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Innovations in plant-based beverages and dehydrated products with added enzymes: from the
analysis of commercial products to laboratory production

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Innovations in plant-based beverages and dehydrated products with added enzymes: from the analysis of commercial products to laboratory production

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To my Helena for being born for me
and for being my sunshine.

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ABSTRACT

This work was the result of an Doctorate of Academic Innovation (DAI) linked to the Postgraduate Program in Pharmaceutical Sciences at the Faculty of Pharmacy at the Federal University of Juiz de Fora with support from the John Bean Technologies Corporation. The aim was to expand knowledge in the area of plant-based beverages and the application of enzymes for producing dehydrated dairy products to generate innovations for both the academia and industry. This work is divided into four chapters: **Chapter 1** Physicochemical aspects of industrial plant-based beverages; **Chapter 2** Physicochemical analysis of thermally treated commercial plant-based beverages with coffee added; **Chapter 3** The effect of transglutaminase on the characteristics of lactose free skimmed milk powder; and **Chapter 4** Study of Maillard reaction markers and color parameters of concentrated milk added with lactase and transglutaminase at different addition times and heat treatment conditions. Chapters 1 and 2 establish promising results for academia and address the lack of literature on commercial plant-based beverages. Furthermore, the two chapters present data that can help industries producing plant-based beverages in the standardization of these products, which is challenging because of the wide variety of vegetables and ingredients used for their formulation in the absence of specific legislation. Chapters 3 and 4 present results applicable to both the academic and industrial environment related to the lactase and transglutaminase enzymes, which can address problems related to the chemistry, technology, and production of dehydrated dairy products using spray dryers. Notably, this work is relevant for advancing our understanding of the transglutaminase enzyme, which still requires unification of its use by regulations at a global level. Its use in dairy products was recently approved in Brazil. Finally, these chapters were or will be submitted and published as articles in international journals and are directly in line with the theme of the MAI/DAI project entitled ‘Technological development and innovation in the food and beverage production chain’ (Call 12/2020 from CNPq).

Keywords: Industrialized beverages; vegetables; ultra high temperature; zero lactose; skimmed milk; drying.

RESUMO

O trabalho foi resultado de um Doutorado Acadêmico de Inovação (DAI) vinculado ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Farmácia na Universidade Federal de Juiz de Fora com apoio da empresa JBT (*John Bean Technologies Corporation*). O objetivo foi ampliar o conhecimento na área de bebidas formuladas à base de vegetais e na área de aplicação de enzimas para produção de lácteos desidratados com intuito de gerar inovações tanto para o meio acadêmico quanto para o meio industrial. O trabalho se divide em 4 capítulos: **Capítulo 1** Aspectos físico-químicos de bebidas industriais à base de plantas; **Capítulo 2** Análise físico-química de bebidas comerciais à base de plantas tratadas termicamente e adicionadas de café; **Capítulo 3** Efeito da transglutaminase na caracterização do leite em pó desnatado sem lactose; **Capítulo 4** Efeito da ação das enzimas transglutaminase e lactase nos marcadores da reação de Maillard e parâmetros de cor em leite concentrado submetido a diferentes tratamentos térmicos. Os capítulos 1 e 2 estabelecem resultados promissores para o meio acadêmico em virtude da ausência de literatura sobre o tema bebidas vegetais comerciais. Além disto, os dois capítulos resultaram em dados que podem auxiliar as indústrias produtoras de bebidas vegetais à base de plantas na busca pela padronização destes produtos, principalmente, pela grande variedade de vegetais e ingredientes que podem ser utilizados para formulação com ausência de uma legislação específica. Já por meio dos capítulos 3 e 4 pode-se obter conhecimentos para o meio acadêmico e industrial relacionados à aplicabilidade das enzimas lactase e transglutaminase as quais podem resultar em problemas e soluções em nível de química, tecnologia e produção de lácteos desidratados via *spray dryer*. Em associação, os trabalhos podem ser relevantes pela expansão da ciência sobre a enzima transglutaminase que ainda necessita de uma unificação do seu uso pelas regulamentações em nível mundial, além do que, foi recentemente liberado o seu uso para lácteos no Brasil. Por fim, os capítulos estabelecidos foram ou serão submetidos e publicados como artigos em revista de cunho internacional e vão diretamente ao encontro do tema do projeto MAI/DAI intitulado “*Desenvolvimento tecnológico e inovação na cadeia produtiva de alimentos e bebidas*” (Chamada 12/2020 do CNPq).

Palavras-chave: Bebidas industrializadas; vegetais; temperatura ultra alta; zero lactose; leite desnatado; secagem.

SUMMARY

1 INTRODUCTION.....	13
2 AIM.....	15
2.1 GENERAL AIM.....	15
2.2 SPECIFIC AIM.....	15
3 LITERATURE REVIEW.....	16
3.1 PLANT-BASED BEVERAGES.....	16
3.1.1 BIBLIOGRAPHICAL REFERENCES.....	17
3.2 DEHYDRATED PRODUCTS WITH ADDED ENZYMES.....	19
3.2.1 BIBLIOGRAPHICAL REFERENCES.....	22
4 CHAPTER 1: PHYSICOCHEMICAL ASPECTS OF INDUSTRIAL PLANT-BASED BEVERAGES.....	24
4.1 INTRODUCTION AND AIM.....	24
4.2 MATERIAL AND METHODS.....	25
4.3 RESULTS AND DISCUSSION.....	28
4.4 CONCLUSION.....	41
4.5 BIBLIOGRAPHICAL REFERENCES.....	41
5 CHAPTER 2: PHYSICOCHEMICAL ANALYSIS OF THERMALLY TREATED COMMERCIAL PLANT-BASED BEVERAGES COFFEE ADDED.....	44
5.1 INTRODUCTION AND AIM.....	44
5.2 MATERIAL AND METHODS.....	45
5.3 RESULTS AND DISCUSSION.....	47
5.4 CONCLUSION.....	57
5.5 BIBLIOGRAPHICAL REFERENCES.....	57
6 CHAPTER 3: THE EFFECT OF TRANSGLUTAMINASE ON THE CHARACTERIZATION OF LACTOSE FREE SKIMMED MILK POWDER.....	60
6.1 INTRODUCTION AND AIM.....	60
6.2 MATERIAL AND METHODS.....	63
6.3 RESULTS AND DISCUSSION.....	70
6.4 CONCLUSION.....	84
6.5 BIBLIOGRAPHICAL REFERENCES.....	85
7 CHAPTER 4: STUDY OF MAILLARD REACTION MARKERS AND COLOR PARAMETERS OF CONCENTRATED MILK ADDED WITH LACTASE AND	

TRANSGLUTAMINASE AT DIFFERENT ADDITION TIMES AND HEAT TREATMENT CONDITIONS.....	88
7.1 INTRODUCTION AND AIM.....	88
7.2 MATERIAL AND METHODS.....	89
7.3 RESULTS AND DISCUSSION.....	92
7.4 CONCLUSION.....	108
7.5 BIBLIOGRAPHICAL REFERENCES.....	109
8 GENERAL CONCLUSIONS AND FUTURE CONSIDERATIONS.....	111
9 ATTACHMENTS.....	112
9.1 SCIENTIFIC DOCUMENTS PUBLISHED OR ACCEPTED DURING THE DOCTORATE PERIOD.....	112
9.1.1 BOOK.....	112
9.1.2 BOOK CHAPTER.....	112
9.1.3 ARTICLES IN SCIENTIFIC JOURNALS.....	112
9.2 WORKS PRESENTED AT SCIENTIFIC EVENTS DURING THE DOCTORATE PERIOD.....	114

1 INTRODUCTION

The Master's Degree in Academic Innovation (MAI)/Doctorate of Academic Innovation (DAI) program is part of the National Council for Scientific and Technological Development (CNPq) and seeks to strengthen research, entrepreneurship, and innovation in higher education institutions by involving undergraduate and graduate students in projects of interest to the business sector through partnerships with companies. The work presented here was the result of a Doctorate of Academic Innovation linked to the Postgraduate Program in Pharmaceutical Sciences at the School of Pharmacy at the Federal University of Juiz de Fora, with support from John Bean Technologies Corporation (JBT) entitled "Technological development and innovation in the food and beverage production chain" (CNPq Call 12/2020).

This study was able to address the objective of the MAI/DAI program (enabling students to carry out academic and industrial work) along with the central theme of the CNPq call 12/2020 (working with innovation in the area of food and beverages). The thesis shows the development of two parts of work. In the first part, including Chapters 1 and 2, the student conducted industrial innovation work in the area of plant-based beverages. It should be noted that this line of research was requested by the company associated with the work (JBT). Therefore, an important stage of the DAI was fulfilled, i.e., to generate results and materials that can strengthen the food and beverage industry.

The student had previously worked on concentrated and dehydrated dairy products with and without added enzymes (lactase):

- DE PAULA, I. L.; TEIXEIRA, E. B. S.; FRANCISQUINI, J. D. A.; STEPHANI, R.; PERRONE, Í. T.; DE CARVALHO, A. F.; DE OLIVEIRA, L. F. C. Buffalo powder dairy products with and without lactose hydrolysis: Physical-chemical and technical-functional characterizations. **LWT**, v. 151, p. 112124, 2021.
- QUEIROZ, E. S.; REZENDE, A. L. L.; PERRONE, Í. T.; FRANCISQUINI, J. D. A.; DE CARVALHO, A. F.; ALVES, N. M. G.; DE OLIVEIRA, L. F. C.; STEPHANI, R. Spray drying and characterization of lactose-free goat milk. **LWT**, v. 147, p. 111516, 2021.
- FRANCISQUINI, J. D. A.; ROCHA, J.; MARTINS, E.; STEPHANI, R.; DA SILVA, P. H. F.; RENHE, I. R. T.; PERRONE, I. T.; DE CARVALHO, A. F. 5-Hydroxymethylfurfural formation and color change in lactose-hydrolyzed Dulce de leche. **Journal of Dairy Research**, v. 86, n. 4, p. 477-482, 2019.

Therefore, to continue with an already consolidated line of research, a strategy for carrying out the second part of the work was established, seeking to deepen the existing knowledge in the area of dehydrated dairy products supplemented with enzymes. An enzyme (lactase), already known and studied by a research group of which the student was a member, was applied. Transglutaminase is an enzyme that has not been studied in spray-dried dairy products. Note that the transglutaminase used is formulated by Ajinomoto Co., Inc. which, at the time the work was carried out, was one of the industrial partners of Quimtec Laboratory, where the work was carried out, and was also linked to the MAI/DAI. Finally, it can be seen that in this part, there was an initiative to use an expertise already developed by the team to leverage knowledge in an area that had not yet been studied. Consequently, the second important stage of the DAI was fulfilled: to generate results and materials that could strengthen academia with literature relevant to the field of food and beverages.

In conclusion, the thesis will have four chapters:

- **Chapter 1** Physicochemical aspects of plant-based industrial beverages.
- **Chapter 2** Physicochemical analysis of thermally treated commercial plant-based beverages with coffee added.
- **Chapter 3** Effect of transglutaminase on the characteristics of lactose-free skimmed milk powder.
- **Chapter 4** Study of Maillard reaction markers and color parameters of concentrated milk added with lactase and transglutaminase at different addition times and heat treatment conditions.

Finally, this work is expected to contribute knowledge and innovations to the food and beverage industry and academia.

2 AIM

2.1 GENERAL AIM

The general aim was to expand knowledge in the area of plant-based beverages and the application of enzymes for the production of dehydrated dairy products to generate innovations for both the academia and industry.

2.2 SPECIFIC AIM

This project is presented as an academic and industrial proposal and is a tool for advancing knowledge and innovation in the area of beverages and food, with the following focuses:

- Conceptualization and brief explanation of plant-based beverages.
- Aiming to characterize the physicochemical aspects of plant-based industrial beverages of different brands and different plants, and to provide novel data at both the academic and industrial levels; this will help regulatory agencies draw up specific legislations that will provide information for standardizing the products available in the market.
- Physicochemical characterization of heat-treated commercial plant-based beverages with coffee added; the aim was to simulate the use of these beverages by consumers and understand the effect of heat treatment and coffee use on the stability of the analyzed products.
- To demonstrate the applicability of enzymes, with a focus on lactase and transglutaminase for dairy products, particularly dehydrated products.
- To understand the effects of transglutaminase on the characteristics of skimmed milk powder with and without lactose, performing analyses ranging from product retention capacity of equipment to determination of compositional, physicochemical, and morphological attributes, particle size, and Maillard reaction markers.
- To confirm and expand on the information obtained in Chapter 3 by studying the markers of the Maillard reaction and color parameters in the model system with lactase and transglutaminase added at different times and under different heat treatment conditions.
- Expanding knowledge and generating innovations for the food and beverage industry and academia.

3 LITERATURE REVIEW

3.1 PLANT-BASED BEVERAGES

Plant-based beverages are suspensions or emulsions made from plant materials, the main sources of which are cereals, legumes, nuts, seeds, and pseudo cereals (SETHI *et al.*, 2016). Substitutes for animal products, such as plant-based beverages, have been gaining increasing market share because of the increase in individuals with a certain degree of lactose intolerance or cow-milk-protein allergy, as well as adherence to diets excluding animal products, including vegetarian, vegan, and flexitarian diets (AYDAR *et al.*, 2020).

The nutritional composition of plant-based beverages varies according to their source and can contain nutrients, such as isoflavones, phytosterols, beta-glucans, arabinose, vitamin E, lauric acid, catechins, lignans, and omega-3, which play important roles in interactions with the intestinal microbiota and antioxidant action (DE BELLIS *et al.*, 2021; AYDAR *et al.*, 2020). Plant-based beverages are generally lactose-free, cholesterol-free and are sold with added calcium and vitamins, such as vitamin B12 (MUNEKATA *et al.*, 2020). The most common sources of plant-based drinks are cereals (oats, rice, corn, and rye), legumes (soybeans, peanuts, and lupins), nuts (almonds, coconuts, hazelnuts, and pistachios), seeds (sesame, sunflower, and pumpkin), and pseudo cereals (quinoa) (MUNEKATA *et al.*, 2020).

From a nutritional perspective, plant-based beverages contain significant levels of bioactive compounds; however, these depend on the type of plant used (MUNEKATA *et al.*, 2020), and include β -glucan, which is associated with lowering blood glucose; phytosterols, which can reduce the risk of cardiovascular disease; isoflavones, which can reduce the risk of cardiovascular disease, cancer and osteoporosis; lignans, which can reduce blood cholesterol levels; and omega-3 fatty acids, which are related to protection against cardiovascular disease development (MUNEKATA *et al.*, 2020; AYDAR *et al.*, 2020).

Among vegetable drinks, soy-based products have been the most important for many years because of their high protein quality and positive health effects, which reduce the risk of neurodegenerative disorders (AYDAR *et al.*, 2020). In a study by Mello *et al.* (2021), soy-based vegetable beverages were found to be the most similar to UHT cow milk in terms of nutritional information on the label as determined using principal component analysis. These drinks are considered beneficial for cardiovascular health, as they are sources of monounsaturated and polyunsaturated essential fatty acids. Among the bioactive compounds already mentioned, these drinks contain isoflavones (effects on cardiovascular and bone health and protection against

cancer) and phytochemicals such as phytosterols (cholesterol-lowering properties). They also contain fiber, minerals (calcium, iron, and zinc), and B vitamins (PAUL *et al.*, 2019). However, because of the presence of unsaturated fatty acids and lipoxygenases, which are involved in the release of off-flavors in most of these drinks, their acceptability is reduced (MUNEKATA *et al.*, 2020; AYDAR *et al.*, 2020). In addition, soy-based beverages have reduced acceptability, especially among men, owing to the presence of isoflavones, which have been erroneously associated with infertility. Notably, these isoflavones have been shown to reduce the risk of prostate cancer development and to inhibit prostate cancer metastasis (MESSINA, 2010). Nevertheless, a growing demand remains for beverages from plant sources other than soy.

Despite the nutritional benefits of the vegetables used to prepare these plant-based drinks, many additives such as stabilizers, emulsifiers, and flavors, are added to improve the aftertaste of these products and provide a more pleasant characteristic to the palate. Consequently, their acceptability can be reduced because an increasing number of people are looking for clean-label products (MELLO *et al.*, 2021).

According to Paul *et al.* (2020), four major areas have been studied to improve the properties of plant-based drinks: stability, removal of unpleasant taste, inactivation/removal of anti-nutritional factors, and increased shelf life. Steps such as peeling, sprouting, blanching, and soaking are performed as preprocessing steps to aid processing.

However, plant-based beverages in the consumer market still suffer from a lack of standardization, mainly because of the sum of three factors: the possibility of using various types of plants, the lack of specific legislation (national/international), and the lack of literature on these products (FRANCISQUINI *et al.*, 2023).

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3.2 DEHYDRATED PRODUCTS WITH ADDED ENZYMES

Dairy products are a part of the diet of the population worldwide because of their high nutritional value, providing increased immune protection and prevention of diseases such as regional enteritis, ulcerative colitis, and malnutrition (CATANZARO *et al.*, 2021). However, poor digestion of lactose, known as lactose intolerance, is highly prevalent worldwide (57–65% of the population) and mainly affects countries such as China and Africa (CATANZARO *et al.*, 2021). Individuals with this condition experience varying degrees of gastrointestinal changes such as gastric discomfort, abdominal pain, diarrhea, and flatulence upon consuming dairy products (SANTOS *et al.*, 2019). To minimize these symptoms, eating habits need to be changed by reducing the intake of lactose-containing foods. One option for these individuals is the intake of dairy products with hydrolyzed lactose, which have been widely produced by the food industry to meet this growing market (QUEIROZ *et al.*, 2021).

To produce dairy products with lactose hydrolysis, the enzyme β -galactosidase (lactase) needs to be used during industrial production. This enzyme is a tetramer of four identical polypeptide chains, with each chain having 1023 amino acids that combine to form five structural domains (SAQIB *et al.*, 2017). Lactase cleaves the disaccharide lactose, to produce galactose and glucose, which participate in glycolysis. In addition to lactose hydrolysis for producing zero-lactose products in the dairy industry, β -galactosidase is also used to produce capsules that are routinely used by individuals with lactose intolerance (HE *et al.*, 2008). It can also be used to solve problems when disposing of whey on a commercial scale. Lactase sources are of microbial, plant, and animal origin, and the choice of source depends on the conditions of the medium in which the enzyme is added, such as pH. Among the microbial β -galactosidase sources used in industries, those obtained from bacteria are characterized by high activity, ease of fermentation, and stability. Also noteworthy are enzymes obtained from fungi, which present an optimum range of activity at acidic pH and higher temperatures (50°C range) (SAQIB *et al.*, 2017).

In addition to the growing market for hydrolyzed lactose dairy products, dehydrated dairy products are a viable alternative for consumers because the drying process facilitates reduced perishability of fluid milk with an extended shelf life and ease of transportation, handling, processing, and formulation (ER *et al.*, 2019; FRANCISQUINI *et al.*, 2020).

However, some technological challenges arise when these two market demands are combined. During the drying of hydrolyzed lactose milk powder, the powder shows greater adhesion to the inside of the drying chamber (even at very low inlet/outlet air temperatures), as

along with a decrease in solubility and modification of the powder microstructure (TORRES *et al.*, 2017). Hydrolyzed lactose dehydrated dairy products are also reported to be more prone to enzymatic browning (Maillard reaction), with a consequent reduction in the nutritional quality of the powder, for example, because of the unavailability of essential amino acids like lysine and the possibility of the formation of mutagenic and carcinogenic compounds (FRANCISQUINI *et al.*, 2020).

The food industry thus needs to adopt strategies to facilitate the drying of hydrolyzed lactose products. One of these strategies, which is frequently used, is based on the use of drying facilitating agents, such as maltodextrin and soluble fibers, which increase the glass transition temperature of the product (DE PAULA *et al.*, 2021).

A recent study reported an improvement in the agglomeration profile of the powder and an increase in the glass transition temperature during the drying of skimmed milk powder by the addition of transglutaminase (TGase) (ER *et al.*, 2019). Thus, the use of TGase could be a new strategy to facilitate the drying of dairy products (especially hydrolyzed lactose); however, this remains to be explored by the industry. In addition, this enzyme is capable of forming intra- and intermolecular peptide bonds in different food proteins such as casein through cross-linking with glutamine (acyl donor) and lysine (acyl receptor) residues, promoting functional changes in foods such as yogurt (foam stability, gelation, thermal stability, viscosity, and water retention capacity). This crosslinking between amino acids can also inhibit the Maillard reaction (MR), but this effect has not yet been reported for spray-dried dairy products (GAN *et al.*, 2009; ROMEIH and WALKER, 2017). Over 30 years ago, Ajinomoto began using transglutaminase in food (discovered by the company in 1987) and established a production process in 1993. Today, it is no longer exclusive to the company, and other competitors produce the same enzyme (AJINOMOTO 2023). Enzyme regulation in Mercosur is relatively new and has only advanced in recent years. In other countries, the situation is similar, with enzymes authorized in different food categories; however, for some food classes, they remain in the approval phase. In Europe, for example, legislation remains country-specific, although the European Union is developing regulations for the use of enzymes in the bloc using a positive list. In the United States and Japan, enzyme use is regulated by the *Food and Drug Administration* (FDA) and *Food Sanitation Act* (FSA), respectively (AJINOMOTO, 2023).

In Brazil, the use of transglutaminase is regulated by ANVISA's RDC 728-2022 (on enzymes and enzyme preparations for use as adjuvants in the production of food intended for human consumption), which seeks to regulate enzyme safety and toxicology. According to this resolution, only transglutaminases from *Strepto verticillium mobaraense* and *Streptomyces*

mobaraense are authorized for use in food (BRASIL, 2022). In Brazil, along with the ANVISA legislation, foods of animal origin are also regulated by the Ministry of Agriculture, Livestock, and Supply (MAPA). This body establishes criteria for the acceptance or non-acceptance of TGase from a technological perspective (mainly fraud through the use of the enzyme). Thus, each food of animal origin, according to its RTIQ or normative instructions, may or may not allow the use of transglutaminase.

Today, in Brazil, this enzyme is used in dairy products such as deserts, *petit suisse* cheese, processed cheese, dairy drinks, and ice cream. In 2023, Mercosur resolution GMC 15/2023 was published, approving the use of transglutaminase in cheese and fermented milk, a category that still had some restrictions on use under MAPA. Discussions on the Mercosur level for fermented milk and cheese with transglutaminase took more than 10 years and sought to harmonize divergent legislation and to modernize the dairy industry, which can now count on new adjuncts to improve its processes and products (BRASIL, 2022; AJINOMOTO, 2023). For the dehydrated or powdered milk category, MAPA Normative Instruction No. 53 of 2018 states that "the use of technology aids authorized in specific legislation is permitted in the preparation of powdered milk, provided they are harmonized in MERCOSUR and incorporated into the national legal system." In other words, it opens possibilities for the addition of this enzyme during manufacture (BRASIL, 2018).

Transglutaminase is considered an adjunct to manufacturing technology because it has no function in the final food product. Therefore, its inclusion in the list of ingredients is not necessary. This enzyme catalyzes peptide reactions between specific amino acids (lysine and glutamine) in the milk protein matrix. This action is subject to time and temperature conditions as well as sufficient substrate to promote the bonds. Transglutaminase loses its activity when denatured or when the sites of action are no longer available because of substrate restrictions. The percentage of transglutaminase to be used in food depends on the type and amount of protein in the matrix (SALUNKE and METZGER, 2023). The amount of TGase must be sufficient to achieve the technological and sensory objectives of the product to be formulated (AJINOMOTO 2023). Therefore, this enzyme is considered safe for use by the food industry; however, the factors of temperature (optimum range between 40°C and 60°C), pH (optimum range between 5–8), and type of food substrate need to be optimized to determine the amount of enzyme to be used in the food (AJINOMOTO, 2023; BRASIL, 2022).

Transglutaminase acts specifically by binding lysine and glutamine (*GL-bound*); when this peptide bond is formed, ammonia is released. The active sites of the enzyme are highly susceptible to oxidation. Therefore, to prevent loss of enzyme activity, some resources, such as

special packaging for protection against atmospheric oxygen or the addition of specific ingredients to the enzyme preparation, can be used to increase its resistance to environmentally present oxidizing agents. In dairy products treated at temperatures below 80°C, application of transglutaminase also requires care in relation to other oxidative enzymes naturally present in milk. Currently, formulation methods, such as encapsulation are available to protect against these oxidizing agents (AJINOMOTO, 2023; DOMINGUEZ *et al.*, 2023).

Studies have reported methods for quantifying GL-bound using gas chromatography (GC), quantifying this binding, and determining whether or not ammonia is released (GHARIBZAHEDI and ALTINTAS). Other ways of detecting GL-bound include the hydroxamate method (BARRETO *et al.*, 2015), the sensitivity method with fluorescent substances, high-performance liquid chromatography (SOKULLU *et al.*, 2008), electrophoresis (LI *et al.*, 2021), and the use of a transglutaminase assay kit (Sigma-Aldrich Corporation) (AJINOMOTO, 2023; OGILVIE *et al.*, 2021).

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4 CHAPTER 1: PHYSICOCHEMICAL ASPECTS OF INDUSTRIAL PLANT-BASED BEVERAGES

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4.1 INTRODUCTION AND AIM

The increased number of individuals presenting a certain degree of lactose intolerance or allergy to cow's milk protein, as well as the increase in supporters of diets excluding animal-based foods (flexitarian, vegan, or vegetarian) has been growing. This situation makes it necessary for the industrial production of substitutes for animal products, such as plant-based foods (AYDAR *et al.*, 2020).

Plant-based beverages can be characterized as suspensions or emulsions produced from plant materials, such as cereals, legumes, nuts, seeds, and/or pseudo-cereals (SETHI *et al.*, 2016). There is significant variability in vegetables that can be used to produce beverages, however, there have been no technical regulations of identity and quality to be followed, resulting in a significant number of non-standard industrialized products on the market. Consequently, these plant-based beverages differ in terms of composition, production, stability, storage, claims, ingredients list, sales denomination, and market price (MELLO *et al.*, 2021).

Previous studies have characterized plant-based beverages and model emulsions prepared in the laboratory (DAI *et al.*, 2018; LI *et al.*, 2021; PADULA *et al.*, 2020; ZHANG *et al.*, 2020; WANG *et al.*, 2017). Mello *et al.* (2021) also evaluated the aspects such as composition, label, and sale prices of industrialized plant-based products.

However, there is a lack of data in the literature on the physicochemical characterization of industrialized plant-based beverages. Obtaining these data will be useful to provide an idea about parameters that can be standardized by regulatory agencies to favor the production of these beverages (ARBACH *et al.*, 2021). To date, there is no specific legislation at the national (Brazil) or international levels.

Therefore, the aim of this study was to perform physicochemical characterization of industrialized plant-based beverages by performing state-of-the-art studies on these beverages.

4.2 MATERIAL AND METHODS

Plant-based beverages of five different vegetables were commercially obtained, with three different brands for each vegetable ($n: 5 \times 3 = 15$ samples). The vegetables were: 1) oat (A1, A2, A3), 2) almond (B1, B2, B3), 3) chestnut (C1, C2, C3), 4) coconut (D1, D2, D3), and 5) soybean (E1, E2, E3). Analyzes were carried out between April and July 2022. For additional information about the analyzed samples, Table 1 shows the list of ingredients and shelf life date of each carton packaging plant-based beverages (all samples with 1 liter package).

pH was determined using a portable digital pH meter (Gehaka PG 1400). Moisture content was determined using the gravimetric method, according to the AOAC International official methods (AOAC, 2005), by drying in an oven (102 ± 2 °C until constant mass, time average of three and half hours). The results were analyzed using mathematical calculations according to Equation 1:

$$[(mf - mt)/ma] \times 100 \quad (1)$$

Where:

mf is the final mass (capsule + glass beads + sample to constant mass)

mt = capsule mass + glass beads

ma = sample mass

Acidity followed the methodology proposed by ISO/TS 11869:2012-IDF/RM 150:2012 (ISO/IDF, 2012). An aliquot of the sample was suspended in water, such suspension was titrated potentiometrically by adding sodium hydroxide solution [$c(\text{NaOH}) = 0.1$ mol/l] until reaching pH 8.30 ± 0.01 , with subsequent mathematical calculation expressed by Equation 2:

$$I = (V \times 10)/m \quad (2)$$

Where:

I = titratable acidity in millimoles of sodium hydroxide per 100 g

V = volume in milliliters of sodium hydroxide solution used for titration

m = mass in grams of the sample portion

Table 1 - Ingredient list and shelf life date of the carton packaging plant based beverages analyzed in this work.

