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Igor Lima de Paula

Microparticulated Whey Protein: Technological Review, Analytical Development, and Innovative Formulation

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Igor Lima de Paula

Microparticulated Whey Protein: Technological Review, Analytical Development, and Innovative Formulation

Thesis presented to the Graduate Program in Chemistry at the Federal University of Juiz de Fora as a requirement for obtaining the title of Doctor of Chemistry. Area of concentration: Chemistry.

Advisor: Prof. Dr. Rodrigo Stephani
Co-advisor: Prof. Dr. Luiz Fernando Cappa de Oliveira

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“Because half of a human being’s life is about surviving the world. The other half is about finding meaning in their existence. For the first, there is work, instinct, and natural evolution. For the second, there is love, faith — and dreams.” adapted from Draccon (2011).

ABSTRACT

The rising incidence of overweight and obesity has led the food industry to seek alternatives for developing products with low caloric content. Among macronutrients, fat has the highest caloric density; however, its removal is not a trivial task, as fat is essential for providing organoleptic and sensory properties to foods. Protein appears as a potential substitute, but its high thermal denaturation rate hinders its application in many products. As a result, the food industry has been actively looking for alternatives to overcome this challenge. Microparticulated protein (MP) is a combination of native and denatured protein that undergoes heat treatment followed by shear processing, which increases its thermal stability while still providing fat-like characteristics. Initially developed by CP Kelco as a fat replacer, microparticulated proteins are already used in a variety of products, such as ice creams, cheeses, salad dressings, and yogurts, with different functional purposes. The first chapter (item 3) of this work aimed to gather information on the production, functionality, and applicability of MPs found in patents, thereby creating a repository of knowledge about this innovative ingredient for the local industry. The second chapter (item 4) focused on developing a methodology to quantify two protein fractions in whey, since understanding the protein denaturation rate is essential for the stability of MPs. For this study, proteins provided by a partner company were used, both before and after microparticulation, at different concentration levels, with and without spray drying. Another emerging demand is related to lactose-free products, especially in countries like Brazil, where the rate of lactose intolerance is relatively high. Therefore, a study (item 5) was conducted to assess whether replacing lactose with glucose and galactose (products of lactose hydrolysis) would alter the characteristics of the proteins and the Maillard reaction. It was found that the presence of carbohydrates in the microparticulation process is more important than the specific type used during production. This study was carried out during a sandwich Ph.D. program in collaboration with Technische Universität Dresden. Finally, the last chapter of this thesis (item 6) aimed to develop an innovative ingredient: hybrid microparticulated protein. These hybrid proteins are produced by combining whey protein with a plant-based protein—in this case, pea protein. To develop this ingredient, two types of acids were tested at three different pH values. It was concluded that the type of acid does not significantly affect the final product; however, the manufacturing pH is crucial for controlling aggregate size and the degree of additional protein denaturation. This final chapter is in the process of

being submitted for patent protection, as it presents a novel product with great potential impact on both the national and international food industries. Thus, these four chapters compose the present thesis and are aligned with the MAI/DAI program from CNPq, which aims to strengthen the ties between the private sector (industry) and the scientific community (universities), fostering stronger partnerships between these two sectors.

Keywords: Microparticulation; whey protein; pea protein; particle size; protein denaturation

RESUMO

O aumento da incidência de sobrepeso e de obesidade faz com que a indústria de alimentos busque alternativas para desenvolver produtos com baixa concentração de calorias. O macronutriente que apresenta maior concentração calórica é a gordura, entretanto sua retirada não é trivial. A gordura é responsável por garantir propriedades organolépticas e sensoriais. A proteína é uma alternativa de substituição, porém sua alta taxa de desnaturação térmica dificulta sua aplicação, com isso a indústria de alimentos vem buscando alternativas para contornar esse problema. A proteína microparticuladas (MP) é uma combinação de proteína nativa e desnaturada que passa por um tratamento térmico seguido de cisalhamento que aumenta a estabilidade térmica da proteína ainda sendo capaz de gerar características próximas aos da gordura. Desenvolvida para ser aplicada como substituto de gordura pela CP Kelco, as proteínas microparticuladas já são utilizadas em diferentes produtos, como por exemplo, sorvete, queijos, molhos para salada e iogurtes com diferentes funcionalidades. O primeiro capítulo (item 3) deste trabalho visou buscar informações sobre a produção, funcionalidade e aplicabilidade das MPs depositadas em patentes, gerando assim um acervo sobre esse ingrediente inovador para a indústria local. O segundo capítulo (item 4) buscou desenvolver uma metodologia para quantificação de duas frações proteicas do soro de leite, uma vez que, saber a taxa de desnaturação proteica é de suma importante na estabilidade das MPs. Para esse estudo, utilizou-se proteínas fornecidas por uma indústria parceira antes e após a microparticulação em diferentes fatores de concentração secas ou não por spray dryer. Outra demanda recente é sobre produtos zero lactose, principalmente em países como o Brasil, uma vez que a taxa de pessoas intolerante é relativamente alta, com isso foi realizado um estudo (item 5) onde avaliou se a substituição de lactose por glicose e galactose (produtos resultantes da hidrólise) modificaria características das proteínas e da reação de Maillard. Foi observado que a presença de carboidratos no processo de microparticulação é mais importante que o tipo de carboidrato utilizado na fabricação. Esse estudo foi realizado durante o doutorado sanduíche realizado em colaboração com a Technische Universität Dresden. Por fim, o último capítulo dessa tese (item 6) visou desenvolver um ingrediente inovador, a proteína microparticulada híbrida. As proteínas microparticuladas híbridas são produzidas através de uma mistura de proteína do soro de leite comum proteína vegetal, nesse caso proteína de ervilha. Para o desenvolvimento desse ingrediente foi testado dois ácidos em três diferentes valores de pH, e foi possível

concluir que o tipo de ácido não interfere no produto obtido, entretanto o pH de fabricação é fundamental para controlar os tamanhos dos agregados formados e a porcentagem de desnaturação proteica adicional. Esse último capítulo está em processo de depósito de patente por se tratar de um produto inovador e que pode gerar grande apreço pelo setor industrial nacional e global. Com isso, esses quatro capítulos compõem a tese e estão correlacionados com o edital MAI/DAI da CNPq que busca estreitar os laços entre o setor privado (indústria) com o setor científico (universidades) visando fortalecer as parcerias entre esses dois grupos.

Palavras-chave: Microparticulação; proteína do soro de leite; proteína de ervilha; tamanho de partícula; desnaturação proteica

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1 INTRODUCTION

With the growing demand for healthier habits and the problems associated with obesity and overweight, the food industry has been seeking alternatives to replace fat in foods without causing the loss of organoleptic characteristics. The main macronutrient that has been gaining attention is protein, as it offers excellent nutritional value. However, its low denaturation temperature can lead to issues such as phase separation and changes in flavour, for example.

Microparticulation emerges as an alternative for the use of proteins, since microparticulated proteins are no longer in their native form, as they are denatured, thus making phase separation of the product more difficult. Another feature is the possibility of using it as a fat substitute, as its particles can provide characteristics similar to fat.

Microparticulated whey protein is used in various products such as yogurt, ice cream, sauces, and cheese. However, the production of microparticulated proteins based on mixed proteins (whey protein and plant protein) is something innovative in the literature.

1.1 MICROPARTICULATION

Over the years, obesity and cardiovascular diseases have become public health problems worldwide, mainly associated with high fat consumption (Godoi, et al., 2021). Projections indicate that by 2030, the majority of the United States population will be overweight or obese. In Brazil, it is estimated that approximately 48% of the population is already in this condition. (Kew et al., 2020, World Obesity Atlas, 2025). Therefore, the demand for reduced-fat foods has been encouraged by government agencies and the food industry (Smirnova; Romanovskaya; Shtrigul, 2013).

Fat is the macronutrient with the highest caloric content per unit mass compared to proteins and carbohydrates (Chung; Degner; Mcclements, 2014; Suárez et al., 2016). Proteins emerge as the main substitute for fat due to their excellent nutritional value and distinct satiety effects compared to carbohydrates (Allahdad; Salmieri; Lacroix, 2023). However, fat removal in foods causes rheological and organoleptic changes, reducing consumer acceptance (Torres et al., 2018).

Microparticulated protein (MP) is an alternative fat substitute in foods (Sun et al., 2016; Shi et al., 2023). MPs are produced through heating and shearing, resulting in

protein denaturation with controlled aggregate size formation (Torres et al., 2018; Hossain et al., 2021). They are formed by mixed colloidal particles of native and denatured proteins (Torres et al., 2016a; Çakir-Fuller, 2015). The aggregates range in size from 0.1 to 100 µm, although aggregates larger than 20 µm may cause a sandy mouthfeel (Hossain et al., 2020; Toro-Sierra; Shumann; Kulozik, 2013).

This controlled protein denaturation leads to greater thermal stability and imparts different functionalities compared to native proteins, modulating emulsifying, gelling, creaminess, and water retention properties (Dissanayake; Liyanaarachchi; Vasiljevic, 2012; Toro-Sierra; Shumann; Kulozik, 2013).

Key factors modulating the size and shape of MPs include protein concentration, carbohydrate and salt content, solution pH, temperature, and shearing (Homer; Lundin; Dunstan, 2021; Tobin et al., 2010; Sánchez-Obando et al., 2020). Literature reports applications of MPs, especially whey protein-based MPs, in various products such as yogurt, ice cream, cheese, sauces and dressings, beverages, and mayonnaise (Hossain et al., 2020; Shi et al., 2023).

The preference for whey proteins over other proteins is due to their ability to form disulfide bonds, providing greater stability to aggregates (Zhang et al., 2020). Microparticulation of plant proteins has been studied in systems based on soy, pea, and potato proteins (Tanger; Ramos; Kulozik, 2021); however, microparticulation of hybrid proteins (whey protein combined with plant protein) has not been reported in the literature.

1.2 WHEY

Currently, the exact origin of whey is not known for certain. However, it is believed to have been discovered around 8,000 years ago. Other studies suggest that whey may have been discovered 3,000 years ago as a result of the enzymatic coagulation of milk, although it did not have any recognized value at the time (Tunick, 2009; Smithers, 2008).

In Brazil, the Ministry of Agriculture, Livestock and Food Supply (MAPA), through its Technical Regulation, establishes the standards of identity and quality (RTIQ) for whey. According to this regulation, whey is obtained from the coagulation of milk used in the production of cheese, food-grade casein, and similar products (Brazil, 2020).

Whey can be divided into two main types (sweet and acid), and its composition consists mostly of water, lactose, minerals, and whey proteins (Rocha-Mendoza et al., 2021). Sweet whey, which is the by-product obtained through enzymatic coagulation during the production of most cheeses, has a pH between 6.0 and 6.8, as well as a lower ash content (Table 1) and a higher protein concentration compared to acid whey (Rama et al., 2019; Wherry; Barbano; Drake, 2019). Acid whey, on the other hand, is obtained through acid coagulation, either by the action of bacteria or by the addition of acids such as lactic, acetic, citric, or hydrochloric acid (Ryan; Walsh, 2016).

Table 1 - Composition of sweet whey and acid whey (g L^{-1}).

Parameter	Sweet Whey	Acid Whey
pH	6.0 – 6.8	< 6.0
Protein	6.0 – 10.0	6.0 – 8.0
Lactose	46.0 – 52.0	44.0 – 46.0
Ash	2.5 – 4.7	4.3 – 7.2

Source: Adapted from Tunick (2009).

Raw whey, although used for animal feed and as an ingredient in the production of other foods, has a rapid spoilage rate due to the fast proliferation of microorganisms (Panesar et al., 2007). As a result, whey processing is employed to improve the product's added value and shelf life, with whey powder being its main form (Kumar et al., 2023).

Another way to utilize whey is by fractionating its components. Whey proteins in powdered form are commonly used in a wide range of applications in the food industry due to their high biological value and technological properties (Yadav et al., 2015).

1.2.1 Whey Protein

The protein composition of whey is quite diverse. Table 2 shows the concentration, molecular mass, isoelectric point, and denaturation temperature of each protein fraction.

Table 2 - Protein composition and characteristics of whey proteins.

Protein	Concentration (% m m^{-1})	Molar Mass (kDa)	Isoelectric point	Denaturation temperature (°C)
β-lactoglobulin (β-lg)	48 – 58	18.3	5.2	75
α-lactoalbumin (α-la)	13 – 19	14.2	4.4	30 – 60
Caseinomacropeptides (CMP)	12 – 20	8.6	<4.1	-
Immunoglobulin (IGs)	8-12	150.0	5.1	-
Bovine serum albumin (BSA)	6	66.4	4.9	60
Lactoferrin (LF)	2	83.0	8.6 – 8.9	62 – 90

Source: Adapted from Bonnaillie; Tomasula (2008); Tavares (2020).

1.2.1.1 β-Lactoglobulin (β-lg)

The main whey protein is β-lactoglobulin, accounting for 48% to 58% of the total protein content. It has a molar mass of 18.3 kDa and contains 162 amino acids in its structure. It is considered an acidic protein due to its isoelectric point of approximately 5.2 and is one of the most heat-stable whey proteins, with a denaturation temperature of 75 °C (Bonnaillie; Tomasula, 2008; De Wit, 1990). β-lg is not found in the milk of certain species, such as camels and humans, and it is associated with cases of cow's milk allergy. The main function of β-lactoglobulin is the transport of nutrients to offspring (Ho; Zou; Bansal, 2022; Sawyer, 2021).

β-lg has more than 10 genetic variants; however, variants A and B are the most common. Amino acids at positions 64 and 118 are responsible for their differences: at position 64, aspartic acid and glycine are found in alleles A and B, respectively, while at position 118, valine and alanine are present (A and B). Additionally, β-lg A is more hydrophobic than β-lg B and can bind small hydrophobic molecules due to the presence of an internal cavity (Fei et al., 2020, Thompson; Boland; Singh, 2009).

It has five cysteine residues, four of which form disulfide bonds that enhance its stability. The free thiol group remains buried within its native tertiary structure, but when this group is exposed, it can form bonds with other molecules, leading to protein denaturation (Croguennec et al., 2014; Yan et al., 2013). Finally, another interesting feature is the modulation of its interactions depending on pH. Between pH values of 3.5

and 7.5, β-lg is predominantly in dimeric form, whereas outside this range, the monomeric form predominates (Tavares, 2020).

1.2.1.2 α-Lactalbumin (α-la)

α-Lactalbumin is the second most abundant whey protein, accounting for 13% to 19% of the protein content. It has a lower molar mass than β-lg, at 14.2 kDa, with a total of 123 amino acids. Like β-lg, it is considered an acidic protein, with an isoelectric point of 4.4, and its thermal denaturation temperature ranges from 30 °C to 60 °C (Bonnaillie; Tomasula, 2008; Golebiowski et al., 2022). α-la is found in the milk of all mammals and is the predominant protein in human milk, produced by the secretory cells of the mammary glands. Its main function is in lactose biosynthesis (Ge et al., 2024).

α-la contains a site that allows binding to calcium ions; the bound form (holo) exhibits greater thermal stability than the unbound form (apo) (Permyakov, 2020). This protein also contains eight cysteine residues that form four disulfide bonds and no free thiol groups, enhancing its stability (Ge et al., 2024).

1.2.1.3 Caseinomacropeptides (CMP)

Caseinomacropeptide is a peptide found exclusively in sweet whey, with a concentration ranging from 12% to 20%. It results from the cleavage of the 105-106 bond of κ-casein by the action of chymosin. CMP is composed of 64 amino acids, has a molar mass of 8.6 kDa, and an isoelectric point below 4.1 (Bonnaillie; Tomasula, 2008; Thomä-Worringer; Sorensen; López-Fandiño, 2006).

CMP presents several variants, although two are most reported in the literature, mainly differing at positions 136 and 148, variant A contains threonine and aspartic acid, while variant B contains isoleucine and valine, respectively (Farrekk et al., 2004). Additionally, CMP can be phosphorylated and glycosylated at different positions, with about half of CMP molecules undergoing these structural modifications (Thomä-Worringer; Sorensen; López-Fandiño, 2006).

1.2.1.4 Immunoglobulins (IGs)

This group of proteins consists of polymers of polypeptide chains linked by disulfide bonds, with the predominant subgroup in bovine milk being IgG, especially IgG1 and IgG2, which are complexes with an average size of 150 kDa (Thompson; Boland; Singh, 2009). Immunoglobulins are antibodies associated with action against pathogenic microorganisms in the gastrointestinal tract and reach the milk through secretory cells after passing through the bloodstream (Ulfman et al., 2018; Hurley; Theil, 2011).

1.2.1.5 Bovine Serum Albumin (BSA)

Bovine serum albumin accounts for about 6% of the total whey protein concentration. It has a molecular mass of 66.4 kDa and consists of 582 amino acids. Like other whey proteins, BSA is also acidic, with an isoelectric point of 4.9 and a denaturation temperature of 60 °C (Bonnaillie; Tomasula, 2008; Topala et al., 2014). This protein is predominant in blood and reaches the milk via the secretory cells of the mammary glands. Its main function is to carry hydrophobic molecules and ions (Kajal; Pathania, 2021).

BSA is found in a monomeric form and contains 17 disulfide bonds in its structure, as well as a free thiol group that allows bonding with other molecules in the medium (Kajal; Pathania, 2021).

1.2.1.6 Lactoferrin (LF)

Lactoferrin concentration in whey is low, approximately 2% of the total protein content. It has a molecular mass of 83 kDa and consists of 689 amino acids. LF is the only basic whey protein, with an isoelectric point ranging from 8.6 to 8.9 and a denaturation temperature varying between 62 °C and 90 °C (Bonnaillie; Tomasula, 2008; Goulding et al., 2021). LF exhibits antimicrobial, immunomodulatory, and anticarcinogenic activities and can increase iron bioavailability. It is produced by the secretory cells of the mammary glands (García-Montoya et al., 2012).

This protein exists as a monomer at low ionic strength; however, with increasing ionic strength, it undergoes dimerization or aggregation (Persson et al., 2010). The apo

form is more stable than the holo form, and all cysteine residues are involved in disulfide bonds (Bokkhim et al., 2013).

1.2.2 Lactose

Lactose is the carbohydrate found in the milk of all species and in the vast majority of dairy products. It is formed by the union of one glucose molecule and one galactose molecule and is classified as a disaccharide (Neves; de Oliveira, 2020; Damodaran; Parkin, 2017). Sweet whey contains lactose concentrations between 46.0 g $100g^{-1}$ and 52.0 g $100g^{-1}$, whereas acid whey has lower values, ranging from 44.0 g $100g^{-1}$ to 46 g 100 g^{-1} (Tunick, 2009).

1.2.2.1 Lactose Intolerance

Currently, it is estimated that around 70% of the global adult population presents some degree of lactose intolerance, with higher prevalence among individuals from South America, Africa, and Asia (Francisquini et al., 2019; Santos et al., 2018). In contrast, in European countries this condition is significantly less common—for example, in the Netherlands, where only about 1% of the population is lactose intolerant (Dekker; Koenders; Bruins, 2019).

Lactose intolerance is characterized by a reduced or absent ability to digest this sugar, due to low lactase enzyme activity in the small intestine (Fialho et al., 2018; Suri et al., 2019; Torres et al., 2017). Various factors can influence lactase presence and activity in the body, such as age, ethnicity, integrity of the intestinal mucosa, and intestinal transit time (Oak; Jha, 2019).

The main symptoms of lactose intolerance include abdominal distension and pain, flatulence, and diarrhea. These symptoms result from the fermentation of undigested lactose by intestinal bacteria, leading to the production of gases such as methane, carbon dioxide, and hydrogen, as well as volatile fatty acids—such as acetic, butyric, and propionic acids—that acidify the intestinal environment, lowering the pH to values below 5.5 (Gasparin; Teles; Araújo, 2010; Torres et al., 2016b).

