



Vegetable Protein Functionality

From Milk Analogue To Fiber

Joana Vanessa Ramos Galante

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Examination Committee

Chairperson: Prof. Duarte Miguel de França Teixeira dos Prazeres (Bioengineering Department, IST)

Supervisors: Prof. Marília Clemente Velez Mateus (Bioengineering Department, IST)

Dr. Ir. Fred van de Velde (NIZO food research B.V.)

Members of the Committee: Prof. Maria Ângela Cabral Garcia Taipa Meneses de Oliveira (Bioengineering Department, IST)

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Abstract

Proteins are important food ingredients, with impact on functionality. Nowadays, consumers are aware of the environmental impact of food production, therefore requiring sustainable food products. Vegetable proteins are more sustainable than animal proteins. In this work, we present a study on the functionality of vegetable proteins, namely of *Eragrostis tef*.

Protein extraction from teff flour was performed with different solvents. The best extraction involved the use of RO-water at room temperature at pH 8.0. Proteins were extracted from teff flour, with and without DTT, and were afterwards characterized by SDS-PAGE and DSC.

A milk analogue from teff flour was developed and improved. Dialysis was proven to be essential in the development of a satisfactory milk drink from teff.

Functional properties of water-soluble proteins from teff flour were also studied. It has been shown that teff flour proteins have high water and oil absorption capacities. This flour could therefore be useful in flavor retention, improvement of mouth feel and extension of shelf life in foods. Moreover, the foams produced by the water-soluble proteins of teff are highly stable, especially between pH 7.0 and 8.0, which can be valuable for product development.

Proteins and polysaccharides are broadly used in food production, since their interactions improve the texture of products. In the present work, the influence of various parameters in the formation of different types of fibers was studied. The best fibers formed from the tested conditions were the unheated fibers with 4% (w/w) Ingredient 1 addition (yield of fiber formation of 23.1%).

Keywords: *Eragrostis tef*, teff, vegetable proteins, polysaccharide, functionality.

Resumo

Proteínas são importantes ingredientes alimentares, com impacto na funcionalidade. Os consumidores estão cientes do impacto ambiental da produção alimentar, exigindo produtos sustentáveis. As proteínas vegetais são mais sustentáveis que as animais. Neste trabalho, apresenta-se um estudo da funcionalidade de proteínas vegetais, nomeadamente de *Eragrostis tef*.

Com diferentes solventes, foram extraídas proteínas da farinha de teff. A melhor extracção envolveu o uso de água obtida por osmose reversa à temperatura ambiente a pH 8.0. As proteínas foram extraídas com e sem DTT e caracterizadas por SDS-PAGE e DSC.

Um análogo de leite a partir de teff foi desenvolvido e melhorado. Foi comprovado que a diálise é essencial para o desenvolvimento de um leite satisfatório a partir de teff.

As propriedades funcionais das proteínas solúveis em água presentes em farinha de teff foram estudadas. Foi demonstrado que estas proteínas apresentam elevadas capacidades de absorção de água e óleo. Esta farinha poderia ser útil em retenção de sabor, melhorias no paladar e extensão da *shelf-life* em produtos alimentares. Além disso, as espumas produzidas pelas proteínas solúveis em água presentes no teff são bastante estáveis, especialmente entre pH 7.0 e 8.0, o que pode ser valioso no desenvolvimento de produtos.

Proteínas e polissacáridos são utilizados em produção alimentar, pois as suas interacções melhoram a textura dos produtos. No presente trabalho, a influência de vários parâmetros na formação de diferentes fibras foi estudada. As melhores fibras formadas nas condições estudadas foram as fibras com adição de 4% (w/w) de Ingrediente 1, sem tratamento por calor (rendimento de formação de 23.1%).

Palavras-chave: *Eragrostis tef*, teff, proteínas vegetais, polissacárido, funcionalidade.

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List of Abbreviations

BD	Bulk Density
CD	Celiac Disease
CF	Concentration Factor
CL	Cross-Linker
CLSM	Confocal Laser Scanning Microscopy
DSC	Differential Scanning Calorimetry
DTT	Dithiothreitol
EA	Emulsion Activity
EC	Emulsion Capacity
ES	Emulsion Stability
FAO	Food and Agricultural Organization
GF	Gluten-Free
GFD	Gluten-Free Diet
GRAS	Generally Recognized As Safe
IP	Isoelectric Point
LGC	Least Gelation Concentration
MWCO	Molecular Weight Cut-Off
OAC	Oil Absorption Capacity
PBD	Packed Bulk Density
PS	Protein Solubility
RO	Reversed-Osmosis
RT	Room Temperature
SDS – PAGE	Sodium Dodecyl Sulfate – Poly Acrylamide Gel Electrophoresis
SPI	Soy Protein Isolate
WAC	Water Absorption Capacity
WPI	Whey Protein Isolate
WSP	Water-Soluble Proteins

1. Introduction

1.1. NIZO food research

NIZO food research is one of the most advanced private contract research companies worldwide. Based in the Food Valley (The Netherlands), it is a small/medium sized company, with 180 employees that help food companies to improve their products, therefore becoming more profitable. NIZO works with international dairy, food and beverage, ingredients and biotechnology industries.

NIZO's expertise and technologies are applied to the following scientific areas: food physics, life sciences and processing technologies. Through this knowledge, improvements in food and ingredients can be applied to various fields, namely flavor, texture or health, cost reduction, processing and food safety. In addition to research and development projects, NIZO has a food-grade Pilot Plant, which is one of the largest in the world and it's available for test productions or even to develop new products at the industrial kitchen facilities. In Figure 1, there is a schematic representation of NIZO's history. There will only be a short reference to the company's history in this report, highlighting its most relevant achievements.

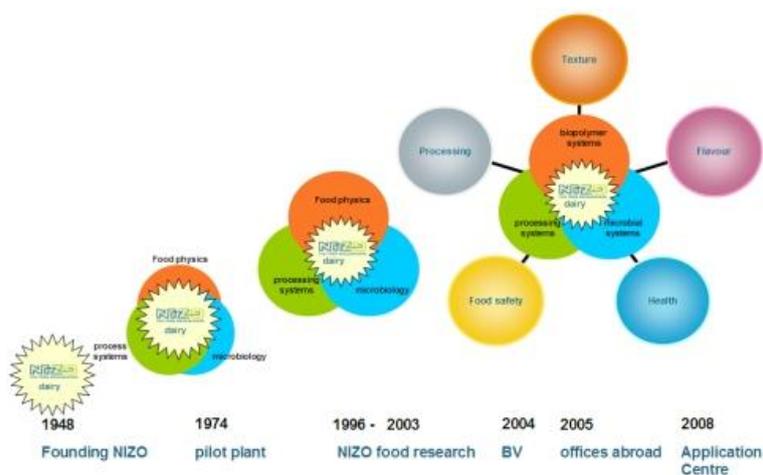


Figure 1 - NIZO's development history.

With more than 60 years of industry experience and having started in 1948 as a quality and food safety control institute, NIZO nowadays applies its knowledge to food in general. In 1974, the food-grade Pilot Plant was built, therefore permitting the translation of NIZO's research to an industrial level. In 2003, NIZO became a private company. In 2005, it established offices in the UK, France and the USA, and in 2007 it also opened an office in Japan.

NIZO's success in the market is related to the deep knowledge of ingredients, their functionality and interactions in the products.

1.1.1. Project

Proteins are an important class of food ingredients, with impact on functionality [1]. Nowadays, consumers are more and more aware of the environmental impact of food production, and therefore require sustainable food products. Vegetable proteins are more sustainable and cheaper but less functional, when compared to animal proteins [1].

NIZO food research has 60 years of experience in dairy protein technology, including molecular characterization, physical functionality, modification and application. In recent years, this expertise has been expanded and also directed to vegetable proteins. Strategic research within the department of Flavor and Texture has shown that fiber-like structures can be prepared from vegetable proteins. These structures, which will be simply denominated as fibers in the current project, can be used in the development of meat alternatives. Strategic research has also shown the possibility of development of a milk analogue based on cereals. The goal of this internship was to study the functionality of vegetable proteins from new sources, such as teff, and development of products thereof, such as meat alternatives and milk analogues.

This project started from the currently developed prototypes of fibers and milk analogues. Basic parameters that influence the quality of the products, such as concentration, temperature and solvent quality were studied for different vegetable proteins.

The next section of this report consists on a literature review, which is mainly focused on teff, describing its relevant properties and applications, and on teff proteins, highlighting the extraction methods and functionality of these proteins. Moreover, a brief review on protein-polysaccharide interactions is presented in the end of the same section. In the second section, the materials and methods of this project are presented. The third section contains the results and the discussion of those results, while the final section encloses the conclusions and recommendations.

1.2. Literature overview

1.2.1. Celiac disease

Celiac disease (CD) is an auto-immune disorder triggered by the ingestion of grains that contain gluten, which is a mixture of storage proteins (prolamins and glutelins) present in several cereals, such as wheat and barley, in genetically susceptible people. Those individuals are, therefore, permanently intolerant to gluten, so if they consume gluten, their intestinal mucosa suffers a continuous damage, more specifically a chronic inflammation, and therefore its functionality can be seriously weakened [2], [3]. This inflammation leads to a deficient absorption of important nutrients, such as iron, folic acid, calcium and fat-soluble vitamins [4].

Celiac disease is the end result of three processes, genetic predisposition, environmental factors and immunologically-based inflammation, culminating in intestinal mucosal damage [4]. CD is one of the most common life-lasting diseases around the World, with an estimated mean prevalence of 1% of the general population [2]. The prevalence of the disease has increased in recent years [3]. There are no available treatments nowadays, apart from a strict lifelong elimination of gluten from the diet of the

affected individuals. The adherence to a strict gluten-free diet (GFD) has been proven to lead to a progressive reduction of the symptoms, along with the healing of the intestinal mucosa [2], [4], [5].

Celiac patients suffer a significant impact on their daily life, due to the dietary changes required to start and maintain a GFD [2], [5]. The main reason for this is that the majority of commercially available staple foods worldwide, such as bread and pasta, contain gluten. Also, gluten-containing wheat proteins or starches are often added to many commercial products, such as ready meals, convenience food products and some medicines, to act as fillers, thickeners, binders or stabilizers [2].

A promising area is the use of alternatives to common gluten-containing grains, like minor or pseudocereals, such as amaranth, buckwheat, sorghum and teff. These grains are also nutrient-dense, so, their incorporation in the GFD could simultaneously add variety and improve the nutritional quality of the products [2], [3].

1.2.2. Gluten-free products

Nowadays, due to the apparent increase in celiac disease (see section 1.2.1) or other intolerances to gluten, the demand for gluten-free products is rising [4].

Gluten contains two protein fractions: glutenin and gliadin. Glutenin is a rubber-like mass when completely hydrated, whereas gliadin gives origin to a viscous and fluid mass when hydrated. Gluten is an essential structure-building protein in flour, and is responsible for the cohesive, elastic and viscous characteristics of the dough. It also contributes to the appearance and crumb structure of several baked products. Also, the gluten matrix greatly influences the important properties of dough, which are extensibility, resistance to stretch, tolerance to mixing and gas-holding ability. Therefore, the replacement of gluten in food products is a great technological challenge, and many commercially available gluten-free products have a low quality, with poor mouth feel and flavor [4].

In recent years, there has been more research and development of gluten-free products, through different approaches which have included the use of starches, dairy products, gums and hydrocolloids, other non-gluten proteins and prebiotics as alternatives to gluten, in order to improve the structure, mouth feel, acceptability and shelf-life of gluten-free bakery products. Starches and gums are often used to improve texture and appearance properties of cereal-based products. Gums, that can be polysaccharides or proteins, are used in gluten-free products for gelling, thickening, water retention and to improve texture [4].

Generally, gluten-free products are not enriched, and are often made from refined flour or starch, so they may not contain the same levels of nutrients as the gluten-containing constituents that they are replacing [4], [6]. Therefore, the enrichment of gluten-free baked products with dietary fiber has been extensively researched, for instance on wheat bread [4]. Other studies have also been carried out on gluten-free products, such as breads, pasta, biscuits and beer, and the level of dietary fiber was increased using different sources, such as corn starch and amaranth [6].

1.2.3. Millets

Millets, also called minor cereals due to its cultivation being only performed in small areas over the world, are tiny cereals, grown mainly in semi-arid regions of Africa and Asia. The major species in terms of area cultivated and total yield is pearl millet (*Pennisetum glaucum*) [7].

Even though millets represent a small part (less than 5%) of the world grain production, they are of paramount importance in the semi-arid and sub-humid zones as staple crops. Millets are a significant staple food in Africa, especially in very dry Savanna areas [7]. This crop can be grown in hot, dry and rainy areas, and also in areas with poor soil, and due to that fact, it is very significant to food security in developing countries. In fact, it is in the low precipitation areas that millets are thought to have the most advantage in relation to major cereals [7]. Millet seeds are very small and can be yellow, white, grey or red [3]. There are two main categories of millet species: pearl millet and the small or minor millets, which include finger millet, fonio and teff [7].

The production of millets ranged from 33.6 to 37.3 million tons in 2001 to 2005, slightly higher than the estimated 29.3 million tons in the 1980s. Quantitatively, the most important millet species, in ascending order, are finger millet, proso millet, foxtail millet and pearl millet [8]. Millets can be sold whole, milled into flour, flakes or grits, puffed or incorporated into pasta, cereals, cracker and other gluten-free formulations [3].

Millets are high energy foods and nutritionally equivalent or superior to other cereals. Generally, millets contain high levels of methionine, they have a high digestibility and their protein content is comparable to those of wheat, maize and rice. Therefore, these foods are especially recommended for children, lactating mothers, elderly and convalescents [7].

The research in this field has been increasing over the years, even though it has been mainly focused on pearl millet. New varieties of millets have been created, with advantageous characteristics such as higher drought resistance, better grain yields, tolerance to diseases and acceptable food quality. The processing methods are also being improved, to develop products more easily accepted by the final consumer, in an attempt to broaden the market [7].

1.2.4. Teff

Teff (*Eragrostis tef*), also known as tef, is a tropical cereal that belongs to the family of *Poaceae*, subfamily *Eragrostoidae*, tribe *Eragrostae*, and genus *Eragrostis*. The word *teff* is thought to have been inspired in the Amharic word *teffa*, whose meaning is “lost”, because the grain has a reduced size and can be easily lost when dropped [9].

Teff is mostly cultivated and consumed in Ethiopia, where it represents 20% of cereal production, and it is also cultivated in Eritrea [7], [8]. Its average annual cultivation area has about 1.4 million ha, producing 0.9 million tons of grain [7]. Teff is the staple food crop and principal source of carbohydrates for the majority of the Ethiopian population [10].

Teff is a gluten-free food, because it contains lower amounts of gluten-like prolamins that might cause problems to celiac patients. Also, in comparison with the commercially-available gluten-free products,

teff contains more vitamin and fiber, which are extremely valuable as supplement in a GFD [5]. For those reasons, this cereal has attracted a great interest in the global market. It has high demand and market value, which gives more revenue to farmers than other crops. Teff straw is used as feed since it is protein-rich, and it is preferred by cattle, making its market price relatively high [10].

Although teff cultivation has started in Ethiopia, the commercial production for international markets in Europe and North America has begun in the USA and South Africa, due to the popularity of *injera* (fermented flat bread made from teff) in Ethiopian restaurants in those markets. Teff is also cultivated in Yemen, Kenya, Malawi and India for food, and in Australia as forage [7].

Teff is resistant to harsh environmental conditions, and it is generally cultivated at high altitudes, between 1700 and 2800 m [8], [9]. Teff can be grown in areas where the average annual rainfall is of about 1000 mm, with relatively high yields [8]. It can be grown in various soil types, even in waterlogged and acidic soils [7]. It adapts to dry conditions, being considered a drought-resistant crop. Despite that fact, one of the major yield-limiting factors in teff production is water shortage [10].

Two types of teff are recognized in Ethiopia, the white and the brown/reddish seed types, but the white is preferred for consume [7], [8]. The grain has an oval shape and a reduced diameter (less than 1 mm). Its starchy endosperm is mainly composed of starch granules and protein bodies. Whilst the protein bodies are not coalescent, the starch granules agglomerate and are polygonal, with a diameter of 2-6 μm [8]. Teff seeds can be stored for long periods of time without being damaged by insects. However, the minimal size of the grain makes planting, harvesting and handling very hard, which poses a limitation to production [7].

This cereal is a very good material for malting and brewing due to its excellent balance of amino acid content, which includes the essential amino acids for humans. Teff is ground into whole-grain flour because the seeds are small, which results in a high fiber and nutrient content. In fact, the nutrient composition of its grain points out to its high potential to be utilized in the food and beverage industry [9].

Teff is particularly useful to augment the hemoglobin level in the human body and also to prevent diseases such as malaria, anemia and diabetes [9].

Recently, the utilization of teff in alimentary products is becoming more and more popular, because it is a gluten-free alternative to wheat products and also a nutrient-rich ingredient, all features that make teff an adequate substitute for wheat and other cereals in food applications, and also a suitable food ingredient to include in the diet of celiac patients [9] (see section 1.2.1). Teff proteins are also thought to be easily digestible, due to the reduced size of prolamins [11]. Despite being gluten-free, teff flour has been reported to produce high-quality leavened flatbread, which is used to produce baked goods and traditional breads, like *injera* [12].

In a study performed in the Netherlands with a significant number of teff-consuming celiac patients, the majority of individuals experienced an important reduction in symptoms, which was not only related to a reduction in gluten-intake, but also to an increase in fiber consumption [5].

1.2.4.1. Properties

Teff has a nutritional composition (see Table 1) similar to other millets, except from the fact that the content of albumins and globulins is higher than that of prolamins, which is unusual. Due to the lower content of prolamins, teff has more protein digestibility than other cereals [8].

Teff is always consumed in the whole grain form, and therefore it has a higher nutritive value than the majority of the consumed cereals, such as wheat, barley and maize [13]. The balance of amino acids is considered to be good, apart from the relatively low lysine content [8]. The main protein fraction in teff (albumin, glutelin and globulin) is the most digestible, so the protein digestibility in teff is high. Teff has a protein content of 9 to 11%, slightly higher than in sorghum, maize and oats. Teff contains more calcium, iron, magnesium and zinc than most cereals, but lower potassium than barley, oats and wheat. The absence of anemia in high teff consuming areas of Ethiopia has been attributed to the high iron content of teff seeds [13].

Table 1 shows the nutritional and essential amino acid composition of this cereal. Apart from having low lysine, isoleucine and threonine, the amino acid composition in teff is well-balanced and can be compared to that of egg [9], [13]. The amino acid balance in teff makes it good for malting and brewing [9].

The average proximate composition of teff is about 9.6% protein, 2.0% fat, 73% carbohydrate, 3.0% crude fiber and 2.9% ash. Teff is relatively rich in energy: 1411 kJ 100 g⁻¹ [9].

The vitamin content of teff is average, for a cereal (Table 1). However, the level of vitamin C is adequate, providing more than 100% of the recommended dietary allowance [13]. Vitamins help in the prevention and treatment of diseases, such as heart conditions, high cholesterol levels and eye and skin problems. In general, vitamins also improve the body mechanism and have unique roles in the body, not performed by other nutrients [9].

The mineral composition of teff is considered adequate (Table 1). The iron and calcium contents are higher than in wheat, barley, sorghum and maize, as shown in Table 2 [9], [13]. The amount of magnesium in teff is also higher than in other cereals, except sorghum [9].

1.2.4.1.1. Carbohydrates

Teff is mostly starchy in composition (73%). In fact, its starch content is higher than the starch content of most cereals. The starch granules in teff are composed of various polygonal simple granules, very small and joint together to form agglomerates [9].

The gelatinization temperature of raw teff has been reported as being in the range of 68-80°C, similarly to other tropical cereal starches such as that of sorghum. During the malting process, there is an improvement of the starch degradability, possibly resultant from changes in the extent of alteration of starch structure and composition. It is known that the degree of gelatinization and the temperature at which starch is degraded depends on other factors, such as fat and amylose content and the size of the granules [9].

1.2.4.1.2. Proteins

The average protein of teff is comparable to the protein content of barley, wheat, maize and pearl millet, and higher than the protein content of rye, brown rice and sorghum. Glutelins and albumins represent the major protein storage components, and their descending order of importance is glutelins (44.55%), albumins (36.6%), prolamins (11.8%) and globulins (6.7%) [9]. More emphasis to teff proteins will be given in section 1.2.5.2.

1.2.4.1.3. Fat

The crude fat content of teff seeds is higher than that of wheat, rye and brown rice, but lower than the content of barley, maize, sorghum and pearl millet. The fat in teff is mainly composed of fatty acids; teff grains are rich in unsaturated fatty acids, which are very important for nutrition, because some of them are not synthesized by humans [9].

1.2.4.1.4. Crude fiber

The crude fiber content in teff is much higher than in most cereals. Many gluten-free foods made with starches or refined flours that have low fiber content may contribute to an inadequate fiber intake by the consumers of these products. This happens because during refining, there is a removal of the outer layer of the grain, which contains the majority of the fiber. However, since teff has small grains, it is mostly made into whole-grain flour, having a higher fiber and nutrient content in comparison to other grains. An adequate consumption of dietary fibers has many health benefits, aiding in the prevention of diseases such as colon cancer and diabetes [9].

1.2.4.1.5. Minerals

In comparison to other cereals, teff is rich in calcium, zinc, magnesium, iron, phosphorus and copper [9]. Mineral composition of teff is summarized in Table 2. Calcium, for instance, influences many bodily functions, and it provides strength to the skeleton and hardness to the teeth. It has been reported that the high intake of calcium in the diet prevents gaining of weight and the accumulation of fat [9]. The intake of iron seems to prevent anemia and malaria. Also, studies performed on athletes indicated that the nutritional quality of teff and its iron content may contribute the most for the good resistance and fitness of Ethiopian athletes [9].

1.2.4.1.6. Phenolic compounds

Phenolic compounds have possible health benefits, including protection against oxidation and prevention of cardiovascular disease and cancer [9], [14]. However, these compounds may contribute to bitterness and aftertaste in food products [15]. Since teff flour is wholegrain, it contains more phenolic constituents than other cereal flours, potentially worsening the problem of bitterness [15]. The major phenolic compound in teff is ferulic acid, but others are present, such as vanillic, coumaric and cinnamic acids, among others [9]. Phenolic compounds are also used as natural antioxidants in the food industry, but they may inhibit digestive enzymes and reduce food digestibility.

Table 1 - Nutrient and amino acid composition of teff [11]. The letter a refers to a pure strain, and b to twelve mixed strains.

Major nutrient ^a		Minerals ^b		Essential amino acids	
Name	g 100 g ⁻¹	Name	mg 100 g ⁻¹	Name	Mean g 16 g-1 N
Moisture	11.0	Calcium	159	Cystine	1.9
Food energy (kJ)	1411	Chloride	13	Isoleucine	3.2
Protein	9.6	Chromium (µg)	250	Leucine	6.0
Carbohydrates	73.0	Copper	0.7	Lysine	2.3
Fat	2.0	Iron	5.8	Methionine	2.1
Crude fiber	3.0	Magnesium	170	Phenylalanine	4.0
Ash	2.9	Manganese	6.4	Threonine	2.8
		Phosphorus	378	Tryptophan	1.2
		Potassium	401	Tyrosine	1.7
		Sodium	47	Valine	4.1
		Zinc	20		
Vitamins (mg 100 g⁻¹)					
Vitamin A (RE)	8				
Thiamin	300.3				
Riboflavin	180.2				
Niacin	250.5				
Vitamin C	88				

Table 2 - Mineral composition of various teff types compared to spring wheat, winter wheat, winter barley and sorghum (mg-1 100 g grain-1) [11]. The letter a refers to a pure strain, and b to twelve mixed strains.

Mineral	Purple teff ^a	Purple teff ^b	White teff ^a	White teff ^b	Spring wheat	Winter wheat	Winter barley	Sorghum
Ca	140	207	160	187	<100	<100	<100	<100
P	415	453	480	440	51	400	480	520
Fe	12.70	23.63	10.60	12.47	7.85	4.00	3.50	6.65
Al	2.25	12.40	<1	1.43	<1	<1	<1	<1
Ba	1.5	2.30	2.17	2.53	0.75	0.60	0.70	<0.1
B	1.60	1.33	1.37	1.30	1.20	1.15	1.10	1.65
Co	0.05	0.06	0.05	0.08	0.06	0.06	0.03	0.03
Cu	4.67	5.86	2.30	4.90	2.00	1.10	1.40	2.35
Mg	155	190	183	190	150	120	130	180
Mn	1.55	2.50	1.67	4.43	5.30	3.60	1.20	2.90
Mo	0.06	0.09	0.07	0.09	0.06	0.06	0.04	0.05
K	570	223	330	<100	370	330	440	440
SiO	Trace	157	Trace	90	Trace	Trace	Trace	Trace
Na	25.20	20.07	22.70	19.73	19.50	16.85	39.20	14.15
Sr	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Zn	5.60	7.57	6.33	7.23	6.00	3.95	4.50	4.40

1.2.4.2. Applications

Teff and other gluten-free ingredients can be used to create gluten-free products, or to substitute wheat in existent products [6]. Teff grains may be mixed with soybean, chickpea and other grains, to produce infant foods [9]. Teff flour may also be used in baby foods, baking products, pastas or extruded products [16].

In Ethiopia, teff is usually ground to flour and used to make *injera*, which is pancake-like bread made from dough fermented for 2 or 3 days. Teff is considered superior to other cereals in the production of *injera*, due to being staling-resistant. It can also be mixed with barley or sorghum flour for the same purpose, but resulting in a decrease in the quality of the bread. Teff flour is also utilized to make porridge and some traditional alcoholic beverages like *tella*, among others [9]. Teff flour can also be used as a thickening agent in products like soups, stews, gravies and puddings. It is considered a gourmet food, particularly in the USA [9].

1.2.5. Vegetable proteins

Vegetable proteins have been more and more studied, typically in the form of protein extracts or seed flours, due to their possible role as an alternative to animal and dairy proteins in food products [17], [18], [19], [20]. These studies are justified with the relatively inexpensive character of vegetable proteins, when compared to other types of proteins [18], [20], [21], [22], [23], [24]. In fact, there is a worldwide demand for economic sources of protein, especially in developing countries [23], [25], [26], [27]. Nevertheless, efficiently using vegetable proteins in the food industry depends on their functionality [18], [19], [20], [24], [28], since functional properties play an important role in the physical behavior of food ingredients during processing and storage [27]. Some of the functional properties studied for these proteins are their solubility, foaming ability and stability, emulsifying activity and stability, gelation and water and oil absorption capacities [29].

Apart from their advantages, vegetable proteins have a low solubility and their seeds/flours may contain anti-nutritional components, which not only reduce digestibility but also confer off-taste and often color to the products made from these proteins, making them less attractive to the consumer [30]. To remove these components, a heat treatment can be performed, which may lead to unfolding and aggregation of proteins, decreasing their solubility and possibly their functionality [31].

Natural vegetable proteins are very important ingredients because they are safe, highly biocompatible and abundant in nature [21], [23], [32], have a high nutritional value and a low production cost [33]. In fact, the improvement on nutrition of products as a consequence of partial replacement of animal foods with vegetable proteins has been reported [19], [34]. Nonetheless, one important drawback is the low digestibility of some vegetable proteins when consumed, due to the shortage of sulfur-containing amino acids [30].

Although most legumes only contain about 2% of protein, some of them, like beans, peas and lentils, are very rich in protein [35]. Most importantly, plant foods are relevant sources of antioxidants, minerals, unsaturated fats, complex carbohydrates, vitamin C and fiber [19], [36], aiding in the prevention of chronic diseases [36].

The main extractions in this project were performed from cereal proteins, so the next paragraphs of this section will be dedicated to these proteins. Cereals are considered the most important crops worldwide, with total annual grain yields of approximately 2000 million tons, while legume seeds have an area of about 250 million tons [37]. Cereal grains contain less protein than legumes, with an average content of approximately 10-12% dry weight, but they still provide more than 200 million tons of protein for the human and animal nutrition [37], [38]. Since cereal proteins have crucial nutritional and functional roles, they have constituted a target of research for years [38].