Vegetable source	Plant based Beverage	List of ingredients	Shelf life date (month-day-year)
Oat	A1	Water, oats, vegetable sunflower oil, tricalcium phosphate and sea salt.	03-25-2023
	A2	Water, organic whole oats, organic sunflower oil, natural calcium and salt.	09-20-2022
	A3	Water, whole oats, soluble fiber, sunflower vegetable oil, calcium, salt, vitamin D, gellan gum stabilizer, flavorings and emulsifiers soy lecithin.	08-23-2022
Almond	B1	Water, sugar, almonds, maltodextrin, calcium and zinc, salt, vitamins (E, B6, folic acid, D and B12), flavoring, sunflower lecithin emulsifier and stabilizers: xanthan gum and gellan gum.	09-29-2022
	B2	Reconstituted almond paste, sugar, calcium and zinc, sea salt, vitamins B2, D2 and B12, stabilizers: locust bean gum and gellan and sunflower lecithin emulsifier.	09-22-2022
	B3	Water, sugar, almonds, maltodextrin, minerals: tribasic calcium phosphate and zinc sulfate, vitamins E, B6, A, folic acid and B12, flavoring, stabilizers: gellan gum (INS418) and xanthan gum (INS 415) and acidity: sodium citrate (INS331iii).	09-01-2022
Chestnut	C1	Reconstituted chestnut paste, calcium (tricalcium phosphate), sea salt, vitamin A (retinyl palmitate), vitamin D2 (ergocalciferol), natural stabilizer gellan gum and natural sunflower lecithin emulsifier.	08-26-2022
	C2	Water and Organic chestnut.	11-22-2022
	C3	Water, organic demerara sugar, organic chestnut, calcium carbonate, sea salt, guar gum thickener and natural flavors.	12-03-2022
Coconut	D1	Water, dehydrated coconut milk, sugar, maltodextrin, calcium, salt, vitamin E, zinc, Vitamin B6 and A, folic acid, and vitamin B12, flavoring, stabilizer: gellan gum (INS 418) and xanthan gum (INS 415), acidity regulator: sodium citrate (INS331iii) and antioxidant: sodium ascorbate (INS 301).	09-19-2022
	D2	Reconstituted coconut milk, calcium and zinc, sea salt, vitamins (B2, D2 and B12), natural flavors, sunflower lecithin emulsifier, locust bean and gellan gum stabilizers and sodium ascorbate antioxidant.	09-17-2022
	D3	Water, coconut cream, sugar, maltodextrin, calcium and zinc, salt, vitamins (E, B6, folic acid, D and B12), emulsifier, esters of mono and diglycerides of fatty acids with citric acid, stabilizers: xanthan gum and gellan gum and flavoring.	09-12-2022
Soybean	E1	Water, soy beans, sugar, minerals (calcium and zinc), salt, vitamins (E, B5, A, folic acid, D and B12) flavoring, stabilizer: sodium citrate, gellan gum and xanthan gum, soy lecithin emulsifier and sacralose sweetener.	07-08-2022
	E2	Water, soy extract, sugar, salt, tricalcium phosphate, carrageenan and carboxymethylcellulose stabilizers, flavorings and sodium citrate stabilizers.	09-20-2022
	E3	Water, soy extract, sugar, polydextrose, salt, mixture of emulsifiers and stabilizers (sugar, emulsifier mono- and diglycerides of fatty acids and carrageenan, stabilizers monobasic potassium phosphate and potassium citrate), stabilizer sodium citrate and flavoring.	08-23-2022

Source: Prepared by the author (2023).

A portable ionic calcium meter (LAQUAtwin, Laqua-cal1) was used to determine the soluble ionic calcium at the original sample pH and at pH 4.3 (determined after the acidification of the sample to pH 4.3, by the addition of 12 M hydrochloric acid). A portable digital refractometer (Milwaukee MA-871) was used to obtain the soluble solid content and an oil bath at 155 °C was used to determine the heat coagulation time (HCT).

The density was analyzed using a Gay-Lussac glass pycnometer (25 mL) calibrated with a thermometer (reference temperature of 20 °C), with the result expressed by Equation 3:

$$d = m/v \text{ (3)}$$

Where:

d = density

m = mass of full sample pycnometer – mass of empty pycnometer

v = volume expressed in the pycnometer (25 mL)

The conductivity was measured using a microprocessor benchtop conductivity meter (Tecnopon, MCA150). An optical microscope (Medilux, MC30) was used for optical microscopy at the magnification of 0.85 (ocular lens) $\times$ 40 (objective lens), performing a total of four fields per analysis performed.

Particle size distribution was determined using a Beckman Coulter LS 13 320 laser diffraction analyzer (Beckman Coulter®, FL, USA) coupled to a liquid analysis module (Beckman Coulter®, FL, USA). The beverages were slowly added to the reservoir containing water at room temperature until an opaque mixture was obtained. Five series of data were collected at 90 s intervals (1.5, 3.0, 4.5, 6.0, and 7.5 minutes [min]). The results (<1 and >1 μm) were obtained using the Fraunhofer approximation for the total solubility.

Finally, the surface zeta potential and particle size distribution were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern, ZEN 3600), with prior dilution of the samples (100x). For the surface zeta potential, water was used as a dispersant with a dispersant refraction index of 1.330, viscosity (cP) of 0.8872, and dielectric dispersion constant of 78.5, and three observations were measured at 25 °C.

R language (version 4.2.1) was used to achieve descriptive statistics, and the Shapiro-Wilk test was performed to verify data distribution for each variable prior to computing calculations. The effect of vegetable was analyzed according to the data distribution as follows:

1) for normal data, an ANOVA and Tukey HSD with 95% confidence and 2) for non-normal variables, the Kruskal-Wallis and paired samples Wilcoxon test were applied to estimate the effect of vegetable.

4.3 RESULTS AND DISCUSSION

Table 2 lists the experimental results obtained for the studied parameters in the physicochemical characterization of the industrially produced plant-based beverages. For comparison, the results found in the literature for each analyzed parameters are also listed in Table 2. According to the Shapiro-Wilk test, 12 out of 15 parameters analyzed did not show a normal distribution; the normal variables were pH, surface zeta potential, and moisture content. Consequently, the mean was calculated as the median and the minimum and maximum values were presented to highlight the variability of the data.

Currently, there is a lack of results in the literature on the physicochemical characterization of commercially produced plant-based beverages, as the previous reports have focused on plant-based beverages produced in the laboratory. Nevertheless, there are differences in the reported values owing to the methodology used in different studies. The present study is based on the current state-of-the-art in plant-based beverages available commercially, and the objective is to provide a comparison for different vegetable sources that will support new product development.

Table 2 - Minimum, maximum, and mean values of physicochemical parameters for industrialized plant-based beverages analyzed in the present study, as well as, the results found in the literature for plant-based beverages or model emulsions laboratory produced.

Code ^a	Parameter	Analyzed Industrialized plant-based beverages ^b			Laboratory plant-based beverage reported results ^{d,e}		
		Minimum Value	Maximum value	Mean value ^c	Value	Reference	Vegetable material
A	pH	6.17	8.01	7.20	3.9	Padula <i>et al.</i> (2020)	Rice and soy
					6.5	Rincon <i>et al.</i> (2020)	Chickpea and coconut
					6.97 to 7.37	Vallath <i>et al.</i> (2021)	Chickpea
B	Acidity (mmol NaOH·100g ⁻¹)	0.07	1.40	0.31	0.78 to 1.39	Rincon <i>et al.</i> (2020)	Chickpea and coconut
C	Heat time coagulation (min)	0.43	10.57	1.74	8.28 to 17.41	Jeske <i>et al.</i> (2019)	Lentil protein solutions and emulsions
D	Moisture content (g H ₂ O·100g ⁻¹)	87.790	98.220	92.200	90.63 to 92.02	Vallath <i>et al.</i> (2021)	Chickpea
E	Density (g·cm ⁻³)	1.023	1.102	1.075	1.13 g·mL ⁻¹	Padula <i>et al.</i> (2020)	Rice and soy
F	Soluble solids (°Brix)	1.70	12.30	7.50	14.5	Padula <i>et al.</i> (2020)	Rice and soy
					4.04	Rincon <i>et al.</i> (2020)	Chickpea and coconut
					4.68 to 7.30	Vallath <i>et al.</i> (2021)	Chickpea
G	Soluble ionic calcium original sample pH (mmol Ca ²⁺ ·kg ⁻¹)	0.0	1.6	0.4	0.00–1252.94 mg·100 mL ⁻¹	Fructuoso <i>et al.</i> (2021)	Plant-based beverages of different vegetable bases ³
H	Soluble ionic calcium pH 4.3 (mmol Ca ²⁺ ·kg ⁻¹)	0.1	37.5	5.0			
*	Released calcium (mmol Ca ²⁺ ·kg ⁻¹)	-1.3	37.0	4.7			
I	Conductivity (mS·cm ⁻¹)	2.08	1897.00	4.81	5.8 to 6.1	Park <i>et al.</i> (2015)	Natural plant-based model emulsion
J	Surface zeta potential (mV)	-39.1	-17.4	-29.1	-25.00 to -50.00	Rahmati <i>et al.</i> (2018)	Speckled sugar been protein and xanthan gum

K	Polydispersity index by DLS	0.090	1.000	0.390	0.13 to 1.29	Rahmati <i>et al.</i> (2018)	Speckled sugar been protein and xanthan gum
L	Z-average size by DLS (nm)	323.00	2293.00	786.25	700.00 to 1072.00	Rahmati <i>et al.</i> (2018)	Speckled sugar been protein and xanthan gum
M	Particle Size < 1µm by LS (%)	1.82	31.00	7.91			
N	Particle Size >1µm by LS (%)	69.00	98.20	92.10			
O	Dv90 by LS (µm)	4.65	120.70	27.51	5.12 to 29.01	Park <i>et al.</i> (2015)	Natural plant-based model emulsion

Source: Prepared by the author (2023).

*Released calcium is computed by subtraction of soluble ionic calcium original sample pH from soluble ionic calcium pH 4.3 and is not presented in Fig. 1.

^aThe codes refer to the ungrouped box-plots presented in Fig. 1 for each parameter.

^bThe reported data consider the group for all plant-based beverages analyzed regardless of the base vegetable material.

^cThe estimated mean was calculated as the median.

^dConsider the possibility of different methodologies and different plant-based beverages in the previous literatures compared to the work formulated here.

^eThe values of literature without unity, follow the same ones of the work formulated here.

As the physicochemical characterization of industrial plant-based beverages has not been previously reported, the results shown in Table 2 are valuable and represent an innovation that can support new product development for plant-based beverages by facilitating comparison. Therefore, the results are relevant for both academic and industrial areas, as physicochemical attributes are fundamental for seeking standardization, improving physical and thermal stability, and increasing the shelf life of products. Despite the increase in the production of plant-based beverages at the industrial level, there is still no specific legislation for this product at the national (Brazil) or international level. Thus, the results presented here can be of relevance, as they highlight the differences between beverages due to the lack of standardization. Specific legislation is necessary for searching physicochemical, nutritional, sensorial, microbiological standardization, shelf life, and permitted ingredients added to beverages regardless of the type of vegetable used.

The mean values for pH, moisture, soluble solids, calcium, surface zeta potential, particle size, and polydispersity index were similar to the reported values. However, acidity, HCT, density, and conductivity were lower than those reported in the literature. The range between the minimum and maximum values in the parameters presented in Table 1 indicates a lack of standardization among the analyzed samples. According to the one-way ANOVA, the vegetable material had an important impact on pH ($p = 0.0153$), moisture ($p = 0.0000$), and surface zeta potential ($p = 0.0008$). However, the Kruskal-Wallis test results suggest that Dv90 ($p = 0.3567$) is the only variable where vegetable does not have an impact. The other non-normal variables presented p -values lower than 0.05, indicating a significant impact of this factor.

Considering the impact of vegetables on plant-based beverage parameters, the physicochemical features cannot be grouped for all vegetables available in the market. This suggests that it is not possible to generate a single list of physicochemical requirements that defines the identity of a plant-based beverage. This demonstrates that it is the limiting factor for specific regulation. Thus, an option to allow partial standardization of the product is to identify trends and subgroups that could generate different types of plant-based beverages. For industrialized plant-based beverages, the absence of specific legislation implies different manufacturing technologies and the addition of different types and quantities of ingredients.

In reference to the reports on laboratory plant-based beverages, the following results can be highlighted. Padula *et al.* (2020) obtained mean values of 3.90 for pH, 1.13

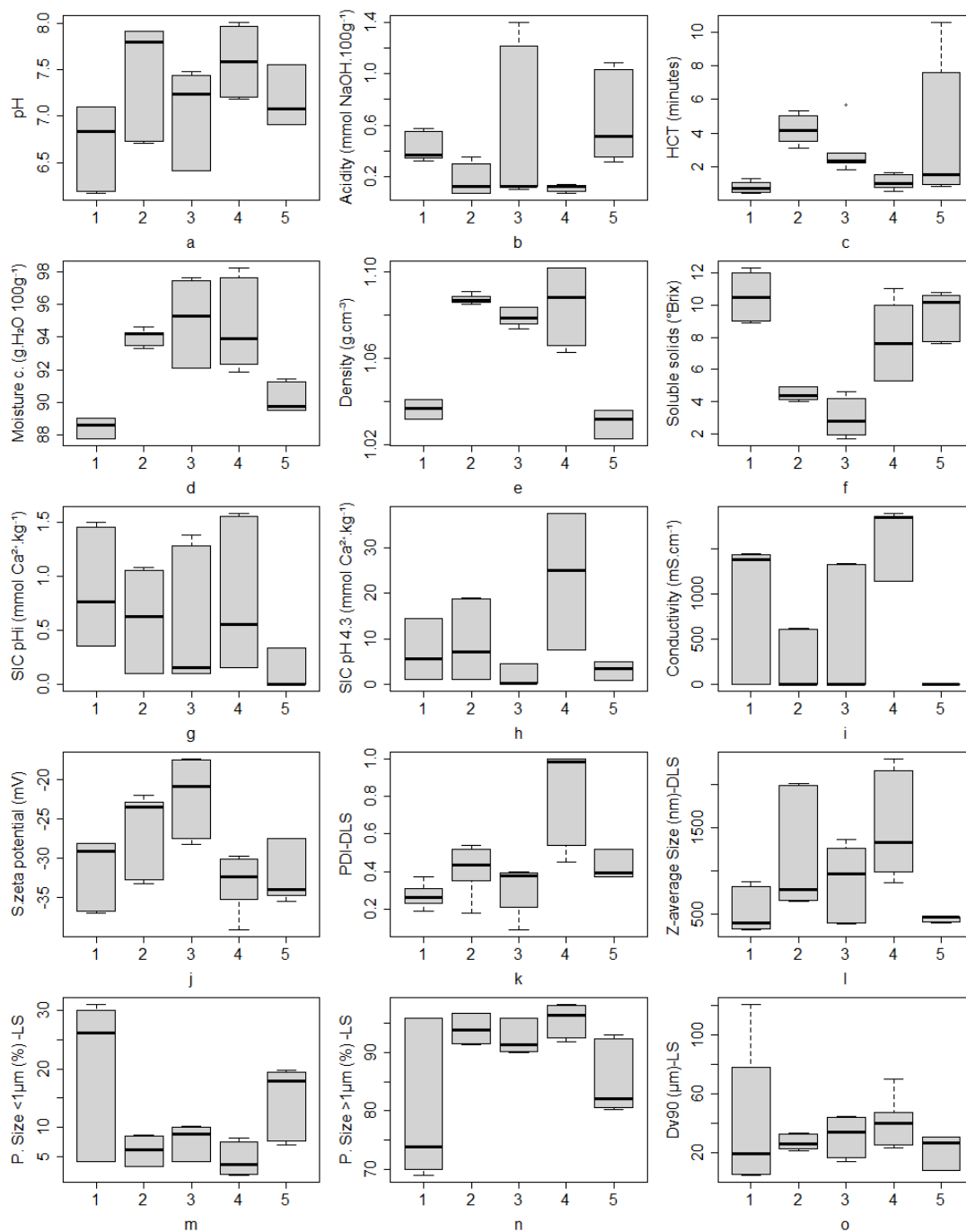
g/mL for density, 14.5 °Brix for solids content soluble in plant-based beverages laboratory-formulated with different concentrations of rice and soy. Vallath *et al.* (2021) evaluated the physicochemical and sensory properties of plant-based beverage developed with chickpea and obtained moisture contents between 90.63 and 92.02 g/100 g, soluble solids content between 4.68 and 7.30 °Brix, and pH between 6.97 and 7.37.

Rincon *et al.* (2020) developed plant-based beverages containing chickpea and coconut. They reported maximum soluble solids content of 4.04 °Brix and pH of 6.50, besides titratable acidity between 0.78 and 1.39 mL NaOH 1 mol/L 100 g. Fructuoso *et al.* (2021) reviewed the nutritional aspects of plant-based beverages and found that the variation in the amount of calcium was 0.00 – 1252.94 mg/100 mL. Jeske *et al.* (2019) studied the effects of high-pressure homogenization and heat treatments on the physicochemical properties of lentil protein solutions and emulsions. They performed HCT analysis and obtained clotting times of 8.28 to 17.41 min.

Rahmati *et al.* (2018) investigated the thermodynamic compatibility and probable interactions between speckled sugar bean protein and xanthan gum (O/W emulsion). In this study, particle size values between 700.00 and 1072.00 nm, PDI between 0.13 to 1.29, surface zeta potential between -25.00 and -50.00 mV were obtained. Park *et al.* (2015) evaluated the effect of high-pressure homogenization on the physicochemical properties of natural plant-based model emulsions. They found the mean particle size was $29.01 \pm 2.96 \mu\text{m}$, which decreased to $5.12 \pm 0.09 \mu\text{m}$ at 100 MPa and the electrical conductivity increased from 0.58 to 0.61 S/m in natural plant-based model emulsion. The authors correlated the particle size with conductivity results. The electrical conductivity of a liquid depends on the number of ions that can freely move in the liquid. Moreover, the increase in electrical conductivity can result in modification of the colloidal phase and dispersion of oil globules in the liquid (PARK *et al.* 2015). In the aforementioned study, an inverse relationship was found between particle size and electrical conductivity, that is, if the size of the particles increased, there was a reduction in the electrical conductivity. However, in this study, no significant statistical correlation was found between the conductivity and particle size, which can be attributed to the fixed particle size in the emulsion (PARK *et al.*, 2015).

Figure 1 shows the impact of vegetable on the studied parameters. Box plots are plotted as a function of each vegetable (oat, almond, chestnut, coconut, and soybean). The objective of this study was to identify similarities and subgroups that would allow further standardization of different types of plant-based beverages.

Figure 1 - Box plots as a function of each vegetable (1 = oat, 2 = almond, 3 = chestnut, 4 = coconut, 5 = soybean). The variables analyzed were: a = pH, b = acidity (mmol NaOH·100 g⁻¹), c = HCT (min), d = moisture c. (content) (g H₂O·100 g⁻¹), e = density (g·cm⁻³), f = soluble solids content (°Brix), g = SIC (soluble ionic calcium) pH_i (original sample pH) (mmol Ca²⁺·kg⁻¹), h = SIC pH_i (soluble ionic calcium at initial pH) in pH 4.3 (mmol Ca²⁺·kg⁻¹), i = conductivity (mS·cm⁻¹), j = S. (surface) zeta potential (mV), k = PDI (polydispersity index) by DLS, l = Z-average size by DLS (nm), m = particle size <1μm by LS (%), n = particle size >1μm by LS (%), o = Dv90 by LS (μm).



Source: Prepared by the author (2023).

As not all the analyzed variables present a normal distribution, it is worth mentioning that the comparisons performed in this section consider a Tukey HSD (using the average) and Paired Samples Wilcoxon Test (using the median), according to the distribution of the variable.

For each vegetable, three commercial products of different brands were considered, and they had different ingredients. Hence, it was possible for one brand, when it was significantly different from the other two, to generate a high amplitude in the box plot affecting the distribution. In this situation, all three brands were analyzed, and the extreme values were not filtered as outliers. As all commercial products are commercially available, they are considered safe for consumption.

The pH (Figure 1a) values ranged between 6.0 and 8.0. The highest median (above 7.5) and highest variability were obtained for the beverages formulated with almonds. However, the lowest median (below 7.0) was found for oats and the lowest for soybeans. According to the Tukey HSD, the results with 95% confidence almond, chestnut, coconut, and soybean did not present significant differences, while oat was marginally different from almond ($p = 0.043$) and significantly different from coconut ($p = 0.015$).

Regarding acidity (Figure 1b), the medians of almond, chestnut, and coconut did not present significant differences according to the Paired Samples Wilcoxon Test. This group was lower than that of oats and soybeans. In addition, the smallest variability was observed for almonds, oats, and coconuts. Notably, excluding soybean, the median values were below $0.4 \text{ mmol NaOH} \cdot 100\text{g}^{-1}$, and it was confirmed that the acidity values were between 0.1 and $0.6 \text{ mmol NaOH} \cdot 100\text{g}^{-1}$ for 13 out of 15 brands analyzed, in which only one brand for chestnut and soybean showed results above the limit.

Coagulation time (Figure 1c) was between 1 and 10 min. The clotting time is extremely important for the stability of the sample during storage. It is relevant that for oats and soybeans, the brand with higher titratable acidity exhibited better HCT stabilities with higher times. It generates the amplitude of the soybean box plot with only brand over 6 min of coagulation. Nevertheless, this trend was not observed for the chestnut brands with higher titratable acidity. In general, the median of almond was higher than that of the other vegetables, being the products with better stability, while oats presented a lower HCT time, and the results were homogeneous in the different brands.

Moisture content can be divided into two groups. The first group comprised of oats and soybeans, which had the lowest median value. The other group consisted of almond, chestnut, and coconut, which had higher moisture content. The highest median

moisture content (Figure 1d) was found for chestnuts (above 95%) and the lowest for oats (below 90%). The beverages formulated based on chestnut and coconut exhibited greater variability, while those formulated based on almond showed the least variability in the results.

The humidity values ranged from approximately 88 to 98 g H₂O·100g⁻¹, which implies that some products with high moisture content do not represent a significant source of nutritional components relevant to the consumer, such as proteins or carbohydrates. Regarding the density (Figure 1e), as well as moisture, the vegetable oat and soybean represent the group with lower density, below 1.040 g·cm⁻³, while almond, chestnut, and coconut presented higher density, above 1.070 g·cm⁻³. The only vegetable with the greatest variability was the coconut.

In reference to the soluble solids (Figure 1f), higher contents were found for oats and soybeans (above 10 °Brix). This group had samples with higher acidity, lower moisture content, and lower density. Intermediate medians and greater variability were obtained for the coconuts (values between 6-8 °Brix). Finally, lower mean for soluble solids content (less than 5 °Brix), as well as lower variability were obtained for almond and chestnut. Soluble solid content is an important indicator of the flavor and market value of plant-based beverages, indicating maturity and quality of consumption. Soluble solids correspond to the total solids dissolved in water (SIRISOMBOON e NAWAYON, 2017). Therefore, the higher the value of °Brix, the greater the concentration of solids in the sample. The highest content of soluble solids was found in the sample based on oats and the lowest based on chestnuts.

The soluble ionic calcium at the original sample pH (Figure 1g) exhibited high variability in the results, regardless of the type of vegetable used. This may be because of the absence of specific legislation that does not stipulate the minimum or maximum concentration of calcium added as an ingredient during the production of commercial plant-based beverages. All the medians in the soluble ionic calcium at the initial sample pH were below 1.0 mmol Ca²⁺·kg⁻¹. The highest mean value was observed for oats (above 0.7 mmol Ca²⁺·kg⁻¹), and the lowest value was observed for soybean (below 0.1 mmol Ca²⁺·kg⁻¹).

Measurement of soluble ionic calcium at pH 4.3 allows the release of calcium bound to sample components, such as proteins, allowing us to obtain the total calcium of a sample. In this case, there was less variability in the results of the soluble ionic calcium pH 4.3 (Figure 1h) compared to the original sample pH. The coconut-based samples

exhibited the greatest variability and the highest median of soluble ionic calcium at pH 4.3 (the only one above $20.0 \text{ mmol Ca}^{2+} \cdot \text{kg}^{-1}$). The chestnut-based samples had the lowest variability and the lowest mean (close to $2.0 \text{ mmol Ca}^{2+} \cdot \text{kg}^{-1}$). Specifically, there is approximately 10 times difference between one vegetable and another. All vegetables had median values below $10.0 \text{ mmol Ca}^{2+} \cdot \text{kg}^{-1}$ (except for coconuts). Soybean showed the lowest concentration of Ca^{2+} in the soluble phase, and the acidic extraction did not release a significant amount of Ca^{2+} , as inferred from the soluble ionic calcium in the original sample pH and soluble ionic calcium sample at pH 4.3. In contrast, the vegetable that released the most important amount of Ca^{2+} was the coconut, followed by oats and almonds. The conductivity values (Figure 1i) were related to the ions in the solution, and the total ion concentrations of soybean, almond, and chestnut were significantly lower than those of oat and coconut. The samples formulated using oats and coconuts had mean values close/or above $1500 \text{ mS} \cdot \text{cm}^{-1}$, excluding one brand for oats. In the other samples (soybean, almond, and chestnut), the mean value was close to zero. The concentration of ions in the aqueous phase was practically null. The dispersion of the data was significantly high for all groups because the values obtained were above $1000 \text{ mS} \cdot \text{cm}^{-1}$ or lower than $5 \text{ mS} \cdot \text{cm}^{-1}$. For soybean, the three brands had values lower than $5 \text{ mS} \cdot \text{cm}^{-1}$.

The surface zeta potential (Figure 1j) indicated that the beverages mainly contained fat, carbohydrates, and proteins, which generated high variability. The median values of the samples formulated with oats, almonds, and chestnuts were above -30 mV . In contrast, for the samples formulated with coconut and soybean, the values were below -30 mV . The amplitudes of all vegetables were similar, and the coconut was less dispersed. In terms of colloidal structures, the surface zeta potential refers to the charge of the particles. Almond and chestnut exhibited a less negative charge, whereas coconut and soybean exhibited a more negative charge. The protein charge depends on the isoelectric point, and there is no correlation between pH and surface zeta potential, which suggests that the protein composition may be different in the vegetable groups. The surface charge is also related to calcium extraction by acidification in the coconut, suggesting that the calcium ions are strongly attached to the proteins, but they are released in acidic media, similar to gastric digestion conditions. In soybean, the ion concentration is lower, and they are strongly attached to the proteins, not being released; therefore, we compared the HCT values (Figure 1c), where soybean is the most stable group.

The monodisperse sample had a high degree of uniformity ($\text{PDI} < 0.4$). In contrast, a polydisperse sample has a low degree of uniformity ($\text{PDI} > 0.4$) (QUEIROZ *et al.*,

2021). Interestingly, when the PDI was higher than 0.7, the distribution was considered to be polydisperse. The only polydisperse population is coconut (mean PDI around 1.00), the other group's results are between 0.20 and 0.40 (Figure 1k). The oat beverage exhibited the lowest PDI (mean of approximately 0.25), which should therefore be considered the most monodisperse. Previous reports have shown that larger the monodispersity of an emulsion, dispersion, or foam, the greater the structural kinetic stabilization. In addition, the presence of larger particles could be related to a pronounced destabilizing effect (DICKINSON, 2015). With this, it can be understood that the higher the PDI of emulsions, as in the case of plant-based beverages, the greater the chance of the product to destabilize, resulting in problems such as phase separation during the storage period, which may imply difficulty in acceptance by consumers. This demonstrates that, depending on the type of vegetable used, there is variation in the monodispersity/polydispersity of the product, which may affect the stability of industrialized plant-based beverages.

The particle size measured using DLS (Figure 1l) exhibited very little variability in the soy-based sample. In association, these samples, as well as the plant-based beverages that used oats, showed mean particle sizes below 500 nm. Intermediate particle sizes have been reported for almonds and chestnuts (500 and 1000 nm). The largest particle sizes were found in the coconut plant-based beverages (>1000 nm). Finally, the greatest variability in the data was found for samples made from almonds and coconuts.

Regarding the parameter smaller than 1 μm (Figure 1m), the variability was more significant in the samples formulated based on oats. The mean values found for the % of particles smaller than 1 μm in the samples were also different among the analyzed vegetables: oat (>25%), almond (close to 5%), chestnut (close to 10%), coconut (lower than 5%), and soybean (close to 20%). The number of particles above 1 μm (Figure 1n), as expected, followed the opposite trend, implying that the increase in the mean followed the sequence: oat (70–75%), soybean (80–85%), chestnut and almond (90–95%), and coconut (>95%).