1.2.2.2 Lactose Hydrolysis

With the significant increase in people diagnosed with some degree of lactose intolerance, the food industry has sought technological alternatives to reduce the presence of this disaccharide in its products. Two main approaches are used for lactose hydrolysis: the chemical pathway and the enzymatic pathway (de Almeida et al., 2015).

Although chemically feasible from a technical standpoint, chemical hydrolysis is rarely applied in the food industry because it requires extreme processing conditions, such as pH below 1.5 and temperatures near 150 °C. These harsh parameters may compromise the nutritional and sensory quality of the final product (Gänzle; Haase; Jelen, 2008).

In contrast, enzymatic hydrolysis stands out as the main industrial choice due to its efficiency and milder operational conditions. Currently, two commercial forms of lactase (β-galactosidase) are used: acidic lactase, more common in dietary supplements for individual use, and neutral lactase, widely employed in industrial processes (Dekker; Koenders; Bruins, 2019).

The enzyme lactase acts by breaking the β-glycosidic bonds of lactose, resulting in the formation of the monosaccharides glucose and galactose (Equation 1), which are easily absorbed by the small intestine (Fialho et al., 2018; Klein; de Jong; Révillion, 2010). However, the presence of these simple sugars after hydrolysis may promote undesirable reactions in the final product, such as the Maillard reaction, since glucose and galactose react with lysine more rapidly than lactose itself, accelerating protein deterioration (Naranjo et al., 2013).

$$lactose \xrightarrow{lactase} D-glucose + D-galactose \quad (1)$$

1.3 PEA

Among legumes, the pea is considered the second most cultivated crop across the continents of Europe, North America, and Asia, due to its strong adaptability, high nutritional value, and excellent economic importance (Robinson; Domoney, 2021). Regarding nutritional value, peas are rich in proteins, dietary fibers, fatty acids, trace

elements, and phenolic compounds (Chen et al., 2023). Among these compounds, pea protein stands out due to its low allergenicity and transgenic safety (Zhang et al., 2023).

Currently, two methods of extracting pea proteins are applied: the first is alkaline extraction followed by isoelectric precipitation, and the second is saline extraction followed by micellar precipitation (Tanger; Mertens; Kulozik, 2022).

A disadvantage of pea protein is its low solubility and limited techno-functional properties, which require modifications via enzymatic treatment, pH adjustment, heating, and high-pressure homogenization to overcome these issues (Yerramilli; Longmore; Ghosh, 2017; Yang et al., 2022).

Another drawback of pea protein is the deficiency in essential amino acids, which has led to the growing use of mixed proteins (both plant- and animal-based) worldwide (Ma et al., 2024; Sim; Moraru, 2020). The combination of proteins can help to overcome the deficiency of this macronutrient found in plant proteins (Zhang et al., 2023).

1.3.1 Pea Protein

The protein composition of pea can be seen in Table 3, divided into four groups: globulin, albumin, prolamin, and glutelin (Lu et al., 2020). A brief discussion of each of these groups follows.

Table 3 - Protein composition and characteristics of pea proteins

Protein	Concentration (% m m^{-1})	Protein	Molar mass (kDa)
Globulin	55 – 65	Legumin	320 – 410
		Vicilin	150
		Convicilin	180- 210
Albumin	18 – 25	Albumin	68.5
Prolamin	4 – 5	Prolamin	n/a
Glutelin	3 – 4	Glutelin	n/a

Source: Adapted from (Lu et al., 2020).

1.3.1.1 Globulin

Globulin is the major group of pea proteins, with an average concentration ranging from 55% to 65%. It is divided into three proteins: legumin, vicilin, and convicilin, which are soluble in saline solution (Zhang et al., 2021; Yang et al., 2022). Legumin is considered a hexameric protein with a molecular mass ranging from 320 to 410 kDa, composed of acidic and basic subunits linked by disulfide bonds (Mertens et al., 2012).

Vicilin consists of trimeric structures with a molecular mass close to 150 kDa, stabilized by hydrophobic interactions (Gatehouse et al., 1982; Sikorski, 2001). Additionally, it has a low concentration of sulfur-containing amino acids and tryptophan, and high levels of basic and acidic amino acids (Lam et al., 2018). Finally, convicilin is composed of trimers with a molecular mass ranging from 180 to 210 kDa,and is rich in sulfur-containing amino acids (Tzitzikas et al., 2006).

1.3.1.2 Albumin

The albumin class consists of water-soluble proteins, with a concentration ranging from 18% to 25% of the total protein content (Lu et al., 2020). This group includes some protease inhibitors, amylase inhibitors, and lectins (Boye; Zare; Pletch, 2010). Another important function of albumins is in the germination process of pea seeds (McCarthy et al., 2016). The cysteine content in albumins is relatively high, ranging from 7.5% to 16.2%, depending on the variant (Eyraud et al., 2013).

1.3.1.3 Prolamin

Prolamin is considered a group of storage proteins in plants, and its concentration in pea is low, ranging from 4% to 5% (Adebiyi; Aluko, 2011). The two main amino acids found in its composition are glutamine and proline (Lu, 2020).

1.3.1.4 Glutelin

Glutelin is an insoluble protein found in the endosperm of pea seeds, present at low concentrations (3% – 4%). It is soluble in dilute acids and bases, reducing agents,

and detergents. Additionally, it is rich in hydrophobic amino acids such as tyrosine, proline, phenylalanine, and valine (Lu, 2020).

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2 GOAL

The main goal of this work was to develop technical-scientific tools regarding microparticulated proteins, aiming to gain a better understanding of the manufacturing steps and to provide support to the Brazilian food industry in comprehending this ingredient. A review article was prepared to compile information from patents previously filed on the topic. Additionally, a method was developed to quantify whey protein fractions using HPLC. The study also investigated the influence of different carbohydrates on the production process of these microparticulated whey proteins. Finally, a production protocol was applied to develop hybrid MPs based on whey protein combined with pea protein at three different pH values, employing two types of acids during their manufacture.

2.1 SPECIFIC GOALS

- Preparation of a review article gathering information on the development of microparticulated proteins in patents;
- Development of a method for quantifying whey protein fractions by HPLC;
- Development of a manufacturing protocol for microparticulated proteins based on whey protein;
- Study of the influence of different carbohydrates on the production of microparticulated proteins;
- Study of the influence of different pH values and different acids on the microparticulation process;
- Application of the microparticulation protocol in the production of hybrid proteins bad on pea protein and whey protein.

3 THE APPLICABILITY OF MICROPARTICULATED WHEY PROTEIN AS AN IN INGREDIENT IN DIFFERENT TYPES OF FOODS AND ITS FUNCTIONALITIES: A CURRENT PATENT REVIEW

This chapter has already been published as an article in the journal European Food Research and Technology whose DOI corresponds to https://doi.org/10.1007/s00217-023-04402-x.

3.1 INTRODUCTION AND AIM

Whey is the main by-product of cheese production, and its composition is given by proteins, lactose, minerals, vitamins, lipids, and water. It is considered a food with significant nutritional and aggregate value, making it a product that has attracted the interest of the dairy and food industries (Masotti et al, 2017; Price, 2018). To make whey an even more valuable food, the researchers at Singer, Yamamoto, and Latella developed the landmark US patent application US4734287A in 1988, which gave rise to the so-called microparticulated whey protein (MWP). According to the patent definition, MWP are particles with a diameter of 0.1–2.0 μm, in which 2% of the proteins can reach a size of 3.0 μm, by subjecting whey to a high shear rate in an aqueous medium at high pH and temperature (Ipsen, 2017; Stankey et al., 2017).

When hydrated, MWP particles have organoleptic properties similar to fats in the emulsified form (Singer et al., 1990). However, given the tendency of the world's population towards obesity and the consequent search by consumers for low-calorie products without altering their organoleptic characteristics, the food industry is continuously directing studies on the use of MWP in different foods, resulting in dozens of new patents (Chudy et al., 2021; Wang et al., 2021).

In addition, with the development of MWP studies, many of its applications in foods have been used to improve their organoleptic and rheological properties (texture, color, flavor, and aroma), to increase its stability and the protein content, as well as, to decrease its fat (Kew et al., 2020). Due to the innovative property of MWP, several patents have been applied with a wide variety of applications. In this way, the search for scientific knowledge in patents becomes essential, as they spread knowledge about new products, processes, and methods. Therefore, the present study aims to review patents application

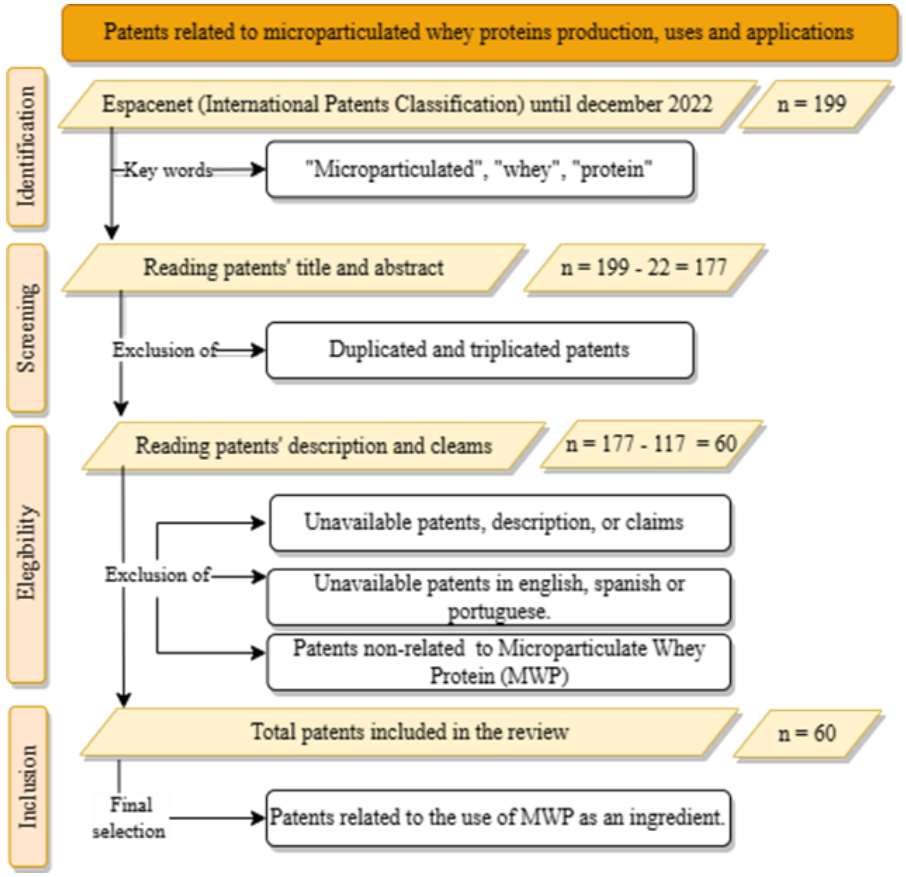
that used MWP as an ingredient and that described its functionalities, such as composition, stability, and organoleptic modifiers in foods.

3.2 METHODOLOGY

The patent search was carried out in the Espacenet database using the International Patent Classification (IPC) system. In the patent search, the keywords “whey”, “protein”, and “microparticulated” were applied to all text fields and titles of patents published until December 2022. Patents with duplicate titles and the same authors were excluded from the search (n = 22) and only the most recent version was retained. Patents that were unavailable (n = 5) and that could not be translated to English, Spanish, or Portuguese (n = 3) were also excluded.

As an eligibility criterion, patents that described MWP as an ingredient in different types of food were selected. This resulted in a total of 60 patents that were analyzed in terms of applicability and functionality of MWP (Figure 1).

Figure 1 - Research methodology flowchart.



Source: prepared by the author.

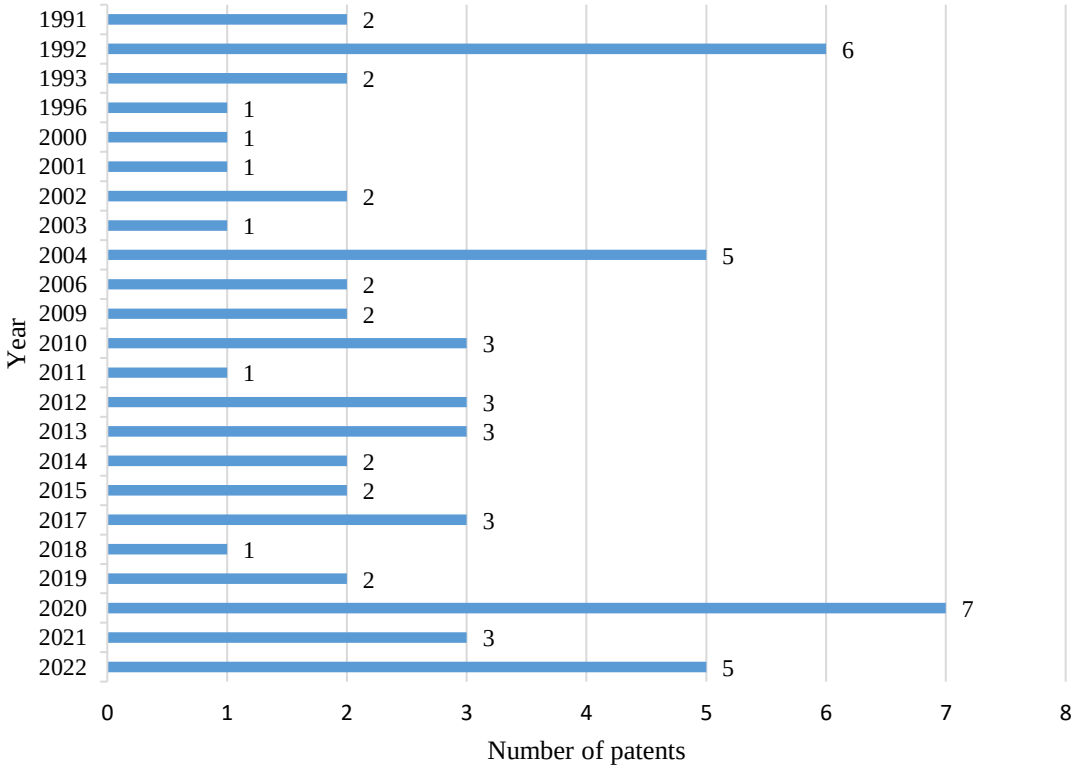
3.3 RESULTS AND DISCUSSION

The information derived from the patents utilized in this study will be discussed below.

3.3.1 Patent applications per year

Following the emergence of technologies to produce MWP in the size range 0.1–2.0 μm, with less than 2% of the total particles exceeding 3.0 μm in diameter, as proposed by Singer, Yamamoto and Latella in 1988, several patents have been filed on the same subject. Figure 2 shows that, on average, 2 patents were published per year. However, in 1992, six patent applications were submitted by Labatt LTD, presenting a new methodology to produce MWPs, followed by applications of these proteins in several food products, such as salad dressing (US5102681A, US5139811A), sour cream (US5096730A), and yoghurt (US5096731A).

Figure 2 - Number of MWP patents applications per year.



Source: prepared by the author.

Five patent applications filed in 2004 can be correlated with the “pioneer” patent expiration, which may have led to an increase in the number of MWP-related patent applications per year. In addition, the increase in patent applications in 2020 can be linked to the updates made by the FDA on the information to be provided on the nutrition labels of packaged foods. This new labelling system is a result of the increase in chronic diseases around the world linked to consumers' poor eating habits. With this label update, manufacturers with annual sales of $10 million or more were required to update their labels by 1 January 2020 (Food And Drug Administration, 2023). As a result, nine patents were filed in 2020, four of which were filed by Arla Foods Amba (Denmark), one of the leading companies in the MWP market with whey ingredients, such as Lacprodan Hydro and Lacprodan ISO Water (Nutraceutical Business Review, 2023).

3.3.2 Patent applications by country

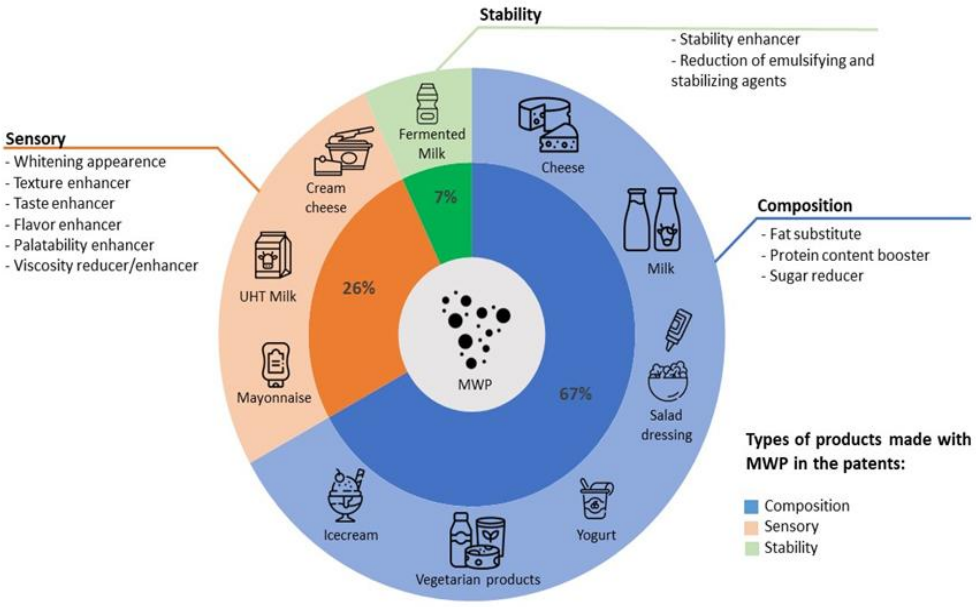
A total of 60 patents were selected, of which 32 were filed in the United States. Countries, such as China, Singapore, Australia, Ireland, Japan, Canada, and Russia, filed less than five patents each. There were also seven patents filed throughout Europe and six patents filed throughout the world.

3.3.3 Functionalities of MWPs

MWP has a wide range of technological properties when incorporated into foods. Its addition as a fat replacer or protein enhancer is very common, mainly due to its versatility as an ingredient, being able to be used in foods whose processing technologies are well known, such as cheese, yoghurts, and other dairy products, but it has also been shown to be included in products with other processing technologies, such as food colouring particles, foaming agents, and instant drinks.

Among the patents reviewed, many of them attributed food technological functionalities to MWP, as composition modifiers, sensory, and/or as stabilizers agents (Figure 3). There was a great interest in including them in general food types, such as sauces, bakery, aerated products, soups, beverages, and even in animal feed, but their inclusion in dairy products was the most striking, with almost all the three functionalities mentioned attributed to MWP.

Figure 3 - Correlation of microparticulated whey protein (MWP) fuctionalities (composition, sensory, and stability) within the different food products proposed in reviewed patents.



Source: prepared by the author.

3.3.4 Composition

Of the patents using MWP to modify the composition of foods, 6 were aimed at increasing protein concentration and 12 were aimed at fat replacement (Table 4). Among food proteins, whey proteins have gained prominence in recent years due to their high nutritional value and functional properties. In addition, studies have shown that these proteins can be used to combat obesity (Pal et al., 2019; Sun et al., 2015). The high consumption of fat in the daily diet of the world's population has led to a worsening of diseases, such as obesity, atherosclerosis, and even certain types of cancer (Melnikova; Stanislavskaya; Korotkov 2015; Tobin et al., 2010). As a result, the world's population is seeking healthier habits and reduced-fat foods (Torres et al., 2018).

Table 4 - Patents that used MWP as an ingredient, describing one product functionality as composition, stability or sensory.

