Functionality-driven fractionation
the need for mild food processing

Marlies E.J. Geerts

Thesis committee

Promotors

Prof. Dr A. van der Padt

Special professor Sustainable Production of Food

Wageningen University & Research

Prof. Dr A.J. van der Goot

Personal chair at Food Process Engineering

Wageningen University & Research

Other members

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Prof. Dr M.A.J.S. van Boekel, Wageningen University & Research

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Functionality-driven fractionation
the need for mild food processing

Marlies E.J. Geerts

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General introduction

1.1. Introduction

The food industry transforms raw materials by physical and/or chemical means into pre-prepared food products. In many cases, a two-step approach is used in which raw materials are first transformed into ingredients, which are then reassembled into final products (Figure 1.1). In the Netherlands, about 70% of the food products available in supermarkets are pre-prepared, processed food products, according to the NOVA food classification system (FoodWatch 2017). These pre-prepared food products are cheap, abundantly available, and convenient for consumers. In addition, pre-prepared food products make transportation and distribution easier.

The ingredients for making pre-prepared food products can be roughly divided into traditional and refined ingredients. Traditional food ingredients, such as flour, eggs and cream, do not have a high component purity and are often multi-functional. For instance, an egg can be used as an emulsifier, a thickening agent, a raising agent and a glazing agent. Novel food ingredients are mostly highly refined with generalized specific functionalities. Refined ingredients, such as starch isolate, protein concentrates, etc., often serve one specific targeted functionality. For instance, protein isolates are added for their emulsification properties and starch for their thickening properties.

The use of refined ingredients has advantages. Refined ingredients are often standardized to meet general specifications. The main advantage of standardization is that natural variation is reduced, which allows global sourcing and easy handling (Ibarz and Barbosa-Canovas 2002). This is important because the quality of well-known branded products must be standardized worldwide. Overall, this development of standardized ingredients has resulted in global sourcing, lower food prices and a larger range of products (Fellows 2009). However, it has also led to an industrial process chain in which the refining of ingredients (pre-processing) and the manufacture of the final consumer product have become disconnected (Boye and Arcand 2013) (Figure 1.1). The quality of refined ingredients is mainly assessed on their purity.

To obtain purity, crops are often fractionated to extract their main component (van der Velde-Koerts et al. 2003), e.g. oil, sugars, starch and protein. Other components are considered as side streams and as a result of the fractionation methods applied, they are often of lower quality or should be considered as waste (Raak et al. 2017). During product assembly, ingredients are combined to create the final product with the appropriate structure. This current setup creates inefficiencies in the food chain (Augustin et al. 2016; van der Goot et al. 2016).

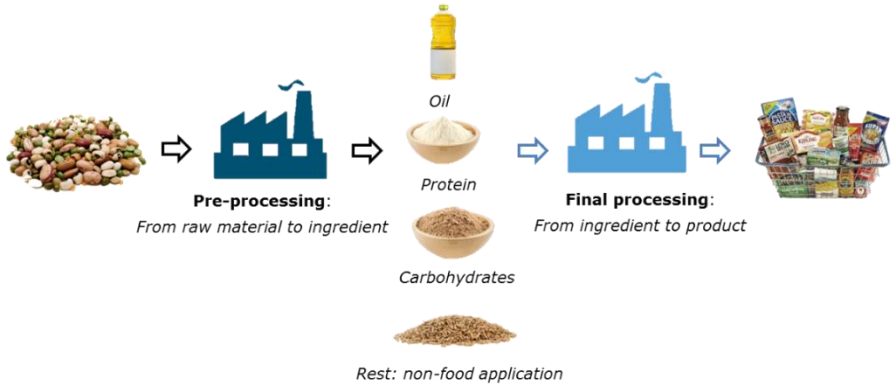

Figure 1.1 The food processing chain from raw material to ingredients and subsequently to final product

Fractionation of plant material into highly refined ingredients requires large amounts of water and chemicals (e.g. solvents, acids, bases) to remove some components (such as oil, whey proteins, sugars, fibres, etc.) and to precipitate and neutralize the desired components (Gueguen 1983; Schutyser et al. 2015). Subsequently, water must be removed to stabilize the components via a drying, which is an energy-intensive process (Dincer 2011; Motevali et al. 2011). In addition, the focus on target components results in the production of by-products (Raak et al. 2017) of a lower quality such that they are not or only partly suitable for human consumption, despite the fact that the initial starting material was fully suitable for human consumption. This explains the efforts to improve the use of by-products by giving them added value. However, as Augustin et al. (2016) indicated, many food processes are sub-optimal, especially when they are energy intensive and do not make optimal use of the components in the agro material that are suitable for human consumption. Subsequently, the question arises if these drawbacks can be tackled from a process engineering perspective.

The consumer perspective is changing towards products perceived as “natural” or “green”. Although refined ingredients allow the production of many cost-effective food products of good quality, these food products are less appreciated by modern consumers, because food production on an industrial scale using additives and well-defined ingredients contradicts feelings of naturalness. In addition, pre-prepared food products, consisting of highly refined ingredients, often have a high energy density. Overall, consumers are moving away from food products with highly refined ingredients, reflected in the demand for “natural products”, often associated with minimal processing and clean labelling, implying limited use of chemicals (Evans et al. 2010; Dickson-Spillmann et al. 2011; Verhoog et al.

2003; Saltmarsh 2015). Bloggers and vloggers are encouraging this trend by posting outspoken views on food and expressing opinions on the healthiness of products and their impact on the environment. This strengthens the need for more “natural” products, because natural is associated with pure and is even considered to be environmentally friendlier, healthier and tastier (Rozin 2005; Rozin et al. 2004). However, to successfully feed the increasing world population with sufficient food of high quality, a more industrialized approach seems unavoidable (Cassidy et al. 2013). The question then is how those industrially produced products can still be produced in a natural way, thereby following the wishes of modern consumers.

1.2. Functionality as the driver during fractionation

The focus on highly refined ingredients has its strengths and opportunities, as indicated in Figure 1.2. Highly refined ingredients are convenient, consistent and allow easy handling. On the other hand, the main negatives of refined ingredients are that processing has a substantial impact on the resourch use efficiency of these ingredients and consumers are again averse to “non-natural ingredients” (Apaiah et al. 2006).

It is therefore not suprising that novel concepts for improving the sustainability of foods point towards limited processing and the use of mildly refined ingredients (van der Goot et al. 2016). This concept also allows milder fractionation of components instead of purification with harsh processing conditions. However, as described in this thesis, this shift implies that the functionality of ingredients should become more important than purity. Agro materials should be fractionated into into a range of valuable fractions, such that the creation of low-value by-products is prevented.

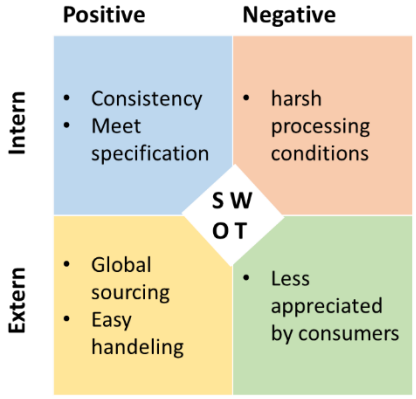

Figure 1.2 SWOT analysis for the highly-refined ingredients

Previous studies indicate that mild fractionation can lead to similar functional properties. In the case of lupine seeds, the functional properties of a lupine protein isolate from aqueous fractionation were not substantially different from those of a conventional lupine protein isolate (Berghout et al. 2014). Papalamprou et al. (2009) and Fuhrmeister and Meuser (2003) showed that the functional properties of a pea protein isolate was enhanced when milder conditions were used. As an example, ultrafiltration improved the solubility, emulsification, and gelation properties of the protein fractions obtained. Furthermore, several starch- and oil-rich legumes showed potential for milder fractionation (de Moura et al. 2011; Pelgrom et al. 2015; Cai et al. 2001). A further step in the fractionation process is to consider the natural structure. Tamayo demonstrated the excellent emulsification properties of thylakoids extracted from sugar beet leaves (Tenorio 2017).

There is increasing interest in the literature in functional fractionation and a function-driven fractionation approach. Not only processing concepts are explored (Berghout 2015; Pelgrom 2015) but also relevant aspects such as interactions between different macro-molecules have been described in recent studies (e.g. protein-polysaccharide blends (Scholten et al. 2014; Van De Velde et al. 2015; Schmitt and Turgeon 2011), starch-fibre blends (Yildiz et al. 2013; Parra et al. 2015; Lai et al. 2011)). These blends are currently created by blending highly refined ingredients, leading to illogical combinations. For instance, Sun et al. (2015) blended highly refined wheat starch and fibres in different ratios. Understanding these interactions is important not only to improve the fractionation of raw materials but also to help explore possible synergistic effects with respect to the functional properties of partly fractionated ingredients. Those blends could probably also be obtained using a function-driven fractionation approach by removing the components of no interest.

1.3. Aim and outline of this thesis

Fractionation processes should be optimized to make full use the raw material, thereby optimizing the amount of natural resources needed. In this thesis, a function-driven fractionation approach is developed to arrive at functional ingredients at best resource efficiency (Figure 1.3). Aqueous fractionation is used as a route to extract starch, proteins and fibres from yellow pea (*Pisum sativum*) and protein from soy beans (*Glycine max*). The functional properties of the enriched fractions obtained were investigated. In addition, applicability was explored in model systems consisting of a thickened oil-in-water emulsion and a fibrous structure for the use in meat analogues. Finally, the resource efficiency of the mildly refined fractions was evaluated using exergy analysis.

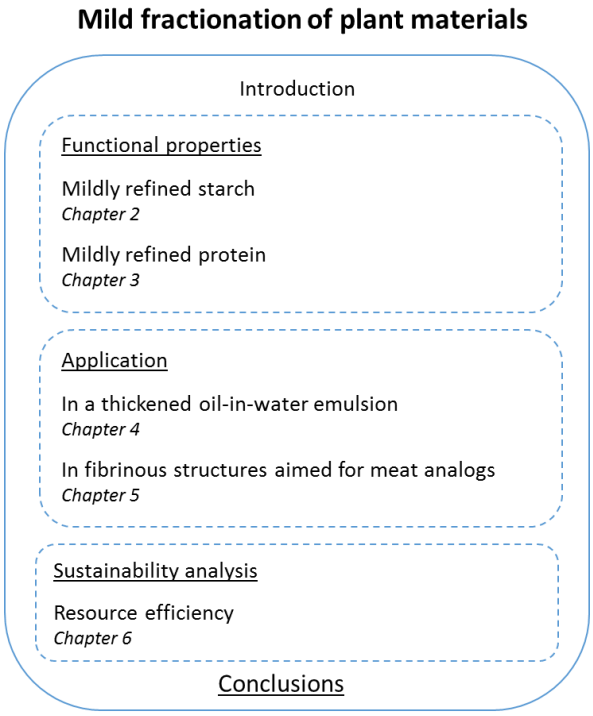

Figure 1.3 Conceptual outline of the thesis

Chapter 2 describes the functional properties of mildly refined starch fractions obtained from yellow pea flour and the influence of other components (mainly fibres). The functional properties investigated include pasting, gel hardness and syneresis. The mildly refined pea starch fraction is compared with commercially available pea starch and a blend of commercially available pea starch and fibres.

Chapter 3 unravels the emulsification properties of a mildly refined protein-rich fraction from yellow pea and the contribution of purification. The emulsification properties of the protein-rich fraction from yellow pea are compared with commercially available pea protein and a dialysed protein-rich fraction from yellow pea. The stability of oil-in-water emulsions was determined at acidic pH, under acceleration forces and a freeze-thaw treatment. Interfacial properties were determined to allow better interpretation of the results.

Chapter 4 explores the applicability of mildly refined fractions from yellow pea (Chapters 2 and 3) in a thickened oil-in-water emulsion. The mildly refined fractions are compared with mixtures of commercially available pea starch and pea protein. The functional properties of a (thickened) oil-in-water emulsion were evaluated based on the stability of the emulsion and its rheological properties.

Chapter 5 explores the applicability of protein fractionation from soy beans for the formation of a fibrous structure for use in analogues. The protein fractions obtained were compared with commercially available soy protein concentrates and isolates. The following functional properties were determined: water holding capacity, nitrogen solubility index, enthalpy of transition, and viscoelastic properties. In addition, the protein fractions were structured with simple shear flow deformation while heating.

Chapter 6 investigates the influence that minimal and mild fractionation has on resource use efficiency. The aqueous fractionation process of yellow pea flour discussed in Chapters 2, 3 and 4 is used as example. The resource use efficiency was evaluated by determining the cumulative exergy consumption, cumulative exergy losses, and exergy efficiency. In addition, the impact of possible alternative scenarios and different ways of allocating exergy consumption on the interpretation of the exergy analysis is discussed.

Chapter 7 concludes the thesis with a general discussion on the main findings and conclusions. Opportunities and constraints of the functional fractionation approach are discussed and the outlook for future research in this area is provided.

2

Understanding functional properties of mildly refined starch fractions of yellow pea

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2.1. Abstract

A route towards the sustainable production of plant based ingredients is the use of milder conditions during fractionation and reduced consumption of chemicals. As a consequence, it becomes more difficult to obtain chemically pure ingredients, instead enriched fractions will be obtained. This paper describes the properties of mildly refined pea starch fractions in comparison to industrially produced and highly refined pea starch. The functional properties investigated are pasting, gel hardness and syneresis upon freezing. The pasting properties of the mildly refined fraction could be well described considering the water binding properties of the main ingredients, which were starch and fibers. The gel hardness was only slightly affected by the presence of fibers in the mildly refined fraction. The fibers mainly acted as a filler. In addition, the mildly refined starch fraction showed a lower syneresis upon freezing, which can be considered as an advantage. The mildly refined starch fraction, and the presence of fibers have slightly different properties compared to highly refined starch. Whether these small differences are advantageous depends on the functionality requested.

2.2. Introduction

The production of food products can be divided in two main parts, primary processing and secondary processing (Boye and Arcand 2013). Primary processing focusses on the conversion of raw materials into specific ingredients, while secondary processes use these ingredients to assemble food products. Nowadays the focus in primary processing is to produce broad applicability of the ingredients standardized functionality, which is obtained by making highly purified ingredients. Secondary processing has the focus on creating a desired (structural) functionality through blending and mixing these standardized ingredients.

The production of highly refined ingredients requires intensive processing (van der Goot et al. 2016). For instance, starch is primarily isolated using a wet milling method (Singh et al. 2014), including numbers of washing steps, thereby using a large amounts of water. Subsequently, part of the initial starch present ends up in side streams. Furthermore, the obtained starch material needs to be dried, requiring high amounts of energy (Hoover and Sosulski 1985). Many consumers perceive highly refined ingredients, and the difficult-to-pronounce names related to those ingredients, as not natural and even unhealthy (Varela and Fiszman 2013). This conception might be true to a certain extent, because refining reduces the dietary fiber and micronutrient content, which have been shown to have several health benefits (Kaczmarczyk et al. 2012; Gangwisch et al. 2015). Clearly, a need exists for a next generation fractionation processes, which have inherently a lower environmental impact through the use of milder conditions. However, those process conditions might lead to enrichment rather than complete purification. A step towards the development of those process is to understand the functional properties of those enriched fractions.

For most legumes, like yellow peas, it is known that a starch and protein rich fraction can be obtained through suspending flour in water and subsequently applying gravitational forces (Czuchajowska 1993; Cai et al. 2001; Pelgrom et al. 2015a). The obtained fraction is rich in starch, which means that, also other components are present. Pelgrom et al. (2015a) found that the mildly refined starch fraction of yellow peas contained approximately 21% fiber. However, it is arguable whether highly purified ingredients are always required taking into account that most foods consist of a mixture of ingredients and recent wished of consumers. It would be more logical to focus on obtaining fractions rather than purity and understanding of their properties based on composition and structure (van der Goot et al. 2016).

Starch is often used as thickener, stabilizer, viscosity builder, or gel former in food products (Ratnayake and Jackson 2008). The functionality of starch is therefore often described through several

characteristics e.g. pasting properties, the gel firmness and syneresis (Lee et al. 2002). The presence of non-starch polysaccharides affects these structural functionalities (BeMiller 2011). In literature, it is found that the pasting and rheological properties of several starch dispersions were significantly affected by the addition of apple pomace (Parra et al. 2015), rice dietary fiber (Lai et al. 2011), wheat fiber (Sun et al. 2015) and oat, pea, lemon and apple fiber (Yildiz et al. 2013a). The magnitude of this effect is related to the nature of the fibers and the extent of starch substitution (Collar et al. 2006). Starch mixed with commercially available pea fibers for instance, showed a lower tendency to retrogradate. (Yildiz et al. 2013a).

Current literature on the influence of non-starch polysaccharides on the structural functionality is mainly obtained through remixing (commercially) available starch and fibers, often from various sources (BeMiller 2011). However, an alternative approach is to obtain those blends of starch and fibers directly from one source using partial fractionation. Those fractions can result from mild fractionation. For that reason, the concept of mild fractionation is explored in this study using pea flour as starting material. The functionality of mildly refined starch fraction was compared to commercially available pea starch isolate, thereby investigating the influence of refining on the eventual structural functionalities desired.

2.3. Materials and methods

2.3.1. Materials

Pre-dried yellow peas (Pisum sativum), were purchased from Alimex (Sint Kruis, The Netherlands). Pea starch isolates (PEA STARCH N-735) and pea fiber isolates (PEA FIBER I50M) containing 50% fibers, were obtained from Roquette (Lestrem, France). For all experiments the water used was purified by passage through a Milli-Q system (Millipore Corporation, Billerica, Massachusetts, USA)

2.3.2. Methods

2.3.2.1. Pea flour production

Dried yellow peas were milled into grits using a pin mill (LV 15M, Condux-Werk, Wolfgang bei Hanau, Germany). The grits were further milled into pea flour with a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany), according to the method of Pelgrom et al. (2015a). The classifier wheel speed of the mill was set at 4000 rpm. The air flow was set at 52 m^3/h, the impact mill speed at 8000 rpm and

the feed rate at 2 rpm. A thermometer inside the mill was used to help controlling a temperature between 16 and 34^{0}C.

2.3.2.2. Mildly refined starch fractionation

The starch fraction was obtained through suspending the pea flour into water using a concentration of 10% (w/v). This suspension was stirred overnight at a temperature of 4^{0}C. A centrifugation step was applied to the dispersions, 1500g for 1 s, to obtain a suspension sediment (pellet) containing starch fraction and part of the fibers. The pellet obtained, containing the mildly refined starch fraction, was freeze dried (Pilot Dryer Epsilon 2-6D, Christ, Germany) before further usage.

2.3.2.3. Isolation of fiber fraction

To determine the specific functionalities of the fibers present in the mildly refined starch fraction as well as in the commercial fibers, the additional starch present was hydrolyzed using thermostable alpha-amylase (Termamyl® 120L, obtained from Novozymes, Bagsvaerd, Denmark). The mildly refined starch fraction and commercial fibers were suspended in water and to both fiber suspensions a surplus of thermostable alpha-amylase was added and incubated in an 80°C water bath for 2 h. Subsequently the solution was centrifuged at 10 000 g for 15 min to separate the soluble sugars from the insoluble fibers. The supernatant was decanted and the pellet containing the fibers was washed by re-suspending it in water and subsequent centrifugation at 10 000 g for 15 minutes. The pellet that was eventually formed was freeze-dried (Pilot Dryer Epsilon 2-6D, Christ, Germany). Freeze dried material was milled using a blade mixer and the residual starch was measured with a Total Starch Amyloglucosidase/a-Amylase Assay Kit (Megazyme International Ireland Ltd, Bray, Ireland) as well as visual inspection using a Phenom G2 Pure scanning electron microscope (Phenom world, Eindhoven).

2.3.2.4. Composition analysis

The protein concentration was determined using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, Interscience, Breda, The Netherlands), using a conversion factor of 5.52 (Holt and Sosulski 1979). The water concentration was determined by drying approximately 1 g of samples in an oven at 105°C overnight. The total starch concentration was determined using a Total Starch Amyloglucosidase/a-Amylase Assay Kit (Megazyme International Ireland Ltd, Bray, Ireland). Ash concentration was determined by AACC official method 08-01 (AACC, 1983). The oil content was determined with a fully automated Büchi extraction system B-811 LSV (Büchi Labortechnik AG, Flawil, Switzerland). Petroleum ether with a boiling range of 40-60°C was used in Standard Soxhlet mode with a sample-to-solvent ratio of 1:6 for 12 h.

2.3.2.5. Rapid Visco-Analysis

Starch dispersions with starch concentrations of 4, 6, 8, and 10 wt.% were subjected to an adapted version of the general pasting method (AACC Method 76-21) using the Rapid ViscoAnalyzer (RVA) (Newport Scientific Pvt. Ltd.,Warriewood, Australia). A measurement cup was filled with 28.5 g of sample, placed into the RVA and stirred at a speed of 960 rpm during the first 10 s to allow complete homogenization of the sample. Subsequently, the stirring speed was adjusted to 160 rpm during the rest of the experiment while using the following temperature profile: heating to 50°C for 60s, followed by heating to 95°C for 3 min and 42 s, held at 90°C for 2 min and 30s, cooled back to 50°C in 3 min and 42 s and finally held at 50°C for 2 min. Thermocline software (Newport Scientific Pvt. Ltd., Warriewood, Australia), was used for analysis of the results.

2.3.2.6. Water binding capacity

Water binding capacity (WBC) was determined for the three starch fractions as well as for the hydrolyzed mildly refined and commercial fibers fraction. 1.0g sample was added to 50 ml water and the suspensions were either heated to 90°C or non-heated, before equilibration overnight at room temperature. Subsequently, a centrifugation step at 3000g for 20 minutes was performed. The supernatant was discarded and tubes were drained for at least 30 minutes on tissue paper. Drained sample was transferred to aluminum trays, weighted and dried at 105°C for 3 days. Water binding capacity was determined by:

$$WBC = \frac{M_{pellet} - M_{dry}}{M_{dry}} * 100\% \quad (2.1)$$

Where M_{pellet} is the mass of the pellet obtained after the centrifugation step and $M_{dry}$is the mass of the pellet after the drying step.

2.3.2.7. Syneresis

5 wt.% starch dispersions were gelatinized using adapted version of the general pasting method, as described in section 2.3.2.5. Samples were stored at either 4°C or -25°C overnight, followed by thawing at 30 °C for 3 h in a water bath incubator. To determine the syneresis, samples were collected after day 1, 2, 3 and 4. The thawed samples were centrifuged at 8 000 g for 10 min and drained on tissue paper for at least 30 min. Syneresis was determined as percentage water separated ($M_{water\ loss}$) to the initial gel weight ($M_{initial\ gell}$), as indicated by the following formula:

$$\%\ Syneresis = \frac{M_{water\ loss}}{M_{initial\ gell}} * 100\% \quad (2.2)$$

2.3.2.8. Rheology

Heated starch suspension with 8 wt.% starch were obtained using the adapted version of the general pasting method, as described under section 2.3.2.5. The heated starch suspensions were poured into metal rings with an inner diameter of 25mm and a height of 10mm. The starch suspensions were cooled at room temperature for at least 2h and stored in a closed box to prevent water evaporation. When cooled down, an amplitude sweep was preformed to determine the linear viscoelastic region, using a controlled stress rheometer Physica MCR502 (Anton Paar, Graz, Austria), equipped with a parallel serrated plate with a diameter of 25mm (PP25) at 20°C. To ensure a uniform contact of the plate with the surface of the gels, all measurements were conducted at a fixed normal force of 0.2N (± 0.05). Subsequently, a frequency sweep was performed at a fixed strain of 0.5%, using a frequency range from 0.1Hz to 10Hz. Storage modulus (G') and loss modulus (G'') were determined using the Rheoplus 32 software, version 2.65 (Anton Paar GmbH). The tan delta (Tan δ) as function frequency was determined, through calculating the Tan δ at the individual frequencies points using the following formula.

$$Tan\ \delta = \frac{Loss\ modulus\ (G'')}{Storage\ modulus\ (G')} \quad (2.3)$$

2.3.2.9. Texture analysis

Gels with 8% (w/v) starch were prepared as described in section 2.3.2.8. Texture profile analysis and single compression tests were performed on the gels using the texture analyzer (Instron-5564 Series Table Model Systems Twin-column design, Canton, USA) equipped with a 100 N or 2000 load cell, at a compression speed of 1 mm/s was used. The texture profile analysis was performed through compressing the sample twice with a deformation of 35% of the unloaded gel height, the test was set to start at a trigger force of 5N. The single compression test was performed through compressing the samples with a deformation of 90% of the unloaded gel height, the compression plate was positioned manually, no trigger force was used. The force-time and the force-deformation curves were recorded with the Bluehill 2 Texture Profile Analysis software (Instron, Norwood, USA).

2.3.2.10. Statistical analyses

All experiments were performed in triplicate unless stated otherwise. A student's t-test was performed to evaluate difference between the samples, the P value was set on P≤0.05 to consider a significant difference.

2.4. Results

The properties of the mildly refined starch fraction were compared to commercial pea starch isolate and to a re-mixture of commercial yellow pea starch and fiber having the same starch/fiber ratio as present in the mildly refined starch fraction (recombined starch). The functional properties tested were pasting properties, syneresis during freezing and cooling, rheological and mechanical properties.

2.4.1. Composition and yield

Table 2.1 shows the composition of the initial pea flour before fractionation, the obtained mildly refined starch fraction and commercial pea starch isolate. The mildly refined starch fraction contains 70.2% starch respectively, whereas the initial pea flour contains 52.5% starch, the commercial starch reach values of 96.2% starch. The mildly refined starch fraction contains a significant amount of non-starch polysaccharides, whereas the protein concentration is substantially reduced compared to the initial pea flour.

The starch-yield of the mildly refined starch fraction is high, approximately 98% of all starch initially present is yielded in this fraction.

Table 2.1 Composition of the mildly refined starch fraction, initial pea flour and the commercial starch isolate to dry matter (DM) ± standard deviation (n = 3)

	Starch (g/ 100g DM)	Non-starch polysaccharide[s] (g/ 100g DM)	Protein (g/ 100g DM)	Oil (g/ 100g DM)	Ash (g/ 100g DM)
Mildly refined starch fraction	70.2±1.5	23.8±3.7	4.6±0.1	0.8±0.2	1.0±0.1
Initial pea flour	52.5±1.1[b]	18.4±1.4[b]	21.4±0.8[b]	2.1±0.3[b]	5.5±0.0[b]
Commercial isolate	96.2±0.8[b]	3.1±0.8[b]	ND	0.6±0.2[b]	0.1±0.0[b]

[a] determined by difference
[b] adapted from Pelgrom et al. (2015a)

2.4.2. Pasting properties

The pasting properties of the three starch fractions were studied through determining the shear viscosity of starch suspensions as function of a certain temperature profile. Figure 2.1 summarizes the results through plotting the peak viscosity at 95°C as function of starch concentration and dry matter concentration. To keep the starch concentration similar in all samples, the total dry matter had to be

adjusted and became larger for the mildly refined and recombined starch fraction due to the presence of the (added) fibers. A similar dry matter concentration implied that the mildly refined and recombined starch fraction contained less starch.

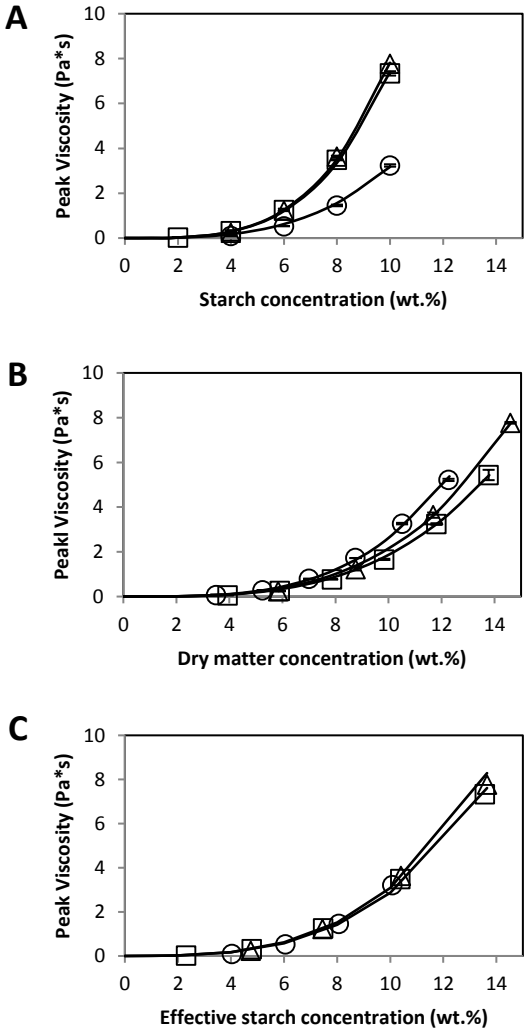


Figure 2.1 Peak viscosity of mild fractionated starch (□), commercial starch (○) and recombined starch (△) as function of starch concentration (A), dry matter concentration (B), effective starch concentration (C) inclusing standard error of the mean (n = 2)

Based on starch concentration (Figure 2.1A), the two fraction containing fibers gave a higher viscosity than the commercial starch isolate. In addition, the results of the mildly refined starch fraction and the recombined starch almost completely overlapped, indicating major similarities between the samples. When determining the peak viscosity as function of dry mater concentration (Figure 2.1B), all three sample show similar behavior. However, the commercial starch sample gave overall the highest viscosity. The curve of the two fraction containing fibers is similar as well, but with, slightly higher viscosity for the recombined starch fraction at high dry matter concentrations.