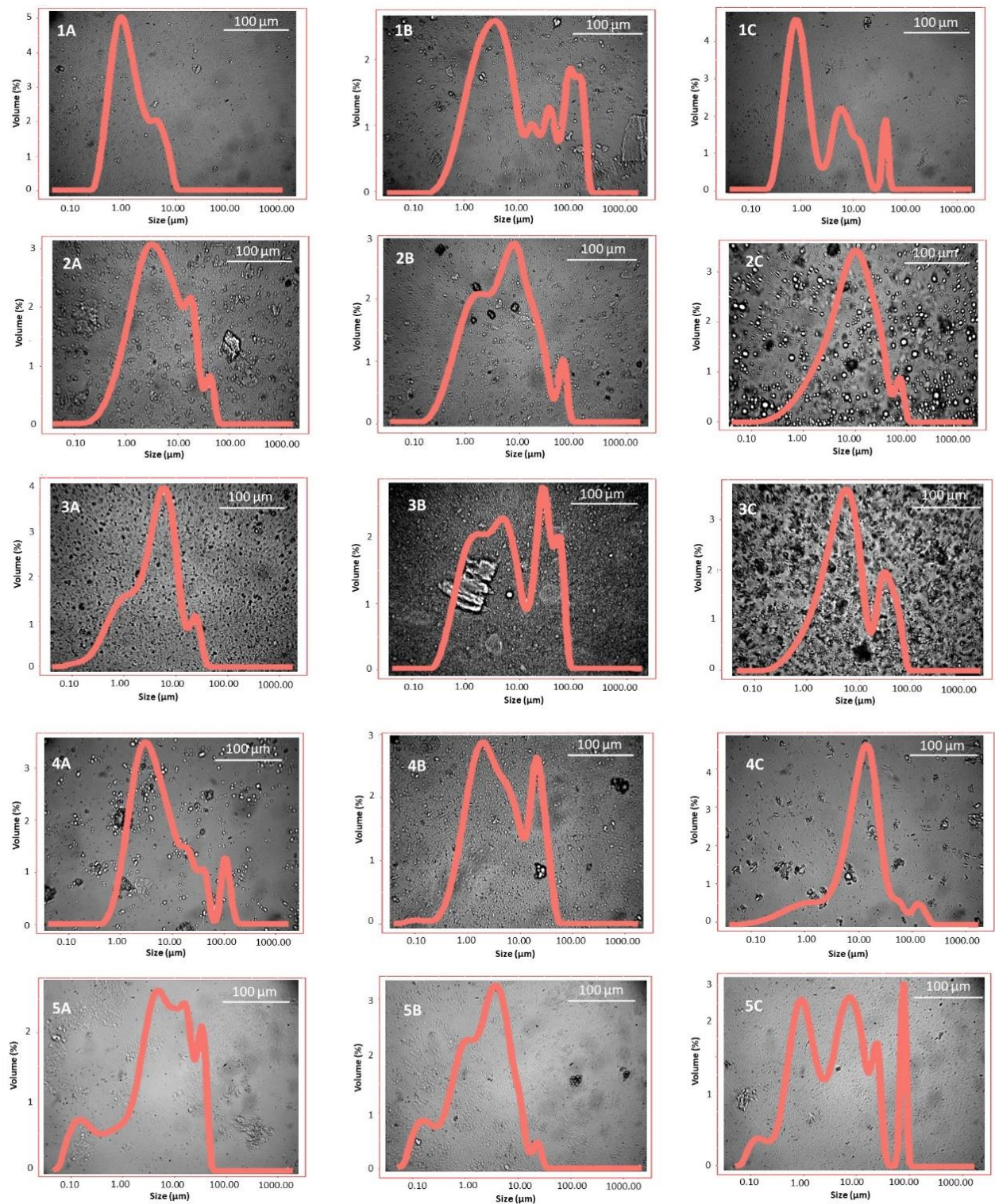
Dv90 corresponds to the fact that the particles have values of 90%, which is equal to or less than the reported results (DE PAULA *et al.*, 2021). For all analyzed vegetables, 90% of the particles exhibited mean values in the region between 20 and 40 μm . In relation to this attribute, plant-based beverages formulated with oats obtained greater amplitude. The other samples varied slightly with the brands analyzed. Notably, the particle size distribution in this case ranged from approximately 5-120 μm . Interestingly,

from the box plot, it can be concluded that oats had the lowest and highest values for the particles.

The two methodologies used to determine particle size and distribution confirmed the same result (in relation to the mean value): sample oats showed smaller particle sizes and sample coconuts showed larger particle sizes. Large particles are usually associated with problems in stability during the storage of beverages. The size of the dispersed phase particles in plant-based beverages is an important factor governing their stability and may thus interfere with the shelf life of the product, as well as its acceptability by consumers.²² In view of the above, it can be concluded that the oat sample (for the most part) is probably the one with the greatest stability against storage, whereas the coconut-based sample (for the most part) will probably exhibit less stability during this shelf life. It is relevant that oats and soybeans represent a group with a higher percentage of particles smaller than 1 μm (Figures 1m and 1n) and a lower z-average size determined by DLS (Figure 1l). This is strongly related to the density of this sample and significantly differentiates this group from almond, chestnut, and coconut. With increasing z-average size, the HCT time decreased, suggesting that the correlation between particle size and stability was significant when more than 90% of the particles had a size greater than 1 μm (Figure 1n).

Figure 2 shows the optical microscopy images of the analyzed samples and overlapping laser diffraction particle size distribution curves (LS). In this section, the aim was to visually present the particle size distribution and morphology of the samples for each vegetable and brand.

Figure 2 - Optical microscopy images and particle size distribution at the analyzed plant-based beverages (1 = oat, 2 = almond, 3 = chestnut, 4 = coconut, and 5 = soybean) of three different brands for each plant (brands A, B, and C).



Source: Prepared by the author (2023).

Optical microscopy showed that the highest variation was due to the differences in the vegetables used. Within the same vegetable, beverages formulated with almond, chestnut, and coconut showed the largest discrepancy among different brands. Beverages formulated with oats and soybeans showed a similar microscopy results, despite the variation in commercial brands. Interestingly, in addition to the physicochemical analysis, the optical microscopy of commercial plant-based beverages presented is one of the first times reported in the literature.

In addition to the results of particle size distribution, an overall mean of 11.16% of particles were in the region below 1 μm and an overall mean of 88.85% of particles were in the region above 1 μm . The sample that showed the highest volume percentage of large particles ($>1 \mu\text{m}$) was coconut (around 98.10%) and the sample with the lowest volume percentage of large particles ($>1 \mu\text{m}$) was oat (approximately 69,00%).

It is possible to infer high variability between samples with regard to the percentage number of particles above and below 1 μm (coefficient of variation of 85.78% for $<1 \mu\text{m}$ and 10.78% $>1 \mu\text{m}$). It was then observed that there was no standardization of the particle size. Plant-based beverages are colloidal dispersions that feature a number of components of different sizes, such as fat globules, ground raw materials, proteins, and carbohydrates. This can contribute to unstable products during storage owing to phenomena such as creaming, sedimentation, and phase separation (DHANKHAR e KUNDU, 2021).

Each sample exhibited different behavior with respect to particle size distribution. Therefore, there was no pattern between vegetables or within the same vegetables. For some foods such as milk, a pattern has already been reported in the literature for this granulometric distribution (FRANCISQUINI *et al.*, 2020). This difference and lack of standards may be due to the different types of vegetables used, as well as different types of ingredients. Nevertheless, a common situation for all analyzed samples was that the particle distribution was in the range of 0.10 and 1000 μm . As mentioned previously, this study represents the state-of-the-art commercial plant-based beverages. Thus, the mentioned fact can contribute to a possible inference of the usual range of the particle distribution.

It is difficult to find previous reports that corroborate the presented results in this study regarding optical microscopy and particle size for industrial plant-based beverages. They were found in studies carried out by Wang *et al.* (2017), Dai *et al.* (2018), Zhang *et*

al. (2020), and Li *et al.* (2021), which showed the results of optical microscopy and particle size. However, in the model, emulsions were formulated based on vegetables.

4.4 CONCLUSION

This study proves to be promising, given the lack of previously reports on the characterization of industrial plant-based beverages. Therefore, the presented research can be considered a state-of-the-art referring to the current state of knowledge about industrialized plant-based beverages commercialized in the market, which can help industries in the development of new plant-based beverages with greater standardization. Additionally it can help in the potential development of specific legislation for these plant-based beverages.

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5 CHAPTER 2: PHYSICOCHEMICAL ANALYSIS OF THERMALLY TREATED COMMERCIAL PLANT-BASED BEVERAGES COFFEE ADDED

This chapter has already been published as an article in the journal *European Food Research and Technology* (<https://link.springer.com/journal/217>) whose DOI corresponds to <https://doi.org/10.1007/s00217-023-04359-x>

5.1 INTRODUCTION AND AIM

Plant based beverages are suspensions or emulsions derived from vegetables. The main sources are cereals, legumes, nuts, seeds, and pseudocereals (ARBACH *et al.*, 2021; SETHI *et al.*, 2016). The lack of specific regulations for these beverages are related to the variability in the plant material, resulting in products without physicochemical, compositional, nutritional, technological, physical/thermal stability, and shelf-life standardization (MELLO *et al.*, 2021). In terms of stability, plant-based beverages can be subjected to different types of heat treatment, the most common being ultra-high temperature (UHT). This process is used to reduce the number of pathogenic or harmful microorganisms, which consequently extends the shelf life of the product (MUNEKATA *et al.*, 2020).

The increasing demand for plant-based beverages is related to the consumption of these products by customers with lactose intolerance, allergies to cow's milk proteins, or practitioners of diets that exclude products of animal origin, such as vegetarians, vegans, or flexitarians (AYDAR *et al.*, 2020; MELLO *et al.*, 2021). A traditional way of using these products is to mimic the use of milk and to make similar culinary preparations such as cappuccinos, lattes, cakes, cookies, and sweets. In these preparations, the plant-based beverages can be subjected to heat treatment and be added of ingredients such as coffee (ZAKIDOU *et al.*, 2022). Due to the inherently acidic character of coffee and the addition of different heat treatments to the UHT plant-based beverages, it may affect its protein stability (GUIMARÃES *et al.*, 2012; KARLSSON *et al.*, 2019). Consequently, this work is based on the hypothesis that when plant-based beverages are used in culinary preparations as heated, boiled, cooked, or added with ingredients such as coffee, the physical or thermal stability may be altered. The effects of these processes have not yet been described in the literature.

Therefore, the aim of this work was to conduct a study with commercial UHT plant-based beverages applying heat treatment and coffee addition to understand the effects on the physical and thermal stability of these products, as well as drawing a comparison of the list of ingredients found on the label of each product with the physicochemical analyzes carried out.

5.2 MATERIAL AND METHODS

Plant based beverages of five different vegetables were purchased in the city of Juiz de Fora - MG and region, considering three different commercial brands of each vegetable ($n: 5 \times 3 = 15$ samples). The vegetables of the plant-based beverages were designated as: oat (brands A1, A2, A3), almond (brands B1, B2, B3), chestnut (brands C1, C2, C3), coconut (D1, D2, D3), and soybean (brands E1, E2, E3). All samples were packed in 1-liter carton packs and were analyzed from April to June 2023. The samples displayed a shelf-life date of the period between July 2022 and March 2023.

An aliquot of 150 ml was taken from each of plant-based beverages and were analyzed at room temperature ($25.0 \pm 1.5^\circ\text{C}$ Treatment 0 – T0); heated at $85.0 \pm 1.5^\circ\text{C}$ for 5 minutes (Treatment 1 – T1); heated at $85.0 \pm 1.5^\circ\text{C}$ for 5 minutes and added of 1 g of soluble coffee (Treatment 2 – T2). In the analyses, it was used soluble coffee powder (coffee brand 3 *corações* extra Strong).

All the heated samples (with and without coffee) had the temperature lowered to $25.0 \pm 1.5^\circ\text{C}$ in an ice bath, then it was proceeded to the determination of pH, heat coagulation time (HCT), optical microscope and particle size distribution.

The pH and HCT were determined using a microprocessor portable digital pH meter (Gehaka PG 1400) and an oil bath at 155°C , respectively. An optical microscope (Medilux, MC30) was used for optical microscopy at a magnification of 0.85 (ocular lens) x 40 (objective lens), performing a total of 4 fields per analysis. The analysis of particle size of these images obtained by optical microscopy using ImageJ version 1.54d software, where the micrographs were transformed to binary images to improve contrast to perform automated particle analysis, identifying from 2000 to 3500 for each image computing the area in square pixels (px^2), according to the image size each squared pixel was equivalent to $8.54 \mu\text{m}^2$, so that assuming spherical particles it was possible to obtain the particle diameter. A frequency table was generated to plot the distribution and quartile distribution

with probability 0.10, 0.50, and 0.90 was computed. The distribution was presented within the images analyzed (Figure 6).

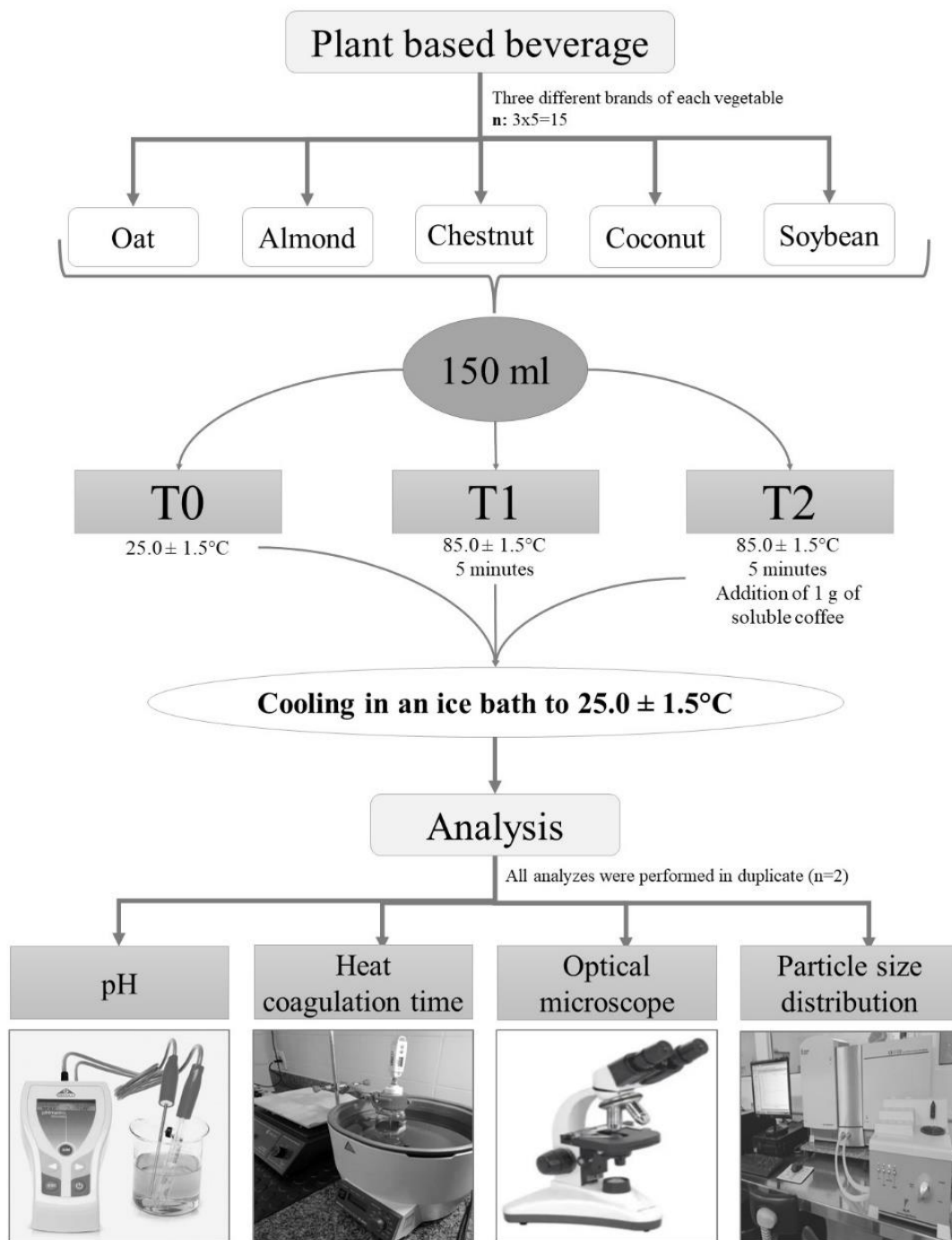
Particle size distribution was determined using a Beckman Coulter LS 13 320 laser diffraction analyzer (Beckman Coulter®, FL, USA) coupled to a liquid analysis module (Beckman Coulter®, FL, USA). Each beverage was slowly added to the reservoir containing water at room temperature until an opaque mixture was obtained. Five series of data were collected at 90-second intervals (1.5, 3.0, 4.5, 6.0 and 7.5 minutes). Results (<1 and >1 μm) were obtained using the Fraunhofer approximation for total solubility.

The pH of the coffee used during the analysis was evaluated by dissolving 1 g of soluble coffee in 150 ml of heated water ($85.0 \pm 1.5^\circ\text{C}$).

The software R (version 4.2.2) was used to achieve descriptive statistics and Shapiro-Wilk test was performed to verify data distribution for each variable aggregating by sample and treatment prior to compute calculations. The effect of vegetable material was analyzed depending on the data distribution as follows: 1) For normal data an ANOVA model and then Tukey test with 95% confidence; 2) for non-normal data the Kruskal-Wallis and Paired Samples Wilcoxon Test were applied to estimate the effect of vegetable material. Graphics were generated using using R programming language package ‘ggplot2’: Elegant Graphics for Data Analysis, 2.0.7.

All analyzes were performed in duplicate ($n=2$) and with the samples at a temperature of $25 \pm 1.5^\circ\text{C}$. Figure 3 illustrates the methodology used in the present work.

Figure 3 - Schematic summary of the methodology used in the work.

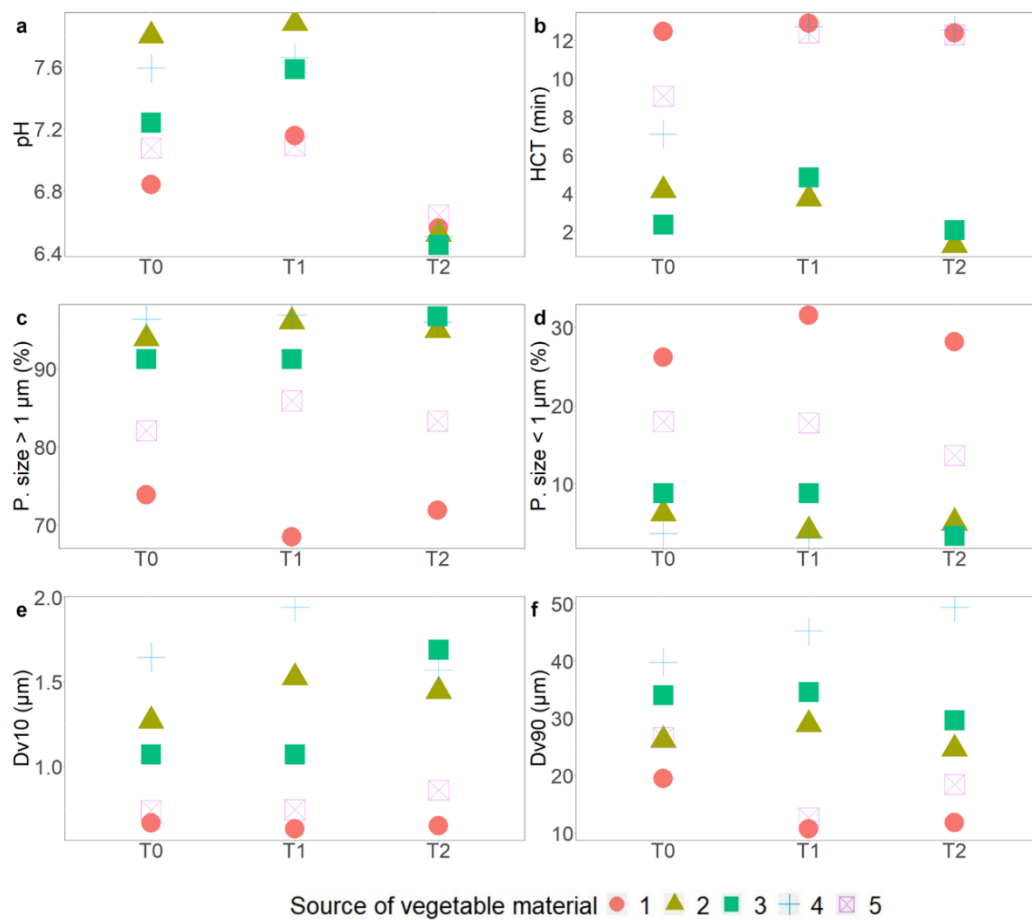


Source: Prepared by the author (2023).

5.3 RESULTS AND DISCUSSION

The median values for the studied parameters in function of the applied treatment being grouped by the source of vegetable material of the commercial plant-based beverages are presented in the Figure 4.

Figure 4 - Effect of the treatment applied in the commercial plant-based beverages grouped by the source of vegetable material (1) oat, (2) almond, (3) chestnut, (4) coconut, and (5) soybean. The treatment applied were T0 (25.0 ± 1.5 °C), T1 heated treatment (85.0 ± 1.5 °C for 5 minutes), and T2 heated treatment added with coffee (85.0 ± 1.5 °C for 5 minutes). The performed analysis were: (A) pH, (B) HCT (min), (C) particle size lower than 1 μm (%), (D) particle size higher than 1 μm (%), (E) Dv10 (μm), (F) Dv90 (μm).



Source: Prepared by the author (2023).

Initially, the effect of the heat treatment (T1) in the pH of the sample was not considered significant as the Tukey HSD test of pairwise comparison applied to the ANOVA model reported no significant difference between the group T0 and T1. In the treatment T0 the median pH values varied between 6.8 and 7.8; in the T1 treatment the pH values varied between 7.0 and 7.9. In Figure 2 it was possible to infer that the

determining factor for the pH reduction was the addition of soluble coffee (T2), this treatment led to a reduction in the pH of all samples. The treatment T2 the pH values varied between 6.4 and 6.6. From the results, it can be inferred that the addition of coffee was the factor that most influenced the pH in the different treatments applied to the plant-based beverages. The pH of the soluble coffee in water was 4.94 and the effect of T2 can be correlated to the fact that coffee is a naturally acidic food that can reduce the pH of the product to which it is added (GUIMARÃES *et al.*, 2012).

In the literature different reports present the pH values for plant-based beverages. Initially, for flavored rice and soybean-based beverages pH values were reported between 3.86 to 3.94 (PADULA *et al.*, 2020). In another report has been presented the development of a vegetable drink based on cashew nuts which pH was 6.49 (LIMA *et al.*, 2021). In the production of a rice-based vegetable drink was reported pH values between 5.7 and 6.1 (PADMA *et al.*, 2018). For a laboratory developed vegetable drink based on chickpea and coconut pH values were reported pH values ranging between 6.25 and 6.50 (RINCON *et al.*, 2020).

Regarding the thermal stability of the samples, evaluated by the HCT, it appears that the products submitted to the thermal treatment without the addition of coffee (T1) obtained a slight increase in the coagulation time by heating. This demonstrates a possible increase in the stability of these samples against heating. Despite this slight increase, the median value in all treatments was within a very similar range: T0 (2 to 12 minutes); T1 (3 to 13 minutes); T2 (1 a 12 minutes). It is worth mentioning that the addition of soluble coffee had no effect on HCT. The effects of homogenization and pasteurization on the formation, stability, and sensory properties of a lentil-based beverage through HCT analysis were reported in literature, presenting results for clotting time from 8.28 to 17.41 minutes (JESKE *et al.*, 2017).

Finally, the particle size ($> 1\mu\text{m}$; $< 1\mu\text{m}$; Dv10; Dv90) was not affected by either the heat treatment (T1/T2) or the addition of soluble coffee (T2). This can be confirmed by the range of values found for the attributes $> 1\mu\text{m}$ (for all treatments between 65 and 95%); $< 1\mu\text{m}$ (for all treatments between 1 and 30%); Dv10 (for all treatments between 0 and 2 μm); Dv90 (for all treatments between 10 and 50 μm).

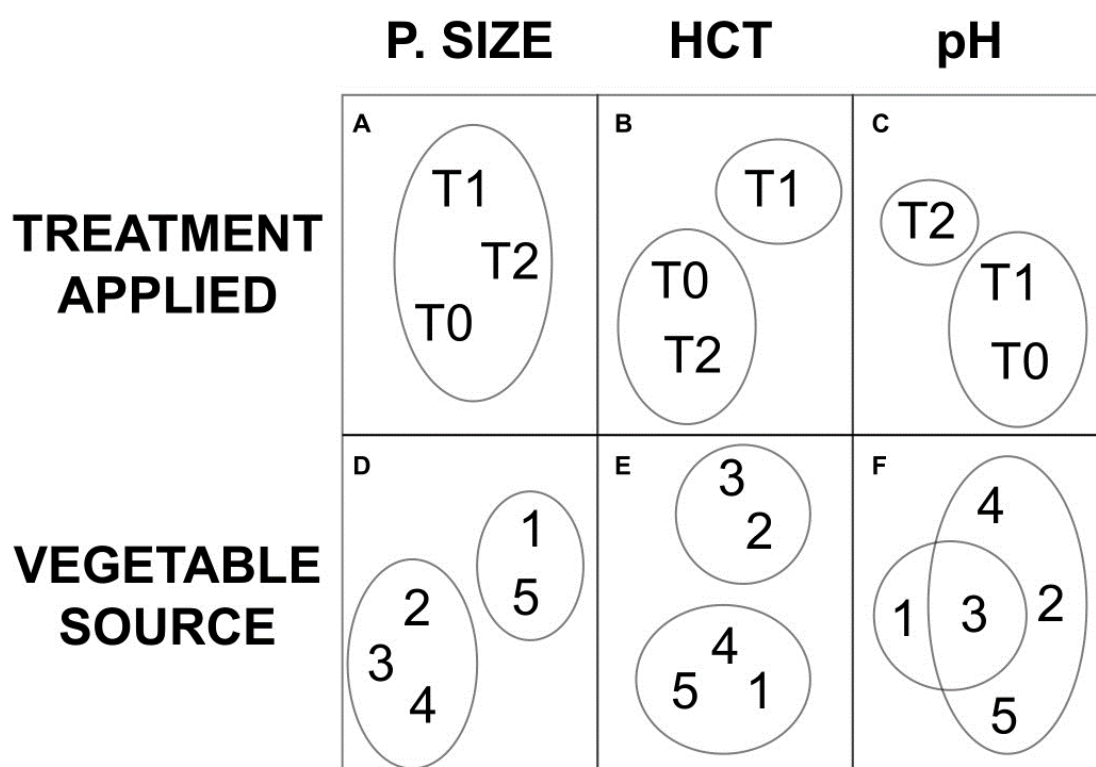
In literature was reported the evaluation of the stability of a soybean drink spiked with konjac glucomannan, in which the particle size distribution ranged from 10 to 1000 nm (AN *et al.*, 2019). In the evaluation of different commercial vegetable beverages the reported particle size measurements showed that the average volume diameters ranged

from 0.60 to 10.51 μm , but the commercial beverage based on quinoa and cashew had values of 81.47 and 29.17 μm , respectively (JESKE *et al.*, 2017). Finally, a report analyzing the physicochemical and acid gelling properties of commercial vegetable beverages compared to cow's milk presented the average particle size for plant-based beverages ranged from 0.55 to 2.08 μm (MÄKINEN *et al.*, 2015).

It is interesting to note that commercial plant-based beverages that were heat-treated (T1) did not result in changes in its physicochemical stability (pH, HCT, particle size), which can lead to the inference that the food has the potential to be used in heating preparations, such as cappuccinos, lattes, cakes, cookies, and sweets.

The impact of the treatment was assessed, in the case of particle size statistics and HCT by applying the Paired Samples Wilcoxon test for pairwise comparison to the Kruskal-Wallis model considering the applied treatment (T0, T1, and T2) as categorical factor, as the particle size distribution statistics and HCT results did not meet the normality assumption in the ANOVA model. In the case of pH, the Tukey test was applied to the ANOVA model using the same categorical factors. The results were plotted and presented in the Figure 5.

Figure 5 - Pairwise comparison results using Wilcoxon rank sum test with continuity correction. (A) particle size, (B) HCT, and (C) pH, in function of treatment applied; (D) particle size, (E) HCT and (F) pH in function of source of vegetable material. The vegetable material source were (1) oat, (2) almond, (3) chestnut, (4) coconut, and (5) soybean. Treatment applied were T0 ($25.0 \pm 1.5^\circ\text{C}$), T1 heated treatment ($85.0 \pm 1.5^\circ\text{C}$ for 5 minutes), and T2 heated treatment added with coffee ($85.0 \pm 1.5^\circ\text{C}$ for 5 minutes).



Source: Prepared by the author (2023).

Regarding particle size, all samples were grouped into a single group, which demonstrates that there was no influence on the particle size of the heat treatment and the addition of soluble coffee on the analyzed products.

The coagulation time by heating implied the division of the samples into two groups: one with treatments T0 and T2 and the other with treatment T1. That is, the heat treatment applied influenced the HCT of the analyzed samples (T1) increasing clotting time. Thus, treatment T0 and T1 are statistically equal and different from T2. That is, the addition of coffee reduced the clotting time of the samples.

