Properties and stability of hybrid pea-dairy protein formulas

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Abstract

The aim of this study was to investigate the functional properties and stability of hybrid pea-dairy protein formulas. To achieve this goal, the combination of pea protein isolate (PPI) with dairy proteins such as micellar casein isolate (MCI), sodium caseinate (NaCas), whey protein aggregate (WPA) was evaluated. Samples stored for 2 and 4 weeks at 4ºC and 20ºC were visually assessed and physicochemical parameters such as color, pH, viscosity, particle size distribution (PSD) and optical microstructure were evaluated.

The results indicated that dissolving the different proteins together, rather than separately, did not display any significant differences in the examined parameters. The most stable sample with optimal viscosity and overall stability, was made with a combination of PPI:NaCas:WPA with 14% total protein in a ratio 60:05:35. This sample was further developed into a hybrid ready-to-drink (RTD) by adding fat and sweeteners.

Apart from the advantage of being able to reach high levels of protein content by combining different protein sources, this study also identified that the hybrid formula fulfilled the nutritional requirements for human growth and development, as it was determined by the protein digestibility-corrected amino acid score (PDCAAS). In addition, the hybrid RTD was found to have a sensorial difference in comparison to the reference made with the same quantity of PPI, as the sample exhibited a milder beany flavor.

This study also considered the packaging selection of the developed hybrid RTD. The packaging chosen was a carton layered with polyethylene and aluminum foil that is used as light and oxygen barrier. This packaging option was chosen due to the potential impact of light and oxygen that can enhance protein and lipid oxidation and due to the susceptibility of high protein products to age gelation that can lower the consumer acceptance.

Finally, various recommendations were made for future research in the area of hybrids, for example to conduct a more comprehensive investigation into the mechanism of protein aggregation in the mixed formulas.

Keywords: properties; stability; hybrid formula; pea-dairy; protein mixtures; development; RTD

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Table of contents

1 Aim & Objectives 13
2 Introduction 14
2.1 Moving forward to hybrid ready-to-drink beverages 14
2.2 Pea protein 16
2.3 Milk proteins 17
2.4 Mixed plant-dairy blends 18
2.5 Parameters affecting the packaging development for a hybrid RTD 20
3 Materials & Methods 21
3.1 First part: Preparation and evaluation of the mixed protein formulas 21
3.1.1 Material selection and trials for the mixed-protein formulas 21
3.1.2 Development of the mixed-protein formulas 23
3.1.3 Visual and physicochemical evaluation of samples 25
3.1.3.1 Visual assessment 25
3.1.3.2 Color 25
3.1.3.3 pH 25
3.1.3.4 Viscosity 25
3.1.3.5 Particle Size Distribution (PSD) 25
3.1.3.6 Optical microstructure 26
3.1.3.7 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis ... 26
3.2 Second part: Development of the hybrid RTD beverage 26
3.2.1 Hybrid RTD development process 27
3.2.2 Physicochemical evaluation 27
3.2.3 Protein Digestibility-Corrected Amino Acid Score (PDCAAS) 28

3.2.4 Sensory evaluation 28
3.2.5 Packaging, Labelling & Claims 28
4 Results & Discussion 29
4.1 First part: Preparation and evaluation of the mixed pea-dairy protein formulas 29
4.1.1 Visual assessment 30
4.1.2 Color 34
4.1.3 pH 36
4.1.4 Viscosity 38
4.1.5 Particle Size Distribution 41
4.1.6 Optical microstructure 44
4.1.7 Conclusions from the trials 47
4.1.8 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis 47
4.2 Second part: Development and examination of the hybrid RTD 49
4.2.1 Analyses results 50
4.2.2 Protein Digestibility-Corrected Amino Acid Score (PDCAAS) 51
4.2.3 Sensory evaluation 52
4.2.4 Packaging selection 53
4.2.5 Labelling & Claims 54
5 Conclusions 55
6 Future recommendations 57
7 References 58

List of Figures

Figure 1 SWOT analysis of mixed animal/plant foodstuff (Guyomarc'h et al., ... 16
Figure 2 Schematic representation of the behavior of plant (green triangles), casein micelles (black/white spheres) and whey (orange spheres) protein blends during heating in different conditions (Hinderink et al., 2021). 19
Figure 3 Processing steps for the mixed-protein samples preparation.................. 24
Figure 4 Processing steps for the hybrid RTD development. 27
Figure 5 Visual assessment of the samples from the trial with 12% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 2.. 31
Figure 6 Visual assessment of the samples from the trial with 14% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 4.. 33
Figure 7 The samples from the trial with 12% total protein after retort (AR) treatment. For sample codes, see Table 2... 34
Figure 8 The samples from the trial with 14% total protein after 2 weeks of storage at 4ºC and 20ºC. For sample codes, see Table 4. 35
Figure 9 Shear viscosity as a function of the shear rate of the samples from the trial with 12% total protein content after retort (AR). For sample codes see Table 2... 39
Figure 10 Average particle size of the samples from the trial with 12% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 2.. 42
Figure 11 Average particle size of the samples from the trial with 14% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 4.. 44

Figure 12 Optical microstructure of the samples S1, S2 and S3 after 2 weeks of storage at 4ºC. The scale bar represents 20 µm. For sample codes, see Table 2. .. 45
Figure 13 Optical microstructure of the samples S4-S9 after 2 weeks of storage. The images depict samples S4, S5, S6, and S9 stored at 20°C, while samples S7 and S8 stored at 4°C. The scale bar represents 20 µm. For sample codes, see Table 2..... 45
Figure 14 Optical microstructure of the samples from the trial with 14% total protein after storage. Sample/Week/Temperature/Scale bar: S20/W4/4ºC/, S16/W4/4ºC/40, S25/W2/20ºC/40, S14/W4/20ºC/40 and S22/W2/4ºC/40. For sample codes, see Table 4. 46
Figure 15 SDS-PAGE profile of PPI, PPI:WPA (60:40), and PPI:NaCas:WPA (60:10:30) in 12% total protein. 48
Figure 16 Visual assessment of the hybrid RTD after 2 weeks at 4ºC and 20 ºC.. 50
Figure 17 Particle size distribution of the sample S16 and the hybrid RTD after 2 weeks of storage at 20ºC. 51
Figure 18 Optical microstructure of the hybrid RTD after 2 weeks of storage at 4ºC and 20ºC. The scale bar represents 20 µm. 51
Figure 19 The mean scores of PPI 12% and Hybrid 14% compared to the reference made with PPI 8,4%. 52
Figure 20 The proposed packaging for the hybrid RTD. 54
Figure 21 Example of label designed for the hybrid RTD. 55

List of tables

Table 1 Raw materials specifications. 21
Table 2 Protein solutions for the trial with 12% total protein. 22
Table 3 Protein solutions prepared for the trial with 14% total protein. 22
Table 4 Samples from the trial with 14% total protein that remained stable after retort. 29
Table 5 Color of the samples from the trial with 12% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 2. 34
Table 6 Color of the samples from the trial with 14% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 4. 36
Table 7 pH of the samples from the trial with 12% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 2. 37
Table 8 pH of the samples from the trial with 14% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 4. 38
Table 9 Viscosity of the samples from the trial with 12% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 2 39
Table 10 Viscosity of the samples from the trial with 14% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 4 41
Table 11 Approximate areas obtained from the plot lanes with ImageJ. 49

Table 12 Summary of the responses received regarding the comparison of the samples with the reference. 53

List of abbreviations

AR	After Retort
α-LA	α-lactalbumin
β-LG	β-lactoglobulin
BR	Before Retort
MCI	Micellar Casein Isolate
Mw	Molecular weight
NaCas	Sodium caseinate
PPI	Pea Protein Isolate
PDCAAS	Protein Digestibility-Corrected Amino Acid Score
PSD	Particle Size Distribution
RTD	Ready-to-drink
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TPC	Total Protein Content
WPA	Whey Protein Aggregates

1 Aim & Objectives

In recent years, there has been a growing interest in the development of hybrid ready-to-drink beverages (RTDs) in the industry. These products offer a unique combination of protein sources, which can provide a more complete amino acid profile and potentially offer additional benefits. However, developing a high-quality hybrid RTD can be challenging, requiring careful consideration of the selection and combination of protein sources, as well as adequate sensory and nutritional characteristics.

In this project, the aim is to develop a hybrid RTD that meets the nutritional needs of consumers while also having appropriate sensory and physicochemical properties. For this reason, the following objectives were set:

- To investigate the behavior of mixed proteins, specifically pea and dairy, in a solution.
- To evaluate the processing parameters that can contribute to the development of a high-quality hybrid RTD.
- To develop a prototype RTD that meets the desired criteria in terms of nutritional, sensory and physicochemical characteristics.
- To identify the potential benefits of hybrid RTD products, specifically with regards to nutritional and functional advantages.
- To propose packaging and labeling of the final product while considering the factors that may affect the hybrid matrix.

2 Introduction

2.1 Moving forward to hybrid ready-to-drink beverages

The plant proteins industry is rapidly growing in recent years as a result of many factors, such as the health benefits, the increased awareness of food security, and the demand for more sustainable and environmentally friendly food sources (Hartzler et al., 2020). Plant proteins are derived from various sources such as legumes, cereals, pseudocereals, seeds, and nuts (Sá, Moreno and Carciofi, 2020) and they are considered to be a healthier alternative to animal proteins due to their lower content in saturated fats and their high amount in fibers (Langyan et al., 2022). Furthermore, the fact that they are easily accessible and have a lower cost for production, in most cases, compared to animal-based proteins, is making them more appealing to a wider range of consumers (Sim et al., 2021).

In addition, the increase in people following plant-based diets has led to a greater demand for plant protein-based products (Clem and Barthel, 2021), such as plant-based milks, ready-to-drink beverages, yogurts, puddings, cheese, and meat alternatives. This, in turn, has generated investments in research and development, with many food companies launching plant-based products to the growing market.

Overall, the growth in plant proteins is a response not only to the changing consumer preferences but also to the increased world population that is estimated to reach 9.5 billion by 2050. Hence, the utilization of alternative protein sources and the development of nutritional and functional plant-based food products is of high importance (Henchion et al., 2017).

However, until plant-based proteins gained popularity, human nutrition was heavily based on animal proteins because of their high quality in terms of nutrition which derives from their complete amino acid profile, the digestibility, and the ability to transport important nutrients such as calcium. Milk proteins play a key role in human nutrition as they are a rich source of essential amino acids, vitamins, and minerals. Casein and whey are highly bioavailable, meaning that they are easily absorbed and utilized by the human body, making them a vital component of a balanced diet (Day, Cakebread and Loveday, 2022). Their high nutritional quality and functional properties make them the key component in a wide range of applications in various food and beverage products, such as dairy desserts, spreads, and nutritional beverages (Andiç and Boran, 2015).

The developments in emerging economies will keep rising and the level of income is highly correlated to the consumption of animal products, including dairy. Hence, dairy protein demand is projected to grow more in the next years as it increased over the last decades (Lagrange, Whitsett and Burris, 2015). This increase is also connected to the rising need for high-quality and nutritious products that support human health and well-being. Furthermore, advances in dairy technology and the implementation of biotechnology, have made it possible to produce high-quality milk proteins in large quantities, making them more accessible and affordable to a wider range of consumers (Nurye Gebeyehu, 2023).

The Adult Nutrition market has experienced a growing interest in products that focus on promoting healthy aging and enhancing physical performance, with protein as the essential nutrient due to its role in building and maintaining muscle mass. This is particularly important for older adults, who tend to experience a decrease in muscle mass and strength below a critical threshold that is called sarcopenia (FrieslandCampina Ingredients, 2023). Products that are relatively high in protein content are therefore in demand, and the quality of the protein is also important, with PDCAAS being a commonly used value to evaluate the protein quality (Schaafsma, 2012).

Moreover, the consumption of ready-to-drink (RTD) beverages has also grown in recent years as a result of urbanization and high-paced lifestyles. RTD beverages will keep giving enormous development opportunities and will be widely accepted due to the convenience of usage and their availability in different flavors (Fortune Business Insights, 2023).

Considering these aspects, the development of a hybrid ready-to-drink beverage that combines plant and dairy proteins can result in a product with several benefits from the consumer's perspective, such as an improved nutritional profile, and better sensory attributes like flavors and textures. In addition, the hybrid beverage has the potential to appeal to a wider range of consumers, including those who follow a flexitarian diet or seek to reduce their consumption of animal-derived foods. This product may also have advantages in terms of development, since the combination of plant and milk proteins can give an improved emulsification behavior to different protein ingredient applications (Khalesi et al., 2022). Additionally, the partial replacement of milk proteins with plant proteins can bring a decrease in the environmental and economic impact (McCann et al., 2018). A SWOT analysis summarizing the main strengths, weaknesses, opportunities, and threats of mixed animal/plant foodstuff is presented in Figure 1. It should be noted that the process of developing a hybrid product can be challenging due to the differences in functionality and solubility of different proteins and thus the interactions that take place in the mixtures should be investigated to find the ideal ratio and to create a highly stable product.

Figure 1 SWOT analysis of mixed animal/plant foodstuff (Guyomarc'h et al., 2021).