2.4.3. Gelation properties

The native starch suspensions formed a gel after heating and subsequent cooling. To determine the rheological properties of 8 wt. % starch gels were subjected to small deformation measurements in the form of a frequency sweep, as depicted in Figure 2.2A. In all cases, G' was found to be higher than G'', indicating that all gels behaved as a viscoelastic solid. The G' and G''–values for the samples containing additional fibers (mildly refined and recombined starch fraction) were higher than those of the commercial starch sample at similar starch concentration. In addition, the highest values for the tan delta (Tan δ) were as well found for the two samples containing fibers (Figure 2.2B).

Subsequently, large deformations were performed on the gel samples using a texture analyzer, Table 2.2. Two types of large deformation tests were performed. A non-destructive deformation of 35% of the initial height was used to determine the hardness and cohesiveness of the gels. Subsequently, a destructive deformation of 90% of the initial height was performed to determine the gel strength. Both the mildly refined and recombined starch gel show a slightly higher hardness value compared to the commercial starch gel at similar starch concentration. Subsequently, the gels formed by mildly refined and recombined starch fraction, showed slightly lower gel strength when based on starch concentration.

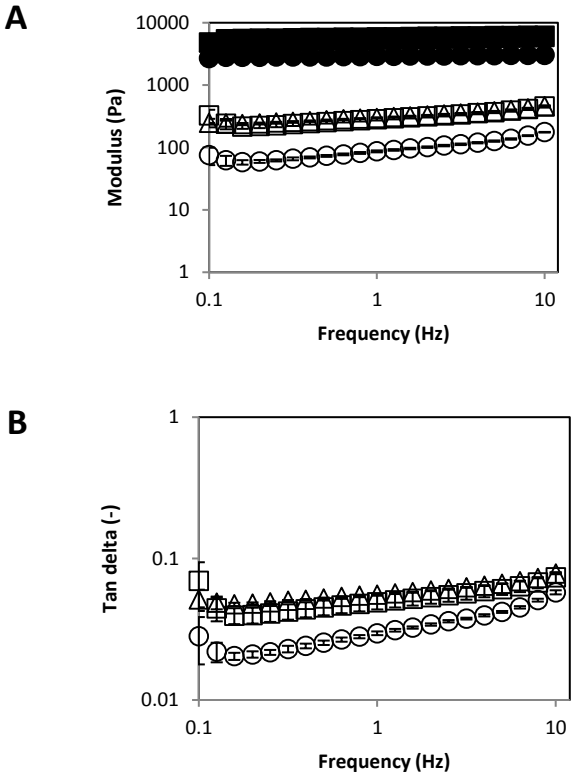


Figure 2.2 Gelation properties of the heated 8 wt.% starch gels, composed of mild fractionated starch (■□), commercial starch (●○) and recombined starch (▲△), A, Frequency sweep sumirized with storage modulus (G' = closed symbole) and loss modulus (G'' = open symbole), B,Tan delta (Tan δ) value obtained from frequency sweep results, including standard error of the mean (n = 3)

Table 2.2 Gel hardness, gel cohesiveness and gel strength based on 8 wt.% starch gel, of mildly refined starch , commercial starch and recombined starch gels, including standard error of the mean (n = 3)

	Non-destructive (35% compression)		**Destructive (90% compression)**
	Hardness	cohesiveness	Gel strength
Mildly refined starch fraction	10.6±0.3	0.87±0.00	24.7±1.6
Commercial starch	8.7±0.3	0.88±0.01	26.9±0.6
Recombined starch	9.8±0.3	0.83±0.01	22.6±0.5

2.4.4. Syneresis

The final property explored was the water holding capacities and syneresis of the starch gels upon storage. The syneresis was measured by storing starch gels at -25°C or 4°C. During storage, it is believed that amylopectin retrogradation causes syneresis (Ratnayake et al. 2002), however limited information is known on how fibers affect the degree of syneresis. That is why experiments were performed with a concentration of 5 wt.% starch, as initial experiments revealed that the main differences in syneresis between the three fractions occurred at low starch concentrations. Experiments were performed at constant starch concentration, meaning that the mildly refined starch and recombined fraction contained more dry matter as extra fibrous material was present.

Figure 2.3 shows the degree of syneresis of the three starch samples as function of freeze-thaw cycles or storage time. The gel samples stored at 4°C as well as -25°C showed an increased syneresis with increasing number of freeze thaw cycles or increased storage time. It was observed that the temperature of storage did not have a significant influence on the degree of syneresis for the samples containing additional fibers (mildly refined and the recombined starch fraction), whereas a higher degree of syneresis was observed for the sample containing only starch when stored at -25°C

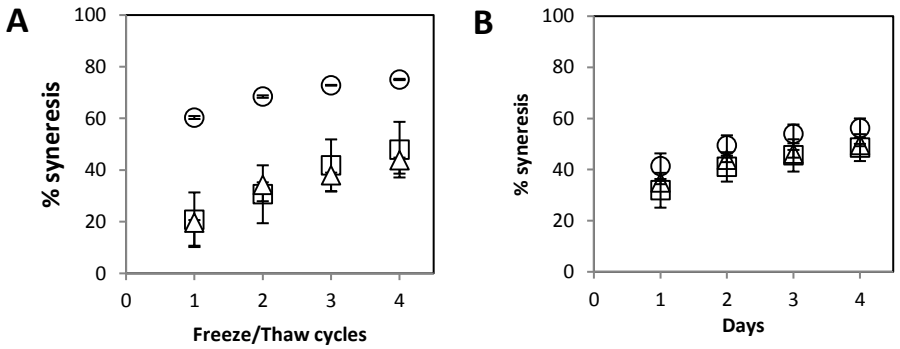


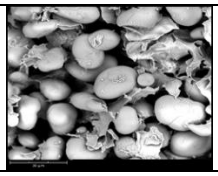
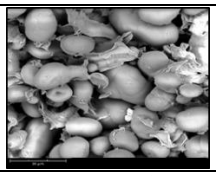
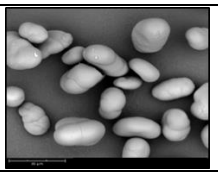
Figure 2.3 Degree of syneresis of 5 et.% starch gel, stored at – 25 °C (A) and 4 °C (B), mild fractionated starch (□), commercial starch (○) and recombined starch (△) gels, including standard error of the mean (n = 3)

2.5. Discussion

The starch concentration in the mildly refined starch fraction was significantly higher than the starch concentration in the original pea flour, indicating enrichment in starch. However, the mildly refined starch fraction was less pure than commercial isolates (Table 2.1). The main other components present in the mildly refined starch fraction are non-starch polysaccharides, most likely fibrous

materials. Peas are known to contain both soluble and insoluble fiber, which have distinct physiological functions and nutritional benefits (Tosh and Yada 2010). The polysaccharide in the mildly refined starch fraction will be mostly insoluble (Pelgrom et al. 2015a), including cellulose, hemicellulose and lignin (Tosh and Yada 2010) due to the fractionation process applied. The three starch fractions in powder form were visually investigated, using SEM pictures, to get more inside in the fiber fraction present (Table 2.3). For commercial starch, only starch granules were visible, which have an oval and irregular shape. Starch granules with the same morphology were also present in the mildly refined starch, indicating that the isolation method did not affect granule morphology. Furthermore, the latter two fractions clearly contained insoluble fiber material, which has similar morphology in both fractions. That observation supports the assumption that both fractions contain similar dietary fiber material.

Table 2.3 Scanning electron micrographs of an unheated dry powders (scale bar represents a length of 30µm) and an overview of the fitting parameter, $\boldsymbol{a_1}$ value, of the pasting properties of mildly refined starch, commercial starch and recombined starch based on starch , dry matter and effective starch concentration

	Mildly refined starch fraction	**Commercial starch**	**Recombined starch**
			
Starch concentration	3.55 ± 0.01[a]	3.18 ± 0.01[b]	3.57 ± 0.00[a]
Dry matter concentration	3.32 ± 0.01[d]	3.47 ± 0.00[e]	3.38 ± 0.00[d]
Effective starch concentration	3.14 ± 0.01[c]	3.17 ± 0.01[c]	3.15 ± 0.00[c]

[a-e] For each pasting property, different superscript uppercase letters show differences between the starch fractions (P<0.05)

To compare the increased peak viscosity as function of starch and dry matter concentration, a power model was plotted through the data points. The power model was used before to describe the effect of fiber concentration on starch pasting properties (Yildiz et al. 2013a), the effect of dry matter concentration on the viscosity of fruit juice (Ibarz et al. 1987), the effect of starch concentration on viscosity (Goksel et al. 2013) and pectin on the viscosity (Kar and Arslan 1999). Equation 2.4 shows the power model, where Y is the pasting property, C is the starch concentration and y_1 and a_1 are both fitting parameters.

$$Y = y_1(C^{a_1}) \tag{2.4}$$

In this equation, y_1 represents the viscosity at a starch concentration of 1% (w/v). We assumed this value constant for pea starch. Following this reasoning, a_1 is the only remaining fitting parameter and is therefore used to compare the individual starch fractions (Table 2.3). A higher a_1 value indicates a stronger dependency of the viscosity on starch or dry matter concentration. When the peak viscosity was based on starch and/or dry matter concentration, the a_1 value indicates no significant differences between the two fraction containing fibers. Whereas the commercial starch sample showed a significant lower or higher dependency.

The two fractions containing insoluble fibers showed a significant higher viscosity value at increasing starch concentration. Most likely, this effect is caused by the additional fibers present in the mildly refined starch and recombined starch fraction. The fibers present have a cooperative effect on the viscosity value, due to their water binding capacity (WBC), which excludes volume from the starch. The WBC was therefore determined for the isolated fibers and for three starch fractions. The isolated fibers were able to bind water already before heating (5 g/g), and the WBC was hardly affected by heating (6 g/g). The three starch fractions on the other hand, showed a relatively low WBC before heating (1-2 g/g), which considerably increased after a heating step (8-9 g/g). The fibers bound already some of the water after suspending the mildly refined and recombined starch fraction in water, prior to starch gelatinization. This water binding leads to an increase in the effective concentration of the starch, which causes the higher increase in peak viscosity during and after heating.

To verify whether this phenomenon is relevant for these starch fiber blends, the peak viscosity was plotted against the effective starch concentration (Figure 2.1C). The effective starch concentration was calculated by subtracting the amount of water that was bound to the fibers before heating from the total volume. The effective volume obtained was used to determine the effective starch concentration. Figure 2.1C depicts the peak viscosity as function of effective starch concentration for the different samples. The similarities in the curves for al samples shows that the peak viscosity could be described by the effective starch concentration, which was confirmed by the statistical analysis based on the a_1 value obtained from the power law model (Table 2.3). In other words, the mildly refined starch and the recombined starch fractions can be considered as a phase separated mixture, in which the water-binding of the fibers influences the effective starch concentration in the continuous phase, which leads to an increased viscosity.

This conclusion is supported by Sun et al. (2015), who found that the peak viscosity of starches to which wheat fiber was added, was significantly higher than the control where no fiber was added. The

wheat fiber was found to have an even higher WBC than the starch, which explained the bigger effect in case of wheat. A similar phenomena is described by Aguilera and Baffico (1997) for whey protein isolate/cassava starch gels. Due to the fact that gelation temperature of cassava starch is lower than the denaturation temperature of whey protein isolate, the cassava starch will swell before the protein are able to gelatinize. This effect increases the effective whey protein concentration and subsequently influencing the strength of the gel formed.

Another way of presenting the results is by considering the total dry matter concentration (Figure 2.1B). The commercial starch sample gave the highest viscosity for all dry matter concentrations investigated, who corresponds with observations by Collar et al. (2006), Sudha et al. (2007) and Goldstein et al. (2010), which found as well a decrease in the shear viscosity when starch was replaced by fibers. The lower viscosity of fiber-containing samples was related to the water holding capacity of the fibers present, which is lower than the water holding capacity of starch.

After cooling, the heated starch suspensions formed a gel. Large and small deformation experiments were performed on these gels. Under small deformation experiments, the gels containing fibers showed the highest storage (G') and loss (G'') modulus at 8 wt. % starch. Similar results were found by Lai et al. (2011), who showed that the addition of rice dietary fiber to rice starch resulted in an increased storage (G') and loss (G'') modulus. Figure 2.2B further shows that the tan delta (Tan δ) was the highest for the two samples containing fibers, indicating that the viscous component was more dominant in these samples. It can therefore be concluded the commercial starch gave the most elastic gel, but that higher storage (G') and loss (G'') moduli were obtained for the samples containing additional fibers.

The large deformation experiment showed that the gels containing additional fibers gave a higher hardness value when performing non-destructive deformation. Sun et al. (2015) found a similar trend after the addition of fibers to a starch gel. Yet, the increase in hardness values when additional fibers were present was less substantial as the increase found by Sun et al. (2015). The cohesiveness value, representing the ability of a sample to retain its structure after compression (Yildiz et al. 2013a), shows that all three starch gels were able to retain, to most extent, their structure and the deformation was non-destructive. The samples containing additional fibers showed the lowest gel strength value when fracturing the gels. The same effect was found by Pelgrom et al. (2015a), where the gel strength of commercial pea starch isolate gels were compared to a pea coarse fraction, containing a comparable amount of fibers as the mildly refined and recombined starch fractions.

It is assumed that the properties of the mildly refined starch fraction can best be explained when considering it as a phase-separated blend, with the insoluble fibers material present having limited gelation properties. The non-starch phase probably acts as filler mainly, explaining the positive influence on the modulus and the hardness value. The reduced elasticity could be caused by the interruption of the interrupt the starch network by the fibers. Pelgrom et al. (2015a) preformed CSLM experiments on a course starch fraction, which is in composition quite comparable to the mildly refined starch fraction. CSLM pictures confirmed that the non-starch materials formed dispersed domains in the starch network and subsequently showed that the starch gel formed by the coarse fraction was interrupted by non-starch material.

Starch gels expel water upon storage. The degree of syneresis depends on the sample type, storage time and -temperature. The commercial starch sample showed a higher degree of syneresis than the samples containing additional fibers when stored frozen. No significant differences were observed when stored at 4^{o}C. The difference in syneresis for the commercial starch sample could be explained by analyzing the freezing and thawing process of the gel. When a starch gel is frozen, the water molecules will form ice crystals. As a result, starch-rich regions are created in the matrix, where the water remains partially unfrozen. The high solid concentration in the starch-rich regions will facilitate the amylopectin chains to associate into thick filaments. When the starch gel is thawed, the ice crystals in the starch-deficient phase will melt and the water can be released from the starch network easily. In this way, the gel network will be damaged, leading to a sponge-like gel structure (Ferrero et al. 1993; Lee et al. 2002; Yuan and Thompson 1998). Upon storage at 4°C, the water phase remains in the liquid state, thereby not facilitating any physical damage to the gel network. The starch gel will thereby (partly) maintain its water holding abilities, explaining the substantial difference for the commercial starch sample between storage at 4^{o}C and -25^{o}C.

The gel containing both starch and fibers showed no significant difference in syneresis between the samples stored at 4^{o}C and -25^{o}C, but the syneresis was lower than of the pure starch gels. The presence of fibers retards syneresis, especially when implementing a freeze-thaw treatment (BeMiller 2011). Similar experiments, in which a hydrocolloid was added to the starch gel, resulted in decreased syneresis as well, which was attributed to the fact that the retrogradation by amylopectin-amylopectin association upon storage was hindered by the hydrocolloids (Ferrero et al. 1993; Wang et al. 2015). Following this reasoning, a possible explanation would be that the insoluble fibers present act as a barrier between the amylopectin molecules, lowering the retrogradation and subsequently

syneresis upon storage. The previously discussed CLSM pictures of Pelgrom et al. (2015a) verify these reasoning, as the non-starch material act as a filler and interrupts the starch gel formed. Furthermore, the fibers present were found to have a relatively good water binding capacity In general, fibers are less sensitive for physical stress. It may therefore be expected that the degree of syneresis is reduced because of the presence of the fibers of which the water holding capacity is hardly influenced by the freeze-thaw treatment (BeMiller 2011). The water originating from the melting ice crystal will can be adsorbed by the fiber material in addition.

2.6. Conclusion

The wish for more sustainable processes drives fractionation towards milder processing conditions. A consequence of the use of those conditions is that no pure fractions are formed, but fractions enriched in certain components. To fully utilize those fractions, it is important to understand their properties based on composition and structure. Here, we explored the properties of mildly refined starch fractions obtained from pea flour. We found that most properties were determined by the starch concentration mainly, but some properties were positively influenced by the presence of other components, mainly fibers. In our case, the viscosity value at increasing starch concertation was enhanced by the presence of fibers. In addition, the starch gels containing additional fibers showed a decrease in syneresis when stored at -25^{o}C. At 4^{o}C, limited differences were observed. Finally, the gel hardness and gel strength were only slightly affected by the additional present of fibers.

The properties of the mildly refined starch fractions could be best explained through considering the fraction as a phase separated system.. The water binding capacity of the fibers present increases the effective starch concentration, which explains higher viscosity increased gel strength and altered syneresis. Depending on the functionality targeted for, the presence of additional fibers could therefore be beneficial. Overall, it can be concluded that mild refinery of pea into an enriched starch fractions leads to an interesting material obtained in a very high yield.

3

Protein nativity explains emulsifying properties of aqueous extracted protein components from yellow pea

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3.1. Abstract

In this paper, the emulsifying properties of a protein-enriched fraction from pea were unravelled. The emulsifying properties of mildly fractionated protein fractions from yellow pea and compared to those of commercial pea protein isolate. The emulsion stability of an oil-in-water emulsion was determined under acidic pH, under acceleration forces and a freeze-thaw treatment. It was found that the emulsions stabilized by the mildly fractionated proteins were less prone to flocculation and coalescence. Those differences were related to the interfacial properties, which indicated that the mildly fractioned proteins were able to form a strong and viscoelastic layer on the interface, providing protection against disruption and high compressive forces. The native state of the mildly fractionated protein was used to explain those results. Denatured protein as obtained after conventional fractionation or after applying an additional heating step resulted in altered interface characteristics, which could explain increased flocculation and droplet coalescence. Overall, the results indicated the relevance of using mild conditions during fractionation. Mild fractionation, thereby shifting the focus from purity to functionality, could be a route to make novel ingredients, with more natural character in a sustainable manner.

3.2. Introduction

Many food product are prepared through mixing pure ingredients, such as proteins, oils and starch. Oil-in-water emulsions are made through dispersion of small oil droplets into an aqueous phase. Those oil-in-water emulsions must be stable during distribution, retail and preparation at home. Often a combination of proteins and surfactants were used for this purpose (Lam and Nickerson 2013). Nevertheless, proteins alone should be sufficient to stabilize an oil-water interface. In case of proteins from plant materials, fractionation process is required that maintains the beneficial functionality of proteins to stabilise emulsions.

Commercial yellow pea protein isolates (CPI) are obtained using an alkaline extraction-isoelectric precipitation method (Salome et al. 2007; Boye et al. 2010b; Stone et al. 2015). In an alkaline extraction-isoelectric precipitation method, protein is fractionated through first solubilising at alkaline condition to remove the insoluble residues. Subsequently, the pH is adjusted to acidic conditions to precipitate protein and to remove dissolved impurities. The pH of the CPI dispersion is adjusted to neutral pH and finally the CPI is (spray-)dried. Overall, this protein fractionation method uses chemicals (acids and bases) and high drying temperatures to allow complete disentanglement of the original structures and extract the individual components. However, pH and the heat treatment are linked to a loss in (native) protein functionality (Arntfield and Murray 1981; Taherian et al. 2011; Wang and Corredig 2011; Denmat et al. 1999), and negative impact on the environment (van der Goot et al. 2016; Schutyser and van der Goot 2011).

In literature, alternative methods are described to obtain plant-based protein isolates, like membrane-based extraction methods. Most of these plant-based protein isolates are reported to have improved emulsification functionality when compared to conventional protein fractionation as described above (Taherian et al. 2011; Alamanou and Doxastakis 1997; Fuhrmeister and Meuser 2003; Lam et al. 2016; Boye et al. 2010a). Further, Fuhrmeister and Meuser (2003) showed that the emulsification functionality of a wrinkle pea protein isolate obtained using ultra filtration was enhanced compared with the conventional fractionation process. Nevertheless, all alternative methods proposed still focussed on obtaining highly purified protein isolates.

Berghout et al. (2014) proposed that purity might not be essential, since most food products contain more than one ingredient and even owe their attractive properties to the presence or/and interactions between different components. The focus on functional characteristics of the ingredients instead of purity also allows the use of mild conditions during fractionation, which better retain native functional properties of the components. A mild fractionation method was recently proposed for

yellow pea yields fractions rich in protein or starch (Geerts et al. 2017a; Pelgrom et al. 2015a), making use of the weak internal structure of the yellow pea. Fine milling of the yellow pea resulted in detachment of the starch granules from the protein matrix. When the yellow pea flour was suspended in water, it could be fractionated through a layer-by-layer separation using centrifugation forces. Similar fractionation process, based on suspending and subsequently settling, were described for other legumes (Czuchajowska 1993; Cai et al. 2001). The obtained soluble protein fraction (SPF) with modest purity (56 g protein/ 100 g dry matter) showed potential as emulsifier. The protein content of the mildly fractioned SPF can be increased through the implementation of a dialysing or ultra-filtration membrane step (Pelgrom et al. 2015a), thereby removing the additional solutes present. However, the question arises whether this additional purification step is necessary for the eventual emulsification properties.

The aim of this paper is therefore to compare the functional properties of mildly fractionated SPF and CPI. The differences in behaviours were related to state of the protein, the dynamic interfacial tension, interfacial rheology and protein composition at the oil-water interface. The emulsification stability was determined at acidic pH, under acceleration forces and a freeze-thaw treatment. Overall, the outcomes provide inside into what extent purification is essential for the emulsification properties.

3.3. Materials and Methods

3.3.1. Methods

Pre-dried yellow peas (Pisum sativum) were purchased from Alimex (Sint Kruis, The Netherlands). Commercial pea protein isolate (NUTRALYS® F85M) was provided by Roquette (Lestrem, France). Sunflower oil was obtained from the local supermarket and used without further purifying, except when determining the interfacial properties. Then, the oil phase was stripped using alumina (MP Alumina N-Super I, MP Biomedicals, Germany) as described by Berton et al. (2011). Sucrose was obtained from Sigma-Aldrich® (Germany). The pH of the protein solutions was adjusted using 1M NaOH and HCl (Sigma-Aldrich®, Germany). For all experiments, milliQ-water was used unless stated otherwise.

3.3.2. Methods

3.3.2.1. Preparation of pea flour

The pre-dried yellow peas were pre-milled into grits by using a pin mill (LV 15M, Condux-Werk, Wolfgang bei Hanau, Germany) at room temperature. The pea grits were then milled with a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) according to the method of (Pelgrom et al. 2015a)). The impact mill was set at a feed rate of 2 rpm, a speed of 8000 rpm, an airflow of 52 m^3/h and a classifier wheel speed of 4000 rpm. A thermometer inside the mill assured that the temperature remained between 16 and 34^{o}C.

3.3.2.2. Preparation protein fractions

Soluble protein fraction (SPF)

A 20 wt.% pea flour suspension was prepared and stirred overnight at 4°C. The suspension was subsequently centrifuged at 10,000 g for 30 min at 20^{o}C. The supernatant containing the soluble pea protein fraction (SPF) was collected. The dry matter content was measured using an infrared moisture analyser (MA35, Saltorius AG, Germany). The supernatant was then diluted to obtain a protein solution of 1wt.%.

Dialysed soluble protein fraction (Dialysed SPF)

The soluble protein fraction (SPF) was prepared as described above and dialysed (cellulose membrane, cut-off 14kD, Sigma-Aldrich®). After dialysis, the sample was centrifuged at 10,000 g at 20°C for 30 min and the supernatant was collected. The dry matter content was measured using an infrared moisture analyser (MA35, Saltorius AG, Germany) and the protein content was adjusted to 1wt.%.

Thermally treated soluble protein fraction (thermally treated SPF)

A SPF solution containing 1 wt.% protein was heated till boiling (100^{o}C). The temperature of the solution was over 90^{o}C for approximately 5 min, which was indicated to effectively denature legume proteins (Palazolo et al. 2011). Subsequently, the solution was cooled to room temperate before further use.

3.3.2.3. Compositional analysis

All SPF samples were dried in a freeze dryer (Christ, Germany) prior to determining the protein, starch, ash and oil content. The protein content was determined by using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, Interscience, Breda, The Netherlands), using a conversion

factor of 5.52 (Holt and Sosulski 1979). The total starch concentration was determined with a Total Starch Amyloglucosidase/a-Amylase Assay Kit (Megazyme International Ireland Ltd, Bray, Ireland). Ash concentration was determined by AACC official method 08-01 (AACC, 1983). The oil concentration was determined with a fully automated Büchi extraction system B-811 LSV (Büchi Labortechnik AG, Flawil, Switzerland). Petroleum ether with a boiling range of 40-60°C was used in Standard Soxhlet mode with a sample-to-solvent ratio of 1:6 for 7 h.

The protein composition was analysed with a size/exclusion Ultimate 3000 HPLC system (Thermo Scientific, MA, USA) equipped with a TSKgel G2000SWxl column (Tosoh Bioscience LLC, PA, USA). A mixture of 30 wt.% acetonitrile, 70 % Milli-Q water with 0.1 % Trifluoro Acetic Acid solution was used as running buffer. The flow rate of the running buffer was set at 1.5 mL/min and the UV detector at 214 nm. The molecular weight was standardized and calibrated by using the following purified proteins: ThyroGlobu (670 kD), β -Globulin (158 kD), Ovalbumin (44.3 kD), α-Lactalbumin (14 kD), Aprotinin (6.5 kD), Bacitracin (1.4 kD) and Phenylalanine (0.2 kD). The aqueous serum phase after emulsification was recovered through applying two consecutive centrifugation steps (3,500xg, 45 min, 20^{o}C and subsequently 18,000xg, 45 min, 20^{o}C). The upper creamed phase was separated from the lower aqueous (serum) phase. The aqueous serum phase was collected and analysed by using HPLC-sec method. Subsequently, the protein content of the serum phase was determined using Dumas method.

3.3.2.4. Differential Scanning Colorimetry (DSC)

Differential Scanning Calorimetry (DSC) measurements were performed using a Diamond DSC (PerkinElmer, Shelton, USA) to determine the transition enthalphy and the denaturation temperature. The DSC analyser was calibrated with indium. An empty stainless steel pan was used as reference. Samples were made by suspending protein in water (20g protein/100g) and heated from 20°C to 140°C at 10°C/min. Nitrogen was used as carrier gas. Measurements were analysed with Start Pyris Software (PerkinElmer, Shelton, USA). The measurements were performed twice.

3.3.2.5. Interfacial properties and rheology

The interfacial properties, both the dynamic interfacial tension and the dilatational interfacial modulus, were determined using an automated drop tensiometer (Tracker™, Teclis-IT Concept, Longessaigne, France) on three protein concentration (0.001, 0.005, and 0.01 wt.%). The interfacial tension was determined for a stripped sunflower oil-water interface. A rising droplet was formed on tip of a stainless steel syringe in a cuvette filled with 25mL protein solution at 20^{o}C. The dynamic

interfacial tension was measured for 14400 second (i.e. 4 hours). Immediately after these 4 hours, a dilatational interfacial rheology measurement was performed. An oscillation frequency and an amplitude sweep were executed in one cycle. First, an oscillation frequency sweep was performed using a volume oscillation amplitude of 5% of the initial drop volume. The measured oscillation frequencies are 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1Hz. Each frequent sweep, consisting of 5 cycles, was followed by a period of rest. The duration of the rest period was equivalent to the length of the five cycles performed before. Subsequently, an amplitude sweep was performed at a constant frequency of 0.02 Hz to determine whether the frequency sweeps were performed in the linear viscoelastic region. The measured oscillation amplitudes were 2, 3, 4, 5, 6, 7, 8, 9, 10 and 15%, each frequent sweep consisted of 5 cycles, followed by a period of rest (250 s).

The dilatational interfacial modulus $|E_d|$ represents the interfacial resistance to a change in area and is defined as;

$$|E_d| = \frac{\mathrm{d}\gamma}{\mathrm{d}\ln A} \tag{3.1}$$

where γ is the interfacial tension and A the interfacial area. The dilatational interfacial modulus $|E_d|$ can be divided into an elastic and viscous component;

$$E_d = E'_d + iE''_d \tag{3.2}$$

where E'_d represents the real part or dilatational elasticity modulus. E''_d represents the imaginary part or dilatational viscous modulus.

3.3.2.6. Preparation of emulsions

10 wt.% oil-in-water emulsions were prepared with a 1 wt.% protein solution. The oil-water mixtures were pre-mixed using an Ultra Turrax (IKA® T25 digital, ULTRA TURRAX®) for at least one minute. Directly afterwards, the solutions were prepared using a homogeniser (LabhoScope™ homogeniser, Delta instruments) at a pressure of 100 ± 5 bar. Subsequently, the pH of the emulsion was adjusted to pH 6.5 or 3.8 using NaOH or HCl.

3.3.2.7. Droplet size distribution

The droplet size distribution was determined by laser light scattering instrument (Malvern Instruments Ltd. 2000, Worchester, UK). The emulsions were diluted 20 times in water prior to measuring. Small aliquots were placed into a measurement chamber containing water until an obscuration rate of 10 to 15% was reached. The particle size distribution was calculated using a refractive index of 1.33 for the aqueous phase and 1.465 for the oil phase. Particle size measurements are reported as surface-weighted mean diameter ($D_{3,2}$) and volume-weighted mean diameter ($D_{4,3}$).

