solubility.

Ergo, the behavioral profile of the samples was achieved through principal component analysis. Three groups were obtained with the division based on the quantity of particles under 1 µm. This value was chosen based on the characteristics of the curve obtained by the particle size distribution analysis. The curve is classified as bimodal, showing two modes with interstice close to <1 µm and >1 µm. The analysis of this result shows a mix of two distinctive population of particles, one being the caseins and the other being fat and other non-hydrated particles.

All in all, it was possible to create a rehydration index in which powders with RI < 5 are the ones with less condition to recover their original conformation after being rehydrated (low dispersion and solubility); RI ≥ 5 and ≤ 20 represents powders with intermediate condition to recover their original conformation after being rehydrated (medium dispersion and solubility); and RI > 20 belongs to the ones with higher condition to recover their original conformation after being rehydrated (high dispersion and solubility). It is worth mentioning that parameters as mixing equipment design, conditions of operation such as agitation and temperature, and chemical composition of the powder affect the rehydration of dairy powders (RICHARD et al., 2013).

Thus, the suggestion of a rehydration index may help the choice of the best powder according to the application. For example, products with RI > 20 should be chosen for direct consumption because the original structure can be achieved more efficiently. On the other hand, powders with RI < 5 or RI ≥ 5 and ≤ 20 could be used as ingredients for the production of other foods (e.g. dairy products, bakery, confectionery, and meat products) without compromising the final characteristics because of its direct application with no rehydration. Therefore, this work stipulates an efficient rehydration index to determine powders dispersion.

At last, Figure 3.c shows the contribution of each resolution channel to the rehydration index intensity. Powders with higher amount of small particles (close to 0.195 µm) presented an increase in RI intensity, while powders with bigger particles (close to 52.6 µm) had a decrease in RI intensity.

The particle size analysis investigated on this work is the result of the combination of 80 resolution channels. Hence, this technique is more accurate and faster, from the analytical point of view, than the official dispersion analysis which comes from a single result. Thereby, the determination of RI through particle size may replace or complement the original analysis of powders dispersion although it is necessary to use an analytical device. The standard dispersibility analysis is manually intense, time consuming and cannot be automated or easily online configured. Thus, alternative techniques have been studied using laser diffraction, sifting (BOIARKINA et al., 2016), particle size distribution (RICHARD et al., 2013) and even the development of a device (LEE et al., 2014) to facilitate the determination of dispersibility.

## CONCLUSIONS

According to morphology and particle size analysis, samples C and F presented contrasting results while the other samples had similar results among each other. The measurement of immediate dispersibility is performed through a manual and laborious test. Therefore, the particle size analysis seems to be a simple and efficient method to determine dispersibility and solubility properties. This work established a rehydration index that makes it possible for the classification of whole milk powder according to dissolution properties for dairy powders.

## ACKNOWLEDGMENT

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# 5. PAPER 3

FRANCISQUINI, Júlia d'Almeida et al. How the heat treatment affects the constituents of infant formulas: a review. **Brazilian Journal of Food Technology**, v. 23, 2020.

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REVIEW ARTICLE

## How the heat treatment affects the constituents of infant formulas: a review

*Como o tratamento térmico afeta os constituintes das fórmulas infantis: uma revisão*

**Júlia d'Almeida Francisquini[1], Lauane Nunes[1], Evandro Martins[1], Rodrigo Stephani[2], Ítalo Tuler Perrone[3], Antônio Fernandes de Carvalho[1*]**

[1]Universidade Federal de Viçosa (UFV), Departamento de Tecnologia de Alimentos, Viçosa/MG - Brasil
[2]Universidade Federal de Juiz de Fora (UFJF), Departamento de Química, Juiz de Fora/MG - Brasil
[3]Universidade Federal de Juiz de Fora (UFJF), Departamento de Ciências Farmacêuticas, Juiz de Fora/MG - Brasil

***Corresponding Author:** Antônio Fernandes de Carvalho, Universidade Federal de Viçosa (UFV), Departamento de Tecnologia de Alimentos, Campus Universitário Viçosa, CEP: 36570-000, Viçosa/MG - Brasil, e-mail: afc1800@yahoo.com

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## Abstract

Breast milk as the children's primary source of nutrition fulfills the babies' needs and can also provide immune protection. In some cases, when mothers are not able to breastfeed, an equivalent substitute is required. Nowadays, the best substitutes of the human breast milk are infant formulas. Different technological routes may be designed to produce infant formulas according to the main challenges: the compromise between food safety and heat treatment damage. This article aimed to review the current scientific knowledge about how heat treatment affects the macro and micronutrients of milk, extrapolating the expected effects on infant formulas. The covered topics were: The definition and composition of infant formulas, industrial methods of infant formulas production, the effects of heat treatment on milk macro and micronutrients.