2.2 Pea protein

Pea protein is a fast-growing plant protein class that derives mostly from dry, whole, yellow peas and it is commercially available as air classified flour, concentrate, isolate, hydrolyzed and texturized. Depending on the process used, pea protein content varies from 48% to 90%. Globulin, albumin and glutenin with a percentage 55%-65%, 18%-25% and 3-4% correspondingly are the main protein types in pea. Globulin is a salt-soluble storage protein that is further classified into legumin (11S), vicilin (7S) and convicilin (7S). Legumin (11S) has a Mw of 360 kDa, consisting of 6 subunits, each of them having a Mw of 60 kDa, while each subunit is composed of an α-chain (20 kDa) and a β-chain (40kDa) and they are connected with a disulfide bond. Vicilin (7S) consists of 3 subunits, which have a Mw equal to 50kDa each and convicilin's (7S) Mw is 280 kDa and is a tetrameric with subunits being 70 kDa each. None of them contains disulfide bonds (Sajib et al., 2023). Pea albumin is water-soluble and consists of the low Mw protein PA1 with two polypeptides of 6 kDa and PA2 with two polypeptides of 25 kDa, Unlike, these two polypeptides are not linked with disulfide bonds (Djoullah et al., 2015).

Pea protein has several functional properties that can be beneficial for the food products development, such as oil and water binding capacity, foam stability and expansion, whip ability, gelation and emulsion stability (Sim et al., 2021). However, its low solubility can hinder some of the properties mentioned above and thus different methods have been investigated to improve the solubility of pea protein, such as high-intensity ultrasound and pH-shifting treatment (Zhang et al., 2022).

Pea protein also offers several other advantages due to its non-GMO status and its low allergenicity, but its beany flavor and the presence of Antinutritional Factors (ANFs) limit its applicability (Xiang et al., 2023). However, it has an excellent nutritional value due to the relatively high number of proteins, carbohydrates, fibers,

minerals and vitamins and the low amount of fat (Wang et al., 2022). In addition, the high level of the Branched-Chain Amino Acid (BCAA) leucine, that is around 8,4 g/100 g of pea protein, and not too far to 8,8 g/100 g that the whey protein has, makes pea protein suitable to augment muscle protein synthesis (Banaszek et al., 2019). Finally, pea protein is relatively low in the sulfur-containing amino acids methionine and cysteine and thus its Protein-Digestibility-Corrected Amino Acid Score (DCAAS), which shows the total digestibility of the proteins, is lower compared to animal derived proteins and varies between 0,72-0,9 depending on the protein state and the quality (Boukid, Rosell and Castellari, 2021; János-István, Rawel and Huschek, 2016).

For the pea protein production, companies use different extraction processes and techniques such as alkaline extraction or salt extraction and isoelectric precipitation. Depending on the method, they can get a variety of different compositions of pea protein isolate. The pea protein isolate (PPI) is becoming a common ingredient in the food and beverage industry as an ingredient and is commonly found in many applications such as alternative meats, meal replacements, plant-based milks and bars (Stilling, 2020).

Finally, pea protein has become a popular choice due to its low environmental impact compared to other protein sources. For instance, for the production of 1 kg of pea protein concentrate, only 1,3 kg of CO_2 are produced, while for the same amount of whey protein concentrate 16,4 kg of CO_2 are generated (Heusala et al., 2020)

2.3 Milk proteins

The protein content of milk can be divided into two main groups: casein and whey (globular proteins). Casein is the predominant protein, making up about 80% of the total protein in milk, and it is found in casein micelles. The other 20% of milk's protein is composed of globular proteins, which are present in the serum, and they are commonly called whey proteins (Walstra, 1999).

Caseins consists of four fractions αs1-, αs2-, β- and κ-casein and are presented in milk as casein micelles. The first three are combined to form the submicelles, which are held together by calcium phosphate to create the casein micelle. The role of κ-casein is to be the interface between the hydrophobic submicelles and the aqueous environment and thereby ensuring the stability of the casein micelle (Huppertz et al., 2017).

Sodium caseinate (NaCas) is a soluble form of casein, obtained by precipitating the casein fraction in milk with acid to separate it from whey protein. For the final product the acid casein is neutralized with sodium hydroxide. Sodium caseinate has a desirable flavor and exhibits great water-binding capacity. It has excellent

solubility and can rapidly disperse in aqueous mixtures. Finally, it displays excellent emulsification properties and heat stability (Khwaldia et al., 2004).

Micellar casein isolate is a type of protein produced through low-temperature microfiltration process. One of the main advantages of micellar casein isolate is its neutral taste profile. Similar to sodium caseinate, it has a high heat stability compared to whey proteins, which denature at temperatures higher than 70°C. It also offers a high nutritional quality, making it a popular choice for the manufacturing of high-protein beverages (Garcia, et al., 2023).

Whey proteins include β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), immunoglobulins, lactoferrin and some enzymes. Whey protein aggregates are produced through thermal aggregation and subsequently spray-dried to obtain a powdered form. Due to the small size of their particles <5 μm that resemble the size of emulsion droplet, they can improve the properties of food and beverage products. This characteristic makes them suitable as raw material for fat replacer in different applications (Ipsen, 2017).

2.4 Mixed plant-dairy blends

Although research on the interactions of plant and dairy proteins is limited without a concrete conclusion, some previous findings are worth presenting. The globular plant proteins are able to form a gel when they are used in a concentration above their critical gelation concentration due to the protein denaturation and aggregation.

When it comes to mixtures, two different types of interactions occur, the first between plant proteins and (micellar) casein and the other one between plant and whey proteins. Studies on the interactions between pea proteins and micellar casein have shown that the denaturation temperature of pea protein was increased when micellar casein was present in the mixture and the two proteins did not co-aggregate when they were heated (Schmitt et al., 2019). Another finding suggests that the gels' stiffness in the mixtures was comparable to that of the gels made from single plant proteins gels. Overall, it was concluded that plant proteins and micellar caseins do not co-aggregate neither in acidic nor in neutral conditions, which is easily translated to the formation of independent networks where the stiffness of the gel is affected by the network that the protein in majority forms (Schmitt et al., 2019).

For the mixtures of plant and whey proteins, most of the studies were carried out at a neutral pH. It has been found that in mixtures where whey/soy protein ratio is above 1, the rheological properties obtained were similar to those of whey protein. At lower ratios, the networks were less strong and, depending on the ratio, different types of aggregates of both proteins' subunits were formed. For instance, at low concentrations and neutral pH, heating pea protein and β-LG resulted in the formation of large soluble aggregates, possibly due to the occurrence of both

covalent and non-covalent interactions. These findings were summarized by Schmitt et al., 2019 and are presented in Figure 2 made by Hinderink et al., 2021.

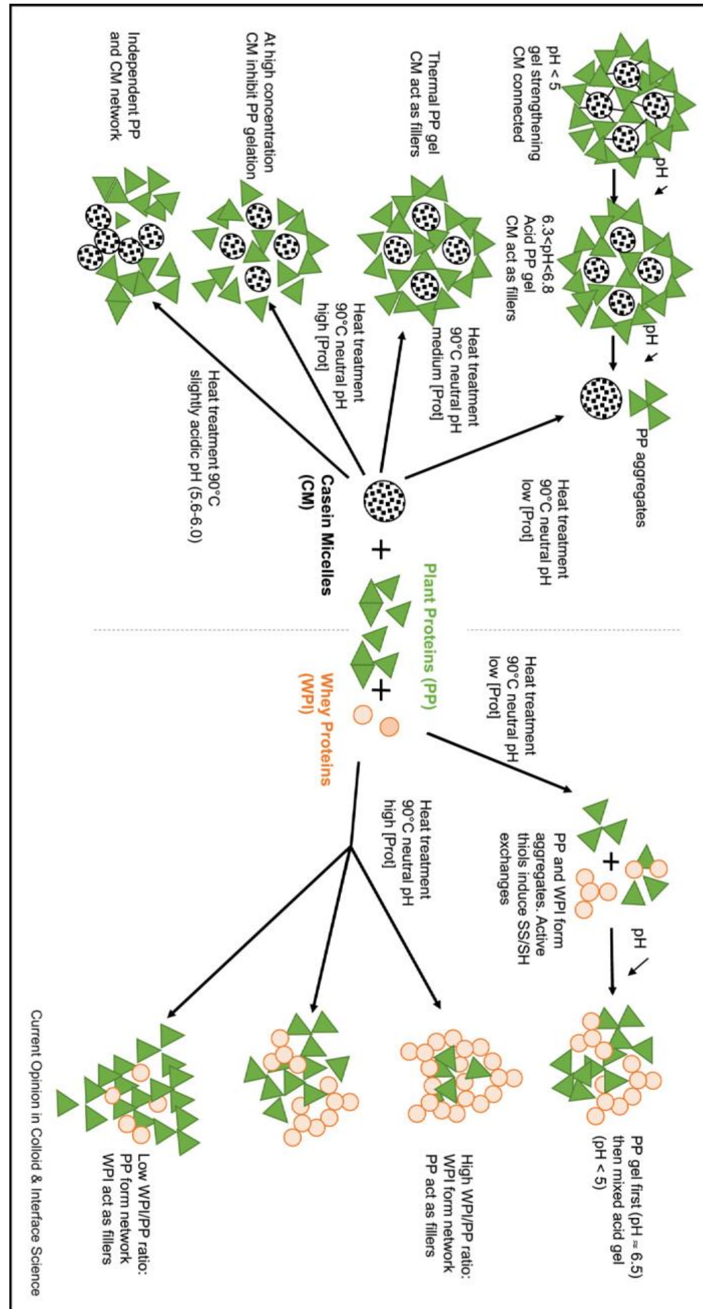


Figure 2 Schematic representation of the behavior of plant (green triangles), casein micelles (black/white spheres) and whey (orange spheres) protein blends during heating in different conditions (Hinderink et al., 2021).

Regarding the interactions between pea protein and NaCas, it was found that they can either exhibit synergistic or antagonistic behavior. Synergistic behavior is observed when the proteins interact with each other in a way that enhances their stability and reduces depletion flocculation, due to mutual interactions between the proteins. On the other hand, antagonistic behavior occurs when the stability of the protein blend decreases, leading to an increase in depletion flocculation. This can happen when mostly NaCas is adsorbed at the interface and other proteins induce depletion flocculation. The occurrence of either synergistic or antagonistic behavior depends on the specific composition and conditions of the protein mixture (Hinderink et al., 2019).

2.5 Parameters affecting the packaging development for a hybrid RTD

The development of a new product should also include the packaging aspect since it serves different functions for a food product, such as containment, protection, communication and convenience (Robertson, 2013). In order to create a package that satisfy these functions, it is important to consider all the factors that can impact the quality of the product being packaged.

One of the important parameters that need to be taken into account is the protein oxidation, which results in modifications of amino acid side chains, fragmentation, and protein cross-linking, which can decrease protein digestibility and negatively affect the sensory and nutritional quality of food products (Hinderink et al., 2020). Protein oxidation is also linked to lipid oxidation in pea protein that is caused by oxygen, light, enzymes like lipoxygenase and changes in the water activity. The lipids can be converted into hydroperoxides leading to volatile and non-volatile compounds that can affect the flavor of the product (Fischer, Cachon and Cayot, 2022). Dairy proteins are also susceptible to oxidation, which can result in lower droplet coalescence stability in emulsion systems, similar to pea protein.

Given that protein powders are prone to oxidation, it can be assumed that products made with high protein content, whether in powder or liquid form, are also susceptible to oxidation during storage. Therefore, it is essential to consider appropriate packaging materials and storage conditions to ensure the stability and shelf life of the product.

3 Materials & Methods

The project consists of two parts, 1st part, the preparation and the examination of the mixed pea-dairy protein formulas and 2nd, the development of the RTD beverage based on the results obtained during the first phase.

3.1 First part: Preparation and evaluation of the mixed protein formulas

3.1.1 Material selection and trials for the mixed-protein formulas

For all the trials that took place during this project, pea protein isolate (PPI), sodium caseinate (NaCas), whey protein aggregates (WPA), and micellar casein isolate (MCI) were used and provided by FrieslandCampina Ingredients. An overview of the raw materials specifications is shown in Table 1.

Table 1 Raw materials specifications.

Ingredient	Protein (DM%)	Moisture (%)	Fat (%)	Ash (%)	Fiber (%)
PPI	87,6	7,3	8,1	3,8	1,9
NaCas	95,9	4,9	0,8	4,1	-
WPA	80,8	4,7	4,6	3,6	-
MCI	88,1	3,3	1,1	7,3	-

The protein content and the ratios were designed for this project based on previous work conducted within the company provided some insights and on preliminary exploratory work carried out at a lab scale during the initial stages of the experiments. The choice of a protein content of 12% as the starting point for the current study was based on the stability demonstrated in previous trials with lower protein content.

Trial with 12% total protein content

For the first trial, the protein content chosen was 12%. Apart from the mixed protein solutions (S4 to S9), the plain protein solutions (S1 to S3) were prepared as references to facilitate the comparisons. The samples E1 and E2 were prepared during the initial phase of the project. The samples are presented in Table 2.

Table 2 Protein solutions for the trial with 12% total protein.

