Functionality-driven fractionation of lupin seeds

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Functionality-driven fractionation of lupin seeds

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Thesis

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof. Dr M.J. Kropff,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Friday 8 May 2015

at 1.30 p.m. in the Aula.

Jacqueline A.M. Berghout

Functionality-driven fractionation of lupin seeds,

166 pages.

PhD thesis, Wageningen University, Wageningen, NL (2015)

With references, with summaries in Dutch and English

ISBN 978-94-6257-228-7

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Chapter 1
Introduction and thesis outline

Highlights:

- Lupin seeds are rich in protein and dietary fibres and contain unsaturated fatty acids

- Simplified fractionation processes for functional fractions

- Focus on functional fractions rather than molecular purity

The growth in the world population requires an increase in the production of protein-rich foods. About two-thirds of the protein in the European diet is obtained through animal-based food products, like meat and dairy (de Boer et al., 2006). The production of these products requires significant amounts of land, nutrients, (fresh) water and valuable plant proteins (Aiking, 2011). For example, the production of 1 kg of animal protein requires 6 kg of plant protein (Pimentel and Pimentel, 2003). It would be more efficient to include these plant proteins in the human diet directly.

Currently, the increasing demand for proteins is met through increased production of tropical crops like soybeans. As a consequence, large scale deforestation and land degradation are enhanced. To encourage a more sustainable use of land, the biodiversity and rotation of crops need to be improved (Aiking, 2011). Besides, the exploration of crops that can be grown in temperate climates should be encouraged. Legumes that can be grown in temperate climates like the north-western parts of Europe are amongst others white beans, red kidney beans, broad beans, lupin seeds, rapeseed, several types of peas, and since recently also quinoa.

1.1 Lupin as a novel crop for plant protein

Lupin seeds have the potential to become a 'novel' plant protein source for food products because they can be grown in temperate climates as in Northern Europe and on different soil types (Berger et al., 2008; Sujak et al., 2006). The potential of lupin as protein source was already recognized around World War I, when banquets with amongst others lupin coffee, lupin margarine and lupin soup being consumed were reported (Becker-Dillingen, 1929). The main drawback of the consumption of lupin seeds was their high level of alkaloids, i.e. bitter compounds that made the seed unpalatable and sometimes toxic for human consumption (Sujak et al., 2006). In recent years, sweet lupin with an alkaloid content lower than 200 mg/kg, and thus less toxicity for humans, have been bred, e.g. Australian *Lupinus angustifolius*, *Lupinus albus* and Andean *Lupinus mutabilis* (Chew et al., 2003; Ranilla et al., 2009; Wu and Sun, 2012). An important advantage of the lupin plant is its ability to fixate nitrogen in the soil and thus to improve the soil fertility, and improve crop yields when rotating the crops with for example potatoes and wheat (Fumagalli et al., 2014; Honeycutt, 1998; Huyghe, 1997). With the upcoming shortage of phosphorus, another advantage of the lupin plant is its ability to acquire phosphorus from soil when grown under phosphor-deficient conditions by a specific root structure: cluster roots (Liao et al., 2012).

Lupin seeds of *L. angustifolius* L. are composed of a hull (25 wt%) and two cotyledons

(75 wt%) (Fig 1). The cotyledons are rich in protein (320 - 337 g/kg with a nitrogen-to-protein conversion factor of 5.7 (Lqari et al., 2002; Oomah and Bushuk, 1983)) and the main proteins found in lupin seeds are conglutin-α, -β, -γ and -δ. The proteins contain several essential amino acids but are deficient in methionine and cysteine, like many other legumes (El-Adawy et al., 2001; Sujak et al., 2006). In combination with cereals, which generally have sufficient amounts of methionine and cysteine, most of the essential amino acids can be supplied by a plant-based diet (Day, 2013). Lupin seeds contain about 80 g oil/kg in the form of oil bodies. Oil bodies have a lipid core that is surrounded by a membrane-like layer composed of phospholipids and proteins, making them physically and chemically stable against the environment (Fisk et al., 2008; Iwanaga et al., 2007). The oil in the oil bodies of lupin seeds is rich in mono- and poly-unsaturated (omega-3 and omega-6) fatty acids (Sbihi et al., 2013). The seeds also contain dietary fibres, like celluloses and pectins, but hardly any starch (Doxastakis, 2000). Additionally, lupin seeds contain biologically active compounds, e.g. phenolic compounds and flavonoids like isoflavones (Ranilla et al., 2009; Siger et al., 2012). Some of these compounds show anti-oxidant activity, which has been related to many health benefits e.g. decreased obesity, decreased insulin resistance and lowering elevated blood cholesterol (Arnoldi et al., 2007). The seeds are low in anti-nutritional factors like phytates, trypsin inhibitors, and lectins, compared with soybeans (Dijkstra et al., 2003) and other grain legumes (Arnoldi et al., 2007), which is advantageous for application in the human diet.

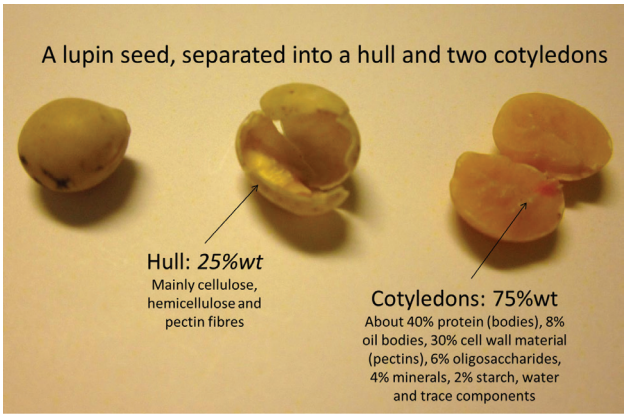


Figure 1 Composition of lupin seeds of *Lupinus angustifolius* (adapted from www.lupins.org)

1.2 Application of lupin

Even though lupin seeds are consumed as a snack in Mediterranean countries, Northern Europeans do not eat the seeds as is (Huyghe, 1997). Ingredients prepared from lupin seeds are however quite commonly applied in food products. Lupin grits and lupin flour are used

in breads, biscuits and pasta for improvement of the appearance and shelf life (Dervas et al, 1999; Doxastakis, 2000). Lupin protein concentrate can be added to fish meal because of its low viscosity and gelling properties (Draganovic et al., 2013). Lupin protein isolate is added to processed meat products like Frankfurters and meat gels, where small amounts of the protein have a beneficial effect on processing and sensory perception (Alamanou et al., 1996; Mavrakis et al., 2003). This is because lupin proteins posess good interfacial properties for emulsions and foams (Pozani et al., 2002). Sosulski et al. (1978) evaluated the potential of lupin as the protein component in imitation milks. Sironi et al. (2005) showed how the individual proteins of lupin can be fractionated and potential applications for these fractions were suggested, like fat-reduced spreads, spreadable cheese, emulsified meat products and salad dressings because of their emulsifying properties and marshmallows and ice creams because of their foaming properties.

Lupin ingredients might become functional ingredients for the food and feed industry, if produced on a commercial scale. The economic feasibility of lupin fractionation processes and the functionality of the fractions obtained was reported to require more research (Sipsas, 2008).

1.3 Fractionation processes

Contrary to soybeans, lupin seeds are not processed for the oil because of the low oil content, which means that the main component of interest is the protein (Doxastakis, 2000). Dry fractionation of lupin seeds yields functional, protein-enriched flours (Pelgrom et al., 2014). Wet fractionation processes were initially developed for soy and then applied to lupin (Doxastakis, 2000). Wet fractionation yields protein concentrates; further purification results in protein isolates (>90% protein). The German Fraunhofer Institute (Freising) produces high-purity lupin protein isolates from lupin flakes on pilot scale (D'Agostina et al., 2006; Wäsche et al., 2001) and on lab scale (Bader et al., 2011; Muranyi et al., 2013; Süssmann et al., 2011). This process usually starts with dehulled seeds being ground or flaked and subsequently defatted with organic solvents, like hexane or petroleum ether (Fig 2).

After oil extraction, acidic and alkaline solubilisation steps are applied to remove anti-nutritional factors and insoluble carbohydrates from the protein-rich supernatant. The protein is then precipitated at its iso-electric point (pI) through addition of ammonium salts or with dilute acids. The pH at which the lupin proteins are least soluble is around 4.5 (Ruiz and Hove, 1976). Wäsche et al. (2001) and Duranti et al. (2008) named this lupin protein

isolate (LPI) 'LPI type E'. This LPI type E did not contain all the proteins present in lupin because e.g. conglutin-γ has a higher pI (around pH 7.6) and therefore does not end up in the precipitated protein pellet (Duranti et al., 2008). This protein fraction can be separated from the supernatant of the acidic extraction step(s) by ultra- and diafiltration and is called 'LPI type F' (Fig 2) (Wäsche et al., 2001). These two fractions have a different functionality because of the different proteins in these fractions, which have a different solubility. After protein extraction, the protein isolates are dried with spray-drying (pilot scale) or freeze-drying (lab scale).

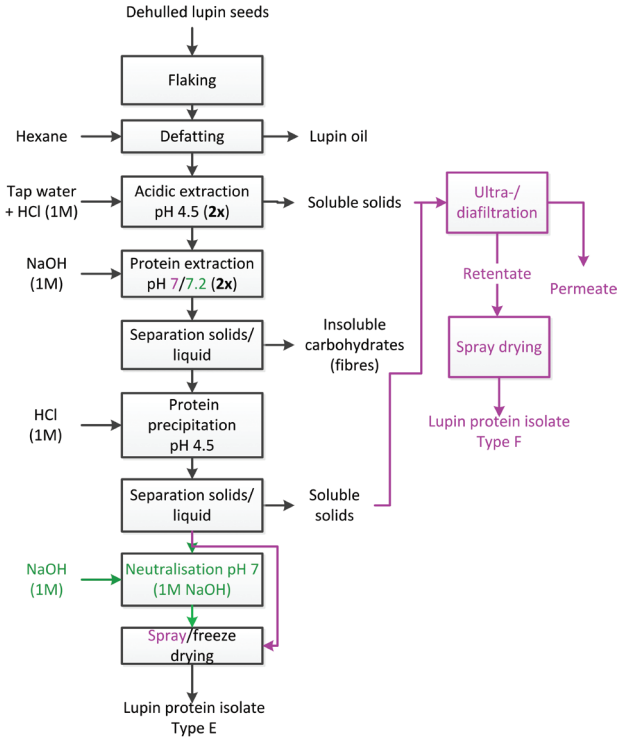


Figure 2 Lupin protein isolate fractionation process based on D'Agostina et al. (2006) (pink lines show variations on the process) and Bader et al. (2011) (green lines show variations on the process)

1.4 Alternative, milder fractionation processes

Due to the extraction and drying steps necessary for the production of the protein isolates, the sustainable image of using plant proteins for food is negatively impacted (Apaiah et al., 2006). Therefore, the isolation process should consume less solvent, less water and less energy. There are several process steps which show inefficiencies: defatting, the actual protein extraction and drying after extraction. From an application point of view, complete purification might not be necessary, which would reduce the need for those inefficient processing steps. For example, products aiming at replacing animal-based products or

animal-based ingredients, may contain other components as well. The plant material that should resemble the composition of animal-based products, has to be rich in protein but may also contain oil and water and should be low in long-chain carbohydrates. Accordingly, fractionation should aim at removing the long-chain carbohydrates instead of the isolation and purification of the protein.

Current fractionation processes that focus on achieving purity make use of organic solvents like hexane or petroleum ether for oil extraction, which is undesired from a sustainability and health perspective. Besides, oil extraction decreases the content of essential amino acids, especially cysteine and methionine, which are already minor in lupin seeds (Schindler et al., 2011). Avoiding oil extraction is thus preferred. Another drawback of the current protein extraction procedure is the use of large amounts of water and chemicals, with sample-to-solvent ratios in the range of 1:8 - 1:20 (w/v). Water needs to be removed from protein concentrates and protein isolates for chemical and microbial stability (and for standardization purposes). However, drying is an energy-intensive process. A third drawback is the use of chemicals to alter the pH, which is not environmentally friendly, usually not regarded organic and the chemicals have to be removed from the ingredients at the end of the process by washing or filtration processes, which requires extra processing steps.

1.5 Novel approach in fractionation of legumes

As stated above, the production of pure ingredients may not be necessary because no food product is composed of a single ingredient. The production of ingredients and structuring into a food product often follows the upper scheme in Fig 3. However, it is not efficient to isolate ingredients with water, evaporate the water for stabilization of the ingredients,

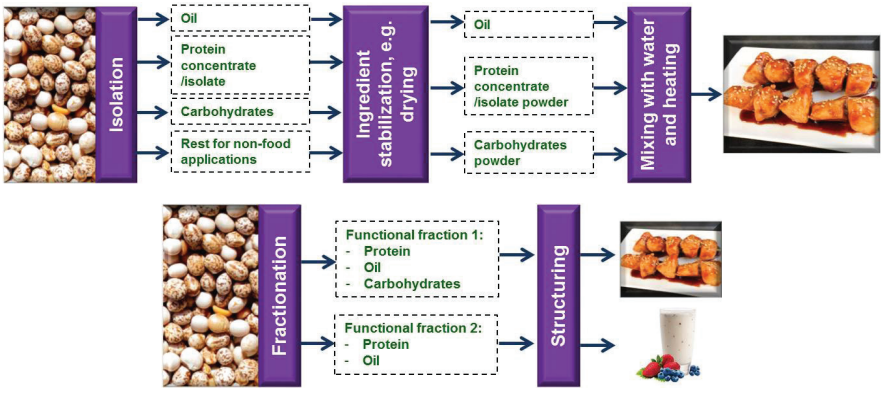


Figure 3 Upper scheme: conventional ingredient production and product processing route, focusing on purity and yield. Lower scheme: new approach, focusing on functionality and sustainability

and then for the final product, mix the ingredients again with water and then evaporate water again through heating to set the structure. It would be more efficient to produce fractions that have the desired composition and functionality for the application in mind as is depicted in the lower scheme in Fig 3. With this approach, less water and energy will be needed and less pure, but functional fractions can be produced (Schutyser and van der Goot, 2011).

In this thesis, dry fractionation and purely aqueous processing are explored as more sustainable routes to obtain protein-rich materials from lupin seeds. Dry fractionation consumes hardly any energy and no water, but produces functional, protein-enriched flours that do still contain other components, such as carbohydrates (Pelgrom et al., 2014). Aqueous fractionation omits an oil extraction step, thus skipping the use of organic solvents. This implies that lupin flour or flaked lupin seeds are dispersed in water and concentrated or isolated under aqueous conditions (Aguilera et al., 1983; Bader et al., 2011; Hojilla-Evangelista et al., 2004; Jung, 2009; Muranyi et al., 2013; Süssmann et al., 2011). The aforementioned authors studied the process parameters of aqueous fractionation and the effect of these parameters on the protein yield and protein content of the protein isolates. The efficiency of aqueous fractionation processes may be further improved by optimising less on purity and yield, and more on functionality of a protein-enriched fraction. This may reduce water and chemical consumption. Additionally, less and milder processing is beneficial for the quality and functionality of the protein. Minimal processing will also be beneficial for the quality and usability of the side streams produced with protein extraction processes. Adding value to the side streams can further increase the sustainability of protein extraction processes because less waste is produced.

Depending on the exact composition but more importantly on the method of isolation, the functional properties of protein isolates may be improved. Wäsche et al. (2001) showed that lupin protein can be a good emulsifier (LPI type E) or foaming agent (LPI type F), but this depends on the composition of these protein isolates. Papalamprou et al. (2009) reported that milder processing techniques improved the functional properties of chickpea protein isolates; milder processing through ultrafiltration increased its solubility, reduced the minimum protein concentration needed for gel formation and improved the gel elasticity. Ultrafiltration was also shown to improve the solubility behaviour of soy protein concentrates and isolates relative to the concentrates obtained with dissolution and precipitation (Alibhai et al., 2006). Ultrafiltration of lupin protein isolates resulted in gels at lower protein concentration than their conventional counterparts (Kiosseoglou et al., 1999).

It is thus interesting to combine these mild processes with aqueous fractionation processes. Since drying consumes a lot of energy, it may be interesting to for example skip drying steps and replace them with membrane filtration processes like ultrafiltration. The chemical, microbial and physical stability of a wet (i.e., not dried) protein isolate may be affected and this has, to our knowledge, not yet been reported.

Aqueous fractionation of lupin seeds implies that oil bodies will remain present during fractionation. Even though oil bodies are known to be physically and chemically stable, processing may influence their stability. Lipid oxidation is one of the primary mechanisms of quality deterioration in foods, e.g. the loss of nutritional value and the formation of unpleasant flavours and odours. The beany, bitter flavour of many legume protein isolates is attributed to the conversion of poly-unsaturated fatty acids to aldehydes, ketones and alcohols (Sessa, 1979) and prohibits incorporation into food products. Even though the presence of oil might be desired from a nutritional point of view, the oxidative stability of the products obtained with aqueous fractionation needs to be assured.

1.6 Aim and outline of the thesis

The overall aim of this thesis was to obtain understanding of the production of functional, protein-rich materials from lupin seeds with reduced environmental impact. The research can be divided into two objectives relating to the fractionation processes for lupin seeds and the functionality of lupin proteins. These topics are interwoven throughout the chapters.

Chapter 2 compares conventional wet and aqueous fractionation (being conventional wet, but excluding de-oiling) processing to obtain lupin protein isolates (LPIs). The compositions of the LPIs were not exactly the same because the aqueous process yielded an LPI that contains 2 wt% oil, but the functionality of the aqueous-fractionated LPI was similar to that of the conventional wet-fractionated LPI. The influence of temperature on the fractionation process and protein functionality is also discussed. **Chapter 3** describes the differences in gelling properties of soy protein isolate (SPI) and LPI. SPI forms a firm gel while LPI forms weak and deformable gels. Even prolonged heating to unfold lupin proteins resulted in deformable gels. Sulfhydryl reactivity and particle size distribution before and after heating were used to explain the differences in the gelling properties between SPI and LPI.

A sustainability assessment of lupin fractionation processes is discussed in **Chapter 4**. Sustainability of processes is assessed with exergy analysis. Exergy is a thermodynamic state variable that quantifies the potential work that can be performed with a specific stream.

Several indicators like exergy efficiency and exergy losses can be used to calculate and visualize inefficiencies in processes or the conversion of raw materials. Dry fractionation was found to be the most sustainable route to obtain protein-enriched flours. Wet and aqueous fractionation processing were used to further increase the protein content of lupin flour or the protein-enriched flour. Wet and aqueous fractionation processing consume water and energy for drying of the LPIs, leading to high exergy losses. To reduce water consumption and exergy losses, dry and aqueous fractionation processes can be combined to obtain LPIs. The consumption of energy can be further reduced by concentrating the LPI to higher protein concentrations instead of drying. It was also shown that utilising all side streams for valuable products is a key factor in improving the exergetic efficiency of fractionation processes.

The technical functionality of LPIs produced with aqueous fractionation that did not receive a drying treatment were compared with the technical functionality of freeze-dried LPIs. **Chapter 5** reports on the viscosity, solubility and swelling properties of wet and freeze-dried LPIs. The heat stability of these LPIs was tested; wet LPIs have a higher solubility, higher swelling capacity and a higher viscosity upon heating. Freeze-dried LPIs are more heat stable than the wet LPIs. The chemical stability of the LPIs and other fractions obtained with aqueous fractionation at two processing temperatures (4°C and 20°C) is described in **Chapter 6**. Lipid and protein oxidation marker values were determined and both processing temperatures resulted in LPIs with oxidation marker values below the acceptability limits. An intermediate heat treatment was applied to inactivate lipoxygenase, but it reduced protein and oil recovery and did not reduce oxidation marker values. This implies that cooling of the process (4°C) and an intermediate heat treatment are not necessary, herewith reducing the environmental impact of the aqueous fractionation process.