Finally, the pH variable also resulted in the formation of two groups. A group was formed with the T0/T1 treatment and another group with the T2 treatment. That is, the

addition of instant coffee led to changes in the pH values. Therefore, treatment T2 differed statistically from treatments T0 and T1.

Regarding particle size, the samples were grouped into two groups, one in which the particles are larger (vegetables 2, 3 and 4), over 90% of particles larger than 1 μm in size. And another in which the particles are smaller (1 and 5), below 90% of particles larger than 1 μm in size. Therefore, the samples formulated based on almond, chestnut and coconut were statistically equal and with larger particle size. While the samples formulated based on oat and soybean differed statistically from the previous ones and had smaller particle sizes.

The HCT also enabled a double grouping: group 1 with longer coagulation time by heating, that is, greater stability against heating (vegetables 1, 4 and 5) and another with shorter coagulation time by heating, that is, less stability when heated (vegetables 2 and 3). Samples formulated with oat, coconut and soybean were the most stable regarding heat coagulation and differed statistically from beverages made from almond and chestnut, in which stability against heat was lower.

Finally, when analyzing the pH variable, two groups were also found, one in which the pH has a lower median values (vegetable 1, maximum value 7.0) and another in which the pHs have higher median values (vegetables 2, 4 and 5, maximum value above 7.2). In highlight, vegetable 3 (maximum value of 7.4) was presented to be in the interface between the two existing groups. Thus, the beverages formulated with oats differed statistically from the others (almond, coconut and soybean). Sample produced with chestnut showed to be statistically equal to oats and other vegetables.

It is worth mentioning that the formulations of commercial vegetable beverages analyzed were different from each other, even those formulated with the same vegetable. Thus, this grouping of vegetables in relation to the studied variables allows a superficial understanding of the behavior of vegetables in relation to particle size, HCT and pH. To add, Table 3 shows the list of ingredients described in each plant-based beverages pack.

Table 3 - Ingredient list of the plant-based beverages pack analyzed reported by manufacturer.

Vegetable source	Plant-based Beverage	List of ingredients (number of ingredients)
Oat	A1	Water, oats, vegetable sunflower oil, tricalcium phosphate and sea salt (5).
	A2	Water, organic whole oats, organic sunflower oil, natural calcium and salt (5).
	A3	Water, whole oats, soluble fiber, sunflower vegetable oil, calcium, salt, vitamin D, gellan gum stabilizer, flavorings and emulsifiers soybean lecithin (10).
Almond	B1	Water, sugar, almonds, maltodextrin, calcium and zinc, salt, vitamins (E, B6, folic acid, D and B12), flavoring, sunflower lecithin emulsifier and stabilizers: xanthan gum and gellan gum (16).
	B2	Reconstituted almond paste, sugar, calcium and zinc, sea salt, vitamins B2, D2 and B12, stabilizers: locust bean gum and gellan and sunflower lecithin emulsifier (11).
	B3	Water, sugar, almonds, maltodextrin, minerals: tribasic calcium phosphate and zinc sulfate, vitamins E, B6, A, folic acid and B12, flavoring, stabilizers: gellan gum (INS418) and xanthan gum (INS 415) and acidity: sodium citrate (INS331iii) (14).
Chestnut	C1	Reconstituted chestnut paste, calcium (tricalcium phosphate), sea salt, vitamin A (retinyl palmitate), vitamin D2 (ergocalciferol), natural stabilizer gellan gum and natural sunflower lecithin emulsifier (8).
	C2	Water and organic chestnut (2).
	C3	Water, organic demerara sugar, organic chestnut, calcium carbonate, sea salt, guar gum thickener and natural flavors (7).
Coconut	D1	Water, dehydrated coconut milk, sugar, maltodextrin, calcium, salt, vitamin E, zinc, Vitamin B6 and A, folic acid, and vitamin B12, flavoring, stabilizer: gellan gum (INS 418) and xanthan gum (INS 415), acidity regulator: sodium citrate (INS331iii) and antioxidant: sodium ascorbate (INS 301) (17).
	D2	Reconstituted coconut milk, calcium and zinc, sea salt, vitamins (B2, D2 and B12), natural flavors, sunflower lecithin emulsifier, locust bean and gellan gum stabilizers and sodium ascorbate antioxidant (12).
	D3	Water, coconut cream, sugar, maltodextrin, calcium and zinc, salt, vitamins (E, B6, folic acid, D and B12), emulsifier, esters of mono and diglycerides of fatty acids with citric acid, stabilizers: xanthan gum and gellan gum and flavoring (19).
Soybean	E1	Water, soybean, sugar, minerals (calcium and zinc), salt, vitamins (E, B5, A, folic acid, D and B12) flavoring, stabilizer: sodium citrate, gellan gum and xanthan gum, soy lecithin emulsifier and sucralose sweetener (18).
	E2	Water, soybean extract, sugar, salt, tricalcium phosphate, carrageenan and carboxymethylcellulose stabilizers, flavorings and sodium citrate stabilizers (9).
	E3	Water, soybean extract, sugar, polydextrose, salt, mixture of emulsifiers and stabilizers (sugar, emulsifier mono- and diglycerides of fatty acids and carrageenan, stabilizers monobasic potassium phosphate and potassium citrate), stabilizer sodium citrate and flavoring (13).

Source: Prepared by the author (2023).

The effect of the heat treatment (T1) in the HCT time was found to be dependent on the composition of the sample and inversely proportional to the particle size of the sample. The oat-based beverages were the group that presented the smallest particle size and the only product that did not report added hydrocolloids such as gums; which impact on the increase in the average particle size in food. Before the heat treatment application (T0) the oat-based beverage presented higher HCT times, followed by coconut and soybean-based beverages, as presented in the Figure 4B. Once the heat treatment was applied (T1) the HCT times of oat, coconut, and soybean-based beverages were not significantly different. It means that the heat treatment impact on the HCT time was not significant when the product does not report hydrocolloids as added ingredients, and when there was not reported the addition of gums the impact is to improve the HCT time to the same level as the oat-based beverages. A literature report evaluated the impact of pulsed electric field application on the different physicochemical and rheological properties of an almond-based beverage and carried out a reduction in particle size, reporting that beverages with lower particle size were those with better stability preventing phase separation (MANZOOR *et al.*, 2019). This idea is considered for oat and soybean-based beverages, as the coconut-based beverage reported the same behavior in terms of HCT time when applying heat treatment, nevertheless its particle size distribution is significantly different having higher particle size. On the other hand, the almond and chestnut-based beverages groups were those that had instability in the face of heating (shorter coagulation time by heating), which may probably be due to their exhibiting particles with a larger size.

The addition of the gums as ingredients is expected to generate different results in the product. In literature was reported the average diameter surface volume (d_{3.2}) for emulsions of commercial gums dispersed in distilled water at a concentration level of 0.5% w/w with 0.05% potassium sorbate, the results had a variation according to the type of gum added, namely: xanthan gum (2.61 μm), arabica (2.48 μm), pectin (2.49 μm), guar (1.14 μm), carrageenan (1.26 μm), and fenugreek (0.72 μm) (HUANG *et al.*, 2001).

When studying the list of ingredients of these vegetables it is possible to understand that the products formulated based on chestnut use in their formulation a smaller amount of stabilizers and / or emulsifiers thus demonstrating a label more "clean" when compared to other vegetables, not existing in the formulation of these additive products that favor stability. The list of almond ingredients is more extensive with the use of different emulsifiers, stabilizers, and other ingredients in the formulations, thus

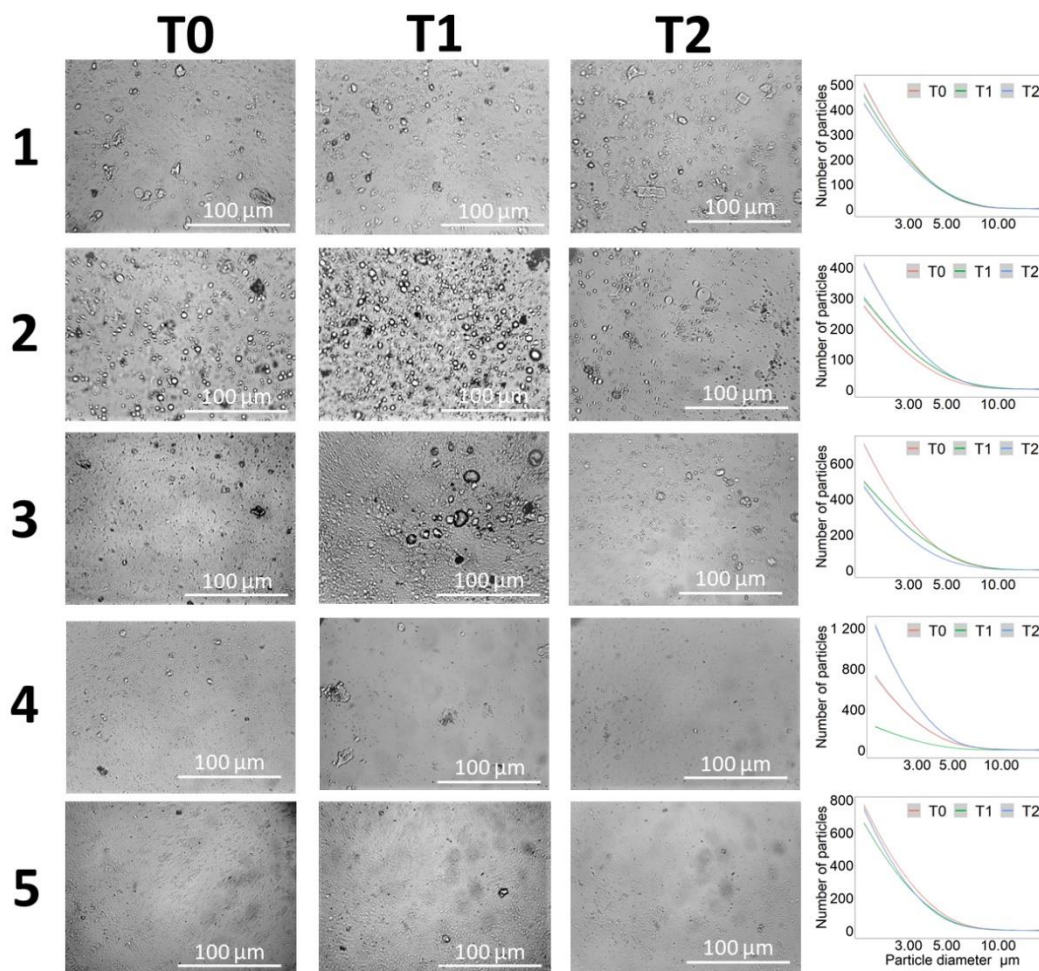
demonstrating the effort of the food industries in trying to stabilize the vegetable drinks formulated with this vegetable, however in this present work they showed less stability. In literature was studied the use of emulsifiers and stabilizers in a peanut-based beverage concluding that the combination of emulsifiers and stabilizers can to increase stability; however, there are few studies describing which is the best combination using different vegetables, the optimal combination in reported for peanut-based beverage was 66% xanthan gum and 34% lecithin at the level of 0.5% by weight of the beverage (GAMA *et al.*, 2019).

It is believed that plant-based beverages are natural foods in practice displaying clean label, however, analyzing Table 3 it can be highlighted that the list of ingredients of these foods is very extensive and can reach 19 different types of substances added among these additives/preservatives. In addition, there is also a great variability of the types of ingredients added, where within the same vegetable there is a great variability resulting in several market products without proper standardization (MELLO *et al.*, 2021). These situations may impact the application of plant-based beverage in area of gastronomy/nutrition. Firstly, the variability between the added ingredients may possibly generate different results the preparation and finalization of culinary dishes with regard to their sensory and/or nutritional characteristics.

It is relevant that the consumer often seeks plant-based foods with the appeal of healthy/natural foods, requirement associated with plant-based beverages since the commercial projects focus on that idea in the description of their products highlighting as an added value. Nevertheless, the analyzed commercial plant-based beverages are not clean label. In the application of the plant-based beverage in culinary preparations is relevant to evaluate the ingredients reported in the product as it can modify the result in terms of taste, odor, flavor, texture, viscosity, and the nutritional characteristics. On the contrary, when the beverage has lower, or no addition of stabilizers or emulsifiers can also affect gastronomic preparations mainly regarding physical/thermal stability (which can be seen in the samples formulated on the basis of Brazilian nuts). Thus, a way to minimize the problems resulting from these impacts on culinary preparations, both from the aesthetic, flavor and/or nutritional point of view, would be the interaction between gastronomes, nutritionists and people specialized in the development of new products in the food industries. It is important to seek more stable vegetable beverages and with less added ingredients to result in culinary preparations with a more pleasant sensory characteristic and with greater appeal of healthiness.

Figure 6 shows the optical microscopy of the analyzed samples comparing the different vegetables and the different treatments applied. The optical microscopy of the samples was not modified by the application of different treatments. That is, the heat treatment and the addition of coffee did not influence the particle characteristics of the plant-based beverages. This situation can be corroborated by the fact that the particle size of the analyzed samples was also not affected regardless of the applied treatments (T0, T1 and T2). It is reported in the literature that optical microscopy of commercial plant-based beverages exhibited variation mainly due to the different vegetables used during manufacture (FRANCISQUINI *et al.*, 2023).

Figure 6 - Optical microscopy and graph of the number of particles versus particle diameter in micrometer of the images obtained. Vegetable material: (1) oat, (2) almond, (3) chestnut, (4) coconut, and (5) soybean. The treatment applied were T0 (25.0 ± 1.5 °C), T1 heated treatment (85.0 ± 1.5 °C for 5 minutes), and T2 heated treatment added with coffee (85.0 ± 1.5 °C for 5 minutes).



Source: Prepared by the author (2023).

In addition, analyzing the optical microscopies using the ImageJ software, a frequency table was obtained to find out how many particles there are for each diameter, thus generating the curves shown in Figure 6. It can be seen from these graphs that in all vegetables, the particle diameter was below 15 μm and the highest frequency (number of particles) remained in the region below 5 μm . Still through the analysis of the existing particles in the optical microscopies, the determination of the Dv90 was carried out (vegetables: 1 - 15.95 μm ; 2 - 16.22 μm ; 3 - 16.05 μm ; 4 - 15.24 μm ; 5 - 15.36), Dv50 (vegetables: 1 - 11.17 μm ; 2 - 11.17 μm ; 3 - 11.29 μm ; 4 - 10.15 μm ; 5 - 10.86 μm) e Dv10 (vegetables: 1 - 5.21; 2 - 5.21; 3 - 5.21; 4 - 4.66; 5 - 4.94). There was no statistical difference ($p < 0.05$) between the studied samples, in relation to the size of the particles when the parameters Dv90, Dv50 and Dv10 were analyzed. In summary, applying the analysis of the images of the particles (droplets) of the plant-based beverages, it is verified that, regardless of the vegetable analyzed, the heat treatment applied or the addition of coffee, small sizes of the analyzed particles were found, with no statistical difference between them.

5.4 CONCLUSION

It can be concluded that the addition of coffee as an ingredient in preparations that use plant-based beverages can be a determining factor in the physicochemical stability, contrary to what occurs for the heat treatment. It was also possible to verify that commercial plant-based beverages are formulated with a large amount of ingredients and therefore cannot be considered clean label. The pH was significantly affected by the addition of coffee, while HCT, particle size and optical microscopy were not significantly affected by the different conditions applied in the work. And finally, the variability between the ingredients and the addition of soluble coffee in vegetable beverages, possibly can generate different results in the preparation and finalization of culinary dishes about their sensory and/or nutritional characteristics.

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6 CHAPTER 3: THE EFFECT OF TRANSGLUTAMINASE ON THE CHARACTERIZATION OF LACTOSE FREE SKIMMED MILK POWDER

This chapter has already been published as an article in the journal *European Food Research and Technology* (<https://link.springer.com/journal/217>) whose DOI corresponds to <https://doi.org/10.1007/s00217-024-04476-1>

6.1 INTRODUCTION AND AIM

The lactose maldigestion, known as lactose intolerance, has a high prevalence worldwide (57% to 65% of the population) and mainly affects countries such as China and Africa (CATANZARO *et al.*, 2021). Dairy products are a part of the diet of the world's population owing to their high nutritional value, resulting in increased immunological protection and prevention of diseases such as enteritis, ulcerative colitis, and malnutrition (CATANZARO *et al.*, 2021). Furthermore, the presence of nutrients such as high biological value proteins, calcium, vitamin D, bioactive peptides, conjugated linoleic acid, phosphorus, magnesium, potassium, zinc and B complex vitamins make milk and dairy products important foods to be part of the diet of the world population with a broad role in nutrition and health (SANTOS *et al.*, 2019).

Individuals with lactose intolerance experience different degrees of gastrointestinal changes such as gastric discomfort, abdominal pain, diarrhea, and flatulence (SANTOS *et al.*, 2019). To minimize these symptoms, changes in eating habits are necessary to reduce the intake of lactose-rich foods. One of the possibilities for these individuals is the intake of dairy products with hydrolyzed lactose, which are widely produced by the food industry to meet the growing market (QUEIROZ *et al.*, 2021). Several dairy products with hydrolyzed lactose are easily found on the market, namely: UHT milk, yogurt, cheese, condensed milk, chocolate. However, it is difficult to find dehydrated dairy products such as milk powder (whole and/or skimmed) which are produced without the addition of drying facilitating agents, unlike the case of dairy compounds and infant formulas. Despite dehydrated dairy products are a viable alternative for consumers because the drying process reduces the perishability of fluid milk, prolongs its shelf life, and makes it easier to transport, handle, process, and formulate (ER *et al.*, 2019; FRANCISQUINI *et al.*, 2020).

This situation occurs because, during the drying of hydrolyzed lactose milk powder, in addition to a decrease in solubility and modification of the microstructure of the powder, there is increased adhesion of the powder to the interior of the drying chamber (even at very low inlet/outlet air temperatures) (TORRES *et al.*, 2017). Moreover, hydrolyzed lactose-dehydrated dairy products are reported to have a greater tendency for non-enzymatic browning (Maillard reaction), with a consequent reduction in the nutritional quality of the powder owing, for example, to the unavailability of essential amino acids such as lysine and the possibility of the formation of mutagenic compounds and carcinogens (FRANCISQUINI *et al.*, 2020). These problems mentioned can result in problems for the efficiency of the manufacturing process, such as, for example, increased expenses with electricity, with consumption of water and cleaning materials due to successive stops for clean in place (CIP) of the drying equipment, it is worth mentioning the waste of products because they are not in ideal packaging conditions. In addition, even if there are no problems during manufacturing, there may be problems during storage of the powders, such as caking, difficulty or impossibility of rehydration, as well as damage to their sensorial (yellowing, oxidation) and nutritional aspects (DE PAULA *et al.*, 2021; FRANCISQUINI *et al.*, 2020; QUEIROZ *et al.*, 2021; TORRES *et al.*, 2017). Strategies must be adopted to facilitate the drying of hydrolyzed lactose products by the food industry. One of these strategies, which is frequently used, is based on the use of drying facilitating agents, such as maltodextrin and soluble fibers, which increase the glass transition temperature of the product (DE PAULA *et al.*, 2021).

A recent study reported an improvement in the powder agglomeration profile and an increase in the glass transition temperature during the drying of skimmed milk powder (SMP) through the addition of transglutaminase (TGase) (ER *et al.*, 2019). Therefore, the use of TGase could be a new strategy to facilitate the drying of dairy products (mainly hydrolyzed lactose), although this is yet to be explored by the industry. Furthermore, this enzyme can form intra- and intermolecular peptide bonds in different food proteins such as casein through crosslinking with glutamine (acyl donor) and lysine (acyl receptor) residues, promoting functional changes in foods such as yogurt (foam stability, gelation, thermal stability, viscosity, and water retention capacity). This crosslinking between amino acids can also lead to the inhibition of the Maillard reaction; however, this effect has not yet been reported for dried dairy products (GAN *et al.*, 2009; ROMEI e WALKER, 2017).

The action of the enzyme is subject to time and temperature conditions, in addition to sufficient substrate to promote bonds. Transglutaminase loses its enzymatic activity when denatured or when action sites are no longer available because of substrate restriction. The percentage of TGase that is used in food depends on the type and amount of protein present in the matrix (SALUNKE e METZGER, 2023). This amount of TGase must be sufficient to achieve the technological and sensorial objectives of product formulation (AJINOMOTO, 2023). In summary, this enzyme is considered safe for application in the food industry, subject to the factors of temperature and enzymatic temperature (optimal range between 40°C and 60°C), pH (optimal range between 5 and 8), and type of food substrate, which may interfere in determining the amount of enzyme that will be used in the food (AJINOMOTO, 2023). Although this information is available in the literature, enzyme regulation is a relatively new field that has advanced in recent years, in Europe, for example, legislation remains specific from country to country, despite the fact that the regulation of the use of enzymes in the bloc is being developed by the European Union through a positive list. In the United States and Japan, enzyme use is regulated by the Food and Drug Administration (FDA) and the Food Sanitation Act (FSA), respectively (AJINOMOTO, 2023).

In short, TGase is being considered as a potential solution to produce skimmed milk powder with hydrolyzed lactose due to two factors: its ability to affect casein cross-linking, which can increase the glass transition temperature of the product, facilitating drying technology and its ability to cross-link glutamine and lysine, making amino acids unavailable to be condensed together with reducing sugars and initiate the Maillard reaction (ER *et al.*, 2019; GAN *et al.*, 2009). With this, the present study explored the hypothesis that the application of TGase to milk with and without hydrolyzed lactose minimizes challenges during the drying stage (less adhesion of the powder to the interior of the drying chamber, improvement in the solubility and agglomeration profile of the powder). This may also reduce the intensity of the Maillard reaction (maintaining the color and nutritional quality of the final product).

Therefore, the aim of this study was to evaluate the application of TGase in dairy products with and without hydrolyzed lactose to verify the influence of the enzyme on drying conditions, product adhesion in the spray dryer, compositional and physicochemical attributes, microscopy, particle size, and Maillard reaction markers of the formulated powders to contribute new insights to the application of TGase in the drying process of dairy products.

6.2 MATERIAL AND METHODS

The low heat skimmed milk powder (SMP) obtained from the company Conaprole (Montevideo, Uruguay) was rehydrated using deionized water ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$) to approximately $40\% \text{ m}\cdot\text{m}^{-1}$ of total solids using a digital shaker (Ika RW 20, universal lab mixer) for an average time of 30 ± 5 minutes. Four treatments were performed in triplicate, totaling 12 experiments ($n = 3 \times 4$). The treatments were: Treatment 1 (control group: SMP $40\% \text{ m}\cdot\text{m}^{-1}$), Treatment 2 (SMP $40\% \text{ m}\cdot\text{m}^{-1}$ added with lactase enzyme at $0.20\% \text{ m}\cdot\text{m}^{-1}$), Treatment 3 (SMP $40\% \text{ m}\cdot\text{m}^{-1}$ added with TGase at $0.025\% \text{ m}\cdot\text{m}^{-1}$), and Treatment 4 (SMP $40\% \text{ m}\cdot\text{m}^{-1}$ added with lactase enzyme at $0.20\% \text{ m}\cdot\text{m}^{-1}$ and TGase at $0.025\% \text{ m}\cdot\text{m}^{-1}$). The lactase enzyme was allowed to act on the product for 24 h before drying, and the TGase enzyme was added to the product on the day of drying, and the samples were subjected to refrigeration ($6^{\circ}\text{C} \pm 2^{\circ}\text{C}$) for 5 h. In other words, in treatment 2, the powder was rehydrated and the enzyme lactase was added, which remained active for 24 hours under refrigeration, followed by drying; in treatment 3, the powder was rehydrated and the TGase enzyme was added, which remained acting, also under refrigeration, for 5 hours with subsequent drying; and finally, in treatment 4, the powder was rehydrated with the addition of the lactase enzyme, which remained acting for 24 hours, after this time, the TGase enzyme was added, which remained acting for another 5 hours, both processes under refrigeration and totaling 29 hours of enzymatic action. TGase was provided by the company Ajinomoto (ACTIVA WM, 80 to 120 units per g) and lactase by the company Global Foods (MAXILACT[®] LGI 5000, enzymatic activity 2600 units per g).

To inactivate TGase, a heat treatment of $85^{\circ}\text{C} \pm 2^{\circ}\text{C}$ was performed for 2 min (GUYOT e KULOZIK, 2011). To standardize the methodology, all treatments, regardless of the addition of TGase, were subjected to the same binomial time and temperature. Then, cooling was carried out in an ice bath until the products reached a temperature of $50^{\circ}\text{C} \pm 5^{\circ}\text{C}$, to be subjected to drying via a spray dryer (MSD, LabMaq) with a 1 mm diameter pressure nozzle. The drying parameters used were as follows: Inlet air temperature $170^{\circ}\text{C} \pm 5^{\circ}\text{C}$; outlet air temperature $88^{\circ}\text{C} \pm 2^{\circ}\text{C}$; blower air flow, $2.4\text{m}^3\cdot\text{min}^{-1}$ to $2.6\text{m}^3\cdot\text{min}^{-1}$; compressed air flow, $30\text{L}\cdot\text{min}^{-1}$ to $35\text{L}\cdot\text{min}^{-1}$; and product input flow, $1.08\text{kg}\cdot\text{h}^{-1}$, with each drying process lasting 45 min. The drying parameters were based on literature studies developed with hydrolyzed lactose products (DE PAULA *et al.*, 2021; ER *et al.*, 2019; QUEIROZ *et al.*, 2021; TORRES *et al.*, 2017).

The powder retention capacity is based on the amount of powder that adheres to the drying equipment (mainly chamber and cyclone). The greater the amount of powder adhered to the equipment, the lower the final production yield. In association, the greater the amount of powder adhered to the chamber, the greater the tendency for darkening and the worsening of the powder's rehydration capacity, increasing the residence time during drying submitted in high temperatures. Therefore, determining the powder retention capacity in the equipment (cyclone, chamber, and total) is interesting as it provides information to understand the yield and type of priority powder that will be obtained during the process (cyclone or chamber) of the new product to be formulated.

The retention capacity in the chamber, cyclone, and total (cyclone plus chamber) were calculated. To determine the retention capacity, the total mass of the product injected into the spray dryer was monitored, and the content of total solids of the product entering the equipment, cyclone powder, and chamber powder was determined. The masses of the products injected into the equipment, cyclone, and chamber powders were measured using a semi-analytical balance. The total solids/moisture was determined using a thermogravimetric balance (MA 150; Sartorius). The retention capacity was calculated using Equation 4:

$$\text{Retention capacity} = [(\text{Mass of total solids of product in kg})/(\% \text{ of total solids of product input } m \cdot m^{-1} \times \text{total mass of product injected in kg}/100)] \times 100 \quad (4)$$

Photographic documentation of different parts of the spray dryer was obtained at the end of each drying process. The morphological characteristics of the powders were obtained using a scanning electron microscope (TM3000; Hitachi®) at magnifications of 100×, 250×, 500×, 800×, 1000×, 1500×, 2000×, 2500×, and 3000×. ImageJ software (bundled with 64-bit Java 8) was used to evaluate the diameter of the particles from the images (1000×) obtained with a scanning electron microscope. Thus, the width or range of size distribution based on volume (SPAN) was determined using Equation 5:

$$\text{SPAN} = Dv90 - Dv10/Dv50 \quad (5)$$

Where:

Dv90 indicates that 90% of the particles have values equal to or less than the result obtained, Dv10 indicates that 10% of the particles have values equal to or less than the

result obtained, and Dv50 indicates that 50% of the particles have values equal to or less than the result found.

The SPAN value was used to calculate the polydispersity index (PDI), which refers to the degree of nonuniformity of the particle size distribution (HORIBA, 2017).

To determine the moisture content and water activity, a thermogravimetric balance (MA 150; Sartorius) and water activity analyzer (LabSwift-aw; Novasina) were used. The fixed mineral residue (ash) was obtained after complete incineration of approximately 3g of the sample in a microprocessed muffle furnace (Q318M21) at 550°C with volatilization of organic matter. The ash content was calculated using Equation 6.

$$\text{Ash\%} = [(m_2 - m_1)/m_0] \times 100 \quad (6)$$

Where:

m_2 = crucible mass with sample

m_1 = empty crucible mass

m_0 = sample mass

To determine the percentage of hygroscopicity of the samples, samples (aliquots of 2 g to 3 g) were placed for 21 d in a desiccator containing a saturated NaCl solution, and conditions were maintained at a relative humidity of 75.5% $\text{m} \cdot \text{m}^{-1}$ and temperature of $23^\circ\text{C} \pm 2^\circ\text{C}$. At the end of the experimental period, the samples were weighed again, and the percentage of hygroscopicity was calculated using Equation 7.

$$\text{Percentage of hygroscopicity} = [(\text{Mass of support with product after 21 d in desiccator} - \text{mass of support of sample} - \text{mass of support on day 1 of weighing}) / \text{mass of support on day 1 of weighing}] \times 100 \quad (7)$$

The particle size was determined using a Beckman Coulter LS 13320 laser diffraction analyzer (Beckman Coulter®) coupled to a liquid analysis module. The mixture was then slowly added to a reservoir containing water at room temperature ($21^\circ\text{C} \pm 2^\circ\text{C}$) to obtain an opaque mixture. Five sets of data were collected at intervals of 90 s (1.5, 3.0, 4.5, 6.0, and 7.5 min). The results (particle size $<1 \mu\text{m}$: indicates quantity of particle above this region and Dv90: indicates that 90% of the particles have values equal to or less than the result obtained) were obtained using a refractive index of 1.333 for the

medium dispersant (water) and 1.57 for the particles. Finally, Beckman Coulter software (version 5.03; particle characterization) was applied to obtain data.