About half of the total protein in cereal grains is storage protein. The major storage proteins in cereal seeds are, in most cereals, prolamins [33], [37], [39]. These proteins are a good source of dietary protein and, in wheat, are the major components of gluten, thus influencing the quality of wheat flour for, as an example, making bread. Even though not all of the cereal prolamins are related to each other, they have a property in common, which is their insolubility in water or diluted salt solutions, when in their native state [37], [39].

Gluten is divided into two fractions, one soluble and the other insoluble in aqueous alcohol. These alcohol-soluble proteins were named prolamins, due to their high contents of proline and amide nitrogen. The alcohol-insoluble protein fraction is called glutelin [39].

Cereal proteins are, in general, deficient in some essential amino acids, and thus may be nutritionally complemented by the combination with legume proteins, and then used in animal feeds. Cereal proteins are usually rich in sulphur-containing amino acids and legume proteins in lysine [37].

Many analytical techniques have been used to study cereal proteins, being electrophoresis one of the most utilized methods. Other methods, like HPCE (high-performance capillary electrophoresis), SDS-PAGE and isoelectric focusing have been used to improve the resolution of protein separation [38].

Fractionation, or separation, of cereal proteins can be performed by selective extraction with different solvents. Cereal proteins have been mainly separated based on Osborne's classification, in which proteins are divided into water-soluble proteins (albumins), salt-soluble proteins (globulins), alcohol-soluble proteins (prolamins), and acid or alkaline-soluble proteins (glutelins). Normally, these extractions are performed in the described sequence: albumins are extracted, followed by globulins, and so on [38].

1.2.5.1 Extraction methods

Since the protein extractions in this project were performed from cereals, the literature search was more focused on the extraction methods for cereal proteins. In the literature, the majority of studies found are focused on the extraction of amaranth. The main reagents in the extraction methods found are presented in Table 3. A brief description of method 1 will be provided below.

Total extraction of cereal proteins is challenging, due to their high complexity and heterogeneity. These proteins interact with each other, but also exist in an environment with interfering compounds, such as polysaccharides, lipids and proteases. In order to efficiently extract cereal proteins, SDS or urea-containing buffers should be used, to disrupt the protein aggregates into a solution of proteins that could be better detected in electrophoretic processes [40].

Table 3 - Main reagents in the cereal protein extraction methods found in the literature, along with the respective references.

Method	Reagents	Reference
1	Albumins and globulins extraction: 1.25 M NaCl	[12]
	Prolamins extraction: 60% (v/v) tert-butanol, with or without 0.05% (w/v) DTT at RT	
2	Total protein extraction: 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.0625 M Tris-HCl	[41]
	Albumins and globulins extraction: 1 M NaCl Prolamins extraction: 70% (v/v) aqueous ethanol; 50% (v/v) aqueous propan-1-ol, 2% (v/v) acetic acid and 2% (v/v) 2-mercaptoethanol	
3	Prolamins extraction: 0.0125M sodium borate, 1% SDS, 2% 2-mercaptoethanol (pH 10) in a flour-solvent ratio of 1:10 60% or 70% (v/v) ethanol at RT	[42]
4	Prolamins extraction: 55% (v/v) isopropanol with 4% (v/v) 2-mercaptoethanol Glutelins extraction: 0.125M sodium borate buffer (pH 10) with 3% (v/v) 2-mercaptoethanol and 1% (w/v) SDS	[33]

The first extraction method represented on Table 3 is related to the fractionation of teff proteins. According to the authors [12], albumins, globulins and low-molecular-weight nitrogenous compounds can be extracted from flour with 1.25 M NaCl. The following step is the recovery of the extracts by centrifugation at 6000 g for 10 min, at 4°C. The supernatant is then submitted to a dialyzing process against distilled water for 24 h at 4°C, with three changes of water, and then freeze-dried. This first extract corresponds to the albumin and globulin fraction. As for prolamins, they can be extracted from the precipitate with 60% (v/v) tert-butanol containing 0.05% (w/v) DTT at RT. The remainder of this procedure (extraction of glutelins) can be consulted in the literature [12]. Other alcohols, such as 60 or 70% (v/v) aqueous ethanol (see methods 2 and 3 of Table 3), can be used to extract prolamins.

1.2.5.2. Teff proteins

The seed protein content of teff has been reported as ranging between 8.7 and 11.1%, according to the literature [9]. Prolamin is the major storage protein in teff, as in other tropical cereals. Teff protein is composed of 3-15% prolamins. The amino acid composition of teff shows a low amount of lysine, but high levels of glutamine, alanine, leucine and proline [9], [12].

Prolamin is only poorly extracted by aqueous alcohols in the absence of a reducing agent. SDS-PAGE of fractions extracted by 50% (v/v) propan-1-ol in the presence of a reducing agent showed two or

three major protein bands, with molecular weights in the range of 20 to 26 kDa [13], [41]. With differential scanning calorimetry (DSC), teff prolamin exhibited a single endothermic peak at 69.85°C, which indicated thermal denaturation of teff prolamin. The relatively low polymerization, hydrophobicity and denaturation temperature of teff prolamins are thought to make prolamins functional in the formulation of breads [12].

1.2.6. Functionality of teff proteins

The study of functionality is extremely important in order to efficiently use teff flour in foods [43]. Functionality can be defined as any property of a food component, except its nutritional properties, that has a high impact on the utilization of that ingredient in food systems [17], [24]. Functional properties reflect the physico-chemical features of proteins (such as amino acid composition, conformation, size, hydrophobicity and structure [44], [45], [46]), which may be influenced by interactions with the environment and food constituents (such as water, ions, proteins, lipids and carbohydrates) [45], [46]. However, the broadly used functionality tests are only applied to proteins, neglecting other components. Though this is useful in understanding the properties of proteins, it might fail in predicting performance of proteins in foods, where the presence of different ingredients and processing operations can affect the properties of the final product [29]. Moreover, features such as chemical composition, presence of impurities (such as carbohydrates) [44], [46] and type of sample (whether the product is a protein isolate, concentrate or a flour) may also affect the functional properties of a given product [47].

It has been reported that, in general, proteins have to be in solution or in fine suspension to have good functional properties [48]. This implies that meals and flours will generally have poor functionality, when compared to protein isolates or concentrates.

The functional properties of proteins in flours, protein concentrates and isolates from cereals and legumes have been investigated by many authors, in order to assess their applicability in food systems. Properties such as bulk density, gelation, emulsification and foaming properties, water and oil absorption capacities and protein solubility are crucial in the formulation and processing of foods [17], [32], [46]. Another functional property of vegetable proteins is the ability of forming textured protein structures, known as textured vegetable protein. These structures can be obtained either by fiber formation (normally starting from the protein isolate), and then merging the fibers in layers to attain the desirable texture (resembling meat), or by thermoplastic extrusion (using the flour, protein concentrate or isolate). Texturized protein products are used as meat extenders in products such as meat sauces and fillings. A replacement of up to 30% of the meat by hydrated texturized products can be done without losing quality. Some advantages of these products are their reduced prices and the increased product juiciness they provide [46]. In this project, textured protein structures (fibers) were developed (see section 3.4).

Functional properties can be classified according to the mechanism of action on three groups: hydration-related properties (water and oil absorption, solubility, wettability); protein structure and rheology-related properties (viscosity, aggregation and gelation); and properties related with the protein surface (emulsification and foaming properties, for example) [46]. In this dissertation, in order

to characterize the functionality of the water-soluble proteins present in teff flour, several functional properties were studied: bulk density (BD), water and oil absorption capacities (WAC and OAC, respectively), protein solubility (PS), emulsion activity (EA) and emulsion stability (ES). The results of this study will be discussed in section 3.3.1. Moreover, the foaming and gelation properties of the water-soluble proteins of teff were investigated for the teff extract subjected and not-subjected to carbohydrates removal. Foaming and gelation will be discussed in section 3.3.2 and section 3.3.3, respectively.

Type	Functionality
Beverages	Solubility, grittiness, color
Baked goods	Emulsification, complex formulation, foaming, viscoelastic properties, matrix and film formation, gelation, hardness, absorption
Dairy substitutes	Gelation, coagulation, foaming, fat holding capacity
Egg substitutes	Foaming, gelation
Meat emulsion products absorption	Emulsification, gelation, liquid holding capacity, adhesion, cohesion,
Meat extenders	Liquid holding capacity, hardness, chewiness, cohesion, adhesion
Soups and gravies	Viscosity, emulsification, water absorption
Topping	Foaming, emulsification
Whipped deserts	Foaming, gelation, emulsification

^a In all of these functionalities, flavor is important.

Figure 2 - Types of foods and their related functionalities. Table obtained from [47].

Many functional properties are influenced by the exposure of the hydrophobic groups of proteins on the surface and the interactions of these groups with oil (in emulsions), air (foam formation) or other protein molecules. Emulsification, foam formation and gelation are of paramount importance in food production, for instance in desserts, puddings, amongst others. More specifically, the foam improves the texture of bread, cakes, cookies, ice creams, among others [49]. The most important functionalities in different types of foods can be consulted in Figure 2.

1.2.6.1. Protein solubility

Protein solubility is the expression of the equilibrium between protein-protein and protein-solvent interactions [19]. Solubility is considered a good index to predict the potential applications of food proteins [24], [46]. It has been reported that protein solubility is closely related to other functional properties, such as emulsifying, gelling and foaming properties [1], [24], [32], [46]. Solubility can be influenced by various factors, such as the composition of the protein, processing conditions, pH and salt concentration [32], [50].

Protein solubility is related to the hydrophilic groups on the surface of the protein, which interact with water [1], [46]. Solubility decreases around the isoelectric point (IP) of the protein (the pH at which the overall protein charge is neutral) and, therefore, it is essential to control the pH when determining solubility. Most proteins have low solubility in the pH range of 4.0 to 6.0, which corresponds to their IP [1], [51]. However, outside this range, solubility increases dramatically, up to 80-90% [51].

Most seed proteins generally exhibit a U-shaped solubility profile, with good solubility in both acidic and alkaline pH values [1], [19], [26], [32], [50], and with a maximum observed at the alkaline pH region [24], [50]. There is typically a decrease in solubility when the pH is decreased, with the minimum being observed at the IP of the proteins [1], [19], [47]. This minimum near the isoelectric point is mainly due to the net charge of peptides (which is near to zero at the IP, so there is no electrostatic repulsion amongst the peptides) and to the surface hydrophobicity (that leads to aggregation and precipitation of proteins by hydrophobic interactions) [1], [19]. In other words, the protein molecules are attracted to each other in the IP zone, and that might explain the low protein extractability at that pH region [19].

The influence of pH on solubility has been attributed to the change in the net charges of the protein as the pH is altered [19]. The high net charge of the proteins at both acidic and alkaline pH regions generally leads to an increase in solubility because the protein unfolds, and this unfolding is even more pronounced at high pH values [19]. The occurrence of high solubility in both acid and alkaline pH regions has been reported as being important in food formulation [1], [34]. The high solubility of some proteins in the region of acidic pH values confers these components a potential role in acidic food formulations [34]. As for the high solubility of proteins at alkaline pH values, it makes proteins suitable to be used in the production of beverages [32]. However, since extremely high pH values are prejudicial to protein quality by destroying lysine, optimum pH values to solubilize proteins from different origins have been reported [1].

1.2.6.2. Bulk density

Bulk density (BD) is an important parameter from which the packaging requirements of the products can be determined [24]. A higher BD is advantageous, because it helps reducing the thickness of the paste, which has been reported as being very important in convalescent and child feeding. Therefore, products with high BD can be used to supplement child foods [32].

1.2.6.3. Water and oil absorption capacities

Water absorption capacity can be defined as being the ability to retain water against gravity [46]. Proteins with high water and oil absorption capacities can be used in formulations like meat, sausages, cakes, breads, mayonnaise, beverages and salad dressings [24], [32], [46].

The interactions of water and oil with proteins are essential in foods, due to their effects on flavor and texture. Moreover, there are economic benefits in the addition of water to products priced according to their weight, and also a positive impact on shelf life, and therefore ingredients with high water absorption capacities are preferred in food formulation [52]. Factors such as amino acids composition, protein conformation and surface hydrophobicity affect the water binding character of proteins [24], [32].

The capacity of flours to retain water and oil may improve the binding capacity, the flavor retention and the mouth feel of meat analogues [32], [49]. Moreover, it can reduce moisture and fat losses on those products [32]. Flours with higher WAC contain more hydrophilic compounds, such as polysaccharides, which absorb a substantial amount of water. The difference in oil absorption capacity among flours

might be explained by the presence of non-polar side chains, which could bind the hydrocarbon side-chains of oil among the flours [32], [46].

1.2.6.4. Emulsion properties

Emulsifying capacity is related to the flexibility of solutes and to protein surface hydrophobicity, specifically to the exposure of hydrophobic groups [19], [32]. When proteins denature, their hydrophobic groups are exposed, so the emulsifying capacity tends to be higher at that pH zone [24].

Since proteins are composed of charged, non-charged polar and non-polar amino acids, that confers to proteins the potential to be emulsifiers [24], thereby having an important role during emulsification, especially by contributing to the emulsion stability [19].

The emulsifying properties of vegetable proteins have been discussed by several authors [17], [19], [24], [26], [32], [34], [44], [45], [47], [48], [50]. Emulsion properties are mainly influenced by solubility, pH and concentration, amongst others [34]. A protein-stabilized emulsion is generally affected not only by the molecular features of proteins, but also by intrinsic factors, such as pH, ionic strength, temperature, volume of the oil phase and protein type [46], [49].

Food emulsions are known to be thermodynamically unstable combinations of immiscible liquids, like oil and water [32]. The formation and stability of an emulsion are very important parameters in food systems like, for example, salad dressings [19], [32].

According to the literature, the capacity of proteins to improve the formation and stabilization of emulsions has an important role in many food applications, such as cakes [19], [32], [34], coffee whiteners [32], [34], comminuted meats [19] and frozen desserts [32], [34]. In their production, these foods are exposed to different stresses, so it is of paramount importance for them to possess variable emulsifying and stabilizing capacities [32], [34].

Emulsion capacity (EC), which can also be expressed in percentage as emulsion activity (EA), consists in the ability of a protein solution or suspension to emulsify oil [44]. It reflects the capability of the protein to rapidly adsorb at the water-oil interface when the emulsion is forming, thus preventing flocculation and coalescence [19], [46]. EC is a measure of the maximum amount of oil that the product in question is able to emulsify without breaking down the emulsion, normally expressed in mL oil per g of sample [17], [44]. In order to measure this property, the first step is to form a mixture between the product and water, followed by oil addition with agitation. Then, the breakpoint of the emulsion can be measured by many methods, such as visually or electronically. The amount of oil emulsified just before the breakpoint, which is the emulsion capacity of the product, can be measured by volumetric or viscosimetric methods, amongst others [44].

Emulsion capacity is known to be closely related to other properties, such as solubility in water [48], [50], protein concentration [34], [47], [50] and hydrophobicity [50]. Highly insoluble proteins are not good emulsifiers because they may lead to coalescence [46].

A positive correlation of EC of food proteins with protein concentration has been reported [50]. However, the inverse tendency has also been reported, showing an initial increase in EC with the

increase of protein concentration. At the oil-water interface, the protein orients its hydrophobic groups to the oil phase and the hydrophilic to the aqueous phase, thereby decreasing the interfacial tension and stabilizing the interface [45], [46]. On the one hand, at low protein concentrations, the protein adsorption at the oil-water interface is controlled by diffusion mechanisms, because the protein will spread over the surface before it adsorbs. On the other hand, at high protein concentrations, the protein migration is not diffusion-controlled, due to the activation energy barrier. This explains the decrease in EC when protein concentration is increased [47].

The increase of the EC both with increasing pH [19], [47], [50] and below the IP of proteins have been largely noted [19], [47]. These increments in EC are thought to be related to the increase in protein solubility in those pH regions [19].

A minimum EC at pH 4.0 has been reported for legume flours [19], [47], thought to be due to an increase in the interactions among proteins, thereby lowering the surface hydrophobicity and decreasing the net charge and solubility of proteins.

Emulsion stability (ES) is the ability of the emulsifier to stabilize an emulsion at the interface, after the formation of the emulsion and following different stress conditions. The stress can be an applied force (gravity or centrifugal forces), heat, or a combination of both methods [44]. ES is influenced by pH and ionic strength, but also by physical processes such as flocculation, aggregation and coalescence [46].

The relation of ES to protein solubility is not as straightforward as in the case of EC. On one hand, it has been reported for chickpea protein isolates that in the alkaline pH region, proteins were more soluble than at neutral pH values, and their negative net charges hampered the aggregation of soluble proteins, and that improved the ES [50]. On the other hand, according to Ahmed *et al.*, the highest ES of legume flour was found at pH 6.0, with a significant decrease of ES in the alkaline pH region [19]. Therefore, it is thought to be more probable the existence of a relation between ES and an appropriate balance among the hydrophilic and hydrophobic groups in the product [50]. Moreover, some authors have suggested that the emulsion stability tends to be maximal near the IP of proteins, due to the higher cohesiveness of protein films near that pH value, which have therefore a tendency to be more stable [48].

In this project, only the emulsification properties of teff flour have been studied. According to the literature [17], both the difference in total protein composition (soluble and insoluble protein) and the presence of other components (such as carbohydrates) might significantly affect the emulsion properties of flours.

Products with good emulsification properties might be good emulsifying agents [26], [47] in salad dressings and mayonnaise [34], [47], meat formulations (sausages, baked foods and soups) [45] or used in milk production, pastries and added to frozen desserts [26], [27], [34].

1.2.6.5. Foaming properties

Foams are known to play an important role in smoothness, lightness, flavor dispersion and palatability of products [34]. Therefore, the ability of proteins to form and stabilize foams is relevant in the production of several foods [53], such as bread, whipped toppings, ice creams, frozen desserts and

cakes [34], [46]. In order to be considered good foaming agents, proteins should rapidly form and stabilize foams at low concentration and over a broad pH range [53]. For that, proteins must have the ability to promptly adsorb at the air-water interface, rapidly change their conformation and rearrange at the interface, and form cohesive viscoelastic films by intermolecular interactions [34], [46]. The latter is important in foam stability [34].

Foams are composed by two parts: dispersed gas bubbles surrounded by a continuous phase [29], [53]. Proteins are present in the continuous phase and also at the interface, reducing the interfacial tension between air and water [46], [53]. Three phases can be considered in foaming studies: formation, stability and consumption. Proteins contribute to foam formation by rapidly adsorbing at the air-water interfaces, thus lowering the interfacial tension [25], [29]. Proteins may also play a role in foam stabilization, by forming a strong and elastic film at the interface, thereby preventing coalescence (which is basically the foam collapse, due to the joining of gas bubbles) [25], [29], [34].

It has been reported that free proteins (solubilized) contribute to foaming capacity, as long as the free protein content in the initial solution is enough to permit the foam formation [52], [54]. Proteins stabilize foams by producing a viscous layer in the bubble surface, thus preventing the breakage of the film. Protein hydrophobicity is related to their fast adsorption at the air-water interface, which leads to foam stability [54]. In other words, the amount of hydrophobic groups exposed at the surface of proteins highly influences foaming properties [25].

Foaming properties have been reported to improve for systems where small protein aggregates are present (35-71 nm), when compared to non-aggregated systems. Moreover, with a mixture of both free proteins and protein aggregates, more stable foams can be obtained [54].

The ability to form foams depends on the configuration of protein molecules. This formation relies on the diffusion of soluble proteins to the air-water interface, where both a fast conformational change (unfolding) and rearrangement of these proteins take place [1], [24], [25], [34], [55]. In order to contribute to foam stability, proteins have to form a thick, cohesive and viscoelastic film around the gas bubbles, because intermolecular cohesiveness and elasticity are relevant in the production of stable foams [24], [34], [55].

The foaming capacity (FC) of proteins can be defined as the amount of interfacial area that can be created by proteins. The foam stability (FS) is the ability of proteins to stabilize (foams) against gravitational and mechanical stresses. These two parameters depend on several factors, such as the type of protein and its capacity to retain air, pH, ionic strength, temperature, processing techniques, the presence of other components apart from proteins, viscosity and surface tension [19], [22], [32], [49].

The increase in FC of certain protein isolates may be related to the increase in solubility, rapid unfolding at the air-water interface, limited intermolecular cohesion and flexibility of proteins [19]. Protein solubility requires high net charges, which effect the protein adsorption at the air-water interface, reducing surface tension and thereby improving FC [22], [34]. However, this may not always be verified in the case of FS [22].

A high FC in the alkaline pH region, concordant with the behavior of protein solubility, has been reported, and is thought to be due to the increase in the protein net charge, which turns the hydrophobic interactions weaker but increases the protein solubility and flexibility. This allows a rapid migration of proteins to the air-water interface, thereby improving the foam formation [19], [34], [47]. Conversely, low values of FC at the alkaline pH region, together with high values at acidic pH, have also been reported. The higher FC at low pH values was once more thought to be due to an increased net charge of the proteins [49].

An increase in FC and FS with increased protein concentration has also been reported, because in these conditions there is an enhanced viscosity and an easier formation of a large and cohesive protein film at the air-water interface [1], [26], [47].

A relevant influence of pH in FS has been reported [19], [34]. A minimum FS in the isoelectric pH region has been reported [19]. According to some authors, a higher FS was detected at low pH values rather than at alkaline pH values. That observation can be explained by the formation of a stable protein layer in the low pH region, which stabilizes the foam [19], [34]. However, a high FS at pH values slightly higher than IP has been reported [19], [49], together with a fast decrease in FS at alkaline pH values [19], [34]. That increase near the IP might be due to the smaller repulsion between proteins, which interact and form a film at the interface, improving foaming properties [49]. The decrease in FS at the alkaline pH region may have been caused by charge repulsion between proteins [19], [34], [49], and also by the existence of some protein solubility in this pH range, which diminishes the amount of protein aggregates necessary to stabilize the foam [19]. Conversely, an increase in FS at alkaline pH values was also reported [49] and thought to be due to the lower bubble coalescence resultant from the poorer foam volume in this pH region. Foam stability is important because the success of a whipping agent relies on its ability in maintaining the whip as long as possible [52].

Higher values of foaming properties have been reported in vegetable flours (for example in soy flours) when compared to the respective protein concentrates. These better foaming properties may be due to the generally high starch content of the flours. A better foam stability of flours might also be related to the increase of surface activity of the oil-water interface. In relation to flours, inverse tendencies have also been reported. According to some authors, defatted flours have shown decreased foaming properties when compared to full-fat flours [47]. However, FC has been reported to be higher in defatted flours than in full-fat flours [45]. Low foam ability is possibly related to well-ordered globular proteins, which resist surface denaturation [34].

1.2.6.6. Heat-induced gelation

Gelation capacity is a widely studied functional property, highly applicable to food production [56]. The development of texture in food products relies on network formation. Networks can hold water, flavors, carbohydrates, among others, and also stabilize dispersed phases [46], [56].

Gelation can be defined as the aggregation of protein, which is normally denatured, into a structured matrix, where a balance between protein-protein and protein-solvent interactions exists, and is supported by a balance between attractive and repulsive forces. Because proteins are normally stable

in aqueous solutions, they have to be destabilized or denatured to form gels. This denaturation or destabilization is usually performed by heat treatment, but denaturant agents or pressure can also be applied [57].

In other words, heat-induced gelation of proteins generally occurs as following: the protein is denatured by heat, then a soluble aggregate is formed and, finally, a network which entraps water and other compounds is formed through the interactions between proteins and soluble aggregates [56].

Gels form due to increasing intermolecular interactions, both covalent (mainly disulphide bonds) and non-covalent (hydrogen bonds, hydrophobic and electrostatic interactions). It is the combination of those different interactions that makes it difficult to model protein gelation. A system can be considered a gel when a continuous network of intermolecular linkages is formed, conferring elasticity to that system [29]. Overall, heat-induced gelation is mainly due to protein-protein hydrophobic interactions [57].

Gelation functionality in a particular food is based on the formation of a network overcoming the limitations of food composition and production. It is known that macroscopic properties such as water and fat release are closely related to both the structure of gel network and interactions between proteins and other molecules in the network. In other words, gelation influences functional properties such as oil and water absorption capacity [29]. Furthermore, gelation capacity and gel properties are related to their rheological properties [58].

Protein gel structure is responsible for the texture of foods. For instance, hardness, which is defined as the biting force necessary to fracture a sample, is a well-known sensory attribute of foods with gel structures, such as cheese or gelatin [29]. Gelling property is also important in the production of sausages or tofu [46]. Food protein gels are commonly separated into semi-solids, such as yogurt, and soft-solids, such as cheese. Factors such as protein type and gelation conditions influence the types of structures obtained [29]. For instance, soy flour and concentrates form soft and weak gels, whereas soy isolates form firm and hard gels [46]. Gelation is also influenced by electrostatic conditions, such as pH and salt concentration, and protein concentration [29]. An example of the latter influence is that, to obtain a gel from soy isolates, a minimum of 8% protein concentration is needed [46]. Other influential factors reported on literature are mineral content, processing conditions, extent of denaturation and amount of phenolic compounds present [46], [57]. The least gelation concentration (LGC) indicates the gelation capacity and the lower the LGC, less protein is needed to form a gel so the better the gelling ability of proteins [52], [59].

1.2.6.7. GDL acidification

Acids are used to decrease the pH of food products, thereby improving their microbial stability and influencing their taste. Acids can also be used to chelate iron and copper ions, which may catalyze food spoilage reactions, such as rancidity [60]. The parameters used to characterize acids are their strength and sourness profile. The stronger the acid, the less amount of acid is required to achieve a certain pH. The sourness profile of an acid consists in its intensity (which may be sharp or mild) and

lasting time (short or long). Comparing acetic acid to gluconic acid, the former provides a sharp sourness that lasts a short time, while the latter provides a mild and prolonged sourness [60].

Glucono-delta-lactone (GDL) is an inner ester of gluconic acid, obtained by removal of water during crystallization [60], [61]. When added to aqueous systems, GDL slowly hydrolyzes to gluconic acid, thus acidifying the systems through a controlled drop in pH [60], [61], [62]. This drop in pH depends on GDL concentration in the system [62], [63].

GDL acidification provides an efficient manipulation of the type of texture obtained in the gels, since the acidification speed increases with temperature. Different gel behaviors according to the acidification process (slow or fast) have been reported. Generally, slowly acidified gels have the tendency to form stronger and more elastic gels than rapidly acidified systems. In fact, in slow acidifications, molecular interactions occur while the pH is lowering, thus leading to the formation of a stronger and well-organized structure in the gels [64].

GDL acidification is applied to cheese, yogurt, processed meat and tofu production, where a gradual acidification is needed to obtain high yields and a desirable texture [61].

GDL also improves color formation, because acidification accelerates that process, and that is crucial in the production of sausages, for instance [61]. Furthermore, the mild taste that GDL imparts in foods is beneficial for preservation purposes, since the growth of most food-spoiling and food poisoning bacteria stops below pH 3.5 to 4.0 [60].

In terms of regulation, GDL is GRAS in the USA to be used in foods with no limitations apart from the maintenance of good manufacturing practices. It may be used as a curing and prickling agent, leavening agent, pH control agent and as sequestrant (preservative agent). GDL has also been approved by the Meat and Poultry Inspection Division to be used to acidify meat and poultry products, with GDL below 0.3%. In the EC, GDL is identified as E-575 and approved for use as an acidity regulator and sequestrant [61].

1.2.7. Protein/polysaccharide interactions

Proteins and polysaccharides are natural polymers used as functional ingredients in many food products [57], [65], mostly because they have the ability to change product structure by altering the texture of foods [65].

Proteins and polysaccharides may either interact with each other or be incompatible. When incompatible, there is phase separation into one protein-rich phase and a polysaccharide-rich phase. On the other hand, when proteins and polysaccharides are attracted to one another, normally through electrostatic interactions, an associative phase separation, also called precipitation or coacervation [65], [66], occurs. In coacervation, there is also a phase separation, but one of the phases consists of protein/polysaccharide complexes, while the other contains the solvent [66]. The texture of coacervates has led to their usage as food ingredients, for example as fat substitutes or meat replacers. Protein/polysaccharide complexes can also be used in the formulation of low-fat meat analogues [66].