**Keywords:** Breastfeeding; Infant; Breast milk; Thermal processing; Browning reaction; Nutrients.

## Resumo

O primeiro alimento do bebê é o leite materno, que deve, entre outras funções, atender, principalmente, às necessidades nutricionais e fornecer proteção imunológica. Em alguns casos, mães impossibilitadas de amamentar necessitam de substitutos. Nos dias de hoje, os substitutos que melhor mimetizam o leite humano são as fórmulas infantis. Diferentes rotas tecnológicas podem ser projetadas para produzir fórmulas infantis apresentando o grande desafio: o compromisso entre alimentos seguros e os danos causados pelo tratamento térmico. Este artigo foi elaborado com o objetivo de revisar o conhecimento científico atual sobre como o tratamento térmico afeta os macro e micronutrientes do leite, extrapolando os efeitos esperados para as fórmulas infantis. Os tópicos abordados são: definição e composição das fórmulas infantis, métodos industriais para produção de fórmulas infantis, efeito do tratamento térmico sobre macronutrientes e micronutrientes do leite.

**Palavras-chave:** Aleitamento materno; Criança; Leite materno; Processamento térmico; Reação de escurecimento; Nutrientes.

HOW THE HEAT TREATMENT AFFECT THE CONSTITUENTS OF INFANT FORMULAS: A REVIEW.

COMO O TRATAMENTO TÉRMICO AFETA OS CONSTITUINTES DAS FÓRMULAS INFANTIS: UMA REVISÃO.

**ABSTRACT –** Breast milk as the children's primary source of nutrition fulfills the babies' needs and can also provide immune protection. In some cases, mothers are not able to breastfeed then an equivalent substitute is required. Nowadays, the substitutes that best mimic the human breast milk are infant formulas. Different technological routes may be designed to produce infant formulas according to the main challenges: the compromise between food safety and the heat treatments damage. This article aimed to review the current scientific knowledge about how heat treatment affects the macro and micronutrients of milk, thus extrapolating the expected effects on infant formulas. The covered topics were: The definition and composition of infant formulas, industrial methods of infant formulas production, the effects of heat treatment on milk macro and micronutrients. **KEYWORDS:** Breastfeeding. Infant. Breast milk. Thermal processing. Browning reaction. Nutrients.

**RESUMO -** O primeiro alimento do bebê é o leite materno, que deve, entre outras funções, atender principalmente às necessidades nutricionais e fornecer proteção imunológica. Em alguns casos, mães impossibilitadas de amamentar, necessitam de substitutos. Nos dias de hoje, os substitutos que melhor mimetizam o leite humano são as fórmulas infantis. Diferentes rotas tecnológicas podem ser projetadas para produzir fórmulas infantis apresentando o grande desafio: o compromisso entre alimentos seguros e os danos causados pelo tratamento térmico. Este artigo foi elaborado com o objetivo de revisar o conhecimento científico atual sobre como o tratamento térmico afeta os macro e micronutrientes do leite, extrapolando os efeitos esperados para as fórmulas infantis. Os tópicos abordados são: definição e composição das fórmulas infantis, métodos industriais para produção de fórmulas infantis, efeito do tratamento térmico sobre macronutrientes e micronutrientes do leite.
**PALAVRAS-CHAVE:** Aleitamento materno. Criança. Leite materno. Processamento térmico. Reação de escurecimento. Nutrientes.

## INTRODUCTION

Breastfeeding, is either essential for the baby's growth and development. It is known that diet plays an important role during the first years of the baby's life helping with the formation of eating habits, supporting the development of the immune system and preventing diseases such as obesity, diabetes, hypertension, and atherosclerosis (Andreas, 2015; Zou et al., 2016).

The World Health Organization (WHO) recommends that breastfeeding should be exclusive for the first six months of the neonate's life and then must be gradually supplemented with another diet (FAO, 1981; Zou et al., 2016). Breast milk supplements the intrinsic deficiencies in newborns by providing immunological protection, preventing the onset of pathologies (infections, allergies, malnutrition), and meeting their nutritional needs (Blanchard et al., 2013).

However, in some cases, breastfeeding may be interrupted or not performed, due to postpartum depression, insufficient milk syndrome, social factors (working mothers), or medical conditions (babies with metabolic disorders) (Pereyra et al., 2003). Based on the high risk of morbidity and mortality of non-breastfeeding infants, the WHO recommends the implementation of artificial feeding (milk taken directly from the mother, breast milk from milk banks, or commercial infant formulas - IF) when it is acceptable, feasible, accessible, sustainable, and also safe.

Although it is not always indicated, artificial feeding is a common practice, especially in underdeveloped countries, to provide the baby milk from other species, which may have a different composition compared to breast milk. Therefore, it should not be provided directly to the baby (Euclydes, 2014).

Microbiological contamination may be the main problem associated with infant formula production (Pei et al. 2018; Coppa et al. 2019; Portela et al. 2019; Zhuang et al. 2019). Different technological routes may be designed to produce infant formulas according to the main challenges: the compromise between food safety and the heat treatments damage. This article was build aiming to review the current scientific knowledge about how heat treatment affects the macro and micronutrients of milk, thus extrapolating to the expected effects on infant formulas. The topics covered are: definition and composition of infant formulas, industrial methods for production of infant formulas, effect of heat treatment on macronutrients and micronutrients of milk.