An FT-Raman spectrometer (RFS 100; Bruker) equipped with a Ge detector and a 1064 nm excitation line of a Nd:YAG laser was used. The samples were analyzed using laser emission at a power of 110 mW, with the following spectral parameters: wavelength, 512 nm; spectral resolution, 4 cm^{-1} in the region between 4000cm^{-1} to 50cm^{-1} . OPUS software (version 6.0; Bruker Optik, Ettingen, Germany) was used for data analytics.

To perform exploratory analysis via principal component analysis (PCA), the Raman spectra were evaluated using the programming language R version 4.2.3. The number of main components was evaluated using the eigenvalue graph that allowed the assessment of the contribution of two Raman spectra in the distribution of samples in the graph of points in two spectral regions: from 3200cm^{-1} to 2800cm^{-1} and from 400cm^{-1} to 1350cm^{-1} . For the PCA of the distribution of particle sizes used as descriptive statistics of the curve, the mean and uncertainty as confidence interval were calculated using the Student's *t*-test distribution for the following statistics: mean, median, $d[4.3]$ (the mean diameters of the particle size based on volume-weighted), standard deviation, variance, coefficient of variation, skew, kurtosis, mode, singular spectrum analysis (ssa), Dv10 (indicates that 10% of the particles have values equal to or less than the result obtained), Dv50 (indicates that 50% of the particles have values equal to or less than the result found), Dv90 (indicates that 90% of the particles have values equal to or less than the result obtained), $>1\text{ }\mu\text{m}$ (indicates quantity of particle above this region), and $>10\text{ }\mu\text{m}$ (indicates quantity of particle above this region).

All the above-mentioned analyses were performed in duplicate on days 1 (D1) to 3 (D3) of storage.

A high-efficiency liquid chromatograph (model 1525; Waters) equipped with a binary pump was used to analyze four markers of the Maillard reaction: 5-hydroxymethylfurfural (HMF), 2-furaldehyde, 2-furyl-methylketone, and 5-methyl-2-furaldehyde. The method used to identify and quantify these four markers of the Maillard reaction was adapted from work developed by Stephani *et al.* (2023). A reverse phase high-efficiency liquid chromatography technique was applied using a Waters Spherisorb column ($150\text{ mm} \times 4.6\text{ mm}$; particle size $3\text{ }\mu\text{m}$) and UV-Vis detector (Photodiode Array Detector). The time required for each analysis was 10 min with an injection volume of $20\text{ }\mu\text{L}$, temperature of 30°C , and wave compression of 284 nm . The elution gradient was formulated using a mixture of the two solvents under vacuum at $1\text{ mL}\cdot\text{min}^{-1}$. The mobile

phase consisted of non-solvent acetonitrile (B) and non-solvent ultrapure water (A). The linear gradient program was initiated as follows: 0–5 min, 95.5%–4.5% B; 5–9 min, 80%–20% B; 9–10 min, 95.5%–4.5% B.

The parameters chosen to be analyzed were the CIEL*a*b* color space, which is the most popular and most used to identify, communicate and evaluate colors, correlating color values with visual perception. This CIEL*a*b* color space has the capacity to help check whether there are inconsistencies or deviations from a standard color of a food and can be useful for the development of new products, as it is possible to compare their color with the color of traditional ones (control). Furthermore, these parameters are used to determine the browning index (BI), which is already discussed in the literature by other authors, thus determining the degree of browning of a given food (DE PAULA *et al.*, 2021; OLIVEIRA *et al.*, 2012; QUEIROZ *et al.*, 2021). These analyzed parameters (L*, a*, b* and BI) are relevant to the present study mainly because they are lactose-free dairy products which end up darkening and becoming more yellowish in comparison to traditional products (control) due to an increase the intensity of the Maillard reaction (increased availability of reducing sugars) (DE PAULA *et al.*, 2021; OLIVEIRA *et al.*, 2012; QUEIROZ *et al.*, 2021).

This way, the color of the powders was obtained using a colorimeter (CR400; Chroma Meter) by directly reading the reflection of the coordinates L* (luminosity), a* (positive = red, negative = green), and b* (positive = yellow, negative = blue). The parameters analyzed in the color analysis were used to determine the browning index. The intensity of the brown color of the samples was evaluated using BI and was calculated using Equation 8, which correlates the parameters L*, a*, and b* (OLIVEIRA *et al.*, 2012; QUEIROZ *et al.*, 2021).

$$BI = ([100(x - 0.31)]) / 0.17 \quad (8)$$

Where:

$$x = (a^* + 1.750 \times L^*) / (5.645 \times L^* + a^* - 3.012 \times b^*)$$

Analyses of Maillard reaction markers and color were performed in triplicate at two storage times: days 3 (D3) and 7 (D7).

SDS-PAGE analysis consists of a type of electrophoresis carried out under denaturing conditions so that proteins can be separated, resulting in protein migration

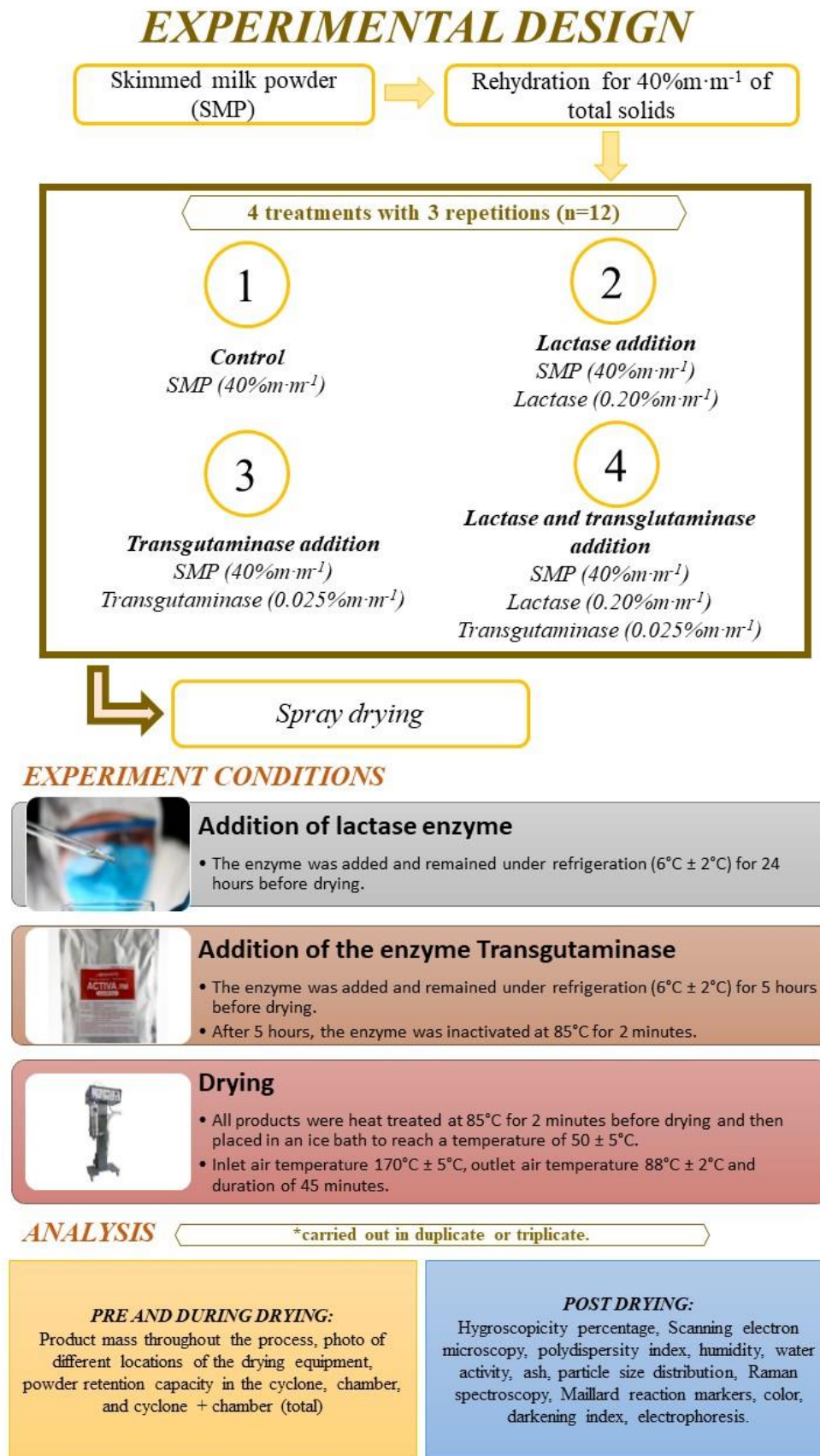
inversely proportional to their molecular weights. Therefore, proteins with higher molecular weight are in the upper part of the gel and those with lower molecular weight in the lower part of the gel. The intention with the gel is to check whether there is the presence of high molecular weight proteins due to the crosslink between lysine and glutamine (GL-bound). However, if these high molecular weight proteins are not found (limitation of the analytical technique) it is also possible to infer that the crosslink was present due to the decrease in the intensity of the casein region (κ , α and β) demonstrating that this protein performed a crosslink if making it high molecular weight (GAUCHE, 2007).

Electrophoresis analysis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis: SDS-PAGE) was performed using a Mini Protean Treta System (Bio-Rad, Hercules). The duration of this run was 2h, and the voltage applied was 100V. The mixture of sample and sample buffer applied to the gel was 1:1 and the powdered products were reconstituted to $9.62\% \text{ m} \cdot \text{v}^{-1}$. In well number 1, approximately 5 μL of the standard protein solution was added and in wells 2 to 5, 89.75 μg of sample was added (T1, well 2; T2, well 3; T3, well 4; T4, well 5). The methodology described by Gauche (2007) was followed with some modification.

The powders from the chamber and cyclone were analyzed to determine the retention capacity, humidity, and particle size distribution (exclusively for PCA determination). For all other analyses, only cyclone powders were analyzed. For statistical analysis of the data, R version 4.2.3 was used with the Tukey (multiple comparison tests and makes comparisons between all pairs) and Duncan tests (identify pairs of means of at least three that differ statistically and requires the calculation of several minimum significant amplitudes) both $p > 0.05$ (value traditionally used to reject the null hypothesis).

Figure 7 summarizes the methodology used in this study.

Figure 7 - Schematic summary of the methodology applied in the present work.



Source: Prepared by the author (2023).

6.3 RESULTS AND DISCUSSION

Table 4 lists the powder retention capacities of the cyclone, chamber, and cyclone + chamber (total) processes.

Table 4 - Powder retention capacity in the cyclone, chamber, and cyclone + chamber (total) in the four treatments (T1, Treatment 1 control; T2, Treatment 2 hydrolyzed lactose; T3, Treatment 3 control with transglutaminase added; T4, Treatment 4 hydrolyzed lactose with transglutaminase added).

Treatment	Powder retention capacity in the cyclone (%)	Powder retention capacity in the chamber (%)	Powder retention capacity in the cyclone + chamber (total) (%)
T1	60.78±6.18 ^a	14.45±2.03 ^a	75.24±8.21 ^a
T2	27.35±2.70 ^b	52.16±1.69 ^b	79.52±1.01 ^a
T3	54.93±17.61 ^a	18.93±22.79 ^a	73.86±5.18 ^a
T4	29.30±2.13 ^b	52.20±3.58 ^b	81.51±5.71 ^a

Source: Prepared by the author (2023).

*Average ± standard deviation, values followed by the same lowercase letter, in the same column, do not differ from each other ($p>0.05$) using the Duncan test.

The highest percentages of powder retention capacity in the cyclone were observed in treatments T1 (average 60.78%) followed by T3 (average 54.93%), that is, products without added lactase. Lower percentages were obtained for treatments T2 (average 27.35%) and T4 (average 29.30), which are hydrolyzed lactose products. Powder quantities obtained in Treatments T1 and T3 were considered equivalent, whereas those of treatments T2 and T4 were similar ($p>0.05$). In contrast, powder quantities obtained with T1 and T3 were significantly different ($p>0.05$) from those obtained with T2 and T4. Regarding powder quantities in the chamber, high powder retention capacities were observed with treatments T2 (average 52.16%) and T4 (average 52.20%). And, they were not significantly different from each other ($p>0.05$). Treatments T1 (average 14.45%) and T3 (average 18.93%) resulted in low powder retention capacities and were not significantly different from each other ($p>0.05$).

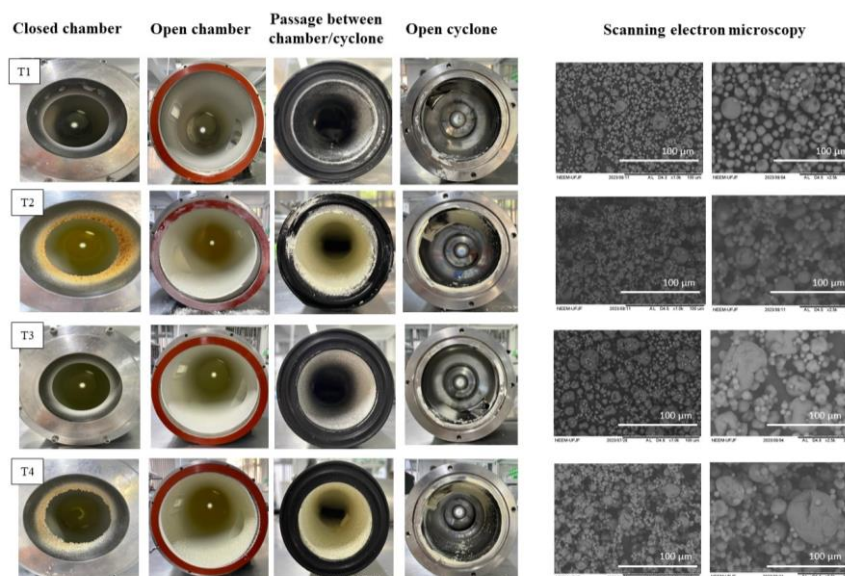
Finally, the powder retention capacity in the cyclone + chamber (total) did not result in a significant difference ($p>0.05$) among treatments T1 (average 75.24%), T2 (average 79.52%), T3 (average 73.86%), and T4 (average 81.51%). It is worth noting that powder retention in T1 was approximately four times greater. And, T3 had approximately

three times more powder coming from the cyclone than from the chamber. In addition, treatments T2 and T4 had approximately two times more powder coming from the chamber than from the cyclone. Although the total powder retention capacity did not differ significantly between treatments. There was approximately two to three times more powder in the drying chamber for treatments T2 and T4 than for treatments T1 and T3.

Changing the location where the powder is stored can result in differences in morphological characteristics, particle size, solubility, and development of the Maillard reaction. Greater powder adhesion in the equipment chamber can result in morphological changes, solubility/particle size distribution, and even intensification of the Maillard reaction in the powders. This situation is commonly reported in the literature for hydrolyzed lactose dairy products owing to the reduction in the glass transition temperature, reduction in lactose, and increase in the concentration of glucose and galactose. Thus, the results of the present study were consistent with these findings, as both treatments involved the addition of lactase (DE PAULA *et al.*, 2021; QUEIROZ *et al.*, 2021; TORRES *et al.*, 2017). As discussed in the material and methods section, it is noteworthy that this information becomes useful for industries producing dehydrated dairy products, resulting in information on the final quality of the product (cost increases during production and storage).

Figure 8 shows the photographs taken immediately after each drying step and the scanning electron microscopy images of the samples in each of the treatments.

Figure 8 - Photographs taken from different parts of the equipment (closed chamber, open chamber, passage between chamber and cyclone, open cyclone) immediately after each drying process and scanning electron microscopy images of powders obtained in the cyclone (1000 $\times$ and 2500 $\times$) of the four formulated treatments (T1, Treatment 1 control; T2, Treatment 2 hydrolyzed lactose; T3, Treatment 3 control with transglutaminase added; T4, Treatment 4 hydrolyzed lactose with transglutaminase added).



Source: Prepared by the author (2023).

Visually, greater adherence of the powders to the equipment (mainly the drying chamber) could be observed in treatments T2 and T4, which are products obtained with hydrolyzed lactose. Furthermore, these products also had a more “brownish” color, indicating a greater intensification of the Maillard reaction. This was confirmed by the retention capacity percentage (Table 4) as these powders constituted the highest percentages of products removed from the drying chamber. Furthermore, per visual inspection, the powder formulated in T4 presented a less “brown” color than that formulated in T2, which may therefore indicate a lower intensity in the development of the Maillard reaction.

In addition, visual analysis of the scanning electron microscopy images indicated that the powders constituted rounded particles typical of products dried via spray dryer. In treatments T2 and T4, the particles appeared to be highly agglomerated (probably owing to lactose hydrolysis). Similar to that in treatments T3 and T4, which exhibited larger particles (probably owing to the addition of TGase).

The PDI values of the samples were significantly different among treatments. The mean $\pm$ standard deviations for the treatments were as follows: T1, 2.74 ± 0.36 ; T2, 1.04 ± 0.77 ; T3, 3.20 ± 0.26 ; and T4, 1.47 ± 0.21 . Treatments T1 and T3 were not significantly different from each other but differed significantly from T2 and T4 ($p > 0.05$). A monodispersed sample has a high degree of uniformity ($PDI < 0.4$), whereas a polydispersed sample has a low degree of uniformity ($PDI > 0.4$) (QUEIROZ *et al.*, 2021). All treatments resulted in samples considered polydispersed. However, samples obtained from treatments T1 and T3 were the most polydispersed, whereas polydispersity of powders from treatments T3 and T4 was less. Therefore, the unhydrolyzed samples (T1 and T3) showed a greater variation in particle size than the hydrolyzed lactose samples (T2 and T4). Owing to the greater agglomeration tendency of the hydrolyzed lactose products, the latter exhibited a more uniform distribution.

The morphological analysis of powders is an interesting analysis because it allows classifying formulated powders in relation to their better or worse rehydration capacity during use by the consumers. The properties of powders can be determined by the type of agglomeration. Lower amounts of fine particles, large individual particles and greater air occlusion result in powders with better rehydration capacity. On the other hand, a greater quantity of broken particles and the presence of fine particles can result in a worse rehydration capacity (FRANCISQUINI *et al.*, 2020).

Table 5 lists the results of the analysis of water activity, humidity, and ash, as well as the percent of hygroscopicity of the powders.

Table 5 - Analysis of water activity, humidity, ash, and percent of hygroscopicity of powders obtained from the cyclone in the different treatments (T1, Treatment 1 control; T2, Treatment 2 hydrolyzed lactose; T3, Treatment 3 control with transglutaminase added; T4, Treatment 4 hydrolyzed lactose with transglutaminase added).

Parameter	Water activity	Humidity (% $\text{m}\cdot\text{m}^{-1}$)	Hygroscopicity (%)	Ash (% $\text{m}\cdot\text{m}^{-1}$)
T1	0.174 ± 0.038^a	3.35 ± 0.41^a	10.35 ± 0.50^a	7.37 ± 0.08^a
T2	0.240 ± 0.044^a	4.42 ± 0.76^a	25.36 ± 0.67^b	7.26 ± 0.12^a
T3	0.232 ± 0.021^a	4.47 ± 0.83^a	9.91 ± 0.43^a	7.39 ± 0.10^a
T4	0.220 ± 0.064^a	4.43 ± 1.36^a	24.89 ± 1.61^b	7.21 ± 0.11^a

Source: Prepared by the author (2023).

*Average $\pm$ standard deviation, values followed by the same lowercase letter, in the same column, do not differ from each other ($p > 0.05$) using the Tukey test.

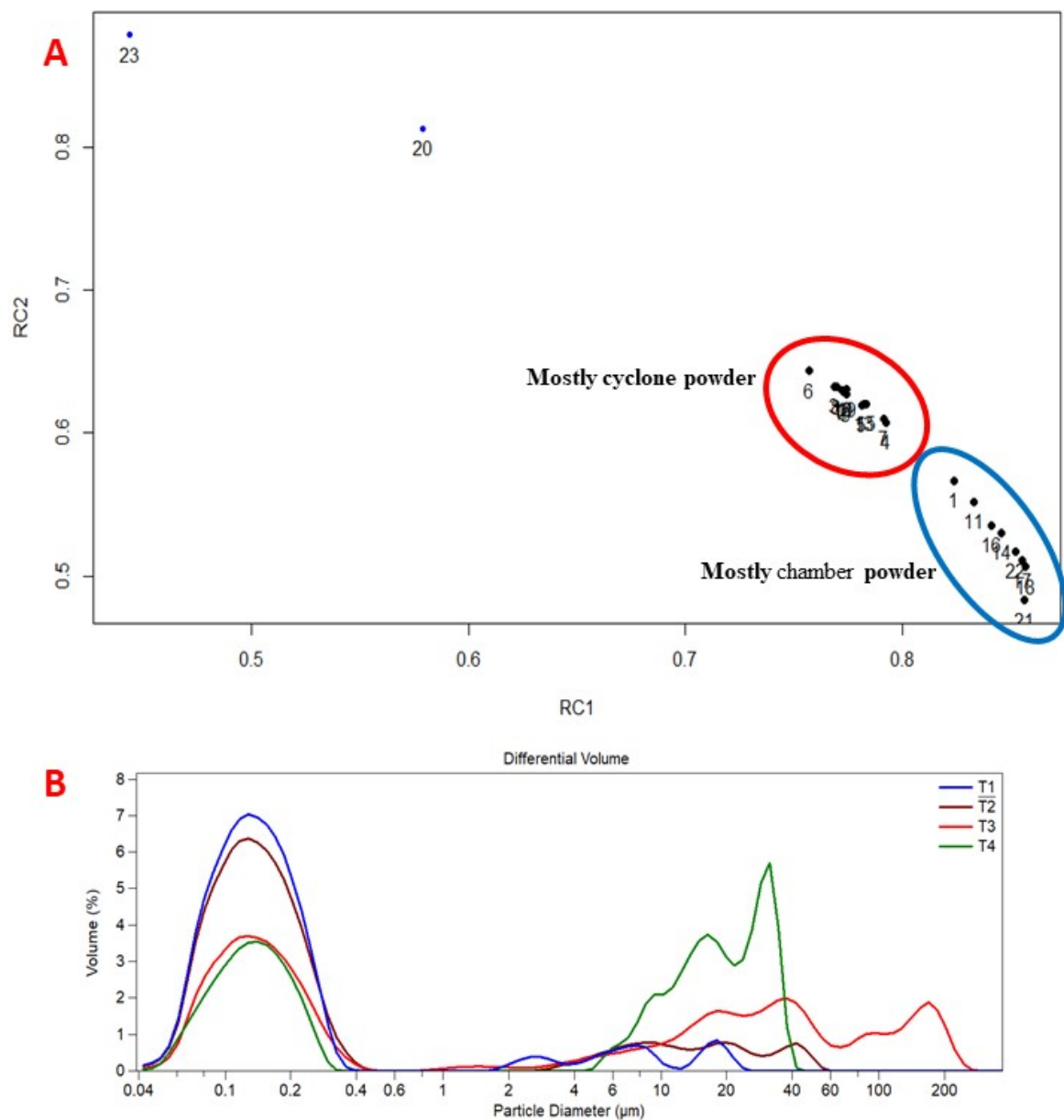
There was no significant difference ($p > 0.05$) between the treatments for water activity (mean; T1, 0.174; T2, 0.240; T3, 0.232; T4, 0.220), humidity (mean; T1, 3.35; T2, 4.42; T3, 4.47; T4, 4.43), and ash content (average; T1, 7.37; T2, 7.26; T3, 7.39; T4, 7.21). These values agree with those reported in the literature, and the water content was within the expected range, avoiding problems with fluidity, stickiness, and stability during storage (DAZA *et al.*, 2016; DE PAULA *et al.*, 2021; QUEIROZ *et al.*, 2021; TORRES *et al.*, 2017).

The hygroscopicity of a powder is based on its ability to absorb water during storage, which can interfere with the shelf-life stability of the sample. According to Schuck *et al.* [18], powders are classified based on the percent hygroscopicity at 75% relative humidity: $\leq 10\%$, non-hygroscopic; 10.1% to 15%, slightly hygroscopic; 15.1% to 20%, hygroscopic; 20.1% to 25%, very hygroscopic; and $> 25\%$, extremely hygroscopic. Therefore, in treatments T1 (average 10.35%) and T3 (average 9.91%), non-hygroscopic to slightly hygroscopic powders were formed, whereas in treatments T2 (average 25.36%) and T4 (average 24.89%) very hygroscopic to extremely hygroscopic powders were formed. The percent hygroscopicity of the powders did not differ significantly ($p > 0.05$) between T1 and T3 and between T2 and T4. In other words, high percent hygroscopicity was observed in samples subjected to lactose hydrolysis.

In a study conducted by Torres *et al.* (2017), humidity and water activity increased with a greater percentage of lactose hydrolysis, which was likely owing to limitations of the analytical technique used, with a greater mass loss accounted for as contributing to percent humidity. In the present study, humidity, independent of lactose hydrolysis, remained constant and was within the range of values reported for dried dairy products, and could therefore be indicative of an alternative and stable analytical technique for hydrolyzed lactose dairy products.

The main components of the particle size distribution of powders obtained from the chamber and cyclone, in addition to the particle size distribution curves of the chamber powders are shown in Figure 9.

Figure 9 - A) Principal component analysis of particle size distribution of powders obtained from the chamber and cyclone with following attributes evaluated: average, median, d[4.3], standard deviation, variance, coefficient of variation, skew, kurtosis, mode, ssa, Dv10, Dv50, Dv90, >1 μm , and >10 μm . B) Particle size distribution curve of powders obtained from the cyclone, from the last rehydration step (7.5 min) in the four formulated treatments (blue curve: T1, Treatment 1 control; wine curve: T2, Treatment 2 hydrolyzed lactose, red curve: T3, Treatment 3 control with transglutaminase added, green curve: T4, Treatment 4 hydrolyzed lactose with transglutaminase added).



Source: Prepared by the author (2023).

Analysis of particle size distribution resulted in the grouping of the powders into two categories: cyclone powders and chamber powders. According to the PCA results, the group in which the cyclone powders were included corresponded to the powders with the best rehydration capacity. In contrast, the group in which the majority of the powder remained in the chamber included powders with the worst rehydration capacity. Thus, depending on where the powder was removed from, an effect was observed on the particle size and, consequently, on the rehydration capacity. Therefore, owing to the powder retention capacity of the equipment, treatments T2 and T4, which had a greater amount of powder in the chamber, were poor at rehydration, that is, the two treatments with lactose hydrolysis.