Chapter 7 concludes with a general discussion of all results presented in the thesis. It starts with summarizing the main findings, after which potentials and bottlenecks of the novel fractionation processes are discussed. The chapter ends with a future outlook on further scientific research on simplified fractionation processes and potential applications.

Aqueous fractionation of protein from lupin seeds was investigated as an alternative to the conventional wet fractionation processes, which make use of organic solvents. The effect of extraction temperature was studied and the consequences for downstream processing were analysed. Omitting the extraction of oil with organic solvents resulted in a protein isolate that contained 0.02 - 0.07 g oil/g protein isolate, depending on the exact extraction conditions. Nevertheless, the protein functionality of the aqueous fractionated lupin protein isolate was similar to the conventional lupin protein isolate. The protein isolate suspension could be concentrated to 0.25 g/mL using ultrafiltration, which provides a relevant concentration for a range of high-protein products. Based on the results, we conclude that aqueous fractionation can be a method to lower the environmental impact of the extraction of proteins from legumes that contain water- and dilute salt-soluble proteins.

Chapter 2

The potential of aqueous fractionation of lupin seeds for high-protein foods

Highlights:

- Aqueous and conventional extracted protein isolates had similar protein functionality
- A lengthy heat treatment increased the water holding capacity of the protein isolate
- Ultrafiltration was successful in concentrating the protein isolate to 0.25 g/mL
- Aqueous processing lowers the environmental impact of protein extraction from plants

This chapter has been published as:

Berghout, J.A.M

Boom, R.M.

van der Goot, A.J.

The potential of aqueous fractionation of lupin seeds for high-protein foods

in

Food Chemistry (2014) vol **159**

pages 64-74

2.1 Introduction

Many protein-rich plants are used as animal feed to produce protein-rich products like milk, eggs and meat. However, the conversion of plant proteins into animal proteins is inefficient (Pimentel and Pimentel, 2003). Even though plant proteins are more abundant, direct consumption of protein-rich plants, beans or seeds is limited (Day, 2013). Previous studies showed that consumers are willing to switch to plant-based products provided that those products have similar taste and texture as their animal-based equivalents (de Boer et al., 2006). Important conditions for the development of plant-based alternatives are the availability of plant protein concentrates and isolates with high functionality and produced in a sustainable manner. Current protein extraction processes are inefficient due to the use of organic solvents, acids and bases, and large amounts of water, as a result of which the environmental gain is less than theoretically possible (Apaiah et al., 2006; Schutyser and van der Goot, 2011).

Lupin is a legume with high protein content and is therefore an interesting raw material for plant-based, high-protein products. Additionally, the seeds of lupin are known to have beneficial health effects, while the plant accepts versatile breeding conditions (Arnoldi et al., 2007; Cerletti and Duranti, 1979; Foley et al., 2011; Fontanari et al., 2012). The sweet variety *Lupinus angustifolius* has a protein content of about 400 g/kg flour and is further composed of carbohydrates (480 g/kg of flour), oil (70 - 100 g/kg of flour), minerals and water. Its proteins have an excellent amino acid composition (El-Adawy et al., 2001; Lqari et al., 2002).

For use in high-protein food products, the proteins need to be extracted from the seeds. Conventional wet extraction processes remove the oil through organic solvent extraction, followed by aqueous extraction steps with varying the pH to obtain an almost pure protein fraction (Alamanou and Doxastakis, 1995; Jayasena et al., 2011; Kiosseoglou et al., 1999; Lqari et al., 2002; Sironi et al., 2005; Süssmann et al., 2011; Wäsche et al., 2001). However, the focus on purity might not be necessary as almost no food product consists of a single ingredient only. In case the plant material should resemble the composition of animal-based food products, it has to be rich in protein, may contain oil and water and it has to be low in long-chain carbohydrates. Consequently, fractionation should aim at removing the undesired insoluble carbohydrates, rather than obtaining pure protein. For this, aqueous fractionation seems to be a suitable method. Aguilera et al. (1983), Chew et al. (2003), Hojilla-Evangelista et al. (2004), Jung (2009) and Bader et al. (2011) studied aqueous processing of lupin and in

the present study we looked into possibilities to make the process more efficient, for instance by using less extraction steps. For example, the low level of alkaloids in *Lupinus angustifolius* (<200 mg/kg, Alimex, the Netherlands) offers the opportunity to omit the acidic extraction step at the beginning of the process. Performing all extraction steps with fewer repetitions will reduce the use of water and chemicals. Aguilera et al. (1983) and Hojilla-Evangelista et al. (2004) obtained protein concentrate fractions with 670 - 790 g protein/kg (N x 6.25), 40 - 66 g oil/kg, and still 60 - 150 g carbohydrates/kg. Jung (2009) studied aqueous processing of lupin flakes where oil and protein were separated. In our case, the presence of oil in the protein-rich fraction might be an advantage for the final product composition. Bader et al. (2011) obtained protein recoveries of only 430 g/kg for conventional fractionation and 420 g/kg for aqueous fractionation, which is lower than protein recoveries of 500 - 600 g/kg that are usually reported (Chew et al., 2003; D'Agostina et al., 2006; Fontanari et al., 2012; Jayasena et al., 2011; King et al., 1985; Ruiz and Hove, 1976; Süssmann et al., 2011). Generally, the protein isolate or concentrate is dried at the end of the process. This might be a redundant step in case the final application contains or requires water, which means that the protein powder needs to be rewetted for post-processing. From an environmental point-of-view, it is interesting to study methods to concentrate the protein isolate instead of drying it to a powder.

This paper investigates aqueous fractionation of lupin seeds in greater detail to obtain protein-enriched fractions that have the potential to be used in plant-based, high-protein foods. As explained above, the presence of a certain amount of oil in the protein fraction is acceptable, which allows the introduction of a simplified fractionation process. In addition to the omission of the oil extraction step, the acidic extraction step at the start of the process is skipped and all extraction steps are performed once. The effects of extraction temperature and pH on protein recovery, chemical composition and techno-functional properties are determined. These properties include the water holding capacity and the nitrogen solubility index. Because high-protein food products contain water, it is also explored how to concentrate the protein. The results are captured in a novel process design for aqueous processing.

2.2 Materials and methods

2.2.1 Raw materials and chemicals

Dehulled, untoasted full-fat lupin seeds (*Lupinus angustifolius*) were obtained from Alimex (the Netherlands). All chemicals and reagents used in this study were of analytical grade.

Tap water was used throughout unless stated otherwise.

2.2.2 Pre-treatment of the seeds

Lupin was pre-milled to grits with a Condux-Werk pin mill LV 15 M (Condux-Werk, Wolfgang bei Hanau, Germany). The grits were further milled into a full-fat flour with a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) with a classifier wheel set at 1000 rpm and the air flow at 80 m^3/h. The screw feeder was set at 2 rpm (corresponding to circa 0.75 kg/h), the impact mill speed was 8000 rpm and the batch size was 1 kg. Part of the full-fat lupin flour was used to prepare defatted lupin flour. To extract the oil from the flour, 45 g of full-fat lupin flour was weighed into a cellulose thimble. Four thimbles (with in total 140 g of full fat flour) were mounted onto the fully automated Büchi extraction system B-811 LSV (Büchi Labortechnik AG, Flawil, Switzerland). The oil extraction was performed with petroleum ether (boiling range 40 – 60°C) in Standard Soxhlet mode for 3 h with a sample-to-solvent ratio of 1:6. The extracted oil and the defatted samples were dried in an oven at 105°C until constant weight. The petroleum ether was removed by evaporation and recovered within the Büchi extraction system.

2.2.3 Preparation of protein isolates

The process conditions used for protein extraction were chosen based on explorative experiments and literature data (Fontanari et al., 2012; Lqari et al., 2002; Süssmann et al., 2011). Seven different protein isolates (PI) were produced. The processing conditions and abbreviations can be found in Fig 4. An overview of the conventional fractionation process and the newly developed aqueous fractionation (AF) processes are also depicted in Fig 4. The conventional lupin PI was produced by dispersing the defatted flour in water using a sample to solvent ratio of 1:15. The pH of the dispersion was adjusted and kept at 9 through addition of 1 mol/L NaOH. The dispersion was stirred at 20°C for 2 h and subsequently centrifuged at 11,000 x *g* and 20˚C for 30 min. The time between centrifugation and decanting was kept as short as possible. The pellet, containing the fibre-rich fraction was freeze dried, while the protein-rich supernatant was collected. The pH of the supernatant was adjusted to 4.5 with 1 mol/L HCl. The dispersion was stirred at 20°C for 1 h and subsequently centrifuged again at 11,000 x *g* at 20°C for 30 min. The supernatant, containing the soluble solids fractions, was collected and freeze dried. The pellet, which contains the lupin PI, was washed with Millipore water twice and subsequently neutralised to pH 7 with 1 mol/L NaOH and kept at 20°C overnight. One-third of the protein suspension was kept for post-processing at 80°C and the other two-third of the protein suspension was freeze-dried.

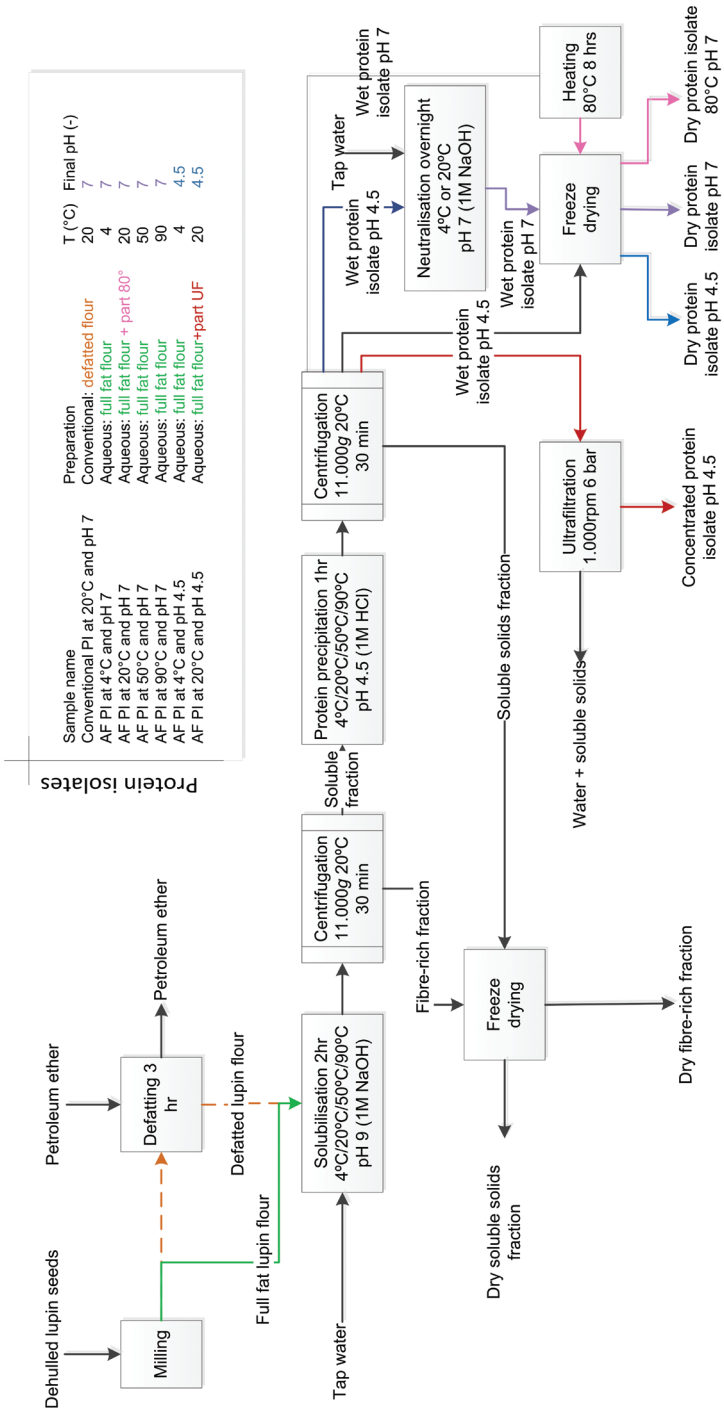


Figure 4 Conventional and aqueous fractionation processes, processing conditions and abbreviations for all protein isolates

The aqueous fractionated lupin protein isolates (AF PIs) were produced by dispersing the full-fat lupin flour in water using a sample to solvent ratio of 1:15. The pH of the dispersions was adjusted to 9 through addition of 1 mol/L NaOH. The dispersions were stirred at 4˚C, 20 ˚C, 50˚C or 90˚C for 2 h, depending on the fractionation method (Fig 4), and subsequently centrifuged at 11,000 x *g* and 20˚C for 30 min. The time between centrifugation and decanting was kept as short as possible. After centrifugation, an oily layer was visible on top of the protein-rich supernatant. This oil could be collected with the supernatant or the pellet, but part of the oil remained stuck onto the tube walls. In all experiments, it was tried to combine the oil with the supernatant, which eventually becomes the protein-rich fraction. The pellets, which contain the fibres, were freeze-dried. The supernatants were collected and the pH was adjusted and kept at 4.5 using 1 mol/L HCl. The resulting dispersions were stirred at 4˚C, 20˚C, 50˚C or 90˚C for 1 h and then centrifuged at 11,000 x *g* and 20˚C for 30 min. The supernatants, representing the soluble solids fractions, were collected and freeze-dried. The pellets, which contain the lupin PI, were washed twice with Millipore water to remove impurities and sodium chloride.

The protein suspensions were split into four parts for further analysis. One of the parts, prepared at 20˚C and pH 4.5 and at 4˚C and pH 4.5, were freeze dried. Another part of the protein suspension, prepared at 20˚C and pH 4.5, was kept for ultrafiltration. The remaining parts of both protein suspensions and the complete protein suspension at 90˚C were neutralised to pH 7. One part of the protein suspension, neutralized to pH 7, was kept for post-processing at 80˚C. The protein suspension, produced at 4˚C, was kept at 4˚C overnight and those produced at 20˚C, 50˚C and 90˚C were kept at 20˚C overnight. All protein extractions were performed in duplicate.

The dried PI was weighed and the protein recovery was calculated as gram of protein in the fraction relative to the gram of protein present in the lupin flour before extraction. Oil recovery was calculated as gram of oil in the fraction relative to the gram of oil present in the lupin flour before extraction. The protein and oil recovery were corrected for the increase in mineral residue by sodium chloride formation.

2.2.4 Post-processing of protein isolate

One part of the wet AF PI at 20˚C and pH 7 and one part of the wet conventional lupin PI were heated at 80˚C for 8 h in a stirring dry bath (2mag magnetic(e)motion, Munich, Germany). Even though water holding capacity was already influenced after 30 min of heating, we chose to make the effect of heating more pronounced. After heating, the samples

were cooled with running tap water and stored for freeze-drying. The heat treatments were performed in duplicate.

2.2.5 Ultrafiltration for protein concentration

Approximately 50 mL of the neutralized AF-PI was kept in its wet state. The solution was subjected to ultrafiltration in an Amicon stirred cell, 50 mL (Millipore Co. Bedford, USA) fitted with a disc membrane with a surface area of 13.4 cm^2 made of regenerated cellulose with a molecular weight cut-off equal to 5 kDa (Millipore Co. Billerica, USA). Pressurized air of 6 bars was put onto the cell and the speed of rotation was set at 1000 rpm to suppress concentration polarization on the membrane.

2.2.6 Chemical analyses

The dry matter content was determined by drying 1 g of sample in an oven at 105˚C overnight. The total ash content was determined with AACC official method 08-01 (AACC, 1983a). The protein content was determined with the Dumas combustion method on a NA 2100 Nitrogen and Protein Analyser (ThermoQuest-CE Instruments, Rodeno, Italy). Methionine was used as standard during the analysis. The protein content was calculated with a nitrogen-to-protein conversion factor of 5.7 that is used for seed storage proteins and a nitrogen-to-protein conversion factor of 6.25 to facilitate comparison with literature. The residual fat content in each fraction was determined with the Standard Soxhlet mode according to AACC method 30-25 (AACC, 1983b). The oil extraction was performed with petroleum ether (boiling range 40 - 60˚C). It was tested that the oil extraction values obtained with petroleum ether were similar to values obtained with hexane. The carbohydrate content of the flour was calculated as the difference of the dry matter content and the other components measured (protein, oil, and mineral residue).

The protein composition of the samples was determined with non-reducing sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. The samples were diluted to a protein concentration of 0.01 g/mL. A sample of 100 µL was mixed with 200 µL of 0.02 g/mL SDS-0.13 MTris–HCl buffer (pH 6.8) solution. The samples were heated at 95˚C for 4 min in a heating block, and cooled to room temperature in about 30 min. Samples were separated on a 12% Tris–HCl SDS-ready gel in a Bio-Rad Mini-Protean cell (Bio-Rad Laboratories Inc., Hercules, USA). The cell was filled with 1 L of running buffer. The running buffer was prepared by diluting a stock solution of 0.03 g/mL Tris base, 0144 g/mL glycine and 0.01 g/mL SDS ten times with Millipore water. Subsequently, 15 µL of sample was applied on the gel. At one of the outside wells of the gel, a marker ranging from 7 kDa to 207 kDa (Bio-Rad

Laboratories Inc., Hercules, USA) was applied. The gels were run at 200 V for approximately 45 min. Afterwards, the gels were washed three times for 5 min with Millipore water to remove the remains of the buffer. Staining was done with Bio-Safe Coomassie Stain (Bio-Rad Laboratories Inc., Hercules, USA) for one hour. Finally, the gels were washed with Millipore water and scanned with an Imagescanner III (GE Healthcare Bio-Sciences, Little Chalfont, UK).

2.2.7 Water holding capacity

The water holding capacity (WHC) was determined according to the official method AACC 56-30 (AACC, 1983c). The approximate WHC value is defined as the maximum amount of water that 1 g of sample will absorb and subsequently retain under low-speed centrifugation (2000 x *g*, 20˚C for 10 min). The sample was placed into pre-weighed 50 mL Greiner tubes and demineralised water was added in small amounts until a supernatant and pellet could be distinguished. This method ensures that no soluble material is extracted from the material.

2.2.8 Nitrogen solubility index

The nitrogen solubility index (NSI) was determined according to the method described by King et al. (1985) and Mohamed et al. (2005). The lupin PIs were suspended in demineralised water at a concentration of 0.01 g/mL. The suspensions were shaken at room temperature for 1 h and then centrifuged (3000 x *g*, 25˚C for 15 min). The supernatants and pellets were dried and analysed for protein content with the Dumas combustion method. The NSI was taken as the fraction of soluble nitrogen to total nitrogen.

2.2.9 Statistical analysis

The results are presented as mean ± absolute deviation on a dry weight basis. Student t-tests were performed to evaluate the differences between the fractionation processes. Differences between means were considered to be significantly different when P was smaller than 0.05.