Basically, the concept of coacervation is that when a protein and a polyelectrolyte hold opposite charges, a complex can be formed by electrostatic interactions. These interactions can be influenced by a variety of physicochemical parameters, therefore influencing the formation of complexes and their solubility [65], [66]. One of these parameters is pH [57], [65], [66], which highly influences the strength of electrostatic interactions by establishing the charge density of the proteins. Another important parameter is ionic strength [57], [65], [66]. For some salts and at some concentrations, the presence of salt may suppress the formation of complexes. However, the addition of small amounts of salt might slightly increase coacervation, because it enhances the solubility of the polymers. The protein concentration is also crucial and ought to be controlled, because self-suppression of the complexation may occur above a certain concentration. There is also an optimum ratio of protein/polysaccharide, which corresponds to an electrically equivalent quantity of each polymer. Finally, other parameters, such as molecular weight [65], [66], temperature, shear or pressure, may influence the coacervation and the stability of complexes [66].

At pH values below the IP of proteins, the proteins are positively charged and thus may interact with carboxylic, phosphate or sulfate groups of the negatively charged polysaccharide to form stable electrostatic complexes [65], [66]. The maximum coacervation is obtained in that pH range. Nevertheless, it has also been reported the formation of soluble complexes above the IP, where the protein bear negative charges, because the protein still contains positively charged areas at their surface [66].

Cross-linking stands for linking polymer chains by bonds, which can be done using a chemical, enzymatic or physical agent that links the polymers [67]. An example of chemical cross-linking agent is glutaraldehyde, which even though it is not used in the food industry due to its toxicity, it has been used in a series of applications that require structural rigidity and stability of the protein, along with resistance to proteolysis [67], [68]. A widely used physical cross-linking agent is heat treatment [67]. The complexes formed after coacervation are loose in the solvent, so the goal of cross-linking the complexes is to aggregate them. This process should protect the fibers from falling apart when the solvent conditions are changed [66]. In this project, the influence of a physical (heating) and a chemical cross-linking agent in the formation of these complexes, which will be denominated fibers in this report, were studied.

1.3. Techniques

1.3.1. Thermal analysis

The thermodynamic state of a material affects its structure, thereby influencing its properties. Using Differential Scanning Calorimetry (DSC), the thermally induced transitions of materials can be studied [69], [70]. DSC measures the difference in the heat necessary to increase the temperature of a sample and a reference. With this method, the stability and folding of bio-molecules as, for instance, proteins, can be evaluated [69], [70]. In this project, the device used to study the thermal transitions of the samples was the DSC Q1000 (TA Instruments), as can be seen in Figure 3.

The functioning of this equipment is based on a constant-rate heating, leading to a detectable heat change of the proteins, which indicates thermal denaturation. These heat changes derive mainly of the differences in the hydration of protein side chains, which instead of being hidden as in their native state, are exposed to the solvent when the protein unfolds due to denaturation [70].

A certain molecule in solution is in equilibrium between the native and denatured (unfolded) state. A denatured protein has a higher heat capacity than the native protein [70]. The transition midpoint (T_m), which is obtained using DSC, is the temperature at which half of the protein is native and the other half is denatured. The higher the T_m , the more stable is the protein, since a higher temperature is needed to have half of the protein denatured [70], [71]. Proteins with higher T_m are less vulnerable to unfold or denature at lower temperatures [70].

With this device, a differential thermogram is obtained, which consists in a graph of heat flow as a function of temperature. Two pans inside a chamber are necessary to perform a measurement: in one of the pans, the sample to be analyzed is added (sample pan); the other pan is called the reference pan, and it is empty. Each pan is on top of a heater, and both heated at a specific constant rate. The pan with the sample inside will heat up slower than the reference pan. The thermogram obtained shows the difference in heat of the two heaters at a certain temperature, as a function of temperature [69].



Figure 3 - DSC Q1000 (photograph taken at NIZO).

1.3.2. Microstructure visualization

Confocal Laser Scanning Microscopy (CLSM) is a technique derived from light microscopy that allows the high-resolution visualization of the microstructure of products [72]. It combines the features of confocal microscopy, allowing the user to obtain images at different sample depths, and of a scanning system that allows image acquisition point-by-point. Generally, the imaging length ranges from 0.5 mm to little mm. The principal advantage of this device is that sample preparation is not needed, because the focus is on one certain point in the sample. Therefore, the sample can be observed without being disturbed [72], [73]. The sample structure can also be viewed in real-time, during heating, cooling or even under mechanical treatment, like during shear and compression tests [72].

The CLSM working principle consists in the passage of a laser beam through a light source opening (pinhole), and that laser beam is focused by an objective lens into a point within or on the surface of the sample (see the left part of Figure 4). The objective lens re-collects the scattered, reflected and fluorescence (emitted by the sample) lights. A part of the light is separated by the beam splitter into the detection device. The light intensity is detected by a photo-detection apparatus (which transforms

the light into an electrical signal that can be recorded by the computer), after passing the pinhole. Sharp images are obtained because the unfocused light is rejected by the pinhole [74].

The staining of the sample is performed with fluorescent dyes, and multiple staining can be done which, along with the possible combination with different lasers, allows the simultaneous visualization of various ingredients of the sample (protein, fat and starch granules) [72], [73].

Quoting a NIZO publication, “The CLSM allows a real-time visualization of complex food matrixes. It helps one understanding the performance of a product or ingredients. It’s a very efficient approach to tackle stability issues, tailor mouthfeel and optimize processes.” [72].

Processing conditions such as heating, mixing, homogenization and aeration may affect the microstructure and, when microstructure is disturbed, product stability may be compromised. By visualizing the microstructure, the product can be efficiently optimized [72].

Some of the CLSM applications are the following: optimization of products, by studying the functionality of ingredients; improvements in product mouth feel; analysis of real-time processes; characterization of different materials, apart from foods; and improvements in product shelf-life, by studying and understanding the causes of product instability [72].

The CLSM utilized in the present project was a Leica TCS SP5 (see the right part of Figure 4). It is composed by the microscope with a laser source, a laser scanning head, and the computer with two screens and a control panel. In this project, this equipment was used to study the microstructure of protein complexes (fibers), as presented in section 3.4. Rhodamin-B and a DPSS laser were used to identify proteins. This equipment covers a wide range of imaging speed, and different laser sources (such as Argon, DPSS and HeNe) can be used depending on the excitation wavelength required. These lasers cover excitation from 350 to 1050 nm.

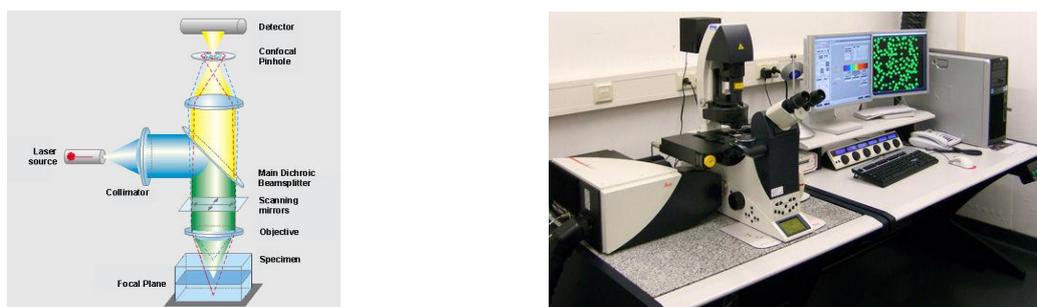


Figure 4 - Scheme of the functioning principle of the CLSM (left part). Photograph of the Leica TCS SP5 CLSM system (right part).

1.3.3. Laser Diffraction

Laser diffraction is being more and more used in the industry for characterization and quality control, by examining the size distribution of systems like emulsions [75], [76]. This method is based on the fact that the diffraction angle is inversely proportional to particle size. Instruments contain two light sources (a He-Ne laser, which emits red light of 633 nm, and a LED that emits blue light of 455 nm [76]), a detector (normally a slice of photo-sensitive silicon with discrete detectors) and a photo-

multiplier tube (together with a signal correlator, this tube is needed to solve the problem of the low intensity of the light at low size ranges, 1nm – 1 µm) [75].

In this project, the device used to determine the particle size of the samples was the Mastersizer 2000 (Malvern), which can be seen in Figure 5. Mastersizer is a flexible particle sizing system which is able to efficiently perform measurements from submicron to millimeter (size range of 0.02 µm to 2000 µm) [75], [76], [77]. Dry powder, suspensions and emulsions may all be correctly measured [77].

The Mastersizer automatically applies the full Mie scattering theory [75], [77], which completely solves the equations for interaction of light with matter. The penalty for this complete accuracy is that the user has to know the refractive indexes of both the material and the medium, and the absorption part of the refractive index has to be known or guessed [75], [76]. However, this is usually not a serious problem for users, since these values are normally known or may be measured [75].



Figure 5 - Mastersizer device used in the present study (photograph taken at NIZO).

The working principle of Mastersizer can be followed on Figure 6. During the laser diffraction measurement, particles dispersed in a suitable medium pass through a focused laser beam [76], [77]. These particles scatter light at an angle that is inversely proportional to their size. Then, the angular intensity of the scattered light is measured by a series of photo-sensitive detectors. It is thus the scattering intensity as a function of angle that is used by the system to calculate the particle size [77].

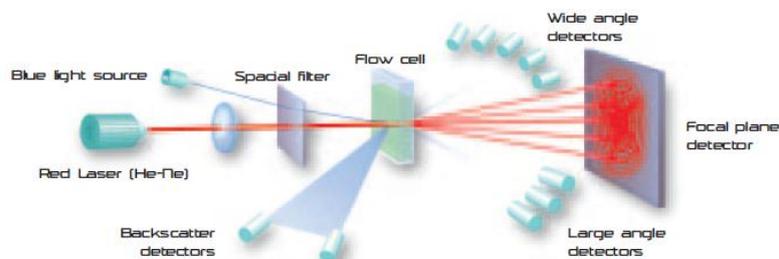


Figure 6 - Working principle of the Mastersizer [81].

The Mastersizer provides important data to the user: $D[3,2]$, which is called the surface area moment mean; and $D[4,3]$, which is the volume or mass moment mean (identical to the weight equivalent mean if the density is constant). The first value shows where the mass of the system is, and that is very convenient to users [75]. The device also provides, in the report of the LD measurements, the particle diameters $d(0.5)$, $d(0.1)$ and $d(0.9)$. The first value is the mass median diameter of the volume of distribution, it is expressed in microns and it signifies that 50% of the sample has a size smaller than that value, while the other 50% has a higher size. The second value suggests that 10% of the sample mass are particles smaller than that value, whereas $d(0.9)$ shows that 90% are smaller and 10% are larger than the indicated value [76].

1.3.4. Texture analysis

Texture is a multidimensional group of food properties, difficult to measure in machines due to the complexity of foods. The common instruments utilized to evaluate the mechanical properties of products measure force in Newton (N) or gram (g) [78], [79]. These mechanical texture measurements normally lead to a destruction of the analyzed material, because the applied force is higher than the strength of the sample [80]. In this project, the TA XT-Plus Texture Analyzer (Stable Microsystems) was used to study texture, as can be seen in Figure 7. The TA uses a load cell fitted to a travelling beam, where a wide variety of test probes and accessories can be attached. The device transmits mechanical energy into the sample, and the resultant forces can be puncture, compression, shear, extrusion, among others. These forces are influenced by probe geometry [79]. The study of rheology in foods provides useful information about the texture of food materials under different deformation conditions. Rheological measurements can replace or complement sensory analysis, helping the user to understand how consumers experience texture [80]. In this project, only penetration tests were performed to the gels, so this literature review will only be focused on that kind of texture measurement test. The test used in this study to measure gel texture, penetration or puncture testing, involves the penetration of the sample with a probe until a defined depth, applying constant load and/or penetration speed. The puncture test is widely used to measure food texture, due to its simplicity and because it can rapidly differentiate between samples [78], [79], [81], [82]. In a puncture test, there should not be any effects from the edges, corners and thickness of the sample on the probe. Moreover, a true puncture test does not occur if the diameter of the food sample is similar to that of the probe; the diameter of the sample should be at least three times higher than the diameter of the probe [78]. The force applied to penetrate the sample is recorded against time or penetration depth (distance, in mm), providing the user with the knowledge of hardness and fracture point of the material [79]. The greater the force, the more resistant the material will be [82]. At the fracture point, a peak in force occurs, after which the force decreases. The results obtained can be correlated to the hardness of the sample. Moreover, the response of the sample to the conditions imposed in the puncture test allows the formation of correlations with the sensory panel or the evaluation of functional properties such as hardness and breakpoint of the sample [79]. When analyzing the texture measurement results, two important parameters can be considered: the puncture force, which is the maximum load applied to the sample to break (in N); and the force/penetration slope of the puncture curve when the maximum penetration force is achieved (in N/mm). The first parameter can measure hardness, whereas the second can indicate structure firmness of samples [83]. However, it should be stated that the point at which the slope should be calculated and the method of measuring it are subject of many interpretations in the literature [84].



Figure 7 - Texture analysis device used in the present study (photograph taken at NIZO).

2. Materials and Methods

2.1. Materials and chemicals

Table 4 - Summary of the chemicals and ingredients used during the internship and the respective suppliers.

Product	Commercial name	Supplier
Ethanol	Ethanol (99.8%)	Sigma-Aldrich
NaCl	Sodium chloride (99.5%)	Merck
NaOH	Sodium hydroxide	AnalaR
Sunflower oil	Sunflower oil	Albert Heijn
DTT	DTT	Sigma-Aldrich
Coomassie Blue	Instant Coomassie Blue	Expedeon
Sample buffer for SDS-PAGE	Laemmli sample buffer	Bio-Rad
SDS-PAGE buffer	10x Tris/Glycine/SDS buffer	Bio-Rad
Pullulanase	Kleistase PL45	Amano Enzyme Inc.
Amyloglucosidase (Bacterial α -Amylase)	Kleistase SD80	Amano Enzyme Inc.
α -Amylase	α -Amylase from porcine pancreas	Sigma-Aldrich

Table 5 - Summary of the equipment and materials used during the internship and the respective suppliers.

Equipment/Material	Commercial name	Supplier
Freeze-drier	Modulyo 4K	Edwards
Water bath	Water bath E-Version	PMT Tamson Instruments
pH meter	Portamess pH meter	Knick
Head-stirring system	Head-stirring system	Joh. Wilten
Ultra-Turrax	Polytron PT 3000	Kinematica AG
Rotary evaporator	Rotary evaporator	Stuart
Pump system for fiber formation	Pump system	Antec Leyden
Centrifuge	Avanti J-26 XP Centrifuge	Beekman Coulter INC VWR
Mastersizer	Mastersizer 2000	Malvern
Differential Scanning Calorimeter	DSC Q1000	TA Instruments
Homogenizer	2 traps Homogenizer	Niro Soavi S.p.A.
	Lab Homogenizer WCFS	Delta Instruments
SDS-PAGE system	Criterion Cell system	Bio-Rad
Confocal Laser Scanning Microscope	Broadband Confocal Leica TCS SP5	Leica Microsystems Ltd.
Light and contrast microscopes	Stereomicroscope DC 300 F	Leica Microsystems Ltd.
	Reichert-Jung Polyvar-Met	
Whipping system	-	-
Texture analyzer	TA XT-Plus Texture Analyzer	Stable Micro Systems
Dialysis membranes	Spectra/POR Dialysis Membrane	Sigma-Aldrich

The materials used in this project were vegetable proteins (called P1 to P3), whey protein isolate (WPI), polysaccharide, food-grade glucono-delta-lactone (GDL), food-grade cross-linker (CL), an artificial sweetener and a food additive (Ingredient 1 – Ing.1). The other chemicals used and the respective non-confidential suppliers are resumed in Table 4. Apart from common laboratory equipment, the equipment and materials utilized and the respective suppliers are resumed in Table 5.

2.2. Optimization of protein extraction from teff

Starting from a method described in the literature and available at NIZO, several trials were performed in this project, in order to optimize the protein extraction from teff flour (TF1 or TF2). These trials included extractions of teff proteins from the flour using ethanol, salt and water, or extraction with water at high temperature. The main results of these trials are presented in section 3.1.1, but only the optimized method will be described below.

The first step of the optimized method consisted in the preparation of a 20% (w/w) suspension of teff flour in RO-water, while vigorously mixing with an Ultra-Turrax (Polytron PT 3000, Kinematica AG) during 10 min at 20000 rpm, cooling with melting ice. Afterwards, the pH was adjusted to 8.0 with 4 M NaOH, followed by mixture with the Ultra-Turrax. The suspension was subsequently centrifuged for 2 min at 2000x g, at 20°C (Avanti J-26 XP Centrifuge, Beckman Coulter), in order to remove fiber and starch fractions from the alkaline dispersion. Samples were collected after the pH adjustment and after centrifugation, for Kjeldahl analysis. Samples of the end product were also collected for SDS-PAGE and DSC. The results of this procedure will be presented in section 3.1.1.

2.3. Development of a milk analogue from teff proteins

After optimization of the protein extraction from teff flour (TF1), a milk analogue was developed. The procedure followed was described in section 2.2, until the centrifugation. The obtained supernatant was then dialyzed overnight at 4°C, using dialysis membranes of MWCO of 6000-8000 Da (Spectra/POR, Sigma Aldrich). Dialysis was done at two conditions: against RO-water or RO-water with 10 mM NaCl. Samples were collected from the two dialyzed solutions, to perform Kjeldahl analysis.

Concentration (CF = 2) with a Rotary Evaporator (Stuart) was performed, for each of the two different solutions, and samples were taken from each concentrate and submitted to total nitrogen content analysis by Kjeldahl. Furthermore, trials with higher concentration factors were performed, in an attempt to raise the protein concentration of the milk analogue. These last trials were considered parallel to the scope of this project, and therefore their results will not be presented. Then, the solutions were submitted to mixing with the Ultra-Turrax between several ingredient additions: 0.05% (w/w) salt (NaCl), 0.75% (w/w) oil (sunflower oil) and 16 droplets of artificial sweetener per L of solution (0.8 g/L of artificial sweetener). The solutions were then homogenized (2 traps Homogenizer, Niro Soavi S.p.A.) at 25°C and 200/20 bar, and a sample of each homogenized solution was taken for Kjeldahl analysis. Afterwards, the solutions were heated at 74°C for 20 s. A part of each solution skipped the heat treatment, to be compared to the heated part in an internal tasting session performed

at NIZO. The results of this procedure will be presented and explained in section 3.2. Pictures from each step of the procedure were taken and will be resumed in Appendix I.

Moreover, a non-dialyzed milk analogue was performed as described above, in order to assess if the elimination of the dialyzing step from the process was possible (see section 3.2). In this trial, samples were collected for size analysis (using the Mastersizer) at different stages of the procedure (after centrifugation, homogenization and heat treatment).

2.4. Fiber formation

The formation of fibers was performed using vegetable proteins from different sources (P1 and P2, where P2 is teff protein), and the results are presented in section 3.4.1 and section 3.4.2, respectively. The comparison between the two types of fibers is described in section 3.4.3. Another protein source (P3) was also used to compare with P1 and P2 fibers. All yields of fiber formation calculated and shown in section 3.4 respect to the wet fibers (after pressing). Fibers yield was calculated by dividing the wet mass of fibers obtained in the end by the weight of the initial fiber solution.

The first step of the fiber formation consisted in the preparation of the polysaccharide solution. The polysaccharide was dissolved in RO-water, stirring during 1 h at RT and then overnight at 4°C. The solution was then heated for 30 min at 80°C in a waterbath, cooled down and stored at 4°C until use.

In the case of protein P1, a suspension was prepared dissolving the protein isolate in RO-water. This suspension was subsequently stirred for 2 h at RT and then overnight at 4°C. Afterwards, the suspension was centrifuged for 15 min at 4000x g, and the supernatant stored until use. In the case of P2, two types of protein solution were used: teff extracts at pH 6.5 and 8.0, and also the extract with ethanol; and a protein solution prepared as described in the former section, but just including the concentration step (section 2.3).

The two solutions (protein and polysaccharide) were mixed with an overhead stirrer. Acid was added at different speeds into the system. In the majority of the trials, the acid addition was performed through a pumping system, at a flow-rate of 0.8 mL/min until pH below the IP was reached. To pump the acid into the system, two syringes filled with acid were connected by a plastic tube to the vessel which contained the system. With lower volumes, the flow-rate was adjusted to 0.4 mL/min, allowing an easier control of the pH. When the desired pH was reached, the suspension was stirred for another 5 min. The fibers were then submitted to heat treatment in a waterbath for 30 min at 80°C, and subsequently cooled down. Then, the fibers were pressed in a sieve to remove the excess of moisture, weighed and some were analyzed using the CLSM.

Various different approaches were attempted, in order to improve the quality of the P1 fibers. After making the fibers, they were incubated with CL (10 g/L of solution) at 4°C or at 50°C, to protect the fibers from falling apart and, simultaneously, to study the influence of the incubation temperature. The influence of the addition of extra ingredients and heat treatment to the fibers was also investigated. For P2, the parameters studied were the influence of heat treatment, protein concentration in the starting solution, the addition of extra ingredients, and the effect of the presence of ethanol in the initial protein solution. CLSM images of P2 fibers are presented in section 3.4.2.1.

2.5. Functionality

2.5.1. Functional properties of teff flour

In order to characterize the functionality of the water-soluble proteins present in teff flour, several functional properties were studied: bulk density (BD), water and oil absorption capacities (WAC and OAC), protein solubility (PS), emulsion capacity (EC) and emulsion stability (ES). All experiments were performed in duplicate, and the respective averages and standard deviations were computed, when applied. The results obtained in this section are present in section 3.3.1, and were compared to the literature whenever possible. The teff flour used in these experiments was TF2, unless specified otherwise.

Bulk density

This experiment was carried out using the procedure of Narayana and Narasinga (1984), as described in reference [34]. A specified quantity (1.0 g) of the flour sample was transferred into an already weighed measuring cylinder (W_1). Packed-bulk density of the flour (PBD) was determined by gently tapping the flour sample to eliminate spaces between the flour; the level was noted as being the volume of the sample and weighed (W_2). Bulk density of the samples was calculated using Equation 1.

$$\text{Bulk density} \left(\frac{g}{mL} \right) = \frac{W_2 - W_1}{\text{Volume of sample}} \quad (1)$$

Protein solubility

From the water/flour mixture

This experiment was based on the procedures described on [28] and [23]. In order to assess the protein solubility of teff flour, 12 suspensions (with duplicates) with a concentration of 2% (w/v) were made from the mixture of 0.2 g of flour and 10 mL of distilled water (initial sample). Then, pH adjustments were performed to pH values ranging from 1 to 12, to investigate the influence of pH on protein solubility of the flour water-soluble proteins. For a better solubilization, the suspensions were stirred for 30 min at RT, using a magnetic stirrer, and the pH was adjusted to the required value with 0.5 M HCl or NaOH. The samples were then centrifuged at 4000x g for 30 min, and the total nitrogen content of the obtained supernatants was determined by Kjeldahl analysis. The solubility profile was constructed by plotting the average values of protein solubility (PS, in percentage) against each considered pH value. The percentage of soluble protein was calculated using Equation 2. The value was adjusted with the dilution resultant from the pH adjustment.

$$\text{Protein Solubility}(\%) = \frac{\text{Amount of protein in the supernatant} \times 100}{\text{Amount of protein in the initial sample}} \quad (2)$$

From centrifuged samples obtained by precipitation or solubilization

The first step was obtaining the teff extract at pH 8.0 (see section 2.2) from TF1. Solubilized proteins were collected as supernatant, which was subsequently submitted to isoelectric precipitation at different pH values in the IP region (pH 4.0, 4.5 and 5.0), or solubilized at pH 7.0; 1 M HCl or NaOH

were used to perform the pH adjustments. After precipitation/solubilization, the proteins were separated by centrifugation at 4500x g for 10 min (at RT). Samples were collected from every supernatant and precipitate obtained after the second centrifugation, and their total nitrogen content was determined by Kjeldahl analysis. Solubility (%) was calculated using Equation 3.

$$\text{Protein Solubility}(\%) = \frac{\text{Protein in the supernatant (g)} \times 100}{\text{Protein in the supernatant (g)} + \text{Protein in the precipitate (g)}} \quad (3)$$

Water absorption capacity

Water absorption capacity (WAC) was determined by centrifugation, using the method outlined by Beuchat *et al.* [85], with modifications [25], [28]. 1 g of sample (teff flour) was added to pre-weighed 15-mL centrifuge tubes. Then, 10 mL distilled water was added to each sample. The suspensions were then mixed with a vortex mixer at maximum speed for 2 min. The samples were allowed to stand at RT for 30 min, and then centrifuged at 3000x g for 20 min. After centrifugation, the supernatant was discarded and the centrifugation tube containing the sediment was weighed. WAC was calculated with Equation 4, and expressed as g of absorbed water per g of sample.

$$WAC = \frac{W_2 - W_1}{W_0} \quad (4)$$

Where:

W_0 – Weight of the dry sample (g)

W_1 – Weight of the tube plus the dry sample (g)

W_2 – Weight of the tube plus the sediment (g)

Oil absorption capacity

Oil absorption capacity (OAC) was determined using the method outlined by Beuchat *et al.* [85], with modifications [25], [28]. The method previously described for WAC was applied for OAC, replacing distilled water with sunflower oil in the same concentration. Immediately after centrifugation, the supernatant was carefully poured into a 10 mL graduated cylinder, and the volume was recorded (V_2). OAC was calculated with Equation 5, and expressed as mL of oil per g of sample.

$$OAC = \frac{V_1 - V_2}{W_0} \quad (5)$$

Where:

W_0 – Weight of the dry sample (g)

V_1 – Volume of oil added to the dry sample (mL)

V_2 – Volume/amount of supernatant poured to a graduated cylinder (g or mL)

Emulsifying properties

This experiment was based on the procedures described on references [34] and [28]. To assess the concentration influence on the emulsifying properties, 1, 2, 3, 4 and 5% (w/v) slurries were prepared from the flour in distilled water, magnetically stirring. In order to study the effect of pH on the emulsifying properties, a part of the prepared suspensions was adjusted to different pH values (4, 6, 8 and 10) while magnetically stirring. Then, 5 g of each protein suspension were homogenized at high

speed for 30 s (Ultra-Turrax, 14400 rpm), and then homogenized again with 5 g of sunflower oil for 1 min. The emulsions obtained were centrifuged at 3000 rpm for 5 min, and the volume of emulsion was measured. Emulsion activity (EA) was calculated using Equation 6 [27].

$$EA(\%) = \frac{\text{Volume of emulsified layer} \times 100}{\text{Total volume in the centrifugation tube}} \quad (6)$$

In order to determine the emulsion stability (ES), emulsions prepared by the above procedure were heated at 80°C for 30 min in a water bath, and then cooled at RT (20°C) and centrifuged again at 3000 rpm for 5 min. ES was calculated using Equation 7 [27].

$$ES(\%) = \frac{\text{Volume of remaining emulsified layer} \times 100}{\text{Total volume in the centrifugation tube before heating}} \quad (7)$$

The controls used in these experiments were 2% (w/v) WPI suspensions at pH 7.0 and at the same values of pH used in the pH influence determination.

2.5.2. Foaming tests

The foaming properties of the flour were not tested, because the aim was to assess the foaming character of teff proteins without much interfering compounds (like fibers or sugars).

Foaming of teff extracts

Foaming properties of teff proteins were also studied. The procedure was based on previous studies performed at NIZO (see Figure 8), and it started with the preparation of the stock solutions that would be submitted to the foaming tests. Protein was extracted from the flour, as described in section 2.2 (with the exception that the centrifugation was longer and at a higher speed: 20 min at 6000x g), and the pH of the supernatant was subsequently adjusted to 4.5 with 2 M HCl, at 4°C. Part of this liquid was used for foaming studies (and part of the liquid already adjusted to 4.5 was then adjusted to pH 7.0 with 2 M NaOH and also used in foaming tests – see orange-colored parts of Figure 8). The remainder solution was gently stirred overnight at 4°C. After the stirring, the solution was centrifuged at 6000x g for 30 min. The precipitate was washed three times with RO water and afterwards dissolved at pH 7.0 in a 6:1 water: precipitate ratio, to achieve a complete removal of any existing contaminant. The resultant suspension was submitted to foaming tests.

Other foaming trial was performed (with shorter centrifugation steps), this time to assess the differences amongst the foaming character of supernatants at different pH values and after an additional centrifugation step. The differences of this procedure, when compared to the above referred, are the following: the first centrifugation was the same as described in section 2.2; the supernatant was adjusted to two pH values, 4.5 and 7.0, prior to the second centrifugation; the second centrifugation was shorter, 10 min at 4500x g and no prior stirring at 4°C was performed.