Total Protein Content 12%			
Solution	**Ratio**	**Sample Name**	**Notes**
PPI	100	S1	-
NaCas	100	S2	-
WPA	100	S3	-
PPI:MCI	80:20	E1	Exploration trial
PPI:WPA	60:40	E2	Exploration trial
PPI:NaCas	80:20	S4	-
PPI:WPA	80:20	S5	-
PPI:NaCas:WPA	60:20:20	S6	-
PPI:NaCas: WPA	50:25:25	S7	-
PPI:NaCas:WPA	60:30:10	S8	-
PPI:NaCas:WPA	60:10:30	S9	-

Trial with 14% total protein content

For the second trial, it was decided to go further with the formula containing pea protein isolate, sodium caseinate, and whey protein aggregates with a total protein content of 14% and examine the behavior of the samples containing different ratios of these proteins. Furthermore, the plain pea protein solution (S11) was prepared as a reference having the same protein content. At this point, it was also tested if the protein dissolvement plays a crucial role in the examined parameters. Thus, some of the samples presented in Table 3 were prepared with a new protocol, where the protein ingredients were dissolved together.

Table 3 Protein solutions prepared for the trial with 14% total protein.

Total Protein Content 14%			
Solution	**Ratio**	**Sample Name**	**Notes**

PPI	100	S11	Proteins dissolved separately and then mixed
PPI:NaCas:WPA	60:2,5:37,5	S20	Proteins dissolved together
PPI:NaCas:WPA	60:05:35	S16	Proteins dissolved separately and then mixed
PPI:NaCas:WPA	60:7,5:32,5	S25	Proteins dissolved together
PPI:NaCas:WPA	60:10:30	S14	Proteins dissolved separately and then mixed
PPI:NaCas:WPA	60:20:20	S18	Proteins dissolved separately and then mixed
PPI:NaCas:WPA	70:05:25	S24	Proteins dissolved together
PPI:NaCas:WPA	70:10:20	S23	Proteins dissolved together
PPI:NaCas:WPA	70:15:15	S22	Proteins dissolved together

3.1.2 Development of the mixed-protein formulas

In the initial steps, the formulas were developed as protein solutions without the addition of sugars, fat, or any other ingredients to examine the behavior of mixed protein solutions. The preparation of the protein solutions followed FrieslandCampina's protocol (FrieslandCampina Ingredients, 2023b).

The first processing step of the initial protocol was the preparation of the protein solutions according to the FrieslandCampina's instructions. For the reconstitution, 10L, 5L, and 3L buckets with lids were used to prevent losses due to evaporation. The solutions were prepared by using a Gronfa water bath (Gronfa, Zutphen, Netherlands) and an overhead stirrer VWR VOS 40 digital (VWR, Germany). Following the dissolvement, the solutions were mixed according to the ratios presented in the previous section. For the solution mixing, a Janke & Kunkel Ultra-Turrax T50 (Janke & Kunkel, Germany) was used. For the new protocol, the only difference was that the proteins were dissolved in the same bucket and thus the mixing step with the Ultraturrax was not needed. The solutions were cooled down to 20ºC in the water bath and then stored in the cooling cell overnight at 4ºC. The next day, the samples were stirred again to 60ºC and then homogenized using a homogenizer Bos MG2-13B (Bos, Hilversum, Netherlands) at 60ºC and 400/100 bar. After the two-step homogenization step, the samples were filled in 100 mL bottles and thermally treated at 121ºC for 8 minutes by using a retort Zirbus HST 6x6x6 (Zirbus, Tiel, Netherlands). Once thermally treated, the samples were stored at 4ºC and 20ºC for 4 weeks.

An overview of the processing can be seen in Figure 3. The samples were measured according to the following parameters: visual assessment, color, pH, viscosity,

particle size distribution, and optical microstructure. For some of the unstable formulas, SDS-PAGE analysis was also performed to investigate which protein fractions were responsible for the instability.

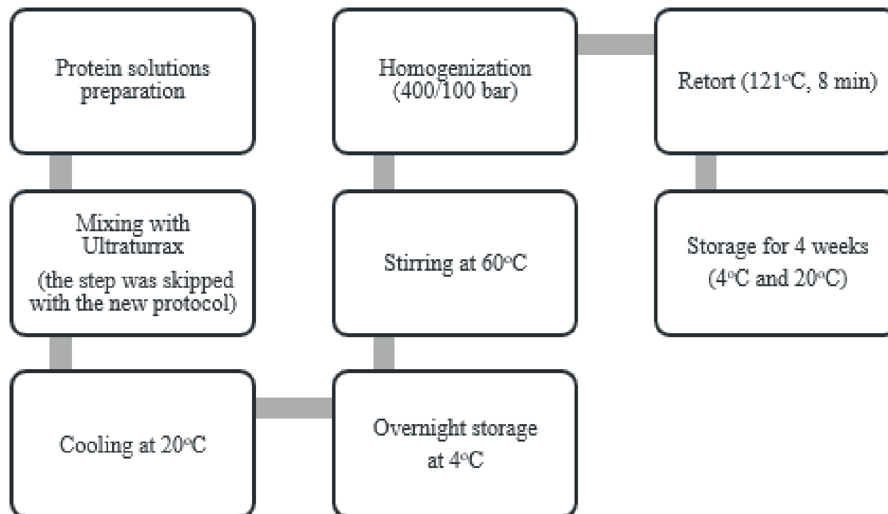


Figure 3 Processing steps for the mixed-protein samples preparation.

All the analyses were performed at the following stages:

- After homogenization
- After retort
- After 2 weeks of storage 4ºC and 20ºC
- After 4 weeks of storage 4ºC and 20ºC

Depending on the performance of some samples after the heat treatment and the storage for 4 weeks, some of the analyses were not performed, since the samples were gelled. SDS-PAGE was performed for some samples that were not stable after storage.

3.1.3 Visual and physicochemical evaluation of samples

3.1.3.1 Visual assessment

The visual assessment of the samples was conducted to investigate possible phase separation, insolubility/inhomogeneity and sedimentation. The appearance of the samples was observed during the preparation process and throughout their storage. For a better interpretation of the results, a black plate was used to investigate the presence of particles in the samples (FrieslandCampina, 2023e).

3.1.3.2 Color

The color of the samples was measured to evaluate the color consistency and their stability. The colorimetric values (L, a, b) were obtained using a CM-5 Konica Minolta Spectrophotometer CM-5 (Konica Minolta, Tokyo, Japan). For the analysis, 20 mL of sample were placed in a transparent plaque and the color data were obtained (FrieslandCampina, 2023a).

3.1.3.3 pH

The pH was measured during the different steps of the preparation process and throughout the sample storage, as an indication of the microbiological stability and the potential aggregation of the samples. For this purpose, a pH meter Mettler Toledo MP220 (Mettler Toledo, Schwerzenbach, Switzerland) was used. The pH meter was calibrated with buffers of pH 4 and 7 before usage. Each measurement was performed in duplicates and thus, the results are presenting the average values ± SD (FrieslandCampina, 2023d).

3.1.3.4 Viscosity

The viscosity was measured to analyze the rheological behavior of the samples, especially in the 100 s^{-1} shear rate that mimics the shear when swallowing. The analysis was made by using a controlled-stress rheometer Paar Physica MCR 302 (Anton Paar, Graz, Austria) with a concentric cylinder CC27 and bob measuring system. The analyses were performed before and after the heat treatment and during storage in different conditions. The shear rate was from 1-200 s^{-1} and the temperature was 20ºC (FrieslandCampina, 2023f).

3.1.3.5 Particle Size Distribution (PSD)

The particle size distribution (PSD) of the samples was obtained to indicate potential aggregation and sedimentation and it was measured with a Mastersizer 3000 equipped with a Hydro 2000G water bath (Malvern Instruments, Worcestershire, UK). The dispersant used was deionized water with a refractive index of 1,330 and the particle size was measured with a refractive index of 1,47. The obscuration was set between 3% and 6%. Each PSD measurement was performed in triplicates and thus, the results presented are average values ± SD (FrieslandCampina, 2023c).

3.1.3.6 Optical microstructure

The microstructure of the samples was observed to determine the existence of aggregation and the size of aggregates and was analyzed using a light microscope Olympus BX-41TF (Olympus, Tokyo, Japan). For each measurement 1 mL of sample and 1 mL of demi water were put in an Eppendorf tube and centrifugated with a centrifuge Eppendorf 5430 R (Eppendorf AG, Hamburg, Germany) for 15 minutes at 5000 rpm. A small amount of sediment was then placed with 50 µl of demi water on a microscope glass slide covered with a glass coverslip. The settings used were those of phase contrast with a magnification x20, x40, x60, depending on the observation, condenser 10 (white), polarizing slide to the right, and prism slide out. The snapshots were obtained with the software CellSens Standard (FrieslandCampina, 2023b).

3.1.3.7 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed for the estimation of the Mw of the protein components, which can be useful in determining whether the protein is aggregating and what the nature of aggregation might be. This was valuable to be performed only for the unstable formulas that were developed during the trials to propose strategies for preventing or mitigating aggregation in future experiments. Briefly, the unstable samples were diluted in 2x Laemmli sample buffer and DL-dithiothreitol (DTT) solution at a final protein concentration of 1 mg/mL. The diluted samples were heated at 95ºC for 5 min (Digital dry bath, Bio-Rad Laboratories, Hercules, USA) and then were left cooling to room temperature. To spin down all the liquid mixture, the samples were centrifuged shortly. Each of the samples (10 µL) was loaded in the wells of a 4-15 % Criterion™ TGX Stain-Free™ Precast protein gel (Bio-Rad Laboratories, Hercules, USA). Stain-free ladder (5 µL) was also loaded to serve as a Mw standard. Electrophoresis was run in a 1x TGS buffer at a constant voltage of 100 V for 5 min which increased to 150 V for 45-50 min until the dye reached ~0.5 cm before the end of the gel. Afterwards, the gel was analyzed in the ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories, Hercules, USA). The intensities of the bands were quantified using the ImageJ software (https://imagej.nih.gov/ij/) (Christopoulou, 2022).

3.2 Second part: Development of the hybrid RTD beverage

The mixed pea-dairy protein formula with the desired criteria was further developed into an RTD beverage, following the protocol that the company has for the plant-based RTDs. The ingredients used for the recipe were: water, PPI, WPA, NaCas, milk fat, sunflower lecithin, sucralose, and acesulfame K.

3.2.1 Hybrid RTD development process

The RTD development started with the preparation of the protein solution according to FrieslandCampina's instructions (FrieslandCampina Ingredients, 2023b). For the dissolvement, 10L buckets with lids were used to prevent losses due to evaporation. The solution was prepared by using a Gronfa water bath (Gronfa, Zutphen, Netherlands) and an overhead stirrer VWR VOS 40 digital (VWR, Germany). Afterwards, it was cooled down at 20ºC in the water bath and then stored in the cooling cell overnight at 4ºC. The next day, the solution was stirred again at 60ºC and the sweeteners were added. The milk fat with the sunflower lecithin was warmed up at 60 ºC and then mixed with the protein solution by using a Janke & Kunkel Ultra-Turrax T50 (Janke & Kunkel, Germany). The mixture was then homogenized with a homogenizer Bos MG2-13B (Bos, Hilversum, Netherlands) at 60ºC and 400/100 bar. After the homogenization step, the samples were processed with OMVE HTST/UHT System HT220 (Omve, De Meern, Netherlands) with a downstream homogenizer at 125ºC for 5 minutes. The products were filled in sterilized plastic containers of 190 mL and were stored at 4ºC and 20ºC for 4 weeks. An overview of the processing steps can be seen in Figure 4.

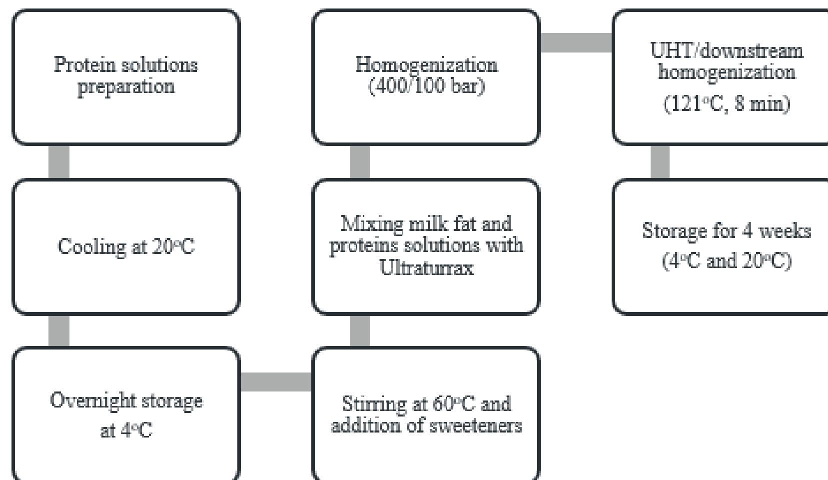


Figure 4 Processing steps for the hybrid RTD development.

3.2.2 Physicochemical evaluation

The RTD was examined according to the same parameters, as the mixed formulas in the first part of the project: visual assessment, color, pH, viscosity, particle size distribution, and optical microstructure. The measurements were taken after 2 weeks of storage at 4ºC and 20ºC.

3.2.3 Protein Digestibility-Corrected Amino Acid Score (PDCAAS)

The theoretical protein digestibility value of the hybrid and the pea RTDs was examined for determining the nutritional suitability of the protein beverages. PDCAAS was calculated by multiplying the most limiting essential amino acid concentration (mg/g protein) with the protein digestibility factor which is 0,95 (dairy reference) divided by the concentration of that amino acid in the reference amino-acid pattern for school children (FAO, 2011). The lowest score obtained is the PDCAAS value (FAO, 2013). The amino acid compositions for the proteins that were used for this project were provided by FrieslandCampina (AOAC , 2018)

3.2.4 Sensory evaluation

Sensory evaluation was carried out within the company, where 20 untrained panelists. The evaluation consisted of two steps. In the first step, the participants were provided with two samples of plain pea protein RTDs, one containing 8,4% PPI and the other 12% PPI. The sample with the lower PPI amount was considered as the reference. In the second step, the hybrid RTD was given to the participants to compare it once again with the reference. Thus, the sensory test followed is described as "Detailed comparison to reference" (FrieslandCampina, 2023g).