3.3.2.8. Emulsion stability under accelerated forces

Emulsion stability under accelerated forces was determined using a LUMifuge (L.U.M. GmbH, Berlin, Germany). 400 µL of the emulsion was loaded into an optically transparent polyamide cell (2x8 mm). The emulsion was centrifuged at 4000 rpm at 20 ˚C. A measurement was performed every 720s. The total duration of the experiment was 8h.

3.3.2.9. Freeze-thaw treatment

The freeze-thaw stability of an emulsion was determined at -25°C. In a number of experiments, sucrose was added to obtain a sugar concentration of 1, 2 and 5 wt.% in the aqueous phase. The emulsions were frozen for at least 18h, after which they were thawed at room temperature. When the emulsion was completely thawed (after approximately 3h), the droplet size distribution was determined.

3.3.2.10. Statistical analysis

All experiments were performed in triplicate, unless stated otherwise. The results were summarised as mean value with standard deviation. A student's t-test was performed to evaluate difference between samples. The P value was set to $P \leq 0.05$ to reveal significant differences.

3.4. Results & Discussion

A soluble protein fraction (SPF) was obtained using mild fractionation and its functional properties were compared with commercial pea protein isolate. The functional properties measured were the denaturation state, dynamic interfacial tension, interfacial rheology and protein composition at the oil-water interface. The emulsion stability was determined at different pH-values, under acceleration forces and a freeze-thaw treatment.

3.4.1. Composition analysis

Yellow peas were milled and suspended in water (Pelgrom et al. 2015a), which resulted in a major part (> 50%) of the protein present in pea to become solubilised. This soluble protein ended up in the supernatant phase upon centrifugation, and is therefore referred to as SPF. A dialysis step was used to remove solutes and consequently to increase the protein content of the SPF. The solutes present in the SPF were quantified previously using NMR, and turned out to be mono- and di-saccharides mainly (Geerts et al. 2017a). A similar NMR analysis of the sample after dialysis confirmed that almost all solutes were removed indeed (data not shown). Now, the protein content became almost similar to the protein content of CPI on dry matter basis, Table 3.1.

Table 3.1 Composition of the soluble protein fraction (SPF), dialysed soluble protein fraction (dialysed SPF) and commercial protein isolate (CPI) in g/100 g dry matter (DM) ± STDV (n = 3)

	Protein Nx5.52 (Nx6.25)	Oil	Starch (and sugars)	Ash	Non-starch Carbohydrates[a]
SPF	55.6 ± 0.6 (63.0)	0.3 ± 0.0	3.0 ± 0.3	8.4 ± 0.1	28.1 ± 1.0
Dialysed SPF	76.6 ± 0.7 (86.7)	2.0 ± 0.7	0.1 ± 0.0	5.5 ± 1.5	15.7 ± 2.8
CPI[b]	78.8 ± 0.2 (89.2)	1.0 ± 0.3	0.3 ± 0.0	6.0 ± 0.0	14.2 ± 0.4

[a] determined by difference
[b] adapted from Pelgrom et al. (2015)

Figure 3.1 shows the chromatographs for the proteins present of the CPI and SPF samples. The CPI, SPF and dialysed SPF showed similarities over the whole range detected. The CPI samples contained a peak around 40 kDa, which was not present in the SPF samples. SPF after dialysis had similar composition as previously described in literature for conventional membrane extraction methods. The proteins obtained in membrane methods are reported to be mixtures of globulins and albumins mainly (Stone et al. 2015; Boye et al. 2010b). Alkaline extraction-isoelectric precipitation used to produce CPI (Salome et al. 2007), yields mainly globulin which can be further subdivided into legumin, vicilin and convicilin (Lam et al. 2016). The additional peaks observed in the CPI sample were therefore related to the difference in globulin composition. Globulins represent up to 80% of the total proteins present. Legumin, vicilin and convicilin in their original configuration have a high molecular weight, being 300-400, 150-170 and 210 kD. The monomers of legumin and vicilin, are expected to be in the size range of 20-70 kD (Lam et al. 2016). Due to the fractionation method applied, the CPI sample includes the alkaline soluble proteins, both vicilin as legumin (Adebiyi and Aluko 2011; Lam et al. 2016). A subunit from legumin (α-subunit) has a molecular weight of approximately 40 kDa and has a relative high pI value (pI of 5.9-6.1) (Dziuba et al. 2014). It was expected that the additional peak present in the CPI was due to a more abundant presence of this subunit of legumin.

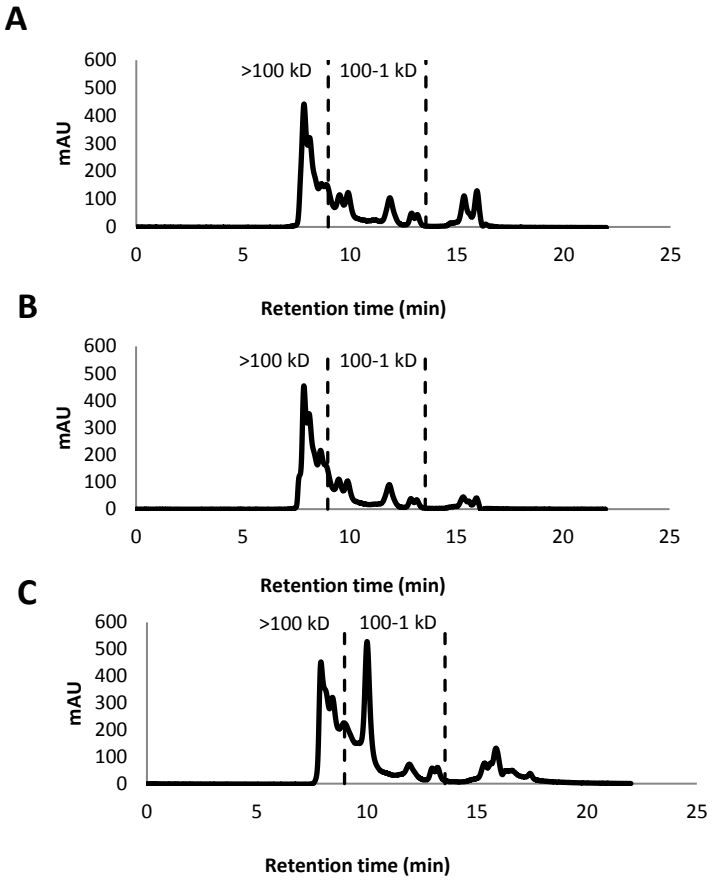


Figure 3.1 Size Exclusive chromatograph showing the differences in protein composition between the SPF (A), dialysed SPF (B) and CPI (C)

3.4.2. Functional properties

3.4.2.1. Protein transition state

Differential scanning calorimetry (DSC) measurements were used to determine the state of the proteins in the different fractions . The SPF samples showed a endothermic peak (ΔH = 4.92 ± 0.5 J/ g protein) around 89.3± 0.7 ^{o}C, which is in line with other values reported for pea protein denaturation temperature (Pelgrom et al. 2015a; Shand et al. 2007; Kozhevnikov et al. 2001). The CPI sample did not show a distinguished endothermic peak, which is reported by more authors for other commercial pea protein isolates (Arntfield and Murray 1981; Shand et al. 2007; Pelgrom et al. 2015a). The absence

of endothermic peak in the CPI indicates that the sample is already denatured during previous processing, whereas the SPF sample is still in their native state.

3.4.2.2. Interfacial properties and viscoelasticity of protein layers

The interfacial properties, both the dynamic interfacial tension and the dilatational interfacial moduli, were determined for the (dialysed) SPF and CPI. Dynamic interfacial tension measurements show the ability of proteins to diffuse and absorb onto an oil-water interface. The dilatational interfacial moduli reveal the characteristics of the interfacial film formed once the proteins are absorbed. The ability of ingredient to rapidly diffuse and re-orient onto the interface and to form a strong interfacial film is considered as an important emulsifier characteristic (Lam et al. 2016; McClements 2015b). Therefore, the dynamic interfacial tension and dilatational interfacial moduli of the (dialysed) SPF and CPI were determined at three different concentrations (0.01, 0.005 and 0.001 wt.%). The dynamic interfacial tension curves were different for the concentration used, for both (dialysed) SPF and CPI (Figure 3.2 A1 and B1). Generally, a dynamic interfacial tension curve can be divided into three different phases when expressed on logarithmic time scale. Those phases are the diffusion (I), adsorption (II) and equilibrium phase (III) (Figure 3.2 A1 and B1) (Beverung et al. 1999). Those three phases could be distinguished for both CPI and SPF indeed when measuring the lowest protein concentration (0.001 wt.%). The (dialysed) SPF showed a shorter diffusion phase. At higher protein concentration, the diffusion phase of the (dialysed) SPF could not be detected, implying that it was shorter than (milli)seconds. In addition, the (dialysed) SPF sample showed a relatively long adsorption phase compared with the CPI sample. Figure 3.3 illustrates the diffusion, adsorption, rearrangement of native and aggregated globular proteins (Tang 2017). Aggregated globular proteins are restricted in their ability to change their structural rearrangements on the interface, which explains the short adsorption phase. Native, globular proteins, on the other hand, will unfold when absorbed onto the interface, resulting in a long adsorption phase (Tang 2017; McClements 2015b).

Dilatational rheology measurements were performed after 4 hrs of protein addition to the oil droplet, which is considered sufficient time to reach an equilibrium at the oil-water interface. The dilatational interfacial moduli of the (dialysed) SPF samples showed a concentration dependency (Figure 3.2 A2). In case of the lowest concentration (0.001 wt.%), the interfacial dilatational elastic modulus (E_d') was dominated over the interfacial dilatational viscous modulus (E_d''), indicating that an elastic film was formed on the oil-water interface. However, an increase in concentration resulted in a decrease in interfacial dilatational elastic modulus (E_d') and an increase in the interfacial dilatational viscous

modulus (E_d''). Those outcomes point towards a more viscous-elastic film being formed at the oil-water interface. In addition, the dilatational interfacial moduli of the CPI samples showed no concentration dependency (Figure 3.2 B2) and the moduli are in the same range as the moduli found by Ducel et al. (2004) for pea protein, despite the fact that they measured at a different pH. For the CPI sample, it was found that the interfacial dilatational elastic modulus (E_d') is higher than the interfacial dilatational viscous modulus (E_d''), indicating that an elastic film is formed at the oil-water interface. Sagis and Scholten (2014) indicated that the change in surface pressure against deformation, plotted in a lissajous curve, provides information about the properties of the film formed at the interface. If the lissajous curve forms a tilted straight line, tilted elipce or circle, the interface has a pure linear elastic, visco-elastic or vicous response respectively. On the lowest concentration (0.001%) a linear elastic response was observed for the dialysed SPF-samples, as revealed by an almost straight tilted line (Figure 3.2 A3). A higher concentration of SPF gave a more linear visco-elastic response as the lissajous curves displayed a tilted ellipse. The lissajous curve of the CPI samples showed no concentration dependency. All concentration gave a linear elastic response, as an almost straight tilted line was obtained (Figure 3.2 B3).

Overall, the dynamic interfacial tension and interfacial dilatational moduli results indicated that a different interfacial layer was formed (Williams and Prins 1996). The DSC results indicated that the CPI sample was almost completely denatured and subsequently proteins in CPI might be aggregated, while the (dialysed) SPF was still native. Those differences can be used to explain differences in adsorption and arrangement proteins at the oil-water interface (Figure 3.3) (Tang 2017). The arrangement of aggregated globular proteins at the oil-water interface is not concentration dependent. Aggregates have a low flexibility, which explains why a similar interfacial layer is formed both at high and low concentration. It can be hypothesized that this layer is thick but inhomogeneous (Tang 2017). The arrangement of native proteins at the oil-water interface is concentration dependent. At low concentration, the native proteins have time to rearrange on the interface and thin elastic layer is formed on the interface. At higher concentrations, the globular protein has limited possibilities to rearrange due to fast adsorption, and subsequently a thick interface visco-elastic layer is formed on the interface (Tang 2017; McClements 2015b). The formation of a thick homogeneous interface visco-elastic layer on the interface is beneficial for the overall emulsion stability and can be considered as an advantageous functional property for the (dialysed) SPF sample.

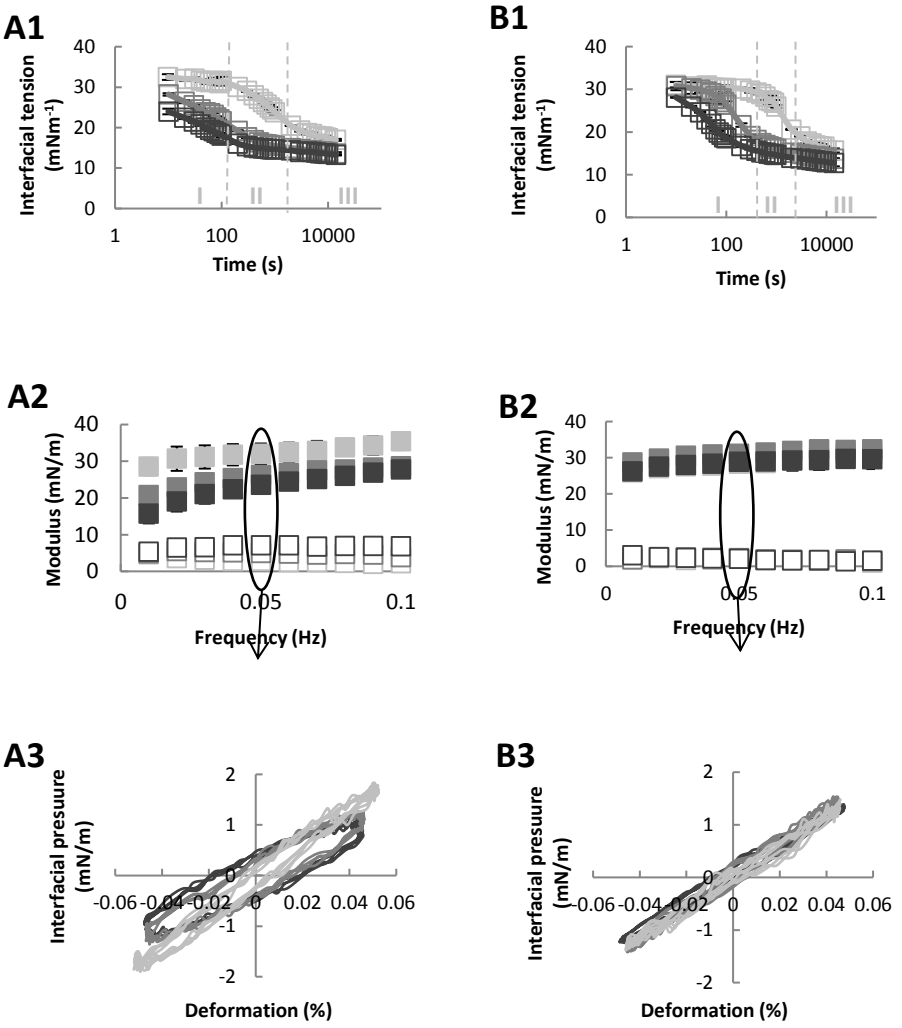


Figure 3.2 Mean dynamic interfacial tension and mean interfacial dilatational moduli of dialysed SPF (A1 and A2) and CPI (B1 and B2) at an oil-water interface, for a 0.01 wt.% (■) , 0.005 wt.% (■) and 0.001 wt.% (■) protein solution. The dynamic interfacial tension (A1 and B1) can deviate in three regimes (diffusion (I), adsorption (II) and equilibrium phase (III)) when expressed on logarithmic time scale, the dotted lines indicate the three regimes that could be discriminated for the 0.001 wt.% protein solution. The mean interfacial dilatational moduli(A2 and B2) was deviating from the interfacial dilatational elastic E_d' (■) and viscous E_d'' (□) moduli. In addition, lissajous curves(A3 and B3) are presented to characterize the properties of complex interfaces. Because, most lissajous curves show great similarity, a single curve is shown (frequency sweep, at 0.05Hz) (n=2)

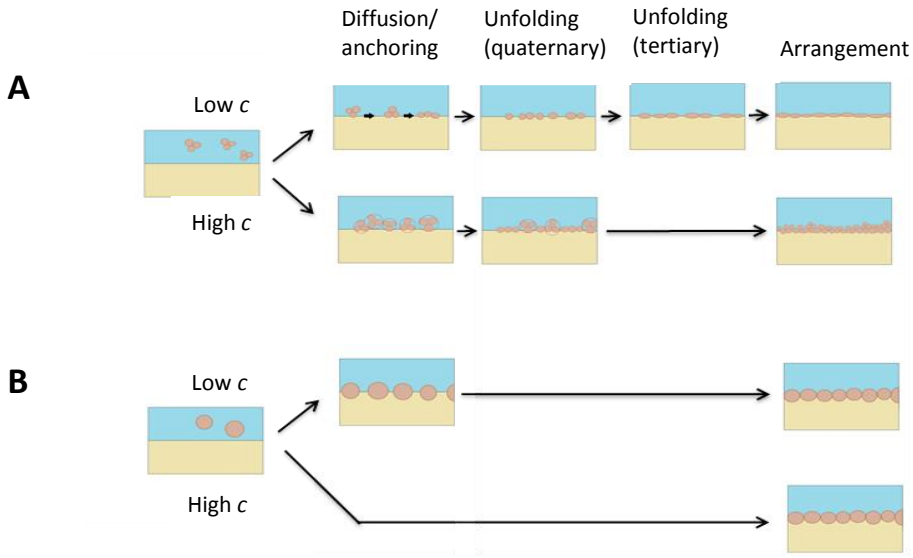


Figure 3.3 Diagram illustrating the adsorption and arrangement of native (A) and aggregated (B) globular proteins at the oil-water interface. The adsorption is deviating in 4 stages; i) diffusion (to the interface)/anchoring at the interface, ii) unfolding, and iii) arrangement of adsorbed proteins vi) arrangement. (Adapted from Tang (2015))

3.4.3. Emulsion stability

A 10 wt.% oil-in-water emulsions was made using a 1 wt.% protein solution. Not all proteins absorbed at the oil-water interface. Characterisation of the protein content in the serum phase after emulsification indicated that approximately 49.5 ± 10.9 % from the initial added protein was present in the serum phase, indicating that approximately 50 % of the proteins present in the SPF samples absorbed at the oil-water interface. For the CPI sample the exact amount could not be estimated, as some material was insoluble. The characterisation of the protein composition in the serum phase indicated that for the CPI sample mostly larger aggregates were present at the oil-water interface, while the composition in the serum phase of the emulsion stabilised by the original SPF samples was almost equal to those in the initial protein solution. These results are in line with the results described before by Keerati-u-rai and Corredig (2009). They indicated that heating a soy protein solution before emulsification resulted in protein denaturation and the formation of protein aggregates. The aggregates mainly adsorbed at the interface, and consequently a reduced amount of larger proteins was found in the serum phase. As expected, the composition in the serum phase for the non-heated samples was almost identical to that of the initial protein solution.

3.4.3.1. Emulsion stability under acidic conditions

Table 3.2 shows the droplet size distributions ($D_{4,3}$ and $D_{3,2}$) of a 10 wt.% oil-in-water emulsion stabilised by SPF or CPI at neutral and acidic conditions (pH 6.5 and 3.8). In addition, oil-in-water emulsions were stabilised by dialysed SPF and thermally treated SPF to investigate the effect of purification and protein denaturation/aggregation. Those pH-values were selected as most food products can be classified in one of those pH categories. At neutral pH, all protein samples stabilized the emulsions. The mean $D_{4,3}$ values found were all in the size range of 1.57 µm ± 0.45 µm. No significant increase in droplet size was observed over a time period of 7 days and microscopic images revealed that flocculation occurred to certain extent. When the pH was adjusted to acidic conditions (pH 3.8), differences between the (dialysed) SPF and CPI samples became evident. Flocculation was observed in all emulsions directly after pH adjustment, but the extent of flocculation was larger in case of CPI, as shown by an increase in $D_{4,3}$. The additional dialysis step performed on the SPF sample had only limited influence on the droplet size distribution. However, a thermally treated SPF sample before emulsification resulted in an increase in average droplet sizes. Now, the $D_{4,3}$ *and* $D_{3,2}$ values became similar to values found for the oil-in-water emulsion stabilized by CPI. Keerati-u-rai and Corredig (2009) indicated that protein aggregates are known to form emulsion with smaller droplets that are more prone to flocculation and coalescence. (Keerati-u-rai and Corredig 2009) We expect that at the interface of the emulsions stabilised by CPI and thermally treated SPF mainly aggregates were present, explaining why those emulsions were more prone to flocculation.

Table 3.2 Mean droplet size distribution, D4,3 and D3,2, of emulsion stabilized by CPI, SPF, dialysed SPF and thermally treated SPF at neutral and acidic pH (pH 6.5 and 3.8), ± standard deviation (n=3)

Sample	**pH 6.5**		**pH 3.8**	
	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$
CPI	1.30±0.03	0.28±0.01	101.6±6.1	19.3±4.2
SPF	1.12±0.11	0.29±0.02	20.3±6.1	9.3±0.9
Dialysed **SPF**	2.16±0.24	0.33±0.03	23.4±5.2	4.3±1.0
Thermally treated **SPF**	1.70±0.22	0.32±0.02	44.8±8.3	22.3±3.2

3.4.3.2. Emulsion stability under accelerated forces

Both the mean droplet sizes and the droplet size distributions did not change for the emulsions stabilized by SPF or CPI at neutral pH when stored for 7 days. Besides, no additional creaming was observed. Centrifugation can accelerate creaming, and is believed to be related to the long term storage stability of an emulsion (Lerche and Sobisch 2011).

Figure 3.4 shows the stability under accelerated forces for the emulsion stabilized by SPF or CPI as a time-related transmission profile over the length of the sample. The first measurement is indicated by the darkest line at the bottom of the graph. It indicates that all samples were non-transmission initially. Each 720 second, the profile was measured and shown in figure 3.4 with lines becoming lighter. The lightest line depicts the final transmission profile. The transmission profiles show that the oil droplets in the samples creamed over time, forming a cream layer that had similar thickness for all samples. However, none of the samples showed oil separation, indicating that hardly any coalescence occurred even at high gravitational forces. Nevertheless, the time in which the thickness of the final cream layer increased differed per sample. The emulsion stabilized through SPF and dialysed SPF creamed almost immediately at these high accelerated forces. The thermally treated SPF showed a reduced creaming rate compared to the original SPF sample, though the creaming rate was still higher than those of the emulsion stabilised by CPI. The emulsions containing CPI showed the slowest creaming. The reduced creaming rate in the CPI and thermally threated SPI sample could be due to an increased viscosity of the continuous phase due to the heat treatment and subsequently the presence of protein aggregates (Peng et al. 2016)

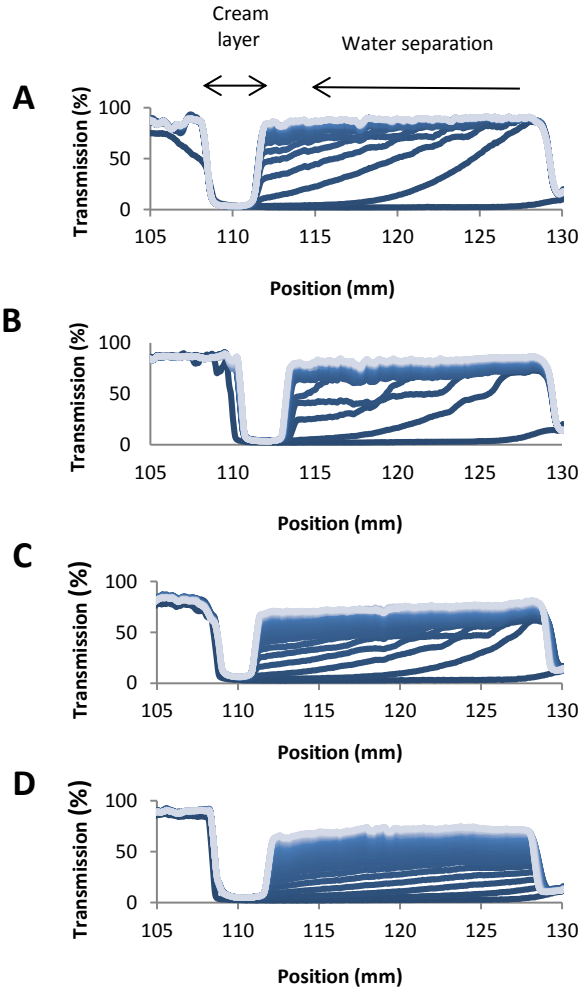


Figure 3.4 Transmission profile of emulsion stabilized by SPF (A), dialysed SPF (B), thermally treated SPF (C) and CPI (D).Local transmission was measured every 720 seconds (12 minutes) for 40 intervals at 4000rpm and 20°C

3.4.3.3. Emulsion stability over a freeze-thaw treatment

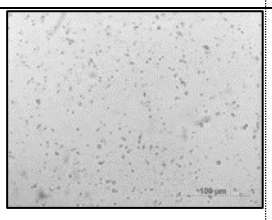
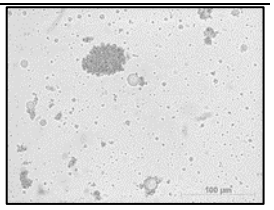
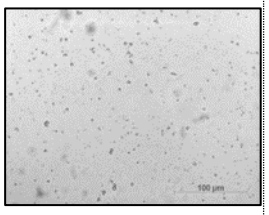
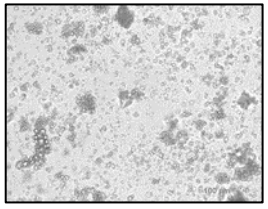
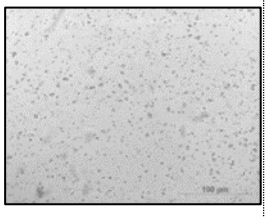
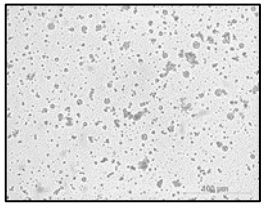
None of the emulsions measured showed oiling off or coalescence when applying accelerating forces. The emulsion stability was therefore also tested under another stressed condition being a freeze-thaw treatment. It was observed that the 10 wt.% oil-in-water emulsion stabilized by CPI was unstable and even showed oiling off ($D_{4,3}$ and $D_{3,2}$ after thawing: 80.8±13.1 µm and 36.2±14.1. µm, respectively). When SPF was used at the same concentration, we observed that the emulsion was able to withstand

a freeze-thaw treatment ($D_{4,3}$ and $D_{3,2}$ after thawing: 1.54±0.2 µm and 0.30±0.02 µm, respectively) (Geerts et al. 2017a). The stabilisation potential of SPF could be strongly reduced by either heating SPF before the emulsification process or through dialysis of the SPF. It suggested that protein denaturation (induced by heating) or removal of solutes (dialysis) negatively influences the potential of SPF to stabilize emulsions.

The role of solutes, such as mono- and di-saccharides, in stabilizing oil-in-water emulsions has been described before (Ghosh and Coupland 2008). The presence of solutes controls compression forces between droplets and hinders coalescence. When such an emulsion is cooled below 0 °C, the aqueous phase will form two phases, being a phase containing pure ice crystals and another phase containing an aqueous liquid enriched in solutes. Most of the oil droplets will be present as well in this aqueous phase (Ghosh et al. 2006; Thanasukarn et al. 2004; Aronson et al. 1994; Magnusson et al. 2011). When the dispersed oil phase in the liquid phase is not able to move freely anymore (i.e. random close packing), instabilities such as aggregation, flocculation and/or coalescence will occur (Ghosh and Coupland 2008). To test this hypothesis, different concentrations of sucrose were added to the aqueous phase (1, 2 and 5 wt. % sucrose in the aqueous phase) and the emulsions were stored at -25^{o}C for at least 24h (Table 3.3). The oil-in-water emulsion stabilized by CPI followed the prediction of Ghosh and Coupland (2008) rather well. The sample that contained 5 wt.% sucrose in the aqueous phase was stable over the full freeze thaw treatment. A lower sucrose concentration resulted in a decrease in stability, as more close contact between the oil droplets will occur at this lower sugar concentration. In addition, less sugar was needed to stabilise an emulsion with dialysed SPF was used as emulsifier. Most likely, the fact that the protein in SPF was still native could make is a better surfactant, requiring less sugar to give a stable emulsion (Table 3.3).

The outcomes above were in line with previous studies, which showed that aggregated protein stabilizes the interface differently than native proteins (Tang 2017). As indicated in figure 3.3, native proteins can form a thick interfacial layer with good visco-elastic properties, whereas, aggregated or denatured proteins mostly form an elastic, and, inhomogeneous interfacial layer, leaving some “holes” possibly. Without sufficient solutes (i.e. low sucrose concentration), high compressive forces are placed onto the oil droplets, which leads to instabilities in case small weak spots are present in the interfacial layer. This explains why emulsions stabilised by aggregated proteins, such as CPI, are more prone to flocculation and the higher stability in case of native proteins, as present in SPF.

Table 3.3 Freeze-thaw stability of the emulsion stabilized by CPI or dialysed SPF is indicating by representing the mean droplet size distribution ($D_{4,3}$ and $D_{3,2}$ ± standard deviation (n=3)) after the freeze-thaw treatment. The close packing value at the different sucrose concentration were estimated using the method proposed by Ghosh and Coupland (2008). In addition, Optical light microscope pictures of the emulsions after thawing are given.