One of the main concerns during the production of powdered dairy products is based on the rehydration capacity of this powder so that it has the ability to return to the "original state" of fluid milk. The rehydration curve for the cyclone powders (Figure 9B) was characteristic of fluid milk, which was to be expected because consumers generally desire the "return" of powdered milk to the original characteristic of fluid milk. Such a curve can be classified as bimodal, showing two modes with an interstice close to $<1\ \mu\text{m}$ and $>1\ \mu\text{m}$ (two distinct populations of particles, one of which indicates presence of caseins and the other indicates presence of fat and other non-hydrated particles) (FRANCISQUINI *et al.*, 2020). When exclusively analyzing the cyclone powders, a significant difference was observed between treatments for the attributes $<1\ \mu\text{m}$ and $Dv90$. For $<1\ \mu\text{m}$ (%), mean $\pm$ standard deviation results for treatments were as follows: T1, 89.49 ± 5.21 ; T2, 80.98 ± 2.89 ; T3, 64.79 ± 20.28 ; and T4, 35.72 ± 13.44 . Significant differences were not observed between treatments T1 and T2, but both T3 and T4 resulted in significant differences ($p > 0.05$). Therefore, the largest quantity of small particles ($<1\ \mu\text{m}$) was found for treatments T1 and T2, that is, without the addition of TGase. In this case, the addition of the lactase enzyme did not affect the particle size of the powders. The largest quantity of large particles ($>1\ \mu\text{m}$) was found for treatments T3 and T4, with added TGase. Therefore, TGase increased the particle size of the powder, probably by crosslinking with glutamine and lysine. The smaller the particle size, the better the rehydration capacity of the powders (FRANCISQUINI *et al.*, 2020). Therefore, the powders containing TGase (T3 and T4) exhibited a lower rehydration capacity than those without TGase (T1 and T2). Furthermore, sample T4 had the greatest quantity of large particles; that is, the size of the particles was influenced to the greatest extent in the product containing both lactase and TGase. This was the first interesting behavior

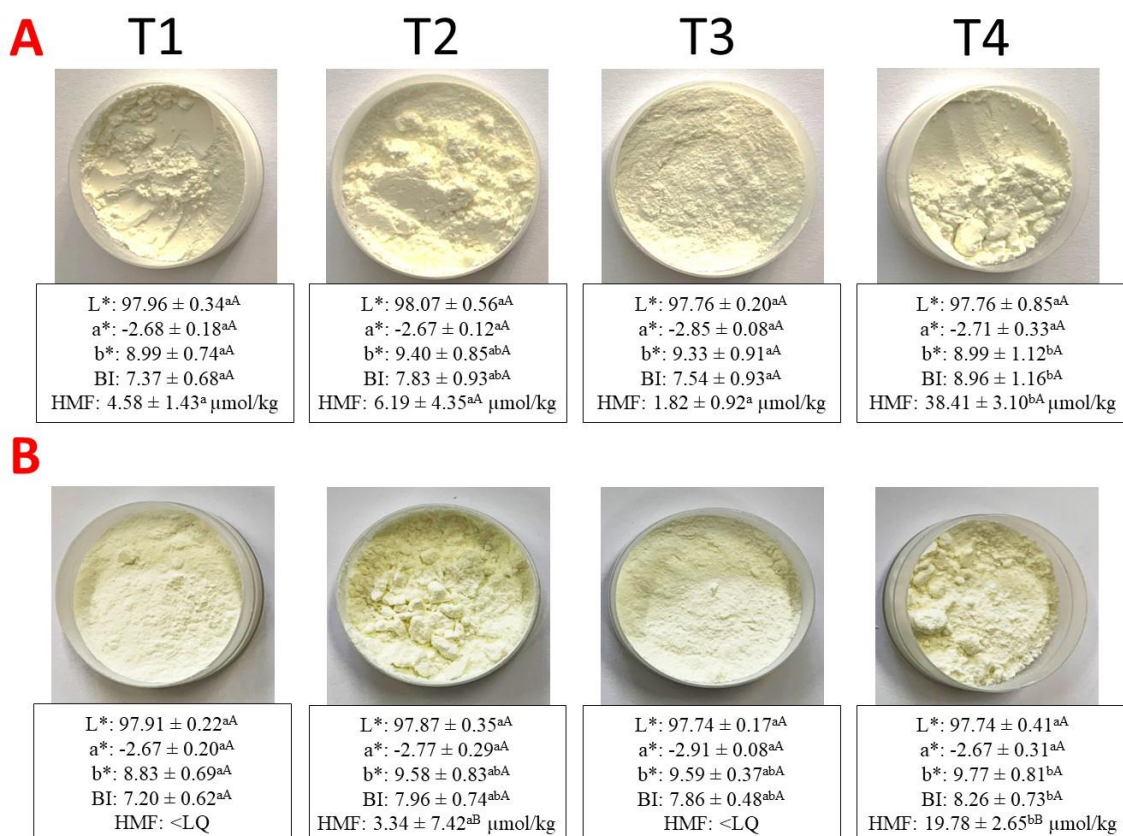
highlighted for this product (T4). In treatments T1 and T2, no increase in the particle size was observed upon the addition of TGase. However, analysis of quantity of particles with size below 1 μm showed that the T4 product had approximately two times less particles in this size category when compared to that in the product without hydrolyzed lactose (T3).

Regarding Dv90 (μm), the mean $\pm$ standard deviation results were as follows: T1, 2.94 ± 0.30 ; T2, 11.07 ± 0.44 ; T3, 9.74 ± 4.79 ; T4, 43.00 ± 7.02 . Here too, the T4 treatment stood out, where 90% of the particles exhibited values equal to or below 43 μm . This value was approximately 14 times higher than that of the T1 treatment and approximately four times higher than that of the T2 and T3 treatments. In summary, the particle size was much larger in the T4 treatment than in the other treatments.

The rehydration capacity is a relevant measure mainly to help in choosing the application of the powder. This helps industries, for example, in choosing which powder will be intended for direct consumption or which will be used as ingredients to produce other foods (FRANCISQUINI *et al.*, 2020).

Figure 10 shows a photograph of the powders as well as the analysis of color, browning index, and the Maillard reaction marker HMF.

Figure 10 - Photograph of the powders with their respective color analysis (L, a*, b*, and BI) and the identified Maillard reaction marker (5-hydroxymethylfurfural) in the powders of the four treatments (T1, Treatment 1 control; T2, Treatment 2 lactose hydrolyzed; T3, Treatment 3 control with transglutaminase added; T4, Treatment 4 hydrolyzed lactose with transglutaminase added). A) and B) correspond to the analysis carried out on days 3 (D3) and 7 (D7) of storage, respectively.



Source: Prepared by the author (2023).

Means $\pm$ standard deviation, values followed by the same lowercase letter, on the same line, do not differ from each other ($p > 0.05$) for the same attribute analyzed (L, a*, b*, BI, HMF). Means $\pm$ standard deviation, values followed by the same uppercase letter, in the same column, do not differ from each other ($p > 0.05$) for the same attribute analyzed (L*, a*, b*, BI, HMF).

** where: BI = browning index; HMF = 5-hydroxymethylfurfural; LQ = limit of quantification.

Luminosity of the powder (L*) and a*, which is based on positive values closer to red and negative values closer to green, showed no significant difference ($p > 0.05$) regardless of the treatment (T1, T2, T3, and T4) or the day of analysis (D3 and D7). The

average for all treatments on D3 was $L^* = 97.89$ and $a^* = -2.73$ and for D7 the values were $L^* = 97.82$ and $a^* = -2.76$. For the b^* attribute, where positive values were closer to yellow and negative values were closer to blue. And for BI, which corresponds to the browning index, significant differences ($p > 0.05$) were found between the treatments (T1, T2, T3, and T4), but not between the days of analysis ($p > 0.05$, D3 and D7). On D3, treatment T4 (mean $b^* = 8.99$ and BI = 8.96) presented high values of b^* and BI, which were significantly different from those of treatments T1 (mean $b^* = 8.99$ and BI = 7.37) and T3 (mean $b^* = 9.33$, BI = 7.54) ($p > 0.05$), but not from values obtained for treatment T2 (mean $b^* = 9.40$ and BI = 7.83). Considering the longer storage period (D7), treatment T4 (mean $b^* = 9.77$, BI = 8.26) did not differ significantly from treatments T2 (mean $b^* = 9.58$ and BI = 7.96) or T3 (mean $b^* = 9.59$ and BI = 7.86) but differed significantly from T1 (mean $b^* = 8.83$ and BI = 7.20) ($p > 0.05$). This indicates that products T2 and T4 were already yellower and darker on the first day of analysis, which is reasonable because the hydrolysis of lactose may have intensified the browning process of the product. With the storage period D7, the T3 product (without lactose hydrolysis, but with the addition of TGase) became more “yellowish” and darker, starting to resemble the hydrolyzed products (T2 and T4), but still did not change compared with the control (T1) group. During the storage period, the product with TGase became yellower and darker, which could be an indication of an increase in the browning of the product.

Among the Maillard reaction markers analyzed, only HMF was identified, that is, the markers 2-furaldehyde, 2-furyl-methylketone, and 5-methyl-2-furaldehyde were below the detection limit. Treatment T4 (average of 38.41 $\mu\text{mol/kg}$) presented the highest HMF values on D3, differing significantly ($p > 0.05$) from the other treatments, which had similar values (average, T1, 4.58 $\mu\text{mol/kg}$; T2, 6.19 $\mu\text{mol/kg}$; and T3, 1.82 $\mu\text{mol/kg}$). The values were approximately 6 to 21 times higher for the product with hydrolyzed lactose with TGase addition (T4) than those of the control (T1), only lactase added (T2), and only TGase added (T3). Considering the storage period, all treatments exhibited a reduction in HMF content at D7, and the amounts differed significantly ($p > 0.05$) when compared with those at D3 (average, T1, below the limit of quantification; T2, 3.34 $\mu\text{mol/kg}$; T3, below the limit of quantification; T4, 19.78 $\mu\text{mol/kg}$). This was possibly due to the transformation of this intermediate indicator into other compounds during the cascade of reactions that occurred throughout the Maillard reaction. At D7 of the analysis, HMF could not be quantified in T1 and T3. On the same day, treatments T2 and T4 differed significantly, with higher HMF content for the treatment with both lactose hydrolysis and

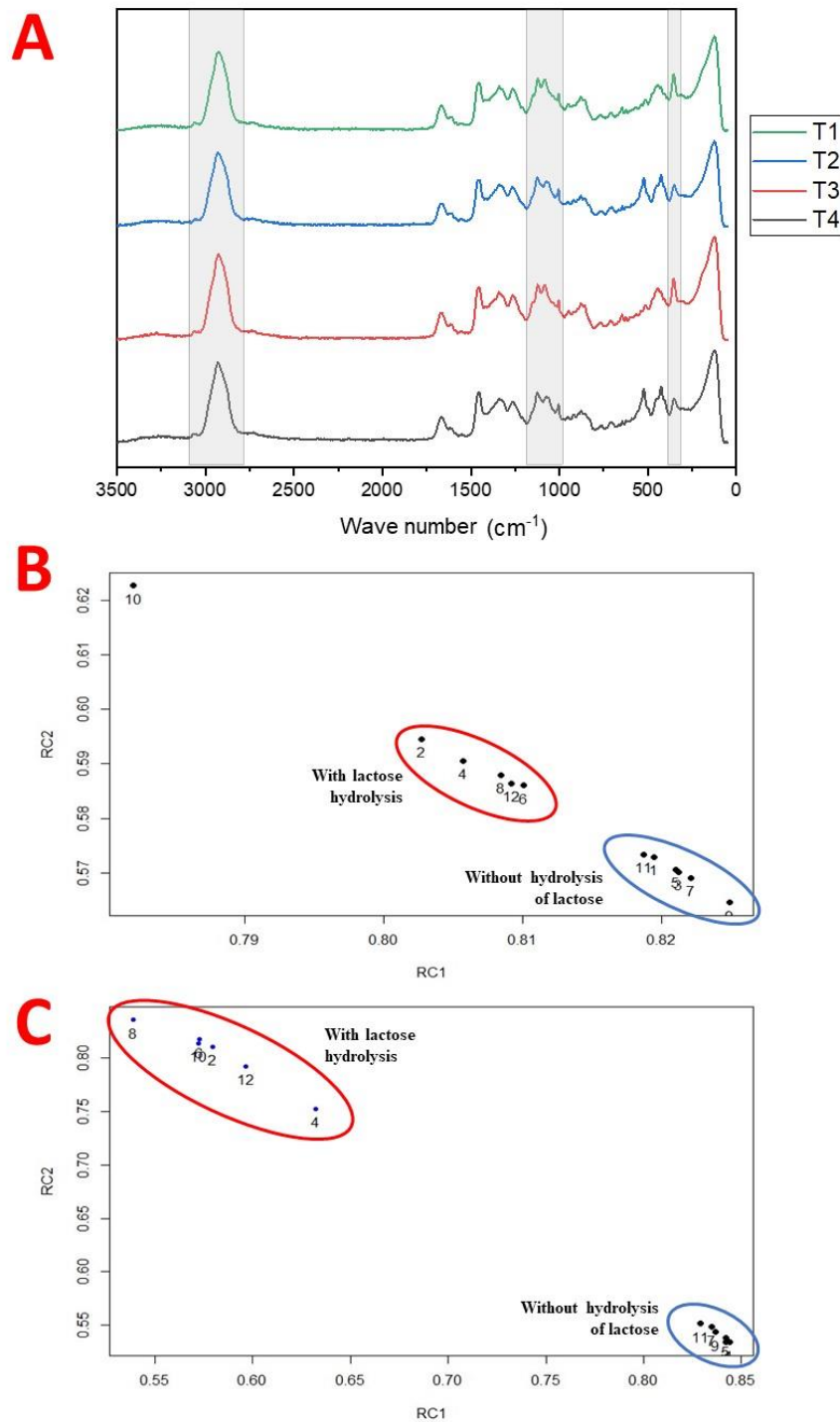
TGase than for the product with only lactase addition. Therefore, the addition of TGase did not allow for a reduction in Maillard reaction markers. The addition of TGase to the dehydrated product without hydrolyzed lactose resulted in a numerical reduction in the HMF values (although the difference was not significant). However, when TGase was added to the dehydrated product containing hydrolyzed lactose, the HMF content increased considerably, intensifying the Maillard reaction.

Few studies related the influence of TGase in dairy products. For certain applications, TGase can be used to slow down the Maillard reaction and crosslinking, as was the case in the study by Gan *et al.* (2009), who observed this phenomenon for soy protein. Therefore, the results obtained for the color and Maillard reaction markers are relevant for understanding the influence of TGase on the color and intensification of the Maillard reaction in dehydrated products with and without hydrolyzed lactose. Given these results, new hypotheses can be developed including the influence of TGase on powders over a longer storage period and at higher storage temperatures, whether enzyme addition was a factor in the increased production of HMF in the product dehydrated with hydrolyzed lactose containing TGase, and the potential effects of adding TGase before lactose hydrolysis.

The color of the product is an important sensory attribute to be analyzed because it is one of the key factors in the choice of powder by consumers. Furthermore, the availability of a product with nutritional quality is essential. It is known that milk and its derivatives are one of the most important sources of proteins and essential amino acids, such as lysine. Therefore, the importance of colorimetric analyzes and Maillard reaction markers becomes evident (CATANZARO *et al.*, 2021; DE PAULA *et al.*, 2021; GAN *et al.*, 2009; QUEIROZ *et al.*, 2021; SANTOS *et al.*, 2019).

Figure 11 shows the Raman spectra of the powders and the grouping of the samples into two different spectral regions using PCA.

Figure 11 - A) Raman spectra of products formulated using four different treatments (T1, Treatment 1 control, T2, Treatment 2 hydrolyzed lactose, T3, Treatment 3 control with transglutaminase added, T4, Treatment 4 hydrolyzed lactose with transglutaminase added). B) Principal component analysis of the spectral region between 400 cm^{-1} to 1350 cm^{-1} . C) Principal component analysis of the spectral region between 2800 cm^{-1} and 3200 cm^{-1} .



Source: Prepared by the author (2023).

As shown in Figure 11A, the spectral region between 2800 cm^{-1} and 3000 cm^{-1} corresponds to symmetric and asymmetric C-H stretching, respectively. Which can identify the state of lactose (amorphous and/or crystalline states), when only lactose is present in the sample. In the case of the present study, lactose remained in an amorphous state in the treatments without hydrolyzed lactose (T1 and T3) (DE PAULA *et al.*, 2021; JÚNIOR *et al.*, 2016; TORRES *et al.*, 2017; QUEIROZ *et al.*, 2021; STEPHANI *et al.*, 2017).

The region between 1500 cm^{-1} and 200 cm^{-1} presents vibrational characteristics where: 1500 cm^{-1} to 1250 cm^{-1} are CH_2 and CH_2OH deformation modes; 1200^{-1} and 950 cm^{-1} are C-C and C-O stretching; 950 cm^{-1} to 800 cm^{-1} deformations of the C-O-H, C-C-H and O-C-H side groups are observed; and finally between 543 cm^{-1} and 410 cm^{-1} for glucose and 477 cm^{-1} and 377 cm^{-1} for lactose correspond to endocyclic and exocyclic deformation modes, respectively. In this region, when the product contained glucose and galactose, an increase in the intensity of the bands at 525 cm^{-1} and 424 cm^{-1} , respectively. Could be observed, confirming lactose hydrolysis in the product with added lactase (DE PAULA *et al.*, 2021; TORRES *et al.*, 2017; SANTOS *et al.*, 2019; STEPHANI *et al.*, 2017; JÚNIOR *et al.*, 2016).

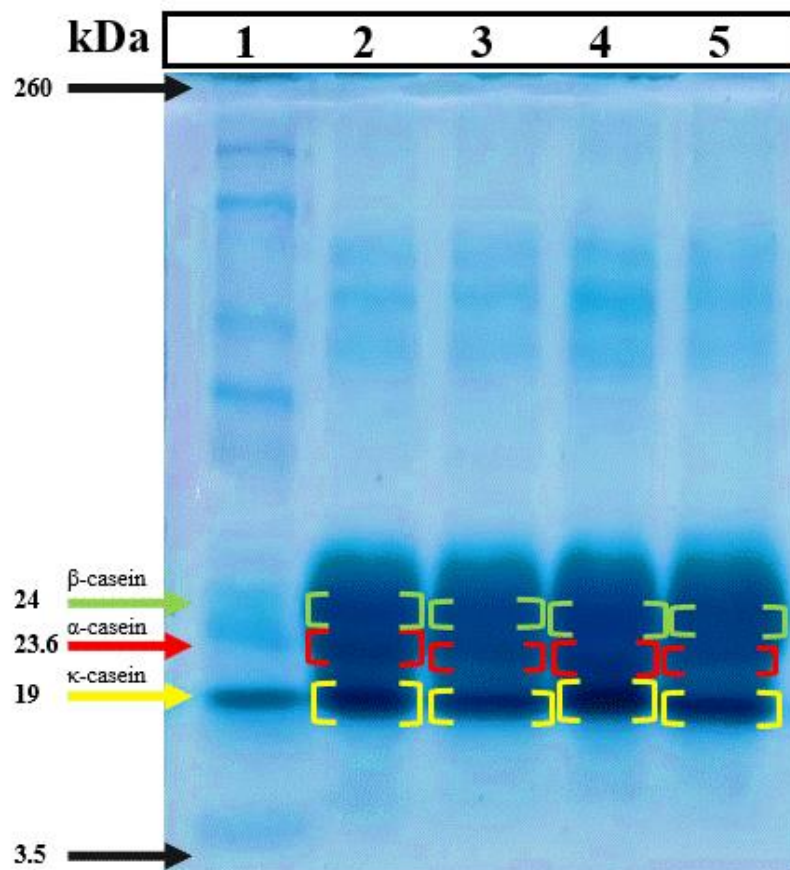
Thus, the characteristic bands of lactose ($2982, 2885, 1086, 876, 851, 479$ and 357 cm^{-1}), glucose ($2947, 2896, 1350, 1125, 1074, 917, 844, 540$ and 405 cm^{-1}), and galactose ($2973, 2939, 2920, 1250, 1071, 890, 833, 704, 661$ and 420 cm^{-1}) can be used to monitor milk powder with and without lactose hydrolysis during its production and aging. Changes in spectral characteristics can indicate the degree of hydrolysis and/or crystallization of lactose (DE PAULA *et al.*, 2021; TORRES *et al.*, 2017; SANTOS *et al.*, 2019; STEPHANI *et al.*, 2017; JÚNIOR *et al.*, 2016).

By grouping the samples using PCA for the spectral regions between 400 cm^{-1} and 1350 cm^{-1} and between 2800 cm^{-1} and 3200 cm^{-1} , the samples could be divided into two main groups: with and without lactose hydrolysis (Figure 5B and 5C). These spectral bands were previously reported to confirm whether the product has been hydrolyzed.

In summary, Raman spectroscopy is an interesting tool to characterize the physical state of food powders (amorphous, crystalline, glass transition). Knowing that the physical state of the powder directly influences its shelf life. Furthermore, carrying out this analysis can help confirm whether or not there is hydrolysis of lactose in the final product, which is relevant for industries, even in compliance with current legislation (STEPHANI *et al.*, 2017; JÚNIOR *et al.*, 2016).

Figure 12 shows results of the electrophoretic analysis carried out for the samples to identify the crosslinking between the amino acids glutamine and lysine (GL-bound) by the addition of TGase.

Figure 12 - Crosslinking between the amino acids glutamine and lysine (GL-bound) by the addition of transglutaminase identified by electrophoretic analysis.



Source: Prepared by the author (2023).

Knowing whether or not the transglutaminase enzyme is active is a priority when using this enzyme. Ways to quantify GL-bound are established in the literature using gas chromatography, hydroxamate method, sensitivity method with fluorescent substances, high-performance liquid chromatography, electrophoresis, and use of a transglutaminase assay kit. Among the established methods, electrophoresis is one of the simplest and normally routine methods in dairy analysis laboratories (GAUCHE, 2007; LI *et al.*, 2013; LI *et al.*, 2021).

During electrophoresis, proteins migrate under the influence of an electric field, and this migration is dependent on factors such as the field strength, network charge, size

and shape of the molecules, ionic strength, viscosity, and temperature of the medium (GAUCHE, 2007). A protein standard was used to observe the protein bands (a total of 12 proteins) with a variation range from 3.5 kDa to 260 kDa, which is described in band 1 of the gel (Figure 12).

Theoretically, a polymerization reaction catalyzed by TGase (GL-bound) results in the formation of high-molecular-weight protein polymers (GAUCHE, 2007). However, identifying the presence of these polymers in the gel was not possible, as the range was likely above 160 kDa. Notably, to visualize this region, the reaction medium conditions (gel and sample preparation, running time, and voltage) could be modified. Two other gels were created with three new sample concentrations (62 μ g, 107 μ g, and 125 μ g) and different buffer:sample ratios (1:1, 1.5:1, and 2.3:1, respectively) in an attempt to observe bands greater than 160 kDa; however, the results did not change.

In a study carried out by Li *et al.* (2013) and Li *et al.* (2021), verification of the formation of high-molecular-weight aggregates in electrophoresis gels was difficult owing to the presence of TGase. However, in both studies, there was a reduction in the intensity of the casein band in the products with the addition of TGase. This was also observed in the present study. There was a decrease in the intensity of the regions where κ -casein (19 kDa), α -casein (23.6 kDa), and β -casein (24 kDa) are found in the products that were added with TGase (lines 3 and 5) (VINCENT *et al.*, 2016).

6.4 CONCLUSION

There are studies in the literature that use the application of transglutaminase to skimmed milk powder. However, no studies have been reported in the literature that apply TGase during the drying of skimmed milk with hydrolyzed lactose. Furthermore, studies that establish the influence on the development of the Maillard reaction by the addition of TGase in dehydrated dairy products, mainly hydrolyzed lactose, are not verified in the literature. What has already been reported in the literature is that the addition of TGase has the ability to inhibit the non-enzymatic browning reaction, in soy products [8]. However, interestingly, it was reported in the present work that TGase considerably accelerated the development of MR in the product with hydrolyzed lactose, when added after lactase.

The characterization of powdered products formulated with or without the addition of lactase and TGase was carried out. Trying to answer the working hypothesis:

the present study explored the hypothesis that the application of TGase to milk with and without hydrolyzed lactose minimizes challenges during the drying stage (less adhesion of the powder to the interior of the drying chamber, improvement in the solubility and agglomeration profile of the powder). This may also reduce the intensity of the Maillard reaction (maintaining the color and nutritional quality of the final product). The results does not support the initial hypothesis of this article, it means that the addition of TGase after lactose hydrolysis didn't minimize the challenges during the drying stage and also didn't reduce the intensity of Maillard reaction in skimmed milk powder.

Finally, the present study clarifies that the use of TGase does not affect the drying conditions of the powders (retention capacity, humidity, water activity, ash), thus demonstrating that the enzyme can be used without affecting the drying technology. Furthermore, despite the increase in particle size of the powders with the addition of TGase, the bimodal conformational characteristics of the fluid milk were retained. The color of the product was not modified regardless of whether TGase was added. The amount of HMF in the product containing only TGase was equivalent to that in the product obtained with the control and only added lactase. In the hydrolyzed product with the addition of TGase, the HMF value was approximately 6 to 21 times higher than that in the other treatments. Lactose hydrolysis was confirmed using Raman spectroscopy. Finally, crosslinking between glutamine and lysine was not detected by electrophoresis, but a decrease in the intensity of the casein bands was observed.

However, the application of TGase in dairy products, especially those in which lactose is hydrolyzed, remains unknown. This work opens the door for future efforts in new projects.

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7 CHAPTER 4: STUDY OF MAILLARD REACTION MARKERS AND COLOR PARAMETERS OF CONCENTRATED MILK ADDED WITH LACTASE AND TRANSGLUTAMINASE AT DIFFERENT ADDITION TIMES AND HEAT TREATMENT CONDITIONS

7.1 INTRODUCTION AND AIM

Transglutaminase is considered an adjunct to manufacturing technology because it has no function in the final food product. This enzyme catalyzes peptide reactions between specific amino acids (lysine and glutamine) in the milk protein matrix. The enzyme action is subject to time and temperature conditions as well as sufficient substrate availability to promote bonds (SALUNKE and METZGER, 2023). Transglutaminase is known to form intra- and intermolecular peptide bonds in different food proteins, such as casein, by cross-linking with glutamine (acyl donor) and lysine (acyl receptor) residues and by promoting functional changes in foods such as yogurt (foam stability, gelation, thermal stability, viscosity, and water retention capacity) (GAN *et al.*, 2009; ROMEIH and WALKER, 2017).

Over 30 years ago, Ajinomoto began using transglutaminase in food (discovered by the company in 1987) and established a production process in 1993 (AJINOMOTO, 2023). The regulation of this enzyme in Mercosul is relatively new and has advanced only in recent years. In other countries, the situation is similar, and it is authorized in different food categories; however, for some classes of food, it remains in the approval phase. For example, the legislation in Europe is still country-specific, although the European Union is developing regulations for the use of enzymes in the bloc using a positive list. In the United States and Japan, enzyme use is regulated by the Food and Drug Administration (FDA) and the Food Sanitation Act (FSA), respectively (AJINOMOTO, 2023; LERNER and BENZVI, 2021).

A recent study that the addition of transglutaminase (TGase) improved the agglomeration profile and increased the glass transition temperature during the drying of skimmed milk powder (ER *et al.*, 2019). Additionally, studies on vegetable proteins reported a reduction in the intensity of the Maillard reaction (ROMEIH and WALKER, 2017). Based on these findings, a new study was carried out to verify the influence of adding TGase to skimmed milk (40% m·m⁻¹ solids), with and without hydrolyzed lactose,

on drying technology and the formation of Maillard reaction products (FRANCISQUINI *et al.*, 2024a).

In the aforementioned study (FRANCISQUINI *et al.*, 2024a), the enzyme was inactivated by heat treatment at $85^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes in a water bath before initiating the drying process. Lactase was added first for 24 hours, followed by TGase for 5 hours under refrigeration ($6^{\circ}\text{C} \pm 2^{\circ}\text{C}$). Some results obtained in this study are highlighted as follows: TGase did not affect drying conditions or technology; particle size was increased by the addition of TGase, despite the powder maintaining the bimodal conformation of fluid milk upon rehydration; the color of the product was not influenced; the intensity of the casein bands was reduced as determined using electrophoresis; and finally, the concentration of hydroxymethylfurfural in the hydrolyzed lactose product supplemented with TGase increased by 6 to 21 fold. This work opened the door for new studies in the search for a better understanding of the influence of concomitant transglutaminase and lactase in dairy products on the formation of Maillard reaction markers.

Therefore, this study aimed to verify the influence of the timing of adding transglutaminase and lactase to concentrated milk on the formation of Maillard reaction markers and color parameters under different heat treatment conditions.

7.2 MATERIAL AND METHODS

The study used a factorial design (three blocks $\times$ six factors $\times$ three levels). Six distinct treatments were established (A1, A2, A3, A4, A5, and A6 where skimmed milk powder reconstituted at $40\% \text{ m}\cdot\text{m}^{-1}$ was mixed with lactase and transglutaminase at different addition times) in three distinct heat treatment conditions (C1, C2, C3) in three replicates totaling 54 samples analyzed ($6 \times 3 \times 3$).

The skimmed milk powder (low heat treatment) was rehydrated using deionized water ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$) to approximately $40\% \text{ m}\cdot\text{m}^{-1}$ of total solids using a digital shaker (Ika RW 20, universal lab mixer). The two enzymes, lactase (company Global Foods MAXILACT[®] LGI 5000, enzymatic activity 2600 units per g) and transglutaminase (company Ajinomoto ACTIVA WM, 80 to 120 units per g), were added at different times to the rehydrated milk powder. The treatments were A1, A2, A3, A4, A5, A6: A1, rehydrated skimmed milk powder (SMP); A2, A1 with lactase ($0.200\% \text{ m}\cdot\text{m}^{-1}$) for 24 hours under refrigeration at $6^{\circ}\text{C} \pm 2^{\circ}\text{C}$ (SMPL); A3, A2 with transglutaminase ($0.025\% \text{ m}\cdot\text{m}^{-1}$) for 5 hours under refrigeration at $6^{\circ}\text{C} \pm 2^{\circ}\text{C}$ totaling 29 hours of combined enzyme

action (SMPLT); A4, A1 with transglutaminase ($0.025\% \text{ m}\cdot\text{m}^{-1}$) for 5 hours under refrigeration at $6^{\circ}\text{C} \pm 2^{\circ}\text{C}$ (SMPT); A5, A4 with the lactase ($0.200\% \text{ m}\cdot\text{m}^{-1}$) for 24 hours under refrigeration at $6^{\circ}\text{C} \pm 2^{\circ}\text{C}$ totaling 29 hours of combined enzyme action (SMPTL); A6, A1 with lactase ($0.200\% \text{ m}\cdot\text{m}^{-1}$) for 19 hours, followed by addition of transglutaminase ($0.025\% \text{ m}\cdot\text{m}^{-1}$) for 5 hours under refrigeration at $6^{\circ}\text{C} \pm 2^{\circ}\text{C}$ totaling 24 hours of combined enzyme action (SMPLT).