2.3 Results

2.3.1 Protein and oil recovery

The full-fat lupin flour contained 945 g dry matter/kg, of which 347 g was protein (N x 5.7), 95 g oil and 26 g mineral residue. Due to the extraction of oil, the protein content of the fullfat lupin flour is lower than that of defatted lupin flour. After oil extraction, the defatted lupin flour contained 929 g dry matter/kg, of which 405 g was protein (N x 5.7) and 35 g

mineral residue while there was no residual oil that could be extracted with a second batch of petroleum ether.

Conventional fractionation from defatted lupin flour resulted in foam formation inside the centrifuge tubes. Apart from the foam, the separation of the fibre-rich pellet from the supernatant afterthe first centrifugation step was difficult, because the pellet was not solid but fluid. The fibre-rich pellet obtained with the aqueous fractionation (AF) process was more solid. The distribution of protein over the fractions of the conventional fractionation process and the AF process at 20˚C and pH 7 was hardly influenced by the type of fractionation process (Table 1), suggesting that both the foam formation and a fluid pellet did not influence the extraction process as such. Most of the protein ended up in the protein-rich fractions, which is in agreement with previous studies (D'Agostina et al., 2006; Fontanari et al., 2012; King et al., 1985; Kiosseoglou et al., 1999). The lowest protein

Table 1 Protein recoveries (g/g) and oil recoveries (g/g) on a dry weight basis of each fraction for each fractionation process

Sample		Protein recovery (g/g)			Oil recovery (g/g)		
Conventional fractionation 20ºC pH 7	Protein-rich fraction	0.61	±	0.01	0.00	±	0.00
	Soluble solids fraction	0.22	±	0.01	0.00	±	0.00
	Fibre-rich fraction	0.22	±	0.01	0.00	±	0.00
Aqueous fractionation 4ºC pH 7	Protein-rich fraction	0.61	±	0.00	0.05	±	0.02
	Soluble solids fraction	0.21	±	0.00	0.00	±	0.00
	Fibre-rich fraction	0.15	±	0.01	0.53	±	0.03
Aqueous fractionation 20ºC pH 7	Protein-rich fraction	0.60	±	0.01	0.07	±	0.00
	Soluble solids fraction	0.18	±	0.03	0.00	±	0.00
	Fibre-rich fraction	0.18	±	0.01	0.68	±	0.01
Aqueous fracionation 50ºC pH 7	Protein-rich fraction	0.53	±	0.00	0.06	±	0.00
	Soluble solids fraction	0.29	±	0.00	0.00	±	0.00
	Fibre-rich fraction	0.14	±	0.01	0.53	±	0.04
Aqueous fractionation 90ºC pH 7	Protein-rich fraction	0.60	±	0.02	0.29	±	0.07
	Soluble solids fraction	0.22	±	0.00	0.00	±	0.00
	Fibre-rich fraction	0.18	±	0.01	0.45	±	0.08
Aqueous fractionation 4ºC pH 4.5	Protein-rich fraction	0.64	±	0.07	0.21	±	0.02
	Soluble solids fraction	0.21	±	0.02	0.00	±	0.00
	Fibre-rich fraction	0.15	±	0.00	0.62	±	0.04
Aqueous fractionation 20ºC pH 4.5	Protein-rich fraction	0.60	±	0.02	0.12	±	0.02
	Soluble solids fraction	0.22	±	0.02	0.00	±	0.00
	Fibre-rich fraction	0.13	±	0.02	0.56	±	0.09

recovery in the protein-rich fraction was found for the AF at 50˚C and pH 7. In this experiment the fluidity of the pellet troubled the separation of the fibre-rich pellet from the protein-rich supernatant. For all other conditions, about 0.60 g/g of the protein was recovered in the protein-rich fractions. The division of oil over the various fractions was influenced by the extraction procedure. The higher the temperature during extraction, the more oil was recovered in the protein-rich fraction and subsequently less oil was recovered in the fibre-rich pellet (Tables 1 and 2). None of the supernatants contained any oil that could be extracted with the Soxhlet method. For oil, the mass balances do not add up completely. Amongst others, this is caused by about 0.5 g of dry matter remaining in the centrifuge tubes after centrifugation, due to the dimensions of the tubes. In case this fraction is mainly oil, it is about 0.19 g/g oil present in the system.

Except for the protein-rich fractions produced at 50˚C and 90˚C, all protein-rich fractions had a protein content higher than 0.90 g/g with a nitrogen-to-protein conversion factor of 6.25 (Table 2), which is the technical requirement for a protein isolate (PI) according to the Codex Alimentarius for vegetable proteins (FAO, 2001). The PI obtained with the conventional fractionation process had a slightly, but not significantly, higher protein content than the AF PI under the same conditions. Removal of the oil from the AF PI prepared at 20°C and pH 7 would give almost exactly the same protein content as the conventional PI. The conventional PI has a slightly lower protein content (N x 5.7) than those reported by Alamanou and Doxastakis (1995) and Süssmann et al. (2011) who used more purification steps, but similar protein content to Rodriguez-Ambriz et al. (2005) when re-calculating their results with a nitrogen-to-protein conversion factor of 5.7. The protein content of the AF PIs was in range with the values reported by Bader et al. (2011) and Jung (2009) when re-calculating their results with the nitrogen-to-protein conversion factor of 5.7.

Next to protein and oil, the PIs consisted of mineral residue and most likely soluble sugars. In contrast to the PIs, the fibre-rich pellet and the soluble solids fraction were not washed. The sodium chloride resulting from the pH adjustment steps is water soluble, and thus adds to the mineral content of those samples. It can be calculated that the mineral content will increase with a factor 10 when the sample is neutralized to a pH of 7.

2.3.2 Composition and functionality

As described above, the protein content and protein recovery were hardly influenced by the changes in conditions during fractionation, except at 90˚C. These extraction conditions also resulted in a strong off-odour, while the addition of hydrochloric acid resulted in

Table 2 Protein content for N x 5.7 and N x 6.25 (g/g), oil content (g/g) and ash content (g/g) on a dry weight basis of each fraction for each fractionation condition

Sample		Protein content Nx5.7 (Nx6.25) (g/g)	Oil content (g/g)	Ash content (g/g)
Conventional fractionation 20ºC pH 7	Protein isolate	0.85 (0.93) ± 0.01	0.00 ± 0.00	0.04 ± 0.00
	Sol solids fraction	0.31 (0.35) ± 0.01	0.00 ± 0.00	0.12 ± 0.00
	Fibre-rich fraction	0.22 (0.24) ± 0.00	0.00 ± 0.00	0.04 ± 0.00
Aqueous fractionation 4ºC pH 7	Protein isolate	0.83 (0.91) ± 0.03	0.02 ± 0.01	0.02 ± 0.01
	Sol solids fraction	0.29 (0.31) ± 0.02	0.00 ± 0.00	0.00 ± 0.00
	Fibre-rich fraction	0.11 (0.12) ± 0.00	0.11 ± 0.01	0.11 ± 0.01
Aqueous fractionation 20ºC pH 7	Protein isolate	0.82 (0.90) ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	Sol solids fraction	0.28 (0.31) ± 0.01	0.01 ± 0.01	0.14 ± 0.01
	Fibre-rich fraction	0.13 (0.14) ± 0.00	0.14 ± 0.00	0.01 ± 0.00
Aqueous fractionation 50ºC pH 7	Protein isolate	0.81 (0.89) ± 0.01	0.02 ± 0.00	0.03 ± 0.00
	Sol solids fraction	0.37 (0.40) ± 0.00	0.00 ± 0.00	0.11 ± 0.00
	Fibre-rich fraction	0.11 (0.12) ± 0.00	0.11 ± 0.01	0.03 ± 0.00
Aqueous fractionation 90ºC pH 7	Protein-rich fraction	0.72 (0.79) ± 0.00	0.09 ± 0.02	0.03 ± 0.00
	Sol solids fraction	0.32 (0.35) ± 0.01	0.00 ± 0.00	0.12 ± 0.01
	Fibre-rich fraction	0.13 (0.14) ± 0.00	0.09 ± 0.02	0.03 ± 0.00
Aqueous fractionation 4ºC pH 4.5	Protein isolate	0.84 (0.92) ± 0.05	0.07 ± 0.01	0.01 ± 0.00
	Sol solids fraction	0.29 (0.31) ± 0.02	0.00 ± 0.00	0.13 ± 0.00
	Fibre-rich fraction	0.11 (0.13) ± 0.00	0.12 ± 0.01	0.02 ± 0.00
Aqueous fractionation 20ºC pH 4.5	Protein isolate	0.85 (0.93) ± 0.00	0.04 ± 0.01	0.01 ± 0.00
	Sol solids fraction	0.33 (0.36) ± 0.00	0.01 ± 0.01	0.11 ± 0.00
	Fibre-rich fraction	0.12 (0.13) ± 0.00	0.12 ± 0.02	0.03 ± 0.01

Means ± absolute deviation (n=2)

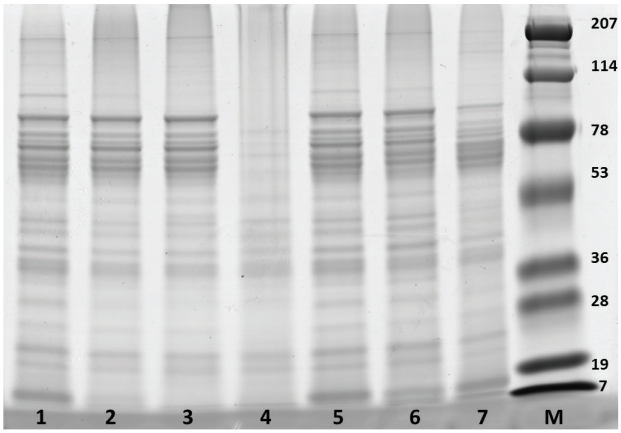


Figure 5 SDS–page on 12% Tris–HCl gel, 1. AF PI 4°C pH 4.5, 2. AF PI 20°C pH 4.5, 3. AF PI 50°C pH 7, 4.AF PI 90°C pH 7, 5.AF PI 4°C pH 7, 6. Conventional PI 20°C pH 7, 7. AF PI 20°C pH 7, M. marker

flocculation of the proteins. The final PI was grey, whereas all other PIs were yellowish. At 4˚C no off-odour was noted and a yellow protein pellet was obtained.

To check the protein composition of the PIs, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–page) analysis was performed (Fig 5). The protein composition of the aqueous protein isolate and the full-fat lupin flour were similar (results not shown). Most protein bands were found within the range of 20 - 90 kDa, which is a common range for lupin proteins (Fontanari et al., 2012; Nikolić et al., 2012). The patterns on the SDS-gel were similar for all PIs, except for the AF PI at 90˚C and pH 7, which had less pronounced protein bands in the higher molecular weight region. This indicated that higher molecular weight proteins had a reduced solubility after extraction at 90˚C and thus did not end up in the supernatant that was transferred onto the gel.

The water holding capacity (WHC) of the conventional and AF lupin PIs at 4˚C, 20˚C and 50˚C with pH 7 could not be determined (Table 3). Even with an excess of water, no distinct pellet was formed after centrifugation. Instead of forming a pellet, the solids remained dispersed in the water after centrifugation, and no gel was obtained. The PIs obtained without the neutralisation step did show a distinct pellet after centrifugation and yielded WHCs of about 1 mL/g protein. Heat treatment of the conventional PI and the neutralised AF PI increased the WHC further to values of about 3.5 mL/g protein. The highest WHC was found for the PI produced at 90˚C. Our hypothesis is that the fact that lupin proteins at pH 7 do not sediment upon centrifugation and remain dispersed could be related to the formation of a fluffy conformational structure. At their iso-electric point (pI 4.5) the charge on the proteins decreases and the protein can adopt a more compact conformation.

Table 3 Water holding capacity (mL/g protein) and nitrogen solubility index (g/g)of conventional and aqueous lupin protein isolates

Sample		Water holding capacity (ml/g protein)	Nitrogen solubility index (g/g)
Conventional PI	20ºC pH 7	Dispersion (no distinct layer after centrifugation)	0.95 ± 0.00
	HT[a] 80ºC 8 hr	4.3 ± 0.4	0.97 ± 0.02
Aqueous PI	4ºC pH 7	Dispersion (no distinct layer after centrifugation)	0.90 ± 0.02
	20ºC pH 7		0.92 ± 0.02
	50ºC pH 7		0.86 ± 0.02
	90ºC pH 7	3.9 ± 0.0	0.76 ± 0.01
	4ºC pH 4.5	1.1 ± 0.0	0.68 ± 0.04
	20ºC pH 4.5	1.3 ± 0.1	0.42 ± 0.03
	HT[a] 80ºC 8 hr	3.5 ± 0.2	0.79 ± 0.00

Means ± absolute deviation (n=2)
[a] HT = Heat Treated

Also upon heating proteins undergo transformations, which cause conformational changes and even association or dissociation. Probably, those transformations are a prerequisite for the formation of a pellet. There was no statistically significant difference in nitrogen solubility index (NSI) between the conventional lupin PI and the AF lupin PI at 20˚C and pH 7. A significantly lower NSI was found for the PI at 90˚C and pH 7, the heat treated PI and for the PIs at pH 4.5. The lowest NSI was found at pH 4.5, where the proteins are at their iso-electric point and therefore least soluble.

2.4 Discussion

The application of protein isolates in plant-based, high-protein foods asks for an environmentally benign method for the production of the protein isolates. Insoluble carbohydrates, like fibres need to be removed and the protein content enhanced. We will here discuss the suitability of aqueous fractionation methods for this aim.

2.4.1 Potential of aqueous fractionation processes for protein isolation

The conventional approach to make plant-based alternatives from legume seeds is to separate the materials into their pure constituents and then blend these into the final composition of the food product. This is not efficient: conventional fractionation of legume materials requires so much chemicals for extraction and energy for drying of the ingredients that the overall potential for improving the sustainability of our food when for example replacing animal proteins is significantly reduced (Apaiah and Hendrix, 2005; Day, 2013). It is more efficient to directly prepare a protein-rich fraction with the desired composition and functional properties for its use in the final products, e.g. the incorporation of aqueous extracted lupin protein concentrates in bread dough (Dervas et al., 1999) and lupin protein extracts for protein-based beverages due to its low viscosity (Chew et al., 2003). In our case, fractionation should aim at removing the undesired insoluble carbohydrates, rather than obtaining pure protein isolates.

Aqueous fractionation (AF) was successfully applied to obtain protein isolates with 0.02 - 0.07 g oil/g protein isolate. The solubility of the protein isolates (PIs) was influenced by the water temperature during extraction. The solubility of the proteins was high at 4˚C, enhancing high protein recoveries and high protein content of the protein isolate. Additionally, the microbial stability of the system at 4˚C will be high and enzymatic reactions are known to be suppressed at this temperature (Yoshie-Stark and Wäsche, 2004). Aqueous fractionation at 90˚C would even inactivate micro-organisms and enzymes.

To produce semi-solid high-protein foods, proteins need to form a gel or even solidify at water holding capacities (WHC's) of about 3 mL/g protein. Interestingly, the water holding capacity of the protein isolate produced at 90˚C lies in the desired range for these high-protein foods. However, during fractionation at 90˚C off-odours were noticed and colour changes were perceived indicating undesired chemical transformations in the PI. Experimentally we found that applying a post-process heating step at 90˚C using the protein isolate produced at 4˚C led to an increase in WHC, which seems a suitable option because this procedure did not give off-odours. Only the yellow colour turned slightly paler. Based on these results and the fact that the use of organic solvents was omitted, we think that the AF process at 4˚C has potential to replace conventional fractionation, while an additional heat treatment can be used to tune the final functional properties.

2.4.2 Towards a novel process design for fractionation processes

In conventional processes, fractionation is followed by a drying step to stabilise the protein isolate. This might not be necessary in case the final application contains or requires water. Therefore, it was explored whether this drying step could be omitted. The AF process gives a PI in suspension with a dry matter content of about 0.04 g/mL. This means that a large part of the water needs to be removed for most applications. Because the PI is completely soluble up to high concentrations (>0.20 g/mL), concentration can be achieved with membrane (ultra)filtration. Ultrafiltration (UF) is more energy efficient than evaporation and has high protein recoveries while small solutes, such as sugars and salts, will be removed to a certain extent. We tested UF at room temperature and observed that it was possible to concentrate the 0.04 g/mL protein suspension into a 0.25 g/mL protein dispersion. This concentration is relevant for high-protein food applications (Purwanti et al., 2010) such as meat analogues, low viscosity protein beverages, ice cream based on lupin or fish feed (Draganovic et al., 2014). Given the short shelf life of products with a high water content (and consequently a high water activity), the protein isolate needs to be processed within short time after extraction. However, in current industrial process chains, spray drying might remain necessary to stabilise the protein isolate and allow storage time before application. The results presented indicate the possibility to separate proteins, oil and carbohydrates using the natural properties of the raw material in an aqueous dispersion, without using organic solvents and minimal use of acid and base. Further, it might be possible to control the oil content in the AF PI fraction without resorting to organic extraction. During the experiments, an oily layer was visible on top of the supernatant after the first centrifugation step, which could be either decanted with the supernatant or retained onto the pellet. If

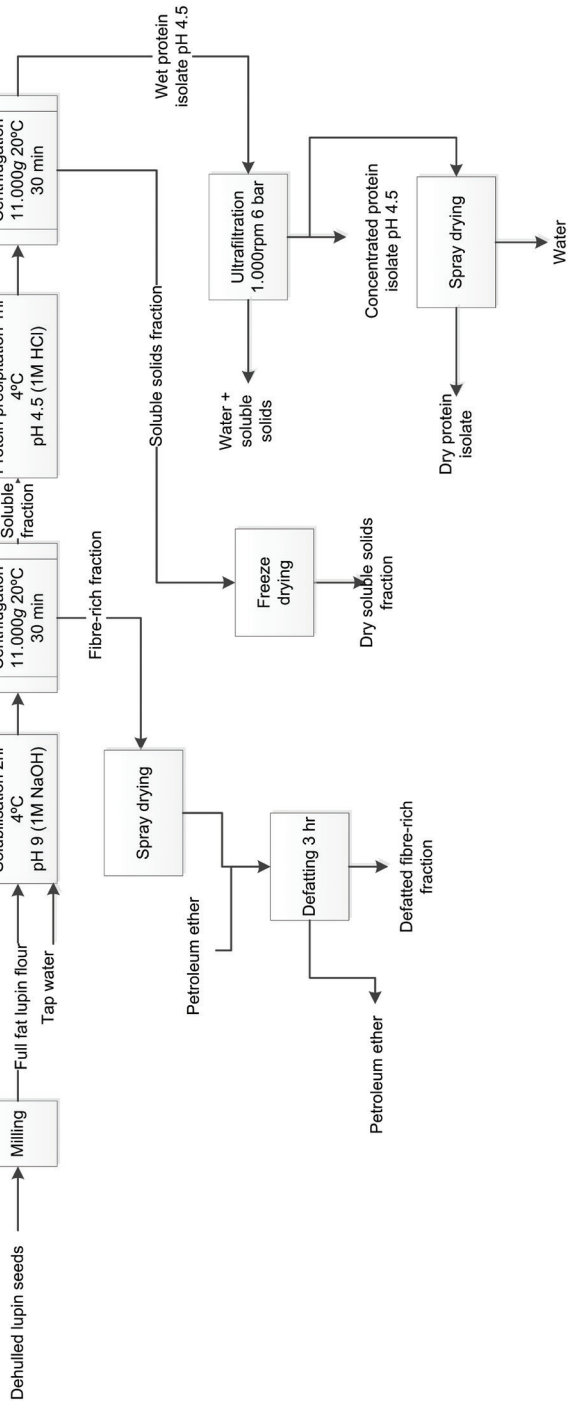


Figure 6 Novel aqueous fractionation process at 4°C with conventional oil extraction for the fibre-rich pellet and ultrafiltration for the protein isolate

desired, the fibre-rich fraction could be defatted to collect the oil that did not end up in the protein isolate, using for example conventional oil extraction. Since the mass of this fibre-rich fraction is only one third of the initial mass of the flour, it will reduce the amount of organic solvents needed for defatting, and avoid any solvent residues in the protein fraction. Consequently, the novel, simplified AF process for lupin protein fractionation can be adapted as is depicted in Fig 6. It can be expected that the process will apply for other legumes or oilseeds that contain water- and dilute salt-soluble proteins as well.