Initially, the samples were prepared, and the participants were invited to try the coded samples. Water was provided between the different tastings for neutralization. The answers were collected in online forms and the results were then interpreted.

3.2.5 Packaging, Labelling & Claims

The selection of packaging was made based on the parameters of protein and lipid oxidation due to the presence of light and oxygen and its suitability will be evaluated in an upcoming section. Nutrition information, particularly the presence of energy and specific nutrients, is a crucial element in food labeling. This information was designed with respect to Regulation No. 1169/2011 (Regulation (EU) Regulation No. 1169/2011, 2011), while the design on nutrition and health claims was based on the guidelines provided by Regulation No 1924/2006 (Regulation (EC) No 1924/2006, 2006).

4 Results & Discussion

4.1 First part: Preparation and evaluation of the mixed pea-dairy protein formulas

To simplify the process of preparing samples, during the trial with 14% total protein, the dissolvement of proteins together and not separately as previously done, was examined. To assess the effectiveness of this new method, some samples were prepared twice, once by dissolving the protein powders separately and once by dissolving them together. The visual assessment, pH values, and viscosity measurements of these samples did not show any significant differences. Therefore, it was concluded that the new protocol was more efficient since it allowed for easier dissolution and faster preparation, and consequently, all subsequent samples were prepared using this protocol.

Due to the higher protein content in combination with the heat treatment, some of the samples did not exhibit the necessary properties for further evaluation of all parameters. For instance, Sample S11, which was made with 14% PPI, gelled after the heat treatment, indicating that PPI cannot be used to produce RTDs with such high protein content. Due to the presence of lumps and a high viscosity following the heat treatment, samples S18, S23, and S24 were not considered suitable for storage for four weeks and were consequently discarded. Sample S25 was monitored for 2 weeks, but it turned into a gel in the 4th week. Therefore, the only samples that will be considered for visual assessment and other parameters are S14, S16, S20, S22, and S25, and are presented in Table 4.

Table 4 Samples from the trial with 14% total protein that remained stable after retort.

Total Protein Content 14%		
Solution	**Ratio**	**Sample Name**
PPI:NaCas:WPA	60:2.5:37.5	S20
PPI:NaCas:WPA	60:05:35	S16
PPI:NaCas:WPA	60:7.5:32.5	S25
PPI:NaCas:WPA	60:10:30	S14

PPI:NaCas:WPA	70:15:15	S22

4.1.1 Visual assessment

Trial with 12% total protein content

The visual assessment of all the samples was made with a black plate and is presented in Figure 5. The composition of these samples is presented in Table 2. The samples S1-S3, that were prepared with single proteins, PPI, NaCas, and WPA, respectively, had a different performance since they derive from different sources, and they have different properties. Samples S1 and S2 showed no phase separation throughout the storage. On the other hand, for sample S3, it was noticed that the protein powder could not be dissolved during the preparation and even after the homogenization step, the solution had many particles that led to protein aggregation with big mass formation after retort and sediment throughout the storage. It is known that one of the whey proteins, β-lactoglobulin is thermally very unstable and thus the stability of the sample was affected (Dutson and Orcutt, 1984).

Samples E1 and E2 were prepared during the exploration phase of this project and contained PPI with MCI and WPA in a ratio of 80:20 and 60:40 respectively, had big particles even before retort and especially in the case of E2, there was mass formation similar to sample S3. Although these mixtures were considered unstable and were not further analyzed, it is reported that either whey protein can form a continuous network with pea protein aggregates being incorporated or they co-aggregate. Similar results were presented in studies with soy and whey protein, where it was proposed that whey protein formed the primary protein network with soy protein incorporated as particulate fillers (Kornet et al., 2021).

A less negative effect due to WPA was observed in sample S5, with a decreased amount of WPA since it was seen that the sample remained stable after retort, even though there were few particles present that transformed to lumps after 4 weeks of storage in both conditions.

The interaction between denaturated pea legumin and vicilin upon heating leads to the formation of pea protein aggregates that can further interact with as1- and β-casein through hydrophobic bonds and with κ-casein via disulfide-thiol exchange (Chuang et al., 2019; Mession, Roustel and Saurel, 2017). The latter is limited due to the lack of free thiol groups in legume globulins (Hinderink et al., 2021) as well as in κ-casein. Due to these hydrophobic interactions and under homogenization, caseins are disordered, and they form a coating around the PPI fractions. Therefore, the solubility of the mixture is increased, and this prevents further aggregation (Yerramilli, Longmore, and Ghosh, 2017). It is also known that casein micelles possess chaperone-like functions, which means that they protect plant globulins

against unfolding, and they inhibit gelation at high concentrations and in neutral pH (Hinderink et al., 2021).

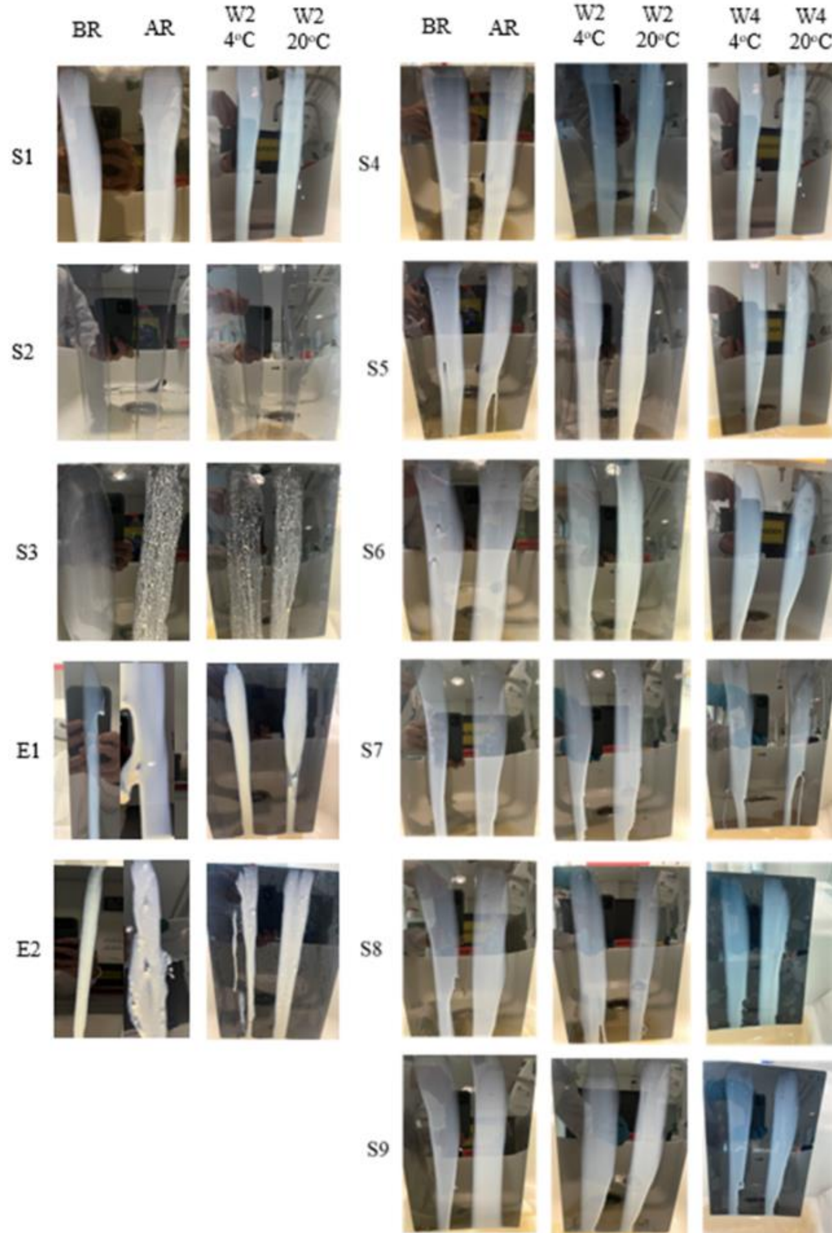


Figure 5 Visual assessment of the samples from the trial with 12% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 2.

These findings led to examining the combination of the three different proteins, PPI, NaCas, and WPA within different ratios in samples S6-S9, which showed high stability, even after storage for 4 weeks. It seems that the addition of NaCas to a protein solution containing PPI and WPA can lead to improved stability by providing an additional stabilizing effect to the WPA, while the WPA stabilizes the PPI by creating a continuous network.

Trial with 14% total protein content

The initial visual assessment of the samples, which is shown in Figure 6, indicated that all were stable and free from lumps before undergoing heat treatment. However, the observation after retort revealed that the samples S14, S22, and S25 exhibited lump formation while S16 and S20 remained stable.

It appears that the composition of the samples played a significant role in their stability. Samples S14 and S25, which had identical PPI compositions, exhibited different levels of NaCas and WPA content. Meanwhile, samples S16 and S25 had different amounts of NaCas and WPA content, with S16 containing 5% NaCas and 35% WPA, and S25 containing 7,5% NaCas and 32,5% WPA. Additionally, sample S22 had a 10% increase in PPI content and the same amount of NaCas and WPA at 15%.

During storage, all the samples, except S16, became increasingly viscous and formed lumps, with sample S25 even becoming gelled after four weeks.

As previously mentioned, the addition of NaCas to a protein solution containing PPI and WPA may enhance stability by providing an additional stabilizing effect to the WPA, which in turn stabilizes the PPI by creating a continuous network. However, it is important to note that as protein content increases, the composition becomes crucial. While certain combinations with the mentioned ratios worked well at 12% TPC, increasing the protein content resulted in increased viscosity, making them unsuitable for further evaluation.

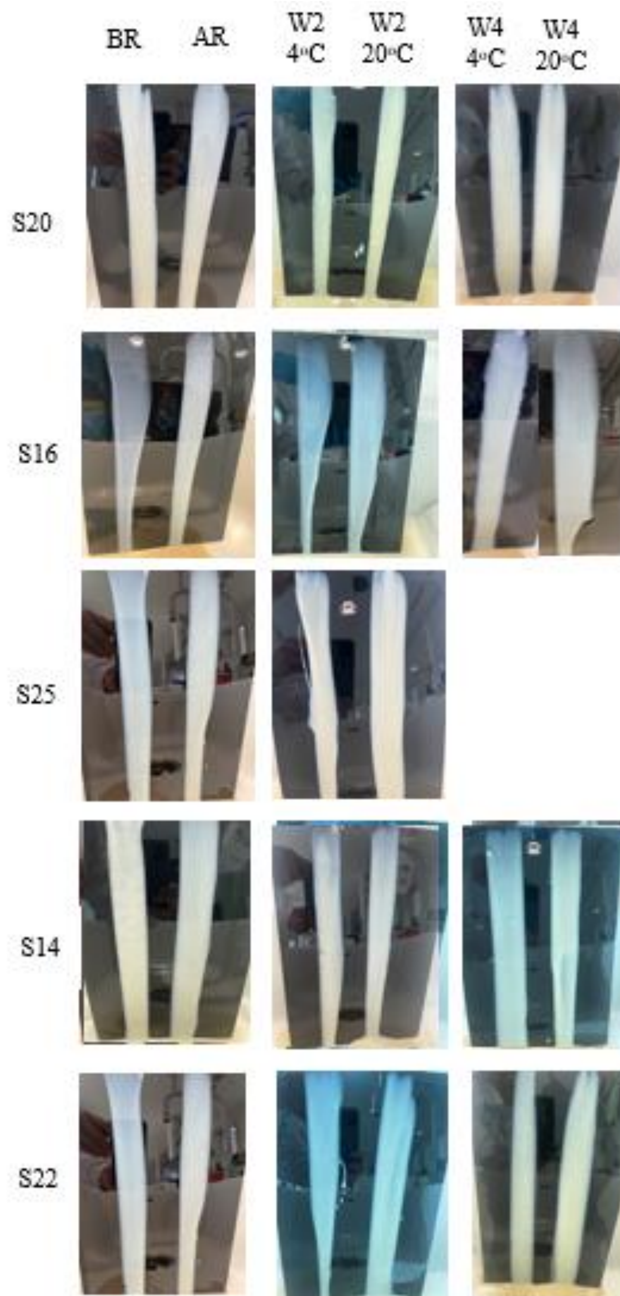


Figure 6 Visual assessment of the samples from the trial with 14% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 4.

4.1.2 Color

Trial with 12% total protein content

Based on the color measurements obtained, the samples S4-S9 have colors that are closer to S1, especially in terms of the L* value that represents the lightness. This similarity is attributed to the use of PPI as the protein with the highest proportion in the samples. Another observation is that all the samples have the lowest values for the three parameters, L*, a*, and b* after retort, which is translated to a darker color. This could be an indication of the Maillard reaction between the amino acids and the lactose derived from the milk proteins (Lund and Ray, 2017). Furthermore, it appears that the color of all samples was stable, with no significant changes in the values at different temperature conditions during storage. Figure 7 depicts the samples after retort and Table 5 presents the colorimetric results for the trial with 12% TPC.