	Year	Country	Publication number	Products made with MWP in the patent	Functionality	Reference
Composition	1992	USA	US5098728A	Food in general	Fat substitute	Singer et al., 1990
	1992	USA	US5102681A	Salad dressing	Fat substitute	Singer; Latella; Yamamoto, 1992d
	1992	USA	US5096730A	Sour cream	Fat substitute	Singer; Latella; Yamamoto, 1992b
	1992	USA	US5096731A	Yogurt	Fat substitute	Singer; Latella; Yamamoto, 1992c
	1992	USA	US5139811A	Salad dressing	Fat substitute	Singer; Latella; Yamamoto, 1992a
	1992	USA	US5158798A	Desserts	Fat substitute	Fung; Miller; Wuesthoff, 1992
	2000	USA	US6113953A	Pressed cheese	Fat substitute	McMahon; Oberg, 2000
	2004	USA	US2004156979A1 US7018668B2	Dairy and non-dairy cream and drink	Fat substitute	Oldrup, 2006; Villagran; Baughman, 2004
	2006	WIPO	WO2006058538A1	Drinks	Fat substitute	Villagran; Baughman, 2004
	2010	USA	US2010092608A1 US8486476B2	Yogurt and cheese	Fat substitute	Gutknecht; Ovitt, 2006
	2011	USA	US2011046048A1 US8815797B2	Drinks	Protein content booster	Minor; Weel; Hotrum, 2011
	2012	USA	US2012034337A1	Dairy products	Fat substitute	Venema et al., 2012
	2014	WIPO	WO2014140023A1	Food in general	Fat substitute	Auty; Abhyankar; Wang, 2014
	2019	Canada	CA3088565A1	Concentrated MWP	Protein content booster	Badayev; Antoshyn, 2019

	2019	European Patent	EP3542635A1 EP3542635B1	Yogurt	Protein content booster	Jensen; Hansen, 2019
	2020	USA	US10729150B2 US2016262412A1	Acidified fruit drinks and vegetable yogurt	Protein content booster	Pedersen; Tingleff, 2016
	2020	USA	US10834934B2	Acidified drinks and yogurt	Protein content booster	Mikkelsen et al., 2020b
	2022	WIPO	WO2022210231A1	MWP	Protein content booster	Hirofumi; Mayumi; Atsuhiro, 2020
Stability	2020	USA	US2020268645A1	Dyes	Stability enhancer	Firoozmand; Hartel, 2020
	2021	Japan	JP2021035332A	Fermented dairy product	Stability enhancer	Takemoto; Asada, 2021
Sensory	1993	WIPO	WO9325083A1	Food in general	Whitening appearance and taste enhancer	Selinger; Laaman, 1993
	2002	European patent	EP1051081A2 EP1051081B1	Mayonnaise, instant tea, cream tea.	Palatability enhancer	Vilagran et al., 2000
	2004	USA	US6673384B1	MWP	Palatability enhancer	Villagran; Baughman, 2004
	2009	European Patent	EP2111117A1 EP2111117B1	UHT milk	Taste enhancer	Dyck, 2009
	2009	European patent	EP2094099A1 EP2094099B1	Instant drinks	Aroma and taste enhancer	Zeller et al., 2009
	2013	USA	US2013273202A1 US9775366B2	Cream cheese	Flavor and texture enhancer	Wolfschoon-Pombo et al., 2013
	2022	USA	WO2022157611A2	Cream cheese	Flavor and texture enhancer	Hirofumi; Mayumi; Atsuhiro, 2020

Source: prepared by the author.

Proteins are considered good candidates to replace fat in foods as they promote satiety and have a low caloric content, 4 kcal g^{-1} instead of 9 kcal g^{-1} for lipids (Chung; Degner; Mcclements, 2014). However, formulations with high protein content may have some limitations, such as instability during production or shelf life, as heat treatment in these foods can cause denaturation of proteins, leading to phase separation (Çakir-Fuller, 2015). With microparticle technology, it is possible to circumvent the problem caused by the lack of stability in these products, as the aggregation of these proteins is carried out in a controlled manner, ensuring the use of higher concentrations of proteins in foods without loss of product properties (Dissanayake; Kelly; Vasiljevic, 2010).

In the study conducted by Çakir-Fuller (2015), the addition of high levels of MWP in emulsions was tested to observe whether these proteins could be used in higher concentrations compared to whey proteins. The results of the study showed that MWP presented greater heat stability at higher concentrations compared to whey protein concentrates (WPC), suggesting that this type of protein can be used in beverages with increased protein value. Another study showed that the MWP produced by the superfine milling technique had the potential to increase protein concentration in emulsions, as they showed greater stability compared to those made with WPC (Sun et al., 2015; Sun et al., 2016).

Patent EP3542635A1 used MWPs to increase the protein content of yoghurt. This patent disclosed a combination of whey proteins, one part of which had particle sizes in the range 1 to 10 μm and another part obtained from acid gelled whey protein aggregates. The patent described the production process of MWP at a pH varying between 5 and 8, under shear rate, thermal treatment, and addition of calcium salts. The inventors found that yoghurts made with the developed proteins had better organoleptic properties than yoghurts made with non-microparticulated whey proteins. A similar invention was also proposed by US2011046048A1 to obtain MWPs that can be added or not to other ingredients to ensure a better result in the microparticulation process and to be used in the production of enteral nutrition.

Patent CA3088565A1 demonstrated a method to produce MWPs using a continuous-flow high shear reactor with simultaneous thermal and mechanical treatment. Proponents of the patent report that the particles produced by this equipment can be micrometric and nanometric in size and can be used in various dairy and non-dairy products to increase the protein content of these foods.

Regarding the use of MWP as a fat substitute, it was found that yoghurts with MWP added had whey protein in relation to denatured protein, providing products with low-fat content and viscosity and creaminess close to those of conventional yoghurt (Torres et al., 2018). Another product produced using MWP as a fat substitute was Petit-Suisse cheese, in which the authors reported that all reduced-fat products had rheological characteristics close to the control product. Therefore, in addition to guaranteeing the same quality of cheese, it was possible to increase the nutritional value of this type of food, making it more attractive to the consumers (Sánchez-Obando et al., 2020).

Patents US5102681A and US5139811A were developed by Norman, Joseph, and Shoji, in which they demonstrated the production process of these proteins and their use in partially or totally fat-reduced salad dressings. The MPWs were prepared using acidified whey with a protein content between 50 % and 55% on a dry basis and the mixture had a total solids' content of 37%. The pH was adjusted to 4.4 ± 0.05 and then the product was transferred to a shear rate system to obtain the microparticulated proteins. The patents also described a similar methodology for production using whey. As an application, the developers described a method for producing low-fat salad dressing using the previously obtained MWP. The same developers of the above patents also used MPWs produced as fat replacers in foods in general (US5098728A), sour cream (US5096730A), and yoghurt (US5096731A).

Patent US2010092608A1 is another example of the use of MWPs as fat mimetics in food. The proteins in this patent were used in yoghurt cheese. The patent uses CPKelco's, commercially available as Simplesse® 100 microparticulated protein, with approximately 53.2% protein by weight for product development. The resulting product did not present syneresis after 15 h at approximately 23 °C.

Patent US2012034337A1 demonstrated the method of producing protein particles, which may or may not contain oil, for use as a fat substitute in dairy products. The inventors reported that the preferred size of these particles was 1–10 μm, in which these particles had an external structure like fat globules, thus imparting a fat-like mouthfeel. The inventors also reported that these products were helpful in reducing calorie intake in a diet.

3.3.5 Stability

Patent US2020268645A1 claimed a dye or pigment useful for food compositions or food ingredients in which MWP was included as an insoluble dispersant (Firoozmand; Hartel, 2020). The dye is adsorbed in MWP as a safer alternative to metal salt lake substrates, which are commonly used despite their aluminium content and possible effects to cause Alzheimer's disease (Yokel, 2012). In that patent, MWP was used as a stabilizer (Table 4) and was a harmless insoluble dispersant of food colors, due to its ability to interact with the pigment and its stability in aqueous medium.

In addition to nutritional properties, some studies have found a positive effect on emulsification properties, foam stability, and gelling ability when MWP is included, even when MWP acts as an inert filler, as in cheese, or when it interacts with the rest of the matrix, as in fermented milk (Dissanayake; Kelly; Vasiljevic, 2010). For example, in patent JP2021035332A filed by Megmilk Snow Brand Co., Ltd., a novel whey protein material was developed that suppresses water separation and imparts hardness to fermented milk and yoghurts.

3.3.6 Sensory

In addition to their functionality, such as reducing fat and increasing protein content, MWP can show improvements in the sensory characteristics of the final product, such as improve texture, flavour, viscosity with increased water retention, creamy mouthfeel, foam formation and preservation of organoleptic characteristics even after thermal treatments such as ultra-high temperature (UHT) (Hossain et al., 2020; Li et al., 2021). In the present article, 32 patents used MWP as an ingredient to improve or modify sensory properties of the products.

Some studies reported the reduction of fat content in foods as a practice due to the worsening of cardiovascular diseases and obesity (Wang et al., 2021). In the microstructure of low-fat stirred yoghurt produced with MWP, it was observed that the reduction or elimination of fat, through changes in formulation and control of parameters during the process, resulted in a modification of the microstructure of the gel, which was reflected in the texture and sensory perception of this product. Some of these defects were rough texture, low viscosity due to low total solid content, undesirable mouthfeel, and severe whey syneresis (Torres et al., 2018; Fang, Guo, 2019).

However, due to its good functional properties, MWP has been used as a fat substitute and texture enhancer in yogurt and other products. In low-fat yoghurt, the use of a whey protein concentrates and MWP resulted in increased firmness and similar textural properties compared to full-fat yoghurt (Li et al., 2021). Another product in which the use of these proteins has shown benefits in terms of sensory attributes was cheese. The addition of three commercially available MWPs (Simplesse®, DairyLo®, and Protelo®) resulted in reduced firmness, improved sensory acceptability, and restoration of proteolysis and opacity (El-Aidie et al., 2020).

Another advantage of MWP was the preservation of organoleptic characteristics even after UHT treatment. The high processing temperatures required to give products a long shelf life, commonly destabilizes whey protein, causing the protein to clump together, which in turn gives the UHT product an undesirable granular texture (Çakir-Fuller, 2015).

In the patent EP1051081A2, it was addressed the need for a creamy mouthfeel agent for ready-to-eat/drink foods and beverages, such as instant drinks, mayonnaise, salad dressings, and puddings. Such an agent comprised as a mixture of a water-soluble component and a water insoluble component. The insoluble component comprised particles with an average diameter of 0.1–3.0 μm, 70% of which is a microparticulate component. The microparticles were substantially spheroidal in shape and had fat-like mouthfeel characteristics. The particles were not aggregated and exhibited the substantially smooth organoleptic character of an oil-in-water emulsion. A similar agent was also presented in patent US6673384B1, in which the microparticulate component include Simplesse®100 and DairyLo®, both derived from whey protein.

The Patent EP2111117A1, entitled “Method and device for improving the taste of long-life milk”, presented a process to improve the flavour of long-life milk, since UHT milk is characterized by a flavour so-called cooked, which the consumer does not seems to like. Another fact is that the practice of reducing the milk fat content, which ultimately has a negative effect on the taste of this product, since fat has a positive effect on the body and the sensation in the mouth. The authors have therefore developed a method that allows the fat content to be reduced while maintaining a taste comparable to that of pasteurized milk. This method consists of using microparticulate proteins with a degree of denaturation of 80% and a particle-size distribution of 1–10 μm, which causes a reduction in the cooking flavor of UHT milk, simulating a full-bodied mouthfeel, with the consequence that fat can be replaced, and whiteness increased.

MWP showed another interesting sensory property as an aroma release enhancer, patent EP2094099A1, which claimed a method for the preparation of instant beverages in which the usual processing conditions damage the volatile key compounds responsible for the desired aroma. The MWP was mentioned to act as a non-fat foaming agent and to reduce residual flavour, even with increased foam thickness.

The patent WO9325083A1, applied by FMC Corporation MWP, also described MPW as a protein opacifying material for food products. It was suggested the use of MWP as a whitening agent, replacing inorganic additives such as titanium dioxide, and allowing greater consumer acceptance, because it comes from a natural source.

However, there are still challenges in the use of MWP in many matrices. Ipsen (2017) showed the importance of the type of microparticulated protein ingredient (MWP or non-microparticulated whey protein), which plays a significant role when it comes to fermented dairy products. Among these, the size of MWP fat replacer particles would be larger than milk fat globules, resulting in higher phase separation and lower firmness in low-fat yoghurts containing MWP. Surface reactivity was another important aspect in the perception of graininess, as it makes MWP a similar part of the yoghurt matrix during the fermentation process. Water-holding capacity also showed differences depending on the particle size and native protein percentage, promoting effective interaction with other proteins, and giving stronger gels (Celigueta et al., 2016). As a strategy, the combination of MWP with polysaccharides as fibers was proposed to improve and mimic emulsions of full-fat foods in reduced-calorie counter parts (Chung; Degner; Mcclements, 2014).

Apart from the above, there was an important aspect that influenced the final perception of MWP in a food product, which is the identification of the best moment to add MWP as an ingredient, taking into account all the subsequent process steps it should be exposed to, for example, when added before fermentation (Ipsen, 2017; Torres et al., 2018).

3.3.7 Patents using MWP for more than one functionality

In addition to individual functionalities, MWP is commonly added to foods or beverages to improve more than one technological or sensorial aspect at the same time. Often, the addition of protein is associated with an increasement in the nutritional properties, such as the reduction of fat in foods, and rheological and sensorial properties

such as texture, palatability, and stability. In this review, 30 MWP patents described to have more than one functionality, as shown in Table 5.

3.3.7.1 Composition and sensory

In the present work, it was observed that 13 patents presented an interaction between composition and sensory functionalities. Patent EP0535728A2 presented a process to produce cheese (with fat or reduced fat) that preserves the texture and flavour of naturally matured cheese without the need for long ripening periods. The reduction in fat content was achieved by blending the initial unripen cheese with a paste containing a low-fat protein source, the MWP. In addition, the cheese became softer as the addition level of MWP increased.

Patent IE20080473A1 described a natural cheese with a low-fat content (3% or less fat by weight) and a manufacturing process that preserved its organoleptic characteristics. Thus, it was added MWP to the cheese to obtain a reduced fat content. A cheese modified with natural enzymes was added to the whey protein mixture, in which the flavour development was accelerated by the addition of microbiological cultures in a controlled temperature environment. The result was a cheese with better flavour and aroma.

Another example of the use of MWPs to simultaneously improve composition and sensory properties was provided by patent US2020008439A1, that described a process for producing a protein-rich liquid dairy. It was also related a protein/mineral composition and its use to produce liquid dairy products with higher protein content and lower viscosity. The MPW composition, together with minerals, is an important nutritional supplement for people who need extra macro and micronutrients, such as the elderly.

Table 5 - Patents that used MWP as an ingredient, describing more than one product functionality as composition stability and sensory.

	Year	Country	Publication number	Products made with MWP in the patent	Functionality	References
Composition and sensory	1991	Canada	CA2060655A1	Food in general	Fat substitute	Fang; Snook, 1991
	1993	Canada	CA2097316A1	MWP powder	Fat substitute Sensory enhancer	Bringe, 1993
	1996	European patent	EP0535728A2 EP0535728A3 EP0535728B1	Cheese	Fat substitute Texture enhancer	Bodor et al., 1993
	2004	USA	US2002037353A1 US6730336B2	Instant flavoured drinks	Protein content booster Sensory enhancer	Villagran et al., 2002
	2004	USA	US2004062846A1 US6824810B2	Dairy and non-dairy creams	Protein content booster Palatability enhancer	Argent et al., 2004
	2010	Ireland	IE20080473A1	Cheese	Fat substitute Palatability enhancer	Lehane, 2010
	2013	Russia	RU2011130529A RU2487550C2	Cheese	Fat substitute Texture enhancer	Burger, 2013
	2014	USA	US2014377439A1	Frozen confectionery product	Protein content booster Sensory enhancer	Eriksen; Baldursson, 2014
	2015	WIPO	WO2015059245A1	Yogurt and powdered food ingredient	Protein content booster Viscosity reducer	Mikkelsen et al., 2020
	2017	Russia	RU2615441C1	Desserts	Protein content booster Fat substitute Sensory enhancer	Shabalin, 2017

	2020	USA	US10709146B2 US2016255848A1	Yogurts	Protein content booster Viscosity reducer	Mikkelsen et al., 2020a
	2022	USA	US2022142209A1	Chocolate cream	Fat substitute Palatability enhancer	Aharonian, 2020
	2022	USA	US2022225629A1	Dairy product	Protein content booster Maintenance of product viscosity	Christensen et al., 2020
Composition and stability	2002	USA	US2002119232A1	Floury bakery products	Fat substitute Viscosity enhancer	Grazela et al. 2002
	2013	Australia	AU2006321056A1 AU2006321056B2	Emulsions containing MWP and lipids	Protein content booster Stability enhancer	Nordvi et al., 2007
	2018	Russia	RU2016124962A RU2654588C1	Sweet concentrated dairy products	Sugar reducer Protein content booster Stability and rheology enhancer	Gnezdilova; Muzykantova; Vinograava, 2017
	2022	USA	US2022000161A1	Protein-rich liquid nutritional compositions	Protein content booster Stability enhancer	Çakir-Fuller et al., 2021
Sensory and stability	2001	USA	US6180159B1	Instant drinks	Palatability and flavour enhancer Stability enhancer	Villagran et al. 2002
	2004	USA	US2003087001A1 US6759078B2	Products derived from cream and/or emulsified butter, fat or oil	Palatability and flavour enhancer Stability enhancer	Stuchell, 2003
	2006	Ireland	IE20060055A1	Cream liqueur	Sensory enhancer	Murphy; Murphy, 2006

					Stability enhancer	
	2020	USA	US2020245633A1	Yogurt and sour cream	Sensory enhancer Stability enhancer	Rajakari; Myllärinen, 2020
Composition, sensory and stability	1991	USA	US5064660A	Cheese	Fat substitute. Reducer of emulsifying and stabilizing agents Preservation of texture, taste, and flavour	Silver, 1991
	2003	European patent	EP1199948A1 EP1199948B1	Flavored instant coffee	Protein content booster Palatability enhancer Stability enhancer	Butterbaugh; Sargent, 2002
	2012	USA	US2012121776A1 US8840945B2	Aerated food products	Protein content booster Texture enhancer Stability enhancer	Arnaudov et al., 2012
	2015	Singapore	SG11201500497QA	Food in general	Fat substitute Texture enhancer Overrun enhancer	Kashiwagi et al., 2015
	2017	Russia	RU2607035C1	Fermented dairy product	Protein content booster Rheology enhancer Stability enhancer	Melnikova; Losev; Stanislavskaya, 2017
	2020	European patent	EP3662755A1	UHT food	Protein content booster Stability enhancer Sensory enhancer	Melnikova; Stanislavskaya; Korotkov, 2015
	2020	Japan	JP2020145926A	Cheese	Protein content booster	Takemoto et al., 2020

					Sensory enhancer Stability enhancer	
	2021	China	CN107853676A CN107853676B	Foaming agent	Protein content booster Sensory enhancer Stability enhancer	Zhang et al., 2018
	2021	USA	US2021007376A1	Floury product	Protein content booster Viscosity reducer Stability enhancer	Povlsen; Langvig; Jensen, 2021
Functionality (not declared)	2010	USA	US2010047423A1 US8889209B2	MWP	Not declared	Kruesemann; Nordanger, 2010
	2012	USA	US8133524B1	Food for blood-sucking insects	Not declared	Acar et al., 2012
	2017	USA	US2017013853A1	Cottage cheese	Not declared	Luo, 2017

Source: prepared by the author.