2.5 Conclusion

Modern fractionation processes should be environmental friendly and focus on functionality rather than molecular purity. Besides, when connected to the application, complete drying of the protein isolate might not be necessary and can be replaced with a concentration process, for example through the use of membranes. This was the motivation to study the potential of aqueous fractionation to prepare a protein-enriched fraction from lupin seeds. The procedure omits the use of organic solvents and yields protein isolates that contain 0.02 - 0.07 g oil/g protein isolate. The protein content of the aqueous protein isolate is similar to that of protein isolates obtained with conventional extraction processes. Moreover, the functional properties such as water holding capacity and nitrogen solubility were in the same range and could be adjusted using an additional heat treatment. The use of ultrafiltration after protein extraction at 4˚C yields stable protein dispersions of up to 0.25 g/mL in water, which might be relevant for a range of high-protein food applications. To increase the shelf life of the protein isolate, UF may be followed by spray drying. If desired, the oil,which ends up for 0.50 - 0.60 g/g in the fibre-rich fraction, can be recovered by an additional oil extraction step. Overall, it seems that scope exists to lower the environmental impact of the extraction of water- and dilute salt-soluble proteins from legume materials. this is required to facilitate the transition from animal-based protein foods towards plant-based protein foods in a sustainable manner.

Acknowledgements

The authors thank The PEAS Foundation and the Institute of Sustainable Process Technology (ISPT) for their financial and project support.

The gelling properties of lupin protein isolate (LPI) were compared with those of soy protein isolate (SPI). It was found that LPI behaves fundamentally different than SPI, evidenced by the formation of weaker and deformable gels. Further investigation shows that both protein isolates can be considered particle gels and that LPI particles do not swell as much as SPI particles inside the network. Besides, heating hardly affects LPI particles while SPI particles show additional swelling. To explain the differences, the sulfhydryl reactivity of LPI was tested. The amount of free sulfhydryl groups on LPI was higher than the amount of free sulfhydryl groups on SPI. Upon heating the amount of free sulfhydryl groups on LPI increases. We hypothesize that the compact, heat stable structure of the protein particles suppresses the intermolecular bonding through disulphide bridge formation and favours intramolecular crosslinking. The small sulphur-rich proteins that are not incorporated within the particles but are present in the surrounding solution cannot strengthen the particle network, due to their low concentration. LPI did not form gels of similar consistency as SPI and may therefore be less useful for solid food products. The thermal stability of LPI could offer opportunities for high-protein foods that require low viscosity after heating.

Chapter 3

Understanding the differences in gelling properties between lupin protein isolate and soy protein isolate

Highlights:

- Lupin protein isolate forms weaker heat-induced gels than soy protein isolate
- Lupin protein isolate and soy protein isolate form particle gels
- Lupin protein particles are heat stable
- The sulfhydryl reactivity of lupin protein isolate increases upon heating
- The concentration of free SH groups on soluble proteins is too low for strong gels

This chapter has been published as:

Berghout, J.A.M

Boom, R.M.

van der Goot, A.J.

Understanding the differences in gelling properties between lupin protein isolate and soy protein isolate

in

Food Hydrocolloids (2015) vol **43**

pages 465-472

3.1 Introduction

Legume seed proteins have gained increased attention due to their favourable nutritional and functional properties for modern food production (Batista et al., 2005; Day, 2013; Makri et al., 2005). The traditional raw materials for many plant-based alternatives to animal-based foods are soybeans and wheat and lately also peas and lupin can be found in these alternatives. Animal-based foods are mainly composed of protein, water and oil. Soybeans and lupin seeds are rich in protein, contain oil and are low in starch, while peas and wheat are high in starch. Soybeans do not grow in temperate areas though and therefore rely on a long supply chain while lupin can be grown in moderate climates areas as Northern Europe. Therefore, more research focuses on legumes that can be grown in moderate climate countries, like pea and lupin (Batista et al., 2005; Cai et al., 2002; Dijkstra et al., 2003; Drakos et al., 2007; Fontanari et al., 2012; Hojilla-Evangelista et al., 2004; Kiosseoglou et al., 1999; Makri et al., 2005; Mohamed et al., 2005; Swanson, 1990). Lupin seeds are interesting as food ingredient because of their high protein content, which is at least similar to that of soybeans. Currently, soy protein isolates and concentrates are mainly used in plant-based products because of their excellent gelling and structuring behaviour (Banerjee and Bhattacharya, 2012; Day, 2013). Many other legumes and oilseeds do not possess these functional properties naturally and that is why soybeans are taken as a benchmark. For example, pea and lupin protein isolates are reported to form weaker heat-induced gels than soy protein isolates (SPI) (Batista et al., 2005). The low gelling capacity made lupin an ideal protein source for replacing fish meal in fish pellets (Draganovic et al., 2014).

Food gels can be considered high-moisture, 3D polymeric networks that resist flow and retain their distinct structural shape upon deformation (Banerjee and Bhattacharya, 2012). Food gels are a continuous network of assorted macromolecules or interconnected particles dispersed in a continuous liquid phase, for which the properties are determined by the components present in the network. For example, differences in gel strength and deformability are related to differences in protein molecular weight and the hydrodynamic size of the polypeptides in the gel (Renkema, 2001; Totosaus et al., 2002). Gel formation of plant proteins can be induced through heating, which leads to transformations such as molecular unfolding, dissociation-association and aggregation (Batista et al., 2005; Damodaran et al., 2008). An unfolded protein exposes functional groups on the surface of the protein such as hydrophobic, hydrogen, electrostatic and sulfhydryl groups. After protein unfolding, protein aggregates are formed through hydrophobic interactions and strengthened further due to the formation of disulphide bridges (Wang and Damodaran,

1991). The role of disulphide bridges in protein gelation is related to their ability to increase the protein molecular weight and hence the chain length, rather than acting as an initial network stabilizer (Clark, 1998; Wang and Damodaran, 1990).

Soy and lupin flour both contain globular proteins, more specifically salt-soluble globulins and water-soluble albumins in a ratio of 9:1. During the production of protein isolates part of the water-soluble albumins are lost, enriching the protein isolate in globulins (Berghout et al., 2014; Lqari et al., 2002). Batista et al. (2005) established a relationship between the gelling ability of soy, pea and lupin protein isolates and their resistance to thermal unfolding. SPI formed strong gels, which was associated with more protein unfolding during and after thermal treatment. LPI formed only weak gels and the authors stated that this was because the unfolding of LPI upon heating was not significant due to its high denaturation temperature. However, it remains interesting to explore the nature of those differences. The reduced thermal unfolding of LPI might be related to the ratio of polar and non-polar amino acids present in LPI and SPI. Fisher (1964) introduced the polarity ratio p, which is the ratio of polar to non-polar volume of amino acid residues. This ratio is 1.7 for lupin flour and 1.4 for soy flour, which means that both have very polar proteins. The small difference between lupin and soy flour probably does not explain the major differences in gelling properties of SPI and LPI. The accessibility of sulfhydryl groups on LPI upon heating might play a role, though this has not been reported yet.

In this study we further explore the differences between LPI and SPI's functional properties and investigate the effect of an altered gelling process, such as prolonged, high temperature heat treatments, on LPIs gelling properties. Small deformation rheology is used to identify the differences between LPI and SPI dispersions and gels for 12 - 30% (w/v) protein and at 95°C. The swelling behaviour of LPI and SPI on macroscopic scale is studied with light microscopy and laser scattering. The differences between SPI and LPI on microscopic scale are investigated by the determination of the size of their protein subunits and by quantification of the amount of free sulfhydryl groups for disulphide bridge formation.

3.2 Materials and methods

3.2.1 Materials

Soy protein isolate (SPI), Supro 500E IP, was kindly provided by Barentz, the Netherlands. This product contained at least 90% protein (N x 6.25) and was not chemically modified after isolation according to the manufacturer's specifications. Lupin protein isolate (LPI)

with a protein content higher than 90% (N x 6.25) was prepared in-house, with the aqueous fractionation method as described previously by Berghout et al. (2014), from untoasted lupin seeds (LI Frank, Twello, the Netherlands). All reagents used were of analytical grade unless otherwise stated.

3.2.2 Methods

3.2.2.1 Preparation of protein dispersions and gels

Prior to gelling, the protein isolates were dispersed into Millipore water in 15 mL Falcon tubes at room temperature. The pH of the SPI dispersions varied between 7.1 and 7.2. The pH of the LPI dispersions varied between 6.8 and 7.0. The protein dispersions were stirred with a glass rod until completely wetted. The concentrations used were 12, 15, 18 and 24% (w/v) for SPI and 12, 15, 18, 24 and 30% (w/v) for LPI. After viscosity measurements (see 3.2.2.4 Small deformation rheology), the dispersions were heated in a water bath at 95°C and kept for 30 min. The dispersions were cooled with running tap water and subsequently stored at 4°C for 24 h. Two additional LPI dispersions of 30% (w/v) were prepared; the first dispersion was heat-treated in a water bath at 80°C and kept for 30 min, the second dispersion was heat-treated at 80°C and kept for 8 h. Both protein dispersions were cooled with running tapwater and stored at 4°C for 24 h. The protein dispersion heated at 80°C for 30 min was re-heated to 130°C in an in-house developed shearing device (van der Zalm et al., 2012) for about 10 min and then cooled down to 10°C. All dispersions and gels were prepared in duplicate.

3.2.2.2 Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were performed with a Diamond DSC (PerkinElmer, USA) using stainless steel pans. About 10 mg of sample was weighed into the pans. The DSC analyser was calibrated with indium and an empty pan was used for reference. Samples were scanned between 20°C and 130°C with a heating rate of 10 °C/min. Measurements were analysed for peak temperature and enthalpy of denaturation.

3.2.2.3 Light microscopy

An upright microscope Axioscope (Carl Zeiss Microscopy, LLC, United States) with camera was used to inspect the samples. The LPI and SPI powders were dissolved in Millipore water at 1% (w/v) and mixed at 900 rpm for 1 h on a Multi Reax vibrating shaker (Heidolph, Essex, UK). One LPI dispersion and one SPI dispersion were heated at 90°C for 30 min and cooled under running tapwater. The samples were prepared on a glass slide at room temperature and covered with a cover slip. Snapshots of 100 x, 200 x and 400 x magnification were taken.

3.2.2.4 Small deformation rheology

The protein dispersions were transferred to a rheometer (Anton Paar Physica MCR301, Graz, Austria) using a cone-plate geometry (CP-20-2). The samples were equilibrated for 5 min; subsequently the flow properties were determined at 25°C using a shear rate range from 1 to 100 s^{-1}. The flow properties of the protein gels were determined with plate-plate geometry (PP-25/P2) under the same conditions as the protein dispersions. Amplitude sweeps were performed to find the linear viscoelastic region of SPI and LPI gels. A frequency sweep test was performed on the protein gels with cone-plate geometry (CP-20-2) at constant strain (0.1%) and increasing angular frequency (0.1 - 10 rad/s) at 25°C. The gels were equilibrated for 10 min. Tangent delta (tan $\delta = G''/G'$) was calculated from frequency sweep data at 1 rad/s (within LVE).

3.2.2.5 Static laser scattering

For particle size analysis, 1% (w/v) protein isolate was dispersed in Millipore water in a 15 mL Falcon tube. For each protein isolate, five tubes were prepared: one tube was kept at room temperature, three tubes were heated at 75°C, 85°C or 95°C for 30 min, and one tube was heated at 80°C for 8 h. Additionally, one tube of 1% (w/v) LPI was heated at 90°C for 8 h. The particle size distribution was estimated by laser diffraction with a Mastersizer-2000 particle size analyser (Malvern Instruments Ltd., UK) with a wet module (Hydro SM). The Mastersizer measured the percentage volume particle size distribution (PSD) from which we calculated the percentage cumulative volume PSD.

3.2.2.6 High Performance-Size Exclusion Chromatography

The method used for High Performance-Size Exclusion Chromatography (HP-SEC) was based on a method previously used for soy proteins (Kuipers et al., 2006). Samples were dissolved in 500 mL of 0.15M Tris-HCl pH 8, containing 8M guanidine chloride and 0.1M 1,4-dithiothreitol. The final protein concentration was 20 mg/mL. Samples were mixed for 45 min. To the samples, 215 mL of acetonitrile containing 2% (v/v) trifluoretic acid (TFA) was added. After mixing for another 45 min, samples were centrifuged (1000 x *g*, 10 min, 20°C). The supernatant was pipetted into Eppendorf tubes and 10 mL was separated using a Phenomenex BioSep-SEC-S 4000 300 x 7.8 mm column (Phenomenex, Torrance, USA) by HPLC (Thermo Scientific, Sunnyvale, USA) operated with Chromeleon software (Dionex Corp., Sunnyvale, USA). The flow rate was 0.5 mL/min and the absorbance was monitored at 280 nm. The running buffer was a solution of 6M urea + 1% (v/v) TFA. The column was calibrated using various proteins in a molecular mass range of 1.35 kDa - 670 kDa. To determine the

ratios of small and large protein subunits, the peaks were split up into four areas: 5 - 10 kDa, 10 - 20 kDa, 20 - 50 kDa and 50 - 500 kDa. The surface areas under the chromatograms were used to estimate the relative amount of protein subunits.

3.2.2.7 Sulfhydryl content

The sulfhydryl reactivity was measured according to the method described by Van Horn et al. (2003), Alting et al. (2003) and Purwanti et al. (2011) with some modifications for insoluble particles. Ellman's reagent or 2-nitro-5-mercaptobenzoic acid (DTNB) was used as a reagent for spectrophotometric analysis (Ellman, 1959). In a 3 mL cuvette, 2.55 mL of 50 mM Bis-TRIS buffer (pH 7.0) was added to 0.25 mL DTNB solution (0.1% (w/v) Bis-TRIS buffer). Samples were diluted with deionized water to final protein concentrations of 2% (w/w), and then 0.2 mL of sample was added to the cuvette with Bis-TRIS buffer and DTNB solution inside. The mixtures were transferred into Eppendorf tubes, wrapped with aluminium foil and mixed for 10 min, after which the Eppendorf tubes were centrifuged at 10,000 x *g* for 1 min. After centrifugation, the supernatants were transferred into cuvettes and stored under aluminium foil for a few min. After a total incubation time of 15 min, the absorbance was measured at 412 nm with a spectrophotometer UV-vis Beckman Coulter DU-720 (Woerden, the Netherlands). The number of sulfhydryl (SH) groups was calculated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ for DTNB using the following formula (Eqs (3.1) and (3.2)):

$$Number\ of\ thiol\ groups\ (\mu mol) = \frac{\ln \frac{I_z}{I_0}}{-\varepsilon \cdot z} \cdot df \cdot volume\ sample \qquad \text{(Eq 3.1)}$$

$$Concentration\ thiol\ groups = \frac{Number\ of\ thiol\ groups}{Protein\ content\ sample} \qquad \text{(Eq 3.2)}$$

where *Iz/I0* is the transmittance, ε the molar extinction coefficient, *z* the path length and *df* the dilution factor. The values of the blanks were subtracted from the absorbance value to calculate the net absorbance value.

3.2.2.8 Statistical analysis

All rheological measurements were performed in duplicate. The figures show the mean value of duplicate experiments. The errors in the measured viscosities and stress values were determined as the ratio of the absolute deviation and the average value, multiplied by 100%. The uncertainty in the storage moduli and loss moduli for duplicate samples may be large, but due to their dependence, the uncertainty in the tangent of delta (tan δ) is small

(in the range of 3%). The protein subunit size and sulfhydryl content are expressed as mean ± absolute deviation based on duplicates. The protein subunit size and sulfhydryl content were evaluated with one-way analysis of variance (ANOVA) for significant differences ($P < 0.05$).

3.3 Results and discussion

Isolation of protein generally denatures protein, but DSC analysis showed that LPI was not fully denatured after protein isolation. SPI was fully denatured. We chose to study gelling properties of the SPI and LPI powders at their natural pH. The pH of SPI and LPI were close to neutral and close to each other (6.8 - 7.2) and we therefore expect little influence of pH on the gelling properties we studied.