## DEFINITION AND COMPOSITION OF INFANT FORMULAS

The Codex Alimentarius Committee (Codex), established by United Nations Organization is the main global regulatory agency for infant formulas, jointly by Food and Agriculture Organization (FAO) and the World Health Organization (WHO) in 1963. IF can be considered complete (adequate proportions of macronutrients, micronutrients, electrolytes, and trace elements) and may also use isolated proteins from cow's milk and intact or hydrolyzed soybeans. All other constituents/nutrients can be added separately regulated by law (Euclydes, 2014).

The formula composition should vary according to the child's growth and it can be further adjusted. Under the current regulations, Infant formulas can be classified into premature infants, infants (first six months), infants monitoring (from the age of six months), early childhood monitoring (from twelve months to three years old) and special formulas. (Figure 1) (FAO, 1981; Blanchard et al., 2013).

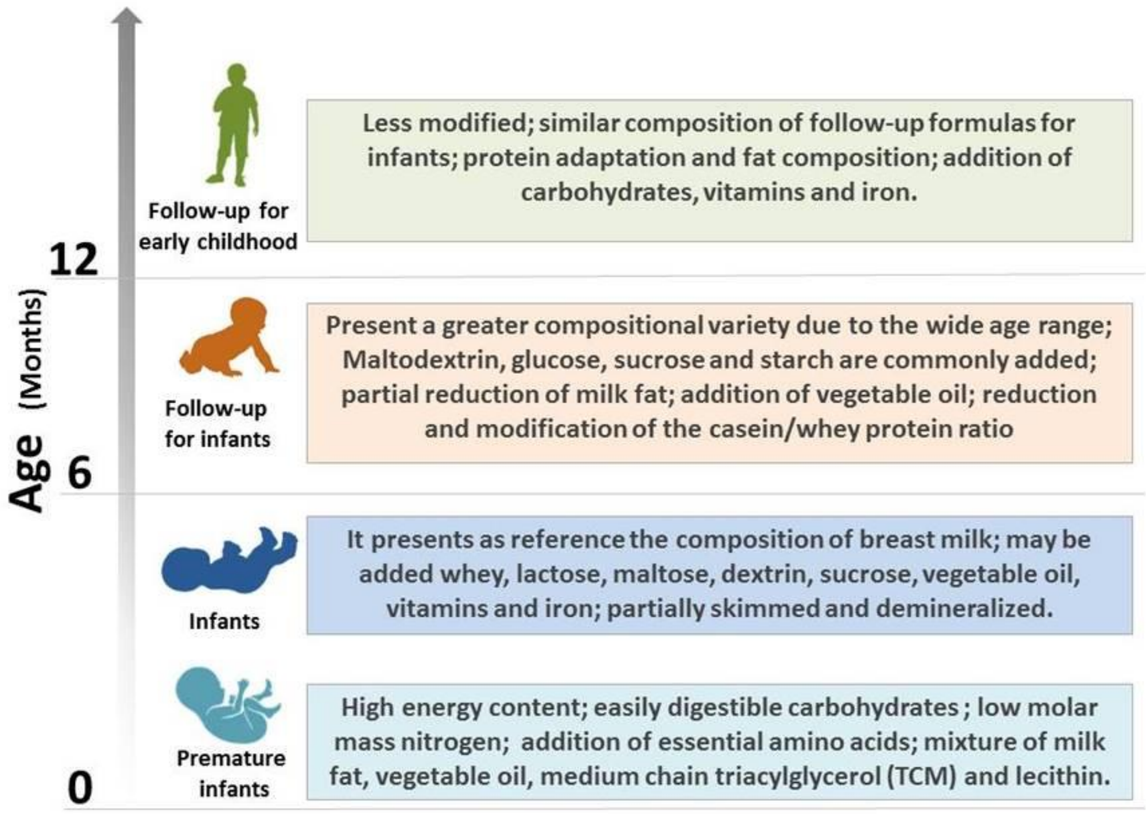


**Figure 1 -** Infant formula characteristics and functions according to the age of the lactant Adapted from (FAO 1981; Blanchard et al., 2013; Euclydes, 2014; Guo, 2014).

Cow's milk, whey protein concentrate, demineralized whey, carbohydrates (lactose, maltodextrin, or sucrose), and vegetable oils were used as raw materials for infant formulas. If necessary, minerals, such as iron may be added to the formulas. This type of formula must contain, in 100 ml of the product ready for consumption, energy values between 60 and 70 kcal, protein (1.8g·(100 kcal)$^{-1}$ to 3.0g·(100 kcal)$^{-1}$), total fat (4.4g·(100 kcal)$^{-1}$ to 6.0g·(100 kcal)$^{-1}$) and total carbohydrates (9.0g·(100 kcal)$^{-1}$ to 14.0g·(100 kcal)$^{-1}$) (FAO, 1981). The most common used Infant formulas (initial stages and the subsequent/following stages) and their compositions are shown in Table 1 (FAO, 1981).