3.3.7.2 Composition and stability

According to the study, there were four patents that used MWP to modify and improve composition and stability. Patent US2002119232A1 covered the use of MWP as a carrier, emulsifier, and fat substitute capable of promoting viscosity for the baking of farinaceous products, mainly cakes. The patent stated that the use of the emulsifier/carrier formulation in bakery products provided better dough performance in the baking process as it improved dough aeration, providing greater volume, improving texture, and the storage qualities.

Another example was patent AU2006321056B2, which dealed with oils, such as omega 3, presenting the disadvantage of fast oxidation and, therefore, does not present a long shelf life. The patent provided an alternative to encapsulating oils to prevent their oxidation in a short period of time. In this context, MWP was used as an encapsulating agent capable of providing stability to the oil, increasing its shelf life and at the same time enriching the oil with the essential amino acids contained in whey proteins. As a result, blending these oils with MWP could be used as an ingredient in a wide range of foods and beverages.

As a final example, patent RU2654588C1 applied MWP to obtain a sweet concentrated dairy product with low sugar content and stability to increase the shelf life of the product. The patent also stated that the product obtained has the characteristics of a traditional sweet milk concentrate.

3.3.7.3 Sensory and stability

It was also described in patents that MWP can be used to simultaneously improve stability and sensory characteristics. A patent using MWP for this purpose was US6180159B1, developed by Procter & Gamble. MWPs were used in instant and ready-to-drink flavoured beverages such as coffee, hot chocolate, tea, creamy juices, milkshakes, and nutritional drinks. The authors reported that MWPs can improve foam stability by reducing surface tension to form continuous films through intermolecular interactions, preventing bubble bursting. In addition, MWPs provided sensory improvements in creaminess and thickness at a lower solid dosage without sacrificing viscosity.

Patent US2020245633A1, developed by Valio LTD, described a method for producing MWPs using an ideal whey. The authors claimed that these MWPs could be used in the production of various dairy and non-dairy products. The MWP developed in this patent was used in the manufacture of yoghurt, presenting a thicker texture than standard yoghurt. In that way, it was also reported that these MWPs could be used as a replacement for a thickening agent such as pectin, guar gum, xanthan gum, and arabic gum, ensuring an improvement in the stability of the yoghurt produced.

The use of MWPs to improve stability and sensory was also described by patent US2003087001A1, which produced a cream containing MWPs to replace of cream in recipes. This new cream resulted in an improved texture, increased creaminess, and ensured greater stability of the emulsion formed.

3.3.7.4 Composition, sensory, and stability

As Table 5 shows, MWP can play many roles when incorporated as an ingredient in a food or beverage. Patent SG11201500497QA claimed a low fat or fat-free air entraining emulsion containing MWP to ensure air entrapment (overrun) in ice cream. Some food properties are affected when fat is reduced, overrun being one of them. Fats are important components that influence food properties, and when reduced, there is often deterioration in the shape retention, melting resistance, smoothness, and mouthfeel perception of ice cream. Therefore, MWP can help correct these undesirable defects by acting as a mimetic of the physical and sensory properties of fats when the size of the aggregated particles is correct (Akbari; Eskandari; Davoudi, 2019). This patent was filed by the Japanese dairy company Meji Co., Ltd. They proposed a low-fat ice cream formulation containing MWP with 50% of particles which allows formation of air bubbles of a diameter of 20–50 μm, in which the overrun was increased without adding other stabilizers and emulsifiers.

Sometimes, MWP can have so many functionalities that they can interact with each other; for instance, in patent US2012121776A1, a method was developed to produce MWP with reduced surface activity. This reduction allowed the addition of MWP as a protein ingredient without suppressing aeration or the amount of air in food products. Due to its high surface activity, MWP could interfere with emulsifiers and foam stabilizers present in aerated products.

On the other hand, whey proteins can be incorporated into many foods, reducing their production costs, making dairy production more environmentally friendly using the whole milk matrix, and improving the rheology, texture, and shelf life of the food. All of this could be seen in patent RU2607035C1, in which the authors proposed a process to produce cultured dairy products using MWP to obtain functional dairy products.

The MWP attributes, such as composition, stability, and sensory properties, arose from the need to offer low-fat products or to increase protein content without affecting or improving the physical properties of the final product. In this way, it was possible to increase consumer acceptance of these products. All these advantages are desirable for functional or modified foods, especially to the industry that is always seeking for high-quality products without losses and with high consumption interests. Nevertheless, this review demonstrated the great versatility of MWP applications in the food industry.

3.3.8 Products derived from patents

Among the patents presented in the article, several have a company as the inventor, including companies, such as Arla Foods, Labatt Ltd, Franklin Foods, and others. The interest of large companies to invest in MWP is growing by patenting compounds, products, and production methods.

Table 6 shows that Arla Foods is listed as an inventor of 7 patents, developing products, such as instant drinks, yoghurts, and protein milk drinks. One example is Nutrilac® FO 7875, a clean label yoghurt with up to 11% protein and only 0.8% fat. Another Arla Foods product on the market is a protein milk with 25 g of protein and 40% less sugar compared to other milks.

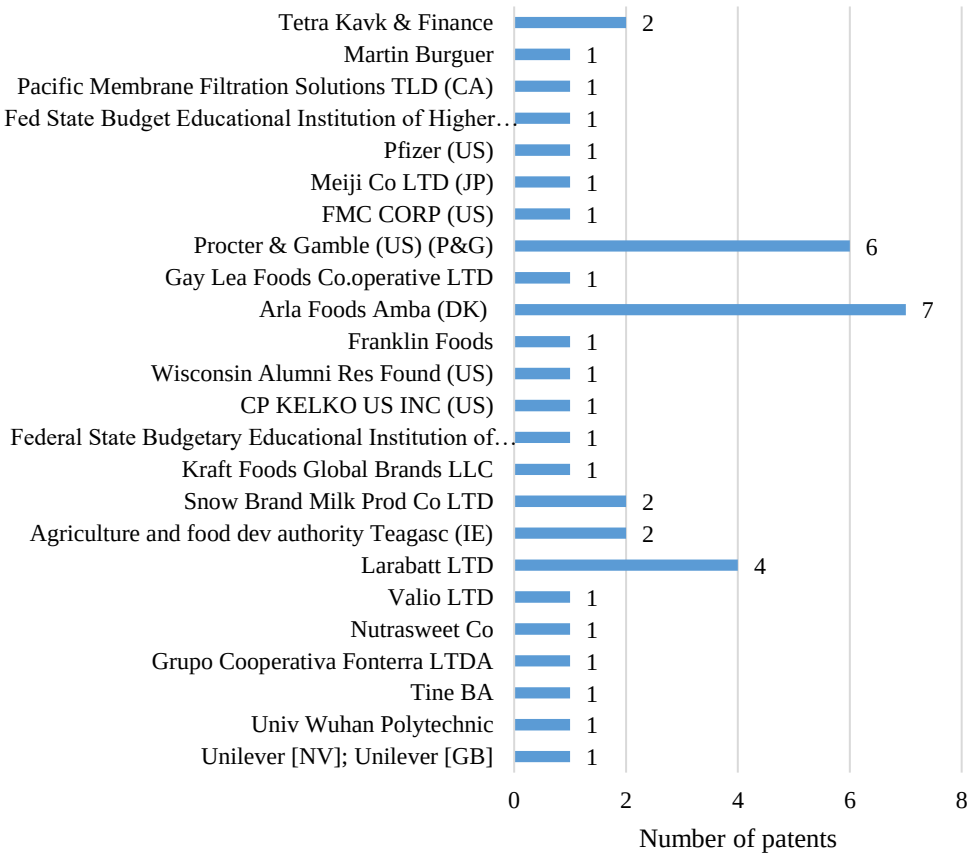
CP Kelco US Inc introduced Simplesse®, the first MWP concentrate that offers heat and pH stability, creaminess, texture, and a wide range of applications in low-fat products. Procter & Gamble was responsible for 6 patents. Of the 24 companies (Figure 4) that applied for a patent, it was listed six products that are on the market.

Table 6 - Number of patents per company and some products on the market.

Patent number	Company	Number of Patents	Products	Reference
EP3542635A1/ EP3542635B1 US2021007376A1 WO2015059245A1 US10709146B2/ US2016255848A1 US2020008439A1 US10834934B2 US2014377439A1	ARLA FOODS AMBA (DK)	7	MWP Powder, Nutrilac® FO-7875, Protein Pudding, Milk Protein Drink, Sour Drinks and Yogurt	ARLA FOODS INGREDIENTS
US20003087001A1 US6759078B2	CP KELCO US INC (US)	1	SIMPLESE microparticulated whey protein concentrate	CP KELCO
EP2094099A1 EP2094099B1	KRAFT FOODS GLOBAL BRANDS LLC	1	Maxwell House Cappuccino Instant Coffee Powder 1 x 750g Maxwell House Cappuccino Milka 335g	AMAZON
SG11201500497Q	MEIJI CO LTD (JP)	1	Meiji High Protein Pasteurized Vanilla Flavoured Milk	CP-MEIJI CO
JP2021035332A JP2020145926A	SNOW BRAND MILK PROD CO LTD	2	Camembert cheese	SNOWBRAND
AU2006321056A1 AU2006321056B2	TINE BA	1	Strawberry and Banana YT® Protein Shake	TINE

Source: prepared by the author.

Figure 4 - Number of patents and companies that presented MWP products.



Source: prepared by the author.

3.4 CONCLUSION

Over the last 3 decades, MWP has been used as an ingredient or compound to influence the composition, sensory, and stability of products. In the present work, 60 patents were reviewed in which MWP was presented as a fat substitute, as an ingredient to improve texture, flavour, viscosity, and as a stabilizer for the most diverse products, such as yoghurt, cheese, salad dressings, and acidified drinks, among others. The current review revealed that 24 of the 60 applicants for an MWP patent were companies, of which at least six have food products already on the market reporting the use of these microparticulated proteins or a higher protein value in the food.

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4DEVELOPMENT AND VALIDATION OF A METHODOLOGY FOR QUANTIFICATION OF WHEY PROTEIN FRACTIONS IN MICROPARTICULATE INGREDIENTS

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4.1 INTRODUCTION AND AIM

Whey is obtained from the coagulation of milk used in cheese production. It comprises water, proteins, lactose, minerals, and fats (Schuck et al., 2004). Whey is widely used in the food industry because it contains all essential amino acids, improves blood pressure, prevents cardiovascular diseases, and has antioxidant properties (Corrochano et al., 2019; Pal et al., 2019). Whey protein can be divided into β-lactoglobulin (ranging from 48% to 58%), α-lactoalbumin (13% to 19%), caseinomacropeptide (12 to 20%), immunoglobulins (8 to 12%), bovine serum albumin (6% to 7%), lactoferrin (1% to 3%), and other proteins (0.5% to 1%) (Tunick, 2009).

One of the main applications of whey proteins in food products is whey protein concentrates (WPC) (Çakir-Fuller, 2015). However, these proteins are thermally destabilized due to their low denaturation temperatures, this denaturation can lead to product defects, as phase separation and loss of sensory characteristics may occur. For example, α-lactoalbumin has a denaturation temperature ranging from 30 to 60 °C, depending on its variant (Bonnaillie; Tomasula, 2008; Tavares, 2020).

Microparticulation is an alternative method to enhance the thermal stability of whey proteins (Kerche; Weterings; Beyrer, 2016). In this process, heat is applied to denature and aggregate the proteins, followed by high-shear steps to break the aggregated proteins into smaller, more uniform particles (Shewan; Stokes, 2013).

With this process, microparticulated proteins combine denatured and native proteins with controlled particle sizes ranging from 0.1 to 10 μm (Renard et al., 2002; Sun et al., 2015). This native and aggregated protein combination increases thermal treatment resistance because it contains fewer free thiol groups, which are responsible for protein denaturation (Dissanayake; Vasiljevic, 2009).

Microparticulation modifies the whey protein structures, resulting in products with functional characteristics distinct from native whey protein, such as emulsifying

activity and gelation properties (Considine et al., 2007; Chung; Degner; Mcclements, 2014). These products can provide textural sensations similar to fat and are used as fat substitutes in food products, including concentrated fermented milk, ice cream, desserts, and processed cheeses (Sandoval-Castilla et al., 2004; Torres et al., 2011).

Several studies have quantified the WPC proteins used as ingredients, which are the main focus of this study. One study quantified WPC to understand how pasteurization, membrane concentration, and spray drying modified the protein concentration in the final powder obtained (Muuronen et al., 2021). Another sought to understand how heat treatment can modify the microstructure, denaturation, and functional properties of goat whey proteins (Zhao et al., 2020). Finally, Buggy et al. (2018) studied how protein concentration and pH interfere with WPI (why protein isolated) protein denaturation under heat treatment. However, a methodology that quantifies β-lactoglobulin and α-lactoalbumin via high-performance liquid chromatography (HPLC) before and after microparticulation has yet to be reported.

Based on this premise, this study sought to develop an HPLC methodology to quantify the two main WPC protein fractions in liquid and powdered form before and after microparticulation and at various protein concentrations. Additionally, we calculated the denaturation percentage of each protein fraction.

4.2 METHODOLOGY

The methodology development process and the quantification of whey protein fractions are presented below.

4.2.1 Reagents, chemicals, standards

Analytical standards of β-lactoglobulin (CAS number: 9045-3-2) and α-lactoalbumin (CAS number: 9051-29-0) were obtained from Sigma-Aldrich (São Paulo, Brazil), trifluoroacetic acid (TFA) and acetonitrile from Merck (São Paulo, Brazil). Water was purified using the Milli-Q system (Millipore, USA).

4.2.2 HPLC equipment

The analytes were separated and quantified using HPLC (Waters, model 1252, Milford, USA), a UV-Vis detector (DAD, diode array detector), and a binary pump. The chromatographic separation employed a Phenomenex column Luna (5 μm, 250 mm × 4.6 mm), kept at 40 °C. The injection volume was 20 μL; β-lactoglobulin and α-lactoalbumin were detected at a wavelength of 214 nm.

4.2.3 Chromatographic conditions

Gradient elution was performed using a mixture of two solvents at a flow rate of 0.5 mL min^{-1}. The mobile phase comprised of 0.1% TFA in ultrapure water (solvent A) and 0.09% TFA in 90% acetonitrile:10% ultrapure water (solvent B). A gradient program was initiated as follows: 0-3 min, 20-40% B; 3-5 min, 45-45% B; 5-7 min, 45-50% B; 7-9 min, 50-80% B; 9-14 min, 80-20% B; and 14-15 min, 20-20% B.

4.2.4 Validation procedure

For method validation, the selectivity, linearity, precision (intra- and inter-assay), accuracy, limit of detection (LOD), and limit of quantification (LOQ) were evaluated following the criteria of RDC No. 166, from July 24, 2017, Brazil (Anvisa, 2017). Selectivity was proven by the retention time equivalence for β-lactoglobulin and α-lactoalbumin as external standards and by the peak area increase in fortified samples. Selectivity was also evaluated for the solutions used in sample preparation and mobile phase; the absence of analytical response at the retention time of β-lactoglobulin and α-lactoglobulin proved it. The analytical curves were constructed using five points, each measured three times, to a concentration ranging from 100 to 300 μg mL^{-1}. The LOD and LOQ were calculated based on the standard deviation of the response and slope using the following formulas:

$$LOD = 3.3 \times \frac{\sigma}{S} \quad (2)$$

$$LOQ = 10 \times \frac{\sigma}{S} \quad (3)$$

where σ is the standard deviation of the Y-axis intercept, and S is the slope of the calibration curve.

The sample dilution integrity was validated in the ratio of 1:5 to a final concentration of 200 μg mL^{-1} of β-lactoglobulin and α-lactoalbumin. This parameter was validated according to ICH guideline M10 (European Medicines Agency, 2022).

4.2.5 Samples

WPC samples of various concentrations (WPC45%, WPC60%, and WPC80%) were analyzed before and after microparticulation. Liquid and powder samples were obtained from a partnering company. The samples analyzed are listed in Table 7.

Table 7 - Number of WPC samples analysed before and after microparticulation.

Form	Protein Concentration (% m m^{-1})	Number of samples
Solution	WPC45	2
	WPC60	2
	WPC80	1
Powder	WPC45	8
	WPC60	5
	WPC80	2

Source: prepared by the author.

4.2.6 Sample preparation for RP-HPLC analysis

The liquid samples were centrifuged (22,000 × g) using a Sigma 2K15 (Sigma, Barueri, São Paulo) for 10 min at 5 °C. Solutions of the powdered samples were prepared at 2% concentration (m v^{-1}) and then centrifuged under the same conditions as the liquid samples. After centrifugation, all samples were filtered through 0.22 μm pore syringe filters for subsequent HPLC analysis. Depending on the protein fraction content determined by analysis, the samples were diluted when necessary to meet the working range of the validated analytical curve.

4.2.7 Percentage calculation of additional denaturation from microparticulation process

To determine the denaturation percentage for β-lactoglobulin and α-lactoalbumin, the Equation 4 was applied (Çakir-Fuller, 2015):

$$\%\ denaturation = \left(1 - \frac{microparticulated\ whey\ protein}{whey\ protein}\right) x\ 100 \qquad (4)$$

The additional denaturation percentage caused by microparticulation is a relationship between each protein fraction concentration in whey and microparticulated whey. The protein concentration in the whey represents the native protein amount in that product. Whey exhibits some protein denaturation percentage due to pre-processing; this pre-existing denaturation was not considered in the calculation. The protein concentrations in the microparticulate are the native protein amounts remaining after processing.

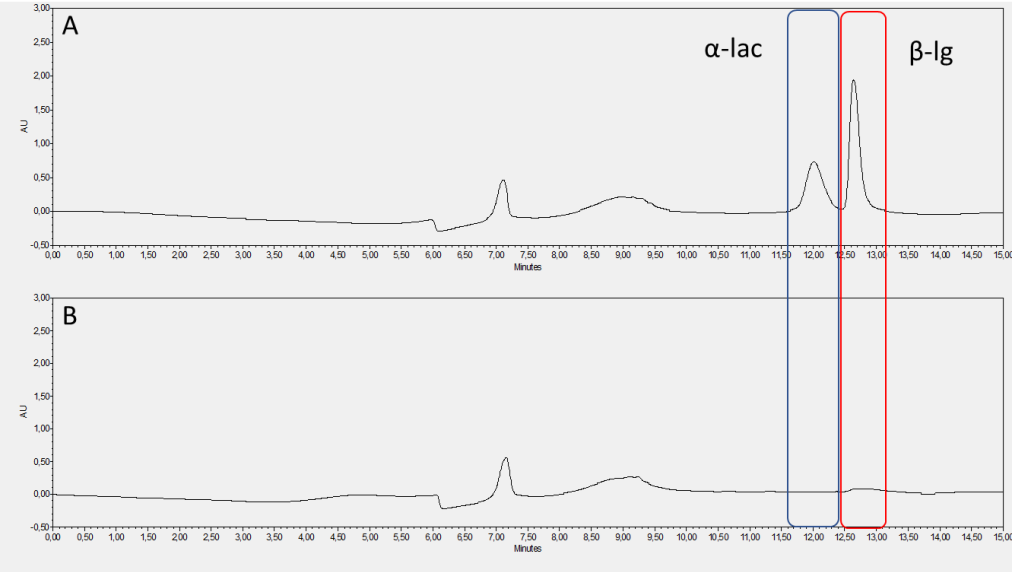
4.3 RESULTS AND DISCUSSION

The results presented below are organized into two sections: the validation of the developed method and the subsequent analyses conducted.