Figure 7 The samples from the trial with 12% total protein after retort (AR) treatment. For sample codes, see Table 2.

Table 5 Color of the samples from the trial with 12% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 2.

Color						
Sample name	Before retort (BR)			After retort (AR)		
	L*	a*	b*	L*	a*	b*
S1	70.50	2.16	16.81	66.61	0.35	12.19
S2	59.14	-3.20	3.80	45.65	-0.38	1.65
S3	89.74	0.13	11.55	85.84	1.76	15.95
S4	70.84	2.25	16.60	65.97	0.30	12.24
S5	75.52	2.69	17.99	71.80	1.14	14.82
S6	76.96	2.22	17.42	71.74	1.44	14.72
S7	77.84	1.77	16.93	73.35	1.19	14.70
S8	73.87	2.14	17.35	69.54	1.27	14.36

S9	77.95	2.13	17.21	74.33	1.26	14.84
Sample name	**W2/4°C**			**W2/20°C**		
	L*	**a***	**b***	**L***	**a***	**b***
S1	66.88	0.19	12.21	67.19	0.37	12.54
S2	46.93	-1.98	2.01	46.18	-0.32	1.98
S3	85.53	1.91	16.02	85.21	2.06	16.05
S4	66.23	0.03	12.64	66.09	0.21	12.29
S5	71.96	1.08	14.90	72.16	1.25	15.03
S6	72.31	1.31	15.07	72.46	1.31	14.99
S7	73.31	1.57	14.94	73.60	1.64	14.94
S8	69.81	1.30	14.70	70.32	1.45	14.95
S9	74.53	1.27	15.06	74.70	1.49	15.17
Sample name	**W4/4°C**			**W4/20°C**		
	L*	**a***	**b***	**L***	**a***	**b***
S1	66.57	0.12	11.98	67.61	0.59	13.17
S2	45.99	-1.85	1.78	45.75	-0.30	1.89
S3	85.21	1.83	16.13	85.75	1.94	16.21
S4	65.93	0.11	12.24	67.75	1.09	14.44
S5	72.88	1.35	15.68	72.84	1.52	15.58
S6	72.47	1.55	15.40	72.93	1.76	15.57
S7	73.50	1.50	15.03	74.27	1.72	15.44
S8	70.22	1.24	14.99	70.53	1.47	15.17
S9	74.60	1.27	15.19	75.02	1.41	15.37

Trial with 14% total protein content

Based on the color measurements presented in Table 6, similar to the trial with 12% TPC, all the samples have lower values for the three parameters, L*, a*, and b* after retort, which is translated to a darker color. This can be explained as an outcome of the Maillard reaction between the amino acids and the lactose derived from the milk proteins. Furthermore, it appears that the color of all samples was stable, with no significant changes in the values at different temperature conditions during storage. Figure 8 depicts the samples after 2 weeks of storage in both conditions. Sample S16 accidentally was not kept before retort and S25 could not be evaluated after 4 weeks of storage since it had gelled.

Figure 8 The samples from the trial with 14% total protein after 2 weeks of storage at 4°C and 20°C. For sample codes, see Table 4.

Table 6 Color of the samples from the trial with 14% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 4.

Color						
Sample name	**Before retort (BR)**			**After retort (AR)**		
	L*	**a***	**b***	**L***	**a***	**b***
S20	78.90	2.07	16.21	76.18	1.20	14.56
S16	-	-	-	75.15	1.21	14.42
S25	77.23	2.24	16.78	74.61	1.47	15.50
S14	77.66	2.60	17.20	73.62	1.33	14.95
S22	73.41	2.60	17.46	70.01	1.25	14.86
Sample name	**2W/4°C**			**2W/20°C**		
	L*	**a***	**b***	**L***	**a***	**b***
S20	76.13	1.22	14.67	76.70	1.30	14.94
S16	75.33	1.15	14.53	75.14	1.29	14.59
S25	74.85	1.59	16.00	75.13	1.70	16.17
S14	74.43	1.34	15.36	74.56	1.60	15.75
S22	70.35	1.54	15.58	70.65	1.65	15.75
Sample name	**4W/4°C**			**4W/20°C**		
	L*	**a***	**b***	**L***	**a***	**b***
S20	76.56	1.45	14.48	76.30	1.21	14.73
S16	74.78	1.07	14.34	75.09	1.21	14.52
S25	-	-	-	-	-	-
S14	74.07	1.49	15.46	74.82	1.49	16.09
S22	70.22	1.52	15.43	70.46	1.64	16.12

4.1.3 pH

Trial with 12% total protein content

As shown in Table 7, the pH values after retort range from 6.44 for the WPA up to 7.18 for the PPI. A pH decrease occurred between the samples after retort, with the highest change being for sample S3 by 0.13. Such changes can be explained based on two facts. Firstly, heat treatment can lead to protein-protein reactions that result in the release of protons, which can lower the pH (Al-Saadi, 2013). Additionally, the heat treatment can lead to the hydrolysis of carbohydrates, in our case, the lactose in the mixed protein samples. The produced D-glucose and D-galactose in alkaline solutions form a series of enediols. From the 3,4-enediol, the pyruvic aldehyde is formed, which at higher temperatures and alkalinity is degraded into lactic acid (Berg, 1993).

None of the mixed samples S4-S9 showed high increase in pH values over a storage period of 4 weeks at both 4°C and 20°C, indicating that they were relatively stable and the addition of different proteins in various ratios did not have a strong impact.

Table 7 pH of the samples from the trial with 12% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 2.

pH						
Sample name	**Before retort (BR)**	**After retort (AR)**	**W2/4°C**	**W2/20°C**	**W4/4°C**	**W4/20°C**
S1	7.18 ±0.06	7.16±0.02	7.18±0.02	7.09±0.02	7.00±0.03	7.07±0.01
S2	6.83±0.01	6.80±0.03	6.77±0.01	6.77±0.01	6.74±0.01	6.79±0.01
S3	6.57±0.01	6.44±0.02	6.47±0.01	6.41±0.03	6.43±0.01	6.45±0.02
E1	6.91±0.02	6.85±0.02	6.92±0.02	6.88±0.01	-	-
E2	6.75±0.01	6.66±0.04	6.76±0.02	6.75±0.02	-	-
S4	7.08±0.01	7.03±0.02	7.03±0.01	7.04±0.01	7.06±0.01	7.05±0.01
S5	6.98±0.02	6.91±0.01	6.90±0.01	6.89±0.01	6.91±0.04	6.84±0.06
S6	6.90±0.01	6.90±0.01	6.90±0.03	6.90±0.01	6.85±0.01	6.86±0.01
S7	6.78±0.03	6.76±0.01	6.76±0.04	6.77±0.05	6.77±0.01	6.76±0.01
S8	6.88±0.01	6.86±0.01	6.90±0.04	6.91±0.01	6.97±0.01	6.90±0.01
S9	6.83±0.02	6.75±0.01	6.80±0.01	6.79±0.02	6.87±0.03	6.79±0.01

Trial with 14% total protein content

As shown in Table 8, the pH values after retort ranged from 6.72 for sample S16 to 6.92 for sample S22. In contrast to the findings of the previous trial, pH increased after retort in a range from 0-0.08 pH units, with the only exception being S14 which displayed a decrease equal to 0.08.

In both periods, 2 and 4 weeks, the samples stored at room temperature presented a lower pH compared to those stored at 4ºC. The pH difference ranged from 0.02-0.07 at 2 weeks-storage and 0.07-0.14 on the 4th week.

Comparing the samples stored in room and fridge temperature for 2 and 4 weeks, it is observed that samples S20 and S16 that were stored in the fridge displayed a decrease ranging from 0.03 to 0.18 respectively. The same pattern was shown for the samples that were stored at 20ºC, which displayed a pH decrease that ranged from 0.03-0.11. Sample S16 accidentally was not kept before retort and S25 was evaluated after 4 weeks of storage since it was gelled.

Table 8 pH of the samples from the trial with 14% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 4.

pH						
Sample name	**Before retort (BR)**	**After retort (AR)**	**2W/4°C**	**2W/20°C**	**4W/4°C**	**4W/20°C**
S20	6.67±0.02	6.74±0.03	6.83±0.01	6.77±0.01	6.80±0.02	6.66±0.04
S16	-	6.72±0.01	6.86±0.01	6.80±0.02	6.68±0.04	6.64±0.03
S25	6.81±0.01	6.83±0.02	6.80±0.01	6.74±0.01	-	-
S14	6.85±0.04	6.77±0.03	6.74±0.04	6.76±0.01	6.80±0.01	6.73±0.01
S22	6.88±0.01	6.92±0.03	6.94±0.01	6.87±0.03	6.97±0.02	6.88±0.02

Overall, it can be concluded that there were no big differences in the pH values for the samples with 14% TPC when stored for 4 weeks.

Finally, the sample that displayed the biggest increase after retort and during storage was S22, which had the highest PPI content and the lowest WPA content, resulting in a higher pH value compared to the other samples. This could be explained by the fact that PPI had a pH value of 7.16 and WPA had a pH value of 6.44, as indicated in Table 5.

4.1.4 Viscosity

Trial with 12% total protein content

The viscosity values measured at a shear rate of 100 s^{-1} (20ºC) are presented in Table 9. Increasing the temperature up to the protein denaturation point during manufacturing can cause a decrease in the viscosity (Himmetagaoglu, Erbay, and Cam, 2018). Such a viscosity reduction is noticeable in both the S1 sample that contains PPI and the mixed protein samples S5-S9, which may show that the aggregation occurring is limited. However, the viscosity for S1 is higher compared to S5-S9. Sample S1 contains 12% PPI, while the percentage of pea protein is lower in the mixed samples. PPI particles are larger and can aggregate easily due to low viscosity, which leads to a higher volume fraction in protein suspensions and subsequently increases the apparent viscosity (Kristensen et al., 2021; Oliveira et al., 2022).

Table 9 Viscosity of the samples from the trial with 12% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 2.

Viscosity at 100 s^{-1} shear rate (mPa*s)						
Sample Name	**Before retort (BR)**	**After retort (AR)**	**W2/4°C**	**W2/20°C**	**W4/4°C**	**W4/20°C**
S1	97.28	84.98	120.50	142.39	133.83	185.7
S2	194.97	154.00	167.55	190.88	-	-
S3	7.48	160.32	6.32	8.76	-	-
S4	90.08	141.59	169.34	228.99	201.54	254.39
S5	91.13	61.36	77.38	93.19	86.88	109.23
S6	51.06	42.68	51.86	60.98	49.98	63.98
S7	30.01	21.92	29.76	28.97	32.42	30.52
S8	50.64	51.72	74.69	76.94	78.39	92.67
S9	38.20	23.54	30.57	30.00	32.94	32.02

Regarding sample S2 containing NaCas, it is reported that most of the casein solutions show a similar flow to the one found during the experiments, and their apparent viscosity is not significantly affected by heat treatment, which is expected due to their high stability in thermal treatments. In addition to that, only S2 displayed a Newtonian flow which means that the viscosity was not affected by the shear rate, which is also confirmed by the literature (Beliciu and Moraru, 2011). All the other samples had a shear-thinning behavior, as can be seen in Figure 9.

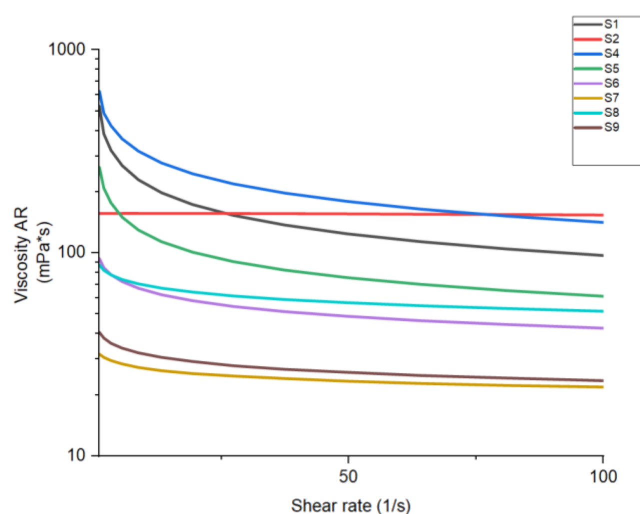


Figure 9 Shear viscosity as a function of the shear rate of the samples from the trial with 12% total protein content after retort (AR). For sample codes see Table 2.

Sample S3, containing WPA, showed a significant increase in viscosity after retort implying that many whey protein molecules were aggregated (Benoit et al., 2013), which was also confirmed by the visual assessment of this sample in Figure 5.

Sample S4 did not display a lower viscosity value after the thermal processing. The comparison with S5, since both contain 80% of PPI and 20% of milk protein, shows that the samples had the exact opposite behavior. It is reported that emulsions made with whey protein presented lower viscosity compared to those prepared with NaCas (Himmetagaoglu, Erbay, and Cam, 2018). Hence, it is hypothesized that this also reflects in the mixed solutions.

Samples S6-9 that were prepared with the combination of PPI:NaCas:WPA showed low viscosity values after retort, even though the total protein content reached 12%. Another critical observation is that the higher the amount of WPA in the mixture, the lower the viscosity. The samples made with 30, 25, 20, and 10% of WPA displayed viscosities equal to 24, 22, 42, and 52 mPa*s respectively. This comes in contrast with the literature when for blends of PPI and whey protein and in ratios 20:80 and 50:50, it was found that the higher the whey protein, the higher the viscosity. Although there is such a difference in the blends, it is a common finding that the mixed solutions have a lower viscosity compared to the PPI solution (Kristensen et al., 2021). The difference in our results may be attributed to the presence of NaCas, but this needs to be further evaluated.