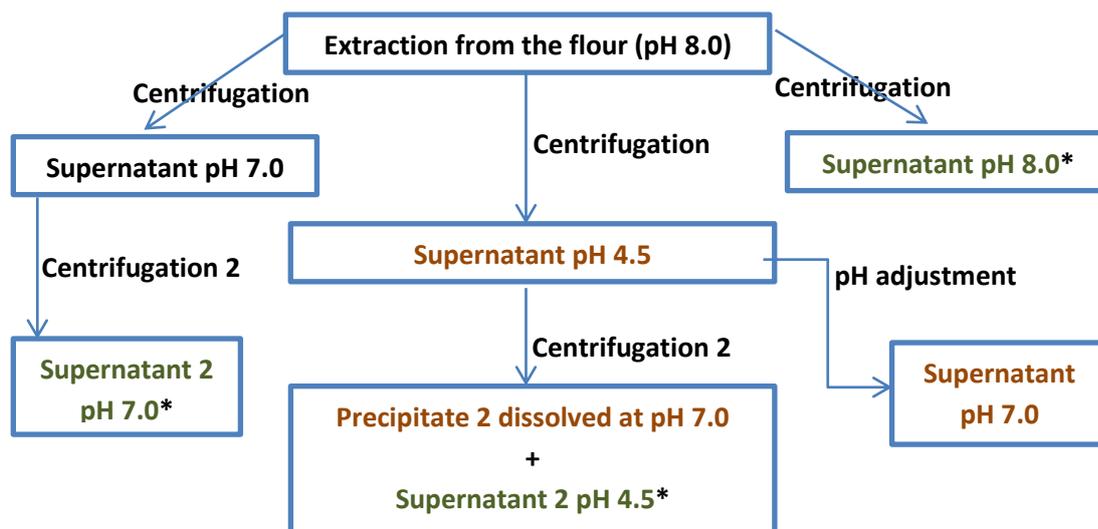


Figure 8 – Schematic representation of the sample preparation for the foaming tests of teff extracts. The samples marked with asterisk and green-colored were submitted to shorter centrifugation steps, as referred in the text above. The foaming character of the orange and green-highlighted samples was analyzed.

The foaming protocol was also based on previous experiments at NIZO. A specified amount (50 mL) of 1 to 2% (w/w) protein concentration suspensions was adjusted to both pH 4.5 and 7.0 and submitted to foaming trials. The supernatants and precipitates represented in Figure 8 that were tested are discriminated below, but are also highlighted at orange and green in the scheme, for the long and short centrifugation conditions, respectively. The samples were whipped for 70 s with a whipping device connected to a tunable power supply which allows controlling the speed of stirring. The settings used were intensity of 1.65A and voltage of 10V. The speed was gradually attained at the beginning and decreased at the end. After the immediate transferal of the foam to a 250 mL graduated cylinder, a stopwatch was set to start counting the time. The volume of liquid, together with the total volume and the level of the foam (initial foam volume), were also recorded. The total volume after whipping was noted, in order to calculate foaming capacity (Equation 8). The foam volume remaining after 20, 30, 60 and 180 min was noted for the study of foaming stability. FS (%) can be calculated as shown in Equation 9. All calculations were based on the literature [34].

$$FC(\%) = \frac{(Total\ volume\ after\ whipping - Total\ volume\ before\ whipping) \times 100}{Total\ volume\ before\ whipping} \quad (8)$$

$$FS(\%) = \frac{Foam\ volume\ after\ time\ (t) \times 100}{Initial\ foam\ volume} \quad (9)$$

The control solutions for these foaming studies were 2% (w/w) WPI and SPI in RO water at two pH values: 7 and 4.5. The analyzed solutions with the first centrifugation conditions were: supernatant pH 4.5 (after the pH adjustment step and before the stirring overnight); supernatant pH 4.5 adjusted to 7.0; and precipitate from the second centrifugation at pH 7.0 (see Figure 8, orange part). As for the shorter centrifugation settings, the analyzed solutions were: supernatant at pH 8.0 (native pH after centrifugation); and supernatants from the second centrifugation at pH 4.5 and 7.0 (see Figure 8, green part). The main results of all these foaming trials are presented in section 3.3.2.

Foaming of starch-devoid samples

To remove the sugars (which are mainly starch, in teff) from the teff extracts, the following procedure (resumed on Figure 9), based on NIZO experience, was followed. Protein was extracted as described in section 2.2. Then, a pH adjustment to 6.0 was performed. Prior to enzymatic treatment, the solution was cooled down to 4°C. Two different conditions of enzymes addition were then tested: condition 1 included the addition of 0.01% (w/w) Pullulanase and 0.02% (w/w) Amyloglucosidase; condition 2 comprised the addition of 0.01% (w/w) Pullulanase, 0.02% (w/w) Amyloglucosidase and 0.02% (w/w) Amylase. The enzyme-treated solutions were stirred overnight at 4°C. Then, dialysis (Spectra/POR Dialysis Membrane, with MWCO of 6000-8000 Da) against RO water was performed during 24 h, with two changes of water. The resultant product was frozen for 24 h at -10°C and subsequently freeze-dried (Modulyo 4K, Edwards). The product was then used for foaming and gelation tests. Samples were subjected to Kjeldahl and sugar determination analysis.

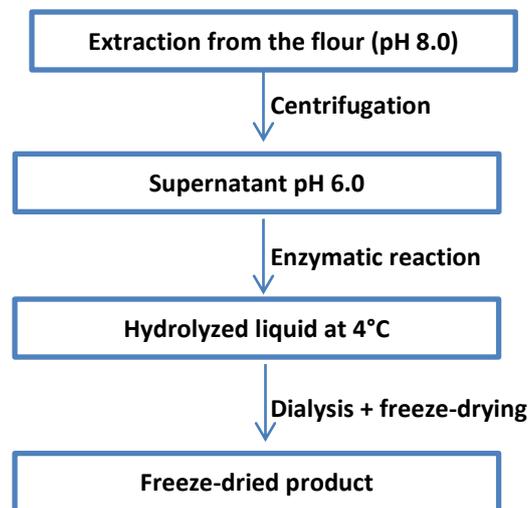


Figure 9 – Schematic representation of the starch removal procedure from teff supernatant.

The foaming tests were performed as described above for the teff extracts. The controls used in these foaming studies were 2% (w/w) protein of SPI in RO-water at pH 7.0 and 4.5. Only the freeze-dried product made with the Condition 1 enzymes was used in the foaming tests.

2.5.3. Heat-induced gelation

Preliminary trials

Preliminary gelation trials were performed based on a recent publication [43], using solutions similar to those of the foaming tests of teff extracts submitted to the most pronounced centrifugation conditions (supernatant at low and high pH values and precipitate at neutral pH). These solutions were heated at 90°C for 30 min, and then rapidly cooled down under running cold tap water. The formation of gels was visually evaluated, by turning the tubes upside down and observing whether the suspension would slip from the tube walls or not. Apart from the previous suspensions, a 10% (w/w) dry matter solution at pH 7.0 was prepared from teff flour, in order to assess the gelation characteristics. This suspension was afterwards heated at 90°C for 30 min, and then immediately cooled under running cold tap water. The formation of gels was visually measured as referred above.

Starch-devoid samples

Gelation tests were also performed to the samples that were submitted to enzymatic treatment (as described in the previous section). Sample suspensions with 1 to 5% (w/v) protein concentrations were prepared from the freeze-dried products in distilled water. From teff flour and from SPI, samples with different concentrations were also prepared to serve as controls. The samples were maintained at their natural pH and heated at 90°C for 30 min, and then cooled rapidly under cold running tap water. The tubes were further kept at 4°C for 2 h, and the least gelation concentration (LGC) of the samples was noted as the lower concentration at which the sample from the inverted test tube did not fall or slip [21], [43]. In terms of gel or non-gel behavior, the samples were classified with different strength degrees, based on the time that each sample would take to slip or fall from the walls (see section 3.3.3). After 24 h at 4°C, the firmness of all samples was studied with a texture analyzer (TA XT-Plus, from Stable Micro Systems), using a 3 mm diameter probe and a 5-kg load cell. The method used to analyze the samples texture is resumed in section 2.6.6. The main results of all gelation trials are presented in section 3.3.3. Texture analysis results are comprised in Appendix II.

2.5.4. GDL acidification

The first step of this procedure was the preparation of the teff extract at pH 8.0, as described in section 2.2. Then, GDL was added to the obtained supernatant in different concentrations: 0.2, 0.3, 0.4, 0.5, 0.7, 1.0 and 1.5% (w/w). The samples were magnetically stirred for 5 min after adding the GDL, in order to get a homogeneous distribution. Samples were then allowed to stand at RT overnight, without stirring. After 16 h, the pH of each sample was measured. Moreover, the strength of each sample was analyzed by visual inspection (by turning the containers upside down and measuring the time it would take for each sample to slip from the bottom of the container).

2.6. Analytical methods

2.6.1. SDS-PAGE

SDS-PAGE was performed using the Criterion Cell system from Bio-Rad, with 12% and 18% Criterion TGX Precast gels. Prior to electrophoresis, the protein solutions (3 to 5 mg/mL) were diluted in Laemmli buffer with DTT (54 mg/mL) in a 1:1 ratio, and were afterwards heated at 90°C for 10 min and cooled at RT. The non-homogeneous samples were centrifuged at 14000 rpm for 5 min. Then, 15 μ L of each sample were loaded into the gel, which was left running for 45 min at 200 V. The protein marker used was a Protein ladder Precision Plus Protein Standards (Bio-Rad), with molecular weights ranging from 10 to 250 kDa. The staining procedure followed was the one from Instant Blue (Expedeon), based on which Coomassie Brilliant Blue was applied to the gels during one hour, followed by two changes of water.

2.6.2. Differential Scanning Calorimetry

Approximately 20 mg of protein dispersion (1 to 5% (w/w) protein) were sealed in aluminum hermetic pans in the DSC equipment (DSC Q1000, from TA Instruments). As a reference, a sealed empty pan was used. The samples were analyzed from 20°C to 120°C, at 5°C/min.

2.6.3. Confocal Laser Scanning Microscopy

CLSM (Leica TCS SP5, from Leica Microsystems Ltd.) was used with the aim of studying the microstructure of the samples. Rhodamin-B staining was used to visualize protein, using the laser DPSS 561 (with an excitation wavelength of 561 nm). P1 and P2 samples were stained with 0.2% Rhodamin-B in water (20 μ L per ml of sample) and afterwards visualized with the CLSM. The 20x immersion and 63x1.2 water corrected objective lenses were utilized, and the digital images obtained had a resolution of 1024x1024. All images shown in this report were acquired with zoom 1.0 and 2.0.

2.6.4. Mastersizer

With the aim of comparing the particle size distribution at different stages of milk development (see section 2.3), namely after the samples have been submitted to centrifugation, homogenization or heat treatment, some droplets of protein solutions were poured into the Mastersizer device (Mastersizer 2000, from Malvern).

2.6.5. Protein content determination

In the present study, the protein content determination was performed through Kjeldahl analysis. This method is widely used to determine the total amount of nitrogen in a sample. It can be used in a broad variety of samples, like food and beverages, meat, grain and soil [86].

The method comprises three steps: digestion, distillation and titration [86]. The first consists in the addition of concentrated sulfuric acid and a catalyzer to the sample, followed by heat treatment. The acid decomposes the organic sample by oxidation, thus liberating the reduced nitrogen as ammonium sulfate. Then, the obtained sample is distilled with base, in order to convert the ammonium salt into ammonia. Finally, the ammonia content is determined by titration [86]. Since this method measures the total amount of nitrogen in a sample, it also measures the non-protein nitrogen. Therefore, the utilization of a nitrogen-protein conversion factor, which is different amongst each type of protein, is required. In this project, a conversion factor of 5.71 [87] was used for teff proteins (see Appendix III).

2.6.6. Texture analysis

In this project, texture analysis of samples previously submitted to gelation was assessed by the performance of single penetration tests, using a TA XT-Plus (Stable Microsystems). The tests were conducted with a flat-topped probe of circular section and diameter of 3 mm, at a point acquisition rate of 100 PPS (points per second) and with a trigger force of 0.01 or 0.1 N. Moreover, a 5-kg load cell was used. Mean maximum force achieved during sample penetration to a depth of 20 mm was used as a measure of hardness. Structure firmness of the samples examined was determined calculating the ratio of force applied to the penetration depth (slope of the puncture force/distance curve). When it was not possible to determine the slope, the ratio of the maximum force achieved during sample penetration and the distance of penetration into the sample was used to estimate firmness.

3. Results and Discussion

3.1. Extraction and characterization of teff proteins

In this project, no fractionation of teff proteins was performed, because all procedures were ultimately focused on product development.

3.1.1. Optimization of the extraction

The protein fractions and respective relative quantity in teff are described in the literature review [9] (section 1.2.4.1). With that in mind, and also based on the methods presented on Table 3 (section 1.2.5.1), different extraction methods were tested throughout this project (represented in Table 6). The trials performed (Table 6) included extraction of teff with ethanol, salt and water, or with water at high temperature. The amount of protein extracted with each method was determined by Kjeldahl analysis and the protein yield was obtained dividing the amount of protein present after centrifugation by the amount of protein present in the quantity of flour used (see Table 7).

Table 6 - Designation given to each extraction method.

Designation	Method
A	Neutral pH at RT (1 st trial)
B	pH 8.0 at RT (2 nd trial)
C	Neutral pH with 60% (v/v) ethanol at RT
D	Neutral pH with 1M salt at RT
E	pH 8.0 at 45°C

The extraction A was the least successful. This result was attributed to the fact that the extraction was performed at neutral pH instead of pH 8.0.

The main result from the extractions A and B is the confirmation that the prolamins are not soluble in water (as shown in the SDS-PAGE results, section 3.1.1.1), and this is why only a low amount of prolamins is recovered in the supernatant. That was expected because prolamins are not soluble in water, only in alcohols. However, comparing the water-soluble protein extraction to the literature (33.3% [12]), 53.6% of the albumins present in the flour were extracted (which are the water-soluble proteins), so the extraction of water-soluble proteins in B can be considered successful (and it is a little bit higher than the percentage of albumins extracted at pH 6.5, which was 50.2%). In extraction B, it is possible that some of the glutelins (base-soluble proteins) have also been extracted, because the extraction was performed at pH 8.0.

Overall, the extraction with ethanol (C) was not considered to be successful. However, when only the amount of prolamins extracted is taken in account, 66.1% of the prolamins present in the flour were extracted from TF1. So, we could conclude that albumins, globulins and glutelins are not soluble in ethanol, as expected.

The extraction with salt (D) was better than that with ethanol, but it was not considered to be successful. In D, almost 14% of the initial protein was extracted, in terms of protein yield (as seen in Table 7). This includes all the globulins (salt-soluble), along with some of the albumins (since they are water-soluble, some of them should have been extracted). Considering that only albumins were extracted, 52.4% of the albumins were considered to be extracted from teff flour. When globulins are included, 44.3% of albumins and globulins were considered to be extracted from the flour. Comparing to the literature [12], more protein was extracted (44.3% compared to 33.3%).

Extraction E was not considered advantageous in relation to extraction B (6% of total protein extracted instead of 10%, in terms of protein yield). However, when only the amount of albumins extracted is taken in account, about 52% of the albumins were successfully extracted from the flour, which means that the extracted protein using this method is slightly lower than in extraction B (in which it was about 54%).

Table 7 - Total amount of protein (Nx5.71 [91]), amount of water-soluble protein (WSP) extracted and visual observations relative to the supernatant of each extraction trial.

Method	Total amount of protein (%)	Total protein (g)	Protein yield (%)	WSP Extracted (%)	Stability of the supernatant
A	0.32	0.85	7.4	50.2	Precipitation
B	0.51	1.35	10.3	53.6	Precipitation
C	0.10	0.26	6.4	N/A	Precipitation
D	0.23	0.62	13.7	52.4	Precipitation
E	0.50	1.32	6.2	51.8	No precipitation

Overall, the best procedure was the extraction with water (B), due to the conjugation of its better results (more protein extracted), one of the highest protein yields and its higher applicability to industry. Therefore, it was decided to perform the further extractions using this procedure.

3.1.1.1. SDS-PAGE profiling

SDS-PAGE was performed as described in section 2.6.1. Figure 10 resumes the results obtained from all the extraction trials (identified as mentioned in Table 8). The band thought to correspond to prolamins is indicated in the figure.

In relation to lane A, the bands aren't easily distinguished, possibly due to the presence of impurities (fat and glycoproteins). Bands of high molecular weight can be detected (at 150 kDa, for example), which might correspond to protein aggregates. Even though prolamins should not have been extracted in this trial, very thin prolamin bands are thought to be present in lane A (at about 20 and 25 kDa). The other bands present in the gel are thought to be from water-soluble proteins. These results confirm that the extraction was not very successful, probably because the extraction was not performed at pH 8.0 (and the extraction at higher pH values is normally more effective).

Table 8 - Identification of the samples present in each lane of the SDS-PAGE gel. All samples were prepared with DTT, except B2.

Lane	Sample
S	Protein standard ladder
A	1 st trial (pH 6.5 at RT) supernatant
B1	2 nd trial (pH 8.0 at RT) supernatant with DTT
B2	2 nd trial (pH 8.0 at RT) supernatant without DTT
B3	Dissolved proteins from teff flour
C	Extraction with ethanol – supernatant
D	Extraction with salt – supernatant
E	Extraction at high temperature – supernatant

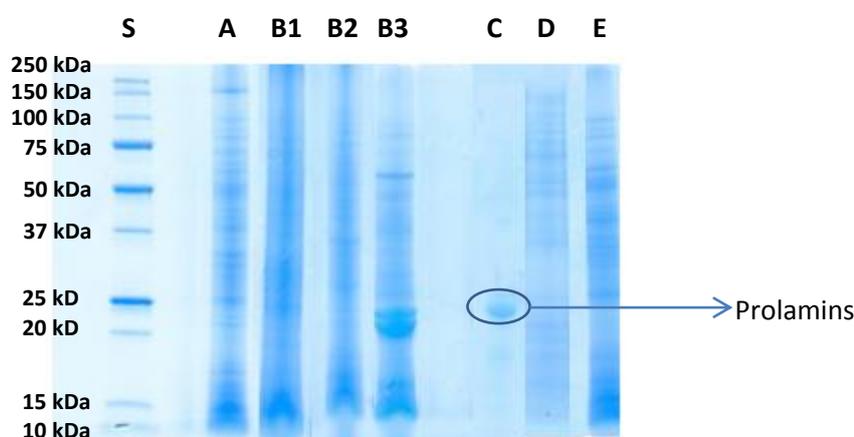


Figure 10 - Electrophoresis gel obtained from the analysis of samples from various extraction trials.

Regarding the lanes correspondent to the samples extracted at RT and pH 8.0, with and without DTT addition (B1 and B2, respectively), the bands are also not very distinguishable, as mentioned above. However, there are pronounced similarities of these bands with the ones present in lane A, which might also arise from WSP. Moreover, very thin bands at about 37 and 75 kDa are also detected in B2 (reported in the literature as 36.1 and 66.2 kDa, respectively [41]). These bands are absent when reducing conditions were used (B1), which possibly indicates that they were polypeptides linked by disulphide bonding and might be prolamin oligomers [41].

Both in reducing and non-reducing conditions (B1 and B2), the 22.5 and 25.0 kDa bands are only slightly visible, and at similar intensities. Compared to the prolamin bands in B3, there are no prolamins in the water soluble fractions (B1 and B2).

In lane B3 (dissolved proteins from teff flour – 3 mg/mL protein content), the major prolamin bands, as reported in the literature [12], are present (between 20 and 25 kDa, comparable with the reported bands at 22.5 and 25 kDa). Moreover, bands at 50 and 75 kDa can also be detected; in the literature [12], similar bands were reported (at 50.2 and 66.2 kDa, respectively), but under non-reducing conditions. The bands of low molecular weight (below 15 kDa), present both in A and all B lanes, are thought to be contaminating proteases, but no similar bands have been previously reported.

In lane C, the prolamin bands between 20 and 25 kDa that have been reported [12], [41] are clearly detected. This was expected because this extraction was performed with ethanol, and prolamins are

alcohol-soluble, so these proteins should be the only extracted proteins under these conditions, as it happened.

Lane D shows similar bands as lane A. However, some of these bands may correspond to globulins (salt-soluble proteins), but since these bands are not reported in the literature, no conclusions can be taken.

Finally, the extraction at higher temperature (E) shows clear bands, similar to those in lane A but slightly more concentrated.

3.1.1.2. DSC profiling

In this project, the DSC was used to verify the denaturation temperature of teff proteins (endothermic peak in the resultant graphs), comparing it with the reference temperatures present in the literature (see section 1.2.5.2). According to Taylor *et al.* [12], only one endothermic peak was expected, at 69.85°C. This peak has been reported to correspond to the denaturation of prolamins.

In this project, two DSC profiles were performed (present in Figure 11 and Figure 12). The samples analyzed by DSC in the first and second trials are shown in Table 9 and Table 10, respectively.

Table 9 - Samples analyzed by DSC and respective designation (matching with that of Figure 11).

Designation	Sample
1	Supernatant (1 st trial) with 1% (w/w) protein concentration
2	Teff flour/water suspension with 5% (w/w) protein concentration
3	Previous suspension after centrifugation (precipitate and supernatant)

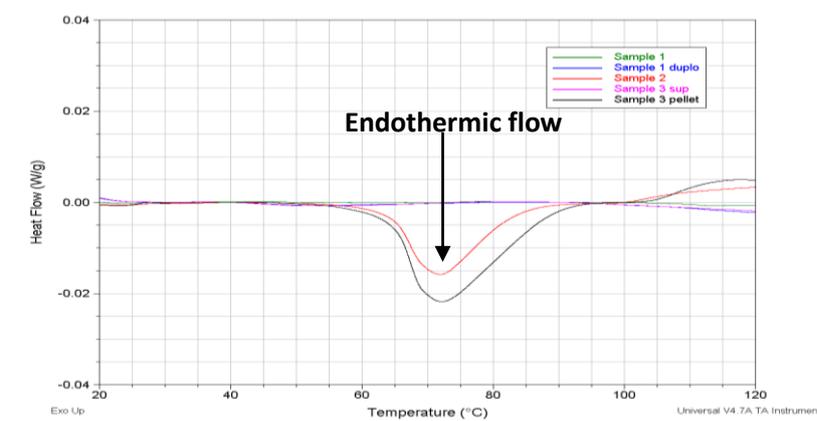


Figure 11 - DSC analysis of teff samples present in Table 9. Sample 1: 1% protein; Sample 2: 5% protein; Sample 3: 5% protein centrifuged. The temperature increments adopted were of 5°C/min.

In the first trial, an endothermic peak for sample 2 was observed at 71.95°C, which is comparable to the results reported [12]. Samples 1 and 3 (supernatant) have not produced any peak. However, in the sample 3 precipitate (black curve in Figure 11), the expected behavior was observed. The fact that nothing was detected in the supernatant and that the correct behavior was observed for the precipitate may be due to the higher protein concentration in the precipitate, or even due to the presence of different albumins in the supernatant, which make the determination of denaturation temperature difficult. From this first DSC study, it can be concluded that the endothermic peak obtained was

concordant with the literature [12], and may therefore be due to the prolamin, which is mainly present in the pellet (because the albumins remained soluble in the supernatant).

In the second DSC trial (Figure 12), a similar behavior was observed. In this case, the endothermic peak for sample 4 was around 72.39°C.

Even though a denaturation temperature similar to the literature has been obtained in the DSC experiments, the DSC operating conditions were not optimized throughout this project.

Table 10 - Samples analyzed by DSC and respective designation (matching with that of Figure 12).

Designation	Sample
1	Supernatant (2 nd trial) with 1% (w/w) protein concentration
2	Supernatant duplicate
3	Precipitate (2 nd trial) with 2% (w/w) protein concentration
4	Precipitate duplicate

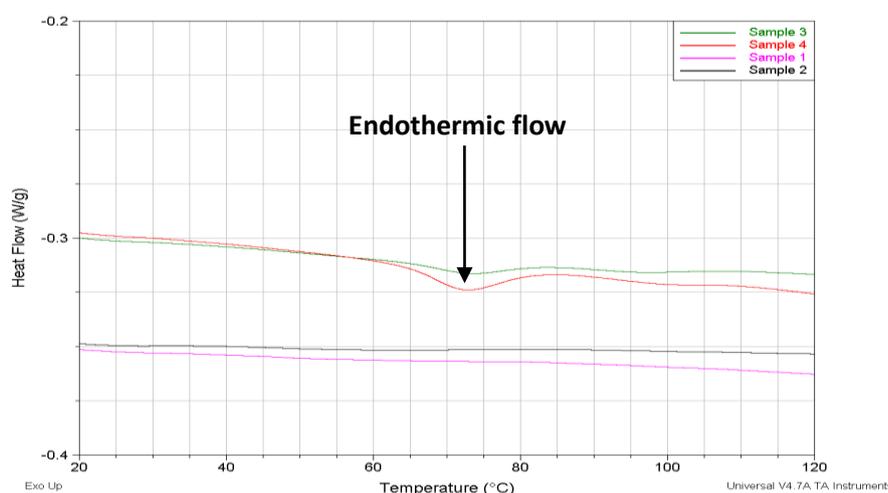


Figure 12 - DSC analysis of teff samples present in Table 10. Sample 1: 1% protein supernatant; Sample 2: supernatant duplicate; Sample 3: 2% protein pellet; Sample 4: pellet duplicate. The temperature increments adopted were of 5°C/min.

3.2. Milk analogue development

After the optimization of the protein extraction from teff flour, a milk analogue was developed, as described in section 2.3. Before the milk development trials, tests were performed to assess which dialyzing method was preferential in terms of product stability and taste: dialysis against RO-water or against RO-water with 10 mM NaCl. No significant differences were observed, so it was decided to test the two conditions in milk formulation.

It should be stated that the dialysis is a common step in milk formulation because more than only proteins can be extracted: polyphenols, salt and sugars may be extracted, and dialysis is used to remove as much of these compounds as possible from the product. As for the concentration, it was an important step in this milk formulation because the protein extracted was low and the product was also diluted after dialysis. Finally, heat treatment was performed to pasteurize the milk.

Influence of method of dialysis in the final product

Protein extraction was performed as described in section 2.2, and two methods of dialysis were compared, as referred above: dialysis against RO-water or against RO-water with 10 mM NaCl. Table 11 comprises the calculated protein yields and losses relative to all relevant steps of the procedure.

Table 11 - Summary of the protein contents and yields relative to each step in the procedure. The protein contents were determined by Kjeldahl analysis (N-protein conversion factor of 5.71 [91]).

Step	Protein content (%)	Total protein (g)	Protein yield (%)	Protein yield per step (%)
Teff flour addition	11.4	17.2	-	-
Centrifugation	0.60	2.59	15.10	15.10
Dialysis with water	0.40	1.93	11.24	74
Dialysis with salt	0.44	1.95	11.36	75

The dialysis with salt seems to be more advantageous than that with water, in terms of protein content of the dialyzed product (see Table 11), but especially in terms of taste (see Table 12). However, it would have to be evaluated whether or not performing the dialysis with salt would be profitable at the industrial level.

From the informal tasting sessions performed at NIZO (see Table 12), it was verified that the overall characteristic flavor of teff milk was slightly beany, resembling soy products. Moreover, the overall acceptability of the milk was good, but less artificial sweetener could have been added. No off-flavors were detected in any of the final products.

Influence on the final product of the absence of dialysis

Protein extraction was performed as described in section 2.2, and no dialysis was done. From the informal tasting sessions performed at NIZO (see Table 12), the non-dialyzed milk was considered too bitter. The product obtained after heating was even bitterer and acquired a brown coloration. When polyphenols (which were not even partly removed because dialysis was not performed in this trial) undergo heat treatment, aggregation can occur, and the formed aggregates may be bitterer than the initial compounds present in solution. Moreover, the solution may be getting browner and bitterer after heating due to a possible Maillard reaction of sugars with proteins [88].