3.3.1 Rheological behaviour of lupin protein isolate dispersions and gels

Visual observation and manual deformation of the LPI and SPI gels showed different consistencies. The LPI gel was easy to deform after gelation and did not reform (i.e. no elastic behaviour). The SPI gel was a self-standing gel that ruptured or broke upon deformation. The concentration at which the dispersions did not flow anymore was 15% (w/v) for the SPI gel and 18% (w/v) for the LPI gel. Dispersing 30% (w/v) SPI in water was not possible because the powder was not fully hydrated at this moisture content. LPI could be readily dispersed and hydrated at 30% (w/v). Fig 7 shows that the SPI dispersions had a much higher viscosity than the LPI dispersions at similar protein concentration. The LPI and SPI dispersions were shear thinning, but the SPI dispersions showed stronger shear thinning behaviour than the LPI dispersions. At low shear rates, 1 - 10 s^{-1}, LPI showed shear thinning

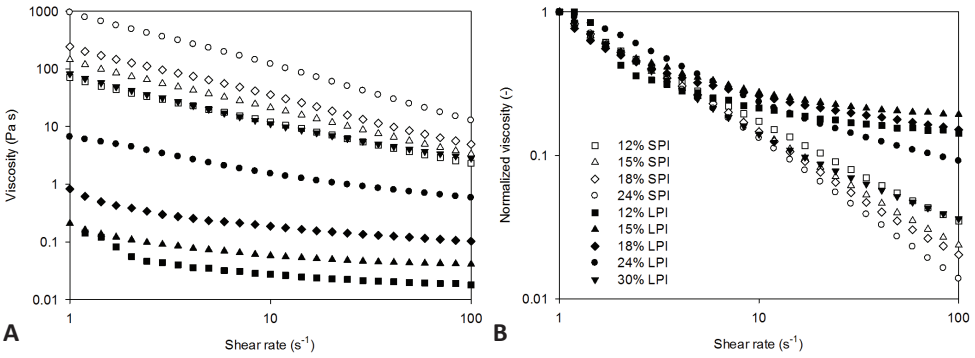


Figure 7 Viscosity as a function of shear rate at 25°C of SPI and LPI dispersions: (A) non- normalized flow curves, (B) normalized flow curves. Protein concentrations are given in the legend. The maximum error for shear rate sweeps was 33.7% for 12% (w/v) SPI and 24.6% for 12% (w/v) and 24% (w/v) LPI dispersions

behaviour, indicating the presence of a network, which was broken down at higher shear rates. At 30% (w/v) the viscosity as well at the shear thinning behaviour of the LPI dispersion were in the same range as the 12% (w/v) SPI dispersion. Fig 8 shows the small deformation results of the SPI and LPI gels that were heated at 95°C. SPI gels did not show frequency dependent behaviour (Fig 8A). According to Clark and Ross-Murphy (1987) weak gels show more frequency dependent behaviour than strong gels. Even though the loss modulus G'' fluctuated slightly at low frequency for the LPI gels at 15% (w/v) and 18% (w/v), no strong frequency dependent behaviour was observed (Fig 8B), which contrasted the visual observations and manual deformation of the weak LPI gels. The storage modulus of SPI was higher than the storage modulus of LPI for each protein concentration (Fig 8C). At high protein concentration (30% w/v) the storage modulus of LPI gels was similar to the storage modulus of the 24% (w/v) SPI gel, but the deformability of the gel remained. Fig 8D shows

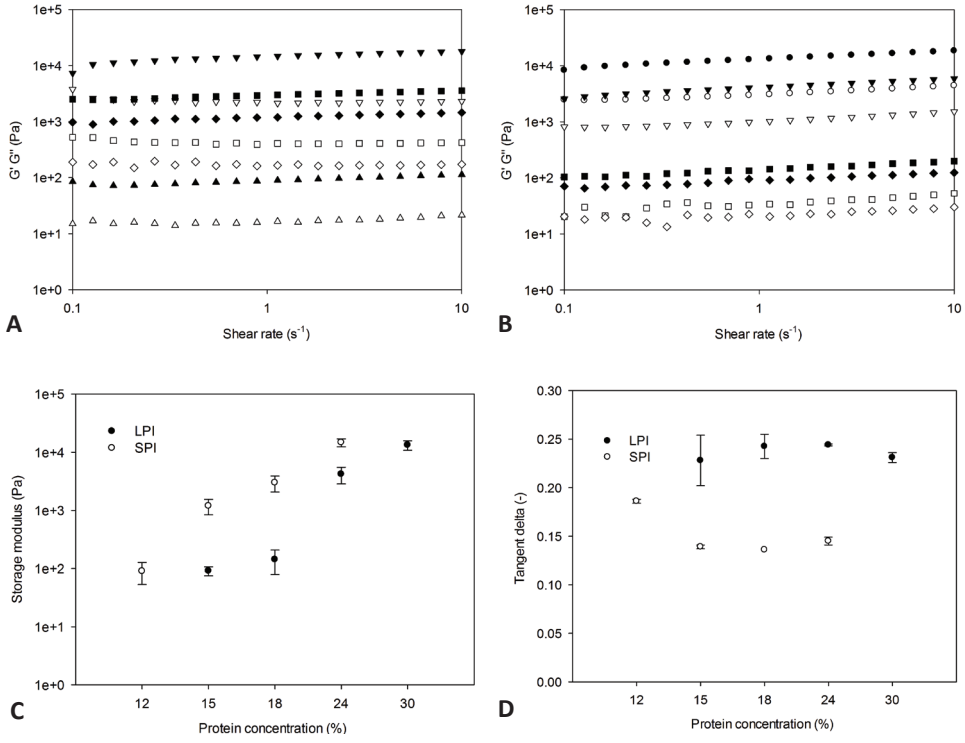


Figure 8 Gel properties of heat-induced SPI and LPI gels heated at 95°C: (A) storage (G' closed symbols) and loss modulus (G'' open symbols) of all SPI dispersions (v 24%, □ 18%, ◊ 15% and Δ 12%) as a function of angular frequency, (B) storage modulus and loss modulus of all LPI dispersions (○ 30%,v 24%, □ 18% and ◊ 15%) as a function of angular frequency, (C) storage modulus and loss modulus at 1 rad/s as a function of protein concentration (%) and (D) tangent delta at 1 rad/s as a function of protein concentration (%). Vertical Vertical error bars represent absolute deviation of the mean

that the tan δ of all SPI gels was lower than the tan δ of LPI gels. High storage moduli values are indicative of stronger inter-molecular networks and increased interactions between proteins, while low tan δ values indicate more elastic networks (Sun and Arntfield, 2010). It can be concluded that LPI formed weaker, less elastic gels than SPI at the same conditions.

3.3.2 Swelling of protein particles

Mixing of LPI and SPI with water resulted in a dispersion with protein particles. Obviously, the final drying process in the isolation process did not allow for complete dissolution of the protein particles. This was revealed by microscopy and the particle size distribution (PSD) analysis (Figs 9 and 10). In case of a particle dispersion, the formation of a gel can be achieved through strong inter-particle interactions or through particle jamming, which is enhanced by particle swelling (van der Sman and van der Goot, 2009). Fig 9 shows that the structure of LPI particles (A) upon heating (B) hardly changed, whereas SPI particles (C) changed considerably upon heating (D). Particle size changes with temperature due to association, swelling or dissociation of particles. The heated SPI particles seemed more

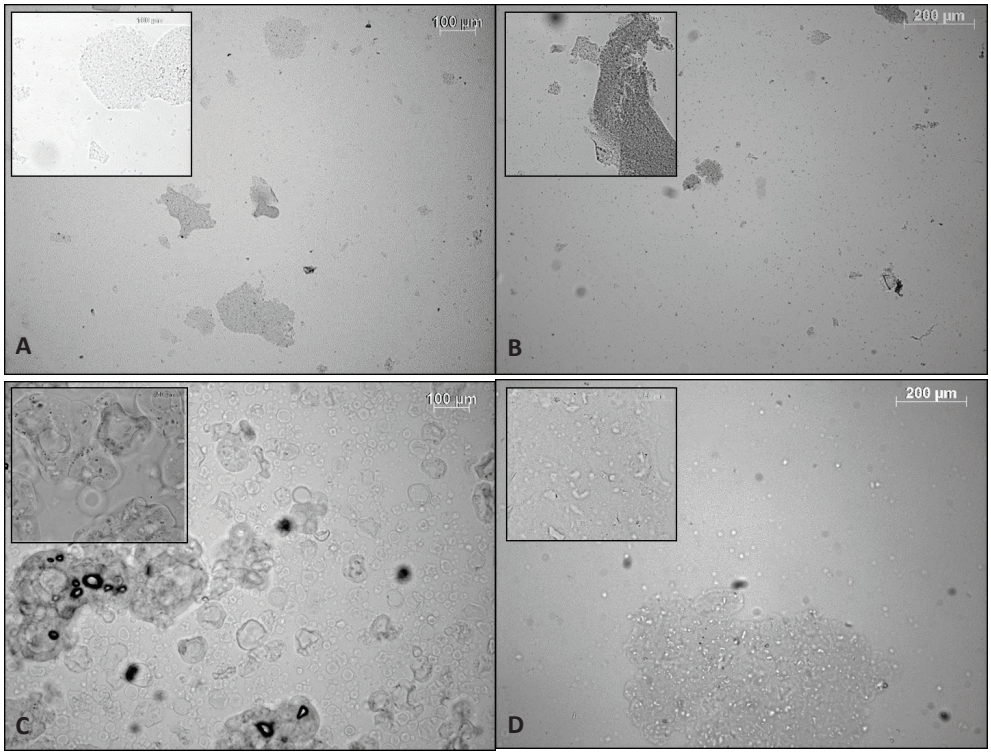


Figure 9 Microscopic images: (A) LPI dispersion unheated, (B) LPI dispersion heated at 90°C for 30 min, (C) SPI dispersion unheated and (D) SPI dispersion heated at 90°C for 30 min. The scale bars correspond to 100 µm in (A) and (C), and to 200 µm in (B) and (D). In the small pictures the scale bars correspond to 100 µm in (A) and 50 µm in (B), (C) and (D)

swollen and deformable than LPI particles, which is probably due to the absorption of water. The observations made in the microscopic studies were in line with the cumulative volume PSD calculated for LPI and SPI upon heating (Fig 10); after heating the PSD for SPI changed, while for LPI hardly any difference was observed in the PSD. SPI particles swelled or aggregated upon heating until 95°C for 30 min and started to dissociate upon longer heating times as can be seen from the increase in the smaller particle size range in Fig 10. Fig 10 shows little change in particle size with an increase in temperature for LPI particles, indicating limited particle swelling or association. Even prolonged heating did not show an increase in smaller particle sizes, thus dissociation of particles was limited. The lack of change in particle size of LPI particles with temperature indicates that the particles have a high thermal stability. Sousa et al. (1995) found the 7S globulin of lupin to be more heat stable than the 7S globulin of soy, which is in line with our results. This heat stability could explain some of the differences in gelling properties of LPI and SPI.

Fig 11 compares non-heated LPI dispersions of 30% (w/v) with LPI dispersions that were heated for 30 min and for longer time (8 h). It turned out that the absolute viscosities of the heat-treated dispersions were higher than the viscosity of the unheated LPI dispersion.

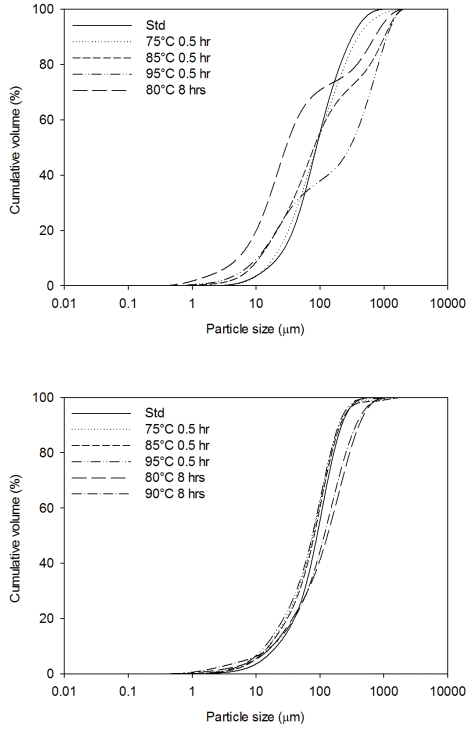


Figure 10 Cumulative volume particle size distribution (mm) of (A) SPI dispersion unheated (std = standard), SPI dispersions that were heated at 75°C, 85°C, 95°C for 30 min and an SPI dispersion heated at 80°C for 8 h, and (B) LPI dispersion unheated (std = standard), LPI dispersions that were heated at 75°C, 85°C, 95°C for 30 min and LPI dispersions that were heated at 80°C and 90°C for 8 h

The shear thinning behaviour did not change upon prolonged heating. Short and prolonged heating increased the viscosity to a value similar as an unheated 24% (w/v) SPI dispersion. Nevertheless, Figs 9B and 10 indicated limited particle swelling upon heating, which could explain a limited effect on viscosity. Even an additional heat treatment at 130°C (re-heating and re-cooling) of the 30% (w/v) LPI gel resulted in a weak, deformable gel. To conclude: LPI remains a weak, deformable gel independent of its thermal history.

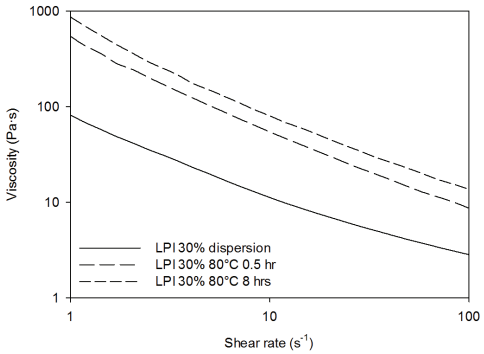


Figure 11 Viscosity as a function of shear rate at 25°C of 30% (w/v) LPI dispersion (un- heated), 30% (w/v) LPI gel that was heated at 80°C for 0.5 h and 30% (w/v) LPI gel that was heated at 80°C for 8 h

3.3.3 Particle gels and jamming

As stated previously, both protein isolates resulted in particle dispersions rather than protein solutions. Thermal treatment altered the particle structure of SPI, leading to the formation of a firm gel. Upon particle swelling, particle interactions increased (most likely due to jamming effects), explaining the increased viscosity and the shear thinning behaviour of SPI. LPI particles were nearly stable upon heating and remained compact. Only at a concentration of 30% (w/v), a marked increase in viscosity was observed suggesting that particles interact due to jamming effects.

Fitting the viscosity data with the Krieger and Dougherty equation would indicate that jamming occurs at mass fractions of 0.3 for LPI, assuming no water absorption. The assumption of a density of 1.35 g/cm^3 for LPI protein (Fischer et al., 2004) leads to a volume fraction of 0.24 for jamming of LPI particles. For monodisperse systems, jamming normally occurs at volume fractions of 0.64 and for polydisperse systems this value can be higher (Walstra, 2003). This suggests that LPI has to absorb about 1.6 - 1.7 times its weight in water to obtain particle volume fractions larger than 0.6. SPI absorbs much more water, leading to larger particles and jamming effects at much lower concentrations. Those observations are in line with the water holding capacity of SPI: 4 mL/g (corresponding to a φ_{max} of around 0.16) and the amount of water that was retained by LPI (±1.3 mL/g, indeed agreeing with a

φ_{max} of around 0.24), just before a viscous supernatant appeared on the pellet (Berghout et al., 2014).

3.3.4 Cross-linking ability of LPI and SPI

The amount of sulfhydryl groups in protein is determined by the amount of sulphur-containing amino acids: methionine and cysteine. The approximate amino acid composition and molecular sizes of lupin and soy proteins were reported before (Belski, 2012; Duranti et al., 2008; Fontanari et al., 2012; Utsumi et al.,1997). Table 4 gives an overview of the protein fractions of soy and lupin and their molecular sizes. We determined the ratio of large to small proteins and the amount of free sulfhydryl groups in LPI and SPI. Dissociating HP-SEC showed that the molecular size distributions of LPI and SPI were different (Fig 12). As expected, SPI had more protein subunits in the size range of 50 - 500 kDa while LPI had more protein subunits in the smaller molecular size ranges: 5 - 10 kDa, 10 - 20 kDa and 20 - 50 kDa.

Table 4 Comparison of SPI and LPI on proteins, protein size, amount of disulphide bridges and sulfhydryl reactivity

		Protein	% in bean or seed	Non reduced Mr (kDa)	Reduced Mr (kDa)	Amount of disulphide bridges	Free sulfhydryl groups (µmol/g)[e]
LPI	11S	Conglutin α[ac]	35-37	330-430	42-45 and 20-22	6	19.5 and increases upon heating
	7S	Conglutin β[a]	44-45	143-260	53-64 and 25-46 and 17-20	0	
		Conglutin γ[a]	4-5	200	29 and 17	2	
	2S	Conglutin δ/ albumin[a]	10-12	13	4 and 9	4	
SPI	11S	Glycinin[bd]	52	300-360	63.5-67.2 and 47.8	2	10.6 and decreases upon heating
	7S	β-Conglycinin[bd]	33-35	150-200		0	
	2S		15	8-22		0	

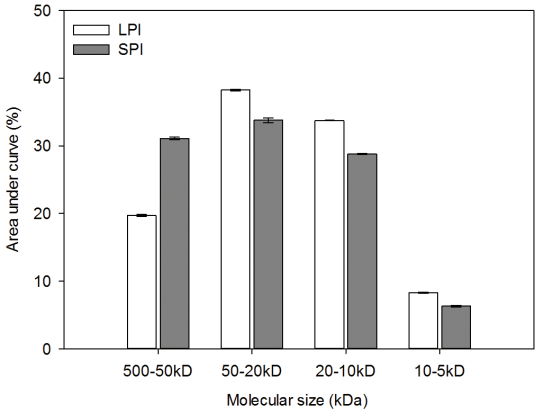


Figure 12 HP-SEC (dissociating conditions) elution profiles of SPI and LPI. Vertical error bars represent absolute deviation of the mean (n = 2)

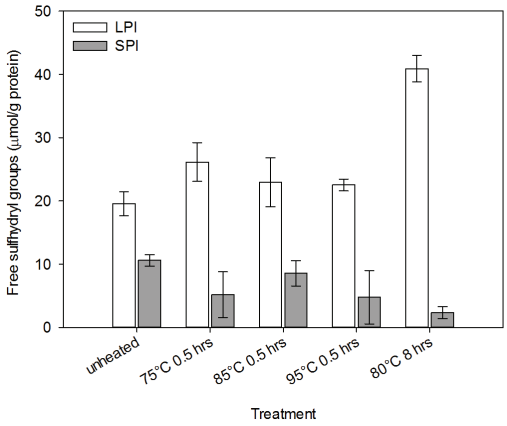


Figure 13 Free sulfhydryl groups of unheated and heat-treated LPI and SPI. Vertical error bars represent the absolute deviation of the mean (n = 2)

The amount of free sulfhydryl groups quantified for SPI and LPI before and after heating at different temperatures was always higher for LPI than for SPI (Fig 13), which was expected from the amount of sulphur-containing amino acids in LPI and SPI (Table 4). For SPI, the value of free sulfhydryl groups decreased with increasing temperature, suggesting that new disulphide bridges formed upon cooling. For LPI, this value increased with increasing temperature, indicating that no new disulphide bridges could be formed. This suggests that sulfhydryl groups cannot react into disulphide bridges, confirming the idea that the particles are compact, with limited mobility of the proteins inside those particles. Our measurements showed that LPI consists of smaller protein molecules than SPI but that the smaller LPI molecules are more reactive than the larger SPI molecules. A change in protein configuration is accompanied by a rearrangement of disulphide bridges. These rearrangements can occur once the temperature is reached to overcome the activation energy for disulphide bridge opening. Sulfhydryl groups in SPI are present on glycinin (11S) (Table 4), which are known to aggregate into larger polymers (15S), strengthened by the formation of additional disulphide bridges (Adachi et al., 2004; Speroni et al., 2009). LPI has more disulphide bridges (Table 4) and the chance that those will open at the same time will decrease with the number of disulphide bridges present, thereby reducing the ability for the protein to change configuration. We suspect that this effect could explain the thermal stability of LPI. The thermal stability is further enhanced by the compact protein particles that form upon drying, where short-range cross-linking dominates over longer range cross-linking. Rector et al. (1989) and O'Kane et al. (2004) respectively, stated that re-heating a whey protein isolate and pea protein isolate gel would increase the amount of disulphide bridges being formed, consequently enabling more extensive short-range crosslinks upon re-cooling. Our

experiment with re-heating to 130°C and re-cooling showed no improvement in the gelling properties of LPI, and thus deviates from the behaviour of whey and pea protein isolate.

We hypothesize that the thermal stability of LPI particles is related to their high sulfhydryl content, resulting in a large number of intramolecular bonds that leads to a stable structure. Part of the free sulfhydryl groups are present on the smallest proteins in LPI (2S albumin and conglutin δ) that might not be incorporated inside the particles but are present in the liquid surrounding the particles. In that case, we postulate that the small size (<23 kDa) of these proteins prevents them from strengthening the whole network (Wang et al., 1990). Whey protein isolate (WPI) however, contains 65% of β-lactoglobulin with a molecular size of 18.2 kDa, is rich in disulphide bridges (Walstra et al., 2006), and forms self-standing gels upon heating (Purwanti et al., 2010). Similar to LPI, the amount of free sulfhydryl groups in WPI increases upon heating (Adachi et al., 2004; Alting et al., 2003) but decreases upon cooling, probably due to disulphide bridge formation. The concentration at which β-lactoglobulin is present in WPI, is much higher than the concentration of conglutin δ and 2S albumin in LPI. It can therefore be concluded that the concentration of conglutin δ and 2S albumin in LPI is too low to obtain a strong protein gel with LPI.

3.4 Conclusion

The aim of this paper was to investigate whether LPI could have similar gel properties as SPI. SPI was taken as reference material because of its wide applicability in many semi-solid food products, amongst others those aimed at meat replacement. Given the similarities in composition of LPI and SPI, LPI is considered an attractive alternative for soybeans because lupin seeds can be grown in moderate climate areas. This paper showed that LPI behaved fundamentally different than SPI, as a result of which it is not able to form gels with similar consistency and deformability as SPI gels. However, LPI offers opportunities for high-protein food and feed products that require a protein source with a high dispersability and lower viscosity after heating at high concentration.