4.3.1 Separation, identification, and quantification of serum proteins

Selectivity was the first parameter evaluated during method validation by comparing the retention time of the diluent solution chromatograms with that of the fortified samples, ensuring that the peak response was exclusively for the compounds of interest, as shown in Figure 5.

Figure 5 - Chromatogram of solution containing α-lactoalbumin (α-lac) and β-lactoglobulin (β-lg) standards (a) and chromatogram of blank showing absence of interferents (b).



Source: prepared by the author.

Linearity of the analytical standards β-lactoglobulin and α-lactoalbumin was determined by constructing analytical curves, from which the respective coefficients of determination (R^2) were obtained (Table 8). All analyses were performed in triplicate.

Table 8 - Parameters determined from analytical curve.

Analyte Linear Range	Line Equation	R^2	LOD ($\mu g\ mL^{-1}$)	LOQ ($\mu g\ mL^{-1}$)
β-lactoglobulin	y = 32054x - 610871	0.9953	18.4	55.9
α-lactoalbumin	y = 46826x - 638918	0.9971	7.2	22.1

R^2 = coefficient of determination; LOD = limit of detection; LOQ = limit of quantification.

Source: prepared by the author.

The LOD and LOQ were calculated according to Equations 1 and 2 and determined by a method based on the response of three replicates of an analytical curve (Table 8).

Precision parameters were evaluated using intra- and inter-assays. The intra-assay was undertaken by analyses of six analytical standard replicates at identical

concentrations of 125, 225, and 275 μg mL^{-1} on the same day under the same experimental conditions. For the inter-assay, solutions with identical concentrations were analyzed on two different days. The data obtained were expressed by estimating the relative standard deviation (RSD) to be less than 5%. The accuracy was in the range of 90-110% associated with precision values, as shown in Table 9.

Table 10 shows the effect of sample dilution on the theoretical concentration compared to the analyzed concentration. The European Medicines Agency (2022) stipulates that the correlation coefficient should be < 15%. The results for both analytes were below the specified values, indicating that the dilution effect produced no interference in the samples.

Table 9 - Parameters determined from precision, expressed through repeatability (Rep.) and intermediate precision (Int. prec.).

Analyte	Concentrations (μg mL^{-1})	Rep. RSD (%)	Rep. Accuracy (%)	Int. prec. RSD (%)	Int. prec. Accuracy (%)
β-lactoglobulin	125	3.63	100.23 - 107.37	3.40	100.23 – 108.16
	225	3.83	93.84 – 100.84	2.44	96.23 – 100.95
	275	4.16	90.69 – 97.40	4.25	90.69 – 102.50
α-lactoalbumin	125	2.60	99.56 – 104.31	3.96	95.65 – 104.31
	225	1.11	99.46 – 101.54	2.25	96.34 – 101.54
	275	1.54	95.65 – 98.48	1.85	95.65 – 100.79

Source: prepared by the author.

Table 10 - Dilution integrity for β-lactoglobulin and α-lactoalbumin.

Analyte	Theoretical concentration (μg mL^{-1})	Real concentration (μg mL^{-1})	Dilution integrity (%)
β-lactoglobulin	200	205.96 ± 9.15	4.44
α-lactoalbumin	200	220.29 ± 3.94	1.79

Source: prepared by the author.

4.3.2 Quantification of β-lactoglobulin and α-lactoalbumin in WPC

WPCs in solid and liquid forms were analyzed before and after microparticulation at protein concentrations of 45, 60, and 80% (m m^{-1}). The number of specific samples for

each concentration is presented in Table 1 and described in “Sample preparation for RP-HPLC analysis” sub-section. Table 11 shows the protein fraction concentrations for each analyzed sample and their denaturation percentages.

Table 11 - Concentration and protein denaturation percentage in analyzed samples before and after microparticulation.

Sample		β-lactoglobulin			α-lactoglobulin		
		WP ($g\ 100g^{-1}$)	MWP ($g\ 100g^{-1}$)	% of protein additional denaturation	WP ($g\ 100g^{-1}$)	MWP ($g\ 100g^{-1}$)	% of protein additional denaturation
Soluion	WPC45%-1	2.51 ± 0.01	0.72 ± 0.01	71.50	0.77 ± 0.02	0.38 ± 0.01	50.74
	WPC45%-2	3.96 ± 0.20	1.89 ± 0.02	52.31	0.76± 0.02	0.52 ± 0.01	31.35
	WPC60%-1	8.52 ± 0.33	1.06 ± 0.02	87.60	1.08 ± 0.02	0.65 ± 0.03	40.06
	WPC60%-2	10.02 ± 0.27	1.48 ± 0.03	85.19	1.26 ± 0.02	1.02 ± 0.09	18.48
	WPC80%-1	5.97 ± 0.01	1.19 ± 0.03	80.11	1.67 ± 0.12	0.87 ± 0.04	47.59
Powder	WPC45%-1	19.66 ± 0.14	8.10 ± 0.27	58.80	5.57 ± 0.11	4.59 ± 0.15	17.63
	WPC45%-2	19.66 ± 0.14	5.81 ± 0.14	70.47	5.57 ± 0.11	3.68 ± 0.07	33.97
	WPC45%-3	21.58 ± 0.49	8.27 ± 1.22	61.66	3.87 ± 0.12	3.67 ± 0.13	5.24
	WPC45%-4	21.58 ± 0.49	7.03 ± 0.25	67.41	3.87 ± 0.12	3.12 ± 0.13	19.35
	WPC45%5-	23.37 ± 0.34	5.46 ± 0.27	76.65	4.92 ± 0.31	2.28 ± 0.16	53.66
	WPC45%-6	21.39 ± 0.35	4.70 ± 0.30	78.02	4.26 ± 0.18	2.19 ± 0.25	48.63
	WPC45%-7	21.39 ± 0.35	4.68 ± 0.06	78.13	4.26 ± 0.18	2.27 ± 0.01	46.55
	WPC45%-8	24.58 ± 3.74	24.22 ± 1.79	1.44	5.34 ± 0.43	4.85 ± 0.08	9.02
	WPC60%-1	27.80 ± 2.66	6.03 ± 0.34	78.31	8.23 ± 0.24	5.24 ± 0.18	36.27
	WPC60%-2	30.76 ± 0.50	5.11 ± 0.30	83.38	7.03 ± 0.65	4.09 ± 0.02	41.89
	WPC60%-3	17.26 ± 1.08	5.77 ± 0.18	66.57	6.13 ± 0.09	4.08 ± 0.09	33.42
	WPC60%-4	21.46 ± 0.30	6.42 ± 0.04	70.07	6.25 ± 0.09	3.68 ± 0.14	41.21
	WPC60%-5	26.98 ± 1.05	9.00 ± 0.66	66.63	7.42 ± 0.19	3.84 ± 0.09	48.32
	WPC80%-1	31.77 ± 0.34	14.28 ± 0.75	55.06	8.69 ± 0.21	5.75 ± 0.03	33.87
	WPC80%-2	31.77 ± 0.34	7.27 ± 0.16	77.11	8.69 ± 0.21	3.81 ± 0.02	56.20

Source: prepared by the author.

After powder sample reconstitution (2% m v^{-1}), higher protein fraction concentrations were observed compared with the liquid products. The β-lactoglobulin concentration (48-58%) in the samples was higher than that of α-lactoalbumin (13-19%) Bonnaillie; Tomasula, 2008; Tavares, 2020).

The concentration of each protein fraction in the samples after microparticulation decreased because of protein denaturation (Sánchez-Obando et al., 2020). Differences in the sample concentrations before microparticulation can be attributed to the raw materials used. However, the difference in the sample protein concentrations after microparticulation may have resulted from variations in the methods employed, as the collaborating industry did not provide these details.

The denaturation percentage was calculated using the values obtained for each protein fraction. In general, β-lactoglobulin showed a higher denaturation percentage than α-lactoalbumin, except in the WPC45%-1 sample. The denaturation values for α-lactoalbumin ranged from 5.24 to 56.20% and for β-lactoglobulin from 1.44 to 87.50%. This significant variation in the denaturation percentage may be related to microparticulation process factors, such as pH, protein and salt concentrations, heat treatment, and shear rate (Toro-Sierra; Schumann; Kulozik, 2013). Therefore, our development of an analytical method to quantify α-lactoalbumin and β-lactoglobulin can contribute as a guide to the microparticulation process.

4.4 CONCLUSION

We developed and validated a methodology for quantifying α-lactoalbumin and β-lactoglobulin in commercial and laboratory-produced microparticulated WPC samples. Statistical analyses demonstrated that the method was accurate, selective, linear, and precise, with a recovery rate exceeding 90%.

The commercial samples exhibited significant variations in the denaturation percentage during microparticulation, which can be attributed to the microparticulation parameters employed.

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5 INFLUENCE OF CARBOHYDRATE ON THE MICROPARTICULATION PROCESS OF WHEY PROTEINS: STRUCTURAL MODIFICATIONS AND INDICATORS OF THE MAILLARD REACTION

The results obtained in this section will be published as an article in a journal related to the topic.

5.1 INTRODUCTION AND AIM

Whey is obtained from milk coagulation during cheese production and, for many years, was considered a by-product of this process, often used as animal feed or even discarded into water bodies, thus causing environmental impacts (Smithers, 2015). Over time, growing environmental concerns have compelled the food industry to seek alternative uses for whey (Marwaha; Kennedy, 1988; Smithers, 2008).

Among the components of whey, whey proteins stand out for their high nutritional value and potential applications in the production of other ingredients (Kilara; Vaghela, 2017). These proteins are divided into β-lactoglobulin (48%–58%), α-lactalbumin (13%–19%), immunoglobulins (8%–12%), bovine serum albumin (6%–7%), lactoferrin (1%–3%), and other proteins (0.5%–1%) (Tunick, 2009).

One of the major challenges in processing whey proteins is their low denaturation temperature, which can cause issues during formulation and throughout the product's shelf life (De Paula et al. 2025). For example, the denaturation temperature of β-lactoglobulin is around 75 °C, while that of α-lactalbumin ranges from 30 to 60 °C (Bonnaillie; Tomasula, 2008).

An alternative to overcome this disadvantage of whey proteins is the use of microparticulation. This process involves the controlled aggregation of proteins through heat treatment combined with mechanical action that modulates the size of the aggregates formed by thermal denaturation (Toro-Sierra; Schumann; Kulozik, 2013). The resulting aggregates have particle sizes ranging from 0.1 to 10 μm and are composed of both native and denatured proteins (Lieske; Konrad, 1994; Renard et al., 2002). Several factors can influence the microparticulation process, such as pH, protein concentration, salt content, and the presence of carbohydrates (Nunes; Tavares, 2019; Sampson; Cooke, 1992).

The presence of different concentrations of lactose has already been studied in relation to its impact on the thermal denaturation of β-lactoglobulin, with concentrations

up to 7.5% m m^{-1} resulting in protein denaturation greater than 95% (Spiegel, 1999). With the increasing number of people who are lactose intolerant, the use of dairy products containing hydrolyzed disaccharides has gained prominence over the years, especially in countries in South America, Africa, and Asia (Paula et al., 2021; Queiroz et al., 2021). The development of a lactose-free ingredient based on microparticulated whey protein could lead to broader applications of these proteins, which are already used in products such as ice cream, protein beverages, hard cheeses, and yogurt (Sánchez-Obando et al., 2020; Sun et al., 2015).

Based on this premise, the present study aims to develop microparticulated proteins using two types of carbohydrates: the first with lactose (WL), and the second with glucose and galactose in the proportion resulting from lactose hydrolysis (WGG). Additionally, a formulation without added carbohydrates (WOC) will be produced in order to understand the necessity of sugar use in the microparticulation process.

5.2 METHODOLOGY

The methodologies employed in this chapter are detailed below.

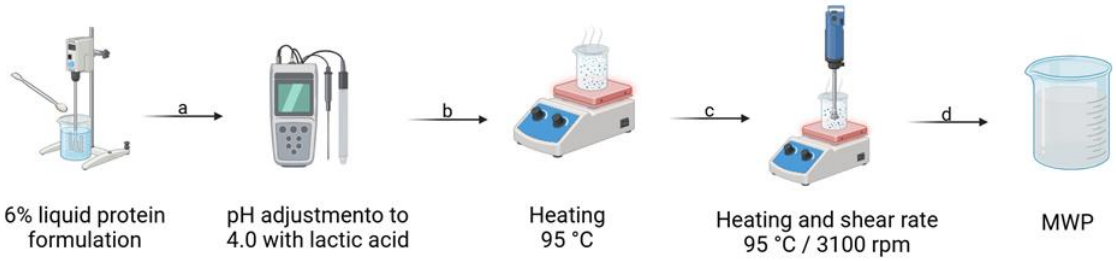
5.2.1 Sample preparation

Initially, three different samples were prepared. The first consisted of a solution containing only WPI with 6% (m m^{-1}) protein and no added carbohydrates (WOC). The second sample contained the same protein concentration but was supplemented with 4.18% (m m^{-1}) lactose (WL). Finally, the third product maintained the same protein content as the other two samples and was supplemented with 4.18% (m m^{-1}) of glucose and galactose, with 2.09% (m m^{-1}) of each sugar (WGG). After solubilizing the powders (WPI and carbohydrate) under agitation for 1 hour, the samples were stored in a controlled environment at 5 ± 2 °C for approximately 24 hours.

After the rehydration period, the sample was heated to 22 ± 1 °C and the pH was adjusted to 4.0 using lactic acid. Subsequently, the mixture was heated to 95 °C, and upon reaching this temperature, a shear rate of 3100 rpm was applied under continued heating for 10 minutes, resulting in the three microparticulated products (Figure 6). Samples were collected at each stage of the process (a, b, c, d). All samples were produced in triplicate.

Two sets of analyses were performed. The first focused on studying protein modifications, including particle size analysis, additional protein denaturation percentage, centrifugation, deconvolution, free sulfhydryl groups, and surface hydrophobicity. The second set of analyses aimed to understand the modifications related to the Maillard reaction, measuring carboxymethyllysine, furosine, lysine, pyrraline, and dicarbonyl compounds.

Figure 6 - Flowchart of the production process for microparticulated whey proteins (MWP).



Source: prepared by the author.

5.2.2 Protein Modification

The analyses conducted to investigate protein modifications included particle size measurement, supplementary protein denaturation, sedimentation and gel formation, infrared spectroscopy, quantification of sulfhydryl groups, and assessment of surface hydrophobicity.

5.2.2.1 Particle Size

A Malvern Panalytical Zetasizer Nano ZS dynamic light scattering equipment was used to measure particle size and size distribution. The samples were diluted in bidistilled water, with 1 mL of sample solubilized in 99 mL of water. Each sample was then placed in a cuvette and analyzed using the equipment. The analyses were performed in triplicate.

5.2.2.2 Additional Protein Desnaturation

Size exclusion chromatography (SEC) was performed to evaluate the molecular weight distribution of soluble proteins extracted from samples. Protein extracts were prepared by homogenizing the samples in phosphate buffer pH 6.8, 50 mM to a concentration of 2 mg protein mL^{-1}. Following centrifugation at 10000g for 10 min at 4 °C. The supernatant was filtered through a 0,45 μm filter prior to injection. Chromatographic separation was performed using a Superdex™ 200 increase 10/300 GL column (cytiva), connected to a KNAUER preparative chromatographic system with pump P 4.1S, detector and autosampler 3950 (KANUER AZURA®). The mobile phase consisted of buffer phosphate 50 mM containing 0.1 M NaCl (pH 6.8) with flow rate of 0.5 mL min^{-1}. A 20 μL sample volume was injected and protein elution was monitored at 280 nm using a KNAUER UVD 2.1S detector. The column was calibrated with protein standards (β-lactoglobulin, α-lactalbumin and bovine serum albumin). Data were analyzed using ClarityChrom® CDS.

To determine the additional denaturation percentage for whey protein, the Equation 5 was applied (Çakir-Fuller, 2015):

$$\%\ denaturation = \left(1 - \frac{microparticulated\ whey\ protein}{whey\ protein}\right) x\ 100 \qquad (5)$$

The additional denaturation percentage caused by microparticulation is a relationship between protein concentration in whey and microparticulated whey. The protein concentration in the whey represents the native protein amount in that product. Whey exhibits some protein denaturation percentage due to pre-processing; this pre-existing denaturation was not considered in the calculation. The protein concentrations in the microparticulate are the native protein amounts remaining after processing.

5.2.2.3 Sedimentation and Gel Formation

The amount of residual mass was obtained after centrifuging approximately 15 g of each sample at 3100g for 15 minutes using a Centrifuge 5804 (brand: Eppendorf). After centrifugation, the liquid phase was removed with the aid of a pipette, and the tube was inverted for 2 hours before the residual mass was quantified. Results are expressed as

mass per 100 g of sample (de Souza et al., 2024). The analyses were performed in triplicate.

5.2.2.4 Fourier Transform Infrared (FTIR) Spectroscopy

Infrared spectroscopy coupled with the ATR system can be used to monitor changes in the secondary structures of proteins. (Ma et al., 2024a). A Nicolet iS5 spectrometer (brand: Thermo Scientific) was used, with a scan range from 500 to 4000 cm^{-1}, a spectral resolution of 2 cm^{-1}, and 128 scans. The amide I region, from 1600 to 1700 cm^{-1}, was evaluated to determine the secondary structure of the proteins (DEVI et al., 2013). The spectral data were processed using OriginPro and FityK software, where baseline correction, normalization, and curve fitting were performed.

Spectral assignments were made as follows: β-sheet (1600–1640 cm^{-1} and 1687–1700 cm^{-1}), random coils (1640–1650 cm^{-1}), α-helix (1650–1660 cm^{-1}), and β-turn (1660–1687 cm^{-1}) (Savadkoohi et al., 2016; Zhang et al., 2021).

The percentage of the protein secondary structure was calculated according to Equation 6:

$$\%\ of\ secundary\ structure = 100 \times \frac{area\ of\ each\ secondary\ structure}{total\ area\ of\ secondary\ structure} \quad (6)$$

5.2.2.5 Free Sulfhydryl Groups (SH)

The measurement of total free sulfhydryl groups and exposed free sulfhydryl groups was determined using the Ellman method (Bveridge; Toma; Nakai, 1974). A 500 mL Tris-glycine buffer was prepared by dissolving 5.20 g of Tris, 3.45 g of glycine, and 0.60 g of EDTA in bidistilled water. The buffer pH was adjusted to 8 with 2 mol L^{-1} HCl. The Ellman reagent was prepared by dissolving 24 mg of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in 6 mL of the Tris-glycine buffer.

For the determination of exposed SH groups, 0.06 g of sample was added to 7.0 mL of Tris-glycine buffer. Then, 70 µL of Ellman reagent was added to the sample solutions, which were incubated for 1 hour.

For the determination of total free SH groups, urea (8 mol L^{-1}) and 0.05% m m^{-1} sodium dodecyl sulfate (SDS) were added to the Tris-glycine buffer to denature the

protein. Then, 0.06 mg of the sample was added to 7.0 mL of the Tris-glycine buffer containing urea and SDS. The solutions were supplemented with 70 µL of Ellman reagent and incubated for 1 hour.

The prepared solutions were analyzed using a UV-Vis spectrophotometer Specord 50 (brand: Analytik Jena) at a wavelength of 412 nm, using a reagent blank. Additionally, a sample blank was prepared by mixing the samples with the Tris-glycine buffer. The concentrations of SH groups were calculated using the equation 7 (Ma et al., 2024b).

$$SH\ (\mu mol.g^{-1}) = \frac{73.53 \times (A^1_{412} - A^2_{412}) \times D}{C} \quad (7)$$

Where A^1_{412} is the absorbance of the sample at 412 nm; A^2_{412} is the absorbance of the blank at 412 nm; D is the dilution factor (1.008); and C is the protein concentration of the samples (mg mL^{-1}).