Over 2 weeks of storage, the viscosity was increased and this increase was higher at 20°C for almost all the samples. The interactions change during storage and thus may lead to aggregation over time when the proteins are exposed to increased temperatures and relative humidity (Gillman, 2014). In addition, proteins are susceptible to age gelation since they create a 3-dimensional network. The age gelation has 4 stages, starting with the rapid first one of product thinning, followed by a stage of product thinning but with a slight viscosity shift. During the third, the gelation leads to a viscosity increase, while in the fourth one, a slight viscosity decrease can be displayed due to the gel breakdown that will lead to syneresis (Singh et al., 2022). Sample S3 did not display a viscosity increase, since the formation of the aggregates after retort led to phase separation, and the sample that was analyzed was not representative. Samples S6-S9 did not display significant differences while being stored in these temperatures, thus, making the combination of the three proteins or the addition of NaCas the key component for stabilizing the solutions and limiting the aggregation.

Over 4 weeks of storage, the viscosity further increased, almost for all the samples but within a range that is still acceptable It can be estimated that by producing mixed protein solutions, the stability of the samples can already be evaluated after 2 weeks of storage.

Trial with 14% total protein content

The viscosity values at a shear rate of 100 s^{-1} (20ºC) are presented in Table 10. The decrease observed in the viscosity after the heat treatment is similar to the trial with 12% TPC and has been explained in one of the previous sections. Although S20, S16, S25, and S24 contain the same amount of PPI, significant differences have been observed in the viscosity measurements. Samples S20, S25, and S14 displayed viscosity values between approximately 137 mPa*s and 197 mPa*s, while S16 had a viscosity equal to almost 34 mPa*s after retort.

Table 10 Viscosity of the samples from the trial with 14% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 4.

Viscosity at 100 s^{-1} shear rate (mPa*s)						
Sample Name	**Before retort (BR)**	**After retort (AR)**	**2W/4°C**	**2W/20°C**	**4W/4°C**	**4W/20°C**
S20	292.42	192.36	238.59	300.57	266.55	277.53
S16	-	33.86	42.11	49.156	51.62	57.46
S25	296.77	197.09	291.09	312.31	-	-
S14	303.40	137.47	183.48	183.24	174.90	218.59
S22	203.64	173.98	227.04	267.05	270.84	306.65

The viscosity of all the samples, except S16, showed an increase after a storage period of 2 weeks, with a larger increase observed in the samples stored at 20°C. After 4 weeks of storage the viscosity was further increased, displaying again higher values for samples stored at 20ºC. In contrast to the behavior of the samples, S16 retained lower viscosity values, equal to approximately 34 mPa*s after retort, and ranged from 42 to 49 mPa*s after 2 weeks and 51 to 57 mPa*s after 4 weeks depending on the storage conditions. This result gave valuable insights by underlying the suitable composition of PPI, NaCas, and WPA within 14% of total protein content. However, the differences in composition cannot explain the significant differences in viscosity without any additional analyses.

4.1.5 Particle Size Distribution

Trial with 12% total protein content

To better examine the aggregation and evaluate the stability of the samples, particle size analysis was performed. PSD gives different measurements correlated to the size and the quantity of the particles in the sample. For a better understanding of the results, the values for D_{90} and D [4,3] represent the diameter of the particles below

which 90% of the population falls and the volume mean diameter respectively, will not be taken into account since the samples contain several fibers that are typically larger, and this can lead to an overestimation of the particles which is crucial when examining aggregation. Thus, the focus will be on D_{50} which represents the average particle size. The results for the mixed samples are shown in Figure 10.

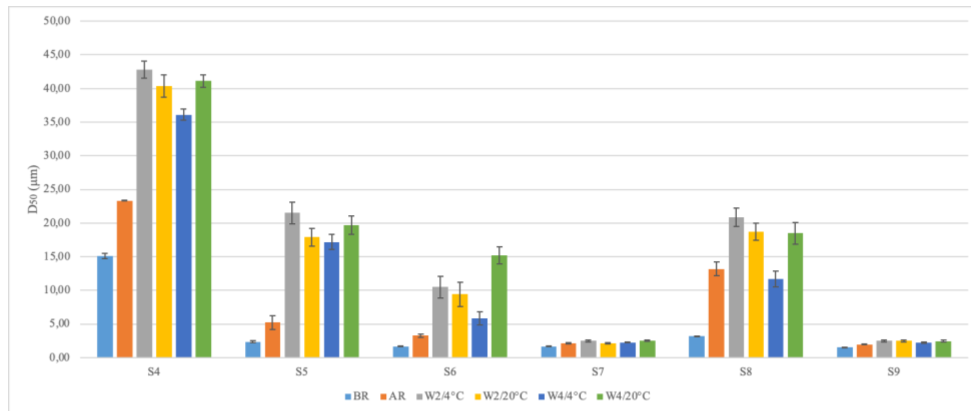


Figure 10 Average particle size of the samples from the trial with 12% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 2.

As it may be observed, the heat treatment caused an increase in the average particle size in all the mixed protein samples S4-S9. The particle size of sample S8 exhibited the highest increase after retort, increasing from 3.2 μm to 13.2 μm, followed by S4, whose size was increased from 15.1 to 23.3 μm. The rest of the samples' particle size after retort ranged from 1.9-5.2 μm. For the samples S1-3 made with single proteins, S3 showed the highest particle size that reached 43.2 μm after retort, confirming the presence of aggregates. Particle size for S1 containing PPI also showed relatively high values probably due to the high pea protein concentration and the presence of 1.9 g of fiber per 100 g of PPI. Generally, larger particle sizes are associated with decreased heat stability and higher viscosity (Liang et al., 2016). This was not the case for sample S2 which underwent a reduction in particle size after retort from 0.9 μm to 0.1 μm. This may be explained by the fact that the surface of NaCas molecules become less hydrophobic when they are subjected to heating since some of the amino acid residues that provide hydrophobicity are hidden and new hydrophilic groups are created during protein hydrolysis. Hence, the self-association of caseins may be changed leading to an effect on particle size (Liang et al., 2017).

During storage for 2 weeks, the particle size has been further increased especially for samples S4 and S5. Sample S4, which had an average particle size of 23.3 μm after retort, reached the size of 42.8 μm and sample S5 from 5.2 μm, the size of 21.5 μm at 4ºC. Samples S6 and S8 had initial particle sizes of 3.3 μm and 13.2 μm,

which increased to 10.5 µm and 20.9 µm, respectively after being stored. The least affected samples after 2 weeks of storage were S7 and S9 indicating that the aggregation was limited. While there was not significant difference in particle size between the samples stored at 20°C for 2 weeks and those stored in a cold environment, the particles in the former were observed to be smaller.

After being stored for 4 weeks at 20°C, all the samples showed a minor reduction in particle size when kept at 4°C, and a slight increase in particle size compared to their size on the second week when stored at 20°C. Similarly, to the cold storage, S7 and S9 displayed the lowest average particle size with 50% of their particle distribution being at values 2.4 µm and 2.5 µm respectively, when stored at 20ºC.

Overall, it seems that to obtain a low particle size and stability throughout storage, it is needed to keep the PPI quantity less than 80% and the amount of WPA higher than 10% in mixed solution with this total protein content. Hence, it can be explained why samples S7 made with PPI:NaCas:WPA 12% in a ratio of 60:10:30, and S9 made with PPI:NaCas:WPA 12% 50:25:25, had the best performance throughout storage and did not display unwanted aggregation.

Trial with 14% total protein content

Particle size distribution was utilized as an additional analytical technique to complement the other results obtained in this trial. As with the previous trial, the emphasis will be placed on D_{50}, which represents the average particle size. The results are presented in Figure 11.

As it may be observed, the heat treatment caused an increase in the average particle size in all the samples. This increase was 1.0 µm for S20, 1.2 µm for S25, and 1.6 µm for S14. After undergoing retort, S22 showed the highest increase in size, going from 3.7 µm to 16 µm. Sample S16 was not kept before retort and thus its results are not presented in the Figure. The size of all the samples, except S22, did not significantly increase after the heat treatment. Similar results had been obtained in the previous trial for sample S4 made with PPI:NaCas 12% (80:20) that had a size of 23.3 µm after retort. In both samples, the ratio PPI/NaCas concentration is 4/1.

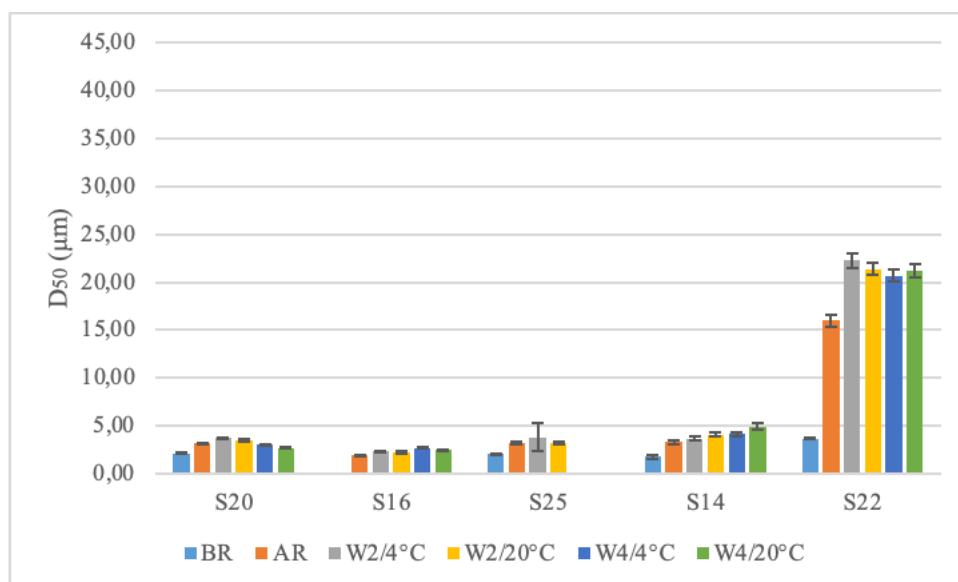


Figure 11 Average particle size of the samples from the trial with 14% total protein before retort (BR), after retort (AR) treatment and after storage at 4°C and 20°C for 2 and 4 weeks. For sample codes, see Table 4.

Over the storage for 2 weeks, the particle size has been further increased but not significantly. For instance, S20 average size increased by 0.5 µm, S16 by 0.3 µm, S25 by 0.5 µm and S14 by 0.4 µm, when stored at 4ºC. Similar size values were obtained when the samples were stored at 20ºC for 2 weeks or in both conditions for 4 weeks. The only exception among these samples was S22 which showed increased protein aggregation. Sample S16 accidentally was not kept before retort and S25 was evaluated after 4 weeks of storage since it was gelled.

4.1.6 Optical microstructure

Trial with 12% total protein content

Based on Figure 12, it can be observed that there are variations in the aggregate sizes of the individual protein solutions S1-S3 after a storage period of 4 weeks. Sample S1, which was prepared using PPI, showed the presence of aggregates with a size of 40 µm. On the other hand, sample S2, which was made with NaCas, exhibited smaller particles with a size of 2 µm. Sample S3, containing WPA, had

large aggregates, as expected, which were visible under the microscope, with a size of up to 80 μm.

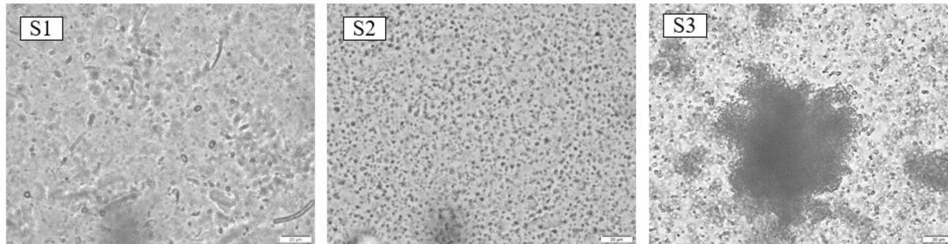


Figure 12 Optical microstructure of the samples S1, S2 and S3 after 2 weeks of storage at 4°C. The scale bar represents 20 μm. For sample codes, see Table 2.

Regarding the microscopic analysis of samples S4-S9, which were prepared using different protein sources, in Figure 13 it is observed that samples all the samples presented aggregates. Some aggregates were captured for samples S6 and S9 during the second week of storage that ranged from 20-40 μm and 30-40 μm respectively. Samples S5, S7 and S8 displayed aggregates of 30 μm in the fourth week of storage, while in S4 there were also some aggregates of 40 μm. Interestingly, samples S7 and S8 showed the densest population of aggregation, even though their viscosity values were similar to S9 which displayed visible aggregates. The results obtained are consistent with previous findings in the literature regarding mixtures of pea protein isolate and whey protein isolate, where the presence of large clusters of up to ~100 μm was observed at higher concentrations of pea protein (Kornet et al., 2021).