Acknowledgements

The authors thank Stefano Risso for his contribution to the experimental work, Herman de Beukelaer for the DSC analysis, Nicolas Hardt and Dr. Paul Venema for fruitful discussions on rheological properties and the peas foundation and the Institute for Sustainable Process Technology (ISPT) for financial and project support through the Intensified Protein Structuring for More Sustainable Food project.

Traditional ingredient production focusses on high purity and yield, resulting in energy- and resource-intensive fractionation processes. We explored alternative fractionation routes for oilseeds by focussing on functionality and optimal resource use. Lupin seeds were taken as model material because they are rich in protein and oil and they can be grown in moderate climate conditions. Dry fractionation yields functional protein-enriched flours without using water, consumes the least energy and exergy losses are low. Purer protein fractions are obtained via wet or aqueous fractionation, but these processes require large amounts of water and an energy-intensive drying step. With the use of exergy analysis, we demonstrate that water and energy consumption can be reduced by replacing drying steps with concentration steps and by combining dry and aqueous fractionation processes. Finally, by valorising side streams, the exergetic efficiency of all fractionation processes increases.

Chapter 4

Sustainability assessment of oilseed fractionation processes: A case study on lupin seeds

Highlights:

- Aqueous fractionation consumes fewer resources than conventional wet fractionation
- Chemical exergy has a bigger impact on process sustainability than physical exergy
- Combining dry and aqueous fractionation decreases water use during protein isolation
- Adding value to side streams improves process sustainability

This chapter has been published as:

Berghout, J.A.M

Pelgrom, P.J.M.

Schutyser, M.A.I.

Boom, R.M.

van der Goot, A.J.

Sustainability assessment of oilseed fractionation processes: a case study on lupin seeds

in

Journal of Food Engineering (2015) vol **150**

pages 117–124

4.1 Introduction

Plant-based diets are more sustainable than animal-based diets (Pimentel and Pimentel, 2003). To replace animal-based protein products, plant-based materials are needed that meet the nutritional, functional and textural properties of animal-based products (Hoek et al., 2011). Animal-based products consist of water, protein and fat. Pulses, legumes and oilseeds contain these components as well, but they also contain carbohydrates, like dietary fibre, starch and sugar, and oil. Promising oilseeds for plant-based materials are soybeans, lupin seeds, canola meal, and sunflower seeds or sunflower meal because they are rich in protein, oil and they are low in starch (Day, 2013). Especially lupin seeds are of interest because they have the highest protein content in comparison with other oilseeds and they can be grown in moderate climate areas like Northern Europe. To better mimic animal-based products, the carbohydrate content has to be reduced, which leads to the necessity of refining. This is generally carried out by wet fractionation, aiming at complete separation and high purity of the components. Unfortunately, conventional wet fractionation processes consume solvents, energy and water, which negatively impact the sustainability of the production of plant-based materials (Apaiah et al., 2006). It leads to a need for milder fractionation alternatives that consume less solvents and energy.

A milder wet fractionation method is aqueous fractionation, where the use of organic solvents is omitted (Aguilera et al., 1983; Campbell et al., 2010; Jung, 2009). In a previous paper we showed that aqueous fractionation of lupin seeds yields protein isolates with 2 - 7 g oil/100 g protein isolate that has similar functionality as protein isolates obtained with conventional wet fractionation (Berghout et al., 2014). Wet and aqueous fractionation of lupin seeds yield wet protein isolates that have to be stabilized to prevent microbial and chemical spoilage, which is often accomplished through drying steps. Drying consumes a lot of energy while it can detrimentally affect protein functionality (Hu et al., 2009; Joshi et al., 2011; Liao et al., 2013). A less energy intensive process is ultrafiltration, however this leads to a reduced microbial and chemical stability and therefore the protein isolates have to be processed shortly after fractionation to prevent spoilage. We investigated the potential of replacing the drying step in the process with a concentration step. Dry fractionation is a sustainable alternative for wet and aqueous fractionation, because it avoids the use of water and consumes less energy (Schutyser and van der Goot, 2011). In addition, the fractions have been processed in a milder way because they have not been wetted and subsequently dried, and therefore retain their original functionality (Pelgrom et al., 2013 and 2014; Schutyser and van der Goot, 2011). Dry fractionation involves fine milling and

air classification. The classification is based on particle size and density differences of the particles obtained after milling. Pelgrom et al. (2014) showed that dry fractionation of lupin seeds provides protein-enriched flours with protein contents of 53.5 g protein/100 g protein concentrate (N x 5.7) and unique protein functionality. The protein-enriched flours can be further refined into protein isolates by aqueous fractionation in case a higher protein content is desired. Separating the raw materials into pure ingredients is however not efficient, because no end product is composed of one pure ingredient. It may be more efficient to produce less pure fractions that can be directly used for an application, consequently avoiding the need for other pure ingredients. Focusing on molecular purity, as is done with protein isolates, also increases the amount of side streams that are produced. These side streams still contain valuable ingredients that can potentially be used for other (food) applications, which can contribute to a more sustainable future (Aiking, 2011; Mirabella et al., 2014). That is why we evaluated the effect of valorising these streams on sustainability of fractionation processes.

Exergy is an environmental indicator that is demonstrated to be useful for development of sustainable products and processes, like for the minimization of the use of resources and the emissions of processes (Bastianoni et al., 2005; Dincer and Rosen, 2004; Rosen et al., 2008). Exergy, also called available work, is a thermodynamic state variable that is based on the second law of thermodynamics (Apaiah et al., 2006) and it shows the potential work that can be done by exchange with a reference environment (Rosen et al., 2008). Rosen et al. (2008) showed there is a positive relation between exergy efficiency and environmental impact.

The objective of this paper is to assess the impact of producing protein-enriched fractions using dry, wet and aqueous fractionation. The production routes are evaluated for mass of solvents used, for energy consumed and for exergy losses and efficiencies. It is evaluated whether a combination of dry and aqueous fractionation processes increases the sustainability of fractionation of lupin seeds. In addition, coupling fractionation and product application, i.e. skipping the final drying step, is discussed. A final optimization of individual unit operations was outside the scope of this study.

4.2 Material and methods

4.2.1 System boundaries

Fig 14 shows the materials and products for all fractionation processes chosen for analysis.

The impact of the processes was evaluated based on the main differences between them. This means that for comparing dry and wet fractionation, the use of water is evaluated and for the conventional wet and aqueous fractionation process the use of an oil extraction step is evaluated. The combination of both processes will be evaluated as well because the products obtained with dry fractionation are not as pure as with wet fractionation.

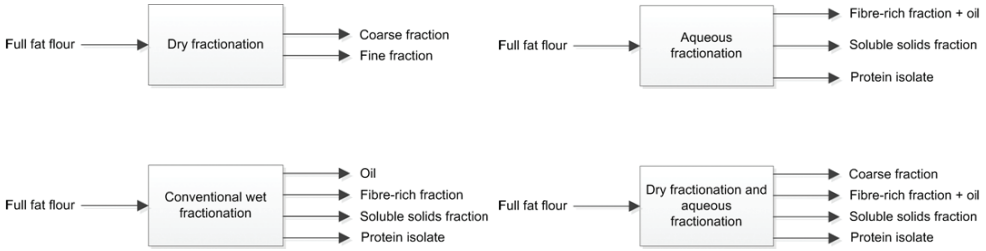


Figure 14 Fractionation processes: dry fractionation, conventional wet fractionation, aqueous fractionation and a combination of dry and aqueous fractionation

4.2.2 Fractionation processes

Dry fractionation was performed as described by Pelgrom et al. (2014). Lupin seeds (*L. angustifolius*) were obtained from LI Frank (Twello, the Netherlands). The seeds were pre-milled into grits with a pin mill LV 15 M (Condux-Werk, Wolfgang bei Hanau, Germany) and were then milled into flour with a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) with classifier wheel speed set at 1000 rpm, mill speed of 8000 rpm, and air flow at 80 m3/h. The flour was fractionated with an air-classifier set at 10,000 rpm and air flow at 80 m3/h, which resulted in a fine and a coarse fraction.

Conventional wet fractionation was performed as described in Berghout et al. (2014). Full fat lupin flour was defatted with petroleum ether (boiling range 40 - 60°C) with a sample-to-solvent ratio of 1:6 on a fully automated Büchi extraction system B-811 LSV (Büchi Labortechnik AG, Flawil, Switzerland). The defatted lupin flour was then dispersed into tap water using a sample-to-solvent ratio of 1:15. The pH of the dispersion was set to 9 with 1 mol/L NaOH and the dispersion was stirred at 20°C for 1 h. The dispersions were centrifuged at 11,000 x *g* and 20°C for 30 min. The supernatant was separated from the pellet and the pH of the supernatant was adjusted to pH 4.5 with 1 mol/L HCl. The protein-rich supernatant was stirred at 20°C for 1 h and subsequently centrifuged at 11,000 x *g* and 20°C for 30 min. The supernatant was separated from the pellet and the pellet was rinsed twice with 50 mL Millipore water and kept at 20°C for 1 h and then centrifuged at 10,000 x *g* and 20°C for 10 min to remove impurities. The protein pellet was stored in the freezer at -20°C for freeze-drying.

Aqueous fractionation omits the oil extraction step used during the conventional wet fractionation process and was performed as described previously by Berghout et al. (2014). The full fat lupin flour was dispersed into tap water using a sample-to-solvent ratio of 1:15. The rest of the fractionation process was the same as for the conventional wet fractionation process, except that the whole process was performed at 4°C. Aqueous fractionation was also performed with the fine fraction that was obtained after air classification. This process is the same as described for the full fat flour.

4.2.3 Data collection

Compositions and masses of all fractions are based on the experimental work. The amount of protein isolate produced was set to 1000 kg (1 ton). Aqueous fractionation yields protein isolates that contain oil, a fibre-rich pellet that contains most of the oil and a soluble solids fraction that does not contain oil. For ease of comparison, it was assumed that the protein isolates (in contrast to the protein-enriched flours) obtained with the different fractionation processes had similar composition, i.e. 83 g protein/100 g, 5 g water/100 g, 10 g carbohydrates/100 g and 2 g mineral residue/100 g. Data of equipment like a spray drier, an oil extraction system and a cooling system are based on equipment often used on pilot scale. The ratio of sample-to-solvent in the oil extractor is based on lab scale experiments performed at the German Fraunhofer Institute in Freising (Bader et al., 2011).

4.2.4 Protein recovery and purity

The protein content of each fraction was calculated from the nitrogen content (N x 5.7), determined with the Dumas combustion method on a NA2100 Nitrogen and Protein Analyser (ThermoQuest-CE Instruments, Rodeno, Italy). Methionine was used as a standard for the analysis. Dry matter content was determined by drying each fraction in an oven at 105°C overnight. The protein recovery in each fraction of each fractionation process was calculated with Eq. 4.1 and the purity (protein content) of each fraction was calculated with Eq. 4.2,

$$Protein\,recovery = \frac{m_{fraction} \cdot x_{p,fraction}}{m_{seeds} \cdot x_{p,seeds}} \cdot 100\% \qquad \text{(Eq. 4.1)}$$

$$Purity = \frac{x_{p,fraction}}{x_{dm,fraction}} \cdot 100\% \qquad \text{(Eq. 4.2)}$$

where m is mass (kg), x_p is mass fraction protein and x_{dm} is mass fraction dry matter.

4.2.5 Calculations

The mass and energy flows were calculated with Eq. 4.3 and Eq. 4.4,

$$\sum m_{in} - \sum m_{out} = 0 \quad \text{(Eq 4.3)}$$

$$\sum (mh)_{out} - \sum (mh)_{in} = Q - W \quad \text{(Eq 4.4)}$$

where m is mass (kg), h is enthalpy (kJ/mol), Q is heat (kJ) and W is work performed by the system.

The exergy taken into account is based on the chemical exergy of the raw material (i.e., the work that could be done by converting this material into environmental components), products and processing aids (Eq. 4.8) and physical exergy (thermal, Eq. 4.5 and pressure exergy, Eqs. 4.6 and 4.7) of the selected processes. For dry fractionation, the electrical exergy was included as well (Eq. 4.9), while thermal and pressure exergy were neglected. The physical exergy refers to potential work that can be performed by heat or expansion, or any other physical exchange with the environment, and is related to inefficiencies in process steps. The chemical exergy refers to potential work of a stream and is related to efficiencies in the use of materials. Exergy losses are calculated with Eqs. 4.10 and 4.11. The exergy efficiency calculations are based on the chemical exergy of streams; the chemical exergy efficiency (CEE) is defined as (1) total exergy out over the exergy that enters a process (total chemical exergy efficiency; Eq. 4.12) and as (2) useful exergy out over the exergy that enters a process (useful chemical exergy efficiency; Eq. 4.13). Useful exergy is defined as the chemical exergy of the fractions that may theoretically find an application, like the protein isolate (PI), the oil, the coarse fraction, the fibre-rich pellet (FP) and the soluble solids fraction (SSF). The sum of the chemical exergy of all these fractions equals the total exergy out.

$$Ex_{physical} = Ex_{thermal} + Ex_{pressure} \quad \text{(Eq 4.5)}$$

$$Ex_{thermal} = m \cdot c_p \cdot \left[\left(T - T_0 \right) - T_0 \cdot \ln\left(\frac{T}{T_0} \right) \right] \quad \text{(Eq 4.6)}$$

$$Ex_{pressure} = \frac{R \cdot T}{M_x} \cdot \left[\ln\left(\frac{P}{P_0} \right) \right] \quad \text{(Eq 4.7)}$$

$$Ex_{stchem} = m \cdot \sum_{i=1}^{n} (b_{stchem,i} \cdot x_i) \quad \text{(Eq 4.8)}$$

$$Ex_{electrical} = Q \cdot f \quad \text{(Eq 4.9)}$$

$$Ex_{loss} = Ex_{in} - Ex_{out} \quad \text{(Eq 4.10)}$$

$$Ex_{loss} = Ex_{waste} + Ex_{destruction} \quad \text{(Eq 4.11)}$$

$$Total\ chemical\ exergy\ efficiency = \frac{Total\ Ex_{chem}out}{Total\ Ex_{chem}in} \quad \text{(Eq 4.12)}$$

$$Useful\ chemical\ exergy\ efficiency = \frac{Useful\ Ex_{chem}out}{Total\ Ex_{chem}in} \quad \text{(Eq 4.13)}$$

where Ex is exergy (kJ), m is mass (kg), c_p is specific heat capacity (kJ/kg·K), T_0 is the reference temperature (K), T is temperature (K), R is the ideal gas constant (kJ/mol·K), M_x is average molar mass of the stream (kg/mol), P is pressure (Pa), P_0 is the reference pressure (Pa), Q is heat (kJ/kg), f is a quality factor (=1 for electrical exergy), b_{stchem} is standard chemical exergy (kJ/kg) for which the values can be found in Appendix Table A2, and x_i mass fraction of component i (-).

4.2.6 Mass and exergy flow visualizations

Mass and energy flows were visualized with Sankey diagrams and exergy flows were visualized with Grassmann diagrams, using e!Sankey 3.1 (ifu Hamburg GmbH, Hamburg, Germany).

4.2.7 Assumptions

For consistent results, exergy analysis relies on the use of a standardised environment, which models our real environment and provides a fixed framework of reference.

- The standard environment has a temperature of 20°C (293.15 K) and pressure of 101.325 kPa.
- Water is available at 293.15 K.
- Natural gas is used to heat up the air for spray drying.
- The specific heat capacity (c_p) (kJ/kg·K) is temperature-dependent but the effect has little influence on the calculated heat values and we thus assumed the cp to be temperature-independent.
- The gas burner has an enthalpic efficiency of 0.65 (i.e. 35% of the heat is lost).
- Chemical exergy values of lupin seeds, water, hexane, sodium hydroxide, hydrochloric acid and steam were considered.
- Waste streams can be emitted to the environment without additional processing.
- There is no accumulation or loss in the process.
- The cooling equipment for aqueous processing has an efficiency of 0.5.
- Heat exchange was assumed to be ideal. Heating duty is provided by steam (403.15 K, 271.8 kPa) and cooling duty is provided by cooling water (288.15 K).
- The maximum amount of hexane wasted, may not exceed 0.8 kg hexane/ton (soybean) seeds, as specified in European law (European Commission, 2008).

4.3 Results and discussion

4.3.1 Dry fractionation versus wet fractionation

Dry and wet fractionation processes differ in their separation principle, the use of resources, the unit operations needed, and the yield and composition of fractions obtained. The efficiency of these fractionation processes is evaluated through calculating and visualizing mass, energy and exergy flows.

4.3.1.1 Protein yield and protein content

Dry fractionation by fine milling is based on the physical disentanglement of protein bodies from fibres and other cellular components, which allows their subsequent separation by air classification (Boye et al., 2010; Pelgrom et al., 2013). Wet fractionation is based on the differences in solubility of the different components in organic solvents, water and saline solutions. Dry fractionation of lupin seeds leads to protein-enriched flours (>50 g protein/100 g). Wet fractionation of lupin seeds can yield protein concentrates (>70 g protein/100 g) and further fractionation leads to protein isolates (>90 g protein/100 g). Fig 15 illustrates that the protein yield usually decreases with increasing protein content for wet fractionation: the amount of protein isolate obtained is generally smaller than the amount of protein concentrate obtained.

Dry fractionation yields a fine fraction and a coarse fraction. Depending on the exact parameter settings of the mill and the air classifier, the process results ideally in about 33

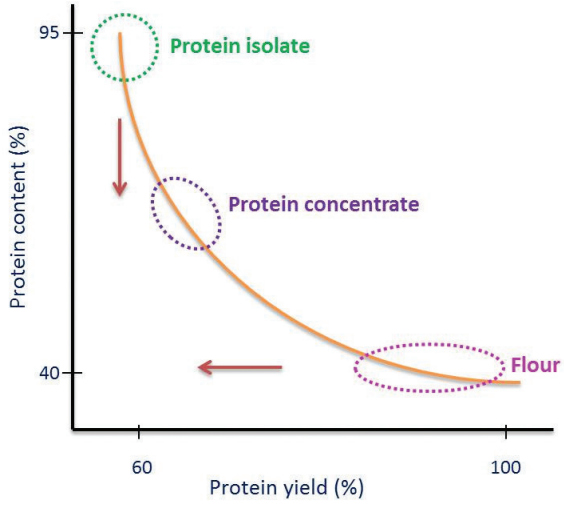


Figure 15 Schematic representation of protein content as a function of protein yield upon wet fractionation of protein

g fine fraction/100 g flour and 67 g coarse fraction/100 g flour for lupin seeds. The fine fraction is enriched in protein (to max. 53.5 g/100 g fine fraction) while the coarse fraction is enriched in fibres and has a protein content of 28.9 g/100 g coarse fraction (Pelgrom et al., 2014). Thus, 48 g/100 g of protein that was initially present in the flour is recovered in the fine fraction, and 52 g/100 g of protein ends up in the coarse fraction. The large volume and the high protein content of the coarse fraction are due to the cut-off between small and large particles in lupin (Pelgrom et al., 2014). The protein yields and protein contents for dry fractionation will follow a different curve than the curve for wet fractionation in Fig 15; half of the protein originally present in the flour shifted to the fine fraction and the other half to the coarse fraction. Wet fractionation on lab scale yielded a protein isolate (28 g/100 g of the seed) with a protein content of 82.6 g/100 g protein isolate. This means that about 63 g/100 g of the protein that was initially present in the flour can be recovered with wet fractionation.