		(dialysed) SPF			CPI		
Sucrose (wt. %)	Estimated close packing value	$D_{4,3}$	$D_{3,2}$		$D_{4,3}$	$D_{3,2}$	
1	*0.936*	2.6±1.0	0.3±0.0		19.7±6.6 *	0.8±0.2 *	
2	*0.864*	2.1±0.2	0.3±0.0		8.9±6.0	0.5±0.1	
5	*0.571*	2.1±0.2	0.3±0.0		1.6±0.0	0.3±0.0	

** Shows oiling off*

3.5. Conclusions

The fractionation process of yellow pea influences the emulsification properties of the proteinaceous fraction obtained. Most of the differences in functional properties could be related to the native state of the proteins present in the mildly fractionated ingredients. The presence of small solutes in that fraction showed limited effect on the functionality, as the SPF and dialysed SPF fraction generally had similar properties. The differences in the degree of denaturation in SPF and CPI influenced the diffusion and adsorption and interfacial characteristics of the proteins and subsequently the emulsification properties. Mildly fractionated proteins were able to form a strong viscoelastic layer on the interface. The presence of a viscoelastic layer at the interface protected oil droplets against disruption and high compressive forces. In addition, protein denaturation and subsequently aggregation, occurring during conventional fractionation or induced through a heating step will influence the interfacial characteristics. The interfacial film formed by denatured protein was more prone to flocculation and coalescence at high compressive forces.

Overall, the results indicated the relevance of mild conditions during fractionation A certain level of impurities may not be detrimental when focusing on the emulsification properties of protein fractions obtained. In addition, mild fractionation allows functionalities to be tuned, as the native state of the proteins could be readily altered. Overall, mild fractionation, thereby shifting the aim from obtaining a highly pure to functional ingredient could assist to increase the naturalness of food ingredients and sustainability of food products.

4

Mildly refined fractions of yellow peas show rich behavior in thickened oil-in-water emulsions

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4.1. Abstract

Conventional fractionation processes aim at high ingredient purity, leading to large water, chemicals and energy consumption. However, as most food product consist of mixtures of ingredients, it is questionable if this high purity is always be necessary .

Mild fractionation makes use of the natural organization of the main components present. In this paper, those components are detached through milling, soaking in water and subsequently using centrifugation forces. Two fractions, soluble protein fraction (SPF) and starch fraction (SF), were studied in a thickened oil-in-water emulsion. The soluble pea protein fraction could be used to make a good emulsion, which remained stable upon environmental stresses (e.g. heating and freezing). Furthermore, the viscosity of the mildly refined fractions indicated a cooperative effect between the protein network and the starch gelation. These results indicate that the mild fractionated complexes have promising features and can become an attractive alternative for conventional ingredients.

Industrial relevance: In this manuscript we aim at understanding the functional properties from fractions that are obtained via mild fractionation concepts. While most focus is now on understanding properties of well-defined, pure ingredients, modern trends, such as increased sustainability and health, point in the direction of less or milder fractionation of plant materials. In this paper, we show that less refined fractions can give rise to interesting properties and can be used to provide functionality now provided by pure ingredients. Overall, this novel approach on the fractionation of ingredients gives a new prospective on prerequisite ingredients must comply.

4.2 Introduction

Food ingredients, like proteins, starch and other biopolymers are used as emulsifiers and structuring agents in multiple food products like sauces, desserts and dressings (Sikora et al. 2008; Román et al. 2015). Generally, the functionality of these eventual food structures is not created by a single ingredient only, but through a combination of ingredients (Dickinson 2007; Almeida-Riveraa et al. 2007). This leads to the question whether ingredient purity is essential to obtain the desired end-product functionalities (van der Goot et al. 2016; Berghout et al. 2014). Nevertheless, current fractionation processes of food ingredients are mostly focussed on obtaining a certain purity of a single ingredient. To obtain high purity, generally harsh fractionation conditions are implemented, including the extensive use of water, chemicals, physical stresses, etc. (Schutyser and van der Goot 2011; Berghout et al. 2015b; Hammond and Jez 2011). Many processes involve multiple dilution steps to extract and purify the ingredient of interest. Water removal through drying, which is an energy intensive process, is necessary to reach stable and safe ingredients and allow global sourcing (Dincer 2011; Motevali et al. 2011). Furthermore, increased purity goes at the expense of yield for most fractionation methods (Berghout et al. 2015b). In the case of fractionation of protein isolates using conventional protein precipitation methods, only around 60 wt.% of initial proteins are recovered in the isolate (Swanson 1990; Alibhai et al. 2006). The remaining 40 wt.% ends up in the various side-streams of the fractionation process. It is clear that a novel paradigm is needed when fractionation biomaterials, focusing on Fractionation processes that are milder to the ingredients leaving those with native-like properties, contrary to current processes, (Arrese et al. 1991; Wagner et al. 2000; Joshi et al. 2011). The need for milder fractionation conditions coincided with a modern consumer's trend, which is that those consumers are getting more reluctant to accept the intensive processing needed to obtain high purity (Saltmarsh 2015). Fractionation methods are preferred with less interference with respect to processing and the use of chemicals, thereby preserving the perception of naturalness (Verhoog et al. 2003).

Literature describes a fractionation phenomenon for yellow peas (*Pisum sativum*) that follows the concepts of mild processing and limited use of chemicals (Pelgrom et al. 2015a; Cai et al. 2001). Here, mildly refined fractions of yellow peas, enriched in a specific component, were obtained through milling of yellow peas and subsequently suspending the flour in water (Pelgrom et al. 2015a). Upon centrifugation, phase separation of the main component can be achieved. The top layers are enriched in protein, whereas the bottom layer is rich in starch. It has been reported that a similar process can be applied to wheat flour (Czuchajowska 1993) and garbanzo bean (Otto et al. 1997). The purities of

the produced fractions do not reach those of conventional purification methods, but the presence of other ingredients, such as non-starch polysaccharides could influence the viscosity and emulsifying properties of the enriched fraction (Alamanou and G.Doxastakis 1997; Yildiz et al. 2013b). So far, only the gel forming properties of the mildly refined fractions of yellow peas is described (Pelgrom et al. 2015a). The application of these fractions in other food systems using an additional functionality has not been further discussed. However, isolated pea proteins showed interesting emulsification properties, comparable to soy protein isolate (Aluko et al. 2009). To increase viscosity, starch is often added to those emulsions. For that reason, the suitability of the soluble protein fraction (SPF) as emulsifier and the starch fraction (SF) as a thickening agent was analyzed in a thickened oil-in-water emulsion. The functionality of the fractions obtained (further referred as functional fractions) in the model systems was described by investigating the emulsifying and rheology properties (Santana et al. 2015). Commercial pea protein isolates (CPI) and starch isolates (CSI) were used as reference materials.

4.3. Materials and methods

4.3.1. Materials

Dried yellow peas (Pisum sativum) were purchased from Alimex (Sint Kruis, the Netherlands). The yellow peas were specified by the supplier to contain 10-15% (w/w) water, 23.0% (w/w) protein, 62.0% (w/w) carbohydrate (of which 44.0% (w/w) starch), 2.0% (w/w) oil, and 3.0% (w/w) ash. Commercial pea protein isolates (NUTRALYS® F85), pea starch isolates (PEA STARCH N-735), were obtained from Roquette (Lestrem, France). 3-(Trimethylsilyl)propionic-2,2,3,3-d4 acid (TSP), Ethylenediaminetetraacetic-d12 -acid (EDTA-d12), Trifluoro Acetic Acid, acetonitrile, KH2PO4, NA2HPO4, NaOH and HCl were purchased from Sigma Aldrich (USA). Difluorotrimethyl-silanyl-(methyl)phosphonic acid (DFTMP) was obtained from Bridge Organics (USA). Sunflower oil was obtained from a local supermarket (Albert Heijn, The Netherlands).

4.3.2. Sample preparation

4.3.2.1. Preparation of the mild refined fractions

Dried yellow peas seeds were pre-milled at room temperature using a pin mill (LV 15M, Condux-Werk, Wolfgang bei Hanau, Germany). After that, the pea grits were further milled using a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany). The classifier wheel speed was set at 4000 rpm and the

impact mill speed at 8000 rpm. The other settings were a feed rate of 2 rpm and an air flow of 52 m^3/h based on previous literature (Pelgrom et al. 2013).

A final concentration in protein of 1.8 ± 0.1 g/100 g water and in starch of 5 ± 0.2 g/100 g water was obtained by suspending 11.4 g of the pea flour in 100 g demi-water. The pea flour suspension was stirred at room temperature for at least 1 h. Subsequently, the pH was adjusted to pH 6.8±0.2 using NaOH or HCl. The pea flour suspension was subjected to two centrifugation steps, as shown in Figure 4.1. The first centrifugation step (1500 g, 1 s, 20 °C) separates the solid starch fraction (SF) from the rest of the solutes. The supernatant was subjected to a second centrifugation step (10000 g, 30 min, 20 °C), which resulted in a separation of a rich-in-soluble protein fraction (SPF) and a rich-in-non-insoluble protein fraction (NSPF). The soluble protein fraction (SPF) and the starch fraction (SF) were not dried but cooled down to 4°C and stored at this temperature. The SPF and SPF were used within 24 h after preparation.

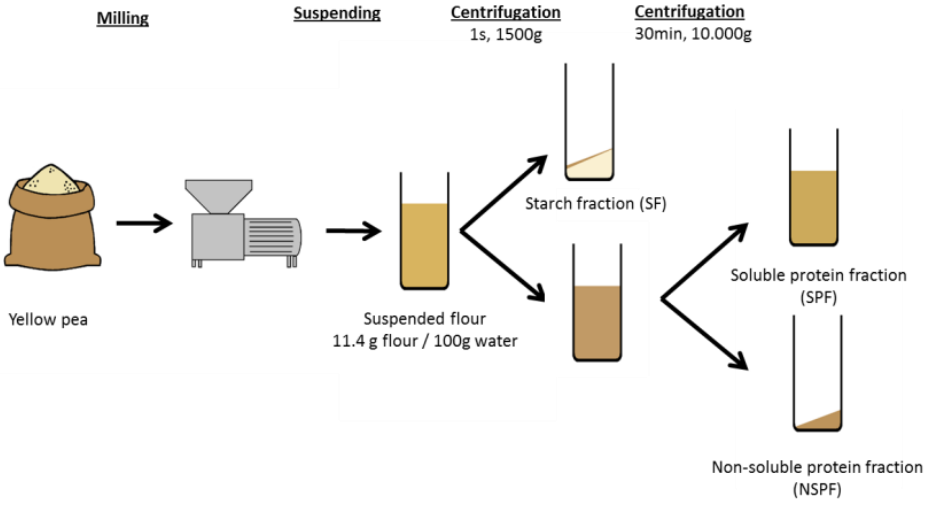

Figure 4.1 Schematic overview of mild aqueous fractionation process

4.3.2.2. Composition analysis

The protein content of starch, soluble and non-soluble protein fractions were determined using a Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, Interscience, Breda, The Netherlands) with a protein conversion factor of both 5.52 (Holt and Sosulski 1979) and 6.25 to allow good comparison with previous studies. The total starch content was determined using the Total Starch Amyloglucosidase/α-Amylase Assay Kit (Megazyme International Ireland Ltd, Bray, Ireland). A

fully automated Büchi extraction system (B-811 LSV, Büchi Labortechnik AG, Flawil, Switzerland) was used to determine the oil content. Petroleum ether was used as extraction-solvent. Ash content was determined using AACC official method 08-01 (AACC, 1983).

The protein composition was determined using Size Exclusion Chromatography (SEC) equipped with a TSKgel G2000SWxl column (Tosoh Bioscience LLC, PA, U.S.A.). The running buffer consisted of acetonitrile and 70% Milli-Q water with 0.1% Trifluoro Acetic Acid (TFA). The flow rate of the running buffer was 1 mL/min and the UV detector was set at 214 nm.

The residual components in the soluble protein fraction (SPF) were analyzed using Quantitative 1H NMR spectroscopy. A 1 wt% SPF was dried using a vacuum-dryer. The dried SPF was re-solubilized in deuterated water and chemical shift indicator (TSP and DFTMP), EDTA-d_{12} solution and a phosphate buffer at pH 7.4, was added and subsequently the samples were mixed thoroughly.
1D 1H-NMR spectra were recorded using a NOESYGPPR1D pulse sequence on a Bruker Avance III 600 NMR spectrometer, equipped with a 5-mm cryo probe at 25 °C. The pribe was tuned to detect 1H resanances at 600.25MHz. Thirty-two scans were collected in 57 k data points with a relaxation delay of 10 s, an acquisition time of 4 s and a mixing time of 100 ms. Low power water suppression (16 Hz) was applied for 0.1 s.
For targeted profiling, 1D 1H-NMR spectra were imported and analyzed in Chenomx software (Chenomx NMR Suite Professional v7.11, Edmonton, Alberta, Canada).

4.3.2.3. Oil in water emulsion

The commercial protein isolation (CPI) solution was prepared by suspending a precisely determined amount of CPI (1.8% (w/w)) in demineralized water and heating at 60 °C for 1 h. Subsequently, the dispersion was mixed using a high performance dispersing unit (Ultra Turrax, IKA) for 10 min and then stirrer overnight to increase solubility. The pH of the solution was adjusted to pH 6.8 ± 0.2 using NaOH or HCl.
A series of oil-in-water emulsions with different oil contents (5-30 % (w/v) oil) were prepared using 1.8 g/100 g water protein solutions (CPI as well as SPF) as emulsifier. Prior to homogenisation, the oil-in-water mixtures was pre-mixed using a an high performance dispersing unit (Ultra Turrax, IKA) at 6000 rpm for ±1 min. The pre-mixed dispersions were homogenised by passing approximately 10 times through a high-pressure homogeniser (LabhoScope, Delta Instruments, Drachten, The Netherlands) at 100 ± 5 bar.

4.3.2.4. Model system

The model system with commercial ingredients was prepared through mixing a quantified amount of starch (5 g/100 g water) in the prepared emulsion. For the mildly refined fraction, the model system was created by blending the starch fraction (SF) provided by the SPF emulsion to obtain a starch content of 5 g/100 g water. The suspensions were heated inside the rapid visco-analyser using a defined heating profile, which ensured starch gelation.

4.3.3. Sample characterisation

4.3.3.1. Particle size

The droplet size distribution of the emulsions was determined by laser light scattering instrument (Malvern Instruments Ltd. 2000, Worchester, UK). The emulsions were diluted in water prior to measuring. Small aliquots were added into a measurement chamber containing water until an obscuration rate of between 10 and 15% was reached. The particle size distribution was calculated using a refractive index of 1.33 for the aqueous phase and 1.465 for the oil phase. Particle size measurements are reported as surface-weighted mean diameter (d_{32}) and volume-weighted mean diameter (d_{43}).

4.3.3.2. Viscosity

The viscosity of the emulsions was determined using a rheometer (Anton Paar PhysicaMCR301, Graz, Austria) with double gab (26.7 mm diameter) or plate-plate (diameter 25 mm, gap 1 mm) geometry. Viscosity profiles of the samples were measured with a shear rate sweep ranging from 0.1–100 (1/s) at 20^{o}C. The final viscosity profile of the emulsions was reported at 20 s^{-1}, to mimic the shear rated food perceive in the mouth during mastication (Chung et al. 2012).

4.3.3.3. Heat treatment

The stability of the oil-in-water emulsions over a heat treatment was determined, through heating a 30 % (w/v) oil-in-water emulsions on a hot plate at 90 °C for 30 min. Small aliquots were taken after 0, 5, 10, 20 and 30 min heating, the droplet size distribution was determined using the method described under subheading 2.3.1.

4.3.3.4. Freeze thaw

The stability of the oil-in-water emulsions over a freeze thaw treatment was determined, through freezing g a 30% (w/v) oil-in-water emulsions at -25 ^{o}C for at least 18 h. After freezing, the samples

were defrosted in a water bath of approximately 30 °C for 30 min and were visually checked for phase separation. The defrosted emulsions were subjected to particle size distribution tests as described in 2.3.1.

4.3.3.5. Rapid visco-analyser

The model systems including starch were subjected to an adapted version of the general pasting method (AACC Method 76-21) using the Rapid ViscoAnalyzer (Newport Scientific Pvt. Ltd.,Warriewood, Australia). 28.5g of sample was transferred to a measurement cup. The temperature was kept at 50°C for 1 min, inclined to 95°C in 3 min 42 s, kept at 95°C in 2.5 min, cooled back to 50°C in 3 min 42 s, and finally kept at 50°C for an additional 2 min. The stirring speed was set to 960 rpm in the first 10 s, followed by stirring at 160 rpm during the rest of the experiment. The peak viscosity and final viscosity were determined using the Thermocline software (Newport Scientific Pvt. Ltd., Warriewood, Australia).

4.3.3.5. Microscopic analysis

The emulsions stabilized by CPI (CPI emulsions) and SPF (SPF emulsions) were visualized using light microscopy (Axioscope, Zeiss, Germany) using 100x magnification with 0-100x dilution depending on the oil concentration of the emulsion. Subsequently, a confocal scanning laser microscope (TCS SP5 spectral scanning confocal microscope, Leica Microsystems, Wetzlar, Germany) was used to visualize the model system. A small aliquot was stained with 1% (w/v) Nile Blue prior to imaging. The microscope sides were exposed to ultra-violet light under vacuum for 90 s to prevent attachment of oil droplets to the glass. A HCX PL APO CS 40.0x1.25 OIL UV objective was chosen to take the images. The emission spectrum was detected at 488 and 633 nm wavelength. The images obtained were captured and further analyzed using digital image processing software (LAS AF Version 2.7.4, Leica, Wetzlar, Germany).

4.3.3.6. Statistical analysis

The composition analyses were performed in triplicates at least to obtain the mean value and standard deviation. All other measurements were performed in duplicate and the mean value and absolute deviation were calculated. To test differences between the samples a student t-test were performed, the P value was set on $P \leq 0.05$ to consider a significant difference.

4.4. Results and discussion

The yellow peas (*Pisum sativum*) were milled using similar settings as described by Pelgrom et al. (2015c). The milled pea flour showed a bimodal particle size distribution, with no particles being larger than starch particles, indicating that the starch granules were detached from the protein bodies and other cellular material. The bimodal distribution of the pea flour originated from the average size of the starch granules and protein bodies (Pelgrom et al. 2013), which were around 22 µm for starch granules (Gujska et al. 1994) and 3 µm for the protein bodies (Pernollet 1978; Varner and Schidlovsky 1963). Three different fractions were obtained after suspending the milled pea flour in water and subsequent settling using gravitational forces. As is indicated in Figure 4.1, the first fraction, being the first pellet, consists of starch mainly (here further referred as starch fraction, SF), while the second pellet is enriched in insoluble protein (further referred as non-soluble protein fraction, NSPF). Soluble protein is obtained from the supernatant (further referred as soluble protein fraction, SPF). The starch fraction (SF) and the soluble protein fraction (SPF) directly used in a model system without prior drying.

4.4.1. Composition analysis

The protein and starch content of the functional fractions were significantly different from the original pea flour (23 and 44 wt% protein, respectively) (Table 4.1), indicating that the mild aqueous fractionation method gave considerable enrichments in protein and starch. The results are in line with the results described by Pelgrom et al. (2015a). The fractions obtained had a lower purity compared with commercial isolates, which was according to expectations.

Soluble protein fraction (SPF) contained most of the soluble components, which were previously identified as to be proteins and soluble polysaccharides (Pelgrom et al. 2015a; Czuchajowska 1993). In total, approximately 50 wt% of the total protein present in the pea flour ended up in this fraction, making it interesting for potential further usage. Subsequently, the relatively high protein content made this fraction relevant as emulsifier in an oil-in-water emulsion.

Table 4.1 The composition of the soluble protein fraction (SPF), Starch fraction (SF) and commercial protein and starch isolate (CPI and CSI) in g/100g dry matter ± standard deviation (+ calculated value), a determined by difference, b adapted from Pelgrom et al. (2015a)

	Protein N x 5.52 (N x 6.25)	Starch	Oil	Ash	Other components[a]
Soluble protein fraction (SPF)	53.8(60.9)±0.5	3.3±0.3	0.3±0.0	9.4±0.1	33.2±0.8
Starch fraction (SF)	4.4(5.0)±0.1	67.3±1.5	0.8±0.2	1.0±0.1	26.4±1.3
Commercial protein isolate (CPI)[b]	78.8(89.2)±0.2	0.0±0.0	1.0±0.3	6.0±0.0	14.2±0.4
Commercial Starch isolate (CSI)[b]	0.0(0.0)±0.0	96.2±0.8	0.6±0.2	0.1±0.0	3.1±0.8

Size exclusion chromatograph revealed that the proteins present in the SPF and CPI showed clear similarities, as the same peaks were observed over the whole size range detected. In addition, quantitative NMR analysis was performed to more extensively analyze the residual low molecular weight compounds present, currently indicated as “other components”. The quantitative NMR analysis identified approximately 32 different low molecular components. The identified compounds represented a substantial amount of the total residual components presents in the SPF, however, not all peaks could be quantified using the Chenomx database. In addition, most identified components were only present in small quantities. Only 10 components were present up to sufficient amounts (see data in appendix). These 10 components could be classified in two main groups; metabolic compounds such as 4-aminobutyrate, choline, 2-hydroxyglutarate, 2-oxoglutarate, myo-Inositol, trigonelline and citrate and sugars such as galactose, glucose and sucrose. Galactose, glucose and sucrose represented over 80% of the total mass of the identified components, indicated that the “other components” are mono- and di-saccharides mainly. Most likely these components formed a broad range of soluble oligo- and polysaccharides as well, of which the individual concentration could not be quantified due to the method used.

The starch fraction was rich in starch and contained approximately 98 wt% of all starch present the original pea flour. Apart from starch, insoluble fiber material formed most of the rest of the pellet composition, including cellulose, hemicellulose and lignin (Tosh and Yada 2010), originating from the cell walls.

4.4.2. Functional properties of the protein stabilized emulsions

The functional properties of the protein fractions (SPF and CPI) were tested as stabilizer of oil droplets dispersed-in-water. The effect of heating or freezing on stabilizing properties of SPF and CPI were describing by the change in droplet size and bulk viscosity.

4.4.2.1. Initial droplet size distribution

The emulsification properties of both SPF and CPI were determined at different oil concentration. Figure 4.2 presents the d_{32} values for the emulsions stabilized by the soluble protein fraction (SPF) and by the commercial protein isolates (CPI) at increasing oil content. The initial emulsions showed mono-modal particle size distributions, as indicated in Figure 4.3. For both the SPF as the CPI emulsions, the d_{32} increased with increasing oil content. This observation is in accordance with data found by Floury et al. (2000), who found a similar trend between oil content and droplet size, when whey protein was used as stabilizer. This increase in droplet size upon oil content is slightly higher for the CPI emulsions compared to the SPF emulsions, though this difference was not significant. Overall, the initial emulsions stabilized by SPF and CPI show limited differences. In addition, microscopic analysis showed limited floculation within the overall sample.

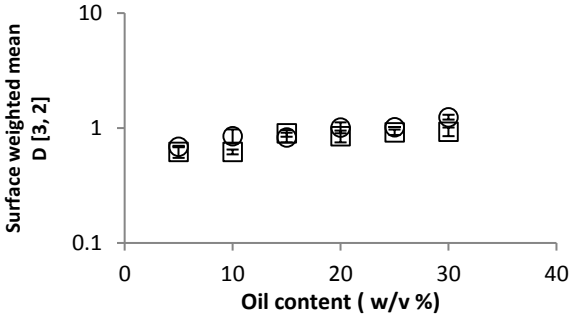

Figure 4.2 Influence of oil content on the droplet size ($d_{3,2}$) of oil-in-water emulsions stabilized by 1.8 g protein/100 g water, derived from the soluble protein fraction (SPF) (□) and commercial pea protein isolate (CPI) (○) ± absolute deviation (n = 2)

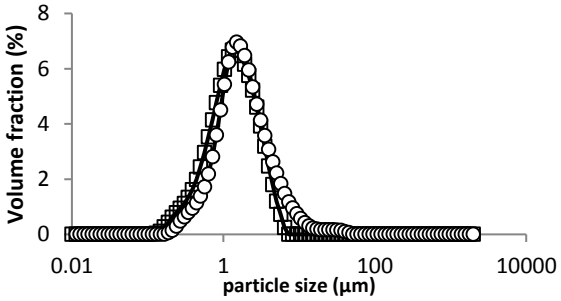

Figure 4.3 Droplet size distribution in the initial oil-in-water emulsion stabilized by CPI (○) or SPF (□), at an oil concentration of 30 % (w/v)

4.4.2.2. Influence environmental stress on droplet size distribution

Most food products are subjected to thermal processing during preparation (cooking) or for shelf life enhancement (pasteurisation or sterilisation). Therefore, the heat-stability of emulsions is an important quality factor when determining the functionality of the emulsifier used. For that reason, the influence of heating on the droplet size of 30 % (w/v) oil-in-water emulsions was determined, and results are presented in Figure 4.4.

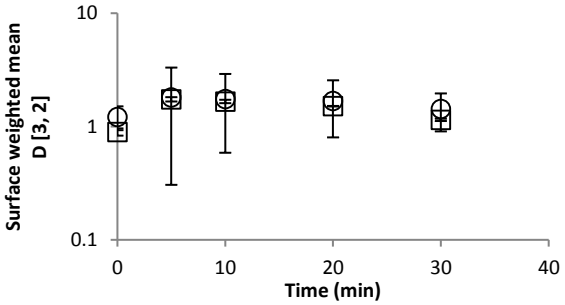

Figure 4.4 $d_{3,2}$ for SPF (□) and CPI (○) emulsions after different heating times (0, 5, 10, 20, 30 minutes) ± absolute deviation (n=2)

The d_{32} was relatively stable upon heating for both the CPI and the SPF emulsions. CPI and SPF emulsions did not show any clear difference after heating. However, minor differences between the time points were observed in case the emulsion was stabilized by SPF. The largest change in diameter was observed after 5 min heating. Then the droplet size gradually stabilized over time, probably due to structural rearrangement of the proteins, which can stabilize the oil droplets.

Previous research showed that commercially available proteins are largely denatured due to the production process (Pelgrom et al. 2015a). As a result, it can be expected that the heating treatment will have minor influence on the configuration of the proteins absorbed onto the oil-water interface. It is assumed that the globular proteins present in the SPF are properly still in the native state (Pelgrom et al. 2015a), implying that heating can result in a change in protein configuration. Palazolo et al. (2011) described that globular proteins from soy being totally denatured after a heat treatment of 5 min at 90 °C. It may therefore be assumed that full heat denaturation is reached after about 5 min heating at 90 °C for the native pea protein present in the SPF fraction. It also explains why prolonging the heating time will have limited effect on the protein configuration. It was therefore expected that only a significant difference in d_{32} value was found between 0 and 5 min heating for the SPF containing emulsion.

Next to a thermal treatment, freezing of food products is often used to increase shelf-life. However, most food emulsions show instabilities over freezing. Therefore, an emulsion with good freeze-thaw stability will have broader applicability (Ghosh and Coupland 2008). For that reason, the 30 % (w/v) oil emulsions were subjected to a freeze thawing procedure and the visual change and change in droplet size were determined (Table 4.2). The CPI emulsions showed creaming and strong flocculation close to destabilization of the emulsion, whereas the emulsions stabilized with SPF were stable over full procedure.

Table 4.2 $d_{4,3}$ and $d_{3,2}$ for SPF and CPI emulsions with an oil content of 30 % (w/v) before and after freeze-thaw treatment of at least 18 hours (n=2), and pictures of the overall emulsion after freezing and thawing.

		$d_{3,2}$	$d_{4.3}$	SPF emulsion	CPI emulsion
SPF emulsion	Before	0.98±0.03	1.09±0.05		
	After	0.81±0.04	7.79±1.34		
CPI emulsion	Before	1.24±0.07	2.12±0.17		
	After	36.18±14.05	80.84±13.13		

Table 4.2 presents the d_{32} and d_{43}-values before and after the freeze-thaw cycle, and pictures of the overall emulsion after freezing and thawing. The emulsions made using the SPF as emulsifier showed hardly any instability over freeze-thaw cycles, as evidenced through the similar d_{32}-values. The ratio between the d_{32} and d_{43} indicated some flocculation within the sample, though this flocculation did not result in creaming. The CPI emulsions showed a high increase in d_{32} and d_{43}, which corresponded with the observed flocculation and creaming.

We hypothesize that the differences described above are due to the presence of soluble components, amongst other sugars, in the SPF. As described above, the emulsions were kept at -25°C for at least 18 h. During this freezing process, the aqueous phase freezes and forms ice crystals that force the oil droplets into the remaining non-frozen liquid voids. Subsequently, the dispersed oil phase probably crystallised, as the freeze point of sunflower oil is between -16°C and -18°C (Grompone 2011). The presence of cryoprotectants, like small solutes, is hypothesized to have a beneficial effect on the stability of an oil-in-water emulsion, in which the continuous phase freezes before the disperse phase (Palazolo et al. 2011; Ghosh and Coupland 2008; Thanasukarn et al. 2004; McClements 2004). The presence of small components, like sugars, decreases the freezing point and suppresses the formation of ice crystals (Thanasukarn et al. 2004). It leads to a large amount of non-frozen liquid voids in the

emulsion. A larger unfrozen liquid fractions leads to a lower oil droplet concentration in this phase which reducing the ability of the oil droplets to form aggregation (Ghosh and Coupland 2008). The SPF contains small solutes, such as mono and di-saccharides, as shown by NMR measurements. Even though, individually these compounds are present in small amounts the total amount of small solutes is considerable. The total amount of small solutes explains why the emulsion stabilized with SPF was able to withstand the freeze thaw procedure.