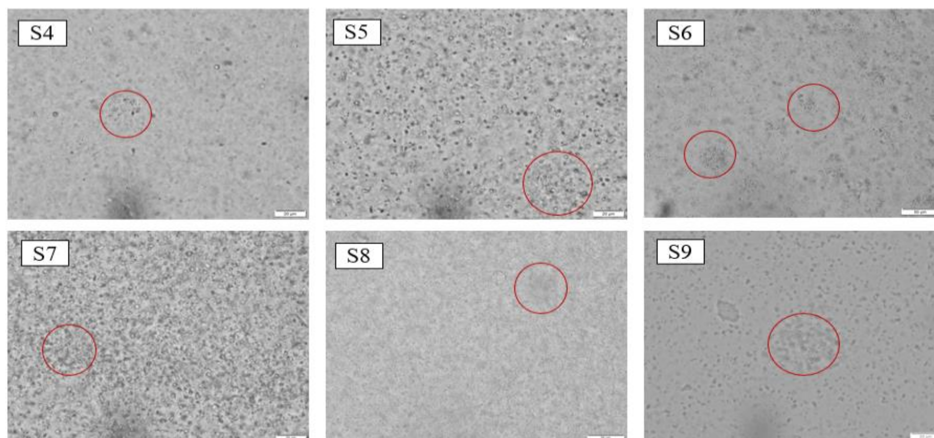


Figure 13 Optical microstructure of the samples S4-S9 after 2 weeks of storage. The images depict samples S4, S5, S6, and S9 stored at 20°C, while samples S7 and S8 stored at 4°C. The scale bar represents 20 μm. For sample codes, see Table 2.

Based on the results, it appears that the addition of dairy proteins to the plant protein did not have a significant impact on the size of the protein aggregates. This is supported by the fact that in sample S1, which only contained pea protein isolate, the size of the protein aggregates was observed to be up to 40 µm. Maybe the distribution of protein aggregates in a solution is more important in determining the consistency and stability of the sample. Even if the size of the protein aggregates is similar between samples, differences in their distribution throughout the solution can affect the overall properties of the sample, such as its viscosity.

Trial with 14% total protein content

The optical microstructure of the samples is presented in Figure 14. As is observed, all the samples presented aggregates of different sizes throughout storage. Throughout the storage period, all the samples displayed aggregates of varying sizes. Sample S20 exhibited aggregates of 50 µm, while the aggregates in S16 ranged from 5 to 20 µm. During the second week of storage, for S25 no large aggregates were found, but small aggregates were densely distributed throughout the solution, which could account for the gelled behavior at the end of storage. Aggregates measuring 30 µm were observed in sample S14, and those in sample S22 ranged from 20 µm upwards. It appears that differences in storage temperature did not have any effect on the size of the aggregates observed.

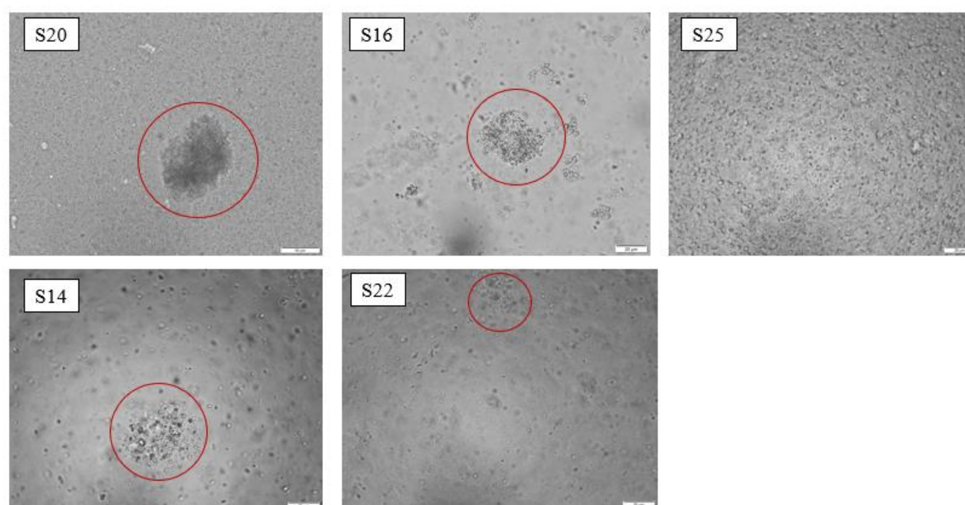


Figure 14 Optical microstructure of the samples from the trial with 14% total protein after storage. Sample/Week/Temperature/Scale bar: S20/W4/4°C/, S16/W4/4°C/40, S25/W2/20°C/40, S14/W4/20°C/40 and S22/W2/4°C/40. For sample codes, see Table 4.

Based on the results, it can be assumed that the differences in the quantity of NaCas or WPA in the samples did not significantly affect the size of the aggregates, but

maybe the distribution of them within the sample can affect other parameters like the viscosity.

4.1.7 Conclusions from the trials

Trial with 12% total protein content

The findings from the trial with 12% total protein content confirmed that the combination of PPI, NaCas, and WPA in specific ratios can result in relatively small protein aggregates and low viscosity values, which is a critical factor for the stability of protein-based RTDs. Therefore, it seemed valuable to further explore the optimal combination of these proteins in different ratios while increasing the total protein content towards 14%.

Trial with 14% total protein content

It appears that despite minor differences in composition among the various solutions examined, only one of them, sample S16 exhibited the desired properties necessary for further development as a hybrid RTD. While the variations in the formulas were not significant, it is important to conduct more specific analyses to determine the underlying reasons for the differences in performance.

It is worth mentioning that there was an attempt to increase the protein by 1% to see how the formulas perform. However, this was not feasible since all the samples were gelled after the heat treatment. The examined formulas with 15% TPC had the following compositions: PPI and PPI:NaCas:WPA (60:2.5:37.5, 60:05:35, 60:10:30, 60:20:20, 70:05:25, 70:15:15). The increasing viscosity of these samples may be related to the fact that casein micelles are disrupted due to the heat treatment, and this can cause an increase in the serum phase viscosity and this increase is higher for increased casein concentrations (Zhao and Corredig, 2014). In addition, the increase in the concentrations of PPI and WPA can lead to an increase in the solids mass fraction, which in turn, results in an increase in the volume fraction (Kornet et al., 2020). Both changes contribute to the overall increase in viscosity, and this can explain why the samples were gelled.

4.1.8 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

During the exploration phase, sample E1 made with PPI:WPA 12% in a ratio of 60:40 displayed phase separation with big particles being present after retort and during storage. However, the addition of 10% NaCas in the mixture in sample S9 had as a result the high stability of this sample. SDS-PAGE was run for these protein solutions to identify any differences in the protein composition. The results of the

gel are presented in Figure 15. A sample of PPI 12% was also prepared and analyzed as a reference to facilitate the band comparison.

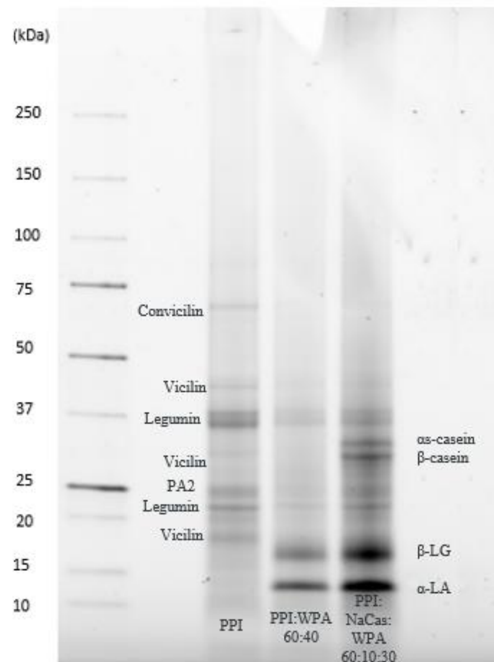


Figure 15 SDS-PAGE profile of PPI, PPI:WPA (60:40), and PPI:NaCas:WPA (60:10:30) in 12% total protein.

Some of the vicilin polypeptides that normally have a Mw of 47-50 kDa and form trimers undergo post-translational cleavage. This has results that α-, β- and γ-vicilin are obtained, of 20 kDa, 13 kDa, and 12-16 kDa respectively (Tzitzikas et al., 2006). The creation of monomeric subunits, like α+β or β+γ, explains why vicilin gave bands with this Mw in the gel.

Based on the observations that the bands of convicilin and vicilin do not appear in the mixed blends on the SDS-PAGE gel, it seems possible that these fractions were lost or degraded during sample preparation or storage.

The intensities measured with ImageJ are presented as areas in Table 11. Given the variation in protein content between the samples, it is clear why the levels of globulins and albumins were higher in the sample containing only PPI compared to the mixture of PPI and WPA. By comparing PPI:WPA and PPI:NaCas:WPA, it appears that the latter has a higher content of globulins and albumins, which was not expected since it contains 30% of WPA instead of 40% that the first one. The same was observed with whey proteins. This can be explained in different ways. One possibility is that the particles formed in the unstable sample may have trapped a certain amount of protein, potentially leading to inaccuracies during the analysis.

Another aspect is that PPI and PPI:WPA were freshly prepared and analyzed, while for the PPI:NaCas:WPA, the sample S9 that was prepared in the first trial was used. Additionally, the use of different batches of raw materials may have contributed to these differences in the composition.

Finally, the big differences in the intensity of the pea and the whey protein bands may be related to the tryptophan levels since the intensity of the protein bands in the Stain-Free gel used is increased linearly as the mass of tryptophan increases (Ladner et al., 2004). Indeed, the pea protein fractions, albumins and globulins, contain approximately 1,23% and 0,97% of tryptophan, respectively (Leterme, Monmart and Baudart, 1990). The tryptophan levels in whey proteins are relatively higher compared to pea proteins, since α-lactalbumin contains 6%, whereas b-lactoglobulin contains 2.2% tryptophan (Kelly et al., 2009; Smedegaard et al., 2021). These differences in tryptophan can explain the varying intensities observed between pea and whey protein fractions.

Finally, it can be assumed that due to the presence of αs-caseins and β-casein in the sample, it was stable, and no particles or mass formation was observed.

Table 11 Approximate areas obtained from the plot lanes with ImageJ.

	PPI 12%	**PPI:WPA 12%**	**PPI:NaCas:WPI 12%**
Ratio	**100:0**	**60:40**	**60:10:30**
Globulins	2349,82	475,24	745,82
Albumins	347,16	75,31	144,14
Whey proteins		3120,76	5333,00
Caseins			626,48

4.2 Second part: Development and examination of the hybrid RTD

The hybrid RTD was formulated by using sample S16 as a basis with the addition of milk fat, sunflower lecithin, and sweeteners. The RTDs were treated with Ultra-High-Temperature (UHT).

4.2.1 Analyses results

The visual assessment of the produced hybrid, which is presented in Figure 16, showed that there was no phase separation, sedimentation, or any other indications of instability through storage.

The color of the produced RTD appeared to be lighter since the L* value is 80,03 instead of 75,33 which was for sample S16, and this could be related to the addition of fat or the difference in viscosity that can affect the way that the light is reflected.

In addition, the pH values of the RTD did not show any significant differences when compared to sample S16. Sample S16, which was stored at 20°C for 2 weeks, had a pH of 6.80, while the pH of the RTD was measured to be 6.72. This difference can be attributed to the presence of milk fat in the RTD.

Figure 16 Visual assessment of the hybrid RTD after 2 weeks at 4°C and 20 °C.

Regarding the viscosity, it displayed higher values when compared to S16, reaching 133,52 and 129,92 mPa*s after 2 weeks at 4°C and 20°C respectively. The increased viscosity is related to the addition of fat that probably increased the solids content and volume fraction of particles in the sample.

Furthermore, from Figure 17, it appears that the PSD curves for the hybrid RTD are slightly shifted on the left of sample S16, which means that its particle size is smaller. This is explained by the second homogenization step that took place before the UHT treatment and contributed to a further particle size reduction.

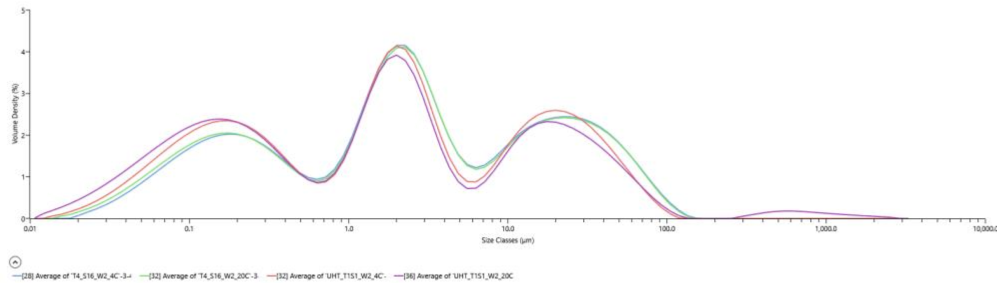


Figure 17 Particle size distribution of the sample S16 and the hybrid RTD after 2 weeks of storage at 20°C.

Finally, the microstructure of the hybrid in both conditions, which is presented in Figure 18, displayed the presence of small aggregates that had a maximum size of 5 µm. However, a higher number of aggregates that were more densely distributed in the sample were observed in the hybrid RTD stored at 20°C.