The results of size determination by Mastersizer are presented on Figure 13. In Figure 13, it can be observed that the particles are uniform in size (narrow distribution). For the sample after homogenization, we can see that the mean diameter is about 2 μm , and that the system contains a small number of agglomerated particles with a diameter of about 25 μm . So, as expected, there was a higher volume of small-sized particles (less than 2 μm) after homogenization, in comparison to the volume of small particles in the product after centrifugation. In the heated sample, there was a shift to higher sizes when the mean diameter of the samples was compared (approximately 10 μm), which may indicate the heat-induced formation of protein aggregates.

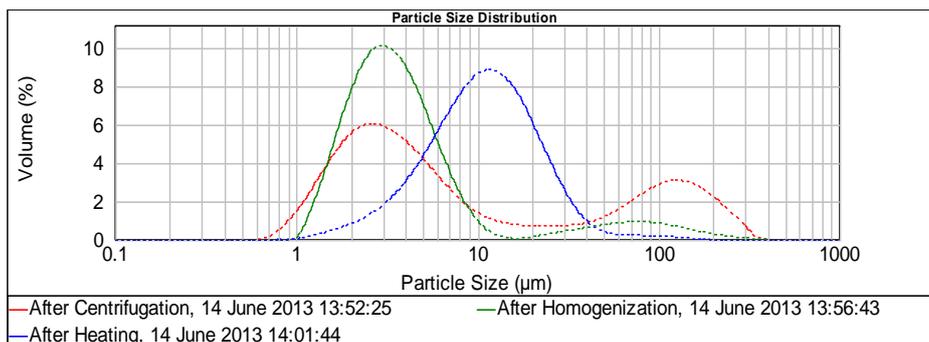


Figure 13 - Particle size distribution of teff solutions (average of two measurements). Red: teff supernatant; green: homogenate; blue: homogenate after heat treatment at 74°C for 20 s.

Comparison of the two milk analogues formulated

The milk analogues formulated were compared, in terms of stability and taste. Comparing the tasting results of the non-dialyzed product with those from the dialyzed products (see Table 12), it can be concluded that dialyzing is indeed necessary to remove the majority of bitterness from the product. Therefore, to get acceptable milk analogues from teff, dialysis would have to be included in the processing.

The preferred product from the informal tasting sessions (see Table 12) was the product dialyzed against salt and that did not receive heat treatment. Moreover, the unheated product that was dialyzed against water was also preferred when compared to the similarly produced heated milk. Since the unheated products were better than the heated ones, further optimization of the heating step could be done, by changing the temperature and/or the time of heat treatment.

Table 12 - Comparison of the milk analogues produced in this project. The asterisk indicates the preferred product in the informal tasting sessions at NIZO.

Product	Dialysis method	Heat treatment	Taste
Dialyzed milk	RO-water	-	Sweet but pleasant
		Yes	Bean-like and astringent
	RO-water + 10 mM NaCl	-	Good mixture of sweet and salt*
		Yes	Astringent
Non-dialyzed milk	-	-	Bitter
		Yes	Very bitter and more aftertaste

3.3. Functionality study of teff proteins

3.3.1. Functional properties of teff flour proteins

Bulk density

BD of teff flour water-soluble proteins was determined as described in section 2.5.1. The results showed that the packed bulk density of teff flour is, on average, 0.396 g/mL (with a standard deviation of 0.011). A higher bulk density was reported as desirable, because it helps in the reduction of the paste thickness, which is of paramount importance in convalescent and child feeding [1], [32].

Protein solubility

From the water/flour mixture

PS of teff flour water-soluble proteins was determined as described in section 2.5.1. Solubility is a very important functional property in proteins, because it affects other properties, such as emulsifying character, foaming and gelation. The U-shaped pattern reported in literature, typical of seed proteins [1], [34], [50], was visible in these results, as shown in Figure 14.

It is known that, in general, proteins are not soluble when the pH of the suspension is the value referent to their isoelectric point (IP), where proteins have a zero net charge. Above and below this pH, a protein usually has the highest solubility. The IP of teff albumins, according to these results, seems to be between pH 3 and 5 (because it is the pH range at which the solubility is lower). This could explain why teff proteins don't precipitate at pH 4.5 in the foaming trials, as presented in section 3.3.2 (the solution remains clear when the pH is adjusted to 4.5, which might mean that the proteins are still soluble).

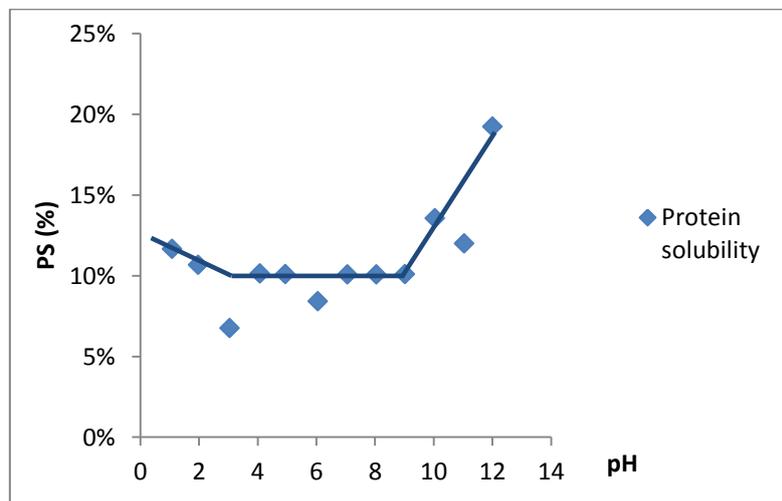


Figure 14 - Solubility profile of teff flour suspensions, obtained by plotting the averages of protein solubility (%) against the average pH, drawing a line to fit the data.

Comparing the solubility of teff proteins with the literature, the results from this experiment show a very low solubility of the flour: the maximum solubility is at high pH values, as reported, but it is less than 20%, instead of the 80-90% that should be expected [89]. However, similarly to previous reports [1], [89], the minimum solubility of teff proteins was found at pH 4.0, and there was a higher solubility at alkaline pH values. Based on the solubility profile presented in Figure 14, we can affirm that teff proteins, like the majority of vegetable proteins [1], [26], are acidic in nature and that their isoelectric point can be approximately 4.0. A minimum solubility was also observed at pH 6.0, so this could also be the IP of some of the teff proteins (since there is a mixture of proteins in teff flour, there can also be a mixture of IP values).

As referred above, teff flour proteins showed higher solubility in both acid and alkaline pH regions, and that has been stated as important in food formulation [1], [34]. However, as reported earlier [1], solubility at high pH values was higher than in the acidic pH region. To solubilize teff proteins in an

effective way without using extremely alkaline pH values, pH 8-10 seems to be an appropriate range. The high solubility of teff flour at alkaline pH values makes it suitable to be used in the production of beverages [32]. As for the high solubility of the flour in the region of acidic pH values, it confers a possible utility to the flour in acidic food formulations, such as protein-rich carbonated beverages [34].

From centrifuged samples obtained by precipitation or solubilization

Solubility of teff flour water-soluble proteins from samples obtained by precipitation or solubilization was determined as described in section 2.5.1. In Figure 15 (orange-colored samples), a scheme of all the samples submitted to solubility determination is presented, to provide more clarity to the reader. Figure 16 presents the solubility results for the samples submitted to isoelectric precipitation and subsequent centrifugation, and for the solubilized and subsequently centrifuged sample at pH 7.0.

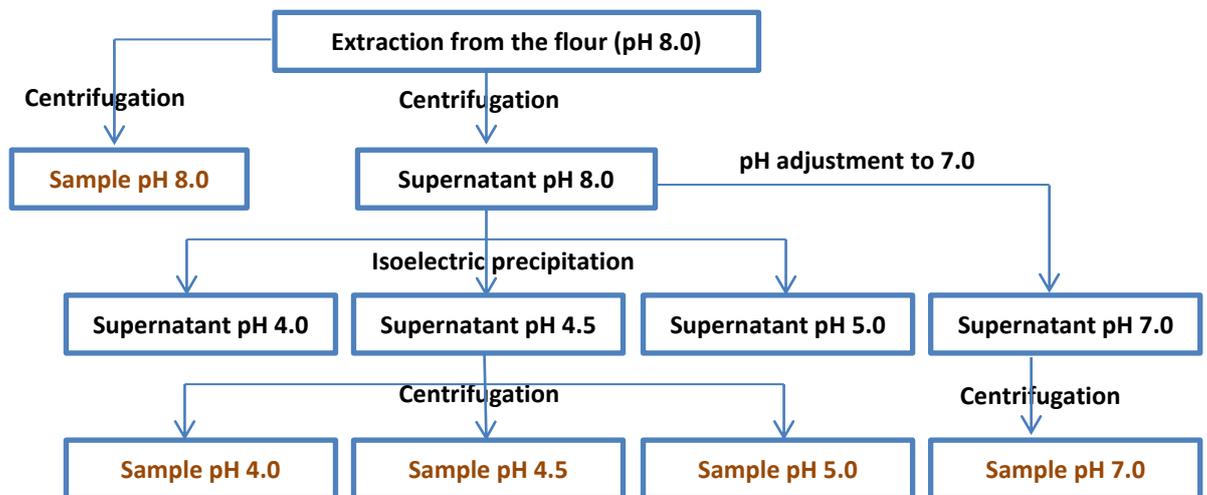


Figure 15 - Schematic representation of the procedure performed to determine the solubility of precipitated or solubilized teff samples. Orange-colored samples (supernatant + precipitate) were submitted to Kjeldahl analysis and their solubility was calculated.

Isoelectric precipitation was performed to verify if a high amount of teff proteins would precipitate by that method, but also to discover if teff proteins could be used in acidic drinks, for example sports drinks. According to the results shown in Figure 16, protein solubility was pH dependent. Solubility increased with increasing pH, and it was extremely higher at pH 7.0 than in the isoelectric zone (pH 4.0 to 5.0). This will be discussed with more detail below.

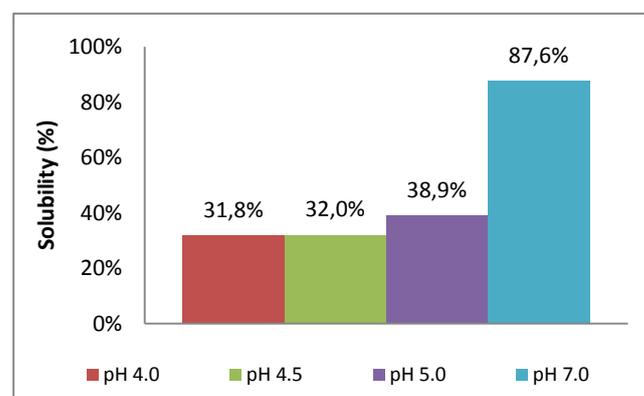


Figure 16 - Influence of pH in the solubility (in %) of water-soluble proteins from teff samples obtained by precipitation or solubilization.

As shown in Figure 16, low protein solubility was verified in the isoelectric pH range (4.0 to 5.0), which might indicate that the isoelectric point of teff water-soluble proteins is in this pH range. So, teff proteins can be precipitated by isoelectric precipitation, but these proteins cannot be used in acidic drinks, because there is about 70% of precipitation at this pH range.

Finally, a very high solubilization of the water-soluble proteins was verified at pH 7.0 (87.6%). At pH values higher than the isoelectric point, the protein molecules are charged, and water might interact with these charges, thereby contributing to protein solubilization [16]. The fact that the protein solubility at pH 7.0 is high is seen, in the food industry, as an advantage in the formulation of products such as beverages [32]. This result reinforces the fact that teff is promising for the development of new beverages.

Water absorption capacity

WAC of teff flour water-soluble proteins was determined as described in section 2.5.1. The results showed that the WAC of teff flour is, on average, 2.34 g/g (with a standard deviation of 0.08) for distilled water suspensions, which is higher than that of chickpea [89], cowpea and soybean flours [16]. In the literature, teff flour had already been reported as having high water absorption capacity, which was attributed to the higher swelling and possibly to the reduced and uniform size of teff starch granules. The small size of the granules provides an increased surface area, thus enhancing water absorption [15].

Hydration is a very important step in conferring acceptable functional properties to proteins in foodstuffs. Interactions of water with flours are critical in food systems, since they affect the flavor and texture of foods. The difference in protein structure and the presence of different hydrophilic components may cause variation in the WAC of flours. The inherent proteins in the flour may also lead to higher water absorption capacity [17]. Flours with high WAC have been reported to contain more hydrophilic compounds, such as carbohydrates [17], [32]. Therefore, it can be suggested that the proteins in teff flour contain more hydrophilic parts than the chickpea flour referred above, thus being able to bind more water [43].

Oil absorption capacity

OAC of teff flour water-soluble proteins was determined as described in section 2.5.1. The capacity of flours to absorb water and oil has been reported to have influence on the improvement of the binding capacity, of flavor retention and mouth feel of food products. Moreover, it may help reduce moisture and fat losses of meat analogues [32], [43]. The results showed that the OAC of teff flour is, on average, 1.29 mL/g for sunflower oil suspensions (with a standard deviation of 0.02). The oil present in the flour affects the OAC (there is already oil in the flour, so the oil absorption will naturally be less), so these results are more comparable to those of whole-fat flours. The OAC of teff flour is higher than that of chickpea flours [17], [89]. For that reason, teff flour might be used in products like meat, sausages or mayonnaise [47], or even in formulation of baked goods such as cake and cookies [43].

Emulsion properties

WPI was chosen to be the control of this set of experiments due to the good emulsification properties of whey proteins. It is important to refer that the emulsion capacity of the control suspension was high at all pH values tested. Moreover, the layers formed in the emulsion studies were, from top to bottom of the centrifugation tube, oil, emulsion, water and solids. High emulsifying activity or emulsifying stability was generally observed when a minimal oil layer was observed.

The controls tested in this experiment had emulsion activity of 50% at all pH values. As for the emulsion stability, it suffered a reduction at pH 4.0 and 8.0 (Figure 17). A preliminary trial has shown a similar trend, but if one is focused on applications that justify working in the pH range of 7.0 to 10.0, more pH values in this range should be tested, to conclude that pH 8.0 is indeed to avoid when protein stabilization is aimed.

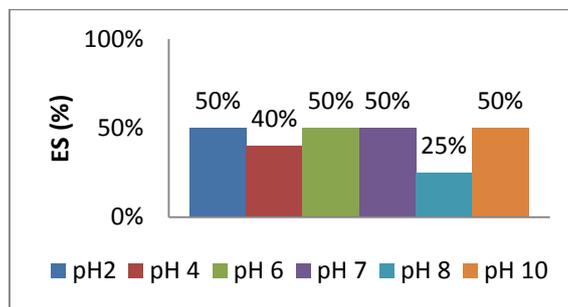


Figure 17 - Influence of pH on the ES (%) of 2% (w/v) WPI suspensions.

In 1% and 2% (w/v) teff flour suspensions, there was no emulsion formation. At 5% (w/v) teff flour suspensions (see Figure 18), the emulsion was also reduced, with only a small layer on the top of the tube, but it was quantified.

Analyzing Figure 18, at pH 6.0 and even more at 7.0, there was a reduction on the emulsion activity of 5% (w/v) teff flour suspensions, when compared to high pH values. There was no emulsion activity detected at pH 2.0 and 4.0, which were therefore excluded from Figure 18.

The results for the emulsion stability of the same suspensions showed an inverse tendency (see the right graph of Figure 18). There was a reduced ES at pH 6.0 and at high pH values. Therefore, these emulsions were not considered stable.

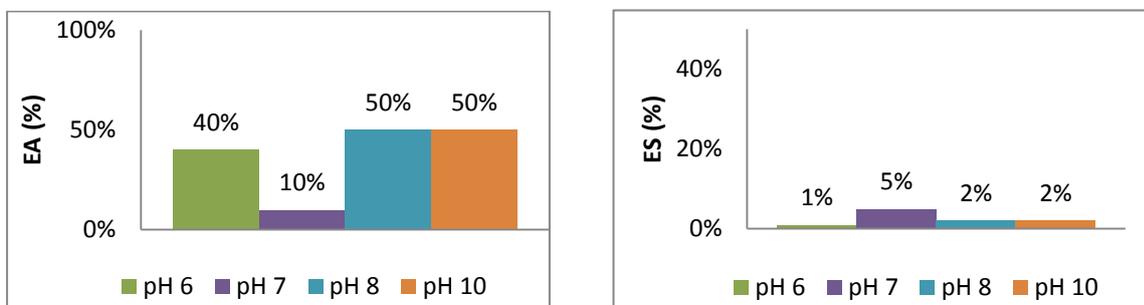


Figure 18 - Influence of the pH on the EA (%) (left graph) and ES(%) (right) of 5% (w/v) teff flour suspensions.

In 10% (w/v) teff suspensions, EA had a maximum at neutral pH values and suffered a reduction at pH 10.0, as can be seen in Figure 19. Moreover, even at the relatively high concentration of 10% (w/v),

the emulsion stability of the tested samples was not significant (Figure 19). In fact, when low values of pH were tested, EA and ES values were close to zero. Therefore, it was decided to do the 10% (w/v) trials only at extreme values of pH. It should be added that, in the 10% (w/v) at neutral pH, there was a reduced emulsion layer in the middle of the other layers in the tube, but a lot of non-emulsified oil on top. Moreover, at pH 10.0, there was even a smaller emulsion layer in the middle of the tube than at pH 7.0.



Figure 19 - Influence of the pH on the EA (left graph) and ES (right) (in %) of 10% (w/v) teff flour suspensions.

Influence of pH on emulsion properties of teff flour suspensions

For 10% (w/v) suspensions, the results show a peak in EA at pH 7.0, as can be seen in Figure 20. For the 5% (w/v) suspensions, the highest EA is observed between pH 8.0 and 10.0. It has been reported [19] that the EA of legume flours, as verified in the present experiment, improves above pH 4.0 and it is higher at alkaline pH than at acidic pH values, with a maximum EA observed at pH 8.0. In other data from the literature, relative to corn gluten meal, it has been reported that the highest emulsifying activity and stability of the emulsions were obtained at pH 7 to 8, which corresponds to the isoelectric point of the meal (where the meal has zero or minimum net charge) [48]. In this experiment, a minimum solubility was also observed at pH 6.0, apart from the minimum solubility verified at pH 4.0. Since the EA of proteins has been reported to be associated with protein solubility [50], the isoelectric point of teff can only be affirmed to belong to the pH range of 4.0 to 6.0. In the analyzed samples, there is a mixture of proteins (because no isolation and/or purification were performed to the samples), so it is possible that an IP range is verified, instead of an IP value (as stated above for the protein solubility).

From pH 2.0 to 4.0, the emulsion activity of 5% (w/v) teff flour suspensions was poor (below 10%, as can be seen in Figure 20). Above pH 4.0, the EA increased substantially until neutral pH, where it was significantly reduced, and then it increased again. It is thought that EA is higher in the alkaline pH region due to high solubility of the flour at the same pH range, as reported in the case of legume flours [19].

Similarly to data reported in the literature [19], [50], the emulsion activity of the samples increased with increasing pH, lowering again when pH 10.0 was reached (Figure 20). In the present experiments, there was an exception at pH 7.0, where a decrease in EA was verified for the 5% (w/v) samples. No explanation was found for this result.

Regarding the emulsion stability of 5% (w/v) suspensions, the results show a reduced ES at low and high pH values (see Figure 21). However, the highest ES is observed at pH 7.0. At alkaline pH (8.0 to 10.0), similarly to what has been reported by Ahmed *et al.* [19], the ES of the flour suspensions decreased. As for the 10% (w/v) suspensions, they show an inverse tendency at higher pH values, but show similar results at neutral pH and near the IP. The controls show a reduction in ES at pH 4.0 and 8.0, and a peak at neutral pH, similarly to the 5% (w/v) samples.

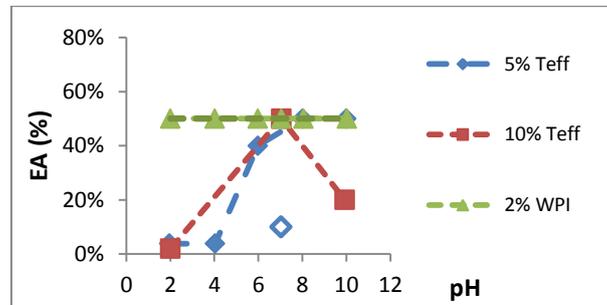


Figure 20 - Influence of pH on the EA (%) of teff flour suspensions at different concentrations. The control used was 2% (w/v) WPI at different pH values.

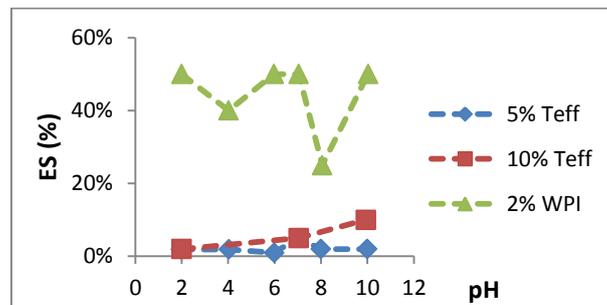


Figure 21 - Influence of pH on the ES (%) of teff flour suspensions at different concentrations. The control used was 2% (w/v) WPI at different pH values.

According to the literature [50], the greatest ES of a protein is not necessarily associated with a higher protein solubility. It is thought that ES is related to an appropriate balance between the hydrophilic and hydrophobic groups. For the 10% (w/v) teff flour suspensions, at pH 10.0, all protein samples were more highly soluble than at pH 7.0, and their negative net charges were not favorable for the aggregation of soluble protein, contributing to emulsion stability, as reported by Liu *et al.* [50]. For the 5% (w/v) suspensions, no association of ES to solubility at higher pH values can be established.

Influence of concentration of the suspension on emulsion properties of teff flour suspensions

EA and ES determined in these experiments were concentration dependent, as shown in Figure 22. Generally, the EA increased as the concentration of flour in the solution increased. According to the results (see Figure 22), the maximum emulsion activity of teff flour suspensions corresponds to the most concentrated suspension tested, 10% (w/v). A positive correlation between increasing emulsion capacity and increasing protein concentration has been reported in the literature [50]. As shown in Figure 22, a minimum concentration of 5% (w/v) is needed in the suspension (which corresponds to 0.6% protein). An acceptable emulsion is obtained at 10% (w/v) (1% protein), because more protein is available to form the emulsion.

At 2% (w/v), there was no emulsion activity verified, probably because the nitrogen solubility results of this experiment were very low (between 1 and 3%). It has been previously reported that there is a close relationship between the emulsion capacity of food proteins and their respective protein solubility and hydrophobicity [50].

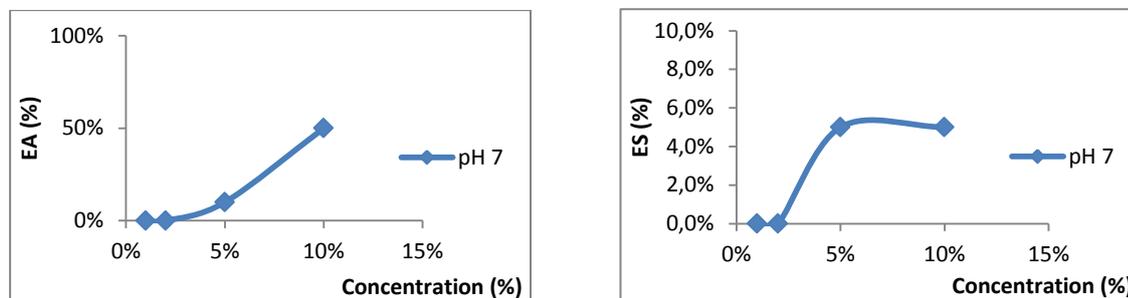


Figure 22 - Influence of the concentration on the EA (left graph) and ES (right) (in %) of teff flour suspensions at neutral pH.

At pH 7.0, both EA and ES of the control solution were 50%. In the case of teff, there was a maximum of emulsion stability in 5% (w/v) suspensions at neutral pH values. However, even the maximum ES of teff emulsions was lower than 10%. Therefore, it has been proven that, at the conditions tested, the stability of teff emulsions was quite low.

High values for EA and ES of teff flour could be useful in food formulations such as sausages, baked foods, frozen desserts and soups [34], [45]. In these products, varied emulsion activities and stabilities are required, due to the different compositions and stresses to which the products are exposed [34].

Resume of functional properties of teff flour

The results of water and oil absorption capacity, bulk density and emulsifying properties of teff flour are shown in Table 13. A comparison of functional properties of teff flour with relevant flours from the literature will be performed in this section.

Table 13 - Average values of functional properties of teff flour. The emulsification data represented is relative to 5% teff flour suspensions at neutral pH. All values are averages \pm standard deviations of duplicate analyses.

Functional property	Teff flour
Water absorption capacity (mL/g)	3.03 (\pm 0.05)
Oil absorption capacity (mL/g)	1.29 (\pm 0.02)
Emulsion activity (%)	10.0 (\pm 0.00)
Emulsion stability (%)	50.0 (\pm 0.00)
Bulk density (g/mL)	0.40 (\pm 0.01)

Compared to other types of sample (such as concentrates and isolates), teff flour has low functionality, as expected. Teff flour contains more carbohydrates than protein concentrates and isolates, which negatively influences the functional properties when compared to those protein samples [89]. As referred in section 1.2.6, it has been reported that, in general, proteins have to be in solution or in fine suspension to possess desirable functional properties [48]. Compared to the literature, teff flour has a lower bulk density (see Table 13) than cowpea flour [45] and lupin flour [52].

The solubility profile for teff flour is comparable to that of lima bean and chickpea flours [26], [89], although presenting much lower solubility values (less than 20% instead of 80-90%). Nevertheless, when the albumins extracted in the solubility trial were calculated, instead of the total protein extracted, the solubility results were different. As shown in Figure 23, the WSP extracted from the flour ranged from 34.7 to 73.4%. The lack of electrical charge in the IP negatively influenced the solubility of proteins (34.7%), as expected. In the optimum pH for the development of milk products (between pH 7 and 9), the albumins extraction was acceptable (more than 50%). This value is comparable with the one resultant from the extractions with water, both at pH 6.5 and pH 8.0.

Compared to data from the literature, teff flour has higher water and oil absorption capacity than that of chickpea [17], cowpea and soybean flours [16]. For that reason, teff flour may have potential as an ingredient in meat, bread, soup and cake formulations [34], [47].

The higher oil absorption capacity of teff flour compared to chickpea flours [17], [89] is probably due to the presence of more available non-polar side chains in teff proteins than in those of chickpea flours [17], [47]. These chains may bind the hydrocarbon side chains of oils among the flours, which may result in higher oil absorption [47]. It is known that teff has a high carbohydrates content (see section 1.2.4.1), so that could explain the obtained results. The higher WAC in teff flour when compared to chickpea flours [17], [89] and lupin flours [52] can be explained by the presence of more hydrophilic parts in carbohydrates, such as polar or charged side chains, which may increase water absorption [47].

The EA of teff flour at neutral pH was 10%. In other studies, it was reported that the emulsion capacity of wheat and sunflower meals were in the range of 10.1 to 25.6% [47]. The EA of teff meal was pH-dependent, with minimum values at pH 4.0, as previously reported [47]. However, the ES of 5% (w/v) suspensions showed an inverse tendency at high pH values, contradicting what was reported in the same study [47]. This result was attributed to a possible influence of gelation (caused by the heat treatment performed) with the emulsion stability.

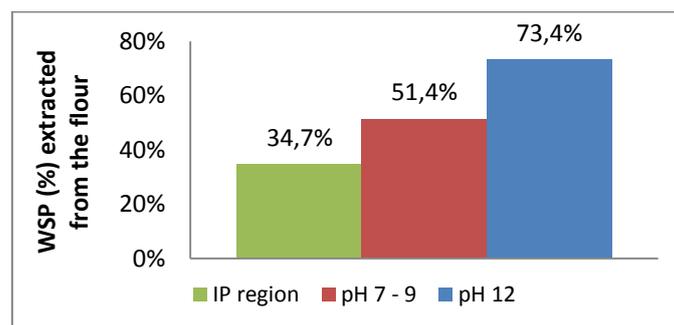


Figure 23 - Extracted water-soluble proteins (in %) as a function of pH, in the protein solubility tests. The percentages represented are referent to the estimated WSP content of the teff flour.