4.4.2.3. Influence environmental stress on bulk viscosity

The techno-functionality of a food emulsion is not only described by the emulsifying properties, but also by the rheological behaviour (Santana et al. 2015). For that reason, the shear viscosity of the CPI and SPF emulsions were determined at shear rate of 20 s^{-1} to mimic the condition within the mouth when chewing (Chung et al. 2012). The initial emulsions, both the CPI as the SPF, showed a similar relation between oil content and viscosity (Figure 4.5). Comparable results were observed by Chung et al. (2012) and Rothwell (1966) for WPI stabilized emulsions and for cream. In an emulsion with limited droplet-droplet interaction and mainly droplets in the same size range, a substantial higher oil content is needed before an increase in shear viscosity is observed, clarifying the almost negligible increase in viscosity at increasing oil concentrations for the initial emulsions (Derkach 2009; McClements 2015a).

Figure 4.5A shows that the heat treatment at 90 °C for 5 min hardly influenced the viscosity of the emulsion stabilized by CPI. This observation is in line with the fact that the pea proteins present in the CPI emulsion are mostly denatured. The heat treatment had a substantial influence on the viscosity profile of the emulsions stabilized by SPF (Figure 4.5B), which effect became even more pronounced at higher oil concentration. The applied heat treatment will most likely result in heat denaturation of the more native proteins still present in the aqueous continuous phase and at the oil-water interface (Palazolo et al. 2011). These results are in line with the findings of Van Vliet (1988) and Dickinson and Chen (1999), who found a similar trend between the viscoelastic behaviour and the oil content for emulsion (droplets). They distinguished droplets as reactive and non-reactive particles. For the non-reactive particles, like the emulsion droplets stabilized by CPI, the heat treatment did not have a positive influence on the viscoelastic behaviour, indicating limited interaction between the oil droplets. For reactive particles, like the emulsion droplets stabilized by SPF, there was a substantial positive effect between the viscoelastic behaviour and oil content. This was explained by the hypothesis that larger particles aggregate faster than smaller ones according to the orthokinetic flocculation theory (Van Vliet 1988). Heat treatment reduces the steric stabilization due to protein

denaturation in the aqueous phase and on the oil-water interface. Due to the size difference between the oil droplets and proteins, the protein stabilizing the oil droplets will aggregate faster than the protein in the aqueous solution. Subsequently, the volume of the aggregate exceeds those of the sum of the individual oil droplets. This increase in effective fraction is responsible for the significant increase in viscosity as function from the oil content (Van Vliet 1988; McClements 2015a).

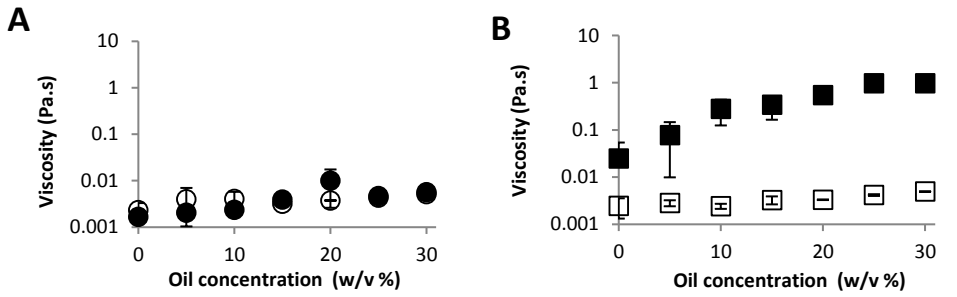

Figure 4.5 The viscosity (Pa.s) at a shear rate of 20 (1/s) for A) unheated CPI emulsion (○) and heated CPI emulsion (●) and B) unheated SPF emulsions (□) and heated SPF emulsions (■) at different oil concentrations %(w/v) at a shear rate of 20 (1/s) ± absolute deviation (n = 2).

4.4.3. Properties of the thickened oil-in-water emulsion: viscosity profile

The functionality of the total thickened oil-in-water emulsions, including the starch fraction, was evaluated by the viscosity profile. The thickened oil-in-water emulsions were heated at 95°C for 2.5 min profile and the peak viscosity at 95°C was used to compare the data using 5 g/100 g aqueous starch suspension as reference (Figure 4.6A).

The viscosity profile obtained for the thickened oil-in-water emulsions made from the commercial ingredients showed hardly any viscosity increase upon increased oil concentration. The profile obtained was similar to the bulk viscosity when only starch was present at 95°C. It can therefore be concluded that the viscosity in the model system with commercial ingredients was mainly determined by the starch present. In contrast, the model system containing functional fractions showed a pattern that was comparable to those previously observed Chung et al. (2012). The viscosity effect could be caused by the presence of a significant amount of fibers in the functional fraction. However, the viscosity at 0 % (w/v) oil did not significantly differ from the model system composed of commercial ingredients, which suggests that the present of these fibers could not be the main explanation.

Chung et al. (2012) suggested that the correlation found between the oil content and the shear viscosity was mainly attributed to the distribution of oil droplets within the regions between the starch network. A higher oil concentration can eventually lead to close packing of the oil droplet, resulting in

a strong increase in viscosity. Following this reasoning, one expects that the viscosity of both model systems would show a positive trend with the oil content, as both the emulsion stabilized by SPF as CPI were stable during the heat treatment. Clearly, this was not the case. The model system containing the functional fractions showed a significant difference with the reference starch suspension especially at higher oil contents.

Figure 4.6B presents confocal scanning laser microscope pictures to visualize the distribution of protein and oil phase in the thickened oil-in-water emulsion after heating. The oil and protein phase present in the emulsion with commercial ingredients were homogeneously distributed, suggesting limited droplet-droplet interactions. The use of functional fractions led to a heterogeneous distribution of oil droplets. The difference observed with the confocal scanning laser microscope (Figure 4.6B) could explain the difference in viscosity profile observed (Figure 4.6A). CSLM-pictures of the emulsion containing functional fractions revealed the formation of a network, which influences on the shear viscosity (Van Vliet 1988). The swelling of the starch granules and the protein network formation, incorporating the oil droplets present, probably have a cooperative effect on the viscosity, resulting in a positive correlation between oil content and viscosity value observed.

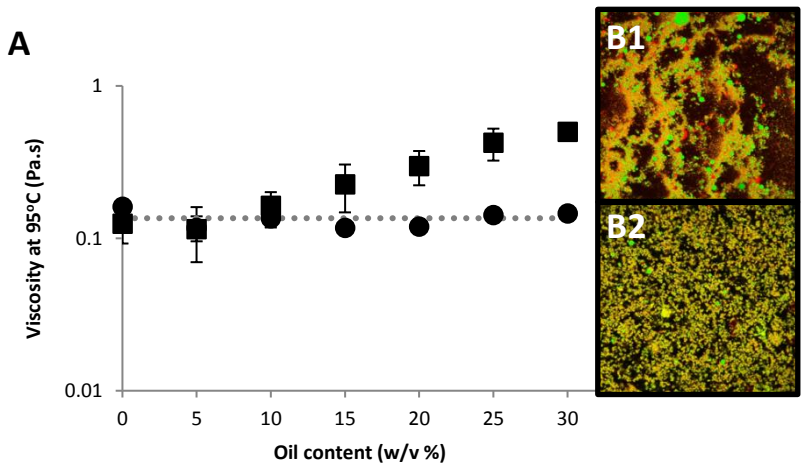

Figure 4.6 A) Peak viscosity at 95°C of the model system conduced from functional fraction (■) and commercial isolates (●) at different oil concentrations, 5g/100g water starch suspension (...) as references ± absolute deviation (n = 2). B) Confocal micrographs of model system containing 30% (w/v) oil, composed form the functional fractions (B1) and commercial ingredients (B2). Nile blue is used to visualize the oil (green) and protein (red) phase.

4.5. Conclusion

The application of mildly refined fractions of yellow peas in a model emulsion was explored and compared with commercially available pea starch and pea protein. The functional properties of (thickened) oil-in-water emulsion were evaluated based on emulsion stability and rheological properties of that emulsion. Overall, it seems that functional fractions showed a richer behaviour in functional properties, meaning that functional fractions can lead to similar properties as the commercial ingredients at least.

Soluble protein obtained after mild fractionation showed good emulsification properties, which resulted in a mono-modal particle size distribution at all oil contents. The emulsions were stable upon heat and freeze-thaw treatments. In that respect, they differed from the commercial ingredients, which gave emulsions with less stability over freeze-thaw treatment.

Prior to heating, all emulsion had a comparable viscosity profile, similar to those of cream. Heating the emulsion composed of commercial protein isolate did not affect the viscosity profile. Heating the emulsion containing the mildly refined faction showed a positive effect on viscosity, which effect was enhanced by increased oil content. Those results hint towards the possible formation of a network that incorporated the oil droplets present. Depending on the final application, these differences can be useful or less desired.

When starch was added to the emulsions, the viscosity profile slightly increased for the model system composed of commercial ingredients, but showed no significant relation with the oil content. The model system composed of functional fractions showed a positive increase with increasing oil content, indicating that the viscosity was caused by a cooperative effect between the close packing of the swollen starch granules and network formation of the protein-stabilized oil droplets.

Overall, it can be concluded that mild fractionation can lead to fractions with functionalities that are partly similar to commercial ingredients, but are also different when considering other aspects. The differences could be attributed to the presence of native protein conformations upon mild fractionation and the presence of other components, such as small solutes. Depending on the final applications, those differences can be useful and even advantageous. Overall, it can be concluded that functional fractions can become a sustainable alternative for highly purified ingredients, and further research is necessary to explore their full potential.

4.6. Supplementary information

Table 4.S1 Quantitative concentrations (mg/g) of the identified low molecular weight compounds present in the samples and identified using Quantitative 1H NMR spectroscopy and present in the Chenomx database. A relative error of 10% should be taken into account.

	Compound	CPI	SPF
Metabolistic compounds	Adenine		<0.01
	Adenosine	<0.01	<0.01
	Guanosine		<0.01
	Uracil		<0.01
	Uridine		<0.01
	4-Aminobutyrate		0.04
	AMP	<0.01	
	Choline	<0.01	0.04
	2-Hydroxyglutarate		0.10
	2-Oxoglutarate		0.03
	myo-Inositol	<0.01	0.13
	Trigonelline		0.04
	Lactate	<0.01	<0.01
	Citrate	0.04	0.07
	cis-Aconitate		<0.01
	Formate	<0.01	
	Fumarate		<0.01
	Malate	<0.01	
	Succinate	<0.01	
Mono and di-saccharides	Galactose		0.21
	Glucose		0.45
	Sucrose	0.14	1.31
Others	Acetate	0.07	<0.01
	Ethanol	<0.01	<0.01

5

Aqueous fractionation processes of soy protein for fibrous structure formation

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5.1. Abstract

Desired properties of ingredients differ for various applications. Here, we use a reverse engineering approach to obtain soy protein fractions targeted for the application of meat analogs. Aqueous fractionation was used to produce these soy protein fractions, which were structured with simple shear flow deformation while heating. The water holding capacity (WHC), nitrogen solubility index (NSI), enthalpy of transition, and viscoelastic properties were determined. We found that a soy protein fraction / full fat flour blend resulted in distinct fibrous structures but only when the soy protein fraction was toasted at 150°C. At this optimum toasting temperature (150°C), the protein fractions had a high WHC, intermediate NSI and its viscoelastic property was characterized as G^* between 1 - 10 kPa. These functional properties were shown to be key for fibrous structure formation, whereas, the influence of the state of the proteins was limited.

Industrial relevance: The market for meat analogs is growing. Nowadays, most of the meat analogs are produced with soy protein concentrates and isolates. These concentrates and isolates are obtained with conventional fractionation processes that involve organic solvents to extract the oil first. As a result, the application of these ingredients is limited, e.g. the product cannot be classified as organic. In this study, we therefore investigated aqueous fractionation of soy to obtain a soy protein fraction with desired functionality that can be used for the application of meat analogs and satisfy the values of consumers.

5.2. Introduction

Ingredient production often aims at general applications, which requires defined chemical composition, and a stable product form such as powder. Traditionally, solubility is the targeted functional property for protein ingredients to allow applicability in drinks, emulsions and doughs (Zayas 1997). Currently, meat analogs are a growing application area. For this application proteins should not solubilize in water, but bind water to allow the creation of a structure. Hence, considering a specific application, one could end up with other or additional requirements for functional properties. Therefore, it can be stated that modern fractionation methods should be designed while keeping a final application in mind. Here, we use fibrous protein structures, which could form the basis for meat analogs as an example to demonstrate a reverse engineering approach to develop ingredients. Soy flour is taken as starting material, because soy-based ingredients are used in many meat analog products currently on the market (Malav et al. 2015; Boland et al. 2013)).

Nowadays, meat analogs that are mimicking the fibrous structure of meat are produced with two extrusion processes; low moisture or high moisture. The extruder is used to form the fibrous structure, which is further processed into a full meat analog by freezing and frying (Giezen et al. 2014). Another innovative technique based on simple shear flow deformation while heating was introduced a decade ago to produce fibrous structures from caseinate (Manski et al. 2007). This concept was later applied to structure soy protein concentrate with a relatively high moisture content (55 wt.%) (Grabowska et al. 2016). For fibrous structure formation with high moisture content, it is known that water absorption and gelling are important properties (Asgar et al. 2010; Singh et al. 2008). Besides protein, other components can contribute to this functionality as well, and might even be required, given the hypothesis that a two phase system is a needed for structuring plant proteins into fibers (Cheftel et al. 1992; Grabowska et al. 2016).

Meat analogs that are currently on the market consist of commercially available protein concentrates and isolates. These concentrates and isolates are mostly prepared with a conventional fractionation process of soy beans, which primary aim was the extraction of oil (Islas-Rubio and Higuera-Ciapara 2002). Oil is extracted from the soybean meal with organic solvents, but the consumer acceptance of those solvents is decreasing, amongst others, for environmental reasons (Dunn et al. 2010). The defatted soy flour is then further processed into protein concentrates or isolates for food applications (Day 2013; Mulder et al. 2016). Partial oil extraction can also be achieved using aqueous fractionation, which might be preferred by current consumers of meat analogs, who are caring about the environment (Hartmann and Siegrist 2017).

Aqueous fractionation is a method in which milled soy flour is mixed with excess water. The application of a centrifugation step yields three phases; *i)* a cream layer, which is rich in oil, *ii)* a liquid phase, which is rich in protein, and *iii)* a pellet, rich in insoluble fibers. The aqueous phase can be used as starting material for protein fractionation by acid precipitation (de Almeida et al. 2014; Russin et al. 2011; Campbell et al. 2011). Soy beans or soy flour are often toasted to decrease the enzyme activity and anti-nutritional factors (Kakade et al. 1974), but this toasting step also lowers the solubility (Wu and Inglett 1974; Onimawo and Akpojovwo 2006). Clearly, reduced solubility is undesired in the first steps of the fractionation process, because the method is based on solubility (Berk 1992).

In this study, an aqueous soy protein fractionation process was designed specifically targeted to make fractions that can be structured into fibers for the application of meat analogs. The aim of this study was to reveal the essential functional properties of ingredients required for fibrous structure formation. To reveal these functional properties, a reversed engineering approach was used. Various soy protein fractions were mixed with water, and structured with a high temperature shear cell. We determined the functional properties of fractions in terms of: water holding capacity, nitrogen solubility, and enthalpy of transition (state of the proteins). The viscoelastic properties of the soy protein fractions in water were determined at similar conditions as used during the structuring process. The fractions were compared with commercially available soy protein concentrates (SPC) and isolates (SPI).

5.3. Materials and Methods

5.3.1. Material

Soy protein isolate (SPI, SUPRO® 500E IP) and soy protein concentrate (SPC, Alpha 6 IP) were both obtained from Solae (Europe S.A.). The manufacturer's specifications indicated that the SPI contained at least 83.4 wt.% protein and SPC contained at least 63.1 wt.% of protein (Nx5.7). The pH was adjusted using HCl and NaOH, both purchased from Sigma Aldrich (Germany).

5.3.2. Methods

5.3.2.1. Preparation of soy flour

Soybeans were pre-milled into grits using a pin mill (LV 15M, Condux-Werk, Wolfgang bei Hanau, Germany). Subsequently, the soy bean grits were milled using a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany). The impact mill was set at a feed rate of 2-5 rpm, a speed of 8000 rpm, an airflow

of 80 m^3/h and a classifier wheel speed of 2500 rpm. A thermocouple inside the mill was used to monitor the temperature, which remained between 16 and 34°C.

5.3.2.2. Aqueous fractionation

A soy protein fraction was prepared by suspending soy flour in water (20 wt.%) and adjusting the pH between 8 and 9 with 1 mol/L NaOH solution. The suspension was stirred for 1 hour and subsequently centrifuged (10,000g; 30min; 20°C). The aqueous samples were poured through a cheese-cloth to separate the cream layer from the supernatant. The supernatant was collected and the pH was adjusted to 4.8 with 1 mol/L HCl. The solution was stirred for at least 1 hour and subsequently centrifuged (10,000g; 30min; 20°C). The pellet was collected and neutralized (pH 6.5-7) with 1 mol/L NaOH and subsequently freeze dried (Christ, Germany). In this study, the supernatant was discarded.

5.3.2.3. Toasting

Soy flour and the soy protein fractions were toasted by spreading the powders over an oven tray ensuring an equal distribution of the flour of around 5-10 mm thick. The oven tray was placed in an Heratherm oven (Thermoscientific, USA) at various temperatures (50-200°C) for 15 minutes. Subsequently, the samples were cooled till room temperature and stored in a closed container at 4°C up until further use.

5.3.2.4. Shear-induced structuring with a high temperature shear cell

A high temperature shear cell was used to structure the soy protein fractions. The soy protein fractions alone and soy protein fractions mixed with soy flour powder in a ratio 70/30 were used for structuring. For the preparation of the sheared samples, salt (1 wt.% NaCl in the total blend) was dissolved in demineralized water. The powders were then added to the salt solution and thoroughly mixed to obtain a mixture with 44% dry matter. A hydration time of 30 min was used. The hydrated materials were placed into a preheated high temperature shear cell (HTSC) at 140oC, and sheared for 15 min at 30 rpm. The HTSC was developed in house (Peighambardoust et al. 2004; Grabowska et al. 2016). It consists of a rotating bottom cone and a stationary cone. An oil bath (JULABO LH46, USA) filled with Thermal H10 oil (JULABO, Germany) was used to heat and cool the cones, while a thermocouple measured the temperature inside the cone. A Haake drive (Haake Polylab QC, Germany) was used to control rotation speed (at 30 rpm). The HTSC was cooled down to room temperature within 5 min, before the samples were taken out. Samples were kept at room temperature for one hour prior to tensile strength analysis.

5.3.2.5. Tensile strength analysis

The degree of anisotropy was determined by cutting tensile bars parallel and perpendicular to the shear flow. From each sample, three tensile bars were taken in parallel and perpendicular. A texture analyzer (INSTRON 5564, USA) was used to deform the samples with a constant deformation rate of 1 mm/s. Samples were placed between two sand-coated clamps at a distance of 15.5 mm. From the stress-strain curve we determined the Young's modulus, tensile stress and tensile strain at rupture. The ratio between the measures (Young's modulus, tensile stress, tensile strain) in parallel and perpendicular direction is referred to as the anisotropic index (AI), which is used as an indication of fibrousness of the material.

5.3.2.6. Composition analysis

The protein content was determined using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, The Netherlands), using a conversion factor of 5.7. Ash content was determined by AACC official method 08-01 (AACC, 1983). The oil content was determined with a fully automated Büchi extraction system B-811 LSV (Büchi Labortechnik AG, Flawil, Switzerland), using petroleum ether as extraction solvent. The duration of the extraction step was set at 7 hours. The carbohydrates were determined by difference.

5.3.2.7. Water holding capacity & nitrogen solubility

The water holding capacity (WHC) and nitrogen solubility index (NSI) of the soy protein fractions and full fat flour were determined with a 2 wt.% dispersion. The dispersions were thoroughly mixed and shaken overnight. Next, the dispersions were centrifuged (10,000g, 30 min, 20oC), and the supernatant and pellet were separated. The tubes were drained on tissue paper and the pellets were weighted. Subsequently, the pellet and supernatant were freeze dried (Christ, Germany). The nitrogen content was determined using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, The Netherlands). The WHC was calculated by the ratio of the wet pellet weight over the initial weight of the sample. The NSI was determined by the ratio of soluble nitrogen over the total amount of nitrogen initial present in the sample.

5.3.2.8. Differential scanning calorimetry

The protein denaturation temperature and the enthalpy of transition of the soy protein fractions and full fat flour were determined with differential scanning calorimetry (DSC) (Diamond DSC, PerkinElmer, Shelton, USA). The DSC was calibrated with indium, and an empty stainless steel pan was used as reference. The soy protein fractions were dispersed in water (20 g sample/ 100 g total). The

samples were heated from 20°C to 150°C at 10°C/min and heating cycle was repeated two times. Nitrogen was used as carrier gas. Measurements were analyzed with Start Pyris Software (PerkinElmer, Shelton, USA).

5.3.2.9. Viscoelastic properties

The viscoelastic properties of the soy protein fraction and soy protein fraction-full fat flour blends in water were determined with a closed cavity rheometer (CCR, RPA elite, TA instruments, USA). Samples were prepared in a similar way as for structuring experiments (section 5.3.2.4). The samples were hydrated in vacuum sealed bags. Subsequently, approximately 5 gram sample was placed between two plastic films and measured in the CCR. An oscillation time sweep was performed at a high frequency (10 Hz) and strain (80%) for 15 minutes at 140 °C. A down pressure of 4.5 bar was applied to close the CCR, and therefore prevent water evaporation.

5.4. Results and Discussion

In this study, we used aqueous fractionation process to obtain soy protein fractions from toasted and non-toasted soy flour. The developed soy protein fractions were examined on their ability to form fibrous structures when processed in a high temperature shear cell. The most successful samples in fiber formation where further analyzed/examined on the most important functional properties with the reverse engineering approach (Otto and Wood 1998).

5.4.1. Aqueous fractionation

The aqueous fractionation process differs from the conventional fractionation process of soy protein since the organic solvent extraction step is replaced by an aqueous fractionation step. The supernatant, rich in water soluble proteins, was further refined with a protein precipitation step to obtain a soy protein fraction. Yield and composition were determined for fractions obtained with aqueous fractionation from toasted and non-toasted flour (table 5.1).

Table 5.1 Composition analysis and protein yield (percentage of proteins present in soy protein fractions of the initial soy flour) of the soy protein fraction, soy protein concentrate, soy protein isolate ([a] determined by difference, mean value ± standard deviation (n=3)).

	Protein Nx5.7	Oil	Ash	Carbohydrates [a]	Protein yield (%)
Soy flour	36.6±0.4	20.2±0.1	5.0±0.1	38.3±0.5	-
Soy protein fraction from					
non-toasted soy flour	75.4±3.4	3.4±0.1	1.9±0.1	19.3±3.5	37.2±0.7
toasted soy flour	68.3±1.8	3.6±1.9	0.1±0.0	28.0±3.7	12.1±1.0
Commercial SPC	63.0±0.2	0.8±0.7	5.8±0.0	30.4±0.9	-
Commercial SPI	83.3±0.7	0.0±0.0	3.4±0.0	13.3±0.7	-

Both soy protein fractions obtained from toasted and non-toasted flour contained still some oil. The fact that some oil remains in the protein fractions was reported before (Campbell et al. 2011). Protein yield from non-toasted soy flour was similar to the yield reported by de Moura et al. (2011). The soy protein fraction obtained from toasted soy flour had a substantially lower yield (12 wt.% of the total protein present in the initial soy flour) compared to soy protein fraction obtained from non-toasted soy (37 wt.% of the total protein present in the initial soy flour). In addition, toasting of the soy flour resulted in reduced separation giving a lower protein content. The decrease in protein content and yield was related to decreased solubility of the toasted material (Wu and Inglett 1974). In further experiments, we therefore explored the possibility of toasting the soy protein fraction in the final processing step after drying the material, because toasting is normally performed in a dry state.

5.4.2. Shear-induced structuring

The soy protein fractions (non-toasted and toasted) were structured in the high temperature shear cell (HTSC) to investigate its potential to form fibrous structures. The advantage of using a high temperature shear cell in this study is that relatively small amounts are needed (40 gram). In previous studies, it was shown that fibrous structures were obtained with commercial soy protein concentrate when processing it in a high temperature shear cell, whereas soy protein isolate alone yielded isotropic structures (Grabowska et al. 2016). Therefore, we benchmarked the soy protein fractions against commercial soy protein concentrate and isolate.

Structures obtained with the soy protein fraction only, both toasted and non-toasted, yielded a layered structure (Figure 5.1A), which was similar to structures that were obtained when soy protein isolate was used (Dekkers et al. 2016). Since the soy protein fraction had a higher protein content than commercial soy protein concentrate, we also mixed the soy protein fraction with soy flour in a ratio of 70:30 to obtain a comparable protein content as commercial soy protein concentrate. It must be noted that the main constituent of commercial soy protein concentrate are proteins and polysaccharides, whereas soy flour contains a considerable amount of fat as well. Hence by mixing soy protein fraction with soy flour, we incorporated fat. Nevertheless, similar fibrous structures were obtained.

The non-toasted blend resulted in an isotropic gel (Figure 5.1B), whereas toasted (at 150^{o}C) soy protein fraction /soy flour blend yielded distinct fibrous structures (Figure 5.1C). These structures are similar to soy protein concentrate presented in literature (Grabowska et al. 2016). Toasting the soy

protein fraction /soy flour blend at higher temperatures (175-200^{o}C) resulted in incoherent and brittle structures, without any fibrous appearance.

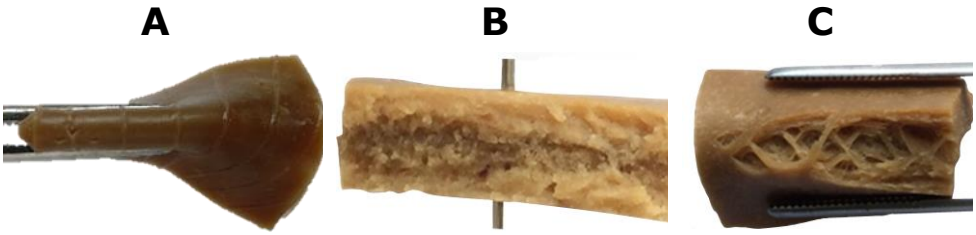


Figure 5.1 Visual appearance of structured samples made with A) 44 wt.% non-toasted soy protein fraction B) 44 wt.% non-toasted soy protein fraction / full fat flour blend (70/30), and C) 44 wt.% toasted soy protein fraction / full fat flour blend (70/30)

Figure 5.2 shows the results of the tensile strength analysis of structures in which we compare soy protein fraction alone with the soy protein fraction mixed with soy flour, both toasted and non-toasted. We compare thesSe results with commercial soy protein isolate and concentrate. The tensile stress and strain differences between parallel and perpendicular were small for the toasted and non-toasted soy protein fraction alone, resulting in a low anisotropic indices (AIσ/AIε = 1.13-1.35). Blending non-toasted soy protein fraction with full fat flour resulted in a decrease of the tensile stress and strain, resulting in even lower anisotropic indices (AIσ/AIε = 0.97-1.14). The toasted soy protein fraction /soy flour blends resulted in a large difference in tensile stress and strain for samples taken in parallel and perpendicular, and hence in a higher anisotropic index (AIσ/AIε = 1.77-1.88). This corresponds to the visual fibrous appearance of this sample. The effect of blending the soy protein fraction with full fat flour had little effect on the Young's modulus and also toasting hardly affected the Young's modules. The anisotropic index of the toasted soy protein fraction / full fat flour blend was higher compared to commercial soy protein concentrate. The absolute tensile stress and strain of the soy protein fractions/soy flour blend were in between the strength of soy protein concentrate and soy protein isolate. Hence, the anisotropic indices of the samples prepared with the fractions obtained with an aqueous fractionation process were similar than those obtained with commercial soy proteins concentrates. Similar tensile strength analysis were performed on beef as reported by Krintiras et al.; the tensile strength of the fibrous structures prepared with the toasted soy protein fraction / full fat flour blend were similar values reported for raw beef (Krintiras et al. 2014).