4.3.1.2 Energy requirements for evaporation of water

Dry fractionation is often praised for the mild disclosure of particles, for the absence of water in the process, and the low energy use. Bond's empirical model indicates that the energy requirement for dry fractionation and air classification is about 1 MJ/kg lupin protein-enriched flour, which is in line with the values estimated by Schutyser and van der Goot (2011). Conventional wet fractionation processes require the following process equipment: oil extractor, mixers, decanters, solids separators and spray driers (i.e. water evaporation). Oil extraction and spray drying are the most energy consuming steps, while mixing and decanting consume less energy. The mass and energy flows are visualized with Sankey diagrams in Fig 16. The energy requirement for drying a wet protein isolate from 30 g dry matter/100 g to 95 g dry matter/100 g in a spray drier was calculated to be 6 MJ/kg evaporated water, which is in concurrence with the values reported by Filková et al. (2007). In other words, this single unit operation requires already 6 times more energy than the total dry fractionation process. In case the fibre-rich pellet (FP) and soluble solids fraction

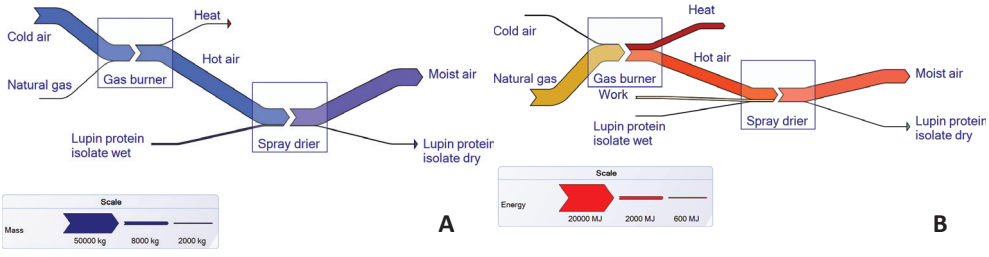


Figure 16 Sankey diagrams of spray drier: (A) Mass flows (kg), (B) Energy flows (MJ)

(SSF) also need to be dried, the drying duties will at least double, considering the water content in those streams.

4.3.1.3 Exergy losses for dry fractionation and spray drying

The chemical and physical exergy flows for the spray drier were calculated and the physical exergy flows were visualized (Fig 17A). The chemical exergy of the natural gas flow was not visualized in the diagram because this stream is much larger than the other streams masking relevant details. The natural gas flow accounts for the biggest exergy loss of the spray drier; 12.8 MJ/kg protein isolate (Fig 18). The physical exergy loss is linked to water vapour coming out of the spray drier and is about 12% of the chemical exergy loss (Fig 18). The physical exergy loss of dry fractionation is calculated to be 1 MJ/kg protein-enriched flour and thus about 6% of the total exergy loss (chemical + physical) for spray drying. Those outcomes clearly demonstrate that dry fractionation is the most efficient method from an environmental point of view as no water is added and no water needs to be evaporated, provided that the protein-enriched flour is a product of interest, i.e. for its functionality. If a higher purity is required, dry fractionation alone is not enough.

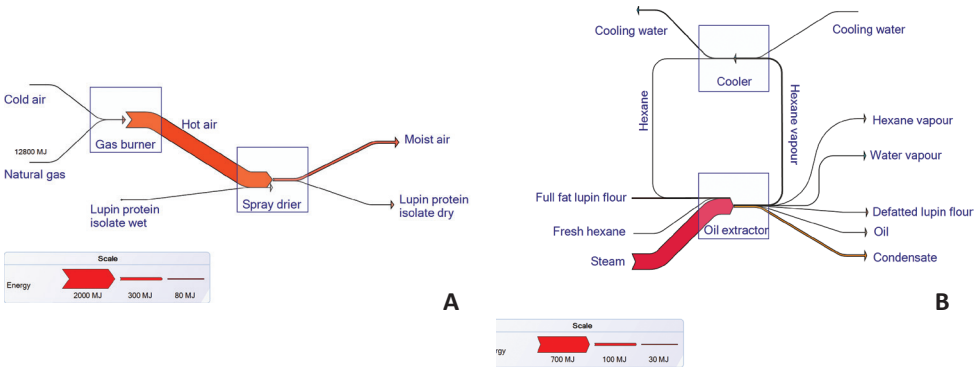


Figure 17 Grassmann diagrams: (A) Spray drier, chemical exergy of the feed, product and natural gas are left out, (B) Oil extractor, chemical exergy of feed, product and hexane are left out

4.3.2 Aqueous versus conventional wet fractionation

Protein fractions with higher purity (protein content) are produced with wet or aqueous fractionation. Conventional wet fractionation usually starts with an oil extraction step with organic solvents, i.e. hexane or petroleum ether, for which the mass and energy flows are visualized with Sankey diagrams in Fig 19 Alkaline solubilisation is performed to separate protein from insoluble fibres, and iso-electric precipitation is performed to separate soluble sugars from protein. Water is used for solubilisation, for pH adjustment with 1 mol/L NaOH

and 1 mol/L HCl, and for washing of the protein pellet to remove excess mineral residue and soluble sugars. The total water consumption adds up to 87 kg water/kg protein isolate, of which most ends up as waste.

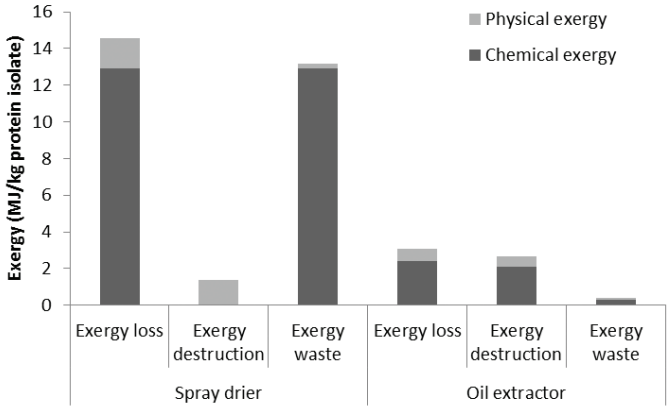


Figure 18 Physical and chemical exergy loss, divided in exergy destruction and exergy waste, for spray drying and oil extraction

4.3.2.1 Energy requirements for oil recovery

Conventional wet fractionation of lupin protein assumes that oil is extracted for food purposes. However lupin oil is not available commercially because of the small amount of oil that is extracted from the seeds (Doxastakis, 2000). An advantage of oil extraction is that the oxidative stability of the remaining biomass produced increases. Lupin seeds contain oil bodies that are well protected by proteins, but pH switches and milling might lead to rupture of oil bodies in lupin, though the extent of this effect is not described yet. Especially (poly-) unsaturated fatty acids are sensitive to lipid oxidation, which are abundant in lupin (Lqari et al., 2003). Oxidation is catalysed by the oxidative enzyme lipoxygenase

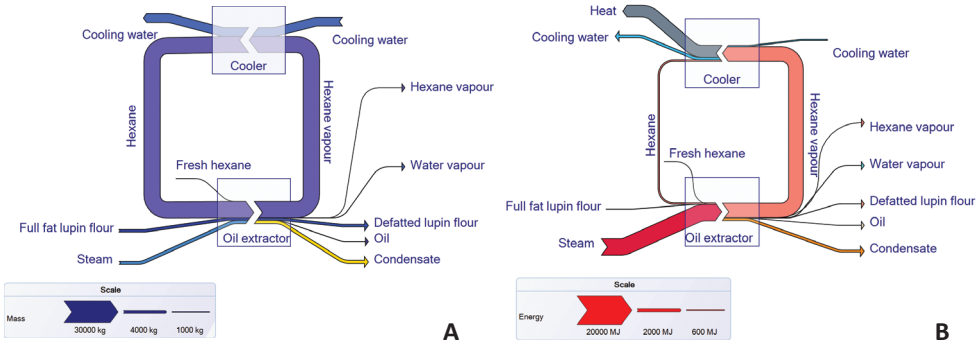


Figure 19 Sankey diagram of oil extractor: (A) Mass flows (kg), (B) Energy flows (MJ)

(LOX) that reacts with unsaturated fatty acids once the oil body is damaged by processing. Generally organic solvents, e.g. hexane or petroleum ether, are used to extract the oil, but these organic solvents are or will become undesired from an environmental point of view, so their use should be reduced or even omitted. To produce 1 ton lupin protein isolate, 22.4 ton of hexane is needed, of which 3 kg is lost as vapour (European Commission, 2008) (Fig 19). The evaporation and condensation of hexane requires 12.9 MJ/kg protein isolate. Since LPI is not produced on industrial scale, it was chosen to evaluate the sample-to-solvent ratio used for lab scale experiments. It should be noted that on pilot scale the sample-to-solvent ratio is much higher and we thus underestimate the amount of hexane used by a factor of 1.67 and on industrial scale the sample-to-solvent ratio is smaller and we thus overestimate the amount of hexane used by a factor of about 3.

4.3.2.2 Aqueous fractionation

Previously, the potential of aqueous fractionation to make protein isolates with a certain amount of oil was described. The protein isolates obtained had similar functionality to a defatted protein isolate (Berghout et al., 2014), which indicates that the oil extraction step with organic solvents is not always necessary. The protein isolate contained 82.6 g protein/100 g protein isolate and represents 63 g/100 g of the seed protein, similar to conventional wet fractionation. Since oil is not removed prior to protein purification, the oil might be oxidized during processing, having a negative effect on the quality of the protein isolate obtained. A route to prevent oil oxidation is to suppress the activity of the enzyme LOX by carrying out the extraction process at 4°C. Cooling the extraction process consumes about 5.4 MJ/kg protein isolate (see Appendix A). The energy for cooling the fractionation process is about 50% of the energy required for oil extraction. Additionally, aqueous fractionation requires 10% less lupin flour than conventional wet fractionation to produce 1 ton protein isolate (Table 5) because the oil is not extracted before processing. For ease of

Table 5 Yield (%), protein recovery after isolation (%), total protein recovery (%), protein in dry state (%), amount of water, NaOH and HCl needed for isolation (kg/ton protein isolate)

	Raw material yield	Protein recovery after isolation	Total protein yield	Protein in dry state	Amount of water used for isolation	Amount of NaOH	Amount of HCl
	% of initial weight flour	% protein of initial amount protein in starting material	%	%	kg/ton protein isolate	kg/ton protein isolate	kg/ton protein isolate
Aqueous fractionation	100	66.4	66.4	0	51000	36.6	45.6
Dry and aqueous fractionation	67	65.7	44.0	52.7	35404	16.3	19.2

comparison, we assumed that the protein isolates obtained with all processes have similar composition even though 5-7 g/100 g of the oil initially present in the seeds ends up in the protein isolate with aqueous fractionation. The chemical exergy value of the protein isolate will increase somewhat because of the oil, but it does not affect the exergy efficiency of this process significantly.

4.3.2.3 Exergy losses for oil extraction

The physical exergy flows for the oil extractor were calculated and visualized (Fig 17B). Chemical exergy flows were also calculated and left out of these diagrams because of their large magnitude compared with physical exergy flows. Fig 18 shows that the contribution of chemical exergy loss is larger than that of the physical exergy loss. The exergy loss can be divided into exergy waste and exergy destruction (Szargut et al., 1988). Exergy waste can possibly be minimized by recuperating streams and reclaiming their exergy, like water vapour or condensate. Exergy destruction is a consequence of the irreversible changes occurring during processing. As a consequence, exergy destruction is unavoidable and cannot be minimized.

Spray drying leads to the biggest exergy losses in the total fractionation process. The oil extraction is the second processing step with respect to large exergy losses. In case of spray drying, most of the losses originate from exergy waste production, which can be minimized by recuperating streams, e.g. water vapour and condensate. Exergy waste in the spray drier can only be minimized if air is heated up by something else than natural gas or when the latent heat in the evaporated moisture can be recuperated. This could be done by for example not using hot air for spray drying, but using superheated steam. As demonstrated above, the exergy indicators (exergy loss, waste and destruction) can help to determine the most inefficient step in the fractionation process and provide routes for optimisation.

4.3.3 Dry fractionation as a pre-treatment for aqueous fractionation

Dry fractionation can be used as a pre-treatment for aqueous fractionation. The advantage is that less material has to be processed under aqueous conditions to obtain the same amount of protein isolate. The combination of dry and aqueous fractionation requires about half the amount of water of the aqueous process to produce 1 ton protein isolate (Table 6). A similar reduction in the use of NaOH and HCl can be realised (Table 6). A disadvantage is that the combination of processes requires about double the amount of flour of the aqueous fractionation process (Table 5). More flour is needed for the combination of processes because only the fine fraction (enriched in protein) is used for further processing while the

Table 6 Masses of starting material and products produced in kg (and as % of initial mass of flour), for conventional wet fractionation, aqueous fractionation and the combination of dry and aqueous fractionation

	Mass in kg (mass % of initial mass flour)		
	Conventional wet fractionation	Aqueous fractionation	Dry and aqueous fractionation
Full fat flour	3739 (100)	3400 (100)	7152 (100)
Oil	265 (7)	-	-
Coarse fraction	-	-	4792 (67)
Fibre-rich pellet	1365 (37)	1577 (46)	782 (11)
Soluble sugars	994 (27)	676 (20)	491 (7)
Protein isolate	1000 (27)	1000 (29)	1000 (14)

coarse fraction is not (yet). This can also be an advantage, because 52 g/100 g of the protein originally present in the seeds is still in a dry, native state in the coarse fraction and can be a valuable, functional fraction for other applications.

Performing aqueous fractionation with the fine fraction as starting material results in an altered distribution of protein and carbohydrates over the various fractions (see Appendix A Table A1 for exact values) because the fine fraction was already enriched in protein. The FP and the SSF of the combined process have fewer carbohydrates than the FP and the SSF of the aqueous process, because the fibres were separated from the fine fraction by air classification. Additionally, the sizes of these side streams decreased with the combination of dry and aqueous fractionation compared with purely wet or aqueous fractionation (Table 5). These results are valuable when looking for applications for the side streams of the fractionation processes and thus for selecting a sustainable fractionation process.

4.3.4 Valorisation of side streams

Minimizing the amount of waste streams increases the sustainability of the fractionation process. The chemical exergy efficiency (CEE) of all processes was calculated and visualized in Fig 20. The CCE was calculated for each process for raw material, products and processing aids used (alkali, acids, and water). For conventional wet fractionation, only the hexane that was lost is taken into account. Fig 20 shows that when only the protein isolate (PI) is seen as a useful product (CEE useful 1), aqueous fractionation has the highest CEE. Obviously, when more products are seen as useful product, such as the coarse fraction, the oil, the FP and the SSF (Fig 20), the CEE's of all processes increased. In case all products are regarded as useful products, the useful CEE equals the total CEE. The CEE's of the combination of dry and aqueous fractionation depend largely on the coarse fraction. These results shows that

purification of a single ingredient reduces the exergetic efficiency of the fractionation process considerably and that adding value to side streams is crucial for increasing sustainability of fractionation processes in general. According to current bio-refining concepts, making use of the whole crop should not only focus on the potential of fractions for food, but also on the potential for feed, chemicals, materials and energy (Aiking, 2011; Mirabella et al., 2014).

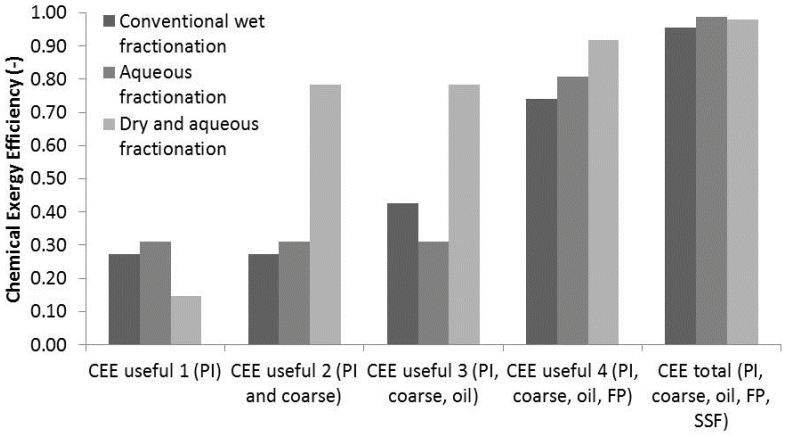


Figure 20 Chemical exergy efficiency (CEE) for different scenarios of useful products for conventional wet fractionation, aqueous fractionation and the combination of dry and aqueous fractionation: raw material, products and processing aids taken into account. PI = Protein Isolate, FP = Fibre-rich pellet, SSF = soluble solids fraction

Lupin oil is not used for commercial purposes because the total yield of lupin oil is small, but making use of the oil increases the CEE of the conventional wet fractionation process with at least one-third. The FP contains valuable nutrients; it contains about 12 g protein/100 g, 65 g carbohydrates/100 g and about 16 g oil/100 g on a dry basis (see Appendix A Table A1). Depending on the animal species this fraction can be used for animal nutrition (Drew et al., 2007; Zhang et al., 2012). In case the oil content limits the processability of the fibre-rich pellet, conventional oil extraction can be used. In this case, less hexane will be consumed because the mass of the FP is about one-third of the mass of flour. Now the FP becomes a raw material for feed production, and therefore the chemical exergy of this fraction can also be allocated to feed production (Draganovic et al., 2013), which improves the exergy efficiency of the fractionation processes described in this paper.

The mass of the coarse fraction in the dry fractionation process is larger than the mass of the fine fraction. The coarse fraction is a dry stream, has a protein content of 29 g/100, contains oil and is enriched in dietary fibre (see Appendix Table A1). The nutritive value of lupin fibres is high (Bähr et al., 2014; Kapravelou et al., 2013). Fechner et al. (2013) showed

that the addition of 50 g dietary fibres (of *Lupinus angustifolius*) to the human diet per day has beneficial physiological benefits; it improved colonic function and reduced risk factors for colorectal cancer. The SSF of the process contains protein, sugars and minerals. Reducing the sugar and mineral content with e.g. dialysis will increase the protein content and this fraction may have unique functionalities because of high levels of sulphur-containing amino acids in the proteins in this fraction (Berghout, unpublished results).

4.3.5 Coupling fractionation and application

We showed that the energy requirements and exergy losses for (spray-) drying are large because a large drying air flow needs to be heated. Normally, drying is performed to stabilize the product for the final application. However, the drying step at the end of the fractionation process can be omitted in case the time of transport between fractionation and application is reduced. Besides, a product that is still in the wet state may have functional properties that the dry product cannot have, e.g. a higher dispersibility in water. Wet fractionation is usually performed at low solid concentrations and therefore concentration or evaporation of some water will still be necessary. This can be done with solid separators, evaporators, or with ultrafiltration. Ultrafiltration requires hardly any energy, no chemicals, no application of heat, and results in protein isolates retaining their functionality (Dijkstra et al., 2003). The pressure exergy of ultrafiltration is 0.0001 MJ/kg protein isolate, which is small compared with the physical (and chemical) exergy used by spray drying. Additionally, ultrafiltration can be applied to remove impurities from the protein isolate, which would save 37 kg water/kg protein isolate and thus reduce the water consumption of wet fractionation processes with 42%. Fractionation of lupin protein can also be performed at higher solid concentration, but the results (yields, purity) on lab scale became worse when using sample-to-solvent ratios below 1:10. We here showed how sustainability of fractionation processes for lupin seeds could be improved and we expect that this also applies for fractionation processes for other oilseeds.