In this study, EA improved with increased protein concentration, while the stability slightly decreased. In the literature, only an initial increase in emulsifying activity with increase in protein concentration has been reported [47]. At pH 6.0, a high EA was observed in 5% (w/v) teff flour suspensions (40%), comparable to that of bean flours [45].

In this study, it has been shown, through detailed characterization of the functional properties, that the teff flour used possesses high water and oil absorption capacities. This flour could therefore be potentially useful in flavor retention, improvement of palatability and extension of shelf life in meat products [34].

3.3.2. Foaming tests

The foaming properties of teff flour water-soluble proteins were determined as described in section 2.5.2. Two controls were used in the foaming trials: WPI for its exceptional foaming abilities and because its proteins have been widely studied; and SPI because it is from a vegetable source and could be a better comparison with teff.

Foaming of teff extracts

Table 14 summarizes the foaming results of the several samples from the extraction of teff tested in this project. Figure 24 shows the foaming capacity of teff samples submitted both to the normal and the shorter centrifugation conditions (see section 2.5.1).

The better foam capacity of the teff extracts (orange supernatant samples, Table 14) when compared to the teff extracts submitted to an additional centrifugation step can be explained by a possible helping role of the starch content in the flours (see section 1.2.6.5).

From the foaming trials at normal centrifugation conditions (see section 2.5.2 and Table 14), we can conclude that teff proteins have a high foaming capacity, higher than that of the controls tested. The supernatant at pH 4.5 has a higher foaming capacity than the controls and a comparable foaming stability 20 min after whipping the sample. When proteins are closer to their isoelectric point, they partly fold, so they would be expected to foam better at lower pH values. In this experiment, only the foaming stability was better at low pH. This result was attributed to the formation of a stable protein layer in the low pH region, which stabilizes the foam. The supernatant at pH 7.0 had a higher foaming capacity than the controls (and even higher than that of the supernatant at pH 4.5), but had a lower foaming stability when compared to the control samples. This high FC in the alkaline pH region is concordant with the behavior of protein solubility (see section 3.3.1), and is thought to be due to the increase in protein net charge, which weakens the hydrophobic interactions but leads to an increase in protein solubility and flexibility, thereby improving foam formation (see section 1.2.6.5). As for the decrease in FS at the alkaline pH region, it might have been caused by charge repulsion between proteins (see section 1.2.6.1). The pellet dissolved at pH 7.0 had a low foaming capacity, but its foaming stability was relatively high, when compared to the controls at pH 7.0. Its foaming stability is much higher after 60 min than that of the supernatant at pH 7.0 (see Table 14).

Table 14 - Summary of the foaming results for teff extracts submitted to normal centrifugation conditions (orange) or to shorter centrifugation steps (green). All samples were analyzed in duplicate, and the represented percentages are means from the resultant values.

Sample	Visual appearance	Foam ability	FC (%)	FS after 20 min (%)	FS after 30 min (%)	FS after 60 min (%)	FS after 180 min (%)
2% w/w WPI pH 4.5	Turbid	High	114	82	82	-	-
2% w/w WPI pH 7.0	Very clear	High	80	77	76	-	-
2% w/w SPI pH 4.5	Precipitate	Medium	60	78	78	76	66
2% w/w SPI pH 7.0	Very turbid	Low	30	88	88	83	69
Teff supernatant pH 4.5 (0.6% protein)	Slightly clear	High	159	83	80	68	42
Teff supernatant pH 7.0 (0.6% protein)	Turbid	Higher than at 4.5	199	57	47	23	-
Teff pellet dissolved at pH 7.0 (1.09% protein)	Very turbid	Much lower than the previous	29	61	61	61	-
Teff supernatant pH 8.0 (0.6% protein)	Very turbid	Medium	41	-	93	90	90
Teff 2nd supernatant pH 4.5 (0.3% protein)	Slightly clear	Medium(similar to the previous)	75	-	54	43	5
Teff 2nd supernatant pH 7.0 (0.3% protein)	Very turbid	Lower than at pH 4.5	43	-	81	81	22

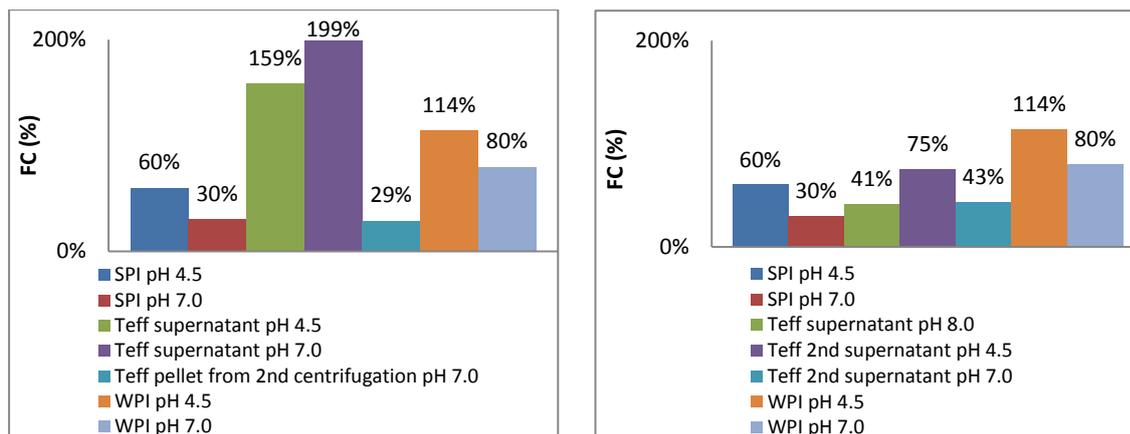


Figure 24 - Foam capacity (in percentage) of teff samples analyzed (left – normal centrifugation; right – shorter centrifugation: see section 2.5.2). The control solutions utilized were 2% (w/w) WPI and SPI at both pH values tested, 4.5 and 7.0.

Regarding the foaming trials at shorter centrifugation conditions (see section 2.5.2 and Table 14), it can be concluded that only the proteins in the sample at pH 4.5 present good foam ability. However, the foam stability of these proteins is very low. This higher FC at low pH values was attributed to an increase in the net charge of proteins. At pH 8.0, the inverse tendency is verified. As referred in section 1.2.6.5, when protein aggregates are present in the system analyzed, more stable foams can be obtained. Finally, at pH 7.0, the results are very similar to those verified for the supernatant of the first centrifugation at pH 8.0. The increase in FS at alkaline pH values (7.0), when compared to the results at pH 4.5, was reported in previous studies (see section 1.2.6.5) and attributed to the lower bubble coalescence that resulted from the lower foam volume formed at pH 7.0. It should be stated that the decrease in FS from 60 to 180 min in the second supernatants at both pH values may have occurred because the protein film formed around the bubbles was not viscous, elastic and resistant enough to inhibit foam coalescence [49].

Comparing the foaming results for teff samples with the SPI controls (see Table 14 and left graph of Figure 24), we can conclude that teff proteins have a high foaming capacity, much higher than that from the controls tested (in the case of the first supernatants). The supernatant at pH 4.5 has a much higher foaming capacity than the controls and a comparable foaming stability 30 min after whipping the sample. This behavior has been explained above. The supernatant at pH 7.0 has a much higher foaming capacity than the controls (and even higher than that of the supernatant at pH 4.5), but has a lower foaming stability when compared to the control samples, both after 20 and 60 min. The pellet dissolved at pH 7.0 has a low foaming capacity, but the foaming stability is relatively high, when compared to the controls at pH 7.0. Its foaming stability is much higher than that of the supernatant at pH 7.0.

Regarding the foaming results for the samples submitted to short centrifugation steps (see Table 14 and right graph of Figure 24), the higher FC was verified at pH 4.5, and it was higher but comparable to the SPI results, but much lower than WPI results. The foaming results for the first and second supernatants at pH 8.0 and 7.0 were similar and higher but comparable to the SPI results, but again much lower than WPI results. Soy protein is more similar to teff proteins than whey proteins, so the

comparison with soy is more relevant, especially in the case of the protein in a purer state (after two centrifugation steps). The higher foaming stability was verified both at pH 8.0 and at pH 7.0 after additional centrifugation, as explained above.

When the low foaming results from the WPI duplicates, which could not be explained, were ignored, a different set of results for these controls was generated (see Figure 25). Comparing now with the WPI results from Figure 25, we can still conclude that teff proteins have a high foaming capacity, higher than that from the controls tested at pH 7.0. The supernatant at pH 4.5 has a lower foaming capacity than the control and a comparable foaming stability 20 min after whipping the sample, as referred above. The supernatant at pH 7.0 has a higher foaming capacity than the controls and the other teff samples (and even comparable to that of the WPI at pH 4.5), but has a lower foaming stability when compared to the control samples. The pellet dissolved at pH 7.0 has similar results as those referred above. Regarding the results presented in the right graph of Figure 25, the results for teff samples are much lower than those verified for WPI samples, as explained above.

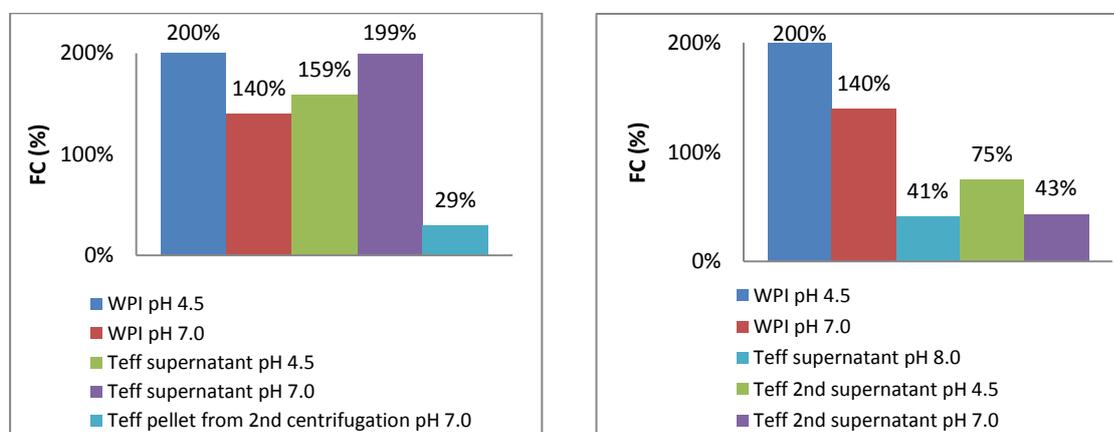


Figure 25 - Foam capacity, in percentage, of teff samples analyzed with the normal centrifugation conditions (left graph) and with the shorter centrifugation conditions (right graph), ignoring the results from WPI duplicates. The control solution utilized was 2% w/w WPI at the tested pH values, 4.5 and 7.0.

The explanation for all the foaming results might be the antagonistic roles of different proteins present in the teff flour (see Figure 26). The protein with good foam ability was denominated protein 1, whereas the protein which seems to hinder foam formation was called protein 2.

At pH 4.5 (IP precipitation + centrifugation), only the protein that has no foaming ability precipitated (protein 2); all of the protein that has foaming capacity (protein 1) stayed in solution, and that may explain the high foam ability of this sample (see Figure 25). When the solubilization is made at alkaline pH values (shorter centrifugation), some of the protein that obstruct foam formation might have stayed in the supernatant (this explains the low results of FC of the supernatant at pH 8.0). Protein 2 negatively influences the foaming, and that might explain why a shorter centrifugation leads to a lower foam formation. When a second centrifugation is performed, more protein 2 seems to precipitate at lower pH values, thereby improving foam formation. The precipitates after the second centrifugation are thought to contain only protein 2, which might explain why the pellet has such low foaming capacity when compared to the other tested samples.

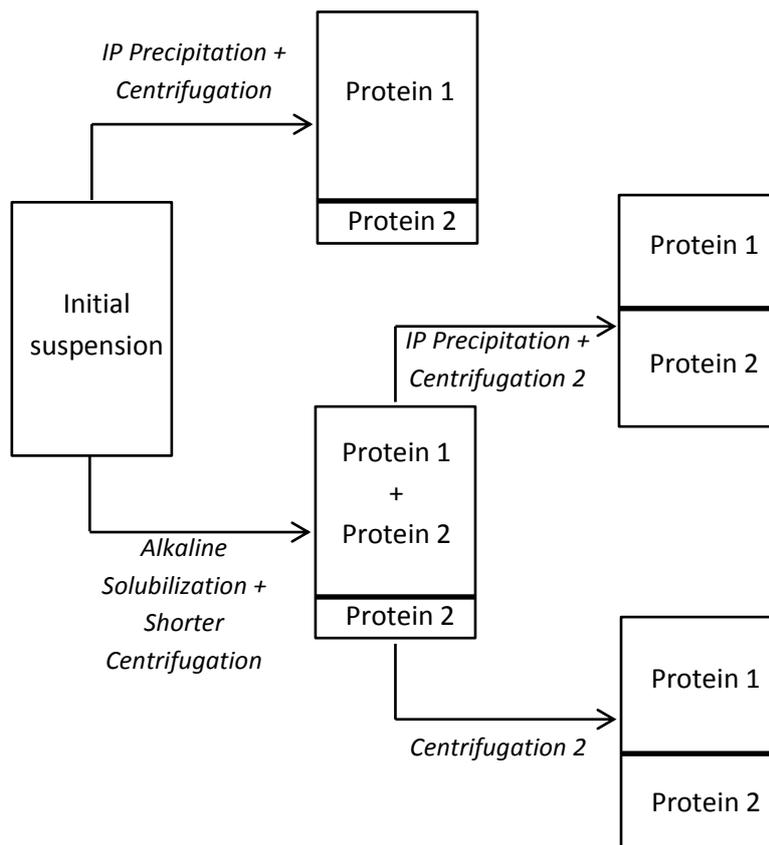


Figure 26 - Protein distribution after each centrifugation step performed. The bold black line separates the supernatant (on top) from the precipitate (on the bottom).

Foaming of starch-devoid samples

Foaming tests of enzymatically treated teff extracts were also performed as described in section 2.5.2, in order to study the influence of sugar reduction in the foaming properties of the samples. The results of the sugar content determination performed to the freeze-dried samples are discussed in the end of this section. Visually, the freeze-dried samples were a thin and loose powder.

The protein content of the teff suspensions was calculated assuming that the protein content after dialysis was the same from the extraction trials, and so the freeze-dried product would have an estimated protein concentration of 77% (w/w). However, since the correct protein concentration in the dialyzed product was actually much lower (about 0.08% instead of 0.37%, as determined by Kjeldahl analysis), instead of 2% (w/w) protein suspensions, the foaming trials were actually performed with suspensions with protein concentrations between 0.1 and 0.4% (w/w). There was no foaming verified in these trials. However, even in further trials where the protein content had been re-calculated knowing the real value after dialysis, no significant foam formation was observed, even though it was not easily destroyed. This indicates that the sample has no foaming capacity.

There are several possible reasons for the loss of functionality of the freeze-dried sample, apart from the extremely low protein concentrations:

- Too much centrifugation (loss of protein, which remained in the pellet). According to the Kjeldahl results, there was a substantial reduction of the protein content when compared to

the extraction and milk development trials. The protein content after centrifugation in the milk development trials was 3 times higher than in the present trials, so the centrifugation time and speed might have been excessive (20 minutes at 6000x g instead of 2 min at 2000x g, like it was performed in the milk trials).

- Proteolytic activity (that could have reduced the protein contained in the samples).
- Loss of protein in the dialysis (even though proteins generally have a MWCO higher than 18000 Da, which means that they shouldn't be removed, and sugars have a MWCO of 500 Da, so they should be removed in the dialysis). According to the Kjeldahl results, there seems to have been a loss of protein due to dialysis, so there might have been some amino acids loss in the dialysis.

Overall, the foaming tests performed to the starch-devoid samples were not considered successful.

Sugar content determination

In order to verify the removal of sugars from the samples, the samples were sent to analysis, to assess their sugar content through high-performance cation-exchange chromatography with a refractive index detector, in an Aminex HPX-87P (Bio-Rad) column. The results showed a presence of 13.84 mg of glucose per g of freeze-dried product. When compared to the sugar content in the flour (2.1% in TF1), only less than 35% of the sugars were removed from the product. A possible explanation for these results is that a protease contaminated the enzymes, taking into consideration that preliminary experience in NIZO group has shown that 12 h at 4°C were sufficient to achieve an efficient sugar removal. To try to confirm this possible explanation, a protease activity assay was also performed (see following section), to understand the decrease of protein after the enzymatic treatment.

From these results, it was confirmed that the removal of sugars was not successful. Previous experiments at NIZO indicated that these sugar removal steps from plants are difficult; such behavior could explain these results. Moreover, the dialyzing procedure could have been less effective than in previous trials. With these results, and assuming a content of 5% (w/w) of water in the freeze-dried product, a composition for the freeze-dried material can be estimated (Table 15). The other components might be plant material, such as fibers or cellulose. In order to remove the cellulose, cellulases could be used, with the purpose of breaking down the plant membranes.

Table 15 - Estimated composition for the freeze-dried product (expressed in g per 100 g of product), assuming 5% of water after freeze-drying.

Protein (g/100 g)	Sugars (g/100 g)	Water (g/100 g)	Others (g/100 g)
16.05	1.38	5.00	77.57

Protease activity assay

The results and calibration curves obtained in the protease activity assay are resumed in Appendix IV. Two of the enzymes used in the sugar removal trial were tested: Pullulanase and Amyloglucosidase (see section 2.1). In the first enzyme, no proteolytic activity was detected at the tested concentrations. However, the proteolytic activity detected in the second product was very high. In fact, the values were

too high to fit into the calibration curve of trypsin: a concentration higher than 500 µg/mL trypsin (proteolytic activity was expressed as trypsin equivalent). From these results, we can affirm that the proteolytic activity of one of the enzymes utilized in the removal of sugars probably influenced the results of the foaming (and gelation, which will be presented in the next section) trials of the enzyme-treated teff extracts.

3.3.3. Heat-induced gelation

Preliminary trials

In the preliminary trials, gelation was performed as described in section 2.5.3. In these trials, there was only gelation in the supernatant at pH 4.5, as can be seen in Figure 27.



Figure 27 - Gelation results for teff supernatant at pH 4.5.

As referred in the previous section, teff supernatant at pH 4.5 produces high and stable foam. Possibly, the gelation results can be explained by this foaming character: the sample is heated, but due to the immense foaming, a gel-like substance remains in the top of the tube, as it can be visualized in the picture from Figure 27.

Starch-devoid samples

After enzymatic treatment to remove the starch from the teff extracts (see section 2.5.2), gelation tests were performed, as described in section 2.5.3. Table 16 shows the results of the gelation trials.

Visual observation results led to the conclusion that almost all samples formed gels (or at least very viscous suspensions). Only low protein concentrations (0.5% (w/v) in the case of teff freeze-dried products and 1 to 5% (w/v) in the case of SPI) did not lead to gel formation (or at least to a very viscous suspension that appeared to be a gel). An increase in protein-protein interactions results in an increased formation of disulphide bonds that would contribute to gel hardness. Also, gelation occurs better at higher protein concentration because of the higher intermolecular contact during heating [34]. So, at higher protein concentration, ordered gel matrixes can be formed [58].

After 2 h in the cold room, the LGC (see section 1.2.6.6) of the samples was determined. As observed in Table 16, LGC of freeze-dried teff was in the protein content range of 1 to 3% (w/v). For the teff flour samples tested, LGC seemed to be 3% (w/v). For the controls (SPI), LGC seemed to be between 5 to 12% (w/v). Below 5%, SPI samples did not seem to have enough protein concentration to form a well-organized and strong matrix. A LGC of 12% (w/v) has been reported for cowpea protein isolate [16].

The gels (and non-gels) discussed above were submitted to texture analysis, as described in section 2.6.6. The texture measurements performed in this project simply provide a rough trend through which the samples can be compared, because the puncture tests were performed with fewer sample than what ideally should have been used (the sample diameter was not three times larger than the probe diameter, as it is theoretically suggested). Texture analysis results are comprised in Appendix II.

Table 16 - Protein concentration (%) and visual observations relative to the gelation tests. Similar codes to those represented in the table were used in Appendix II, where the texture analysis results are shown. The samples marked with asterisks were analyzed twice after gelation trials. The letter *a* in the gel/non-gel column respects to gel strength (see bottom of the table).

Sample	Sample Code	Protein concentration (w/v, %)	Drop or slip	Gel/Non-gel ^(a)	
Freeze-dried product	A	0.5	Yes	+/-	
	Condition 1	B1	1.0	No	+
		B2	1.8	No	+
		X	1.7	No	+
		Y	2.0	No	+
		D	0.5	Yes	+/-
	Condition 2	E	1.8	No	+
		F	2.3	No	+
G		2.7	No	+	
Teff flour		H	1	No	+
	I	3	No	+	
	H	1*	Yes	+	
	I	3*	No	+	
SPI Controls	J	2	Yes	-	
	K	8	Yes	+/-	
	J	2*	Yes	-	
	K	8*	Yes	-	
	L	12*	No	+	
Teff powder	1	5	No	+	
Teff flour	2	1	No	+	
	3	5	No	+	
SPI Controls	4	1	Yes	-	
	5	5	No	+	
	6	10	No	+	

(a) - No gel +/- Very weak gel + Gel

3.3.4. GDL acidification

For a more complete study of the gelation process, it was decided to test the acid-induced gelation through the addition of food-grade GDL and maintenance of the samples at RT for 18 h. The aim of this trial was to verify if gels were obtained after slow acidification of the teff extract (from TF1) at pH 8.0. The results are resumed in Table 17.

Table 17 - Amount of GDL added, along with the pH measurements after 18 h and the visual evaluation of gel strength. The initial pH of the samples was 7.5. Duplicates were prepared.

GDL (w/w, %)	pH (T, °C)	Gel strength (visual observation)*
0.2%	4.28 (24.0)	--
Duplicate	4.28 (24.0)	---
0.3%	3.54 (24.7)	---
Duplicate	3.99 (24.1)	---
0.4%	3.83 (24.8)	---
Duplicate	3.85 (24.2)	--
0.5%	3.92 (24.2)	---
Duplicate	3.99 (23.9)	---
0.7%	3.84 (23.1)	+
Duplicate	3.87 (23.4)	-
1.0%	3.75 (24.2)	--
Duplicate	3.74 (24.2)	-
1.5%	3.48 (24.2)	+/-
Duplicate	3.48 (24.4)	-

* The hardness of the samples was evaluated through codes: ---: very weak gel (it slipped after 2 s); --: weak gel (it slipped after 5 s); -: moderately weak gel (it slipped after 10 s); +/-: slightly weak gel (it slipped after 20 s); +: slightly strong gel (it slipped only after 3-5 minutes).

At GDL concentration higher than 0.7% (w/w), the samples seemed to get thicker, even though they were all soft. Also, in general, it was verified that the higher was the GDL concentration the lower was the final pH, as expected (see section 1.2.6.7).

Figure 28 shows the gel formation in the sample with a GDL concentration of 0.7% (w/w). In these trials, no texture analysis was performed, because the gels were too weak (even the 0.7% (w/w) GDL gel fell down 5 min after the visual evaluation). This result was attributed to the low protein concentration in the samples (less than 1%). There is some indication that gel formation by GDL acidification is possible with teff, but more conditions should be tested in future studies.

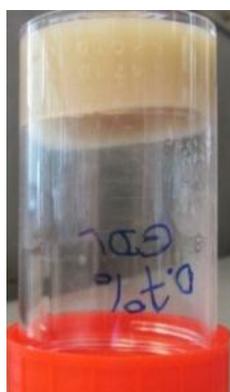


Figure 28 - Visual observation of gel formation in 0.7% (w/w) GDL acidified samples.

3.4. Fiber formation

In order to recreate the meat-resembling fibrous texture, vegetable proteins can be combined with a polysaccharide. The formation of fibers was performed using different proteins (P1 and P2). The comparison between the two types of fibers is described in section 3.4.3.

3.4.1. P1 fibers

a) Influence of heating on the production of fibers

In previous experiments at NIZO, it was verified that performing heat treatment improved fiber formation because it helped to fixate the fibers. Therefore, the effect of heat treatment in fiber formation was investigated in this project. The fibers used in this section do not have any extra ingredients addition; they are produced only from the protein/polysaccharide mixture. Figure 29 shows the difference in the yield of fiber formation amongst heated and unheated fibers from P1. This yield seemed to have a tendency to be higher in the fibers that were subjected to heat treatment (15.5% instead of 14.5%, as shown in Figure 29). That was expected, because the proteins can aggregate during heating, especially near their IP (and the pH was lowered below the IP region for vegetable proteins), where proteins can be denatured and thus more sensitive to aggregation. These values of yield are lower than those obtained in a previous work [90] (between 20 and 25%). However, the conditions used were not the same: the main difference seems to be that not the same vegetable protein was used.

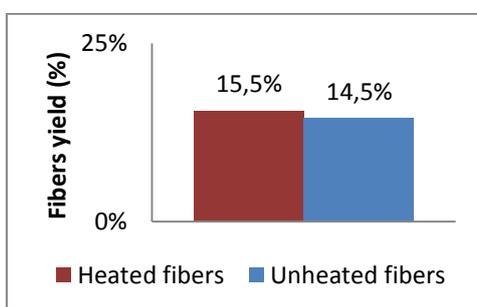


Figure 29 - Comparison between heated and unheated fibers, in relation to the fibers yield.

The fibers obtained in this section (see left picture of Figure 30) were long and cohesive (see CLSM images in section 3.4.1.1). However, their taste was acidic, which might constitute a problem in the incorporation into food products. To overcome this problem, different ingredients were added (see text below).



Figure 30 - Picture of heated P1 fibers (left), P1 fibers with ingredient 1 (middle) and P3 fibers (right).

b) Influence of Ingredient 1 and CL addition on the production of fibers from different protein sources

This trial was an attempt to improve the quality of the fibers, in terms of taste and appearance, because the previous fibers (section a) were acidic and too wet. In this section, the fibers were not submitted to heat treatment, and the acid was added at high speed. In the sample incubated with CL, the incubation at low temperature (4°C) was used. 6% (w/w) of Ingredient 1 was added to the initial protein solution, in one of the batches of fibers.

The P1 fibers with Ingredient 1 had the highest yield of fiber formation, as shown in Figure 31. This yield was higher than the yield from P1 fibers without Ingredient 1 addition. Therefore, the results indicate a positive influence of Ing.1 on fiber formation. Moreover, the incorporation of CL in the process seems to hinder fiber formation, thereby reducing the yield.

The lower yield of the P3 fiber formation (which had no Ingredient 1 addition) possibly supports the idea that Ingredient 1 might improve fiber formation, even though the protein used was different.

In terms of appearance, the fibers with Ingredient 1 are more colored (yellow coloration) than the simple fibers (see Figure 30), and can more easily be incorporated into food products. CL also conferred flavor and a yellow color to the fibers, as this compound is a brown solution (see Figure 37). P3 fibers are much longer and seem to retain more water (Figure 30).

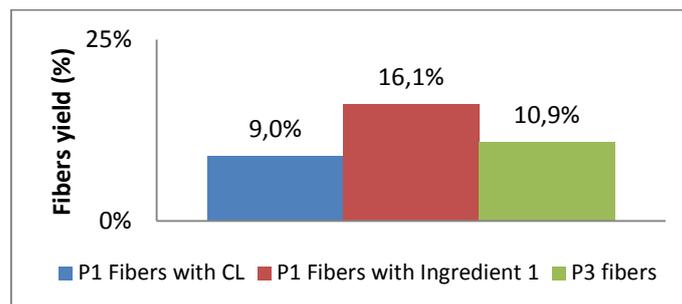


Figure 31 - Comparison between different kinds of fibers, in relation to the final fiber yield (%).

c) Influence of Ingredient 1 content on fiber formation

In this trial, different Ingredient 1 contents were added to the fibers (2, 4 and 6% w/w of the protein solution), and no heat treatment was performed. Furthermore, no CL was added to the fibers in this sub-section.