5.2.2.6 Surface Hydrophobicity (S_0)

Surface hydrophobicity is used to monitor protein denaturation and the extent of hydrophobic interactions in protein aggregates. ANS (8-anilino-1-naphthalenesulfonic acid) was used as a fluorescent probe, binding to hydrophobic groups exposed on the surface of soluble proteins or peptides, while insoluble aggregates may not contribute to surface hydrophobicity (Ma et al., 2024b).

Stock solutions of each sample were prepared with a protein content of 0.12 g $100g^{-1}$, where 1 g of sample was dissolved in 49 mL of phosphate buffer (10 mM, pH 7.0) under continuous stirring overnight. After this time, four solutions were prepared with protein concentrations of 0.0075, 0.0150, 0.0225, and 0.0300 mg mL^{-1} using the same buffer. 3 mL of each protein solution was added to 30 µL of ANS (8.10^{-3} mol L^{-1}), then the solutions were vortexed for 20 seconds and kept in the dark for 15 minutes. Fluorescence intensity was measured using a Cary Eclipse Fluorescence Spectrophotometer (brand: Agilent Technologies), with excitation and emission wavelengths of 365 nm and 480 nm, respectively. The S_0 value was obtained by calculating the slope of the regression curve of fluorescence intensity as a function of protein concentration (Yerramilli; Longmore; Ghosh, 2017).

5.2.3 Maillard Reaction Indicators

The analyses focused on Maillard reaction indicators included the determination of lysine, furosine, CML, pyrraline, and dicarbonyl compound concentrations.

5.2.3.1 Lysine and Furosine

500 µL of each sample was hydrolyzed for 23 hours with 4.5 mL of 7 mol L^{-1} HCl at 110 °C. After cooling in an ice bath, 400 µL of each sample was taken, dried under vacuum, and dissolved in 1 mL of loading buffer at pH 2.2. The samples were then filtered through a 0.45 µm pore membrane filter and analyzed.

Lysine and furosine analyses were performed using an Alpha Plus amino acid analyzer (LKB Biochrom), equipped with a stainless-steel column (200 x 4 mm) packed with ion exchange resin, Ultropac model, with a particle size of 8 µm (LKB Biochrom). The buffers and program used during the run are described by (Henle et al., 1991). Lysine was detected using ninhydrin. The reagent coil temperature was set to 135 °C, and data acquisition and peak integration were performed using Baseline chromatography software (Waters Dynamic Solutions) on an IBM 286 AT (Henle et al., 1991).

5.2.3.2 Nε-(carboxymethyl)lysine (CML) and Pyraline

1 mL of each sample was mixed with 10 mL of a cooled acetonitrile and methanol solution (50/50 v v^{-1}) in a test tube, along with 10 µL of the internal standards solution. This new solution was cooled at -25 °C for 1 hour and centrifuged for 5 minutes at 3000g. The supernatant was transferred to a polypropylene tube and evaporated in a Zymark TurboVap LV evaporator (Hopkinton). The precipitate was dissolved in 1.4 mL of water and 1.4 mL of hexane. The sample was centrifuged at 1000g for 2 minutes, and the organic phase was discarded. The resulting aqueous phase was added with 100 µL of NFPA (nonafluoropentanoic acid). The mixture was applied to a solid-phase extraction cartridge (Waters Oasis HLB 6 cc), previously conditioned by sequential washing with 2 mL of methanol, 2 mL of methanol/10 mM NFPA (50/50 v v^{-1}), and 4 mL of 10 mM NFPA. After sample application, the cartridge was washed with 4 mL of a methanol and 10 mM NFPA mixture (5/95 v v^{-1}), and analytes were eluted with 5 mL of methanol and 10 mM NFPA mixture (90/10 v v^{-1}) into test tubes. The eluate was evaporated,

redissolved in 40 µL of water, and analyzed by HPLC-MS as described by (Schwarzenbolz et al., 2016).

The concentrations of CML and pyralline were analyzed using an Agilent 1200 series chromatographic system coupled to an Agilent 6410 mass spectrometer with electrospray ionization source (ESI-MS, all from Agilent). The gas temperature was set at 350 °C, gas flow at 11 L min^{-1}, nebulizer pressure at 35 psi, and capillary voltage at 4000 V. A combination of two columns was used: ZORBAX 300SB-C18 (2.1 × 50 mm, 1.8 µm) from Agilent and Hypercarb (2.1 × 150 mm, 5.0 µm) from Thermo Fisher Scientific, both operated at a flow rate of 0.2 mL min^{-1}. The chromatographic parameters are described by (Schwarzenbolz et al., 2016).

5.2.3.3 Dicarbonyl Compounds

The 1,2-dicarbonyl compounds were analyzed as their respective quinoxalines after derivatization with OPD (orthophenylenediamine). One milliliter of sample diluted to 30% m v^{-1} was mixed with 0.6 mL of an aqueous OPD solution at 0.2% m v^{-1} and left at room temperature for 12 hours, in the absence of light. The solution was filtered through membrane filters with a pore size of 0.45 µm, and 20 µL of the filtrate was injected into the equipment.

An HPLC system was used, consisting of an HPLC pump K1001, a solvent organizer K1500, a UV detector K2500, and an automatic sampler Basic Marathon (all from Knauer). A flow rate of 1 mL min^{-1} was employed, using a Knauer Eurospher 100 C18 analytical column (4.6 x 250 mm, 5 µm), with a detection wavelength of 312 nm. The analysis gradient was described by Weigel; Opitz; Henle (2004). Data processing was performed using Eurochrom 2000 software.

5.2.4 Statistical Analyses

The statistical analyses were performed at a significance level of 0.05 using the Tukey test, with the aid of R software version 4.1.2 (The R® Foundation for Statistical Computing).

5.3 RESULTS AND DISCUSSION

The results described below are once again organized into two categories: protein modifications and Maillard reaction indicators.

5.3.1 Protein Modification

The results pertaining to particle size, supplementary protein denaturation, sedimentation and gel formation, infrared spectroscopy, sulfhydryl group quantification, and surface hydrophobicity are presented below.

5.3.1.1 Particle Size and Additional Protein Denaturation

Particle size analyses were performed on the product after reconstitution of the ingredients (WPI) and on the microparticulated product (MWP). The three products after reconstitution showed particle sizes of 0.48 ± 0.09 µm, 0.59 ± 0.13 µm, and 0.53 ± 0.11 µm for WPI-WOC, WPI-WL, and WPI-WGG, respectively. Since the microparticulation process results in thermal aggregation and the subsequent breakdown of these aggregates by shear rate, the newly formed aggregates are larger than the initial particles (Toro-Sierra; Schumann; Kulozik, 2013). The aggregates obtained after the microparticulation process had sizes of 2.62 ± 0.51 µm, 2.75 ± 0.73 µm, and 2.57 ± 0.59 µm for MWP-WOC, MWP-WL, and MWP-WGG, respectively. The particle size of MWPs should be between 0.1 and 10 µm; therefore, the results obtained in this study demonstrate that the breakdown of the aggregates formed during shearing was efficient, indicating good standardization of these particles (Lieske; Konrad, 1994; Renard et al., 2002). Non-uniform particle sizes may result in problems during processing and storage of the product, such as modification of sensory characteristics (Sun et al., 2015; Zhang et al., 2023).

Another important factor to determine whether the microparticulation process was efficient is the rate of additional protein denaturation. This rate is determined by quantifying the proteins before and after the microparticulation process. The denaturation rates of the formulated products in this study were 73.96 ± 2.03% for the WOC product, 73.24 ± 1.72% for the WL product, and 72.75 ± 1.42% for the WGG product. These values are higher than average values reported in the literature, such as 62.34 ± 17.10%,

where 20 commercial samples with different protein concentrations were analyzed in both aqueous and solid systems (de Paula et al., 2025). Another study demonstrated that a commercial sample provided by Fonterra Co-operative Group Ltd. for the work presented a denaturation rate close to 74%, which is in agreement with the results obtained in this study (Çakir-Fuller, 2015).

Comparing the microparticulation process among the three samples, it is possible to conclude that microparticulation was effective due to the particle sizes being within the ideal range and the protein denaturation rate being high. Furthermore, it can be observed that the use of glucose and galactose (WGG) does not alter the final product compared to the WOC and WL products.

5.3.1.2 Sedimentation and Gel Formation

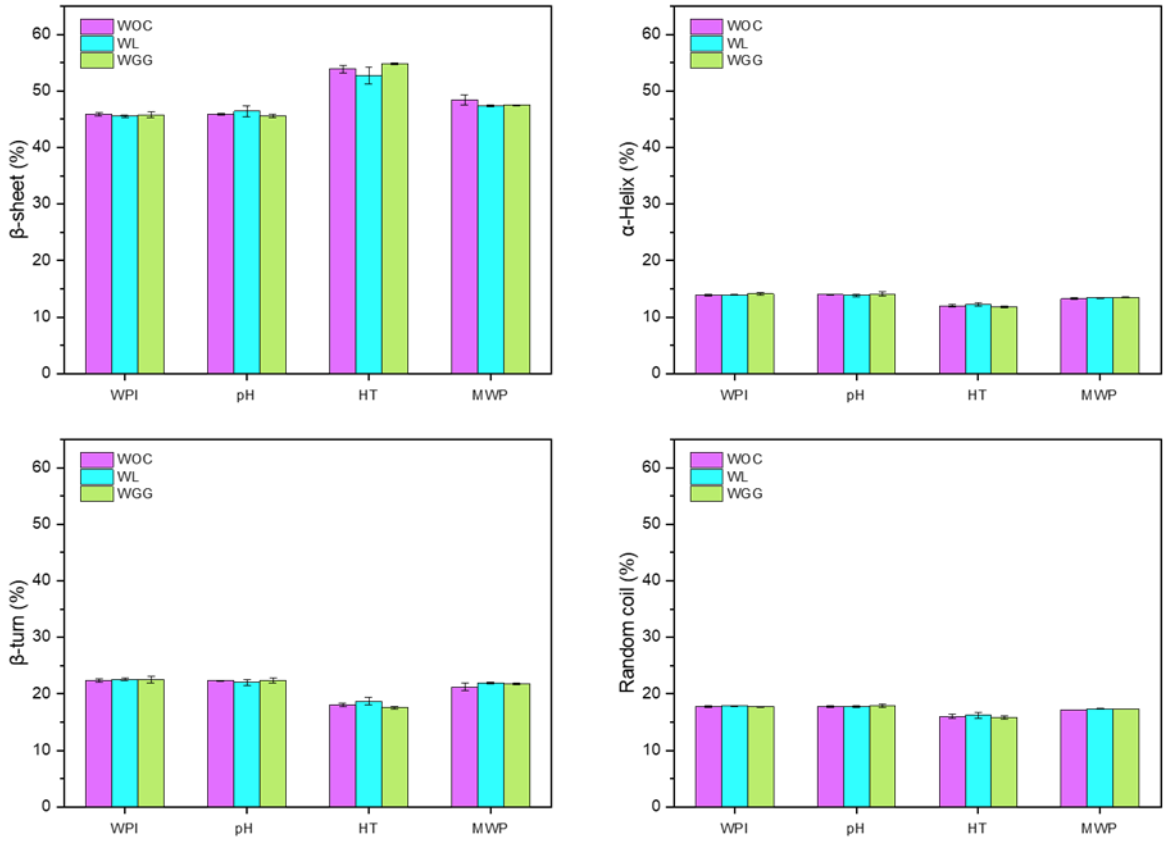
During the first two stages of the microparticulation process, the sedimentation rate remained constant among the three products, indicating no interference from the carbohydrate or the pH adjustment to 4.0 in the formation of precipitates. However, for the products subjected to thermal treatment at 95°C (HT-WOC, HT-WL, HT-WGG), the amount of sediment obtained by centrifugation increased to 33.99 ± 0.60%, 34.04 ± 1.95%, and 34.73 ± 0.32%, respectively. This sediment increase is due to protein destabilization during heat treatment (de Souza et al., 2024).

The microparticulated products, after centrifugation, exhibited gel formation. MWP-WOC showed the lowest gel mass at 79.38 ± 6.35%, while the products with added carbohydrates, WL and WGG, presented values of 87.96 ± 3.13% and 84.58 ± 4.24%, respectively. This indicates a slight influence from the presence of carbohydrates, though not from the type used, on the amount of gel formed. Gel formation may be associated with various factors such as heating temperature, pH, and protein concentration, which, after centrifugation, may become high enough to promote network formation (Bordenave-Juchereau et al., 2005; Sağlam et al., 2012). Additionally, other studies suggest that MWP can form gels, and their stability depends on the treatment applied and the size of aggregates formed during microparticulation (Dissanayake; Liyanaarachchi; Vasiljevic, 2012; Torres et al., 2016).

5.3.1.3 Deconvolution

Figure 7 represents each secondary structure of the microparticulated proteins at different stages of microparticulation in the region from 1600 cm^{-1} to 1700 cm^{-1}.

Figure 7 - Deconvolution of each secondary structure of microparticulated proteins with different carbohydrate compositions at each stage of microparticulation.



Legend: WOC – without carbohydrate; WL – with lactose; WGG – with glucose and glicose; WPI – whey protein isolate; HT – heat treatment; MWP – mcroparticulated whey protein

Source: prepared by the author.

This region was chosen because it corresponds to the Amide I mode, which shows the most significant changes in the spectrum (Zhao et al., 2020). The percentage of each secondary structure changed upon thermal treatment but returned to values close to the initial ones after shear under heating. For the β-sheet structure, the value increased with heating, while the percentages of the other three conformations decreased. It is also

evident that the use of different carbohydrate compositions and concentrations did not affect the secondary structure percentages when each stage was analysed individually. One study showed that heating a WPC solution from 30 °C to 60 °C increased the β-sheet percentage from 40.4% to 62.3%, while the other three structures decreased (Hussain et al., 2012). The main reason for this increase in β-sheet content is due to the denaturation and aggregation of whey proteins, mainly β-lactoglobulin (Panick; Malessa; Winter, 1999).

With the reduction of pH to 4, modifications in the proportions of the protein secondary structures could be expected, since this pH value is below the isoelectric point of some whey proteins. However, the values observed were unchanged. Tomczyńska-Mleko et al., (2014) studied changes in these conformations in WPI by varying the pH from 3 to 10, and statistical differences were only observed at pH 6.

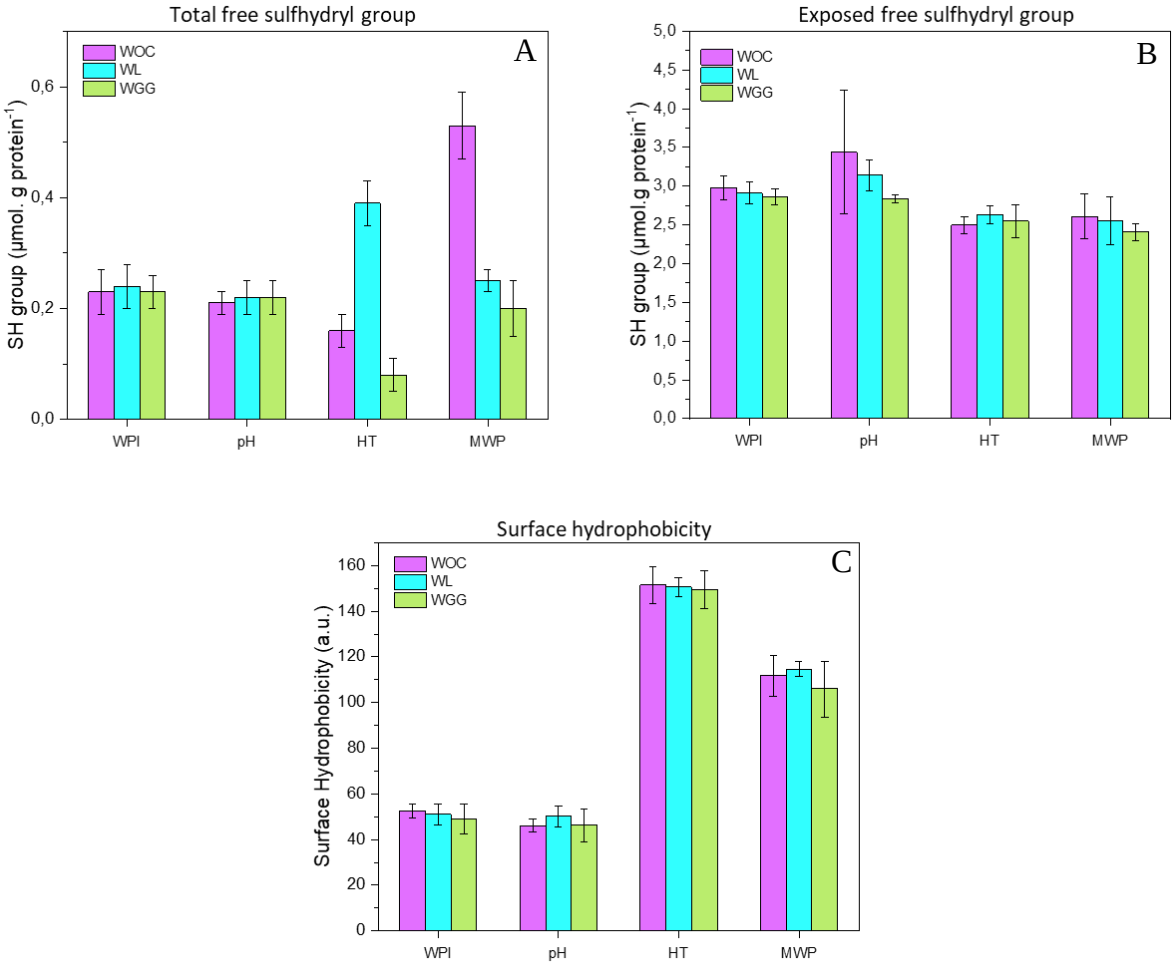
5.3.1.4 Free Sulfhydryl Groups

Free and total sulfhydryl groups were analyzed in all three samples at each stage of the microparticulation process (Figure 8.A and 8.B). The difference between the two analyses lies in the use of urea in the total free SH group quantification, which allows for full unfolding of the protein chain (Ma et al., 2024b). For the total free sulfhydryl groups (Figure 8.A), the first two stages (WPI and pH) showed similar results. However, after heating to 95 °C, changes in sulfhydryl concentrations were observed. The HT-WL product showed an increase in SH group concentration, while HT-WOC and HT-WGG exhibited a decrease, with the reduction being more pronounced in HT-WGG. This decrease may be related to chemical interactions between proteins, whereas in the presence of lactose, the release of sulfhydryl groups is favoured and their participation in disulfide bond formation is reduced (Wang et al., 2012). After applying shear under heating, the carbohydrate-containing products (MWP-WL and MWP-WGG) returned to their initial SH values, whereas the product without carbohydrate (MWP-WOC) showed an increase in exposed sulfhydryl content.

For the exposed free sulfhydryl group (Figure 8.B), pH adjustment resulted in an increase in values for the pH-WOC and pH-WL products, whereas the pH-WGG product remained relatively unchanged. This stability may be attributed to the protective effect of glucose on proteins through non-covalent interactions and steric exclusion (Velusamy; Palaniappan, 2016). Following thermal treatment (HT and MWP stages), a decrease in

exposed free SH groups was observed, particularly in samples without added carbohydrates (HT-WOC and MWP-WOC). This reduction may be associated with an increased formation of disulfide bonds during protein aggregation, as similarly reported by Wang et al., (2012) in studies involving soy protein heated to 90 °C.

Figure 8 - Results of exposed free sulfhydryl groups, total free sulfhydryl groups, and surface hydrophobicity for the microparticulated samples.