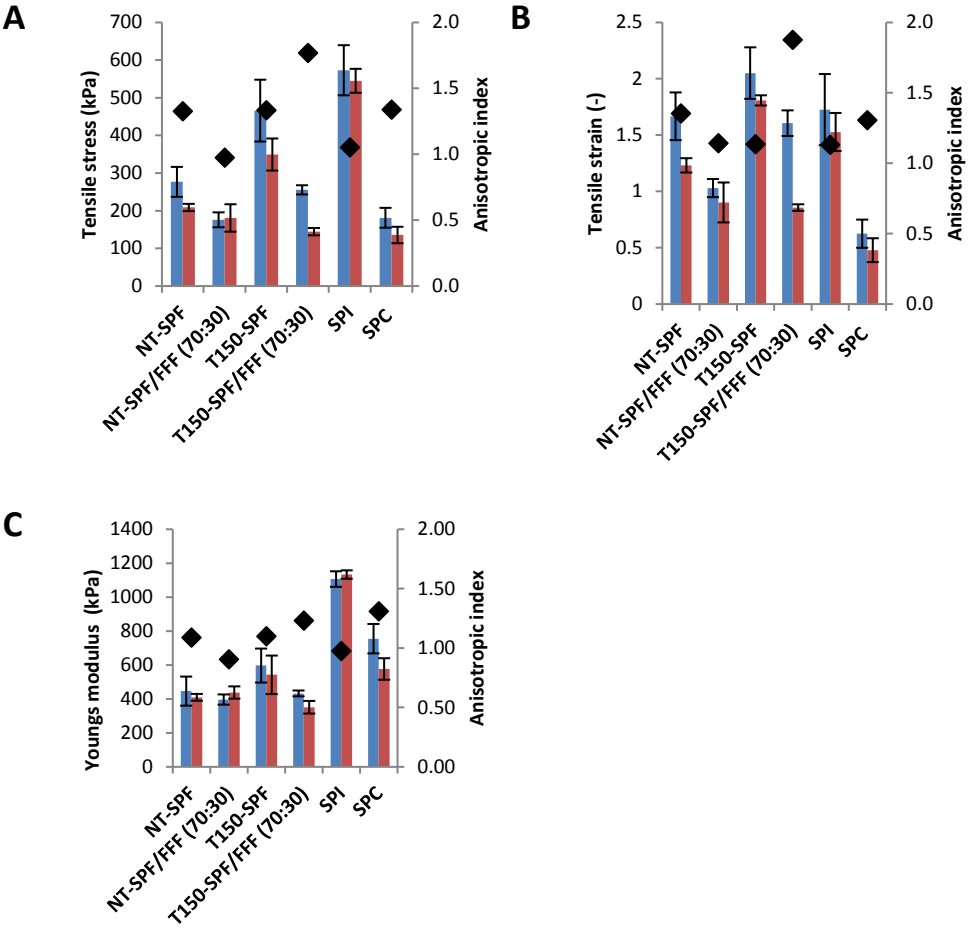


Figure 5.2 Tensile strength analysis results of non-toasted (NT) soy protein fraction (SPF) and SPF / full fat flour (FFF) blend in the ratio 70/30, and toasted at 150°C (T) soy protein fraction (SPF) / full fat flour (FFF) blend (70/30), commercial soy protein isolate (SPI), soy protein concentrate (SPC), A) tensile stress B) tensile strain C) Young's modulus (blue = samples in parallel direction, red = samples in perpendicular direction, black diamonds = anisotropic index, mean value ± absolute deviation (n = 2).

5.4.3. Functional properties of soy protein fractions

5.4.3.1. Water holding and nitrogen solubility

Fibrous structure formation was positively influenced by toasting the dried soy protein fraction at 150°C. The differences between the soy protein fractions due to toasting became apparent when adding water (Figure 5.3). Hydration of non-toasted, toasted at 50 and 100°C soy protein fractions were well dispersible in water and partly dissolved resulting in a paste. Toasting the soy protein

fraction at 125 and 150°C absorbed water, leading to a crumbly texture, which was comparable to commercial soy protein isolate and concentrate. The aqueous soy protein fractions were slightly yellowish. These differences can be related to differences in composition, such as the presence of oil or carbohydrates, and the differences in fractionation processes. Toasting the soy protein fraction at 175 and 200°C resulted in a sandy structure, with a dark brown color and a burned smell indicating that at these high temperatures the samples started to degrade.

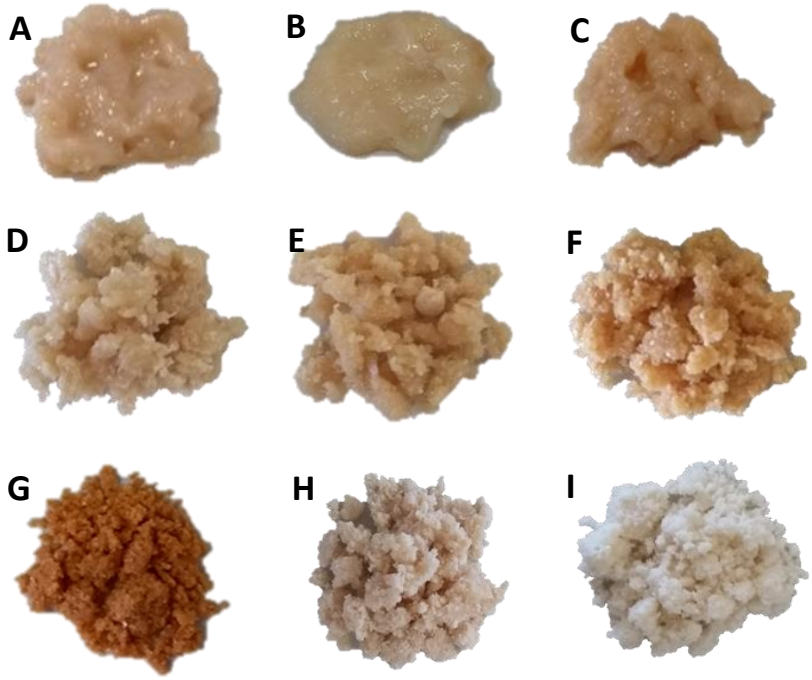


Figure 5.3 40 wt.% dispersion of non-toasted soy protein fraction (A) and the soy protein fractions toasted at 50°C (B), 100°C (C), 125°C (D), 150°C (E) , 175°C (F), 200°C (G), and the commercial soy protein isolate (H) and soy protein concentrate (I).

The differences in hydration properties of the soy protein fractions were quantified by measuring the water holding capacity (WHC) and the nitrogen solubility index (NSI) of the soy protein fractions. In figure 5.4, the WHC and NSI are shown of the soy protein fractions non-toasted and toasted at different temperatures (50-200°C). The WHC increased with toasting temperature up to 150°C. A further increase of the toasting temperature led to a decrease in WHC. The WHC of the soy protein fraction toasted at 150°C was in between the values for SPI and SPC. A relatively high WHC seems important for fibrous structure formation.

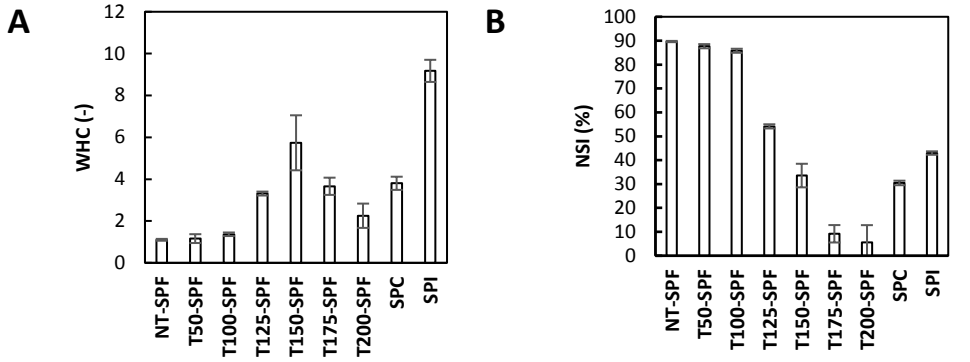


Figure 5.4 A) water holding capacity (WHC) and B) nitrogen solubility index (NSI)) for denatured soy protein fraction non-toasted (NT-SPF) and toasted at various temperatures (50, 100, 125, 150, 175 and 200°C) soy protein fractions (T..-SPF), and the commercial soy protein concentrate and isolate (SPC and SPI) (n=3).

Toasting also resulted in a lower NSI. The commercial soy protein isolate and concentrate had overall a low NSI, which is comparable to the NSI of the soy protein fraction toasted at 150°C. The results suggest that full solubility is not a requirement, or possibly even a disadvantage when the fractions is used to make anisotropic materials.

Overall, the commercial samples as well as the soy protein fraction toasted at 150°C formed upon hydration a crumbly texture, which was assumed to be related water absorption by the protein particles. At lower toasting temperatures, the soy protein fraction formed a sticky texture, which could probably related to the higher NSI and subsequently water was mostly bound interstitially. At higher toasting temperatures, the soy protein fraction formed a sandy structure because almost no proteins were dissolved, and hence there was limited interstitial water present (Peters et al. 2017). Previous studies also showed that toasting or a heat treatment resulted in lowering the protein solubility (Wu and Inglett 1974; Nagmani and Prakash 1997; Petruccelli and Añón 1994; Narayana and Narasinga Rao 1982). A decrease in solubility in thermally treated proteins is often linked to denaturation (López de Ogara et al. 1986) and possibly protein aggregation (Narayana and Narasinga Rao 1982; López de Ogara et al. 1986).

5.4.3.2. State of the proteins

The denaturation temperatures (T_d) and enthalpy of transition (ΔH) of the toasted and non-toasted soy protein fractions was determined to determine whether protein denaturation is indeed causing

the differences in solubility (table 5.2). The commercial soy protein isolate and concentrate sample did not show any peaks, which indicated that most proteins were already denatured during the conventional fractionation process. The soy protein fractions toasted up to 150°C showed two peaks, around 77°C and 95°C, indicating that aqueous fractionation did not result in complete denaturation of the proteins. The two peaks can be related to the denaturation of the two major storage proteins present, conglycinin and glycinin (Hermansson 1986). Yet, the enthalpy of transition (ΔH) slightly decreases with an increasing dry-toasting temperature, indicating that there are transitions occurring in the soy protein fraction. The toasting step was performed in a dry state, which limited the denaturation even at high temperatures (Wu and Inglett 1974). Though, when the soy protein fraction was toasted at a temperature of 175 or 200 °C, no peaks could be identified, which indicated that the sample was mostly denatured. It was already observed that toasting at these temperatures resulted in degradation. Based on the results above, we can conclude that it is not necessary for proteins to be native for successful transformation into fibrous structures. This hypothesis is further supported by the fact that additional experiments showed that heating the soy protein fraction in a wet state resulted in denaturation. Nevertheless, shear-induced structure formation with denatured soy protein fraction / full fat flour blends resulted in isotropic structures similar to non-toasted blends (supplementary information).

Table 5.2 Endothermal differential scanning calorimetry (DSC) peaks (20 wt.% dry matter, heating rate 10 °C/min, n=2)) of several soy protein fraction, and commercial soy protein isolate and concentrate (ND= not detected)

	Peak 1		**Peak 2**	
	T_d (°C)	**ΔH (J/g)**	**T_d (°C)**	**ΔH (J/g)**
Soy protein fraction				
Non-toasted	77.9±0.5	0.22±0.03	96.1±0.0	0.79±0.01
50°C	78.0±0.0	0.28±0.02	95.1±0.0	0.91±0.09
100°C	78.0±0.1	0.27±0.03	95.3±0.1	0.86±0.05
125°C	78.0±0.7	0.17±0.03	95.4±0.3	0.78±0.01
150°C	77.6±0.0	0.19±0.01	95.4±0.3	0.78±0.01
175°C	ND*	ND	ND	ND
200°C	ND*	ND	ND	ND
Commercial SPC	ND	ND	ND	ND
Commercial SPI	ND	ND	ND	ND

5.4.3.3. Viscoelastic properties

The viscoelastic properties of the soy protein fraction toasted at various temperatures were determined during a high frequency and high strain treatment in a closed cavity rheometer at 140°C. These conditions were used to mimic the shear process as applied in the high temperature shear cell. Figure 5.5A compares the complex modulus (*G**) of non-toasted and toasted soy protein fraction at various temperatures. Three clusters in *G** were observed when toasting the soy protein fraction; 50-100°C, 125-150°C, and 175-200°C. These clusters were also found when hydrating the soy protein fraction. It can be seen that toasting at 125-150°C increased the *G** with roughly one order of magnitude (1 - 10 kPa) as compared to non-toasted, resulting in a *G** more similar to the commercial concentrate and isolate (Figure 5.5B). This toasting temperature was found to be optimal for shear-induced structuring. Toasting at even higher temperatures (175-200°C) increased the *G** even further, however this toasting temperature resulted in incoherent, crumbly structures. The initial bump found in the toasted soy protein fraction up to a toasting temperature of 150°C might be related to denaturation of the proteins, since all samples showing this bump also showed two endothermic peaks. In addition, the viscoelastic properties of the soy protein fraction / full fat flour blend were determined and showed similar results (data shown supplementary information).

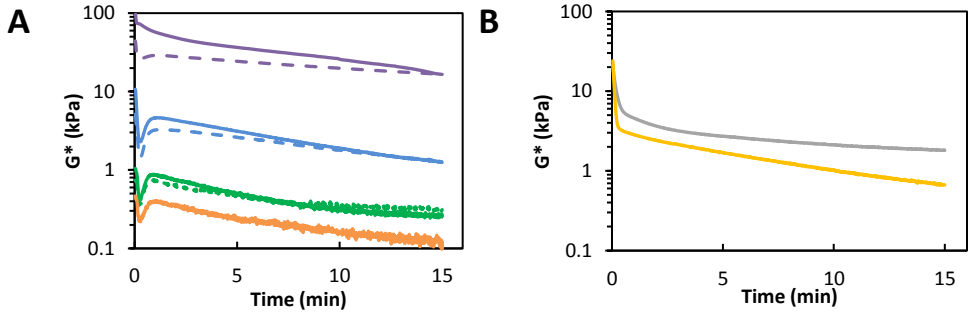


Figure 5.5 Apparent complex modulus (G) measured during a time-sweep experiment at high strain (80%) and high frequency (10 Hz) at 140°C of A) 44 wt.% soy protein fraction non-toasted (—) and toasted at 50°C (...), 100°C (—), 125°C (...), 150°C (—), 175°C (...), 200°C (—), and B) 44 wt.% commercial soy protein isolate (—) and soy protein concentrate (—) (n=3).*

5.4.4 Design of functional properties

A reversed engineering approach was used to design the fractionation process aimed at obtaining functional properties needed for making fibrous structures. The results in this paper indicate that the functional properties needed alter during fractionation and structuring and that relevant functional

properties do not always coincide with properties generally considered. When having the application of meat analogs in mind, it is not necessary to focus on purity optimization, and on classical functional properties such as high solubility. In this study, high water holding and medium nitrogen solubility seem more relevant for fibrous structure formation. With the use of a reversed engineering approach, we therefore adapted the conventional processes (*i.e.* the organic solvent extraction was exchanged with an aqueous extraction step and a toasting step was applied later) and designed an aqueous fractionation process tailored to yield fibrous structures.

In addition, we used minimal and mild fractionated ingredients as starting material to make fibrous structures. Sturtewagen et al. (2016) and Apaiah et al. (2006) indicated that the production of meat analogs is currently not much more efficient than pork meat. This is mainly due to the intensive ingredient preparation of conventional ingredients. It would therefore be beneficial if minimal and mild fractionated ingredients can be used in meat analogs, thereby making a significant contribution in respect to sustainability.

5.5. Conclusions

With a reverse engineering approach, we gained important insights in the essential functional properties of soy protein fractions that are required to obtain fibrous protein structure, which could form the basis for a next generation meat analogs. The essential functional properties of the fractions were obtained by changing process steps. In this study, it was shown that toasting is an important processing step to obtain the functional properties needed for fibrous structure formation. The toasting temperature altered the functionality of the soy protein fractions, and three toasting temperature clusters could be distinguished. The functional properties of the three clusters revealed that soy protein fractions with a relatively high water holding capacity, intermediate nitrogen solubility and viscoelastic properties characterized as G* between 1 -10 kPa were shown to lead to fibrous structures. Most distinct fibrous structures were obtained with a mixture of 70 wt% toasted (150oC) soy protein fraction and 30 wt% native soy flour.

Toasting early in the fractionation process impacted the yield negatively, since it decreased the solubility. High protein solubility was necessary during protein fractionation to obtain a high yield. Hence, the toasting step is recommended to be applied after aqueous fractionation due to the differences in functional properties needed during fractionation and structuring.

Furthermore, the most distinct fibrous structures in this study were formed with a mixture of more intensive and limited fractionated ingredients. This combination is an interesting option for product development. Forthcoming studies could compare this combination of intensive and limited

fractionation with conventional fractionation to determine whether this design is a preferred route for producing more sustainable foods in the future.

5.6. Supplementary information

Table 5.S1 Tensile strength analysis of denatured and denatured and toasted (150°C) soy protein fraction (SPF) / full fat flour (FFF) blends (70:30).

		Tensile stress (kPa)	Tensile strain (-)
Denatured SPF/FFF (70:30)	Par	140 ± 21	0.89 ± 0.05
	Per	92 ± 16	0.51 ± 0.04
	AI	1.53	1.73
Denatured & Toasted 150-SPF/FFF (70:30)	Par	329 ± 61	2.10 ± 0.20
	Per	126 ± 11	0.83 ± 0.03
	AI	2.61	2.53

Table 5.S2 Water holding capacity (WHC), nitrogen solubility index (NSI), denaturation temperature (Td) and enthalpy of transition (ΔH) of denatured and toasted (150°C) soy protein fraction (SPF) (ND = not detected).

	WHC (-)	NSI (%)	T_d (°C)	ΔH (J/g)
Denatured SPF	0.29 ± 0.01	95.4 ± 0.9	ND	ND
Denatured & Toasted 150-SPF	7.14 ± 0.66	11.4 ± 3.3	ND	ND

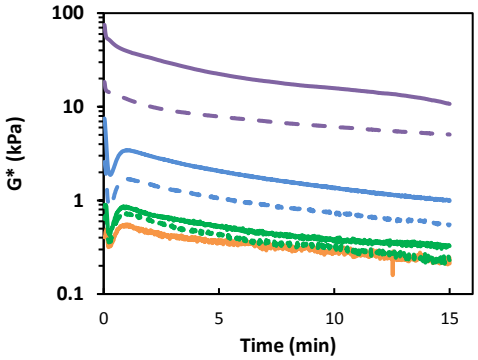


Figure 5.S1 Apparent complex modulus (G) measured during a time-sweep experiment at high strain (80%) and high frequency (10 Hz) at 140°C of A) 44 wt.% soy protein fraction / full fat flour blend (70/30) non-toasted (—) and toasted at 50°C (…), 100°C (—), 125°C (…), 150°C (—), 175°C (…), 200°C (—)*

6

Exergetic comparison of three different processing routes for yellow pea (Pisum sativum): Functionality as driver in sustainable process design

This chapter has been accepted as Geerts MEJ, Veghel A, Zisopoulos FK , van der Padt A and van der Goot AJ Exergetic comparison of three different processing routes for yellow pea (Pisum sativum): Functionality as driver in sustainable process design in Journal of Cleaner Production

6.1. Abstract

Today, the environmental performance of food products and food ingredients is mostly evaluated on the basis of mass (MJ/kg). However, food ingredients are generally added to obtain a specific functionality, such as increased viscosity or modification of the texture. The functionality obtained is not always fully correlated with the amount of ingredients added. This can be especially true when ingredients are produced using different processes. We have investigated how the functionality of ingredients can be included in a sustainability analysis. Here, we have combined exergy analysis and functionality to select the most beneficial process route for fractionation of yellow pea flour. We assess the resource use efficiency of three fractionation processes for yellow pea flour: conventional wet fractionation (CWF), dry fractionation (DF), and a mild wet fractionation (MWF). Exergy analysis based on mass showed that DF has the highest exergy efficiency (99%), due to the (almost) complete use of raw materials, followed by the MWF (54%) and CWF (35%). Interestingly, even though DF is identified as the preferred technology on exergy analysis based on mass, DF is not the preferred option when the results are expressed as MJ/functionality. In that case, more DF starch is needed to obtain the desired functionality, resulting in higher exergy consumption for DF. This study shows that mass-based exergy analysis could result in an exergy efficient process route, whereas for functional application in a food product, this process route is not always the most efficient with regard to resource. This outcome demonstrates the need for inclusion of functionality in sustainability analysis.

6.2 Introduction

The food industry uses ingredients to adjust the properties of food products. The amount of ingredients used is determined by the functionality required, implying that the amount itself is less relevant. Nevertheless, the environmental performance of ingredients is often expressed on the basis of mass or units. Therefore, it is interesting to investigate how the functionality of ingredients can be included in environmental performance analysis. Nutritional value is a functional property that is already studied in life cycle analysis (Sonesson et al., 2017; van der Werf et al., 2014). Here, we propose a method to include technical functionality in a sustainability analysis.

Current fractionation of agricultural materials for the production of protein and starch isolates requires not only large amounts of energy, water, and materials but it also leads to side streams of lower quality than the original raw material (Berghout et al., 2015; Hammond and Jez, 2011; Schutyser and van der Goot, 2011). An alternative approach is to fractionate agricultural materials using mild processing conditions, reducing the number of processing steps and omitting the use of chemicals (Geerts et al., 2017a; Pelgrom et al., 2015). Milder conditions will not deliver pure ingredients, although they can provide fractions that are enriched in, for example, protein or starch. Those enriched fractions might have relevant functional properties, given the fact that, from a functionality perspective, a high level of purity is not always necessary. Mildly fractionated protein-rich and starch-rich ingredients from yellow pea (*Pisum sativum*) showed industrially relevant functionality compared with other commercial isolates (Geerts et al., 2017a; Geerts et al., 2017b). The use of milder conditions can be part of a new approach towards fractionation. van der Goot et al. (2016) proposed several strategies aimed at making the overall food production system more sustainable by, for example, using less refined ingredients, tailoring food ingredient production to specific applications instead of general use, and focusing on functionality rather than purity. Ultimately, it could be interesting to look for other crops instead of fractioning commonly used crops. Albanese et al. (2018) showed that Mediterranean old wheat varieties had relevant properties for brewing beer. Here, we study the environmental performance of three fractionation processes for yellow pea flour. More specifically, we compare the conventional wet fractionation (CWF) of yellow pea flour with mild fractionation (MWF) and dry fractionation (DF) processes. The latter two exploit the difference in density and size of the main components in the flour. MWF is based on differences in solubility, and consequently requires water as a solvent and the need for dehydration steps. DF is a dry fractionation process and, consequently, no additional drying steps are required (Pelgrom et al., 2013; Schutyser et al., 2015). These three processes differ in the number of processing steps, energy use, water use, and the use of

raw material and chemicals. Nevertheless, it is still unclear in what way those processes influence the overall resource use efficiency (RUE) of pea flour especially when considering the functionality of such a fraction in a food product.

As a starting point, we assess the three technology options using the environmental performance based on the product mass by its RUE, which is quantified with the concept of exergy. Exergy is based on the first and second law of thermodynamics and, therefore, it is an objective measure for assessing the RUE of food production chains and processes (Zisopoulos et al., 2017b) by estimating both the consumption and the irreversible destruction of natural resources (Zisopoulos et al., 2017a). Exergy analysis does not provide a direct solution, but it indicates whether an alternative process route or process configuration has higher RUE. Examples in which exergy analysis is studied include beer brewing (van Donkelaar et al., 2016), fish feed formulation (Draganovic et al., 2013) and bread production (Zisopoulos et al., 2015). In this article, we compare the RUE for the three fractionation routes and indicate the most resource-efficient fractionation route. However, many assumptions made for the analysis can influence the results, and therefore a follow-up sensitivity analysis is included as recommended by Zisopoulos et al. (2016). So far, exergy analyses of different food ingredient manufacturing processes have omitted the intended purpose of their useful output streams and their actual use in other food products. Finally, we take functionality (e.g. thickening properties) as the basis for the exergy calculation and evaluate the change in sustainability ranking.

6.3. Materials and Methods

6.3.1. General description of the fractionation processes for yellow peas

We analyse three processes for the fractionation of flour produced from yellow peas (*Pisum sativum*): conventional CWF (with emphasis on isolate production), and the alternative MWF and DF processes. Process flow diagrams for all three fractionation processes are shown in Figure 6.1, including the system boundaries and most relevant input and output streams. In all cases, the starting raw material was yellow pea flour but the product portfolio (output fractions) obtained differed after each fractionation process. All three fractionation processes yielded a fraction rich in protein and a fraction rich in starch. In addition, the CWF yielded an additional fibre-rich product stream and the MWF an additional product stream containing an insoluble protein fraction. Subsequently, as the CWF and DF do not yield a stream containing mainly fibres, the fibres are divided between the protein- and starch-rich streams. The protein content of the initial raw

material (pea flour) and all other streams is estimated using an N-conversion factor of 5.52 (Holt and Sosulski, 1979).

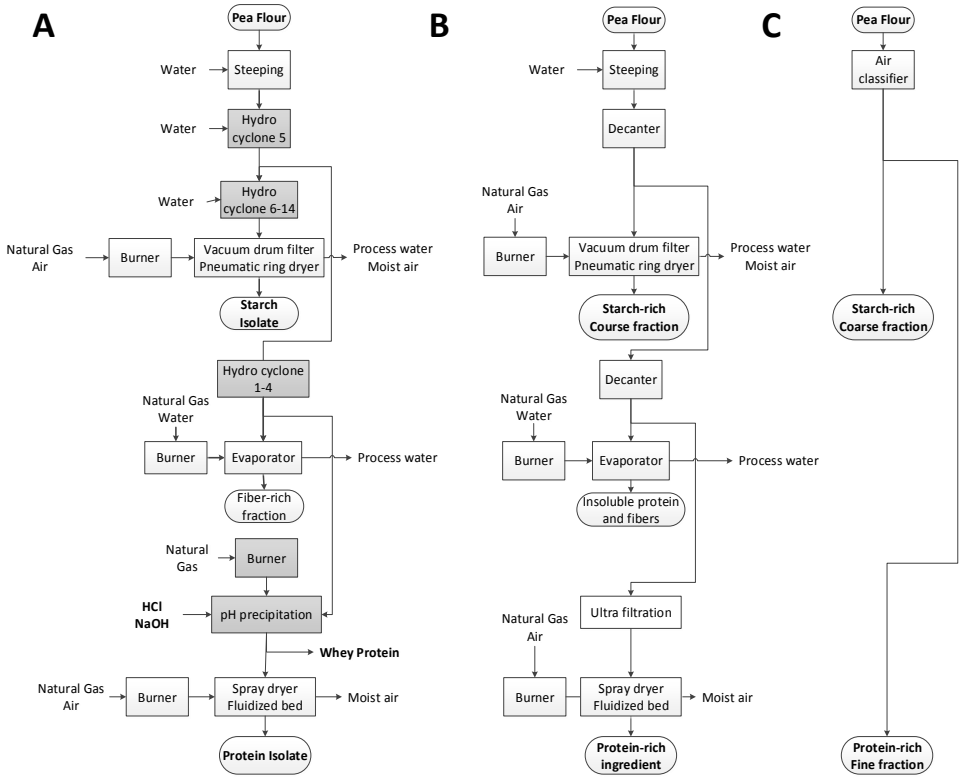


Figure 6.1 Flowchart of the CWF (A), MWF (B), and DF (B) process. The grey boxes in CWF indicate the steps that are omitted or replaced in the alternative wet fractionation.

CWF yields three useful fractions: a starch isolate, a protein isolate and a third, fibre-rich fraction (95%, 76% and 50% purity on a dry matter basis, respectively). In the base case scenario, it is assumed that the starch and protein isolates are suitable for human consumption, and the fibre-rich fraction is used for animal feed. MWF yields three useful fractions: a starch-rich fraction, a soluble protein-rich fraction and an insoluble protein-rich fraction (70%, 54% and 52% purity on a dry matter basis, respectively) (Geerts et al., 2017a; Geerts et al., 2017b). DF yields two fractions: a starch-rich course fraction and a protein-rich fine fraction (67% and 42% purity on a dry matter basis, respectively) (Pelgrom et al., 2015).

MWF and DF lead to fractions of lower purity, but the quantities (yields) of the fractions obtained are higher than those obtained with CWF (Table 6.1).

Table 6.1 The estimated yield, waste and water use of the conventional wet (CWF) and alternative mild (MWF) and dry fractionation (DF) processes.

	CWF		MWF		DF	
	Starch	**Protein**	**Starch**	**Protein**	**Starch**	**Protein**
Mass (kg/1000 kg pea flour)	452	216	665	226 (+ 94)	746	254
Starch or protein (kg/1000 kg pea flour)	381	148	406	103 (+ 42)	428	107
Waste (kg/1000 kg pea flour)	332		15		0	
Water use (kg/1000 kg pea flour)	3260		1147		0	

CWF starts with soaking the pea flour in water. Subsequently, the pea flour suspension enters a series of hydrocyclones where the heavier starch suspension (starch milk) emerges from the bottom of the hydrocyclones and is then dewatered in a vacuum drum filter and dried in a pneumatic ring dryer. The lighter mixture consisting of proteins and fibres emerges at the top of the hydrocyclones and is fed to a centrifugal decanter in which the pea fibres are separated from the protein-rich supernatant. The pH and temperature of the protein-rich supernatant is adjusted to pH 4.5 and 60^{o}C and enters a maturation tank. Subsequently, the precipitated proteins are separated from the soluble whey proteins in a decanter. The soluble whey proteins are dewatered and discard in the CWF process. The pH of the precipitated globular proteins is adjusted to neutral conditions, which leads to solubilization of the proteins again. Finally, the globular proteins are dehydrated in a spray dryer followed by a fluidized bed dryer to obtain a protein isolate (Passe et al., 2012; Salome et al., 2007).

MWF is an alternative fractionation process and is a simplified version of CWF (Geerts et al., 2017a). The grey coloured boxes in Figure 6.1A show the process steps that are omitted or replaced. First, the pea flour is soaked in water, and the pea flour suspension enters a decanter where the starch-rich fraction is separated from the protein-rich fraction. The starch-rich fraction is dewatered by a vacuum drum filter and then dried in a pneumatic ring dryer; the protein-rich fraction is fed into another centrifugal decanter and separated into a soluble and an insoluble protein-rich fraction. The fraction rich in soluble protein is dewatered by ultra-filtration and then dried in a spray dryer followed by a fluidized bed drier to yield a protein concentrate (Geerts et al., 2017a).

DF consists of only one air classification processing step. The pea flour enters the air classifier where the drag forces created by the incoming air flow and the centrifugal forces created by the classifier wheel separate the smaller particles from the larger ones, producing a coarse fraction and a fine

fraction. The coarse fraction is rich in starch, whereas the fine fraction is rich in protein (Pelgrom et al., 2013).

6.3.2. Detailed description of the fractionation processes of yellow peas

This section describes the most relevant assumptions made for the first (further denoted as the base case) scenario.