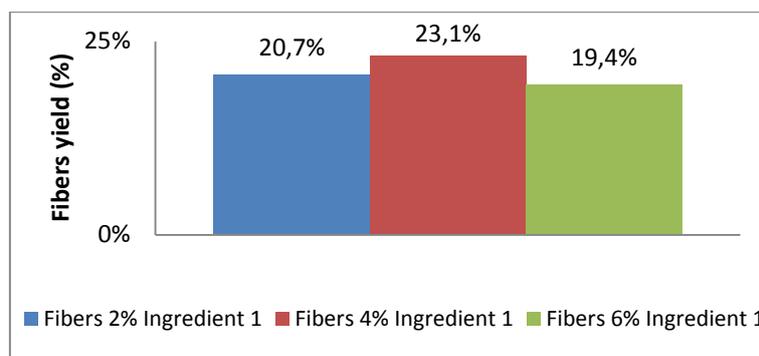


Figure 32 - Influence of Ing.1 concentration in the starting solution on the yield of fibers formation.

The pumping flow rate of acid addition to the fibers was reduced for a better control of the pH values (0.4 mL/min instead of 0.8), because the initial volume used was smaller. The optimal Ing.1 content among those tested was 4% (w/w), as shown in Figure 32. Visually, no difference was detected amongst these fibers.

d) Influence of the presence of salt on fiber formation

In this trial, 0.5% (w/w) NaCl was added to the fibers, after the polysaccharide addition. The fibers with salt were compared to the simple fibers (without any extra ingredients added), without heat treatment. Visually (see Figure 33), P1 fibers with salt seemed to retain more water and to be less cohesive than the normal P1 fibers, without ingredient addition (see Figure 30).

The results suggest a slightly negative influence of the presence of salt in the formation of fibers. It has been reported that the presence of salt at some concentrations may suppress the formation of complexes, depending on the nature and concentration of the salt (see section 1.2.7). At low salt concentrations, there is a salting-in region caused by the favorable interaction between the ions in the media and the surface charge of proteins. In a salting-in region, the salt interacts with the protein, hence increasing protein solubility [91]. However, a high salt concentration may inhibit complexation. At high concentrations, the ions present in solution screen the charges of the polymers, thus reducing their associative interactions and, therefore, the amount of soluble complexes formed [66]. This might explain the small reduction in the formation of fiber complexes.

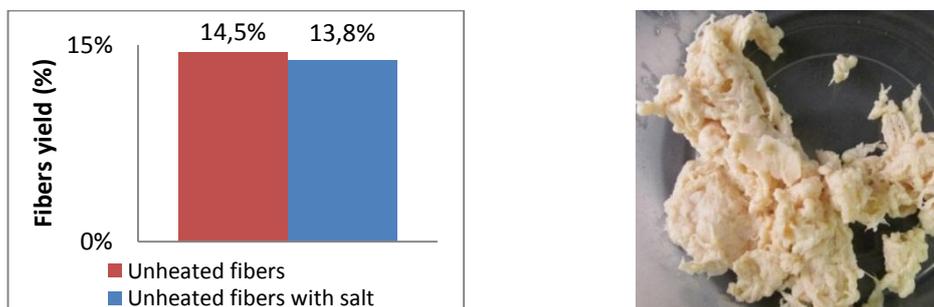


Figure 33 - Influence of the presence of salt on the yield of fibers formation (left). Picture of P1 fibers with 0.5% (w/w) salt (right).

e) Influence of the presence of cross-linking agent on fiber formation

In this set of experiments, fibers with and without incubation (at high or low temperatures) with a cross-linking agent were compared. Moreover, the influence on the fibers yield of heat treatment before the final pH adjustment was analyzed.

- **Cross-linking agent (incubation at 4°C)**

This trial was performed to study the effect of the final pH adjustment in the fibers yield, after incubation at 4°C overnight with CL. A comparison of the yield of fibers with and without final pH adjustment to higher pH values was also performed. Amongst the pH values tested, the pH value that optimizes the fibers yield seems to be pH 5.0 (see Figure 34). Comparing the yield of the fibers at acidic pH (fibers with CL) with that of the fibers at higher pH values, it can be concluded that a pH adjustment in the end of the procedure is advantageous in terms of yield.

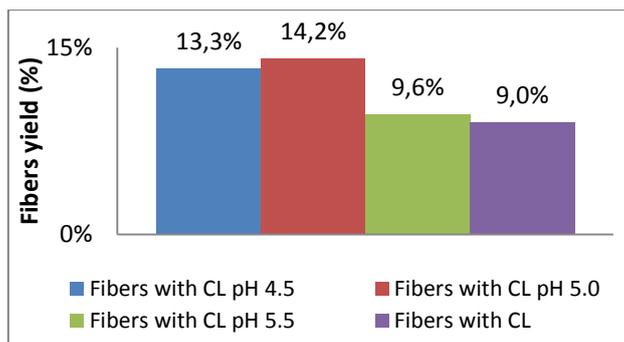


Figure 34 - Influence of final pH on the final amount of fibers obtained.

- **Cross-linking agent and heating step (incubation at 4°C, followed by heating)**

This trial was performed to understand the effect of the heating step on the fibers yield. The heat treatment was performed before adjusting the pH to higher values. The results shown in Figure 35 suggest that there is a higher yield when the heating step is performed, which might mean that the heating process helps to retain water inside the fibers.

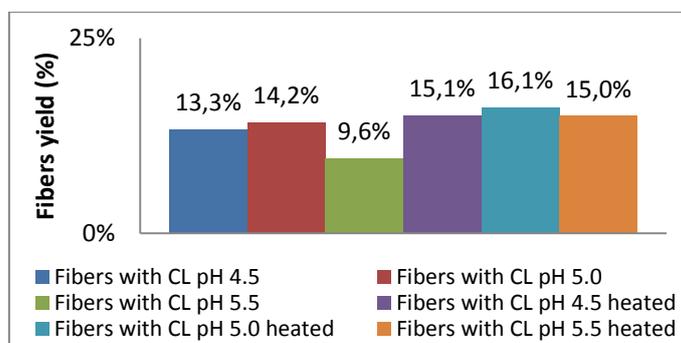


Figure 35 - Influence of heating step on the final amount of fibers obtained (compared to the fibers without heating step).

- **Cross-linking agent, incubation at 50°C and heating step**

This trial was performed to understand the effect of the high temperature incubation step in the fibers yield. The fibers were incubated at 50°C for 1 h with 1% (w/v) CL, instead of being incubated overnight at 4°C with the CL. Comparing all the incubation methods, the highest yield in fiber formation (see Figure 36) is verified when the incubation at high temperature is performed, but the difference in relation to the trial with the heating step is not very significant. The best pH value amongst the tested seems to be 5.0, visually, in terms of texture and taste and also in terms of yield. Therefore, only the samples at this pH were analyzed with the CLSM and produced in further trials. Moreover, only the pictures obtained from the fibers at pH 5.0 are shown (see Figure 37). In these pictures, well-structured fibers can be observed, but the fibers seem watery and soft, and the fibers incubated at 50°C resemble meat, more than the others.

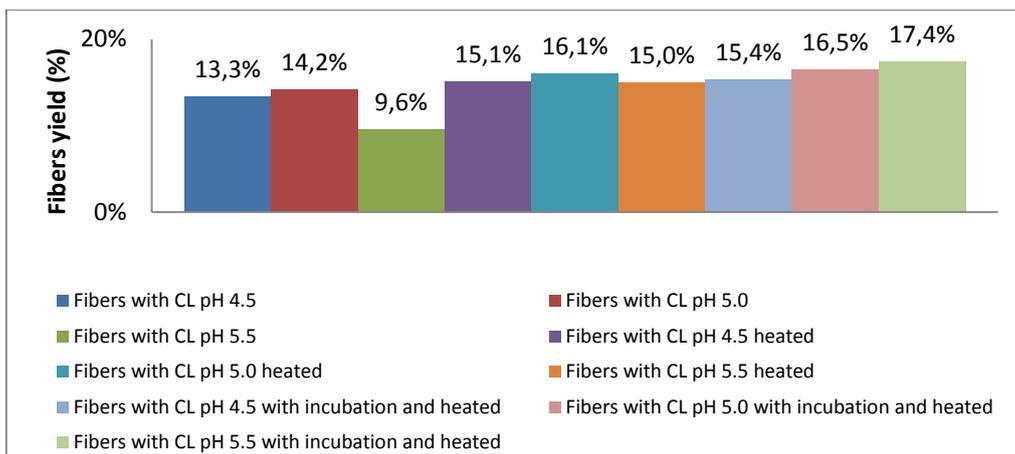


Figure 36 - Influence of the incubation with CL at high temperature on the final amount of fibers obtained (comparison with the fibers with and without heating step).



Figure 37 - P1 fibers incubated at 4°C for 1h with 1% (w/v) CL (left), at 4°C for 1h with 1% (w/v) CL followed by heating (middle) and at 50°C for 1h with 1% (w/v) CL (right), all at a final pH value of 5.0.

f) Influence of the presence of Ing.1, salt and cross-linking agent on fiber formation:

This trial was an attempt to improve the quality of the fibers, by incorporating Ing.1, cross-linking agent and salt one by one and at the same time in the fibers. A pertinent question to be answered was whether or not simply heating, without adding CL, was enough to raise the pH to 5.0, without destroying the fibers. All fibers in this trial were submitted to heat treatment (30 min at 80°C). The fibers produced with CL were incubated for 1h at 50°C. A comparison with similar fibers from previous trials was also performed.

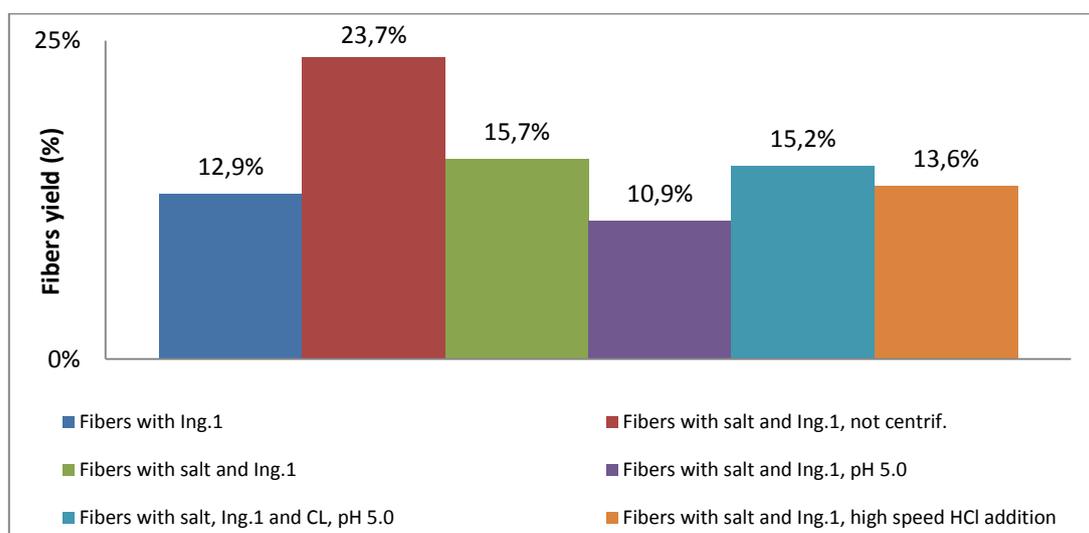


Figure 38 - Influence of the addition of Ing.1, salt and/or incubation with CL at high temperature on the final amount of fibers obtained.

The reduction in fibers yield verified when the acid was added at high speed was not very significant, as shown in Figure 38. Moreover, the appetitive taste and visual appearance of the fibers were not reduced. Therefore, it was decided to apply the high speed addition of acid to further trials.

In this set of experiments, an additional comparison was made: the production of fibers from a non-centrifuged protein solution was compared, visually and in terms of yield, to the regular production (with and without raising the pH to 5.0 in the end of the procedure).

Regarding the results shown in Figure 39, a higher yield was obtained for the fibers produced from a non-centrifuged initial protein solution, which was expected, because the protein concentration is higher when the centrifugation step is not performed. However, the fibers produced by such a procedure may contain impurities, not desirable in a food product. Still in Figure 39, when the pH was adjusted to the optimal pH from the previous trials (pH 5.0, as referred in section e), there was a significant reduction in the fibers yield. This result was attributed to the presence of salt that, as was referred previously, seems to cause a decrease in the fibers yield.

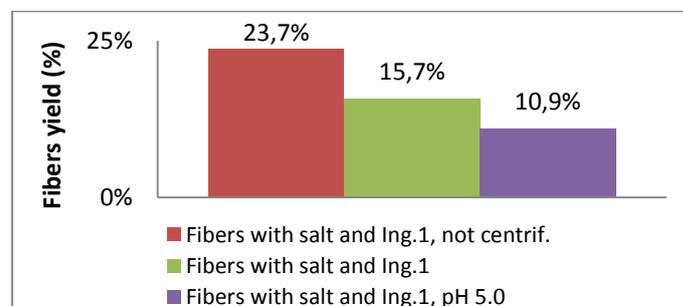


Figure 39 - Influence of the preparation procedure of the initial protein solution and of the end pH on the final amount of fibers obtained. Data extracted from Figure 38.

Based on the yield reduction, it can be concluded that some of the fibers are destroyed when the pH is raised to 5.0 only heating afterwards, instead of adding also CL. The fibers dissolve again, because the binding between polysaccharide and protein becomes weaker. As the pH deviates from the IP, proteins become less sensitive to aggregation and heating, so they could possibly become more soluble (see section 1.2.6.1). CL aggregates the fibers, strengthening the protein disulphide linkages, thereby protecting the fibers from breaking (see section 1.2.7).

Another conclusion is that Ing.1 and salt can be successfully incorporated in the fiber formation from P1, without reducing the yield. Finally, it can also be concluded that with an addition of acid to the solution at high speed while stirring vigorously, the yield is not significantly reduced. So, this procedure can be executed, thereby facilitating the fiber production.

The visual aspect of the different fibers formed in this sub-section is shown in Figure 40. These fibers were also informally tasted at NIZO. As for the fibers represented in the first picture of Figure 40, their flavor was a little bean-like, but the mouth feel and taste were considered good. The fibers represented in the second picture (upper right) had a more neutral flavor, also a good mouth feel and taste, and better structure than the previous fibers. The fibers from last picture (down right) were thinner, weaker and much more humid than the other fibers that were produced. A lot of foam was

released from these fibers when compression was performed, and the fibers were harder to compress (maybe because a lot of protein was lost).



Figure 40 - P1 fibers formed by several methods: fibers with salt and Ing.1 addition, made from a part of the protein solution which wasn't centrifuged (left upper picture); fibers with salt and Ing.1 addition, made with the normal procedure (right upper picture); fibers with Ing.1 addition (left lower picture); fibers with salt and Ing.1 addition, with pH adjustment to 5.0 (right lower picture).

g) Overall conclusions for P1 fibers production:

One important conclusion is that heating improves the fibers yield. Also, the results show that the Ing.1 addition not only improves the mouth feel and taste of the fibers, but also raises the fibers yield (however, as mentioned earlier, the optimal Ing.1 content is not the maximum tested). In fact, the best fibers from P1 obtained in this study were the fibers with 4% (w/w) Ing.1 addition, unheated and without other ingredients added (yield of fiber formation of 23.1%). However, no heat treatment was attempted to these fibers. If these fibers were heated, the yield would be expected to improve even more. Another important remark is that the addition of CL only increases the fibers yield when the pH is raised after fiber formation. The optimum pH was 5.0, and the yield improved when heating was performed, and even slightly more when the incubation was performed at a higher temperature (maintaining the heating step after raising the pH to 5.0). However, the rising in yield might not be significant enough to justify the application of this procedure at the industrial scale.

Homogenization of the initial protein/Ing.1 solution was attempted, but the results suggested that the mixture through Ultra-Turrax was enough to create a good emulsion, so the fibers were made without the homogenizing step. The non-homogenized product was also considered better in the informal tasting sessions, probably because the big drops of Ing.1 improved the mouth feel of the product. Moreover, from the tasting sessions, it was observed that the non-likable flavor seemed to be a bit lost after the heating procedure. For the fibers incubated with CL that were heated, there was a better texture and weaker flavor, maybe due to protein denaturation. Also, when more heating was performed to the CL-treated fibers, it is possible that there was more protein denaturation, and a better gelation, so the fibers obtained were better.

Regarding the optimum pH values after fiber formation, pH 5.0 seems to be the optimum pH. In fact, at pH 4.5, the fibers were too dry and, at pH 5.5, the fibers became too soft.

Finally, it should be stated that, with these fibers, different meat analogues could be produced, such as tofu, chicken meat, quiche, minced meat or sausages.

3.4.1.1. CLSM images

The CLSM images were obtained with a dye solution: 0.2% Rhodamin-B in water. Depending on the dye used, different colors can be displayed in the images. The protein present in the samples can be visualized at orange. All images were obtained at a 10 μm depth into the sample.

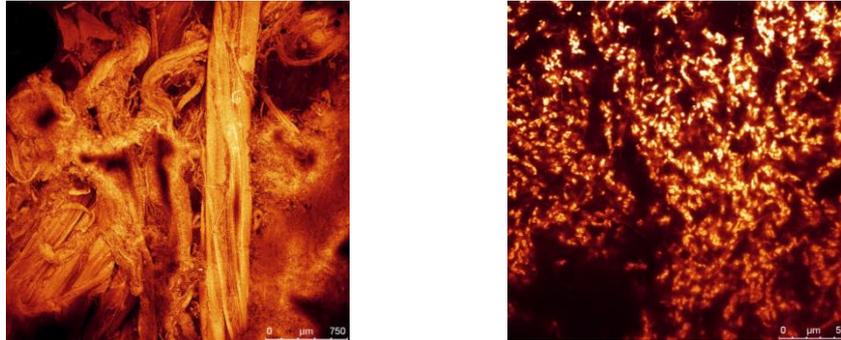


Figure 41 - CLSM images of P1 heated fibers (left) and P1 fibers with 0.5% w/w salt (right). Settings: Objective 20x, zoom 2.0 (left); Objective 63x, zoom 1.0 (right).

At the CLSM, the fiber structures were clearly distinguishable in the P1 heated fibers, as can be seen in the left picture of Figure 41. These fibers were big and cohesive. As for the P1 fibers with salt (right picture of Figure 41), only protein aggregates can be visualized, and not the fiber structures. Nevertheless, the fiber formation was visually evident (see Figure 40).

Figure 42 shows the CLSM images of P1 fibers incubated with CL, at different temperatures and incubation durations, and also with or without heating step after raising the pH to 5.0. It was decided to only analyze the P1 fibers at pH 5.0, since this was the optimum pH in terms of yield and appearance of the fibers. In the first picture (incubation at low temperatures and heat treatment), some fiber structures can be detected, but also some protein aggregates. In the other two pictures, no significant fiber structures can be visualized, only protein aggregates.

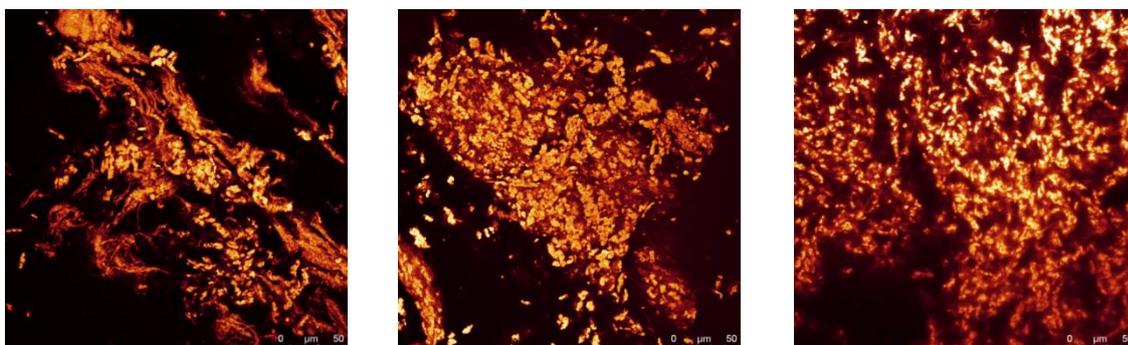


Figure 42 - CLSM images of P1 fibers incubated at 4°C overnight with 1% (w/v) CL at pH 5.0, with heating step before increasing the pH to 5.0 (left) or without heating step (middle), and P1 fibers incubated at 50°C for 1 h with 1% (w/v) CL (right), with heating step before increasing the pH to 5.0. Settings: Objective 63x, zoom 1.0.

3.4.2. P2 fibers

In this set of trials, the objective was to verify if there was fiber formation in the case of P2 (teff protein). If so, it could be possible to produce solid foodstuffs using teff, and not only drinks. P2 fibers were developed from teff extracts (at pH 6.5 and 8.0), instead of being developed using a protein isolate (like P1 fibers were made). The protein isolate has a purified protein (without interfering compounds such as sugars, that are present in the extracts), and that is thought to influence the final fibers formed, because the purer the protein, the more it is available to interact with the polysaccharide during fiber formation. Therefore, P2 fibers were expected to be less strong than P1 fibers.

a) Initial trials:

In these trials, there was formation of P2 fibers. There was no visual difference between the fibers formed in these trials, apart from the amount formed. These fibers were small and thin (see the right part of Figure 43, where only the heated fibers from the extract at pH 8.0 are shown), but that was expected because the protein concentration was very low. In the second trial, there was formation of about twice more fibers than in the first trial, which was expected, because the protein extracted was also twice the amount as in first trial (see section 3.1.1). The yield of fiber formation in these fibers was considered good (24.8%), when compared to a previous work [90] (between 20 and 25%).

The end pH, after fiber formation, was lower in the second fibers (from the extraction at pH 8.0, as described in section 3.1.1) than in the first trial (from the extraction at pH 6.5, as described in section 3.1.1), and maybe that has influenced the higher yield in relation to first trial (as shown in Figure 43). The protein content was almost the double in the second trial than in the first trial (better extraction in the second trial), so a positive influence of the protein content on fiber formation was expected. Regarding the heat treatment, that step did not seem to have any influence on the fibers yield (as shown in Figure 43), in contrast to what was verified in P1 fiber trials (see section 3.4.1, part a).

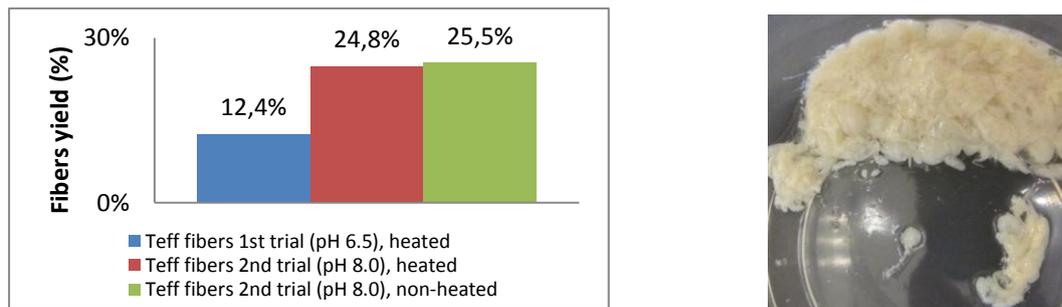


Figure 43 - Comparison between heated and unheated fibers, in relation to the fibers yield (left). Picture from the heated P2 fibers formed from the extract at pH 8.0 (right).

b) Influence of the presence of ethanol on the formation of fibers

This was the first and only trial in the presence of ethanol. The aim of this trial was to verify whether or not there would be P2 fiber formation in the presence of ethanol, and if the intense alcoholic flavor that ethanol confers to the extract could be lost in the end.

There was no fiber formation in the case of the unheated P2 solution with ethanol. One hypothesis for this occurrence could be that the pH was too low for the fiber formation, or the pH might have been

incorrectly measured because ethanol can influence the pH electrode. However, since in this trial there was more polysaccharide available (less protein concentration than in the previous trials, but the ratio between protein and polysaccharide was maintained), more fiber formation than in the previous trials (section a) was expected. Another hypothesis is that there was no fiber formation in this case because there was no heating or, in other words, that the concentration of ethanol is the limiting factor. When the solution is heated, there is evaporation of some ethanol, and then fiber formation occurs. However, the most probable hypothesis is that the prolamins (which are the proteins extracted with ethanol, as explained in section 3.1.1) are not able to form fibers.

The results suggest a negative influence of ethanol in the fiber formation (see Figure 44), since the yield was significantly reduced. Without heating, there was no fiber formation.

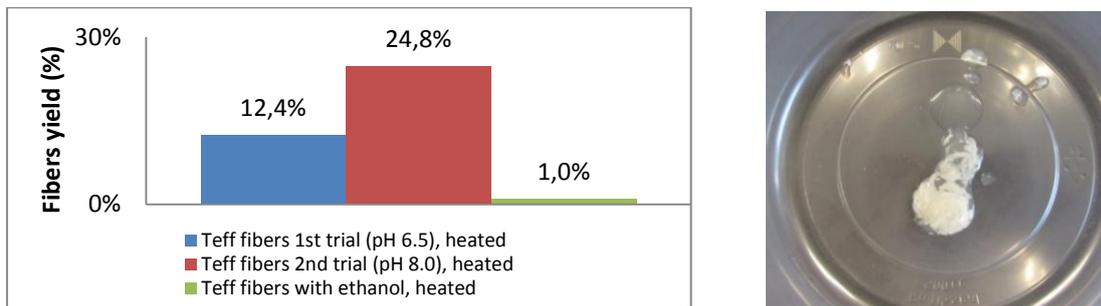


Figure 44 - Comparison between the heated P2 fibers with and without ethanol, in relation to the fibers yield (left). Picture from the heated P2 fibers formed from the extract with ethanol (right).

c) Influence of the protein concentration on the formation of fibers

The objective of the execution of this trial was to verify if there was more fiber formation when the protein concentration was higher (about 1% protein instead of 0.3 or 0.5% of the 1st and 2nd trials, respectively - see section 3.1.1). Also, some ingredients were added prior to the fiber formation (Ing.1 and salt), as it had been done in the case of P1 fibers. A comparison of P2 fibers (teff fibers) with added ingredients to similarly produced P1 fibers was performed.

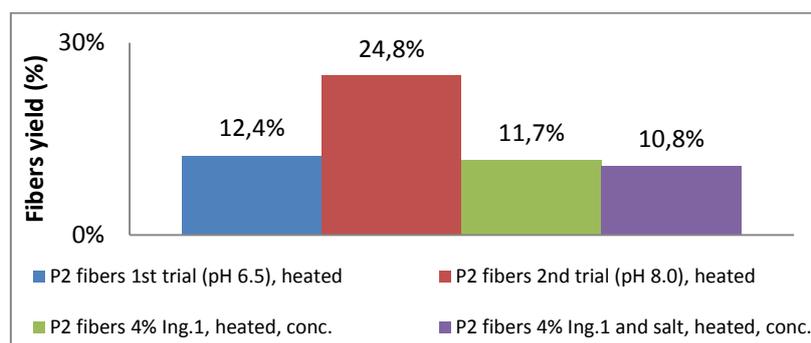


Figure 45 - Comparison between the heated P2 fibers with and without concentration (1% protein concentration, instead of 0.3% or 0.5%, in the 1st and 2nd trials, respectively), in relation to fibers yield.

The lower stirring speed in the present trial in relation to the second trial may have influenced the drop in yield. Even though the yield was expected to increase when we increased the protein content in the initial solution of the fibers, the yield of fiber formation was reduced when Ing.1 was added, and even

slightly more reduced when salt was also added (as shown in Figure 45). In fact, the yields became similar to that of the first trial.

The addition of ingredients was only attempted in the concentrated fibers, so we do not have a suitable control of P2 concentrated fibers to compare these results with. Nevertheless, the yield reductions in this trial were also attributed to the addition of ingredients. The salt addition to P2 fibers was half of the salt addition performed in the P1 fiber trials. Low salt concentrations, depending on the type of salt, usually help in the formation of complexes [66], but the contrary was verified in our results. No explanation was found for that observation.

d) Overall conclusions for P2 fibers production:

P2 fibers were also observed using light and contrast microscopes. The conclusions from these observations are resumed in Table 18. From these fiber trials, it was concluded that P2 is not suitable for fiber making in the tested conditions. The exception was the simple P2 fibers (from the initial fibers trial, section a), which although not very cohesive and watery, had a more neutral taste (see Table 18).

Table 18 - Resume of the most relevant conclusions from the analysis of the initial trials results.