All treatments (A1, A2, A3, A4, A5, A6) were subjected to three different heat treatments C1 (no heat treatment); C2 (heat treatment at $85^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes); C3 (heat treatment at $145^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes). Notably, for condition C3, the Heat Coagulation Time (HCT) methodology proposed by Francisquini *et al.* (2024b) was followed with minor modifications.

For all samples, reaction marker determination and Maillard and color analyses were performed in triplicate. The samples were analyzed after storage at $8^{\circ}\text{C} \pm 2^{\circ}\text{C}$ on D1 (day 1 of storage) and onD7 (day 7 of storage).

A high-efficiency liquid chromatograph (model 1525; Waters) equipped with a binary pump was used to analyze four markers of the Maillard reaction: 5-hydroxymethylfurfural (HMF), 2-furaldehyde, 2-furyl-methylketone, and 5-methyl-2-furaldehyde. The method used to identify and quantify the four markers of Maillard reaction was adapted from that developed by Pinto *et al.* (2024). A reverse phase high-efficiency liquid chromatography technique was applied using a Waters Spherisorb column ($150 \text{ mm} \times 4.6 \text{ mm}$; particle size $3 \mu\text{m}$) and UV-Vis detector (Photodiode Array Detector). The time required for each analysis was 10 min with an injection volume of $20 \mu\text{L}$, temperature of 30°C , and wave compression of 284 nm . The elution gradient was formulated using a mixture of the two solvents under vacuum at $1 \text{ mL}\cdot\text{min}^{-1}$. The mobile phase comprised nonsolvent acetonitrile (B) and nonsolvent ultrapure water (A). The linear gradient program was initiated as follows: 0–5 min, 95.5%–4.5% B; 5–9 min, 80%–20% B; 9–10 min, 95.5%–4.5% B.

The color of the powders was determined using a colorimeter (CR400; Chroma Meter) by directly reading the values of the coordinates L^* (luminosity), a^* (positive = red, negative = green), b^* (positive = yellow, negative = blue), C^* (degree of saturation by Chroma) and h (tonality). The parameters analyzed in the color analysis were used to determine the browning index (BI). The intensity of brown color in the samples was evaluated using BI and was calculated using Equation 9, which correlates the parameters L^* , a^* , and b^* (OLIVEIRA *et al.*, 2012; QUEIROZ *et al.*, 2021).

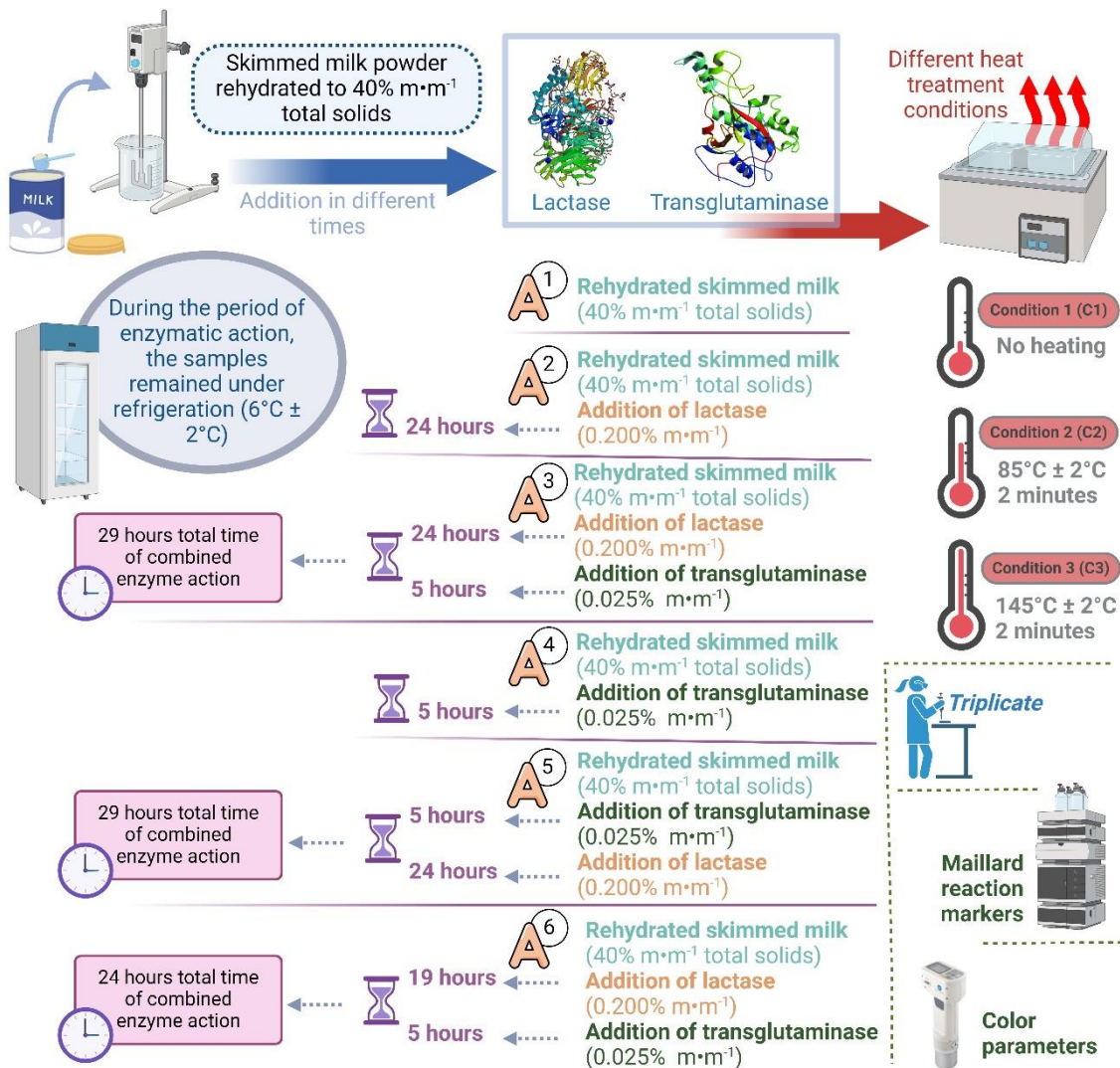
$$BI = ([100(x - 0.31)]) / 0.17 \quad (2)$$

Where:

$$x = (a^* + 1.750 \times L^*) / (5.645 \times L^* + a^* - 3.012 \times b^*)$$

Figure 13 schematically summarizes the methodology used in the present work.

Figure 13 - Schematic summary of the methodology used in this work



Source: Prepared by the author (2024).

The temperature of the rehydrated powdered milk (40% m·m⁻¹ of total solids) was also monitored every 30 seconds, using a digital thermometer with a probe (TP 101), until it reached the target heat treatment. Both condition 2 (85°C ± 2°C for 2 minutes in a water

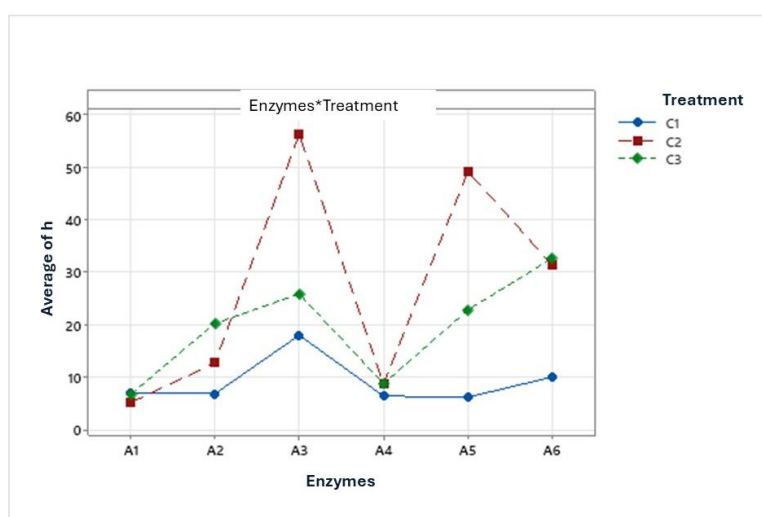
bath) and condition 3 ($145^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes in an oil bath using the HCT technique) were examined. From this monitoring, photographic documentation of the products was carried out before and after the heating, and a graph with the heating ramp (time in minutes x temperature in $^{\circ}\text{C}$) was generated for each of the conditions analyzed using Excel, 2016.

R language (version 4.2.1) was used to perform statistical analysis (mean comparison test) of the HMF results and color parameters (browning index). For factorial planning, interaction graphs of the adjusted means of the analyzed parameters with the heat treatment factors and enzyme addition, score graphs, and loading graphs were obtained using Minitab Statistical Software version 22.

7.3 RESULTS AND DISCUSSION

Figure 14 displays the HMF result in the six treatments performed (A1, A2, A3, A4, A5, A6) with the three analysis conditions (without heat treatment - C1, with heat treatment at 85°C for 2 minutes - C2 and at 145°C for 2 minutes - C3).

Figure 14 - 5-hydroxymethylfurfural content ($\mu\text{mol/kg}$) in reconstituted skimmed milk under different conditions of lactase and transglutaminase enzyme application, on day 1 of analysis.



Source: Prepared by the author (2024).

Figure 14 shows the analysis of the Maillard reaction marker HMF. The first interesting result was that in A1 (without the addition of enzymes), the values were in the

same region of the graph, regardless of the heat treatment conditions (C1, C2, and C3). The values differed when enzymes were added and HMF formation was altered when the product was subjected to different heat treatment conditions. In A2 (addition of lactase only), the highest HMF values were obtained for samples subjected to heat treatment C3 (145°C for 2 minutes), followed by condition C2 (85°C for 2 minutes) and finally condition C1 (no heat treatment). Therefore, for products with hydrolyzed lactose, temperature was the factor that most influenced the development of Maillard reaction markers.

In treatment A3 (addition of lactase, then TGase with a total time of 29 hours of enzymatic action) two aspects could be highlighted by analyzing the data: 1) concomitant use of the enzymes increased the HMF values when compared to those in the products with only one of the enzymes under all heat treatment conditions and; 2) the most discrepant point in the value of the adjusted averages was found in the sequential enzyme addition in heat treatment condition 2 (85°C for 2 minutes).

Treatment A4, which contained only TGase, had very similar adjusted mean scores among the different conditions (C1, C2, and C3), indicating that this enzyme had no impact on the formation of the Maillard reaction products.

In treatment A5, the same phenomenon occurred as in treatment A3. Thus, the combination of TGase and lactase resulted in increased HMF formation (higher values for C2, C3, and C1). Notably, in this case, the time of enzyme addition was changed, i.e., first TGase and then lactase (same enzyme action time of 29 h). This shows that, regardless of when the enzymes are added, the formation of this Maillard reaction marker is increased. The results indicate that the factor that most influenced the development of the Maillard reaction products when both enzymes were added was the time for which the product was exposed to heating and not the temperature, as the product at 85°C for 2 minutes (C2) had the highest HMF values. In this condition, the temperature increased gradually, unlike in the 145°C for 2 minutes condition (C3) where the heating ramp was faster.

For product A1, there was no statistical difference ($p>0.05$) in the HMF results, regardless of the analysis conditions used (C1, C2, C3). In other words, for the control product (skim milk powder), intensification of the heat treatment (C1, C2, and C3) had no effect on the formation of Maillard reaction markers. However, for the A2 product, in D1, the HMF values increased in the sequence C1 (average 6.83 μ mol/kg), C2 (average 12.68 μ mol/kg), and C3 (average 20.19 μ mol/kg). This shows that the increase in

temperature considerably interfered with the intensification of the Maillard reaction in products containing hydrolyzed lactose.

In treatment A3, in D1, the product without heat treatment (C1) was statistically the same as the product heat-treated at 145°C for 2 minutes (C3) and both were significantly different from the product heat-treated at 85°C for 2 minutes (C2). In the A3 treatment, it is worth highlighting the value of C2, which averaged 56.18µmol/kg for D1. The development of Maillard reaction markers in this condition (85°C for 2 minutes) was probably influenced by heating with a gradual increase in temperature (Figure 15A).

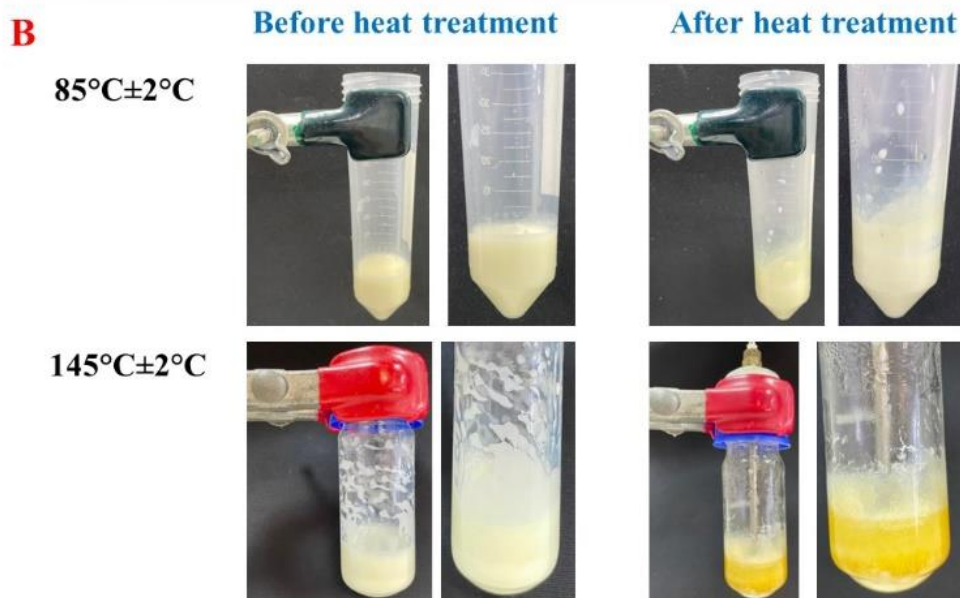
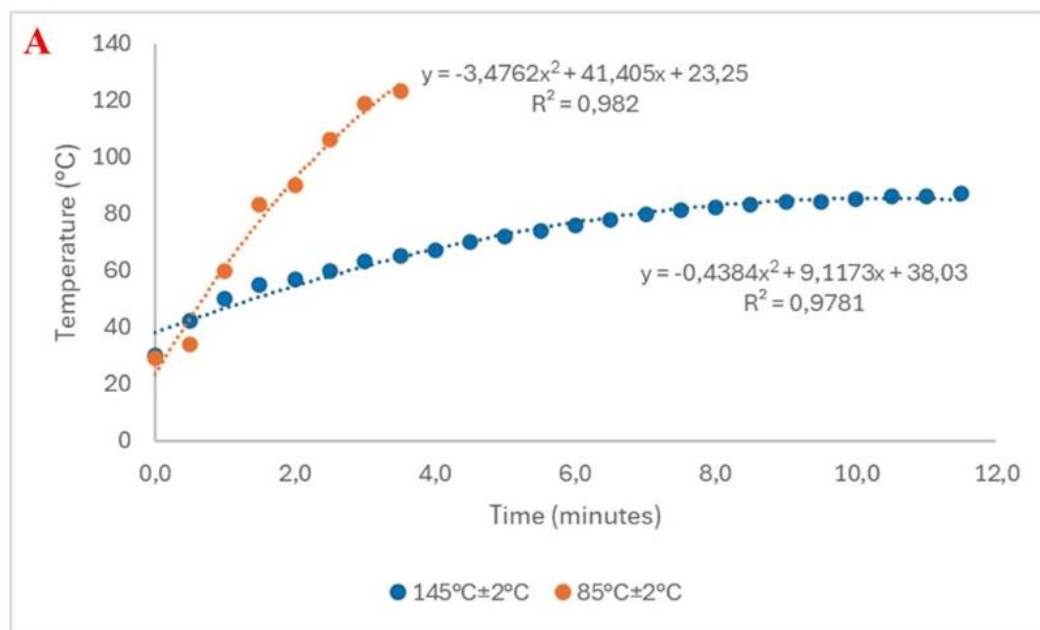
In A4, in D1, HMF formation was greater when the product was subjected to the two heat treatments, i.e., in both C2 (average 8.66µmol/kg) and C3 (average 8.71µmol/kg) the values were higher than those in the sample without heat treatment (average 6.39µmol/kg). Here, heating interfered with the formation of HMF in the presence of TGase.

In A5, HMF in D1 increased under the different conditions (C1, C2, and C3). This treatment produced results similar to A3, i.e., the formation of HMF intensified at 85°C. Therefore, a gradual increase in temperature was inferred to intensify HMF formation in the presence of the two enzymes, regardless of when the lactase and TGase were added (Figure 15A). Finally, in A6 (D1), the samples without heating (C1) were significantly different ($p>0.05$) from those heat-treated under conditions 2 and 3.

Comparing the conditions used in this work (C1, C2, C3) in relation to the HMF value, the products subjected to condition 2 (85°C for 2 minutes) stand out. Under these conditions, the highest HMF values were found compared to those of all other samples analyzed. Among these values, treatments A3, A5, and A6 stood out, all of which added lactase and transglutaminase simultaneously. For A3, the HMF value was approximately 3 times higher compared to that of C1 (no heat treatment) and approximately 2 times higher compared to that of C3 (145°C for 2 minutes). For A5, the HMF value was approximately eight times higher than that of C1 and approximately two times higher than that of C3. Finally, for treatment A6, the HMF value was approximately five times higher in condition 2 than in condition 1, and was statistically similar to that in condition 3.

Therefore, the heating ramp of concentrated milk was monitored to understand the reason for this result (Figure 15).

Figure 15 - A) Graph of time (minutes) × temperature (°C) of the heating ramp of the products subjected to condition 2 (target heat treatment of $85^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes) and condition 3 (target heat treatment of $145^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes); B) Photos of the products before and after heat treatment at $85^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in a water bath (condition 2) and $145^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes in an oil bath (condition 3).



Source: Prepared by the author (2024).

Figure 15B shows a graph of the heating ramp (every 30 seconds) for the rehydrated skim milk, which represents the uncontrolled temperature and time until the target heat treatment is reached. The heating ramp for condition 2 was longer, where the product reached from 30°C to 85°C in 11 minutes and 30 seconds. In contrast, for condition 3, the heating ramp was faster, where the product reached from 30°C to 123°C in 3 minutes and 30 seconds. Notably, the heat treatment of condition 3, 145°C for 2 minutes, corresponds to the HCT technique, which involves an oil bath heated to 145°C–155°C. However, the sample does not reach exactly 145°C, as seen in Figure 3A, where the maximum temperature of the sample was 123°C.

The optimum temperature for enzyme development is between 35°C and 60°C. For lactase prepared by bacteria, the optimum temperature is between 35°C and 40°C and for lactase prepared by fungi, the optimum temperature is approximately 55°C and 60°C (FELLOWS, 2018; FENNEMA, 2010). Notably, this study was performed using a lactase produced from a fungus (*Kluyveromyces lactis*), indicating that the optimum temperature was approximately 55°C and 60°C. Further, the optimum enzymatic temperature for TGase is between 40°C and 60°C (AJINOMOTO, 2023; BRASIL, 2022).

Thus, by analyzing the heating ramp, the product subjected to heat treatment condition 2 was found to spend approximately 3 minutes between 42°C and 63°C. For the product subjected to heat treatment condition 3, the product directly went from 34°C to 60°C, i.e., the product did not remain within the optimum temperature range for enzyme activity. Thus, under condition 2, the heat treatment used was able provided an environment conducive to greater enzyme activity (at the maximum speed of enzyme action). However, under condition 3, the enzyme soon reached the temperature at which the enzyme inactivation process began.

In other words, under heat treatment condition 2, lactase and transglutaminase likely acted with greater intensity. Therefore, there was greater hydrolysis of lactose, making available an even greater quantity of reducing sugars (glucose and galactose), which act as substrates for the Maillard reaction, resulting in increased formation of the intermediate products of the Maillard reaction, such as HMF.

As shown in Figure 15B, the products subjected to heat treatment in condition 2 did not exhibit a significant change in color. However, when the products were subjected to heat treatment under Condition 3, a considerable change was observed in the color of the sample. This situation indicated that when the product was subjected to heat treatment at $145^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes, the intermediate Maillard reaction markers (such as HMF)

may have been transformed into the final Maillard reaction markers, such as melanoidins. Thus, that the type of heat treatment used for condition 3, i.e., the oil bath heating methodology (HCT), was more intense, which accelerated the Maillard reaction and resulted in faster transformation of intermediate products into the final products. This can be explained by the fact that the Q10 of the Maillard reaction is between 2.4 and 5.0, which is the number of times the speed of the chemical reaction increases with each increase of 10°C (WALSTRA, 1984). In this case, when there was a change from 85°C to 145°C from condition 2 to condition 3, there was an increase of 60°C and the Maillard reaction was accelerates from 191 to 15625 times.

It is not necessary that the product with the most pronounced color will have the greatest formation of Maillard reaction indicators; this has also been reported in other studies (DE PAULA *et al.*, 2022; FRANCISQUINI *et al.*, 2019; QUEIROZ *et al.*, 2021). HMF is an intermediate product of the reaction, and quantification of a low-magnitude value may indicate that the reaction has already progressed to advanced stages with the formation of melanoidins (FRANCISQUINI *et al.*, 2019). Interestingly, as shown in Figure 15B, despite having a very rapid heating ramp, the products subjected to heat treatment at 145°C ± 2°C for 2 minutes, visually had a darker, yellowish/brownish color, which may actually indicate the formation of melanoidins.

Melanoidins are high-molecular-weight compounds (66 to 236 kDa) that contain nitrogen in their conformation and exhibit antioxidant, anti-obesity, antimicrobial, anti-hypertensive, anti-inflammatory, and sensory activities. They can be obtained through four different routes. The first route is based on the cross-linking of colored compounds of low molecular weight by free amino groups of lysine or arginine in proteins. The second route involves the formation of repeated furan and pyrrole units that undergo polycondensation. The third route involves the formation of melanoidins from the degradation products of polymerized sugars interconnected by amine compounds during the initial stage of the Maillard reaction. The fourth and final route of melanoidin formation involves proteins crosslinking with Maillard reaction products (melanoproteins) (CÄMMERER and KROH 1995; HOFMANN *et al.*, 1999; FOGLIANO and MORALES, 2011; SHAHEEN *et al.*, 2022).

Based on these, one hypothesis is that in the first phase of the Maillard reaction, the reducing sugar undergoes condensation with amino acids, peptides, or proteins. In the second phase of the Maillard reaction, HMF is formed, which can bind to proteins, resulting in the formation of melanoidins in the third phase of the reaction. Upon adding

TGase, the enzyme can bind to the protein, making it impossible for HMF to bind. Therefore, less melanoidin is formed and more HMF remains in the sample to be analyzed. Therefore, if TGase acts during the first phase of the reaction, less HMF is formed. The results reported here indicate that TGase prevents HMF from reacting with proteins and consequently forming melanoidins in the intermediate phase of the reaction. Thus, transglutaminase inhibits the formation of dark compounds (the final phase of the reaction), resulting in a higher HMF concentration (FOGLIANO and MORALES, 2011; SHAHEEN *et al.*, 2022).

Comparing the treatments in the unheated condition (C1), A1 (skim milk only) can be inferred to have the same HMF formation as that in A2 (skim milk and lactase), A4 (skim milk and transglutaminase), A5 (skim milk, transglutaminase, and lactase), and A6 (skim milk, lactase, and transglutaminase, with a total enzymatic activity of 24 h). These differed significantly from A3 (skim milk, lactase, and transglutaminase with total enzymatic action of 29 h), which had the highest HMF value under these conditions. In A3 (D1), the HMF value was 17.96 $\mu\text{mol/kg}$, showing that the addition of lactase with enzymatic action for 24 hours followed by the addition of transglutaminase with enzymatic action for 5 hours, totaling 29 hours, was the condition that most influenced the development of the Maillard reaction marker (HMF).

When the sample was heat-treated at 85°C for 2 minutes (C2), treatments A1, A2, and A4 were statistically similar to each other ($p>0.05$), with the lowest HMF values. Treatment A3 was statistically similar to treatment A5, which had the highest HMF value. Finally, treatment A6 differed significantly from the other treatments, with an intermediate HMF value. Overall, the highest values were obtained for A3 (skimmed milk, lactase and transglutaminase with 29 hours of total enzyme action), A5 (skimmed milk, transglutaminase and lactase with 29 hours of total enzyme action), and A6 (skimmed milk, lactase and transglutaminase with 24 hours of total enzyme action) in D1 (average A3 of 56.18 $\mu\text{mol/kg}$, A5 of 49.08 $\mu\text{mol/kg}$ and A6 of 31.27 $\mu\text{mol/kg}$).

Upon analyzing condition C3, i.e., 145°C for 2 minutes, D1 treatments A1 and A4 as well as A2, A3 and A5 were found to yield statistically similar results ($p>0.05$). A3 was also statistically similar to treatment A6. The highest HMF values were found in A3 (25.81 $\mu\text{mol/kg}$), A5 (22.76 $\mu\text{mol/kg}$), and A6 (32.58 $\mu\text{mol/kg}$).

In conclusion, when the samples were heat-treated, the highest HMF values were obtained for treatments with both enzymes added concomitantly (lactase and transglutaminase). When comparing the control product (A1 skim milk only) with the

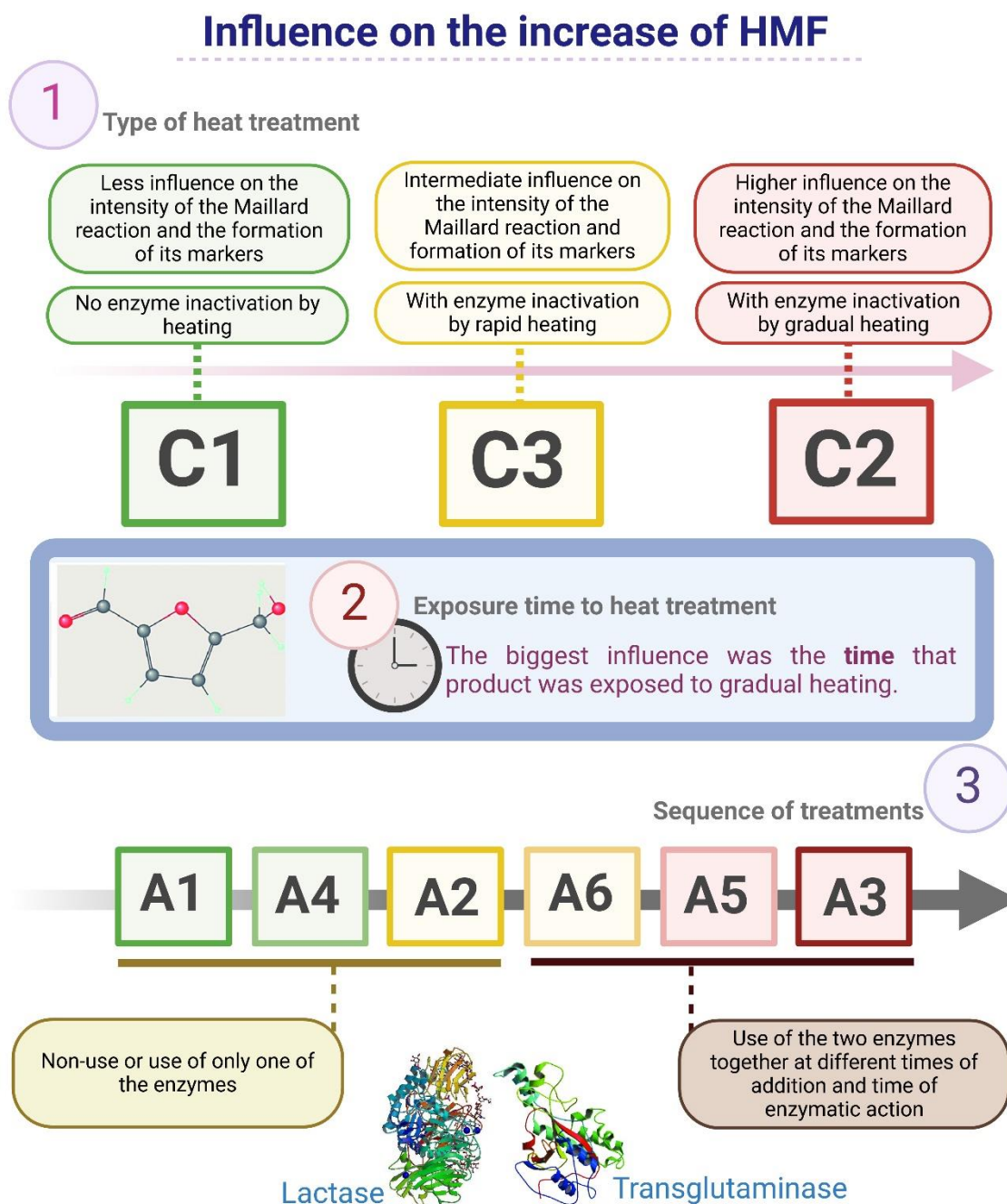
product subjected to heat treatment condition 2, the HMF value increased approximately 2 times with treatment A2, 11 times with treatment A3, 1 time with treatment A4, 10 times with treatment A5, and 6 times with treatment A6. When comparing the control product with the product subjected to heat treatment condition 3, the HMF value increased by approximately three times with treatments A2, four times with treatment A3, one time with treatment A4, three times with treatment A5, and five times with treatment A6. Therefore, the HMF values increased considerably in treatments with concomitant enzyme addition. Thus, regardless of the order in which the enzymes were added and the time of enzyme action, when the two enzymes were used concomitantly, the formation of this intermediate product of the Maillard reaction was intensified.