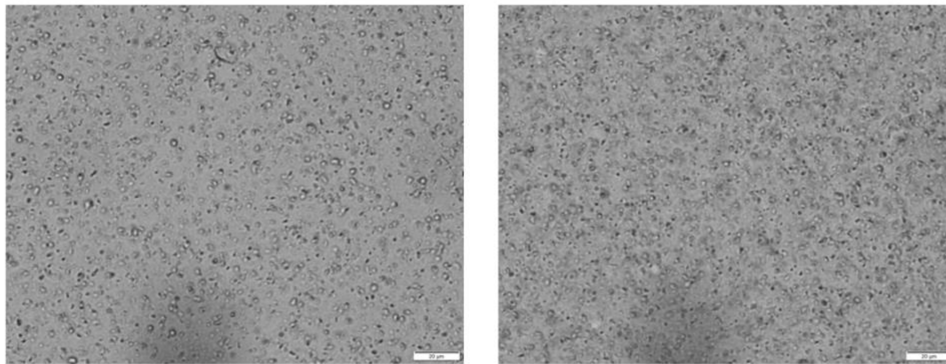


Figure 18 Optical microstructure of the hybrid RTD after 2 weeks of storage at 4°C and 20°C. The scale bar represents 20 µm.

4.2.2 Protein Digestibility-Corrected Amino Acid Score (PDCAAS)

Dairy proteins, such as whey and casein, are highly digestible and have a PDCAAS score of 1, indicating that they contain all the essential amino acids required. On the other hand, plant proteins generally have a lower PDCAAS score due to their inadequate amounts of essential amino acids. Pea protein, for instance, has a PDCAAS score ranging from 0.78 to 0.91 (Hertzler et al., 2020).

The selected protein blend used for developing the RTD showed a PDCAAS score of 1.12, which was truncated to 1. Therefore, it can be concluded that the mixed protein blend is a complete protein source that meets the amino acid requirements for human growth and development.

4.2.3 Sensory evaluation

The sensory evaluation had as its scope a detailed comparison to the reference. A sample made with 8,4% of the protein was prepared since this is the exact amount of PPI that the hybrid RTD contains. The participants characterized its taste as earthly and cardboard-like. Regarding the aftertaste, most of the participants found it bitter, beany and they found again notes that resembled cardboard. It is known that certain compounds such as hexanal, benzaldehyde, and pentanal are associated with beany off-flavors in plant-based proteins (Fischer et al., 2022). Additionally, dimethyl disulphide (DMDS), a volatile sulphur compound, contributes to a rotten odor and has a strong impact on the aroma of pea protein extract (Schindler et al., 2012).

The mean scores of taste and aftertaste compared to the reference are presented in Figure 19. As it is seen, the sample with PPI 12% was found to be from little different to different compared to the reference, with the main comments being that is less sweet and had more cardboard notes in terms of taste. The aftertaste was characterized as nutty and astringent. However, some participants found it milder, less sweet and less bitter. This can be explained by the fact that the sample had an increased viscosity due to the high amount of PPI and this results in a decrease in the intensity perception of volatile and non-volatile compounds (Hollowood, 2002).

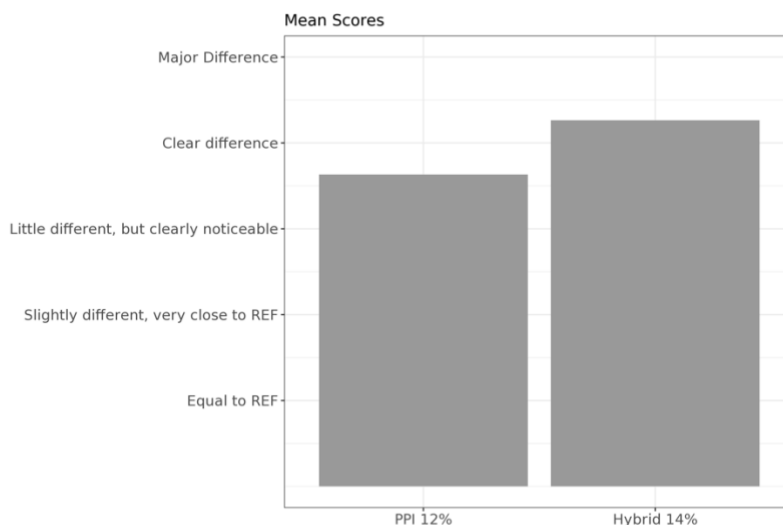


Figure 19 The mean scores of PPI 12% and Hybrid 14% compared to the reference made with PPI 8,4%.

For the hybrid with 14% total protein, it was found to have between clear and major difference from the reference. The taste was evaluated by some as less sweet, maybe justified due to the increased viscosity, and from some others as sweeter probably due to the presence of lactose. It was also a coming finding that the taste was less

beany. The aftertaste was evaluated as sweet with longer duration, milder with fewer pea notes, and milky. The taste and aftertaste characteristics of the samples compared to the reference are presented in Table 12.

Table 12 Summary of the responses received regarding the comparison of the samples with the reference.

Sample	Taste	Aftertaste
PPI 12%	cardboard, less sweet	less sweet, less bitter, nutty, astrigent
Hybrid 14%	less sweet, sweeter, less beany	milky, less beany, sweet

Overall, it can be assumed that the addition of milk proteins has a positive effect but it is not sure if this derives from the increased viscosity due to the high protein content or their ability to mask the pea protein flavors. This aspect needs to be further evaluated by performing a GC-MS analysis to detect all the volatile compounds present in the samples.

4.2.4 Packaging selection

Proper packaging selection can help mitigate the impact of oxygen and light on the oxidation of proteins and lipids in the hybrid RTD. As the project progressed rapidly, it was not possible to conduct a thorough evaluation of the product's shelf-life and to examine the real impact of these parameters on the shelf-life of the product.

In products processed with UHT, the two major problems are gelation and sedimentation. The high-protein concentration in beverages results in accelerated age gelation, which in turn increases the viscosity. This viscosity increase can be perceived as a quality defect. When sedimentation occurs, a compact layer is formed on the bottom of the primary packaging, and it normally consists of insoluble protein aggregates or protein particles of different sizes. It has been shown that sedimentation increases with the storage temperature (Karlsson et al., 2019; Singh et al., 2022).

Combining all this information along with the consideration that the high-protein content in the product will lead to a sedimentation long-term, thus limiting consumer acceptance, the idea is to use a carton package designed for aseptic filling. The package will consist of paper to create stability and strength and aluminum foil that acts as an oxygen, light, and flavor barrier, needed in our case. To protect from external moisture, adhesion between the other materials, and effectively seal in the liquid contents, the other layers of the package will be composed of polyethylene

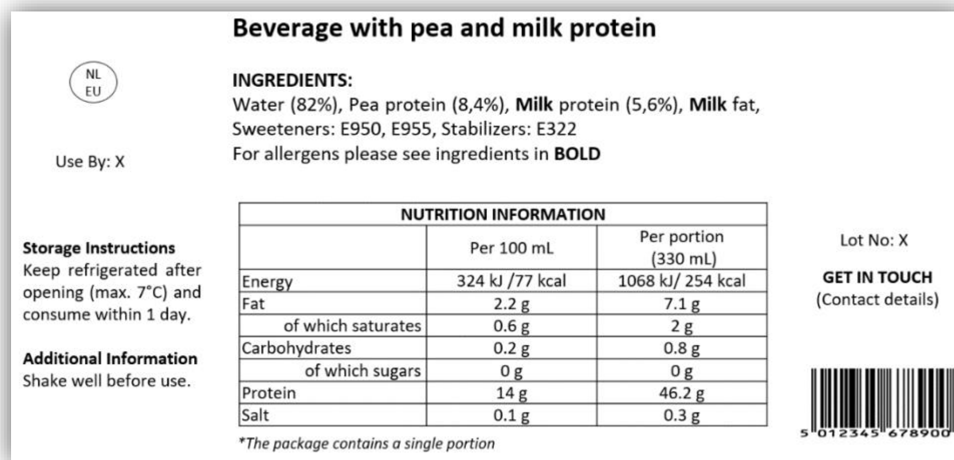
(PE). The lid will be made of high-density polyethylene (HDPE). The proposed package is shown in Figure 20.

Figure 20 The proposed packaging for the hybrid RTD.

4.2.5 Labelling & Claims

The label is one of the most critical aspects in a package along with the chosen material as it provides important information to consumers about the ingredients, the nutritional value, allergens, etc. This helps consumers to make informed decisions and it is also a mean for companies to differentiate their products from competitors since they are compared under the same umbrella. Overall, it creates transparency and helps build a loyal relationship between the company and the customer.

The mandatory information for the label including the nutrition declaration was designed in respect to Regulation No. 1169/2011 (Regulation (EU) Regulation No. 1169/2011, 2011). An example of a possible label that can be used is seen in Figure 21.



NUTRITION INFORMATION		
	Per 100 mL	Per portion (330 mL)
Energy	324 kJ /77 kcal	1068 kJ/ 254 kcal
Fat	2.2 g	7.1 g
of which saturates	0.6 g	2 g
Carbohydrates	0.2 g	0.8 g
of which sugars	0 g	0 g
Protein	14 g	46.2 g
Salt	0.1 g	0.3 g

Figure 21 Example of label designed for the hybrid RTD.

The nutrition and health claims of the product were identified according to the guidelines provided by Regulation No 1924/2006 (Regulation (EC) No 1924/2006, 2006). It can be stated that the hybrid RTD has "High protein" as at least 20% of the energy value is provided by protein. The amount of protein in the hybrid RTD corresponds to 92% of the reference intakes (RI) for adults. Thus, it may also be a suitable product for medical nutrition, but this needs to be further examined considering the recommended dietary allowances (RDAs), estimated energy requirements (EERs), and the recommended micronutrients for specific medical conditions. Finally, based on the calculation using the European Nutri-score system, the hybrid RTD was evaluated with an A score, indicating that it is considered a healthy option.

5 Conclusions

This project was aimed to the development of a hybrid RTD that meets the nutritional needs of consumers and has the suitable sensory and physicochemical properties. The development of the hybrid RTD centered on identifying the

combination of proteins that exhibited the essential characteristics required for this type of product.

The following conclusions were identified:

- In terms of processing, the protein blends can be co-dissolved, but they should be properly hydrated to obtain the full potential of their functional properties.

- Reaching 14% total protein by using only PPI is not feasible , thus making the addition of WPA and NaCas to stabilize the mixture really impactful. When focusing on a higher protein content, it is recommended to keep the PPI content low. As for dairy proteins, they should be used in the optimal ratio to achieve the lowest viscosity possible and ensure long-term product stability with limited unwanted aggregation.

- The most effective blend for the 14% total protein RTD was found to be PPI:NaCas:WPA in a ratio 60:05:35, since when it was examined as a protein solution without any additional ingredients, it displayed low viscosity.

- Regarding the sensory properties, the developed RTD exhibited differences compared to the reference sample made with the same quantity of PPI that was 7,5%. This indicates the possibility of developing products by limiting the beany flavor that derives from pea protein.

- The hybrid RTD reached a PDCAAS equal to 1,12, which shows that is a complete protein source that meets the amino acids requirements for human growth and development.

Finally, it seems that the mixed blends of plant-based and dairy proteins are a combination, which gives several functional properties, such as emulsification, self-stability and homogeneity and at the same time it can lead to a formulation that has a high-quality protein source and improved sensorial properties. By incorporating plant-based protein, the final product will not only offer a most cost-effective alternative to dairy RTDs, but it will also appeal to consumers looking for flexitarian, more sustainable and less-animal derived options.

6 Future recommendations

It is recommended to explore the full potential of the plant/dairy blends and identify the benefits that these mixtures can offer in terms of emulsification, sensorial or any other characteristics. More analytical techniques such as FT-IR can also be used to study the aggregation between pea and dairy proteins to be able to provide more valuable insights on the behavior of these blends.

Regarding the processing parameters, it is recommended to explore the use of cold-homogenization as a method to incorporate insoluble proteins into the casein micelles, an aspect that was recently discovered but could not be studied due to time constraints. Further investigation is recommended to determine the optimal timing and conditions for homogenization in relation to the hydration of mixed protein blends. It would also be valuable to compare samples that are overnight hydrated, as was done in this study, to those that are not, in order to determine if overnight hydration is crucial for mixed blends as it is for some dairy proteins. Finally, from development perspective, it could be also examined what the pH adjustment to certain levels has as result in the stability and viscosity of the mixtures.

As a future step to maintain low viscosity throughout storage, it may be beneficial to explore the use of a lower amount of fat in the RTD formulation. In the case of the PPI:NaCas:WPA protein blend, WPA has the ability to mimic fat droplets, thereby maintaining the emulsification properties of the mixture even with a lower fat content. Furthermore, using the GC-MS methods to analyze the volatile compounds would provide valuable insights into the flavor differences between plant-based and mixed formulations and would help identifying the correlation with the sensory findings.

Moreover, the development of hybrid RTDs has the potential to provide the specific nutritional needs and thus it is recommended to explore the suitability of the hybrid mixtures into medical or infant nutrition. Last but not least, to further enhance the product's positioning, it is recommended to conduct a cost analysis to strengthen the concept of adding pea protein to reduce the cost of the production, while maintaining the same nutritional benefits for human growth and development.

Finally, from a sustainability perspective, it would be beneficial to investigate the differences in the environmental impact of producing plant-based, dairy and hybrid RTDs. This analysis would allow for a better understanding of the ecological impact of each product.

The idea of hybrid blends is a relatively new concept that has not been extensively explored. Therefore, conducting further research is necessary to better understand the potential of hybrid blends. More exploration projects must be undertaken to determine the feasibility and viability of using hybrid blends in different applications and to fully realize their benefits.

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