Trial	Color	Smell	Taste	Fibers formation	Light and contrast microscopy
1 P2 fibers (2nd trial)	White	Acidic	Neutral	Yes	Elongated, small but cohesive fibers: 5 µm diameter
2 P2 fibers with ethanol	White	Alcohol	Alcohol	Just with heating	Non-cohesive and jelly fibers, thicker than those of 2 nd trial: 100 µm diameter
3 P2 fibers + Heating at 80°C for 30 min	White	Neutral	Neutral	Yes	Elongated, small but cohesive fibers: 5 µm diameter
4 P2 fibers + No heating	White	Neutral	Neutral	Yes	Fine fibers, not well-aligned: 40 µm diameter

Table 19 - Resume of the most relevant conclusions in the informal tasting session of P2 fibers with added ingredients.

Sample	Color	Smell	Taste	Mouth feel
Fibers with Ing.1	Not attractive	Very acid and bitter	Very acid and bitter	Not good Prickling
	Brown		Oxidation taste Burned bread	
Fibers with Ing.1 and salt	Similar color	Less acid and bitter than the previous sample	Less intense than the previous sample	Better

The overall conclusions of all P2 fiber trials are summarized in Table 18. From an informal tasting session performed with three individuals, but only to the P2 fibers with added ingredients, the conclusions from Table 19 were assessed.

A significant amount of acidity and bitterness was detected in P2 fibers with added ingredients. The results point to the conclusion that it is possible to form fibers from teff (P2) extracts, but optimization is needed.

3.4.2.1. CLSM images

The CLSM images from P2 fibers were obtained by staining with 0.2% Rhodamin-B in water. At orange, the protein present in the samples can be visualized. All images were obtained at a 10 μm depth into the sample.

The CLSM pictures shown in Figure 46 indicate that P2 fibers are much thinner than P1 fibers. The fibers from the second trial (middle picture) are longer than the ones from the first trial, and had a better alignment. The second extraction was more effective, like previously referred, so it is possible that the high protein concentration in the second trial might explain these differences in the alignment of fibers. Moreover, when the unheated fibers were compared with the heated ones (image not displayed in the report), these were also thin but not really as well-aligned as in the heated trial.

Regarding the fibers formed in the presence of ethanol, these fibers seemed thicker than P2 fibers from the other trials. Visualization with the CLSM was difficult, possibly due to the presence of ethanol in the sample.

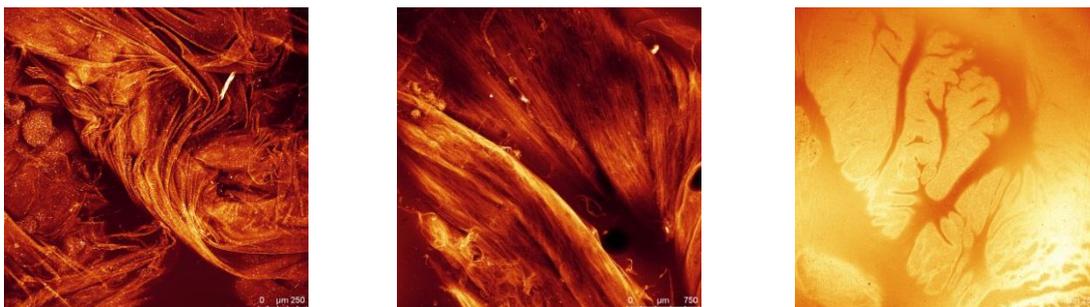


Figure 46 - CLSM images of heated P2 fibers from the first trial (left), from the second trial (middle), and image of the heated P2 fibers formed in the presence of ethanol (right). Settings: Objective 20x, zoom 2.0.

3.4.3. Comparison

In this section, P2 fibers were compared to P1 fibers. The influence of the heating step, ingredients addition and method of production were investigated.

a) Influence of heat treatment

In each type of fibers, the heat treatment did not seem to have had much influence on the yield of fiber formation. However, as shown in Figure 47, the results indicate a significantly higher yield in the case of P2 fiber formation, when compared to that of P1 fibers. It is known that the yield of fiber formation is dependent on the protein used in the procedure, and these results seem to illustrate that dependency. Moreover, having in account that the protein content inside the P2 fibers with 4% Ing.1 (5.3% w/w of protein) is much lower than the protein content of the P1 fibers with 6% Ing.1 (13.1% w/w of protein), the higher yield on P2 fiber formation (when compared to P1 fiber formation) may not turn P2 into the preferable fibers. P2 fibers might have a lot of water enclosed inside, and that would explain their softness and moistness.

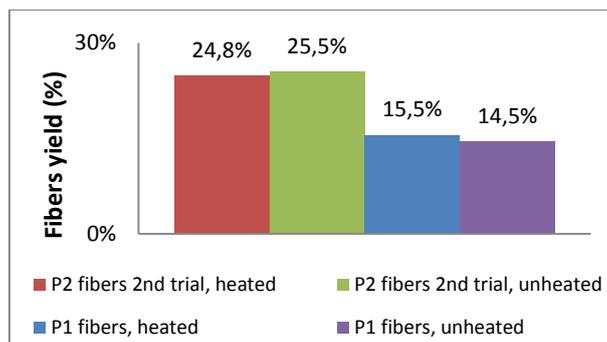


Figure 47 - Comparison between the P1 and P2 fibers with and without heat treatment, in terms of fibers yield.

b) Influence of Ing.1 and salt content

The amount of salt added to P2 fibers was half of that added in the P1 fibers formation. The amount of Ing.1 was 4%, which was the value that yielded the best results in P1 fibers formation.

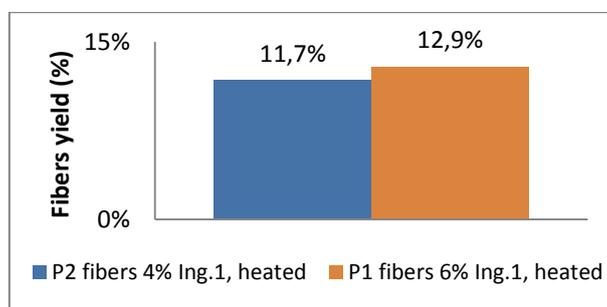


Figure 48 - Comparison between the heated P1 and P2 fibers with Ing.1 addition, in relation to the fibers yield.

As referred previously (part g of section 3.4.1), the optimum Ing.1 content in P1 fibers formation was 4%. Therefore, the influence of the addition of 4% Ing.1 before the formation of P2 fibers was investigated. There was not a significant difference between the yields of fiber formation of the two types of fibers (see Figure 48), even taken as a reference the result for P1 fibers with 6% Ing.1 (which had shown poorer yield on P1 experiments). Moreover, the yield of P1 fiber formation was substantially reduced in relation to that of the P2 fibers without ingredient addition (Figure 47), which led to the conclusion that the Ing.1 addition negatively influenced the fiber formation, especially for P2.

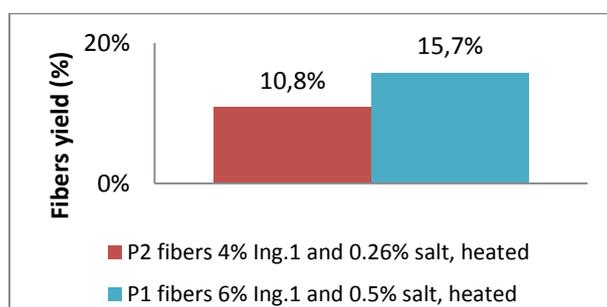


Figure 49 - Comparison between the heated P1 and P2 fibers with Ing.1 and salt addition, in terms of fibers yield.

Analyzing Figure 49, it can be concluded that the addition of ingredients to the P2 fiber procedure negatively influences the yield of fiber formation, when compared to P1 fibers. The results show that the addition of salt reduced the yield, even more than it had been reduced when Ing.1 was added.

c) Influence of the stirring speed and method of acid addition

The results show similarities between the heated P2 fibers with 4% (w/w) Ing.1 and the heated P1 fibers with 6% (w/w) Ing.1 at 175 rpm (see Table 20). The use of magnetic stirring instead of overhead stirring may have resulted in a reducing yield. Magnetic stirring grinds the fibers, making them smaller. The best yield of fiber formation (see Table 20) corresponded to the best fibers obtained in all fiber trials: P1 fibers with 4% (w/w) Ing.1 addition, unheated and without other ingredients added (yield of fiber formation of 23.1%).

Table 20 - Stirring speed, speed of acid addition and fibers yield of different types of fibers produced, from P1 and P2. The cells marked with asterisk correspond to fibers produced while magnetically stirring, instead of using overhead stirrers.

	Type of fiber	Stirring speed (rpm)	Speed of acid addition (mL/min)	Fibers yield (%)
P1 Fibers	4% Ing.1, unheated	175	Low	23,1
	6% Ing.1, heated	175	Low	12,9
	6% Ing.1, unheated	175	Low	19,4
	6% Ing.1, 0.5% salt, heated	175	Low	15,7
	6% Ing.1, heated	250	High	20,6
P2 Fibers	4% Ing.1, heated	200*	Low	11,7
	4% Ing.1, 0.26% salt, heated	200*	Low	10,8

4. Conclusions

Through optimization of the extraction of teff proteins from the flour, it was concluded that the best procedures were the ones using RO-water at RT or at higher temperatures. The main conclusion from the extraction trials was that it is very difficult to extract protein from teff, so one idea could be to use teff flour as such in product formulation, which would imply less additional costs than performing this low yield-extraction from teff.

Regarding the milk development, a milk drink was developed from teff flour, with a fresh and nice taste. In future studies, the addition of ingredients should be adjusted, in order to obtain a desirable salt-sugar balance. Another very important conclusion from the milk development studies was that dialysis was proven to be essential in the development of a satisfactory product from teff flour. In fact, this step was proven crucial in the elimination of off-flavors from the teff milk obtained. Furthermore, the unheated milk products were considered better than the heated products, in terms of taste, so, the heat treatment conditions should also be optimized, either by changing the time or the temperature of the process.

Regarding the fiber formation from vegetable protein extracts, the best fibers from the tested conditions were the P1 fibers with 4% (w/w) Ing.1 addition, unheated and without other additives (yield of fiber formation of 23.1%). Moreover, we concluded that fiber formation from teff (called P2 in the fiber section) is possible, but it requires further improvement. The low amount of fibers obtained from teff is connected to the low protein concentration in the teff extracts. Moreover, we can affirm that several aspects can influence fiber formation: overall protein concentration, presence of additional ingredients in the starting solution of the fibers, purity of the protein, presence of insoluble parts and even molecular properties of each protein.

It has been shown, through detailed characterization of the functional properties, that the teff flour used possesses high water and oil absorption capacities. This flour could therefore be potentially useful in flavor retention, improvement of palatability and extension of shelf life in meat products [34].

Furthermore, the conclusion from the foaming trials performed in this project was that the water-soluble proteins from teff have high foaming capacity and especially stability, which can be further studied and extremely useful in product development. Therefore, teff proteins might be used as whipping agents.

Recommendations

As far as the extraction is concerned, using ultrafiltration in the teff extraction to recover protein should increase solubility, since it has been suggested that changes in the protein during precipitation lead to a decrease in solubility.

Moreover, in addition to the study of teff protein solubility in water that was done in this project, an IP determination using the Zetasizer or isoelectric electrophoresis could be performed in further studies. For this, it might be necessary to purify teff proteins, to reduce possible interferences.

Regarding the fibers, one could investigate the influence of different end pH values in the fiber formation from P2. One could also try to add CL and verify whether or not that addition can mask the majority of acidity and bitterness of P2 fibers.

Regarding texture analysis of gelled teff suspensions, further texture measurements could be performed, at a higher scale and with a higher protein concentration. Having higher amount of sample available (preferentially after a concentration and/or a freeze-drying procedure) would permit the performance of a reliable puncture or compression test, and that could provide the user with more than a rough trend through which the samples could be compared. Furthermore, the experimental conditions of texture analysis could be optimized: both the instrumental settings and the conditions of gel formation.

Regarding the acid-induced gelation (with GDL), in this report, we only studied the influence of GDL concentration in the formation of gels. Another interesting study would be to measure the pH change during acidification, and to assess the influence of the concentration of GDL in that pH change. Moreover, the effect of protein concentration in gel formation could be studied. In addition to the suggested studies, the effect of heating the samples for 30 minutes at 80°C before inducing gelation could also be tested and analyzed through CLSM and texture analysis.

Finally, further studies could also be performed to compare the nutritional and functional properties of teff with those of other gluten-free grains, such as amaranth, sorghum and buckwheat. Since the prevalence of celiac disease has been increasing around the World, there is a major need for the development of alternative GF products to diversify the diet of celiac patients. However, the higher price of these products might constitute a problem, so further research is needed to reduce this economic problem [3].

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6. Appendix

6.1. Appendix I – Milk development pictures

The present appendix contains pictures taken at various stages in the teff milk development procedure (see section 2.4).



Figure 50 - Suspension obtained after mixture with the Ultra-Turrax (left) and after pH adjustment (right).



Figure 51 - Supernatant and precipitate obtained after centrifugation.



Figure 52 - Picture taken to the dialysis membranes after 24h of dialysis against water (left) and against salt and water (right).



Figure 53 - Suspension obtained after concentration of the sample dialyzed with water (left) and the sample dialyzed with salt and water (right).



Figure 54 - Product obtained after homogenization of the sample dialyzed with water (left) and with salt and water (right).

6.2. Appendix II – Texture analysis results

Table 21 - Designation given in graphs from Figures 55 and 56 to each sample analyzed, and respective content.

Sample	Content (protein concentration, in %)
A	0.5% Freeze-dried product of Condition 1
B1	1.0% Freeze-dried product of Condition 1
B2	1.8% Freeze-dried product of Condition 1
D	0.5% Freeze-dried product of Condition 2
E	1.8% Freeze-dried product of Condition 2
F	2.3% Freeze-dried product of Condition 2
G	2.7% Freeze-dried product of Condition 2
H	1% Teff flour
I	3% Teff flour
J	2% SPI
K	8% SPI

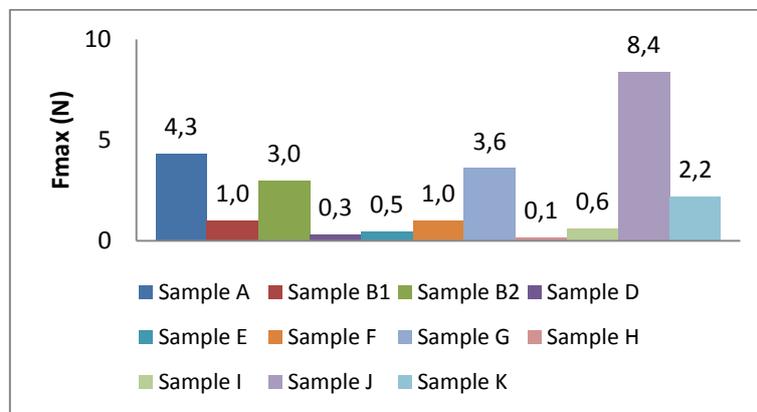


Figure 55 - Maximum force (N) attained in each sample submitted to texture analysis (Table 21).

The results of Figure 1 show a higher Fmax (maximum force) to the samples J, A, G, B2 and K. In the second condition, the sample hardness seems to increase with the protein concentration (samples D to G), and the same to the flour samples (H and I). The remainder results do not have visible trends.

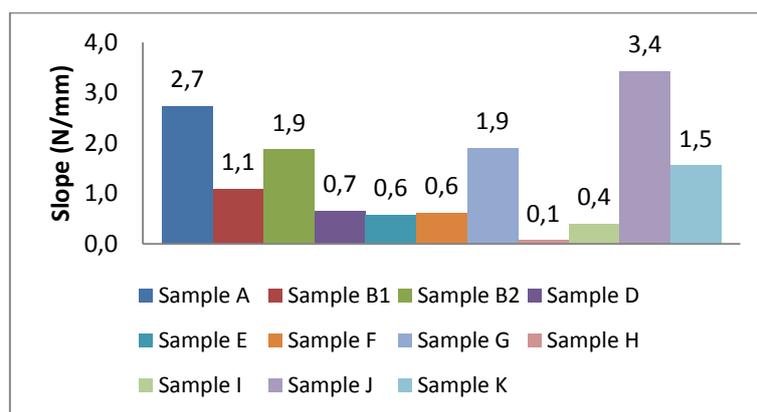


Figure 56 - Slope (N/mm) calculated for each sample submitted to texture analysis (Table 21).

The results show a higher slope to the samples J, A, G, B2 and K (as it was verified to Fmax). The same trends referred above are verified for the slope.

Table 22 - Designation given in graphs from Figures 57 and 58 to each sample analyzed, and respective content.

Sample	Content (protein concentration, in %)
X	1.7% Freeze-dried product of Condition 2
Y	2.0% Freeze-dried product of Condition 2
H	1% Teff flour
I	3% Teff flour
J	2% SPI
K	8% SPI
L	12% SPI

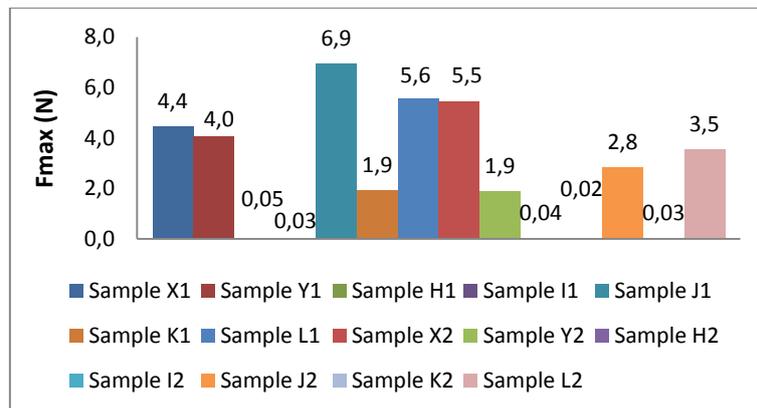


Figure 57 - Maximum force (N) attained in each sample submitted to texture analysis (Table 22).

The results show a higher Fmax to the samples J1, L1, X2 and X1. The difference amongst samples coded with 1 and 2 is the trigger force (0.01 N for the first case and 0.1 N for the second). In the freeze-dried samples, the hardness seems to decrease with the protein concentration (samples X and Y), and the same to the flour samples (H and I). The remainder results do not have explainable trends. If we ignore sample K, the SPI samples hardness seems to decrease with the protein concentration.

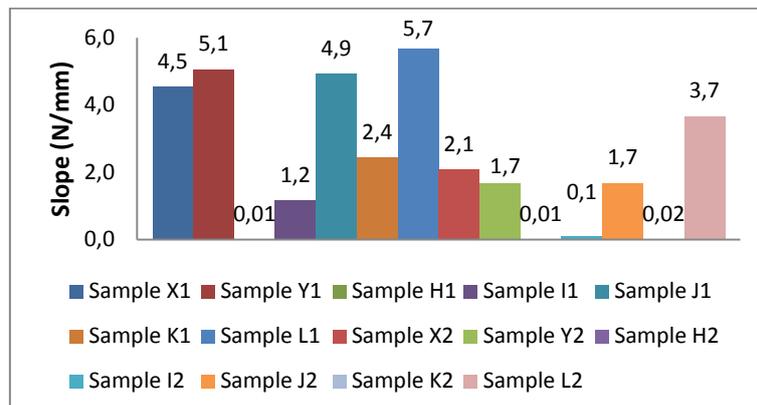


Figure 58 - Slope (N/mm) calculated for each sample submitted to texture analysis (Table 22).

The results show a higher slope to the samples L1, Y1, J1 and X1 (almost like has happened with Fmax). In the teff flour, the sample firmness seems to increase with the protein concentration (samples H and I). The remainder results do not have explainable trends. If we ignore sample K, the SPI samples firmness seems to increase with the protein concentration (inverse trend when compared to Fmax).

Table 23 - Designation given in graphs from Figures 59 and 60 to each sample analyzed, and respective content.

Sample	Content (protein concentration, in %)
1	5% Freeze-dried product of Condition 2
2	1% Teff Flour
3	5% Teff Flour
4	1% SPI
5	5% SPI
6	10% SPI

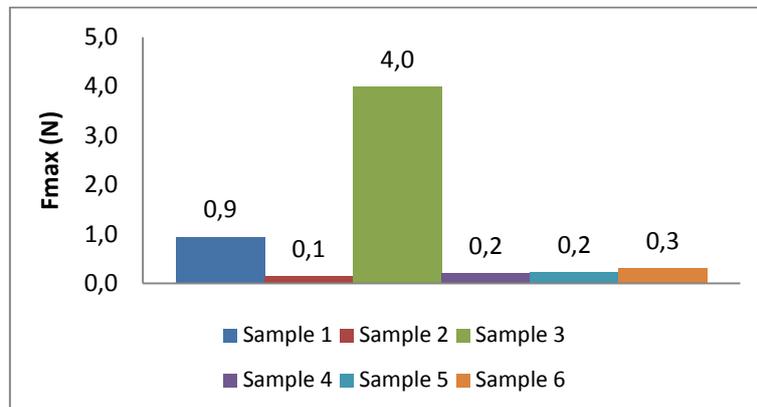


Figure 59 - Maximum force (N) attained in each sample submitted to texture analysis (Table 23).

The results show a higher Fmax to the samples 1 and 3, which are samples from the 5% teff extract and 5% teff flour, respectively. The SPI controls did not show a high hardness, but it increased with the protein concentration. No more trends can be explained.

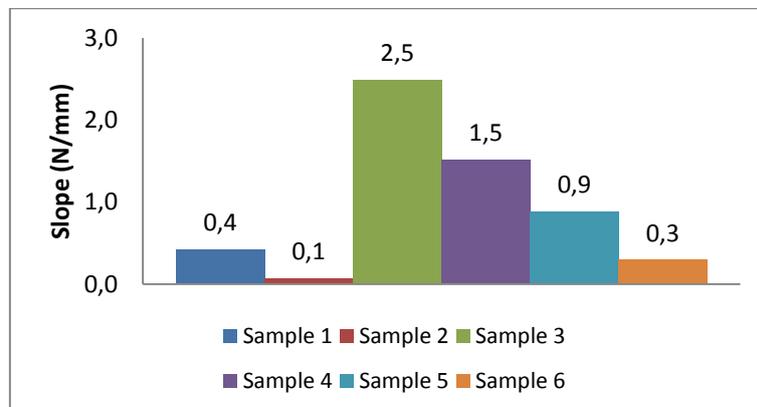


Figure 60 - Slope (N/mm) calculated for each sample submitted to texture analysis (Table 23).

The results show a higher slope to the samples 3 and 4, which are samples from the 5% teff flour and 1% SPI, respectively. For the SPI controls, the sample firmness seems to have decreased with protein concentration. No more trends can be explained.

6.3. Appendix III – Nitrogen-protein conversion factors

This appendix consists of an extract of a table from the Bulletin of the International Dairy Federation [87], which contains total nitrogen to protein conversion factors. The conversion factor used in this project for teff was 5.71 (soy flour and products).

Table 24 - Extract of a table from the Bulletin of the International Dairy Federation, which represents nitrogen-protein conversion factors (present in the literature) of currently available samples of vegetable protein sources.

Product Name/Class	Nitrogen Conversion Factor (NCF)	% N in Protein	Ref No.	References : scientific publication, analytical data, legislation
Soy Flour	5.71	17.51	9	FAO/WHO (1970) FAO Nutritional Study 24, Rome
Soy Products	5.71	17.51	9	FAO/WHO (1970) FAO Nutritional Study 24, Rome
Brazil Nut (<i>Bertholletia excelsa</i>)	6.97***	14.35	12	Chunhieng, T., Goli, T., Piombo, G., Pioch, D., Brochier, J., and Montet, D. (2004) Bois Forets Trop. 280, 91-98
Casava Root (<i>Manihot esculenta</i>)	4.75-5.87***	21.05-17.24	13	Yeoh, H., and Truong, V. (1996) J. Sci. Food Agric. 70, 51-54
Corn (<i>Zea mays</i>)	5.77***	17.33	14	Baudet, J., Huet, J. C., and Mosse, J. (1986) J. Agric. Food Chem. 34, 365-370
Corn (<i>Zea mays</i>)	5.89***	16.98	15	Notheisz, K. (1983) Cereal Res. Comm.11, 57-58
Corn (<i>Zea mays</i>)	6.14***	16.29	15	Notheisz, K. (1983) Cereal Res. Comm.11, 57-58
Corn (<i>Zea mays</i>)	6.39***	15.65	15	Notheisz, K. (1983) Cereal Res. Comm.11, 57-58
Corn (<i>Zea mays</i>)	6.28***	15.92	15	Notheisz, K. (1983) Cereal Res. Comm.11, 57-58
Mushrooms (<i>Basidiomycetes</i>)	4.70***	21.28	16	Mattila, P., Salo Vaananen, P., Konko, K., Aro, H., and Jalava, T. (2002) J. Agric. Food Chem. 50, 6419-6422
Pea (<i>Pisium sativium</i>)	5.52***	18.12	17	Holt, N. W., and Sosulski, F. W. (1979) Can J of Plant Sci. 59, 653-660
Peanut (<i>Arachis hypogaea</i>)	5.46***	18.31	18	Misra, J. B. (2001) Peanut Sci. 28, 48-51

6.4. Appendix IV – Protease activity assay

The method followed to perform the protease activity assay was the one described in the Pierce Protease Assay Kit, from Thermo-Scientific (Product 23263). The proteolytic activity was expressed as trypsin equivalents ($\mu\text{g/mL}$ trypsin).

Results

Table 25 - Proteolytic activity expressed as trypsin equivalent ($\mu\text{g/mL}$ trypsin), for each sample type, at each concentration and time of incubation tested.

Sample type	Sample concentration (%)	Proteolytic activity for 20 min incubation ($\mu\text{g/mL}$ trypsin)	Proteolytic activity for 1h incubation ($\mu\text{g/mL}$ trypsin)	Proteolytic activity for 2h incubation ($\mu\text{g/mL}$ trypsin)	Proteolytic activity for 4h incubation ($\mu\text{g/mL}$ trypsin)
Pullulanase	0.01	0.08	0.05	0.07	0.07
	0.1	0.09	0.06	0.07	0.07
Alpha-amylase	0.02	12.30	112.34	>500	>500
	0.2	>500	>500	>500	>500

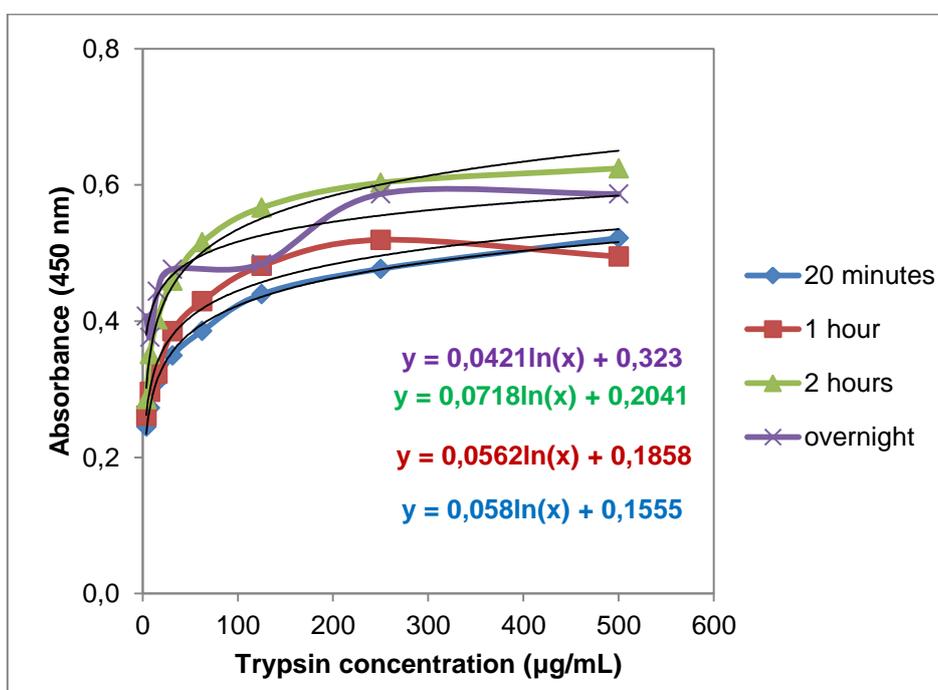


Figure 61 – Calibration curves (absorbance at 450 nm versus trypsin concentration in $\mu\text{g/mL}$) for each incubation time tested. A logarithmic adjustment was performed to each curve, and the resultant equations are displayed in the graph.