Previous studies on isolated soy protein have shown that the addition of transglutaminase reduces the intensity of the Maillard reaction, with a consequent reduction in the formation of its markers (GAN *et al.*, 2009). This was correlated with the crosslinking that TGase catalyzes between lysine and glutamine, making them unavailable for condensation with reducing sugars (ROMEIH and WALKER, 2017). However, in a study by Francisquini *et al.* (2024a), the formation of the Maillard reaction intermediate markers was intensified when TGase was added to the hydrolyzed lactose products. In other words, addition of the two enzymes (lactase and transglutaminase) also considerably intensified the formation of HMF in the dehydrated products with lactose hydrolysis (approximately 6 to 21 times more). These authors added lactase to concentrated skimmed milk with an enzyme action time of 24 hours, and then added transglutaminase for 5 hours, both under refrigeration ($6 \pm 2^\circ\text{C}$). Enzyme inactivation was then carried out by gradually increasing the temperature to 85°C for 2 minutes, so that the product could be spray dried. This condition was similar to the one used in this work, in treatment A3 subjected to condition C2. Overall, A3/C2 was one of the products with the most intensified formation of HMF, in line with the findings of the aforementioned study (FRANCISQUINI *et al.*, 2024a).

Condition C1 (no heat treatment) for treatments A5 (skim milk, transglutaminase for 5 hours and lactase for 24 hours) and A6 (skim milk, lactase for 19 hours and transglutaminase for 5 hours) remained statistically similar ($P > 0.05$) and showed the lowest HMF values. These results open up perspectives for further work in the area of dehydrated and hydrolyzed dairy products supplemented with TGase and their influence on non-enzymatic browning reaction markers.

Figure 16 schematically summarizes the results found here.

Figure 16 - Schematic summary of the main results of HMF analysis in the different treatments (A1: SMP; A2: SMPL; A3: SMPLT; A4: SMPT; A5: SMPTL; A6: SMPLT) and in the different heat treatment conditions (C1: no heat treatment; C2: with heat treatment at $85^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes; C3: with heat treatment at $145^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes).

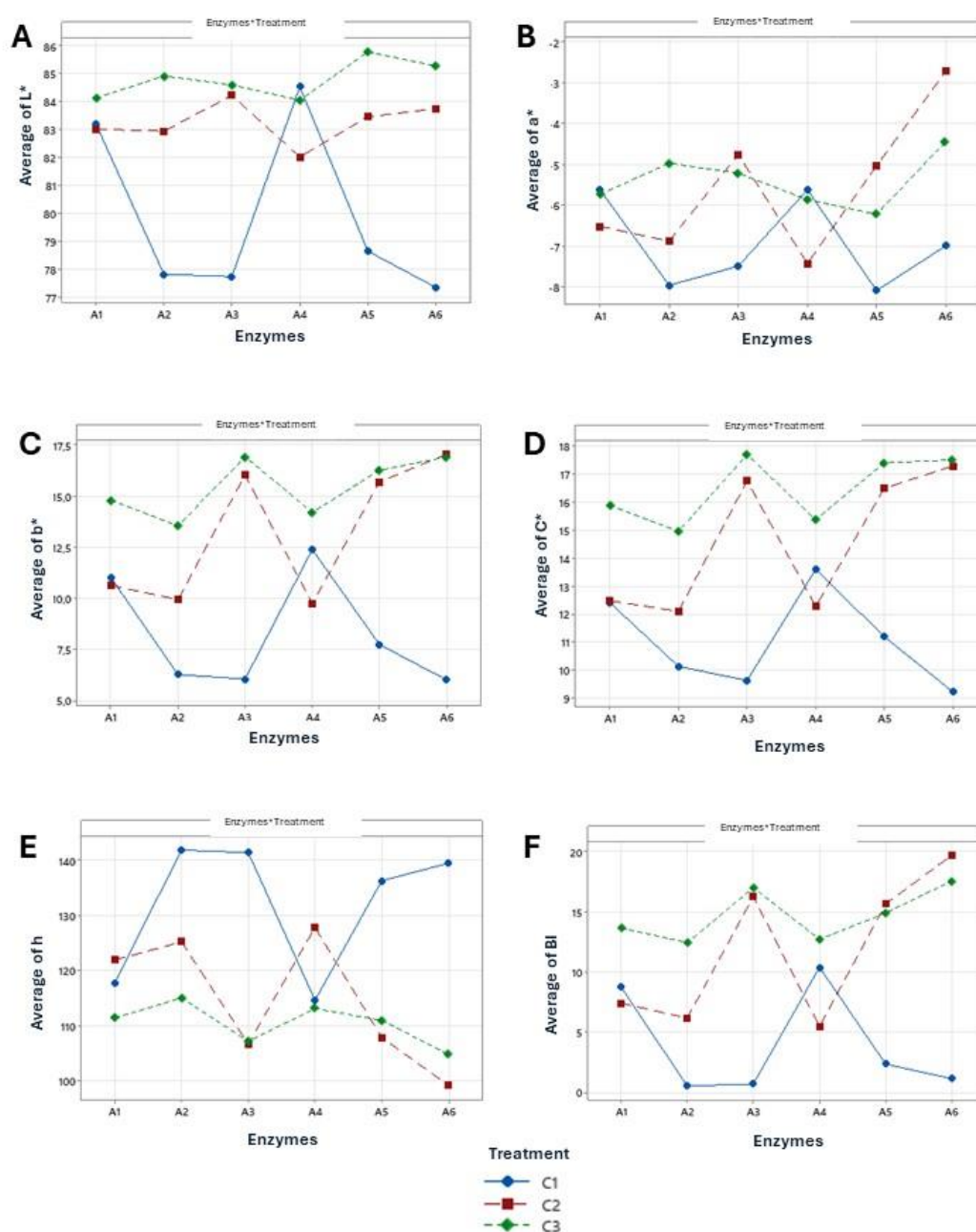


Source: Prepared by the author (2024).

The mean comparison tests for the Maillard reaction markers and color analysis were carried out for the products analyzed on both D1 and D7 of storage. However, only the values reported for D1 are highlighted, as there were no significant changes in the statistical analysis when analyzing and comparing the results with those on D7. Thus, the storage time (7 days at $8^{\circ}\text{C} \pm 2^{\circ}\text{C}$) was sufficient to alter the products in terms of HMF content and color parameters.

To visually show the interaction of different treatments (A1: SMP; A2: SMPL; A3: SMPLT; A4: SMPT; A5: SMPTL; A6: SMPLT) with the different heat treatment conditions used (C1: no heat treatment; C2: with heat treatment of $85^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes; C3: with heat treatment of $145^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes) in relation to all the color parameters (L^* , a^* , b^* , C^* , h , BI), interaction graphs of the adjusted means of these analyzed attributes were drawn. Day 1 of the analysis was chosen for the graph because the values reported on day 7 did not differ significantly from these.

Figure 17 - Interaction graphs of the adjusted means of the analyzed parameters (A: parameter L^* , B: parameter a^* , C: parameter b^* , D: parameter C^* , E: parameter h , F: browning index) with the heat treatment factors (C1: no heat treatment; C2: with heat treatment at $85^\circ\text{C} \pm 2^\circ\text{C}$ for 2 minutes; C3: with heat treatment at $145^\circ\text{C} \pm 2^\circ\text{C}$ for 2 minutes) and addition of enzymes (A1: SMP; A2: SMPL; A3: SMPLT; A4: SMPT; A5: SMPTL; A6: SMPLT) of the samples analyzed on day 1.



Source: Prepared by the author (2024).

The highest values for the L^* parameter were obtained for the products subjected to condition 3 heat treatment, i.e., 145°C for 2 minutes for the treatments (A1, A2, A3, A5 and A6) except for A4 whose highest L^* value was reported for condition 1 (no heat treatment). Therefore, these samples exhibited the highest luminosity. Meanwhile, the lowest L^* values were reported for treatments A1, A2, A3, A5, and A6 subjected to condition 1 (no heat treatment) and for treatment A4 subjected to condition 2 (85°C for 2 minutes). These samples had the lowest luminosity compared with that of the other samples.

For the a^* parameter (Figure 17B), all samples had negative values closer to green. Upon comparing the results, the lowest values (closest to the green region) were obtained for treatments A2, A3, and A5 in heat treatment condition 1. The highest a^* values (closer to the red region) were obtained for treatments A3, A5, and A6 under heat treatment condition 2.

Figure 17C shows the values of the b^* parameter from the color analysis, with all the analyzed samples showing positive results and, therefore, being in the yellow region. The highest values for this parameter (more yellowish samples), in all the evaluated treatments (A1, A2, A3, A4, A5 and A6), were obtained during heat treatment condition 3 (145°C for 2 minutes). The lowest values (samples less yellow and closer to blue) were obtained for treatments A2, A3, A5, and A6 under condition 1 (without heat treatment).

Figure 17D shows the degree of saturation of the samples analyzed by chroma (C^*), which corresponds to the distance from the luminosity axis. The highest values were obtained for the 145°C for 2 minutes condition (C3) in all the treatments (A1, A2, A3, A4, A5 and A6), these being the most saturated samples. Consequently, the least saturation (lowest chroma values) was obtained for the samples without heat treatment (C1) in treatments A1, A2, A3, A5 and A6, and for the sample in the condition of 85°C for 2 minutes for treatment A4.

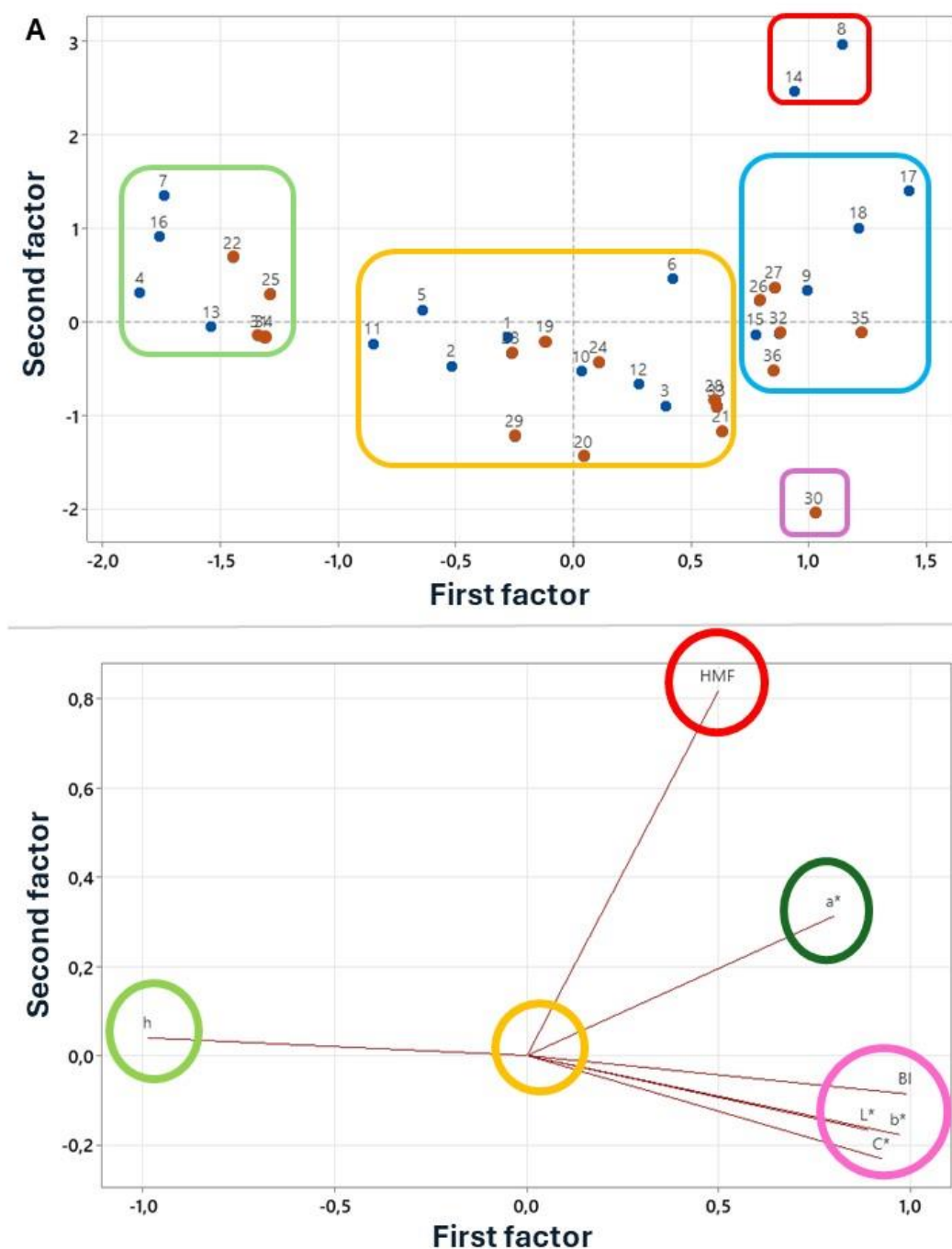
The values of the h parameter are shown in Figure 17E. In this case, the hue of the sample was evaluated, which in all the samples analyzed were in the yellow range (100–140°). Notably, the lowest values were obtained for treatments A1, A2, A3, and A4 under heat treatment condition 3 and for A5 and A6 under heat treatment condition 2. Further, the highest values were reported for the samples subjected to treatments A1 and A4 under heat treatment condition 3, and for the samples subjected to treatments A2, A3, A5, and A6 under heat treatment condition 1. Further, the samples with the lowest h values were those closest to green.

The last parameter related to the product color was the browning index (Figure 17F). The darkest products were from the treatments A1, A2, A3, and A4 in the 145°C heat treatment condition for 2 minutes and treatments A5 and A6 in the 85°C heat treatment condition for 2 minutes. The lightest products, with the lowest BI, were treatments A2, A3, A5 and A6 in condition 1, i.e., without heat treatment, and treatments A1 and A4 in condition 2, i.e., with heat treatment at 85°C for 2 minutes.

Finally, treatment A6 had lower adjusted mean scores than those of treatment A3, indicating that the time for which the enzyme was active (24 h) may have influenced the lower formation of HMF.

Principal component analysis was carried out to group the samples according to their similarity in terms of color and HMF parameters (Figure 18).

Figure 18 - A) Score graph (D1: blue circles; D7: orange circles); B) loading graph (factorial load) of the analyzed samples (Treatment: A1: SMP; A2: SMPL; A3: SMPLT; A4: SMPT; A5: SMPTL; A6: SMPLT and Heat treatment: C1: no heat treatment; C2: with heat treatment at $85^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes; C3: with heat treatment at $145^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes) on days 1 and 7 of manufacture using parameters L, a^* , b^* , C^* , h, browning index, and hydroxymethylfurfural.



Source: Prepared by the author (2024).

The samples were divided into five groups by analyzing the score graph (Figure 18A). Thus, some of the results are highlighted below.

Sample 30 stood out from all the other analyzed samples (lilac-colored group); it was from day 7 of the analysis, condition 3, and treatment 4 (milk with only transglutaminase added). The factors that differentiated this sample from the others, causing it to be grouped differently, were the higher values reported for the browning index (BI) and the parameters C* and b* in the color analysis.

Another group was formed with samples 8 and 14, both on day 1 of the analysis and in condition 2 of heat treatment, and these were grouped together (group with red color) as those with the highest HMF values and high browning index values in relation to all the other samples analyzed. Numerically, the highest HMF value was found for sample 8, which was subjected to treatment A3, where the skimmed milk was first supplemented with lactase (24 hours) and then with transglutaminase (5 hours). The lowest HMF value was reported for sample 14, in which treatment A5 was carried out, where skim milk was first supplemented with transglutaminase (5 h) and then with lactase (24 h).

Three large clusters were formed. The first, green group, only included samples subjected to condition 1 (no heat treatment), both on D1 and D7, and with different treatments (A2, A3, A5, A6). Therefore, the determining factor for this grouping was the absence of heat treatment. With regard to HMF, the values were between 5.38 $\mu\text{mol/kg}$ and 17.96 $\mu\text{mol/kg}$, i.e., intermediate to low. Another highlight was that these were the eight samples with the lowest BI values (between 0.52 for sample 4 and 4.00 for sample 34), the highest values for the h parameter (between 132.24 for sample 25 and 141.85 for sample 4), the lowest values for the C* parameter (between 9.22 for sample 16 and 11.96 for sample 34), the lowest values for the b* parameter (between 6.00 for sample 16 and 8.61 for sample 25), and the lowest values for the L* parameter (between 77.36 for sample 16 and 78.90 for sample 31).

A group of nine samples was formed (blue group), including samples from heat-treated conditions (C2 and C3) and three different treatments (A3, A5, and A6). These three treatments had lactase and TGase added simultaneously. These samples were brought close together by the medium to high values of the L* parameter (between 82.83 for sample 32 and 85.79 for sample 15) and the HMF content (between 11.93 $\mu\text{mol/kg}$ for sample 36 and 32.58 $\mu\text{mol/kg}$ for sample 18), as well as high values for parameters a* (between -2.74 for sample 17 and -6.22 for sample 15), b* (between 15.85 for sample

26 and 17.24 for sample 35), C^* (between 16.69 for sample 26 and 17.68 for sample 9), h (between 99.19 for sample 17 and 110.97 for sample 15), and BI (between 14.90 for sample 15 and 19.86 for sample 35).

The last group (yellow) was the largest, totaling 16 grouped samples. The samples in this region were from the three heat treatment conditions ($C1$, $C2$, and $C3$) and three treatments ($A1$, $A2$, and $A4$). In this case, the samples were grouped together mainly because they displayed intermediate values for the attributes analyzed (HMF , BI , parameters h , C^* , b^* , and L^*).

Samples 1 to 18 were analyzed on day 1 of storage, and samples 19 to 36 were analyzed on day 7 of storage. Thus, by analyzing the score graph, most of the samples analyzed on D1 and D7 were found to be in the same group. This was only true for four sets of samples: 8 and 26, 12 and 30, 14 and 32, and 15 and 33. Samples 8 and 26 were subjected to heat treatment condition 2 and treatment $A3$ (addition of lactase for 24 hours followed by $TGase$ for 5 h). These samples within the score graph were separated by the value reported for HMF , which was 2.7 times higher on day 1 of storage (8) than on day 7 of storage (26). The same situation was reported for samples 14 and 32 (condition 2, treatment $A5$: $TGase$ for 5 hours and lactase for 24 hours), whose HMF was 3.3 times higher for D1 (14) than for D7 (32), as well as for samples 15 and 33 (condition 3, treatment $A5$: $TGase$ for 5 hours and lactase for 24 hours), where the HMF was 2.2 times higher for D1 (16) than for D7 (33). Therefore, for these three cases, in which the same sample was separated but analyzed on different storage days, the HMF value was used. In only one case, samples 12 and 30 (condition 3 and treatment $A4$: addition of only $TGase$ for 5 hours), despite being the same samples analyzed on different storage days, had BI values that were 1.6 times higher and C^* ; b^* values that were 1.4 times higher on day 7 of storage than on day 1.

The loading graph shown in Figure 18B confirms the results presented above, i.e., in the red color group (samples 8 and 14) HMF was the most influential factor in grouping the samples and in the lilac color group (sample 30) the most influential factors were the BI , L^* , C^* , and b^* parameters, distancing this sample from the others. Further, the samples grouped in green color (samples 4, 7, 13, 16, 22, 25, 31, and 34) had the most decisive h values. The grouping of samples 9, 15, 17, 18, 26, 27, 32, 35, and 36 (colored blue) was mainly attributed to the value of the a parameter. Finally, the largest grouping (yellow samples 1, 2, 3, 5, 6, 10, 11, 12, 19, 20, 21, 23, 24, 28, 29, and 33) was because

of the intermediate values of practically all the attributes analyzed (HMF, BI, L*, b*, h, and C*).

7.4 CONCLUSION

In conclusion, heat treatment condition C2 ($85^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes) had the greatest influence on HMF formation in the different treatments applied (A1 to A6). The explanation for this is that lactase and transglutaminase remain at the optimum temperature for enzymatic action for a longer period in this condition.

The samples subjected to treatments A3 and A5 under condition 2 showed the highest HMF formation compared to that in all the other samples analyzed. Transglutaminase and lactase were concomitantly added to both these treatments, with only the time of addition changed. This shows that regardless of the time of addition, when the two enzymes were added concomitantly and then inactivated at $85^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 2 minutes, the intermediate product of the Maillard reaction, HMF, was formed in greater quantities. Under all heat treatment conditions used (C1, C2, and C3), the highest HMF concentrations were also found in the samples subjected to treatments A3 and A5. The TGase enzyme was hypothesized to act on the proteins, preventing HMF from binding to them, because of which this intermediate product was not converted into melanoidins and remained at a higher concentration in the final product.

Regarding the color parameters, the highest L* values and saturation were found for samples subjected to the C3 treatment, whereas the lowest L* values and saturation were reported for the samples subjected to the C1 condition. All samples had an a* characteristic of green-colored products, while the b* and h parameters classified the samples as yellow. The browning index was higher for samples subjected to conditions C2 and C3.

Finally, by analyzing the score graph and loading graph, the samples were divided into five groups. The factors that influenced the grouping of the samples were as follows: the color parameters C*, b*, and the browning index for the lilac group; HMF for the red group; the absence of heat treatment of the sample for the green group; and heat treatment at both conditions C2 and C3 for the blue group. Finally, the green group contained samples that had intermediate values for all attributes analyzed (HMF, BI, parameters h, C, b*, and L*).

Overall, the results reported in this work open up future prospects for studying the influence of concomitant addition of TGase and lactase on the color and Maillard reaction products of dairy products.

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8 GENERAL CONCLUSIONS AND FUTURE CONSIDERATIONS

The DAI enables students to become differentiated professionals because of the integration of practical and academic experiences. Being linked with a private company allows one to experience activities related to professional reality, whereas being linked with a public institution allows one to experience activities of a scientific and academic nature.

In addition, the work undertaken here provides information that helps industries better understand the properties of plant-based beverages, which are in increasing demand in the market. This knowledge can be used to improve manufacturing technology, nutritional profiles, and product standardization, in addition to helping in the development and implementation of specific legislations. This work also facilitates the achievement of results that expand our knowledge in the area of ingredients (enzymes) routinely used by food industries worldwide, making new discoveries available for use.

Finally, this work opens doors for future studies, such as the development of plant-based beverages at the laboratory level with different ingredients added from different vegetables, to develop a formulation that meets the physicochemical and stability requirements along with possessing pleasant sensory characteristics and good nutritional quality (clean label). Further, this work expands new paths to be followed for projects involving the use of transglutaminase and lactase in dehydrated dairy products as well as in model solutions, mainly with regard to the influence of their concomitant action on the development of Maillard reaction markers and color parameters.

9 ATTACHMENTS

9.1 SCIENTIFIC DOCUMENTS PUBLISHED OR ACCEPTED DURING THE DOCTORATE PERIOD

9.1.1 BOOK

- 1) POMBO, A. F. W.; DE CARVALHO, A. F.; PERRONE, I. T.; FRANCISQUINI, J. D. A.; CAMPOS, N. S.; STEPHANI, R. **Fundamentos práticos da fabricação de doce de leite**. Funarbe, 2021.

9.1.2 BOOK CHAPTER

- 1) DA SILVA, R. A. D.; FRANCISQUINI, J. D.; DE CARVALHO, A. F.; STEPHANI, R.; PERRONE, I. T. Química Tecnologia e Inovação em Alimentos e Bebidas. *In: Bebidas vegetais e probióticos: uma revisão Narrativa*. CNPq, 2024.
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9.1.3 ARTICLES IN SCIENTIFIC JOURNALS

- 1) QUEIROZ, E. S.; REZENDE, A. L. L.; PERRONE, Í. T.; FRANCISQUINI, J. D. A.; DE CARVALHO, A. F.; ALVES, N. M. G.; DE OLIVEIRA, L. F. C.; STEPHANI, R. Spray drying and characterization of lactose-free goat milk. *LWT*, v. 147, p. 111516, 2021.
- 2) DE PAULA, I. L.; TEIXEIRA, E. B. S.; FRANCISQUINI, J. D. A.; STEPHANI, R.; PERRONE, Í. T.; DE CARVALHO, A. F.; DE OLIVEIRA, L. F. C. Buffalo powder dairy products with and without lactose hydrolysis: Physical-chemical and technical-functional characterizations. *LWT*, v. 151, p. 112124, 2021.
- 3) FRANCISQUINI, J. D. A.; DA COSTA, J. D. C.; STEPHANI, R.; PERRONE, Í. T. Aspectos nutricionais da gordura láctea: Uma revisão dos benefícios comprovados do seu consumo para a saúde humana. **Research, Society and Development**, v. 10, n. 9, p. e58710918430-e58710918430, 2021.

- 4) MELLO, L. O.; FRANCISQUINI, J. D. A.; DA COSTA, J. D. C.; KHARFAN, D.; PERRONE, Í. T.; STEPHANI, R. Comparison of composition, nutritional aspects and market price between UHT milk and UHT plant-based beverages. **Research, Society and Development**, v. 10, n. 13, p. e128101320860, 2021.
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- 15) FRANCISQUINI, J. D. A.; GOMES, E. R.; VEGA-CASTRO, O. A.; CALDERON, J. C.; ARCILA, E.; COSTA, F. F.; RODARTE, M. P.; CERQUEIRA, A. F.; DE OLIVEIRA, L. F. C.; STEPHANI, R.; DE CARVALHO, A. F.; PERRONE, I. T. Micellar casein and whey protein as encapsulating agents of natural extracts: antioxidant capacity and phenolic compound. *Revista Virtual de Química*, No prelo 1-10. DOI: <http://dx.doi.org/10.21577/1984-6835.20240049>
- 16) Determinação de cálcio iônico por meio de eletrodo de íon seletivo em diferentes condições de tratamento térmico do leite - Accepted for publication in the Journal of Cândido Tostes Dairy Institute.
- 17) Estudo comparativo da composição nutricional e análise da rotulagem de *whey protein* concentrado, isolado e hidrolisado - Accepted for publication in the Journal of Cândido Tostes Dairy Institute. DOI: 10.14295/2238-6416.v79i3.975

9.2 WORKS PRESENTED AT SCIENTIFIC EVENTS DURING THE DOCTORATE PERIOD

- 1) Dairy Talks 2022 – Dairymen’s talk: technology, engineering and innovation – Plant based Beverage! <https://www.inovaleite.com/dairy-talks>
- 2) Nizo 2023 - 14th NIZO Dairy Conference – Use of milk protein for protection of bioactive substances in coffee silverskin extracts <https://www.nizodairyconference.com/>
- 3) Nizo 2023 - 14th NIZO Dairy Conference – Coffee silverskin extract encapsulation by milk protein during spray drying <https://www.nizodairyconference.com/>
- 4) 35th Regional Meeting of SBQ-MG - Comparative analysis of the physicochemical properties of plant-based fermented beverages <https://ersbq2023-dqi.ufla.br/>
- 5) 35th Regional Meeting of SBQ-MG - Analysis of ionic calcium in milk matrix by ion selective electrode <https://ersbq2023-dqi.ufla.br/>

- 6) Slacan 2023 - 15th Latin American Symposium on Food Science and Nutrition – Physicochemical aspects of commercial plant based beverages
<https://2023.slacan.com.br/slacan-2023/page/2489-home>
- 7) Innovadoce 2024 - Technology and Sensory Panel – Formation of 5-hydroxymethylfurfural and color change in dulce de leche with hydrolyzed lactose <https://www.inovaleite.com/innovadoce>
- 8) Whey conference 2024 - 11th International Whey Conference - Whey and dairy based powders lactose free: the effect of drying chamber and drying conditions on the dryability of the products <https://internationalwheyconference.com/>