Legend: WOC – without carbohydrate; WL – with lactose; WGG – with glucose and glicose; WPI – whey protein isolate; HT – heat treatment; MWP – mcroparticulated whey protein

Source: prepared by the author.

5.3.1.5 Surface Hydrophobicity (S_0)

Another technique used to study protein modifications is the quantification of surface hydrophobicity (Figure 8.C). Measurements were performed on the three samples across the four analysis groups. In the first two sample sets, no statistically significant differences were observed. However, with thermal treatment, the surface hydrophobicity value increased approximately fourfold. This increase is associated with protein denaturation caused by the heat treatment, as the hydrophobic groups that were previously buried within the protein structure become exposed after heating (Moro; Gatti; Delorenzi, 2001; Tian et al., 2023).

Studies show that microparticulated products exhibit higher surface hydrophobicity values than their counterparts before microparticulation. Sun et al., (2015) demonstrated that at two different production times (4 and 8 hours), the values increased 2x and 3x, respectively, while in this work the values increased by approximately 3x.

Another interesting factor is the reduction in surface hydrophobicity between the HT and MWP products for all three treatments. One hypothesis is that the aggregates broken down by shear reduce the exposure of hydrophobic groups on the denatured proteins, in addition to causing structural reorganization of the proteins, as observed through deconvolution and in the study conducted on soy protein by Wang et al., (2014)

5.3.2 Maillard Reaction Modifications

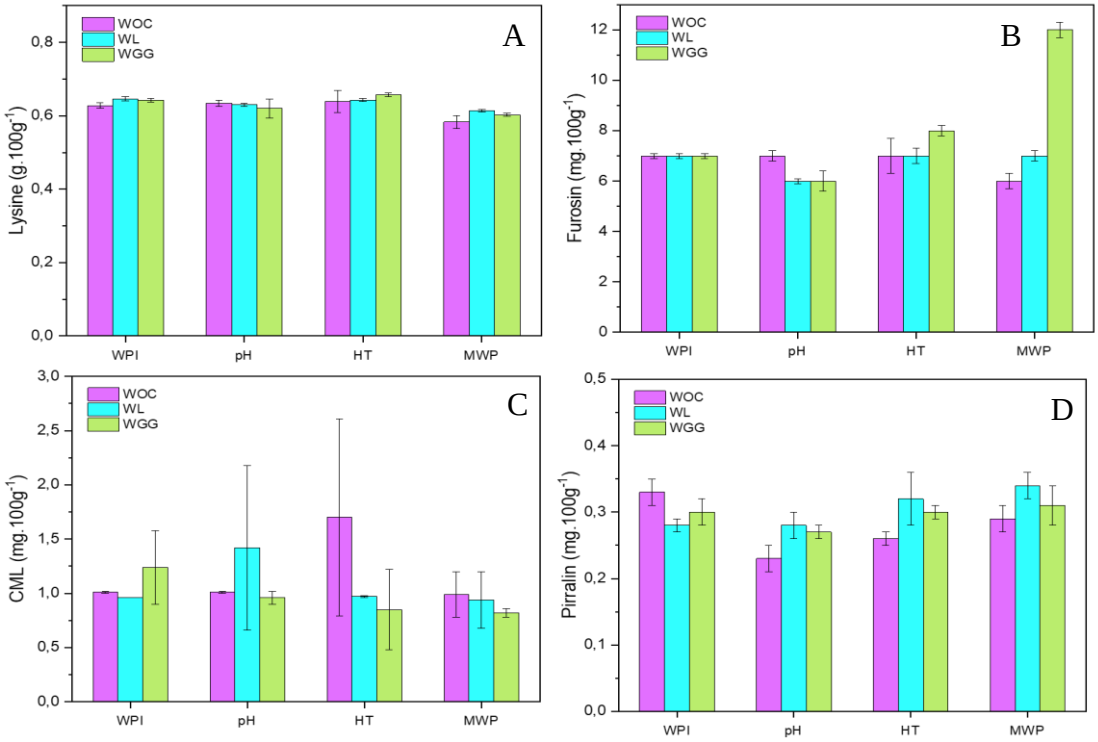
The results regarding lysine, furosine, CML, pyrraline, and dicarbonyl compounds are presented below.

5.3.2.1 Lysine

Lysine concentration was measured in each of the three treatments across the four stages of the microparticulation process (Figure 9.A). Lysine is the main amino group involved in the Maillard reaction due to its high reactivity with reducing sugars (Hemmler et al., 2018). Literature reports indicate that approximately 10% of the amino acid content in whey protein concentrate (WPC) is lysine, which is consistent with the results observed in this study. The protein content of the solutions was 6 g $100g^{-1}$, and the measured lysine concentration was approximately 0.6 g $100g^{-1}$ (Tunick et al., 2016). Lysine levels

remained relatively stable throughout each stage of microparticulation, except in the MWP product, where a slight decrease was observed. This reduction may be attributed to lysine loss during the 10-minute heat treatment, as the Maillard reaction progressed (Desrosiers; Savoie, 1991).

Figure 9 - Results of lysine, furosine, CML, and pyrraline analyses for the microparticulated samples.



Legend: WOC – without carbohydrate; WL – with lactose; WGG – with glucose and glicose; WPI – whey protein isolate; HT – heat treatment; MWP – mcroparticulated whey protein

Source: prepared by the author.

5.3.2.2 Furosine

Another Maillard reaction indicator quantified in this study was furosine (Figure 9.B). This compound is commonly used as a marker of heat damage in various foods and is formed through the interaction of amino groups (such as those found in lysine) with reducing carbohydrates (Erbersdobler; Somoza, 2007). In nearly all treatments, furosine concentration remained relatively constant, ranging from 6 to 8 mg 100 g^{-1}. However, in the MWP-WGG product, the concentration of this marker was nearly double, reaching

12 mg 100 g^{-1}. The higher furosine content in this product may be associated with a greater concentration of reducing sugars and the slight decrease in lysine during heat treatment, which favors the formation of this compound (Desrosiers et al., 1989). Pyo et al., (2016) observed that adding glucose to WPC increased furosine levels from 10.83 mg g^{-1} of dry matter to 14.54 mg g^{-1} after heating at 55 °C for 7 days.

5.3.2.3 Nε-(Carboxymethyl)lysine (CML) and Pyrraline

CML is an advanced glycation end-product (AGE) of the Maillard reaction, formed through the interaction between lysine and reducing sugars present in the matrix. It is considered a reliable marker of this reaction due to its stability (Nguyen; Van Der Fels-Klerx; Van Boekel, 2014). The CML (Figure 9.C) concentrations observed in this study did not differ significantly among samples and remained below 3 mg g^{-1}, indicating minimal formation of this compound. Another advanced Maillard reaction product quantified in this study was pyrraline, which is also used as an indicator of heat damage and protein degradation in foods (Liang et al., 2016). Similar to CML, pyrraline concentrations were low (below 0.4 mg 100 g^{-1}) and showed no significant differences among the products analysed (Figure 9.D).

It was expected that the presence of reducing sugars in the samples would increase concentrations of these compounds after thermal treatment (Nielsen et al., 2022). Moreover, the low levels of both CML and pyrraline may suggest preservation of the nutritional value of the proteins in the samples, particularly lysine, as discussed in Section 3.2.1 (Tamanna; Mahmood, 2015; Xiong et al., 2024)

5.3.2.4 Dicarbonyl Compounds

Table 12 presents the results of the quantification of dicarbonyl compounds at different stages of the microparticulation process for each of the three treatments. The compounds measured were 3-DG (3-deoxyglucosone), 3-DGal (3-deoxygalactosone), 3-DLac (3-deoxylactulosone), and lactulose. The first three compounds are formed through the degradation of Amadori products, which occur during the intermediate stage of the Maillard reaction (Cui et al., 2021). Each compound is associated with the presence of specific carbohydrates: 3-DG is formed in the presence of glucose, 3-DGal with galactose, and 3-DLac with lactose (Hayase, 2000; Hellwig; Degen; Henle, 2010).

Lactulose, on the other hand, is formed during the heating of foods containing lactose, as a result of lactose isomerization. It may contribute to the Maillard reaction, mainly by acting as a precursor of 3-DLac (Adachi; Patton, 1961; Zimmer et al., 2017).

Table 12 - Results of dicarbonyl compound analyses (mg kg^{-1}).

Sample		3-DG	3DGal	3-DLac	Lactulose
WOC	WPI	-	-	2.0 ± 0.1^{c}	-
	pH	-	-	1.9 ± 0.1^{c}	-
	HT	-	-	1.9 ± 0.1^{c}	-
	MWP	-	-	2.0 ± 0.1^{c}	-
WL	WPI	-	-	2.4 ± 0.1^{b}	6.8 ± 0.4^{b}
	pH	-	-	2.3 ± 0.1^{b}	6.9 ± 0.1^{b}
	HT	-	-	2.4 ± 0.1^{b}	6.8 ± 0.1^{b}
	MWP	1.1 ± 0.1^{b}	0.7 ± 0.1^{b}	2.7 ± 0.1^{a}	8.3 ± 0.5^{a}
WGG	WPI	$0.8 \pm 0,1^{b}$	0.7 ± 0.1^{b}	1.8 ± 0.1^{c}	-
	pH	-	-	1.9 ± 0.1^{c}	-
	HT	-	-	1.9 ± 0.1^{c}	-
	MWP	3.3 ± 0.1^{a}	4.1 ± 0.1^{a}	2.0 ± 0.1^{c}	-

Legend: WOC – without carbohydrate; WL – with lactose; WGG – with glucose and glicose; WPI – whey protein isolate; HT – heat treatment; MWP – mcroparticulated whey protein; 3-DG – 3-deoxyglucosone; 3-DGal – 3-deoxygalactosone; 3-DLac – 3-desoxylactosulose

Means followed by the same lowercase index on the same column do not differ from each other ($p>0.05$).

Source: prepared by the author.

In the MWP-WL sample, the formation of 3-DG and 3-DGal was observed, which may indicate the conversion of lactose into glucose and galactose, subsequently transformed into these two dicarbonyl compounds. Another notable finding regarding 3-DG and 3-DGal is that their concentrations increased by 4.1-fold and 5.8-fold, respectively, when comparing the WPI-WGG and MWP-WGG products. This increase is likely attributed primarily to the thermal treatment applied (Aasmul-Olsen et al., 2024).

No significant differences were observed in the concentration of 3-DLac among the products. However, lactulose was only detected in the WL samples, where lactose was added to the formulation. A similar trend was observed for lactulose concentration compared to 3-DG and 3-DGal, with the microparticulated product (MWP-WL) showing higher levels than other stages of microparticulation. This increase may again be associated with the thermal treatment applied.

5.4 CONCLUSION

The aim of this study was to assess whether the presence of different carbohydrates could influence the microparticulation process in terms of protein modifications and the Maillard reaction. All microparticulated samples exhibited similar particle size distribution and thermal denaturation rates, indicating a standardized microparticulation process across treatments. Analysis of total exposed sulfhydryl groups showed that the absence of added carbohydrates preserved these active groups for disulfide bond formation, which may lead to stability and application issues in the final products. Additionally, surface hydrophobicity correlated with protein secondary structure: as β-sheet conformation increased (in HT products), surface hydrophobicity also increased, returning to near-initial values at the end of the microparticulation process (MWP).

Regarding Maillard reaction markers, lysine, CML, and pyrraline concentrations remained stable across all products, suggesting that neither carbohydrate type nor microparticulation stages significantly influenced these compounds. However, furosine concentration was notably higher in the MWP-WGG product, likely due to the presence of reducing sugars such as glucose and galactose, in combination with protein.

Finally, dicarbonyl compounds were quantified, and results showed that the use of glucose and galactose increased 3-DG and 3-DGal concentrations in the MWP-WGG product compared to WPI-WGG. Lactulose was detected only in the WL samples-where lactose was added-and was found in higher amounts in the MWP-WL product, again likely due to thermal treatment.

Overall, these findings suggest that the use of carbohydrates is important for the microparticulation process, but the specific type used does not significantly affect the manufacturing steps. The development of lactose-free MWP may offer a range of applications, particularly in food products targeted at lactose-intolerant consumers.

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6 PRODUCTION OF HYBRID MICROPARTICULATE PROTEIN BASED ON WHEY PROTEIN AND PEA PROTEIN. VARYING HYDROGEN POTENTIAL AND ACID DURING MANUFACTURING

The results obtained in this section will be published as a patent in collaboration with UFJF, due to the fact that the hybrid proteins represent a scientific and industrial innovation on a global scale.

6.1 INTRODUCTION AND AIM

Over the years, there has been an increase in the number of individuals who are overweight or obese around the world. As a result, government agencies have started to seek mechanisms to address these conditions. One alternative is the production of foods with reduced calorie values, with fat being the primary macronutrient to be reduced in these cases (Melnikova et al., 2015; Sun et al., 2015; Tobin et al., 2010).

The removal of fat can lead to texture problems, stability during shelf-life, changes in taste, and the physical appearance of food products. Proteins can be used as a substitute for fat since they can increase satiety, thus reducing food intake (Chung et al., 2014; Sánchez-Obando et al., 2020).

Whey proteins are already widely used by the food industry due to their functional properties. However, they have a high rate of thermal denaturation, which can lead to issues with the product, such as phase separation and precipitation (Çakir-Fuller, 2015; Dissanayake et al., 2012). In light of this problem, the food industry employs the technique of microparticulation to substitute fat with protein, as microparticulation ensures that proteins exhibit characteristics similar to those of fat, including rheological properties (Çakir-Fuller, 2015; Torres et al., 2011).

In the literature, the use of microparticulated whey protein (MWP) in yogurt, cheese, salad dressing, and ice cream has been reported (Sánchez-Obando et al., 2020; Sun et al., 2015). However, there are few studies reported on the use of microparticulated pea proteins (Tanger et al., 2022).

Microparticulated proteins (MP) are produced through thermal treatment, shear rate, pH adjustment, and protein and salt concentration (Nunes; Tavares, 2019; Sampson; Cooke, 1992; Toro-Sierra et al., 2013). MPs are considered a mixture containing native proteins and aggregates formed during the process, with a final size ranging from 0.1 to

10 μm, exhibiting particle sizes similar to fat globules. They are employed as fat substitutes or to increase protein content in foods (Lieske; Konrad, 1994; Renard et al., 2002).

Therefore, the aim of this study is to develop a methodology for microparticulation of whey protein and a hybrid protein based on WPC and pea protein, while varying the pH value and type of acid used. This involves monitoring the particle size distribution at each stage of the process and quantifying α-lactalbumin (α-lac) and β-lactoglobulin (β-lg) before and after microparticulation.

6.2 METHODOLOGY

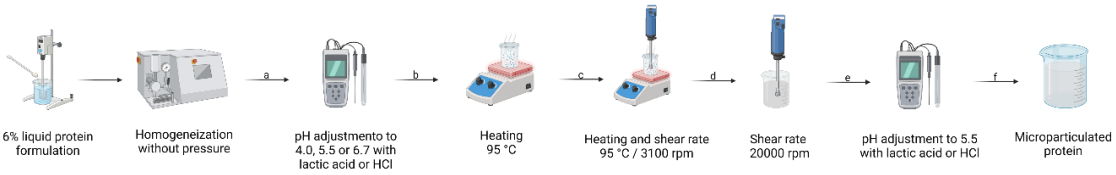
Two types of microparticulate proteins were produced (control and hybrid). For the production of the control, only WPC80% was used as the protein source, while for the hybrid product, WPC80% and pea protein were utilized. The microparticulates were produced at three different pH values (4.0, 5.5. and 6.7). The pH was adjusted using two different acids (37% hydrochloric acid (m v^{-1}) and 84% lactic acid (m v^{-1})), and when necessary, 50% sodium hydroxide (m v^{-1}) was used. A total of 12 products were produced by applying different pH conditions, types of acid, and protein sources, and the experiments were conducted in triplicate.

Initially, 55.17 g of WPC80%, 34.83 g of whey permeate, and 639.17 g of deionized water were weighed for the control. Now for the hybrid were weighed, 27.59 g of WPC80%, 26.69 g of pea protein, 35.73 g of whey permeate, and 639.17 g of deionized water. The powders were mixed with each other and then added to water, maintained under constant agitation for 1 hour. The control solution under these conditions contained 12% total solids (m m^{-1}) and 6% protein (m m^{-1}). It was stored at a temperature of 7 ± 2 °C for 24 hours to ensure better reconstitution of the powders. This procedure was performed in triplicate. Figure 10 represents the microparticulation steps for the products.

The homogenization step was performed to ensure the best reconstitution of the powders. The pH adjustment was carried out using lactic acid, hydrochloric acid, or sodium hydroxide to achieve one of the three target pH levels (4.0. 5.5. and 6.7). After pH adjustment, the solution was heated in a process simulator until it reached a temperature of 95 °C, and then a shear rate of 3100 rpm was applied with heating for 10 minutes. Following this step, the product underwent a shear rate of 20000 rpm for 5

minutes using an Ultra-Turrax T25. Finally, the pH was adjusted to 5.5 (close to the initial solution's pH) using lactic acid, hydrochloric acid, or sodium hydroxide.

Figure 10 - Production of microparticulated protein.



Source: prepared by the author.

6.2.1 Particle Size Analysis

The particle size distribution of the control and hybrid proteins was performed using a laser diffraction particle size analyzer LS 13 320 (Beckman Coulter), coupled with the Aqueous Liquid Module (Beckman Coulter).

The particle size was determined at each of the stages shown in Flowchart 1. Data were collected in the range from 0.375 µm to 2000 µm, with a collection time of 100 seconds. The results were obtained using a refractive index of 1.333 for the dispersing medium. Since the refractive index values of the samples are not yet known, the Fraunhofer approximation was used to obtain the data, ensuring the reliability of the collected data.

6.2.2 Quantification of α-lactalbumin and β-lactoglobulin

The separation and quantification of the analytes of interest were carried out using the high-performance liquid chromatography (HPLC) system Waters, model 1252, detector UV-Vis (DAD – Diode Array Detector) and equipped with a binary pump. The chromatographic separation employed a Phenomenex column Luna 5µ (250 mm × 4.6 mm) 100A, kept at 40 °C. The injection volume was 20 µL, the β-lactoglobulin and α-lactoglobulin was detected at a wavelength of 214 nm (de Paula et al., 2025).

Gradient elution was carried out by a mixture of two solvents at a flow rate of 0.5 mL min-1. The mobile phase consisted of 0.1% TFA in ultrapure water in solvent A and 0.09% TFA in 90% acetonitrile and 10% ultrapure water in solvent B. A linear gradient

program was initiated as follows: 0-3 min, 20%-40% B; 3-5 min, 45%-45% B; 5-7 min 45%-50% B; 7-9 min, 50%-80% B; 9-14 min. 80%-20% B and 14-15 min, 20%-20%.

The samples before and after the centrifugation process were centrifuged using a Sigma 2K15 centrifuge at 22.000 × g for 10 minutes at 5 °C. After the centrifugation process, all samples were filtered through 0.22 µm pore size syringe filters for subsequent analysis on the liquid chromatograph. Depending on the protein fraction content determined by the analysis, the samples were diluted, when necessary, to meet the working range corresponding to the validated analytical curve.

6.2.3 Percentage of additional protein denaturation

The percentage of protein denaturation was calculated based on the results obtained from the quantification of each protein fraction before and after the protein microparticulation process. According to the equation below (Çakir-Fuller, 2015).

$$\%\ denaturation = \left(1 - \frac{microparticulated\ whey\ protein}{whey\ protein}\right) x\ 100 \qquad (8)$$

Considering that microparticulate is the concentration of each protein fraction after the microparticulation process and not microparticulate is the concentration of each protein fraction before the microparticulation process.