**Table 1 -** Composition of infant formulas for infants.

| | **0 to 6 months** | | **6 to 12 months** | |
|---|---|---|---|---|
| **Composition by 100 kcal** | Minimum | Maximum | Minimum | Maximum |
| **Energy, kcal *** | 60 | 70 | 60 | 70 |
| **Protein, g** | 1.8 | 3.0 | 1.8 | 3.5 |
| **Lipids, g** | 4.4 | 6.0 | 4.0 | 6.0 |
| **Acid. Linoleic, mg** | 300 | 1400 | 300 | 1400 |
| **Acid. Linolenic, mg** | 50 | NE | 50 | NE |
| **Relation n-6 : n-3** | 5:1 | 15:1 | 5:1 | 15:1 |
| **Carbohydrates** | 9.0 | 14 | 9.0 | 14 |
| **Vit. A, µg RE** | 60 | 180 | 60 | 180 |
| **Vit. D, µg** | 1.0 | 2.5 | 1.0 | 3.0 |
| **Vit. K, µg** | 4.0 | - | 4.0 | - |
| **Vit. E, mg α-TE** | 0.5 | - | 0.5 | - |
| **Thiamine, µg** | 60 | - | 60 | - |
| **Riboflavin, µg** | 80 | - | 80 | - |
| **Niacin, µg** | 300 | - | 300 | - |
| **Vit. B6, µg** | 35 | - | 35 | - |
| **Vit. B12, µg** | 0.1 | - | 0.1 | - |
| **Ac. Pant., µg** | 400 | - | 400 | - |
| **Folic acid, µg** | 10 | - | 10 | - |
| **Vit. C, mg** | 10 | - | 10 | - |

| | | | | |
|---|---|---|---|---|
| **Biotin, µg** | 1.5 | - | 1.5 | - |
| **Iron, mg** | 0.45 | - | 0.9 | 2 |
| **Calcium, mg** | 50 | - | 50 | - |
| **Phosphor, mg** | 25 | - | 25 | - |
| **Magnesium, mg** | 5.0 | - | 5.0 | - |
| **Sodium, mg** | 20 | 60 | 20 | 60 |
| **Chlorine, mg** | 50 | 160 | 50 | 160 |
| **Potassium, mg** | 60 | 180 | 60 | 180 |
| **Manganese, µg** | 1.0 | - | 1.0 | - |
| **Iodine, µg** | 10 | - | 10 | - |
| **Selenium, µg** | 1.0 | - | 1.0 | - |
| **Copper, µg** | 35 | 120 | - | 120 |
| **Zinc, mg** | 0.5 | - | 0.5 | - |
| **Choline, mg** | 7.0 | - | 7.0 | - |
| **Inositol, mg** | 4.0 | - | 4.0 | - |
| **L- carnitine, mg** | 1.2 | - | - | - |

**Source:** Adapted from FAO 1981.

The majority of the proteins come from cow's milk and whey. It is necessary to adapt the ratio whey/casein proteins to promote a better digestibility, metabolic overload reduction, and the infant nutritional needs. The levels of essential and semi-essential amino acids in the milk formula must be at least equivalent to the reference protein (human milk) (Blanchard et al., 2013).

The human milk fat composition differs from cow's milk and the historical development of infant formulas presented three stages of evolution considering human milk fat substitution over the years: stage 1 - focus on energy replacement (1856 to 1910-1920), stage 2 - focus on energy replacement and fatty acid composition (1910-1920 to 1990), stage 3 - focus on energy replacement, fatty acid composition, triacylglycerol and complex lipids (Wei et al. 2019).

There is a reduction in the amount of saturated fat in the formula as the lipid in this formula will partly be derived from the dairy fat and partly from different vegetable sources such as soybeans, corn, sunflower, and canola. Also, there is a need to improve the digestion and absorption of calcium in order to adjust the composition of essential fatty acids (Euclydes, 2014; Guo, 2014). The content of trans fats in the formulas should be minimal, around 3% of total fatty acids, as recommended by Codex (Thompkinson & Kharb, 2007).

Gastrointestinal disorders (including constipation) in babies are related to the consumption of vegetable oil–based infant formulas which are mainly associated to long chain saturated fats esterified at sn-1 and sn-3 positions (breast milk has fatty acids esterified at sn-2 position) (Mehrotra et al. 2019).

Most of the present formulas require external addition of lactose to match the amount contained in human milk. However, in some cases, the mixtures of some carbohydrates such as sucrose, maltodextrin, glucose polymers, and starch can be supplemented as well. All formulas must be enriched with iron and also must have the same osmolarity as breast milk (< 460 $mOs \cdot ml^{-1}$) (Euclydes, 2014; Andreas, 2015).

Some ingredients can be added to further reach the standards of breast milk, such as nucleotides which help in the maturation of the gastrointestinal tract and invoke immunomodulatory effects, long chain fatty acids (arachidonic and docosahexaenoic), which are responsible for physiological and metabolic functioning; probiotics and prebiotics, associated with pathogenic inhibition and immunomodulation, and amylopectin, used for children with gastroesophageal reflux (Euclydes, 2014).