In CWF and the MWF, the pea flour is first soaked in water to create a 25 wt.% dispersion using approximately 0.1–0.2 MJ/kg feed electricity for mixing (Schutyser and van der Goot, 2011). The pea flour dispersion is transferred to a steeping tank, and subsequently inserted in a decanter or a series of hydrocyclones. In the steeping tank, approximately 3.6 wt.% of the initial pea flour dispersion is lost (Salome et al., 2007). The centrifugal decanter is estimated to use between 1.1 and 3.6 kJ of electricity per kg feed (GEA Equipment, 2017), whereas the hydrocyclones are estimated to use between 1.4 and 2.2 kJ of electricity per kg feed (National Research Council, 1981). Furthermore, optimal operation of the series of hydrocyclones requires approximately 500 kg of water per ton of flour dispersion (Passe et al., 2012; Salome et al., 2007). The dewatered starch-rich fraction exiting the vacuum drum filter has approximately 60 wt.% dry matter, and the electricity use is estimated to range between 74 and 124 MJ/m^3 of material processed (Petit, 2014). The vacuum drum filter is cleaned with water, using typically 5% of the incoming feed. The energy use of a pneumatic ring dryer is estimated to range between 3.2 and 5.4 MJ/kg water removed (van Manen, 2006). The fibre-rich fraction is concentrated in an evaporator with an estimated energy use of between 0.5 and 1 MJ/kg water removed (Fox and Akkerman, 2010).

In CWF, the protein-rich fraction undergoes a protein precipitation treatment where the pH of the protein dispersion is first adjusted to pH 4.8 with hydrochloric acid at 60°C (Passe et al., 2012) and the whey and globular proteins are separated using a centrifugal decanter. Subsequently, the pH is re-adjusted to pH 6.8–7 with sodium hydroxide and the dry matter content is adjusted to 33 wt.%. The protein-rich fraction is dried in a spray dryer and the drying is completed in a fluidized bed dryer. The energy use of a spray dryer is estimated to range between 3.5 and 4.5 MJ/kg water removed and that of a fluidized bed dryer to range between 4.5 and 9 MJ/kg water removed (Fox and Akkerman, 2010). In MWF, the protein-rich fraction after the second decanter step is directly concentrated by ultra-filtration to 33% dry matter. The electricity use of an ultra-filtration membrane is estimated to range

between 1.4 and 2.2 kJ/kg feed (Cheryan, 1998). Subsequently, the protein-rich fraction is dried in a spray dryer and fluidized bed dryer.

In DF, the pea flour is separated into protein- and starch-rich fractions using an air classification step; the electricity use of an air classifier is estimated to be between 1.1 and 1.8 kJ/kg feed. Finally, the energy input for all thermal processing steps (i.e. heating, evaporation, drying) for all processes is assumed to be provided by burning natural gas with a lower heating value of 49.9 MJ/kg (Engineering ToolBox, 2017). The efficiency of the burners is estimated to range between 60% and 80% (Zisopoulos et al., 2015). The process parameters selected for the base case scenario are summarized in Table 6.2.

Table 6.2 Process parameters selected for the base case scenario, including their assumed minimum and maximum values.

	Min	Base	Max	Reference
Electricity use				
Centrifugal decanter (MJ/kg feed)	0.0011	0.0024	0.0036	(GEA-Equipment 2017)
Hydrocyclone (MJ/kg feed)	0.0014	0.0018	0.0022	(National-research-council 1981)
Air classification (MJ/kg) feed	0.0011	0.0015	0.0018	(Hosokawa-Alpine 2009)
Dispersion mixing (MJ/kg feed)	0.1	0.15	0.2	(Schutyser and van der Goot 2011)
Air pump (MJ/kg feed)	0.015	0.019	0.023	(Hosokawa-Alpine 2009)
Vacuum drum filter (MJ/m^3 feed)	74	99	124	(Petit 2014)
Ultra filtration membrane (MJ/m^3 feed)	8.6	10	82.6	(Cheryan 1998)
Efficiency				
Burner (%)	60%	70%	80%	(Zisopoulos et al. 2015)
Fuel energy use in drying processes				
Spray dryer (MJ/kg water removed)	3.5	4.0	4.5	(Fox and Akkerman 2010)
Pneumatic ring dryer (MJ/kg water removed)	3.2	4.3	5.4	(van Manen 2006)
Evaporator (MJ/kg water removed)	0.5	0.8	1	(Fox and Akkerman 2010)
Fluidized bed dryer (MJ/kg water removed)	4.5	6.75	9	(Fox and Akkerman 2010)

There are several ways to evaluate the fractionation process. Therefore, we have analysed six scenarios and compared them with the base case scenario (Table 6.3). In scenario 2, it is assumed that only “green energy” is used (e.g. electricity obtained from wind power generators and use of solar drying processes) (Eswara and Ramakrishnarao, 2013). Even though “green energy” can also be costly in terms of exergy (Koroneos et al., 2003; Park et al., 2014), we simplify green energy to have a zero exergy requirement. In scenario 3, it is assumed that all fractions produced are suitable for human consumption. The fibre-rich fraction in CWF is currently used for animal feed. Nevertheless, the protein- and starch-rich fractions in the DF and MWF processes both contain fibres making them relevant to include the fibre fraction in CWF. In scenario 4, it is assumed that only the two main

fractions produced (starch- and protein-rich ingredients) are suitable for human consumption. Subsequently, the insoluble protein fraction in MWF is considered waste, despite its considerable protein content. In scenario 5, it is assumed that all protein-rich fractions, including the insoluble protein fraction, are spray dried.. In scenario 7, it is assumed that the main protein-rich ingredient in both CWF and MWF is concentrated (33 wt.% dry matter) instead of spray dried, because it has been shown that concentrating can be an energy-efficient alternative for drying (Depping et al., 2017).

Table 6.3 Selected scenarios based on the assumptions made.

Scenario	Changes
Scenario 1	Base case scenario
Scenario 2	Use of "green" energy
Scenario 3	All output streams are useful products
Scenario 4	Only two main ingredients are useful products (starch and protein–rich ingredient)
Scenario 5	All protein-rich ingredients are spray-dried
Scenario 6	The main protein-rich ingredient is concentrated instead of spray dried

6.3.3. Thermodynamic assessment

Thermodynamic assessment of the fractionation processes is done using a classic exergy analysis approach (Zisopoulos et al., 2017b). First, the system boundaries and reference environment are determined, followed by calculation of mass, energy, and exergy flows for the various processes, which are used to construct Sankey and Grassmann diagrams (Soundararajan et al., 2014) with E!Sankey Pro software.

The mass flow analysis is conducted for every process.

$$\sum M_{in,k} = \sum M_{out,k} \tag{6.1}$$

where $M_{in,k}$ (kg) is the mass of a stream entering a process step, and $M_{out,k}$ (kg) is the mass of a stream leaving a process step.

The reference environment is set at 1 atm, 298 K and 0.008 kg water per kg dry air (41% relative humidity). The forms of exergy considered in this study are the material and immaterial exergy (Equation 6.2).

$$\mathrm{B}_i = \mathrm{B}_{i,material} + B_{i,immaterial} \tag{6.2}$$

where B_i (MJ/kg), $B_{i,material}$ (MJ/kg) and $B_{i,inmaterial}$(MJ/kg) are the exergy, material exergy and immaterial exergy of stream i, respectively.

The material exergy of material flows depends on the standard material exergy of its components (Equation 6.3).

$$B_{i,material} = \sum (x_j \cdot b_j^0) \tag{6.3}$$

where x_j $(-)$ is the fraction of component j in stream i, b_j^0 (MJ/kg) is the standard material exergy of component j.

The most relevant forms of immaterial exergy in this study are energy provided by electricity and by burning natural gas (Equations 6.4–6.6).

$$B_{i,immaterial} = B_{i,electricity} + B_{i,natural\ gas} \tag{6.4}$$

$$B_{i,electricity} = E_e \cdot ß_j \tag{6.5}$$

$$B_{i,natural\ gas} = M_{natural\ gas} \cdot LHV \cdot ß_j \tag{6.6}$$

where $B_{i,electricity}$(MJ/kg) and $B_{i,natural\ gas}$ (MJ/kg) are the electrical and natural gas exergy of stream i, respectively. E_e(MJ/kg) is the electrical energy, β_j is a quality factor that relates the energy to the exergy content and is considered to be 1.0 and 1.04 for electricity and natural gas, respectively. $M_{natural\ gas}$ (kg/kg) is the mass of natural gas, and LHV (MJ/kg) is the lower heating value of natural gas.

Any output streams that are considered as waste are given an exergy value of zero. In this study, waste streams include all mass flows that are not meant for human consumption. In addition, it is assumed that water vapour and warm, moist air are not recoverable.

6.3.3.1. Exergetic indicators

The following exergetic indicators are used to evaluate the processes for their performance: cumulative exergy consumption (CExC), cumulative exergy losses (CEL) and exergetic efficiency (η).

The CExC is the sum of all material and immaterial exergy flows consumed in a process (Equation 6.7).

$$CExC = \sum B_{in} \tag{6.7}$$

The CEL (MJ/kg) is the sum of all material and immaterial exergy flows that are lost (i.e. destroyed and wasted) (Equation 8). Here, irreversible losses are considered as inevitable losses occurring due to, for example, heat transfer, phase changes, or inherent inefficiencies in process equipment.

$$CEL = \sum \mathrm{B}_{in} - \sum \mathrm{B}_{out} = \sum \mathrm{B}_{destroyed} + \sum \mathrm{B}_{wasted} \quad (6.8)$$

The rational exergetic efficiency η (%) is the ratio of the exergy of the useful fractions obtained to the CExC (Equation 6.9).

$$\eta = \frac{\sum(\mathrm{B}_{fraction})}{\sum(\mathrm{B}_{in})} \quad (6.9)$$

By calculating the exergy losses in each process, the critical exergy loss points (CEPs), defined as the locations where most exergy was lost, can be identified in each process.

Both CExC and η are used to assess the RUE of the seven scenarios.

6.3.3.2. Sensitivity analysis

A sensitivity index (SI) was calculated to screen for the most influential process parameters on the identification of CEPs (Zisopoulos et al., 2016). A high SI value indicates that the variability of the model parameters can influence the results of the exergy analysis substantially. The SI is defined as

$$SI_i^j = \left| \frac{I_i^{max} - I_i^{min}}{I_i^{Base\ case}} \right| \quad (6.10)$$

where SI_i^j is the sensitivity index for an indicator j at the maximum (I_i^{max}), minimal (I_i^{min}) and base case value ($I_i^{Base\ case}$) of a selected process variable i, with all other process parameters are at their base case values.

In addition, the influence of data variability on the results of the exergy analysis was assessed. The most influential input variables in the model were identified with the SI. The selected input variables were randomly varied in the model between their minimal and maximal values, and all other parameters were kept constant. All input variables were assumed to follow a normal distribution and they were simultaneously and randomly varied for a number of repetitions. The number of repetitions chosen was sufficient to obtain stable mean output distributions of CExC, CExL and η as represented in boxplots.

We then compared the exergy analysis on different bases. More specifically, we calculated the exergy consumption (CExC): (a) to process 1 kg of pea flour (MJ consumed/kg pea flour processed), (b) to produce 1 kg of starch (MJ consumed/kg starch produced), (c) to produce 1 kg of starch-rich fraction (MJ consumed/ kg starch-rich fraction produced), and (d) to induce a desired level of functionality when incorporating the starch-rich fraction in a food product (MJ consumed/functionality induced in the final product). In this study, functionality is considered as the peak viscosity value (i.e. the thickening property) of the starch-rich fraction.

6.4. Results and Discussion

6.4.1. Product mass-based exergy assessment

A summary of CExC and CEL for the base case scenario is shown in Figure 6.2. All three fractionation processes have a similar material CExC (approximately 16 MJ/kg pea flour) because the starting material was the same in all processes. The immaterial exergy consumption CExC is divided into CExC for burning natural gas and using electricity. CExC for burning natural gas is consumed mainly in the drying processes, and the amount consumed is similar in CWF and MWF (approximately 10–12 MJ/kg pea flour). CWF has the highest CExC for electricity (approximately 2 MJ/kg pea flour), because it involves the largest number of processes. Nevertheless, CExC for electricity in MWF is lower but in a similar range.

The exergy consumed for burning natural gas and using electricity is irreversibly lost in all fractionation processes. For that reason, the exergy lost for burning natural gas and using electricity almost equals the exergy consumed. CWF wastes a substantial amount of material and, therefore, material exergy (approximately 6 MJ/kg pea flour). DF has no material exergy losses because it makes complete use of the raw materials. In addition, it uses limited amounts of electricity (approximately 1 kJ/kg pea flour) and, therefore, it has a very high rational exergetic efficiency of approximately 99–100%. MWF has a lower exergy efficiency (54%) because all immaterial CExC is lost. CWF has the lowest exergy efficiency (35%), which can be explained by substantial losses of both material and immaterial exergy.

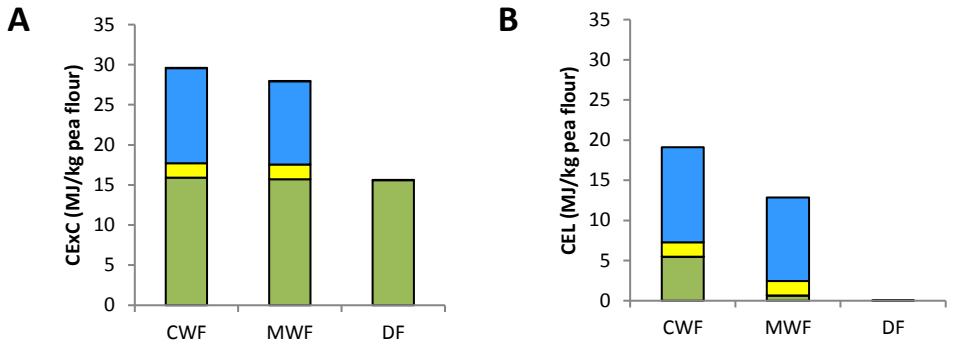


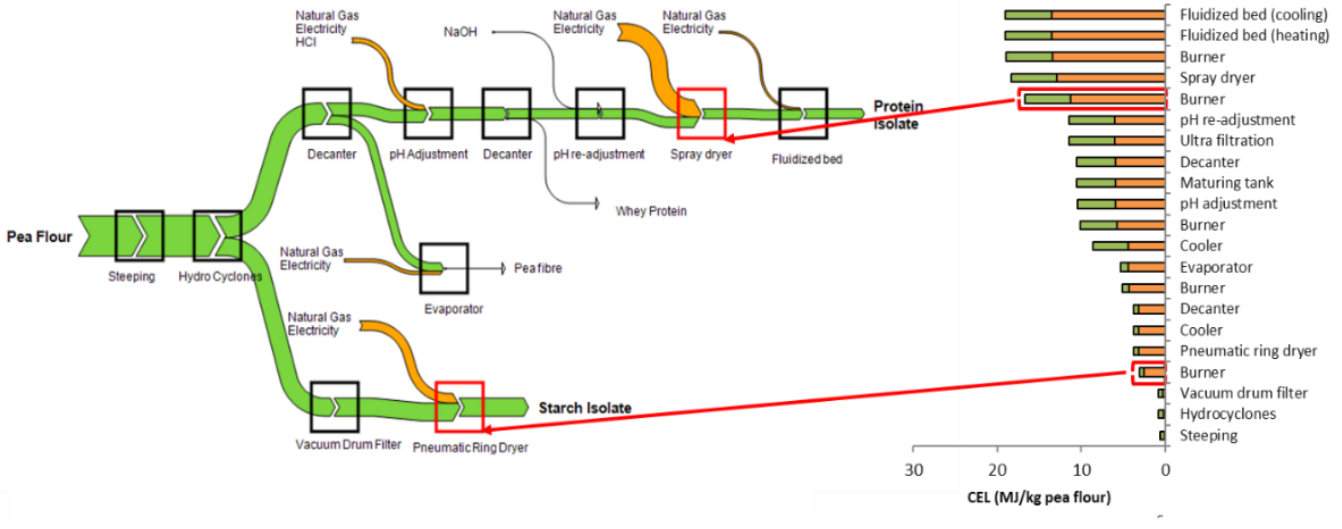
Figure 6.2 Summary of the exergy analysis for CWF, MWF and DF for the base case scenario. A) the cumulative exergy consumption of the fractionation processes, B) the cumulative exergy losses of the three fractionation processes.(material exergy, immaterial exergy is divided in electrical usage exergy and natural gas burning exergy).

6.4.1.1 Critical Exergy loss Points (CEPs)

Figure 6.3 shows the Grassmann diagram for CWF and MWF for the base case scenario. The Grassmann diagram for DF is not shown because it consists of only one processing step. The main CEPs in the CWF and MWF are related to the drying steps (i.e. spray drying and pneumatic ring drying). In CWF, spray drying and pneumatic ring drying account for approximately 40% and 15% of the CEL, respectively. In addition, evaporation is also an exergetically expensive process, accounting for approximately 26% of the CEL. The processing steps involved in pH adjustment account for 10% of the CEL, because the feed stream is heated during the pH precipitation step (Passe et al., 2012; Salome et al., 2007). In this calculation, the embodied exergy cost of producing the required materials (i.e. the acids and bases) is not considered because the focus of the research is on the RUE of the fractionation processes. Nevertheless, this exergy cost is expected to be considerably higher than the standard material exergy as has been shown in the case of the addition of exogenous enzymes used in beer brewing (van Donkelaar et al., 2016).

In MWF, the spray drying and pneumatic ring drying processes account for 57% and 33% of the CEL, and combined they are responsible for over 90% of the total CEL. Overall, these CEPs indicate the importance of reducing the number of drying steps because they are the most exergy-intensive processes (Dincer, 2011; Zisopoulos et al., 2017b).

A


Natural Gas
Electricity
HCl
NaOH
Natural Gas
Electricity
Natural Gas
Electricity
Protein
Isolate
Decanter
pH Adjustment
Decanter
pH re-adjustment
Spray dryer
Fluidized bed
Whey Protein
Pea Flour
Steeping
Hydro Cyclones
Natural Gas
Electricity
Pea fibre
Evaporator
Natural Gas
Electricity
Starch Isolate
Vacuum Drum Filter
Pneumatic Ring Dryer
Fluidized bed (cooling)
Fluidized bed (heating)
Burner
Spray dryer
Burner
pH re-adjustment
Ultra filtration
Decanter
Maturing tank
pH adjustment
Burner
Cooler
Evaporator
Burner
Decanter
Cooler
Pneumatic ring dryer
Burner
Vacuum drum filter
Hydrocyclones
Steeping
30
20
10
0
CEL (MJ/kg pea flour)

B

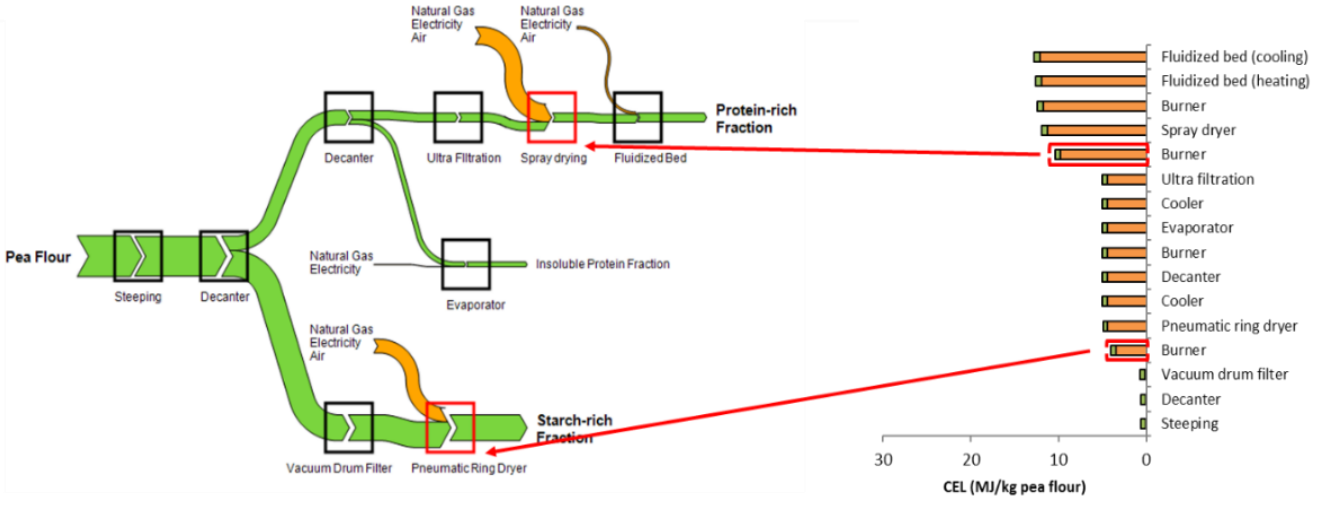


Figure 6.3 Grassmann diagram for the CWF (A) and MWF (B) processes for the base case scenario, depicting the cumulative exergy losses (MJ/kg pea flour) and the critical exergy loss points (CEPs). The Grassmann exergy flows and the cumulative exergy losses were subdivided into material (■) and immaterial exergy (■).

6.4.1.2. Sensitivity analysis

The sensitivity analysis indicates that there is no clear single predominant variable that has a big impact on the outcome of the analysis (Figure 6.4A). All SIs are below 0.11 (i.e. their assumed minimum and maximum values cause up to 11% variation in η). The SI for DF is negligible for all input variables, indicating that they have a limited influence on η. CWF and MWF show a similar SI profile, and only differ in the energy use at the evaporation process. The variability in the electricity use has limited influence on η because the SI of all processes that use electricity is low (i.e. below 1%) compared with the energy use required in the drying processes (i.e. between 1% and 6%). The input variables that have a high SI (burner, spray dryer, pneumatic ring dryer, evaporator and fluidized bed dryer) relate mainly to drying and to the burning of natural gas.

Figure 6.4B shows the influence of data variability on the results of η. The η of DF is not affected because it does not include any drying or natural gas burning processes. Some uncertainty is observed in η for CWF and MWF due to variation in energy use for drying and burning natural gas. The median and the first and third quadrants (indicated by the box) are all close at 35%, 34%, and 36% for CWF, and 54%, 53%, and 55% for MWF; their corresponding lower and upper confidence intervals do not overlap. Therefore, the initial interpretation of the outcome of the exergy analysis can be considered as valid for the assumptions studied and the input variables tested.

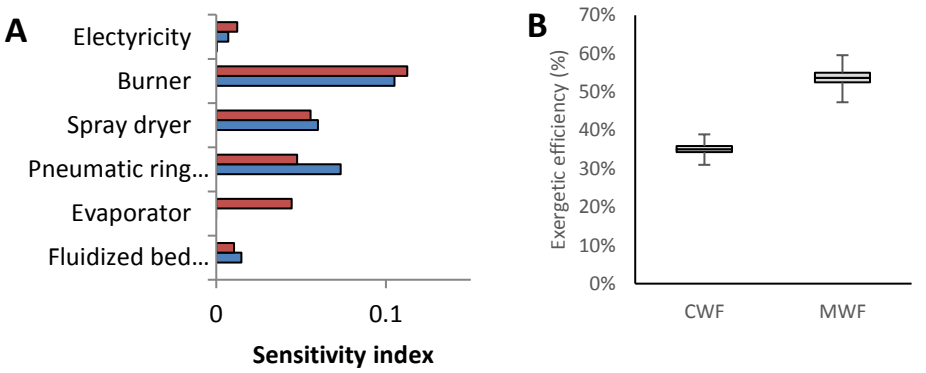


Figure 6.4 A) Sensitivity indexes for the rational exergetic efficiency (%), CWF , MWF and DF processes. B) The influence of the data variability on the results of exergy efficiency (η).

6.4.1.3. The effect of the scenarios

The assessment of the six different scenarios shows that even though the order of η for the three fractionation processes remains unaffected, their values for CWF and MWF vary (Figure 6.5).

In scenario 2, CExC for all the fractionation processes becomes similar, due to the dominant share of material exergy of the pea four; η is mainly affected by waste production. DF and MWF produce a limited amount of waste and therefore have high η. CWF leads to fractions that are not suitable for human consumption, and, therefore, has a lower η.

In scenario 3, the fibre-rich ingredient produced in CWF is not considered as waste, resulting in an increase in η from 35% to 46%. In scenario 4, the insoluble protein fraction produced in MWF is assumed to be not suitable for human consumption, making it waste and leading to a small decrease in η from 54% to 51%.

In scenario 5, the insoluble protein fraction is dried in a spray dryer instead of an evaporator, resulting in a substantial decrease in η from 54% to 42%. The use of an inefficient drying method has a large influence on the immaterial exergy consumption and subsequently on the η and CExC of the technology. Although it is generally assumed that complete use of raw materials is a priority for developing sustainable food process designs (Vandermeersch et al., 2014; Zisopoulos et al., 2015; Zisopoulos et al., 2017b), this analysis showed that it can also be important to focus on the efficient use of immaterial exergy. A comparison between scenarios 4 and 5 indicates that complete use of materials requires more energy-intensive drying processes. The η for MWF is higher in scenario 4 than scenario 5, indicating that wasting the insoluble protein stream is perhaps a more preferable option than drying it in an inefficient manner.

In scenario 6, the production of a liquid concentrate instead of a dry protein powder has a strong effect on the η and CExC for CWF and MWF. For CWF and MWF, the spray drying process is a CEP, accounting for over 57% and 38% of the exergy losses, respectively.

In conclusion, all scenarios point in the same direction: the relative exergy efficiency is best for DF (around 100%), followed by MWF (44–95%) and lowest for CWF (35–65%).

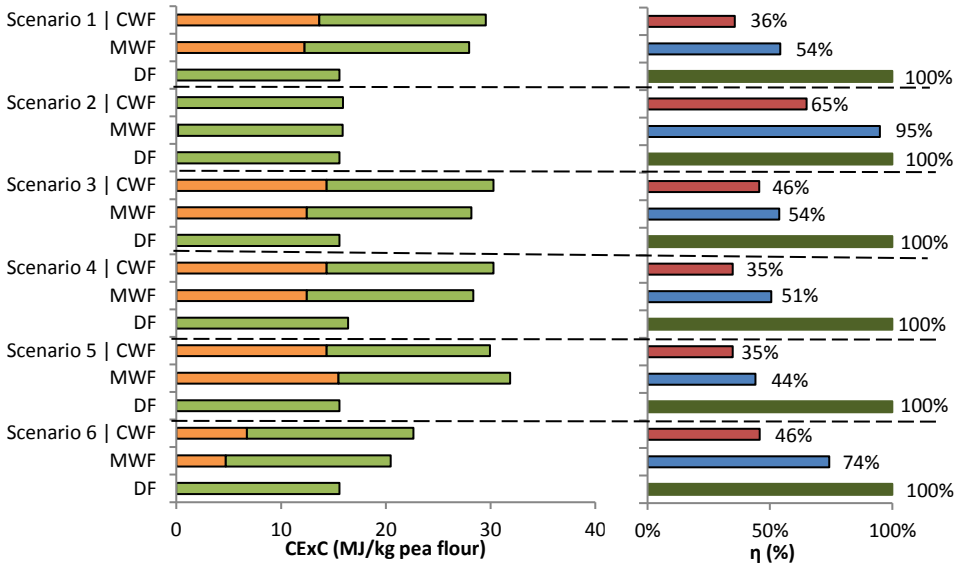


Figure 6.5 The cumulative exergetic consumption (left) and rational exergetic efficiency (right) of the different scenarios. The cumulative exergetic consumption was subdivided into material (■) and immaterial exergy (■) in the ■ CWF , ■ MWF and ■ DF processes.

6.4.2. Functionality based exergy assessment

Until now, we have expressed the results of the exergy analysis on a raw material basis (MJ/kg pea flour). Figure 6.6 shows the results of CExC and η expressed in different units: MJ/kg pea flour, MJ/kg starch, MJ/kg starch-rich ingredient and MJ/functionality. Overall, η is not affected by the unit used, but the CExC itself does change. All three processes start with the same quantity of pea flour and yield similar quantities of starch, however the quantities of starch-rich fraction obtained differ (Table 6.1). Subsequently, the units MJ/kg pea flour and MJ/kg starch have similar ratios for all three processes, however, when expressed per kg starch-rich ingredient, the ratios of the fractionation processes differ. MWF and DF yield more starch-rich fraction than CWF, and consequently their CExC decreases compared with CWF.

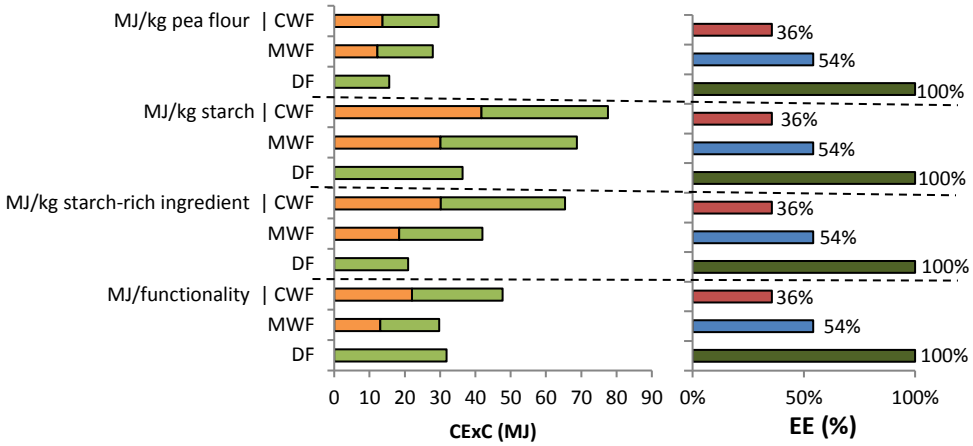


Figure 6.6 The cumulative exergetic consumption (left) and rational exergetic efficiency (right) expressed in different units: MJ/kg pea flour, MJ/ kg starch, MJ/kg starch-rich ingredient and MJ/ functionality (at a viscosity of 4 Pa s). The cumulative exergetic consumption was subdivided into material (■) and immaterial exergy (■) in the ■ CWF , ■ MWF and ■ DF processes.