6.2.4 Statistical Analysis

The statistical analyses were conducted at a significance level of 0.05 using the Tukey test, applied with the assistance of R software version 4.1.2 (The R® Foundation for Statistical Computing).

6.3 RESULTS AND DISCUSSION

The results pertaining to particle size and the HPLC-determined concentrations of whey protein fractions for both ingredients are presented below.

6.3.1 Control product

As seen in Figure 10, as demonstrated in the methodology, six samples were collected at different stages during the microparticulation process, represented by numbers 1 to 6.

Table 13 - Average particle sizes (Dv50) for the control product in µm.

	Hydrochloric acid		
Sample	pH = 4.0	pH = 5.5	pH = 6.7
1	7.04 ± 1.49^{b}	12.28 ± 2.44^{a}	8.73 ± 1.54^{b}
2	8.80 ± 2.38^{a}	10.74 ± 3.64^{a}	9.46 ± 2.65^{a}
3	36.02 ± 2.30^{c}	52.04 ± 2.35^{b}	82.99 ± 18.65^{a}
4	4.45 ± 0.16^{c}	27.20 ± 1.71^{b}	72.26 ± 17.14^{a}
5	4.34 ± 0.14^{c}	10.80 ± 0.51^{b}	14.92 ± 4.41^{a}
6	4.36 ± 0.07^{c}	10.74 ± 0.61^{b}	14.07 ± 2.40^{a}
	Lactic acid		
Sample	pH = 4.0	pH = 5.5	pH = 6.7
1	10.62 ± 3.76^{ab}	11.61 ± 2.59^{a}	7.54 ± 0.83^{b}
2	4.21 ± 2.28^{c}	8.57 ± 0.45^{b}	12.08 ± 1.41^{a}
3	42.42 ± 2.59^{b}	46.83 ± 8.50^{b}	55.93 ± 3.09^{a}
4	4.79 ± 0.27^{c}	28.59 ± 5.81^{b}	61.25 ± 9.61^{a}
5	4.55 ± 0.37^{c}	8.03 ± 0.57^{b}	13.55 ± 0.66^{a}
6	4.88 ± 0.38^{c}	8.47 ± 1.07^{b}	12.93 ± 0.51^{a}

Legend: 1 - after homogenization; 2 - pH adjustment; 3 - reached 95 °C; 4 - shearing at 3100 rpm at 95 °C for 10 min; 5 - shearing at 2000 rpm for 5 min; 6 - after pH adjustment to 5.5.

Means followed by the same lowercase index on the same column do not differ from each other ($p>0.05$).

Source: prepared by the author.

Sample 1 corresponds to the product coming out of the homogenization process, sample 2 is after the pH adjustment to 4.0, 5.5. or 6.7, sample 3 is after heating at 95 °C,

sample 4 is after a shear rate of 3100 rpm with heating, sample 5 is after shear at 20000 rpm, and finally, sample 6 is after pH adjustment to 5.5, thus concluding the process. The particle size analysis aimed to determine the average size of the particle size distribution and the percentage of particle sizes below 20 µm, which is the threshold for sandy perception in the human palate (Toro-Sierra et al., 2013).

Table 13 represents the average particle size distribution for the control product. For the control produced with HCl, it can be observed that sample 1 at pH 5.5 is statistically different from the other two treatments; however, with the pH adjustment, all products are statistically equal. After the heat treatment, the lower the pH value, the smaller the average particle size, indicating that pH can modify the size of aggregates formed during heating. This effect can also be associated with the percentage of protein denaturation during heating. As shear was applied, the average size continued to decrease, but the effect of the solution's pH was still noticeable. At the end of the microparticulation process, all three treatments were statistically different from each other, and the one at pH 4.0 resulted in the smallest average size (Spiegel; Huss, 2002).

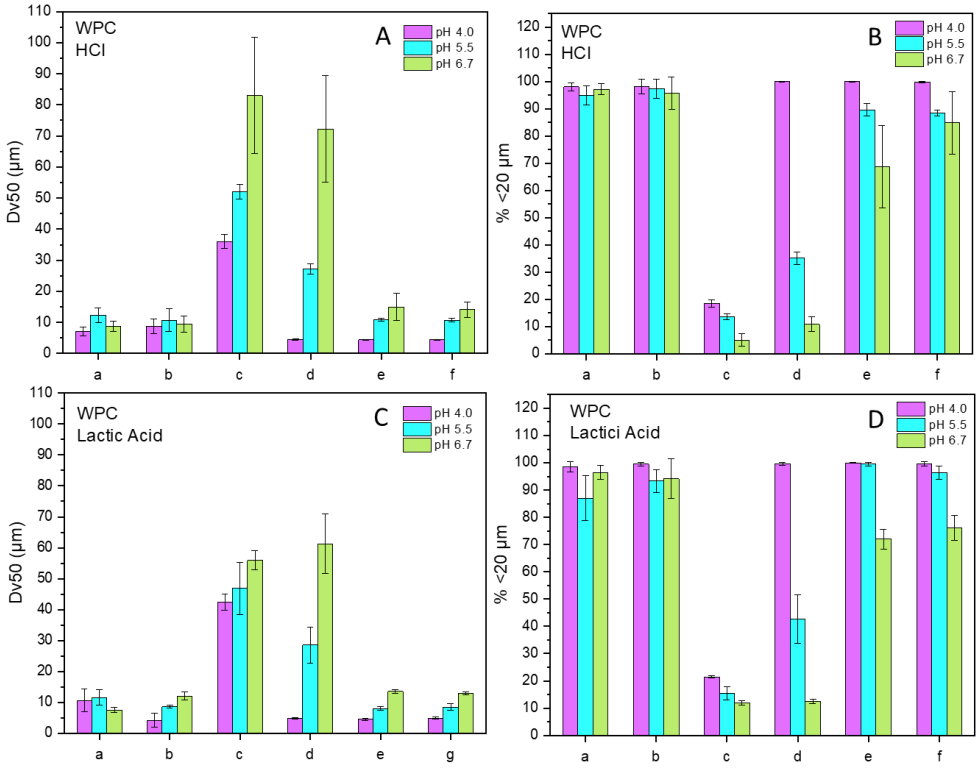
With the product made with lactic acid, the same pattern of particle size distribution was observed, confirming the pH's influence on modulating aggregate size.

With Figure 11, analyzing the percentage of particles with sizes <20 µm, the heat treatment generated approximately 25% of particles below the threshold value for all treatments, which is consistent with the data observed for the average size. After microparticulation completion, the product at pH 4.0 had the best percentage of particles below <20 µm. Therefore, for the control, when considering only the effect of pH in the microparticulation process, it is possible to state that the products at pH 4.0 performed better than those at pH 5.5 and 6.7.

Table 14 represents the concentration of α-lac and β-lg in the control samples before and after the microparticulation process. The concentration of α-lac is lower than the concentration of β-lg, which is expected due to the higher concentration of β-lg compared to α-lac in whey protein (Bonnaillie; Tomasula, 2008; Tavares, 2020). Another factor that has been observed previously is the reduction in the concentration of both proteins before and after the microparticulation process.

The products at pH 5.5 and 6.7 for α-lac showed concentrations below the quantification limit, while at pH 4.0, it was possible to quantify this protein fraction. These results indicate that pH does indeed influence the microparticulation process (Spiegel; Huss, 2002).

Figure 11 - Particle size distribution for the control product in hydrochloric acid (A and B) and in lactic acid (C and D).



Legend: 1 - after homogenization; 2 - pH adjustment; 3 - reached 95 °C; 4 - shearing at 3100 rpm at 95 °C for 10 min; 5 - shearing at 2000 rpm for 5 min; 6 - after pH adjustment to 5.5.

Source: prepared by the author.

For α-lac, the products at pH 4.0 showed denaturation of approximately 62% with lactic acid and around 76% with hydrochloric acid. However, for all the other four treatments, this percentage was over 99%, considering the quantification limit for this calculation, β-lg exhibited similar protein denaturation for all treatments.

With that in mind, it's worth noting that the choice of the best method for this microparticulation process would be the one with pH values varying around 5.5, regardless of the chosen acid, considering both particle size and protein denaturation rate

Table 14 - Concentration and percentage of denaturation of α-lac and β-lg in the control samples.

Sample	α-lac Before (g 100g^{-1})	α-lac After (g 100g^{-1})	α-lac % of protein denaturation	β-lg Before (g 100g^{-1})	β-lg After (g 100g^{-1})	β-lg % of protein denaturation
pH 4.0 LA	6.23 ± 0.31	2.35 ± 0.25	62.33 ± 2.23	26.77 ± 2.22	0.96 ± 0.25	96.42 ± 0.82
pH 5.5 LA	5.86 ± 0.31	<LOQ	>99	24.58 ± 1.49	1.11 ± 0.28	95.46 ± 1.13
pH 6.7 LA	5.65 ± 0.38	<LOQ	>99	24.87 ± 1.93	1.51 ± 0.69	94.05 ± 2.42
pH 4.0 HA	6.95 ± 0.50	2.40 ± 0.63	65.1 ± 9.9	27.62 ± 2.33	0.90 ± 0.07	96.72 ± 0.27
pH 5.5 HA	6.57 ± 0.68	<LOQ	>99	24.89 ± 1.77	1.19 ± 0.22	95.21 ± 0.19
pH 6.7 HA	6.07 ± 0.27	<LOQ	>99	27.13 ± 1.82	1.37 ± 0.51	94.85 ± 1.55

Legend: LA - lactic acid; HA - hydrochloric acid; LOQ - limit of quantification.
Source: prepared by the author.

6.3.1 Hybrid product

As noted for the control product, after homogenization, the samples produced with hydrochloric acid did not show statistical differences. With the pH adjustment, the product at pH 6.7 was statistically different from the others (Table 15).

Upon heating the products to 95 °C, there was a differentiation in the distribution, with the treatment at pH 5.5 having the largest size, indicating the formation of larger aggregates. After the end of shear rate application and the pH adjustment to 5.5, the final average particle sizes were similar to each other and below 20 µm, resulting in efficient microparticulation for all treatments. Analysing the percentage of particles below 20 µm, it can be observed that the treatments that generated the highest percentage were at pH 4.0 and 5.5.

Transitioning to the product made with lactic acid, the same correlation can be observed in the samples after heating, indicating that the acid assists in modulating the aggregates formed. At the end of the microparticulation process, the treatments at pH 5.5 and pH 6.7 were statistically different from each other. Analysing the percentage of particles below 20 µm, it can be observed that the treatments that generated the highest percentage were at pH 4.0 and 5.5 (Figure 12).

For the hybrid proteins, considering only the effect of pH in the microparticulation process, it can be stated that the products at pH 4.0 and 5.5 performed better than the product at pH 6.7.

Table 15 - Average particle sizes (Dv50) for the hybrid product in µm.

	Hydrochloric acid		
Samples	pH = 4.0	pH = 5.5	pH = 6.7
1	14.39 ± 3.40^{a}	16.83 ± 1.50^{a}	16.98 ± 1.28^{a}
2	17.76 ± 0.69^{b}	17.86 ± 4.58^{b}	29.71 ± 5.45^{a}
3	16.61 ± 0.96^{c}	33.90 ± 0.40^{a}	24.44 ± 1.82^{b}
4	12.21 ± 0.31^{b}	12.96 ± 0.28^{a}	10.84 ± 0.55^{c}
5	17.55 ± 1.65^{a}	10.33 ± 0.52^{b}	11.67 ± 0.92^{b}
6	11.81 ± 1.64^{a}	9.95 ± 0.64^{b}	10.78 ± 0.30^{ab}
	Lactic acid		
Samples	pH = 4.0	pH = 5.5	pH = 6.7
1	15.83 ± 4.27^{a}	16.76 ± 0.66^{a}	18.55 ± 2.30^{a}
2	20.44 ± 2.61^{a}	19.11 ± 5.76^{a}	22.88 ± 2.20^{a}
3	15.81 ± 0.77^{c}	31.03 ± 1.92^{a}	27.59 ± 3.57^{b}
4	16.35 ± 1.76^{a}	12.08 ± 1.04^{b}	11.29 ± 0.68^{b}
5	17.66 ± 1.50^{a}	10.23 ± 0.49^{b}	11.03 ± 0.46^{b}
6	11.05 ± 1.54^{ab}	10.55 ± 0.19^{b}	11.97 ± 0.44^{a}

Legend: 1 - after homogenization; 2 - pH adjustment; 3 - reached 95 °C; 4 - shearing at 3100 rpm at 95 °C for 10 min; 5 - shearing at 2000 rpm for 5 min; 6 - after pH adjustment to 5.5.

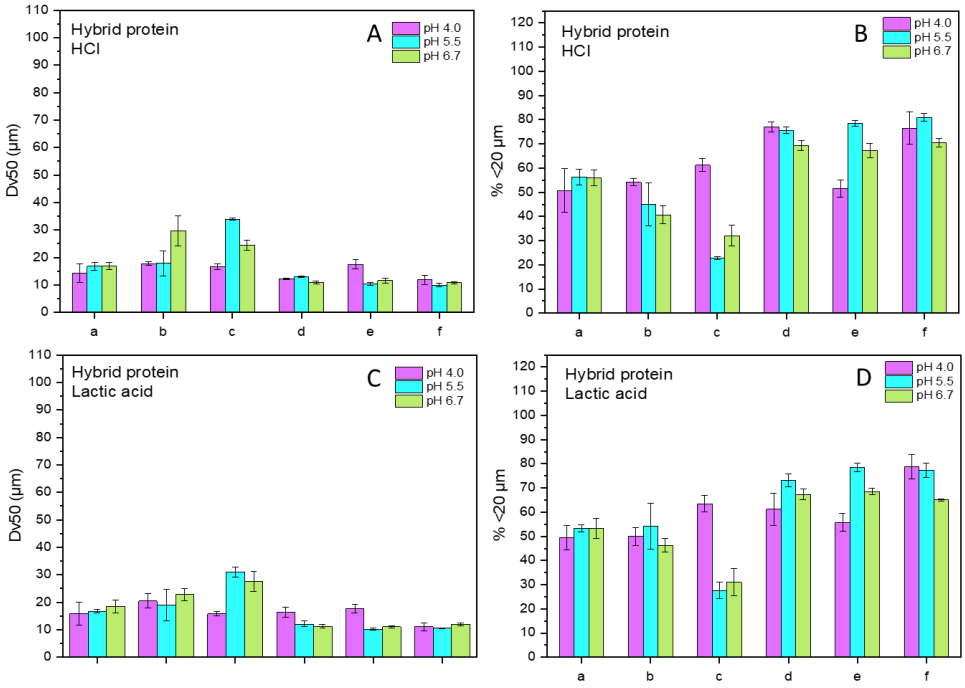
Means followed by the same lowercase index on the same column do not differ from each other ($p>0.05$).

Source: prepared by the author.

The same two protein fractions were quantified for the hybrid proteins (Table 16). It's worth noting that the concentration of each of these fractions was approximately half of the control product before the microparticulation process. This is justifiable because in the control product, the only protein source was WPC, while in the hybrid product, the protein source was divided between WPC and pea protein.

The same patterns reported for commercial proteins and the control product were observed, which include a higher concentration of β-lg than α-lac and a reduction in these proteins after microparticulation.

Figure 12 - Particle size distribution for the hybrid product in hydrochloric acid (A and B) and in lactic acid (C and D).



Legend: 1 - after homogenization; 2 - pH adjustment; 3 - reached 95 °C; 4 - shearing at 3100 rpm at 95 °C for 10 min; 5 - shearing at 2000 rpm for 5 min; 6 - after pH adjustment to 5.5.

Source: prepared by the author.

The denaturation rate for α-lac was approximately 76% for both treatments at pH 4.0, while for the products at other pH levels, it was higher than 99%, similar to the control product. For β-lg, the denaturation rate was similar for all treatments. The conclusion regarding the best choice should be made analogously to the products containing only WPC, as the results showed the same pattern.

Table 16 - Concentration and denaturation percentage of α-lac and β-lg in the hybrid samples.

Sample	α-lac			β-lg		
	Before ($g\ 100g^{-1}$)	After ($g\ 100g^{-1}$)	% of protein denaturation	Before ($g\ 100g^{-1}$)	After ($g\ 100g^{-1}$)	% of protein denaturation
pH 4.0 LA	2.98 ± 0.55	0.66 ± 0.14	76.73 ± 7.69	12.80 ± 1.15	0.44 ± 0.12	96.63 ± 0.65
pH 5.5 LA	3.08 ± 0.19	<LOQ	>99	11.93 ± 0.59	0.48 ± 0.11	95.92 ± 1.04
pH 6.7 LA	3.07 ± 0.30	<LOQ	>99	12.64 ± 1.20	0.37 ± 0.07	97.00 ± 0.71
pH 4.0 HA	3.08 ± 0.28	0.73 ± 0.26	76.55 ± 7.54	13.43 ± 0.85	0.35 ± 0.06	97.40 ± 0.39
pH 5.5 HA	3.16 ± 0.23	<LOQ	>99	12.67 ± 0.74	0.52 ± 0.05	95.86 ± 0.51
pH 6.7 HA	3.18 ± 0.07	<LOQ	>99	12.82 ± 0.54	0.28 ± 0.03	97.81 ± 0.05

Legend: LA - lactic acid; HA - hydrochloric acid; LOQ - limit of quantification.
Source: prepared by the author.

6.4 CONCLUSION

Proteins were microparticulated using only WPC (control) and WPC with pea protein (hybrid). Analyzing particle size and the percentage of denaturation for each of the treatments, it is possible to conclude that the best conditions for microparticulate production are at pH 5.5 using either of the two acids. This is because protein denaturation is higher than 99% for both fractions, and the average particle size is less than 20 μm. Therefore, it can be concluded that pH is a more critical factor in the microparticulation process than the type of acid used. These results are in the process of being patented in collaboration with the Federal University of Juiz de Fora (UFJF).

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7 CONCLUSION

Microparticulated proteins were developed to meet industrial demands, with the primary objective of serving as fat replacers in food products. Among the companies involved, CP Kelco, through its product *Simplesse*, was the first to enter this market. The first article (item 3) shows that the main functions of MWPs are to modify composition, modulate sensory characteristics, and improve food stability. The key attributes to be considered are particle size — which should range from 0.1 to 20 µm — and the degree of protein denaturation, as higher denaturation levels generally lead to greater product stability in future applications.

Given the importance of controlling protein denaturation, the second article (item 4) aimed to develop an HPLC-based methodology to quantify the two main protein fractions in whey. This method was successfully developed and enabled the quantification of additional denaturation rates in microparticulated whey protein samples produced by a partner industry at different concentration levels, both before and after the drying process. This methodology was also applied in other sections of this document, contributing to the development of a laboratory-scale production method for microparticulated proteins.

The third article (item 5) focused on understanding how the presence of lactose and disaccharides, after hydrolysis, influences the characteristics of microparticulated proteins. Overall, it was found that the presence of a carbohydrate in the manufacturing process is more significant than the specific type used, especially when analyzing protein denaturation rate and particle size.

Finally, item 6 of this document aimed to develop a hybrid microparticulated protein based on whey protein and pea protein, with the goal of creating a new ingredient for the food industry. It was observed that the type of acid used did not influence particle size or the denaturation rate of whey proteins. However, the pH applied during the microparticulation process proved to be a key factor in controlling these two main product characteristics.

In general, this work provided a scientific and technological foundation for a process that, although widely known internationally, had not yet been developed, studied, or applied in Brazil. The generation of knowledge between academia and local industries is the main focus of the funding program under which this thesis was developed: MAI/DAI. The MAI/DAI program aims to strengthen interactions between universities

and industry, fostering the development of new technologies in the country with the support of the funding agencies CNPq and CAPES.