## INDUSTRIAL METHODS FOR PRODUCTION OF INFANT FORMULAS

Infant formulas are found in two readily consumable forms: liquid concentrate and powder form. However, the liquid version presents an elevated price due to the production expenses, transportation, and distribution. In addition, a short shelf life requires an appropriate refrigeration and extra care with the packaging avoiding potential contamination (Kliegman et al., 2014).Therefore, the powdered version represent the major formula available in the market which can be manufactured using "dry mix" or "wet/spray" processes (Guo, 2014; Schug et al., 2016).

the ingredients in the dry mix process are first mixed together in powder form and then packaged. This method includes some advantages as lower energy expenditure during production and less investment in equipment, construction, and maintenance. However, the microbiological safety may be questioned since it depends on the quality of the raw materials used to assemble the final product. Moreover, the major problems are associated with post-processing contamination with *Salmonella* and other

*Enterobacteriaceae*, including *Enterobacter sakazakii*, associated with food-poisoning outbreaks (Song et al., 2018).

In order to minimize the microbiological risks associated with dry mixing protocols, the wet method represents the most common used procedure for the powder formula production, using a better monitoring and control of the process steps (Blanchard et al., 2013; Guo, 2014; Schug et al., 2016). Initially, the raw material in powder form are mixed with water and oil, and then pasteurized, homogenized, and concentrated under vacuum until the obtention of a solid concentration between 40% and 50% (Figure 2) (Blanchard et al., 2013; Schug et al., 2016).

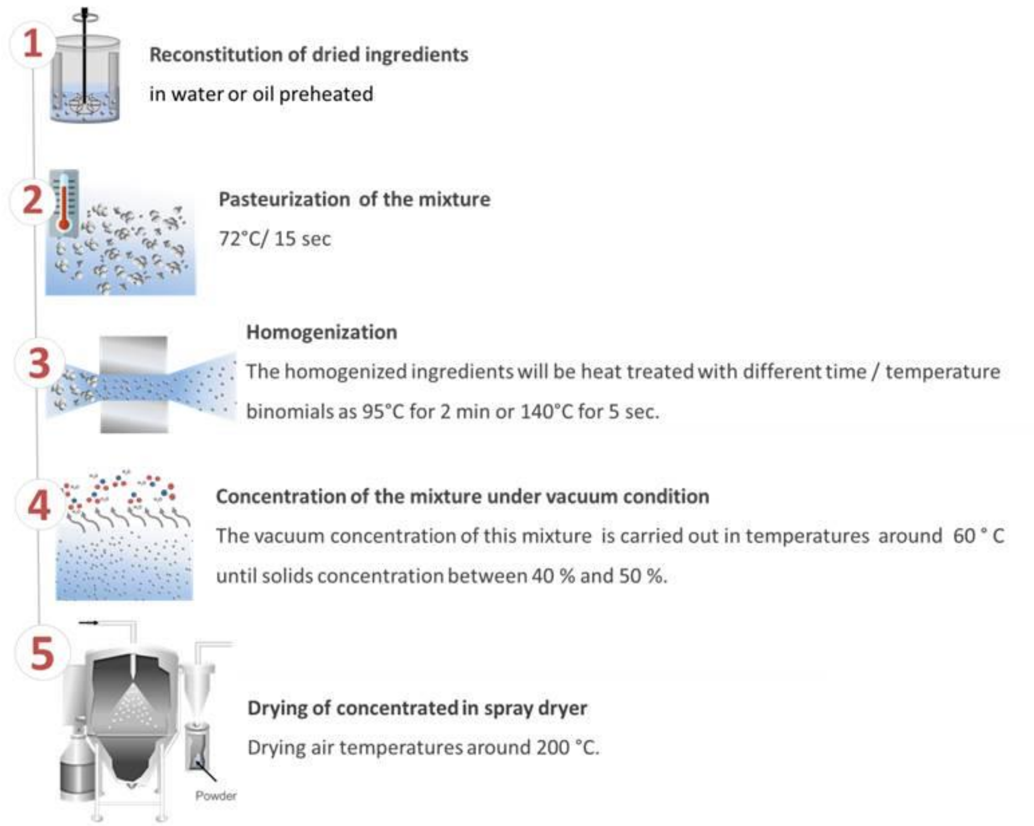


**Figure 2 -** Steps of production of infant formulas by wet via. Adapted from (Zhu & Schuck, 2013; Kliegman et al., 2014; Schug et al., 2016; Song et al., 2018).

Through this process the fluid material is transformed into dried particles by spraying into a heated atmosphere (Figure 2). When the homogenized mixture comes in contact with drying air the evaporation occurs in the droplets until the moisture content becomes too low to diffuse through the dry droplet surface. Finally, the recovery of dry powder is carried out in the cyclone (Keshani et al., 2015). The last stage is coupled to an adequate packaging process with minimal microbiological contamination. The appropriate packaging process improves the product shelf life, avoiding deterioration, oxidation, and agglomeration of particles.