Finally, the amount of starch needed to arrive at the required functionality, here viscosity, is also included in the evaluation. Figure 6.7A shows that the starch-rich fractions produced in CWF and MWF have similar thickening properties (Geerts et al., 2017b), whereas those produced in DF have less thickening effects (Pelgrom et al., 2015). Therefore, a larger amount of starch-rich fraction produced by DF should be added. Because more DF starch is needed for a given viscosity, the CExC expressed as MJ/functionality of DF becomes comparable with that of MWF or even higher when even higher viscosity is required (Figure 6.7B). It might be possible that DF-produced material performs better for other functional properties. In that case, the advantages of DF as fractionation method become even greater. However, in this example, DF is the preferred option when classic exergy units are used because it has the lowest CExC and the highest η. When the results are expressed in terms of MJ/functionality, CExC for DF becomes comparable with that for MWF, despite the highest η.

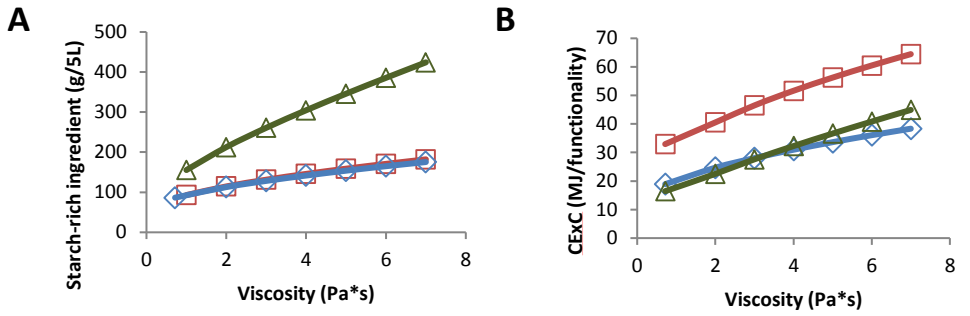


Figure 6.7 A) The viscosity of starch-rich fractions in water (g/L) for CWF (□), MWF (△), and DF (◇) fractionation; adapted from Geerts et al. (2017b). B) The cumulative exergy consumption for CWF, MWF and DF at different viscosities.

6.5. Conclusion

In this study, we compared three different processing routes for the fractionation of yellow pea (*Pisum sativum*) and present functionality as a basis for environmental performance calculations. This extension of exergy analysis allows ranking of process routes according to the minimal exergy needed to arrive at a certain functionality needed for a food product. This analysis swapped the ranking of two mild fractionation methods in our case study. To arrive at this conclusion, we investigated the theoretical RUE of three fractionation processes for yellow peas, and discussed the impact of (a) alternative scenarios and (b) different bases for exergy consumption on interpretation of the results of the exergy analysis.

For all mass-based scenarios, DF outperforms CWF and MWF with a rational exergetic efficiency of 99–100%, due to the almost complete utilization of all fractions of high material exergy (approximately 16 MJ/kg), obtained with a very low physical exergy consumption (approximately 1 kJ/kg pea flour). MWF was more exergy efficient than CWF due to the substantial material losses (i.e. material exergy wastage) occurring in the latter. This result highlights that waste prevention should become a priority in the design of fractionation processes, which is in line with other studies.

CWF and MWF have an exergy efficiency that ranges between 35% and 65%, and between 44% and 95%, respectively, but those values vary substantially when considering the different mass-based scenarios. The different exergy efficiency values indicate the relevance of assessing different possible scenarios. The sensitivity analysis identified that the drying and natural gas burning steps have the

largest influence on the results of the exergy analysis. Nevertheless, the data variability had a minor influence on the results of exergy efficiency.

Finally, it was shown that consideration of exergy consumption to induce a desired functionality (i.e. viscosity) can provide an additional perspective to exergy analysis. More specifically, it was shown that the starch-rich fraction produced in the DF process is less favourable for thickening. This research stresses the relevance of linking the concept of exergy with food ingredient functionality as a means for comprehensive analysis of the RUE of food processing processes.

7

General discussion

Enriched functional fractions can be created by minimal and mild fractionation. Mild processing has the potential to optimize the use of the raw material and to limit the use of water, chemicals and organic solvents. However, enriched fractions still contain other components, and the properties of such enriched fractions may differ from those of the pure ingredients. The thesis aims to investigate the consequences of minimal and mild fractionation on product formulation and the resulting properties. This chapter summarizes the main findings of the previous chapters and discusses the opportunities and constraints of the functional fractionation concept.

7.1. Main findings

Current fractionation processes are set up to obtain targeted components of high molecular purity, despite the high processing intensity required (Augustin et al. 2016). Nevertheless, the fractionation of agro material can often be done in a milder manner, thereby minimizing the processing steps essential for the separation of the main components (e.g. starch, protein, oil). In this thesis, an aqueous fractionation method was used to obtain protein- and starch-rich fractions from yellow pea and soy bean flour. The aqueous fractionation method used was based on the natural organization and immiscibility of the main components. The components were detached through milling, followed by soaking in water and fractionating using centrifugation forces.

In Chapters 2 and 3, the functional properties of the mildly refined fractions were further investigated. The functional properties of the starch fraction were mainly determined by the concentration of starch, but the other components present (mainly fibres) also influenced the functional properties. Depending on the application, the presence of additional fibres could be beneficial; for example, pasting properties are enhanced as a result of the water binding properties of the fibres. In addition, the functional properties of the protein fraction were mainly determined by the state of the protein. The presence of small solutes barely influenced the functional properties, meaning that further purification was not needed from a functionality perspective.

Chapters 4 and 5 explored the applicability of the mildly refined fractions in model systems. The fractions from yellow pea were explored in a (thickened) oil-in-water emulsion and found to have the functional properties of the commercial isolates but with additional functionality. The difference in functional properties can be advantageous, but the potential depends on the final application. The latter indicates the relevance of a strong link between fractionation and application. In addition, the applicability of protein fractions from soy beans was explored for formation of a fibrous structure for

use in meat analogues. The study gave an indication of the most essential functional properties needed to create fibrous structures (e.g. a high water holding capacity (WHC), intermediate nitrogen solubility index (NSI), and a viscoelastic property (*G**) between 1 and 10 kPa) and demonstrated the relevance of a final toasting step on these functional properties. Overall, the study indicated that one should not always strive for classic functionality, such as high solubility. The most promising structures were obtained with a mixture of limited or even unprocessed full-fat flour and a more intensively processed protein fraction. This combination is an interesting concept of increasing the sustainability of the whole by using a blend of native and (mildly) fractionated ingredients.

In Chapter 6, the resource use efficiency of the mildly refined fractions was investigated. The fractionation process for yellow pea flour discussed in Chapters 2, 3 and 4 was used as example. The resource use efficiency was determined using an exergy analysis. Initially, the outcomes of the exergy analysis were expressed on the basis of mass (MJ/kg). However, food ingredients were added to obtain a specific functionality, therefore the outcomes of the exergy analysis were also expressed on the basis of functionality (MJ/functionality). Expressing the outcomes based on functionality instead of mass influenced the interpretation of the exergy analysis; the mildly refined starch fraction had better pasting properties than the starch fraction obtained with dry and conventional wet fractionation processes. Overall, this indicates that fractionation methods should strive not only for optimal (exergetic) efficiency but also optimal functionality.

7.2. Considerations on functionality and fractionation methods applied

In this thesis, functionality was mainly based on the structural properties, but functionality is more than that. Functional properties also include taste, colour, nutritional value, etc. The presence of additional components that are probably present in mildly fractionated ingredients can have a substantial influence on the functional properties; e.g. taste can be affected by the addition of legume-based ingredients (Shehata et al. 1988). This aspect was not considered in this thesis but should be included in future research.

The functional properties of food products, in our case the structural properties, are related to the molecular composition, but products with similar composition can vary in functional properties. For instance, tofu and Greek yoghurt are both high in fat and protein, and even though they are similar in composition, the functional properties of the two products differ. These differences in functional properties are related to the differences and order of the processing methods applied. In this thesis, the conventional fractionation process was minimized and the order of the processing steps was

altered. Subsequently, the mildly refined fractions were compared with conventional ingredients to gain understanding (Figure 7.1), and conventional ingredients and fractionation processes were used as reference. The conventional ingredients and processes helped us to define goals initially, but this approach might restrict the use of more innovative processing steps. For instance, in-house experiments show that pea protein and starch can be separated using simple shear, similar to the separation of wheat gluten and starch from wheat flour (Peighambardoust et al. 2008). In addition, the solubility of plant proteins is influenced by temperature. For example, the solubility of pea protein changed significantly when cooled from room temperature to freezing temperatures, which can be used as a means of fractionation. Therefore, further research should consider these and other potential fractionation processes and compare them with the conventional process.

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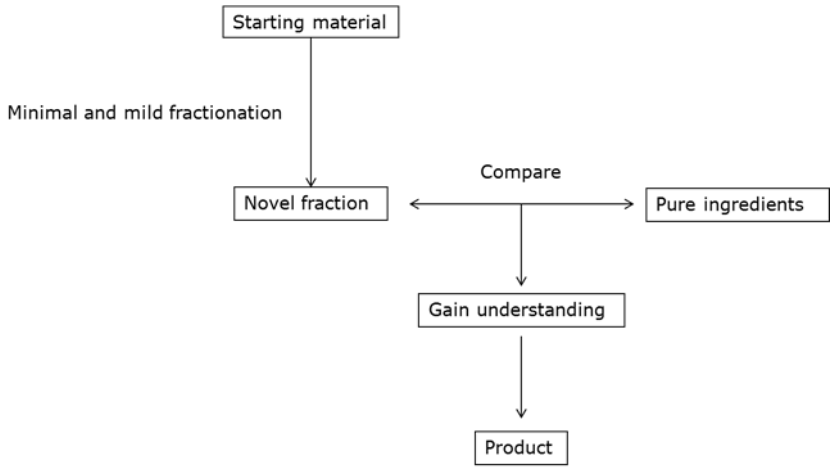

Figure 7.1 The main approach used to evaluate the potential of the novel fractions obtained

Functional properties are affected by the processing conditions during fractionation, for example, the drying method used (Hu et al. 2010; Berghout et al. 2015a). In addition, the drying method has a substantial influence on resource use efficiency (**Chapter 6**) (Raak et al. 2017), as a result of the energy-intensive nature of this process (Fox and Akkerman 2010). In research, a mild drying method is preferred for the main components (e.g. freeze drying), but this drying method has low resource efficiency. Mild drying is generally desired because it renders proteins with high solubility, which is considered to be beneficial. However, high solubility is not always needed; a relatively low solubility is preferable for creating a fibrous texture, as shown in **Chapter 5.** This demonstrates that research in food technology should be more holistic, not only focusing on the purity of ingredients but taking the functionality in the application as the starting point.

7.3. Define functionality instead of purity

Food ingredients are normally used for specific functional properties. For example, starch is added to improve pasting properties, gelation or reduce syneresis (**Chapter 2**). In contrast to pure ingredients, traditional ingredients such as eggs are multi-functional; they can be used as an emulsifier, thickening agent, raising agent and a glazing agent (Moleman 2017). We believe that effective food processing chains are characterized by a strong link between fractionation and application. However, a strong link can only be achieved if the functional properties required are clearly defined.

Functional properties are often not created by a single component. A combination of ingredients is needed. For instance, the creaminess of whipped cream is created by protein-stabilized fat droplets that stabilize in the entrapped air bubbles (Smith et al. 2000). The interactions between protein and fat in the cream are of interest, rather than their individual properties (Bongers 2009; Almeida-Riveraa et al. 2007). The same holds for wheat flour used to create the spongy structure of bread. Here, the interaction of gluten and starch is essential.

In addition, recent research on novel protein structures indicated the relevance of the presence of a natural matrix. In novel protein structures for meat replacers, soy protein concentrate (≈70% protein) showed more potential than soy protein isolate (>90% protein) (Grabowska et al. 2016). Soy protein concentrate consists mainly of protein and polysaccharides. The presence of both components seems to be essential to accomplish the fibrous structure (see **Chapter 5**). Soy beans were fractionated into protein fractions for use in meat analogues. The soy beans were fractionated without the use of any organic solvents, and thus the fractions did not have the highest molecular purity and even contained some oil. This protein fraction led to distinct fibres when mixed with unprocessed soy bean flour.

The mildly refined starch and protein fractions showed similarities with their commercial counterparts (**Chapters 2 and 3)**. Both cases indicated that high purity is not always necessary from an application point of view. This shows that the specific functionality required, not the purity, determines the fractionation route. Therefore, there is a scientific need to understand and clearly define functionality.

7.4. Towards an integrated food system.

7.4.1. Fractionation using natural structures

Agro materials have a naturally high degree of organization. Jørgensen (2015) tried to include the degree of organization in his approach to determine the eco-exergy of organisms. Jørgensen (2015) indicated that the (eco-)exergetic value of agro material is higher than the sum of the main components. This difference was accounted for by a so-called β value, which describes the ratio

between the eco-exergy and the chemical exergy of all components. The fractionation of agro materials in highly purified ingredients reduces the degree of organization, and the (eco-)exergetic value becomes equivalent to the standard specific chemical exergy of the main components. Upon product assembly, these ingredients are structured again, thereby increasing the degree of organization.

Ideally, processing from agro material to ingredient and subsequently from ingredient to food product does not break up structures followed by structure build up, but only alters structures from one form to another (Figure 7.2). This ideal route is presented in most traditional ingredients (such as cream, flour, etc.), where the agro material is not entirely fractionated, but altered from its initial structure to the desired structure.

In legume seeds (and in potatoes, corn, etc.), the main components are organized in structural elements. Preferably, mildly refined fractions make use of and subsequently maintain part of the structures and complexes present in the agro material. It can be hypothesized that maintaining natural structures decreases the gap from the agro material, ingredient and pre-prepared food product. Presumably, a lesser degree of organization has to be destroyed and re-created during fractionation and structuring, resulting in higher research use efficiency.

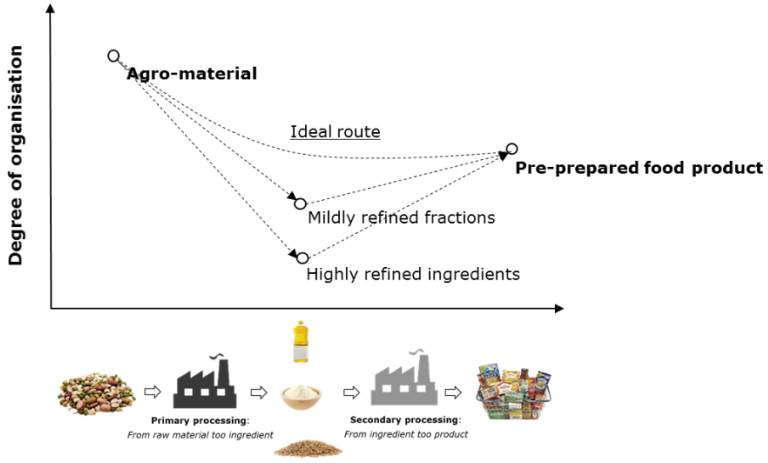

Figure 7.2 Degree of organisation within the food processing chain

An example of a fractionation process in which the native structure and properties are maintained is dry fractionation. In the case of dry fractionation, precise milling increased the fractionation efficiency (Pelgrom et al. 2015a), because macrocomponents (in legume seeds) are often present in well-defined structural elements, e.g. oil bodies, protein bodies, starch granules. Oil bodies are spherical lipid-rich

structures of approximately 0.1–2.5 µm, which are stabilized by triglycerides and specific proteins (oleosin) (Tzen and Huang 1992). Protein bodies are spherical protein-rich structures of approximately 1–20 µm, which contain an amorphous protein matrix stabilized by a single membrane layer (Huang 1985; Plant and Moore 1983). Starch granules are semi-crystalline starch-rich structures approximately 1–100 µm (Jane et al. 1994). These storage organelles are incorporated in cotyledon cells of the legume seeds. The cell walls of those cotyledon cells mainly consists of cellulose, hemicellulose, pectin and non-storage protein.

These structural elements can be detached through milling. The resulting flour can be dry separated through air classification, but it is also possible to suspend the milled flour in water. Upon centrifugation, pea flour separated into three or four layers that can be collected. For starch-rich legumes as well as wheat, it was reported that the top layers are enriched in protein, whereas the bottom layer is rich in starch (Pelgrom et al. 2015b; Czuchajowska 1993; Otto et al. 1997). For oil-rich legumes, phase separation of the main macronutrients occurs as a result of the immiscibility of oil in water. Oil bodies can be collected in a cream-rich phase (Campbell and Glatz 2009).

If required, the enriched fractions can be further purified. For instance, several enzyme-assisted protocols are designed to release the oil out of oil bodies. However, the enzymes used will also affect the proteins present, decreasing their quality. In addition, oil bodies are stable over a wide range of temperature and hydration and are therefore proposed as a potential alternative for conventional emulsions (Campbell and Glatz 2009; Jung 2009; Deckers et al. 2000; Nikiforidis et al. 2012). In other words, more intense purification can make the ingredients more prone to degradation.

7.4.2. Resource use efficiency, avoid food waste

The fractionation of agro material into different fractions creates by-products. This is not directly negative, as long as all fractions can be used in the food chain. Nevertheless, the fractionation of highly refined ingredients is related to the production of by-products with reduced quality (Raak et al. 2017). Reduction of the amount of these by-products is difficult to achieved if the target of fractionation is the production of highly refined ingredients (Augustin et al. 2016). Focusing on highly refined ingredients decreases the yield and increases the amount of side streams created (Berghout et al. 2015b). For instance, in the transformation of soy flour to soy protein isolate, about 40% of the total amount of protein present is lost for human consumption (Gueguen 1983; Berghout et al. 2015b; Alibhai et al. 2006). That is why we suggest using the term “avoidable waste” for that part of the by-products created during fractionation that was originally suitable for human consumption. Maslow’s hierarchy for minimizing food waste indicates that reducing food waste is preferred over re-using, re-

cycling and disposing (Vandermeersch et al. 2014; Papargyropoulou et al. 2014). Recent sustainability analysis points in the same direction, namely that material loss prevention has a key role in improving the environmental impact of the whole process chain (Zisopoulos et al. 2015) (**Chapter 6**).

7.4.3. Effect on Health: risks and opportunities of functional fractions

Pre-prepared food products are often energy dense and low in micronutrient content and fibers, partly due to the use of highly-refined ingredients. The consumption of these pre-prepared food products are assumed to play an important role in the main welfare-related illnesses in the developed world, such as obesity, diabetes, cardiovascular diseases and certain cancers (Augustin et al. 2016; Who and Consultation 2003; Stookey 2001; Vandevijvere et al. 2010; Jebb 2005; Miller and Welch 2013).

The natural matrix of agro-materials is rich in fibers and micro-nutrients. It is known that intensive processing leads to loss and degradation of micro-nutrients, such as vitamins and trace elements (Reddy and Love 1999). In addition, fibers are often removed to increase the purity of a component, even though dietary fibers are known for their beneficial health effect. Removal of fibers leads to higher glycemic index after consumption of for carbohydrate–rich products, which is linked to diabetes and cancer (Kaczmarczyk et al. 2012; Gangwisch et al. 2015).

From a health perspective, mild and minimal refining can be positive as it will result in rendering more fibers and micronutrients in the final food product. However, also anti-nutritional compounds or taste influencing compounds could remain in the product such as protease inhibitors (trypsin inhibitors), amylase inhibitors, lectins, polyphenols, saponin, phytic acid etc. (Van der Poel 1990; Khokhar and Chauhan 1986; Schutyser et al. 2015). Part of those components can be inactivated through toasting or are removed with post processing, like cooking. It should be noted though that those processes will also influence the functionality of the bio-molecules and micronutrients present, though this change is not always negative (Chapter 6). For that reason, a consideration has to be made between nutritional value, taste, functionality, etc., and additional research on novel potential inactivation techniques is needed (Knorr et al. 2011).

7.5. Future research

In past decades, complex processing was applied to obtain simple and pure material streams. However, several new factors, such as the increased importance of sustainability and altered consumer behaviour, have led to a shift towards milder and minimal processing, resulting in more

diverse material streams. This approach will result in ingredients and products that meet the needs of consumers for more sustainable, natural and healthier products.

The research described in this thesis outlines how the potential of mild fractionation can be fully used to strengthen the link between fractionation and application. This coupling will lead to more ingredients with specified functionality, not component purity. Milder processes can be used for the production of these ingredients.

The concept described above requires adaptation of current characterization methods. Current methods and protocols are aimed at general functional properties. Specific functional properties cannot always be fully quantified using these general methods. The consequences of the concept are demonstrated in **Chapter 5**. Here, the functional properties of a soy protein fraction were investigated for use in meat analogues. The soy protein fractions were structured at high temperatures and pressures. A normal rheometer at standard conditions gives only limited information on the structuring properties of soy protein fractions. We therefore proposed the use of a closed cavity rheometer instead, which is more often used in the rubber industry (Burg et al. 1987) to mimic the conditions and subsequently functional properties essential during structuring. Overall, the use of more innovative methods to determine the functional properties will help to get a better understanding and to better define the required functional properties.

In this thesis, an aqueous fractionation method was mainly used. Although aqueous fractionation has its benefits, exploring other mild and minimal fractionation methods could also be interesting. Fractionation methods that minimize the use of water are preferred, such as dry fractionation or fractionation using simple shear (Pelgrom 2015; Peighambardoust et al. 2008). The natural characteristics of the components can be used during fractionation, for instance by understanding the solubility characteristics of protein. It is clear that the improvement from these innovative processes in terms of research use efficiency needs to be quantified to allow evidence-based solutions instead of intuition to determine the sustainability of a process.

The functional fractions obtained are mostly of a lower purity than conventional ingredients. The other components present influence the functional properties of these multi-phase systems. The effect of these components depends on the ratio present in the multi-phase system. Therefore, more knowledge is needed on the influence of these other components on the functional properties by reconstructing functional fractions in model systems and investigating the effect when the ratios in the multi-phase system are altered.

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Summary

Food products often contain highly refined ingredients, with the advantages of mostly constant quality, easy of handling and global sourcing. However, the production of these highly refined ingredients requires harsh processing conditions. In addition, consumers no longer appreciate products containing highly refined ingredients because they are perceived as “unnatural”. This explains why sustainable concepts aim to limit processing or to use mild conditions if processing is unavoidable. Mild fractionation not only gives a more natural impression of products based on these ingredients but also potentially adds to resource efficiency.

In this thesis, we explore the concept of minimal and mild fractionation and the research questions that arise. Here, aqueous fractionation is used as a mild process for the fractionation of starch and protein from yellow pea (*Pisum sativum*) and protein from soy beans (*Glycine max*). The functional properties of the protein- and starch-enriched fractions are investigated and related to the applicability and the resource efficiency of those fractions.

Chapter 2 investigates the functional properties of the mildly refined starch fraction obtained with a mild aqueous fractionation process. The mildly refined starch fraction had a lower purity than conventional starch isolates as a result of the presence of residual fibres. Overall, the mildly refined starch fraction can be best described as a phase-separated system, with the fibres dispersed in a continuous starch phase. The fibres present influence the functional properties. The pasting properties were positively influenced by the presence of the fibres. However, the gel hardness was influenced by interruption of the continuous phase. The results indicate the relevance of a clear view of the functional properties required in the product, because the presence of other components (in this case fibre) can have desirable as well as undesirable effects, depending on the properties required for certain applications.

Chapter 3 investigates the functional properties of the mildly refined protein fraction obtained with a mild aqueous fractionation process. The emulsification properties were compared with commercially available pea protein isolate. The emulsification properties can be best explained by the state of the protein present. The protein in the mildly refined fraction was still native protein, whereas the proteins in the commercial isolate were denatured and (partly) aggregated. The native protein formed a stronger, viscoelastic layer on the oil-water interface, providing protection against disruption and high compressive forces. Overall, emulsions stabilized by the mildly refined protein fraction became less prone to flocculation and coalescence.

Chapter 4 explores the applicability of mildly refined ingredients in a model system of a thickened oil-in-water emulsion, focusing on the mildly refined soluble protein and starch fractions discussed in Chapters 2 and 3. The mildly refined fractions showed similar behaviour to commercial isolates, although their behaviour was richer in the model system. The mildly refined protein fraction, for instance, showed good emulsification properties and was able to thicken the emulsion when a heating step was applied. Depending on the application, these differences can be beneficial. In the model system, for instance, less additional starch would be needed because the protein acted not only as an emulsifier but also a thickener.

Chapter 5 focuses on the importance of a more direct link between fractionation and application. A fractionation process was designed to obtain a soy protein fraction specifically for making highly fibrous structured materials as the basis for a meat analogue. The more direct link between fractionation and application revealed the most essential functional properties for structuring. These functional properties were quite different from the properties normally aimed for. The results indicated that high solubility was not favourable and an added toasting step was required to lower the solubility. In addition, the most promising results were obtained with a blend of intensively fractionated ingredients and native flour. The use of this combination is an interesting route to decrease the environmental impact of the ingredients of a product.

Chapter 6 investigates the influence that the concept of minimal and mild fractionation has on resource use efficiency. The fractionation process for yellow pea flour discussed in Chapters 2, 3 and 4 was used as the basis (here referred to as mild wet fractionation). The process was compared with dry fractionation and conventional wet fractionation. We investigated how functionality could be included in such an exergy analysis and how it influenced the interpretation. Classic exergy analysis outcomes are mostly expressed on the basis of mass (MJ/kg), despite the fact that food ingredients are generally added to obtain a specific functionality. Therefore, we explored whether sustainability analysis can be based on functionality (MJ/functionality). The classic exergy analysis indicated the clear benefits of the dry fractionation process, because of its high exergetic efficiency. Nevertheless, when results were expressed on the basis of functionality, a different interpretation was obtained indicating the benefits of the mild wet fractionation. The starch fractionation obtained with the mild wet fractionation method had better functional properties, indicating that fractionation methods should strive not only for optimal (exergetic) efficiency but also optimal functionality.

Chapter 7 provides a general discussion of the main findings and recommendations for future research. In this thesis, functionality is mainly described by the structural properties, but functionality is more than this. A more holistic view is recommended, taking all aspects into consideration and creating a strong link between fractionation and application. A clear definition of functionality is essential. This will help evaluate the presence of the other components in the mild fractions and determine the necessary purity of the novel ingredient. Subsequently, the degree of fractionation required and possible use of the natural structure present in the agro material seem to be a promising route for further study. Finally, a few tactics are discussed to tackle the research needed to fully use the potential of functionally driven fractionation.

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Knowledge is in the end based on acknowledgement
(Ludwig Wittgenstein)

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About the author

Marlies Geerts was born on 12 January 1990 in Eindhoven, the Netherlands. She attended Rythovius College in Eersel, where she obtained her VWO diploma in 2008, with a major in Natuur en Gezondheid (Nature and Health).

Marlies studied Food Technology at Wageningen University. In her Bachelor thesis at the department of Food Physics department she worked on the structure-functional relation of novel surfactants, obtaining her bachelor degree in 2011. After which Marlies continued with the Master Food Technology, with the specialisation Sustainable Food Process Engineering. She conducted her Master thesis at the department of Food Process Engendering, where she worked on the activated carbon structure for the chromatographic enrichment of bio-active peptides. For her internship, she joined the department of Bio-Process Engineering at the Danish Technical University to study the expression and purification of enzymes for the generation of human milk oligosaccharides.

After completing her Master studies in 2013, Marlies continued working as PhD at the Food Process Engineering department of Wageningen University on the project *Bio-refinery of solid raw materials for food purposes.*

Contact: marliesgeerts@live.nl

Publications

Geerts MEJ, Mienis E, Nikiforidis CV, van der Padt A & van der Goot AJ (2017) Mildly refined fractions of yellow peas show rich behaviour in thickened oil-in-water emulsions. Innovative Food Science & Emerging Technologies. 41, 251-258.

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Overview of completed training activities

Discipline specific activities

Courses

Reaction kinetics in food science	2014
Multivariate analysis for food sciences	2014
Design of Experiments	2014
European school of Rheology	2015
Process Economics & Cost Engineering	2015
Advanced Food Analysis [a]	2015

Conferences

Nederlands Process Technology Symposium (Utrecht, the Netherlands) [a]	2014
EFFoST (Uppsala, Sweden) [a]	2014
Wageningen PhD symposium (Wageningen. The Netherlands)[c]	2015
EFFoST (Vienna, Austria)[b]	2016
EFFoST (Sitget. Spain)[b]	2017

General courses

VLAG PhD week	2014
PhD carousel	2014
PhD carousel	2015
Project and Time Management	2015
Mobilising your scientific network	2015
Effective behaviour in your professional surroundings	2015
Technique for writing & presenting a scientific paper	2016
Scientific Writing	2016
Career Perspectives	2017

Optional courses and activities

Food Process Engineering Group day [a,b]	2014-2017
VLAG PhD council	2014-2016
Wageningen PhD council	2014-2016
PhD study tour, Chile and Brazil [b]	2014
PhD study tour, Germany and Switzerland [a,b]	2016

[a] *Poster presentation*
[b] *Oral presentation*
[c] *convener*

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