## EFFECT OF HEAT TREATMENT ON INFANT FORMULAS

### Effect of heat treatment on macronutrients

Upon heat treatment, a number of irreversible changes in the composition of milk macronutrients can occur (Qian et al., 2017). Milk proteins are among the most heat-sensitive substances. However, caseins rich in proline and naturally unfolded, will be less affected by heat when compared to whey proteins (Raikos, 2010).

Casein micelle size distribution, chymosin coagulation, and thermal stability are normally recovered by milk reconstitution after drying. However, this protein may undergo chemical and structural changes during milk heating process, which may affect the profile of bioactive peptides produced during protein digestion (Souza et al., 2015).

Finally, after intense heating process casein molecules may be cleaved, leading to their dephosphorylation. In addition, severe heat treatment can also cleave peptide chains which produces soluble peptides (Xu et al., 2016).

The glycosylated state of κ-casein inhibits *Helicobacter pylori* in human gastrointestinal mucosa owing to its structural similarity with SIgA. However, κ-casein can be deglycosylated upon heating, thus promoting the loss of its protective function (Lönnerdal, 2017). Besides, at high temperature a great amount of calcium phosphate associated with casein micelles results in increasing dissociation of κ-casein and decreases the product stability (Borad et al., 2017).

Heat treatments result in loss of function of β-casein the mainly responsible for the high bioavailability of calcium and zinc in human milk. Thus, this sub category of casein protein has a great role in the development and growth during the infant by increasing the bioavailability of minerals in human milk. Consequently, the lost of its function would result in future problems for the child (Lönnerdal, 2017).

Whey proteins are more susceptible to heat treatment leading to possible alteration of functional and nutritional characteristics. Denaturation of whey proteins is considered to be the most important reaction during the heat treatment of dairy products. Whey proteins may undergo irreversible denaturation, affecting their threedimensional conformation and functional properties (water absorption, gelling, emulsification, and viscosity) (Golkar et al., 2019).

Whey proteins may also interact with casein micelles, resulting in aggregation/dissociation of micelles (specifically, κ-casein micelles) decreasing the solubility of milk proteins. These associations modify the micellar surface, leading to an alteration of hydrophobicity and functionality of the reconstituted dairy products. Also, as a result of this aggregation, most proteins lose their biological activity, eventually promoting protein coagulation (Souza et al., 2015; Raikos, 2010; Borad et al., 2017; Qian et al., 2017). In addition, a few of whey proteins, especially β-lactoglobulin, bind covalently to membrane proteins of fat globules (Liu et al., 2012).

a-lactalbumin may lose calcium and zinc binding ability followed by decrease in the bioavailability of these nutrients. On the other hand, lactoferrin tends to lose its microbial action due to heating (Golinelli et al., 2014). Although β-lactoglobulin does not exist in human milk, it is present in IF derived from cow's milk and thus can be denatured or even become insoluble during heat treatments (Rafe & Razavi, 2015).

Besides lipids aggregation with casein, heat cycles can favor the hydrolysis and interesterification of glycerides subsequently changing the quality and quantity of short chain fatty acids (C4:0, C6:0, and C8:0) in infant formulas (Pestana et al., 2015). Milk fat globule membrane (MFGM) derives from mammary gland epithelium and it is primarily composed by polar lipids with interspersed membrane-bound proteins, glycoproteins, enzymes, and cholesterol which results in a bioactive molecule that provides some of the important protective features of breast milk. However, the majority of infant formulas do not contain MFGM. Recent breakthroughs in manufacturing technologies permit the concentration of bovine MFGM making it possible to add MFGM into IF. Nevertheless, heat treatment can result in an enhanced permeability of MFGM, thus affecting its stability (Liu et al., 2012).

Heating the milk can also inactivate the enzyme superoxide dismutase in addition to allowing copper to move from the plasma to fat globules membrane. Such situation can lead to dairy products oxidation. The temperature which fat is subjected to bears a significant effect on the amount, composition, and stability of fat crystals. Also, prolonged heating and storage initiates induction of lactose crystallization and increased water activity accelerating the lipid oxidation (Walstra, 2006).

In addition, the heat treatment can also result in the breakdown of phospholipids with consequent increase in the amount of inorganic phosphate (Walstra, 2006). To conclude, lipids can carry liposoluble vitamins (A, D, E, and K) and are precursors for the synthesis of hormones involved in the modulation of immune and inflammatory responses. They are important components of cell membranes, essential for the development of the central nervous system and the children's retina. Lipids also improve the processes involved in information codification, storage, and memory recovery; or psychomotor processes. Thus, they may lose these fundamental functions due to alteration upon heating (Zou et al., 2016).

Apart from what has already been known about these macronutrients, it may be considered that the higher protein content, lower concentrations of long chain polyunsaturated fatty acids, and presumably the lack of insulin sensitizing hormones, along with numerous other biologically active substances in infant formulas, play a pathophysiological role, for example in insulin sensitivity (Klenovics et al., 2013).

Heat treatment can also affect carbohydrates. The isomerization of lactose into lactulose and organic acids is one of the common consequences of heating. Lactulose is a disaccharide, formed by fructose and galactose residues obtained upon the isomerization of lactose, which may occur in the alkaline environment or during the heat processing of milk. Unlike lactose, lactulose cannot be hydrolyzed by human intestinal enzymes. However, it can be fermented by bacterias present in the colon, mainly by *Bifidobacterium sp.,* which acts as prebiotics (Zhang et al., 2010). Lactulose is the main resultant compound formed during the heat treatment of the milk and its formation is relative to the subjected heat intensity. Based on this characteristic, the amount of lactulose in the heated dairy products can be used as an indicator of the intensity of the heat treatment (Walstra, 2006).

Besides forming lactulose lactose can also react with amino acids, peptides, or proteins by Maillard reaction. This non-enzymatic browning reaction is one of the main non-enzymatic reactions responsible for reducing the quality of dairy products when heated and continued to be stored (Deeth & Hartanto, 2009).

The Maillard-induced conjugation can lead to the glycation of proteins and peptides (glucose or lipids bind to the proteins permanently). This leads to changes in physicochemical properties and technological functionality (solubility, heat stability, emulsification, foaming, and gelation properties) of milk proteins and peptides and their derivatives (Torres et al., 2017). The Maillard reaction in an infant formula can result in the formation of products such as furfural and carboxymethyl lysine, which are often associated with the reduction/loss of the nutritional values imparted by the essential amino acids such as arginine, lysine, methionine, and tryptophan, as well as reducing the digestibility of the food for the infant (Mehta & Deeth, 2016). These products, when combined with advanced glycation end products (AGEs) (formed by glycation) can result in an excessive endogenous *pool* in those who ingested infant formulas (Vistoli et al., 2013).

Despite the reported disadvantages Maillard reaction under dry conditions seems to be an effective way of reducing the antigenicity of cow's milk proteins, this is because allergens (proteins) are modified by this reaction (Golkar et al., 2019). It is known that the higher and earlier the exposure and ingestion of such products happen, the higher will be the interference on the child's health. Thus, an excessive endogenous *pool* in childhood may render a pathogenic role in the development and progression of different oxidative diseases in adulthood. These include diabetes, insulin resistance, chronic renal failure, cardiovascular diseases (atherosclerosis), neurological disorders (Alzheimer's disease), aging, oxidative stress, protein damage, reduced blood vessel elasticity, vascular thickening, hypertension, endometrial dysfunction, retinal pathology, osteoporosis, and arthritis (Vistoli et al., 2013).

In short, during intense heating lactose can either be decomposed into lactulose or participate in the Maillard reaction and the main results are changes in flavor and brown color development due to the appearance of melanoidins, which is also followed by loss of nutritional value due to the decrease of lysine to be absorbed by the intestines (Walstra, 2006).

Additionally, caramelization reaction can be mentioned as a common phenomenon with dairy products after short time heating process. In this case, the sugars are pyrolyzed in caramel (high molecular weight and dark degradation products) (Araújo, 2008). Such reactions may result in browning of foods, loss of lysine, modification of flavor, formation of hydroxymethylfurfural and melanoidins, and even carbonization of food, if the heat treatment continues (Fennema, 2010).

**Effect of heat treatment on micronutrients**

The liposoluble vitamins A, D, E, and K and a few of the B complex vitamins like biotin and riboflavin are relatively stable in spite of heat treatment of infant formulas. The water-soluble vitamins like vitamin C and thiamine are the most affected by heat treatment as they are more unstable and naturally oxidized by air (Sucupira et al., 2012). In addition, significant reductions in the levels of niacin, pyridoxine, cobalamin, and folate have been reported under the influence of different milk processing treatments (Asadullah et al., 2010).

Oxidation leads to the loss of ascorbic acid (vitamin C), which acts as an antioxidant, helps in strengthening the immune system and blood capillaries, preventing of flus and infections (Araújo, 2005). Vitamin C also offers resistance to the bones and teeth by helping the absorption of iron, along with regulating the metabolism of a few amino acids. Also, vitamin C, under the effect of heating leads to the formation of dark pigments that promote changes in the color, texture, and flavor of the food (Fennema, 2010). Besides nutrition, vitamin C is added to infant formula with the purpose of contributing technologically by enhancing the chemical stability of the product due to its antioxidative role. If necessary, addition of vitamins (A, D, E) may also be done. The vitamin loss is variable and depends on the time/temperature binomial to which the product is subjected. A prolonged heating time is more responsible in the destruction of vitamins than the temperature itself (Leskova et al., 2006).

Among the minerals, calcium is one of the most affected by heat as it can be internalized in the casein micelles and become unavailable for absorption of the child. Calcium can be lost during heating through fouling in the equipment along with other minerals and proteins (Gaucheron, 2005). Besides calcium, the inorganic phosphate content also becomes very high due to the hydrolysis of phosphate esters of casein and phospholipids, decreasing the availability of phosphorus. In addition, it is a common practice to fortify the milk with iron and copper (Walstra et al., 2006).

## CONCLUSIONS

The main impacts of the heat treatment to macronutrients of milk are: loss of function of β-casein (mainly responsible for the high bioavailability of calcium and zinc in human milk), α-lactoalbumin lose the calcium and zinc binding ability (decrease in the bioavailability of these nutrients), lactoferrin tends to lose its microbial action, quality and quantity of short chain fatty acids (C4:0, C6:0, and C8:0) modifications in infant formulas, breakdown of phospholipids, and Maillard reaction in infant formula may result in the formation of products such as furfural and carboxymethyl lysine (associated with the reduction/loss of the nutritional values imparted by the essential amino acids such as arginine, lysine, methionine, and tryptophan, as well as reducing the digestibility of the food for the infant).

The main impacts of the heat treatment to micronutrients of milk are: oxidation of the water-soluble vitamins like vitamin C and thiamine, liposoluble vitamins A, D, E, and K, biotin and riboflavin are relatively stable, among the minerals, calcium is one of the most affected by heat as it can be internalized in the casein micelles and become unavailable for absorption of the child.

According to the scientific literature there are several damages to milk components caused by heat treatment as those applied in infant formula production. As a consequence, food safety and the heat treatments damage still the big challenge for infant formula production. Therefore, the design of new routes for infant formula production is necessary, based in new non-thermic technologies and membrane filtration. These technologies seek to reduce energy consumption and the damages to the milk components providing new high nutritional value for infant formulas based on milk.

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# 6. OTHER PUBLICATIONS DEVELOPED DURING THE DOCTORATE

## 6.1 PUBLISHED PAPERS

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2. NUNES, Lauane et al. Evaluation of the Maillard reaction in infant formulas after opening. **Journal of Food & Nutrition Research**, v. 58, n. 3, 2019.
3. FRANCISQUINI, Júlia d'Almeida et al. Evolution of soluble solid content and evaporation rate curves during the manufacture of dulce de leche (dl). **Food Science and Technology**, v. 39, n. 1, p. 78-82, 2019.
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5. RENHE, Isis Rodrigues Toledo et al. Obtenção de Petit Suisse com baixo teor de lactose e adição reduzida de açúcares. **Revista do Instituto de Laticínios Cândido Tostes**, v. 73, n. 1, p. 43-50, 2018.
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11. STEPHANI, Rodrigo et al. SIMULATION OF WATER EVAPORATION ON ULTIMONITORED THERMOMIX®: POTENTIAL APPROACH FOR STUDIES ON MILK EVAPORATION. **Revista do Instituto de Laticínios Cândido Tostes**, v. 72, n. 4, p. 227-235, 2017.

## 6.2 PUBLISHED BOOK CHAPTER

1. STEPHANI, Rodrigo et al. Dulce de Leche—Chemistry and Processing Technology. In: **Milk Production, Processing and Marketing**. IntechOpen, 2019.

## 6.3 PUBLISHED BOOK

1. Perrone, Ítalo Túler et al. JD. Doce de Leite: Química e Tecnologia. Cap-Lab, 2019.

# 7. GENERAL CONCLUSIONS AND PERSPECTIVES

The hydrolysis can mainly affect the sensory characteristic of dulce de leche, increasing the difficulty to reach the product point. In addition to resulting in different color (darker), texture (increased viscosity) and flavor (sweeter) characteristics of the traditional product in which lactose is intact. Such modifications have as main cause the intensification of the Maillard reaction and its products formation for the greater concentration of reducing sugars. Therefore, **Paper 1** helps to understand the consequences caused by this hydrolysis, making it possible to create lactose-free dulce de leche as close to the traditional and well-established products in the market.

The International Dairy Federation (IDF) sets the ISO 17758 **|** IDF 087 methodology as reference for dispersibility determination, which is by definition the percentage by mass of the dry matter of the samples that can be dispersed in a defined amount of water. Water content of the sample must be previously analyzed for this determination. However, tests to determine rehydration properties are usually highly dependent on the measurement technique and the classification scale, which in turn force industries to develop their own methods, adapted to specific applications or to meet clients' demands. In view of this, there is currently a classic and little objective method making it essential to develop a new method to perform the analysis of solubility in powdered milk, which was done in **Paper 2.**

There are several damages to milk components caused by heat treatment as those applied in infant formula production. Consequently, food safety and heat treatments damage are still the big challenges for infant formula production. With this, it is essential that the industries develop infant formulas that meet the baby's physiological demands and the legislation, as well as having a microbiological and nutritional quality with less heat treatment interference in the final product. **Paper 3**, therefore, was developed to help industries to develop infant formulas of better nutritional quality (less heat treatment interference), further demonstrating the importance of the product x process relationship.

As future perspectives, we hope to continue with the studies of the technological consequences caused by the hydrolysis of lactose in concentrated and dehydrated dairy products. As well, we intend to proceed with the use of instrumental analytical techniques to replace the classic methods routinely used for dairy products, aiming at obtaining more reliable and standardized results. Finally, we hope to develop new studies, bring possibilities for modifying the process and producing infant formulas to improve nutritional attributes, in addition to highlighting the need for *in vivo* and *in vitro* studies to demonstrate the effects of heat treatment's influence on this product.