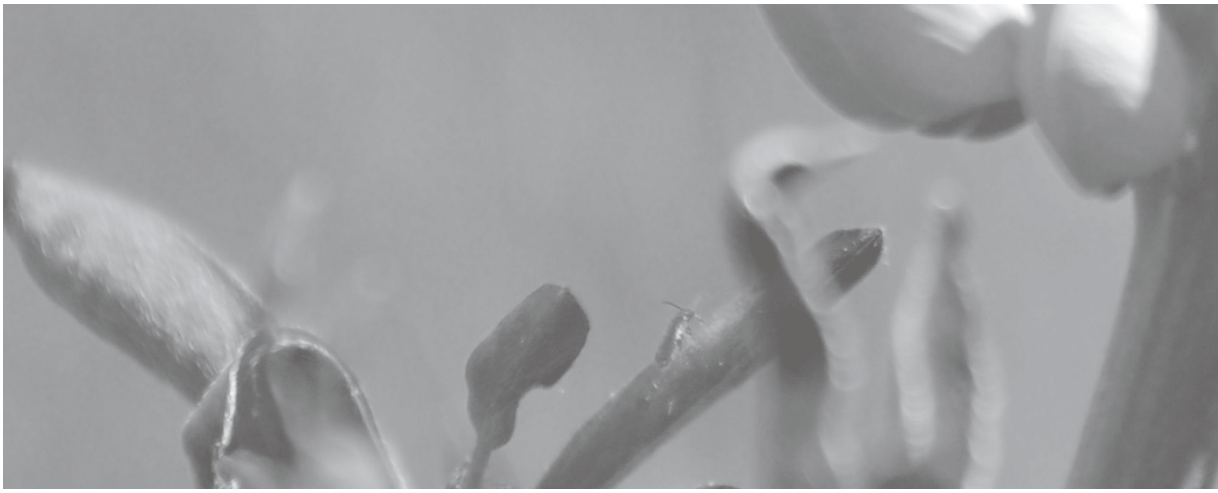
4.4 Conclusion

Producing functional protein fractions rather than pure ingredients reduces the environmental impact, i.e. the use of fewer resources and production of less waste, of fractionation processes for the case of lupin seeds. We assessed the impact of oil extraction and drying on process sustainability. Dry fractionation yields rather impure but functional protein-enriched fractions and requires the least amount of energy and no water. To obtain fractions with higher protein contents, wet and aqueous fractionation have to be used.

Conventional wet fractionation required 22.4 kg hexane/kg protein isolate for oil extraction and 87 kg water/kg protein isolate for further protein fractionation. The extraction of oil is an energy-intensive process, while the presence of oil in the protein isolate did not influence its functionality significantly. Aqueous fractionation omits the oil extraction step, but requires a similar amount of water for protein fractionation. One way to reduce water consumption of the process is by combining dry and aqueous fractionation processes; 34% less water was needed. A disadvantage of this process is that the amount of flour needed to obtain 1 ton protein isolate was double the amount needed for purely aqueous fractionation. However, 51 g/100 g of the protein initially present in the seeds remains in a dry, native state in the coarse fraction and can thus be another functional fraction. Another way to reduce water consumption is by replacing or preceding the final drying step (requiring 6 MJ/kg evaporated water and having large exergy losses) of the protein isolate with a concentration step, e.g. ultrafiltration. Ultrafiltration requires hardly any energy, but the wet protein isolate needs to be heat-treated or stabilized directly after fractionation. Process sustainability could be further increased by valorising the side streams that are enriched in dietary fibres, like the fibre-rich pellet and the coarse fraction.

Acknowledgements

The authors thank Suzanne Overbeek and Francisco Rossier for their contribution to the calculations and discussions and the peas foundation and the Institute for Sustainability Process Technology (ISPT) for financial and project support.



The technical functionality of freeze-dried lupin protein isolates (LPIs) was compared with that of 'wet', non-dried LPIs, both obtained through aqueous fractionation. The wet LPI was concentrated to 10% (w/v) with ultrafiltration, and kept in the wetted state, while freeze-dried LPI was obtained through freeze-drying and re-dispersed at 10% (w/v) in water. The viscosity, nitrogen solubility index (NSI), particle size distribution and the volume fraction of wet and freeze-dried LPI dispersions were compared before and after heating. The NSI of the freeze-dried LPI was lower than that of the wet LPI. This observation was attributed to the presence of large protein aggregates in the freeze-dried LPI, as observed by microscopy and light scattering. The viscosity of the wet LPI dispersions at 10% (w/v) increased after heating at 100°C, while the viscosity of the freeze-dried LPI dispersions at 10% (w/v) remained unchanged. This behaviour could be explained by considering the large protein aggregates found in the freeze-dried LPI and their volume fraction after heating. The volume fraction of the freeze-dried LPI dispersions increased only slightly after heating, while the volume fraction of the wet LPI dispersions increased significantly after heating at 100°C and increased by a factor of 2 after heating at 121°C. Thus, the freeze-drying process induced aggregation of lupin protein, thereby creating heat-stable protein aggregates that did not swell upon heating. The wet LPI may be described as small protein aggregates that swell upon heating and do not aggregate below 80°C, but aggregate further when heated to 100°C and 121°C.

Chapter 5

Freeze-drying induces aggregation in lupin protein isolates

Highlights:

- Aqueous fractionation consumes fewer resources than conventional wet fractionation
- Chemical exergy has a bigger impact on process sustainability than physical exergy
- Combining dry and aqueous fractionation decreases water use during protein isolation
- Adding value to side streams improves process sustainability

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Freeze-drying induces aggregation
in lupin protein isolates

5.1 Introduction

Plant protein isolates can be prepared using wet fractionation techniques. Here, prior to protein extraction from oilseeds, the flour is defatted with organic solvents like hexane or petroleum ether. The defatted flour is then solubilized in water or a buffer at alkaline pH, after which insoluble parts are separated from the protein-rich supernatant. The protein is separated from other soluble solids, like sugars, by iso-electric precipitation of the protein. Aqueous fractionation of lupin seeds without oil extraction results in lupin protein isolates (LPIs) containing a few percent of oil and with functional properties similar to those of wet-fractionated LPIs, which generally does not contain oil (Berghout et al., 2014). The LPIs obtained by wet fractionation techniques have a low gelling capacity and high heat stability (Berghout, Boom, & van der Goot, 2015), which makes lupin protein a potential raw material for plant-based foods with suitable dairy-like attributes. Suitable dairy-like attributes for high-protein beverages are for example high solubility in water and low viscosity after pasteurization and sterilization, which are important properties for consumer acceptability (Chew et al., 2003).

Generally, protein isolates are dried after protein isolation for chemical and microbial stability. Water-based plant protein dispersions are typically dried using freeze-drying, spray-drying or vacuum-drying (Hu et al., 2009; Joshi et al, 2011). Freeze-drying is an expensive drying process, which is typically used for specialty ingredients and is generally perceived to be a relative mild drying process. Spray drying is often applied in industry because of its scalability, continuous operation and standardized quality specifications (Georgetti et al., 2008). Vacuum drying is a low cost process that is performed at low temperature, but requires long residence times (Joshi et al., 2011). An operational disadvantage of all drying methods is the fact that a drying step is energy-intensive (Berghout, Pelgrom, Schutyser et al., 2015). To reduce the energy consumption of protein fractionation processes, the drying steps may be replaced by ultrafiltration to concentrate the protein dispersion up to the concentration relevant for direct product application (e.g. up to 10% (w/v) for high-protein beverages). After fractionation and concentration, the protein dispersion will have to be heated to ensure microbial and chemical stability. The properties of the resulting concentrated LPI dispersions have not yet been reported to our knowledge.

The processing steps, like drying, influence the functional properties of plant proteins, like their solubility, aggregation, water absorption capacity and gelling capacity (D'Agostina et al., 2006; Hu et al., 2009; Joshi et al, 2011; Liao et al., 2013; Roy and Gupta, 2004). A crucial

functional property of protein is its solubility because the other functional properties of a protein depend on the solubility. Papalamprou et al. (2009) reported that milder processing techniques, rather than the composition of the protein isolate, improved the functional properties of chickpea protein isolates in terms of increased protein solubility, reduced minimum protein concentration needed for gel formation, and improved gel elasticity. Ultrafiltration was shown to improve the solubility behaviour of soy protein concentrates and isolates (Alibhai et al., 2006) and resulted in gels at lower protein concentration for LPIs (Kiosseoglou et al., 1999). The effect of the drying method on protein functionality depends on the drying method and on the type of protein. Freeze-drying influences the morphology and size of the protein particles and the surface hydrophobicity of proteins by partial denaturation, due to stresses such as low temperatures, freezing stresses (e.g. phase separation, pH change and ice crystal formation) and drying stresses (Hu et al., 2009; Wang, 2000). Spray drying reduced the solubility of a lentil protein isolate less than vacuum drying (Joshi et al., 2011), but thermally damaged lupin protein isolates (D'Agostina et al., 2006). Since freeze-drying is generally perceived as the mildest form of drying, this drying method was chosen for comparison with ultrafiltration.

The objective of this paper is to better understand the differences in the properties of ultrafiltered, non-dried 'wet' LPI dispersions and freeze-dried LPI dispersions, both obtained by aqueous fractionation. The properties of the wet and freeze-dried LPIs are compared by measuring viscosity, nitrogen solubility index, volume fraction and the zeta potential before and after heat treatment. These parameters reflect the influence of freeze-drying on the structural properties of LPI on a molecular and mesoscopic scale, like solubility and state of aggregation of the protein.

5.2 Materials and methods

5.2.1 Materials

Lupin seeds (*Lupinus angustifolius*) were obtained from LI Frank (Twello, the Netherlands). Tap water (4.8ºdH) was used throughout, unless stated otherwise. All reagents used were of analytical grade.

5.2.2 Protein isolation

Freeze-dried lupin protein isolates (LPIs) were prepared by aqueous fractionation as described by Berghout et al. (2014). In short, aqueous fractionation starts with solubilizing full-fat lupin flour into tap water in a ratio of 1:15 (w/v) and adjusting the pH to 9 with 1

mol/L NaOH. The dispersions were stirred at 4°C for 1 h and subsequently centrifuged, after which a fibre-rich pellet could be separated from a protein-rich supernatant. The protein-rich supernatant was acidified to pH 4.5 with 1 mol/L HCl and stirred at 4°C for 1 h. Then the dispersions were centrifuged and the protein pellet was washed twice with distilled water. The protein pellet was re-dispersed in distilled water and the pH was adjusted to 7.0 with 1 mol/L NaOH. These protein dispersions at pH 7.0 were stored in the freezer at -20°C and subsequently freeze-dried. Wet LPIs were prepared in a similar way, except for the freezing and drying step. Instead, after neutralization to pH 7.0 with 1 mol/L NaOH, the wet LPI dispersions were concentrated using membrane (ultra)filtration (Amicon cell, Millipore Co. Bedford, USA), fitted with a regenerated cellulose membrane having a molecular weight cut-off of 5 kDa (Millipore Co. Billerica, USA). Pressurized air at 4 atm was applied over the cell and the stirring speed was set at 500 rpm. To prevent bacterial growth during concentration, the water jackets surrounding the Amicon cells were cooled with a circulating water bath set at 4°C. All protein isolations were performed in duplicate.

5.2.3 Sample preparation

Protein dispersions were prepared by dispersing the freeze-dried LPI in distilled water at 10% (w/v) and the wet LPI dispersions were concentrated to 10% (w/v) using membrane filtration. The dispersions were at pH 7.0 and were stirred for 1 h before heat treatment. One dispersion was kept at room temperature, the second dispersion was heat-treated in a water bath at 60°C for 30 min, the third dispersion was heat-treated in a water bath at 80°C for 30 min, the fourth dispersion was heat-treated in an autoclave (Systec V-150, Systec GmbH, Germany) at 100°C for 5 min and the fifth dispersion was heat-treated in an autoclave at 121°C for 5 min. The autoclave required 30 min to reach the set temperature, cooling down required 20 min. All bottles containing the protein dispersions were cooled to room temperature with running tap water. Each heat treatment was performed in duplicate.

5.2.4 Sample composition

The dry matter content was determined by drying 1 g of sample overnight in an oven at 105°C. The oil content was determined by a Soxhlet extraction according to AACC method 30-25 (AACC, 1983b) on a fully-automated extractor (Büchi extractor B-811, Büchi Labortechnik, Germany). Oil extraction was performed with petroleum ether (boiling range 40 - 60°C) for 3 hrs. The protein content was determined with the Dumas combustion method on a NA 2100 Nitrogen and Protein Analyser (ThermoQuest-CE Instruments, Rodeno, Italy). Methionine was used as standard and the protein content was calculated

with a nitrogen-to-protein conversion factor of 5.7 that is used for seed storage proteins. The ash content was determined with the AACC official method 08-01 (AACC, 1983a). The chemical compositions of wet and freeze-dried LPIs are shown in Table 7.

Table 7 Chemical composition of wet and freeze-dried protein isolates, presented as mean ± absolute deviation (% on wet basis, 5% moisture)

	Protein	Oil	Ash	Carbohydrates 'by difference'
Wet lupin protein isolate	86.7±1.9	1.5±0.6	6.3±0.0	5.5±1.2
Freeze-dried lupin protein isolate	89.7±0.6	1.5±0.0	5.1±0.1	3.7±0.6

5.2.5 Volume fraction of protein dispersions

To calculate the volume fraction of protein (ϕ) for wet and freeze-dried LPI dispersions, the Einstein expression was used:

$$\eta_{rel} = 1 + \frac{5}{2} \cdot \varphi$$

in which η_{rel} is the relative viscosity ($\eta_{dispersion}/\eta_{water}$) and φ is the volume fraction of the dissolved material (-). The LPI dispersions were diluted with distilled water in a range of 2 - 30 mg/mL, which is sufficiently diluted to use the Einstein expression. The relative viscosity of each dilution was measured using glass capillary viscometers having an internal diameter of 0.63 mm (Ubbelohde). The viscometer was placed in a temperature controlled water bath at 25.00ºC ± 0.01 (TV 4000, Tamson). Each dilution was measured in triplicate and average viscosity values were calculated. The volume fraction of the dispersions could then be calculated with:

$$\frac{\varphi}{c} = \frac{5k}{2}$$

in which c is the concentration (mg/mL) and k the slope (mL/mg) in the relation $\eta_r = 1 + kc$.

5.2.6 Particle size distribution

The particle size distribution was estimated by static light scattering (Mastersizer-2000, Malvern Instruments Ltd., UK). Particle sizes of the LPIs were also measured using a Zetasizer nano series (Zetasizer Nano ZS, Malvern Instruments) using 173° backscattering light from a laser with a wavelength of 633 nm. The LPI dispersions were filtered to remove particles >11 µm with Whatman No. 1 filter paper using a Buchner funnel that was connected to a vacuum pump. The dispersions were subsequently diluted to avoid multiple scattering.

5.2.7 Protein dispersibility

The nitrogen solubility index (NSI) was determined according to the method described by Berghout et al. (2014). Freeze-dried LPIs were suspended in distilled water at a concentration of 1% (w/v) and wet LPIs were diluted to 1% (w/v) with distilled water. The dispersions were shaken at room temperature for 1 h and then centrifuged (3000 × *g*, 25°C for 15 min). The supernatant solutions were separated from the pellets, frozen at -20°C, freeze-dried and analysed for protein content with the Dumas combustion method. The NSI was calculated as the fraction of soluble nitrogen to total nitrogen.

5.2.8 Viscosity

One hour after heat treatment, the protein dispersions were transferred to a rheometer (Anton-Paar Physica MCR301, Austria) using a cone-plate geometry (CP-20-2). Samples were equilibrated for 5 min and flow properties were recorded at 25°C using a shear rate range of 0.01 - 100 s^{-1} and 100 - 0.01 s^{-1}. The wet LPI dispersions heated at 121°C were subjected to a frequency sweep at constant strain (0.1% - within linear regime) from 0.1 - 10 rad/s at 25°C.

5.2.9 Zeta potential

The zeta potential was measured by using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) fitted with an autotitrator (MPT-2, Malvern Instruments). Protein dispersions were prepared at 1% (w/v). Titration started at pH 10.0 and went down to pH 3.0. Measurements were repeated three times.

The zeta potential of the LPIs after heat treatment were measured in a measuring cell. About 1 mL of a 1% (w/v) dispersion was transferred into the measuring cell and five measurements were made of each sample.

5.3 Results and discussion

5.3.1 Functionality of freeze-dried LPI dispersions

Fig 21 shows microscopic images of a wet lupin protein isolate (LPI) and a freeze-dried LPI. The freeze-dried LPI contains large, irregularly shaped aggregates of protein, which were not found in the wet LPI. These irregularly shaped aggregates were found after freeze-drying, but it is possible that these may have been formed either during freezing or during freeze-drying. The LPIs were submitted to a relatively slow freezing process in the freezer at -20°C, which can induce local protein concentration due to ice crystal formation.

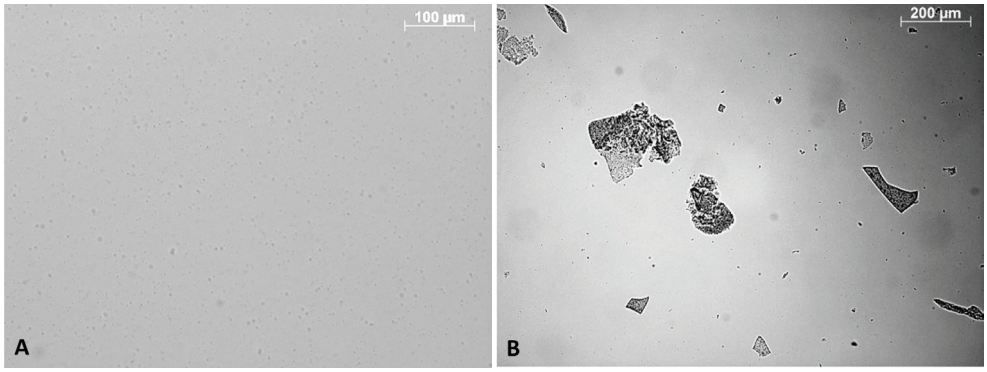


Figure 21 Effect of freeze-drying on the macroscopic structures of lupin protein isolate as observed with the light microscope. Lupin protein isolates were prepared by aqueous fractionation and subsequently concentrated to 10% (w/v) and kept in a wet state (A) or by aqueous fractionation, freezing at -20°C and subsequent freeze-drying of the aqueous protein pellet and re-dispersing the protein pellet at 10% (w/v) in water (B). Samples were diluted to 1% (w/v) with distilled water

The nitrogen solubility index (NSI) was determined after protein isolation. Even though the average iso-electric point (pI) of lupin protein is close to pH 4.5, the individual proteins have a range of pI's (Duranti, 2008). This pI range explains why only part of the lupin proteins ends up in the LPI. The other part was either trapped in the fibre-rich pellet in the first solubilisation step during isolation, or the proteins involved did not precipitate at pH 4.5.

The proteins that precipitate at pH 4.5 end up in the LPI and therefore the NSI of both LPIs is 0% around this pH (Fig 22A). The proteins re-solubilize at a higher or lower pH but the wet and freeze-dried LPIs behave differently. The freeze-dried LPI had a lower solubility than the wet LPI at pH above 6 and below 4: the drying process does not allow complete dissolution of the protein aggregates (Berghout, Boom, & van der Goot, 2015). Incomplete dissolution

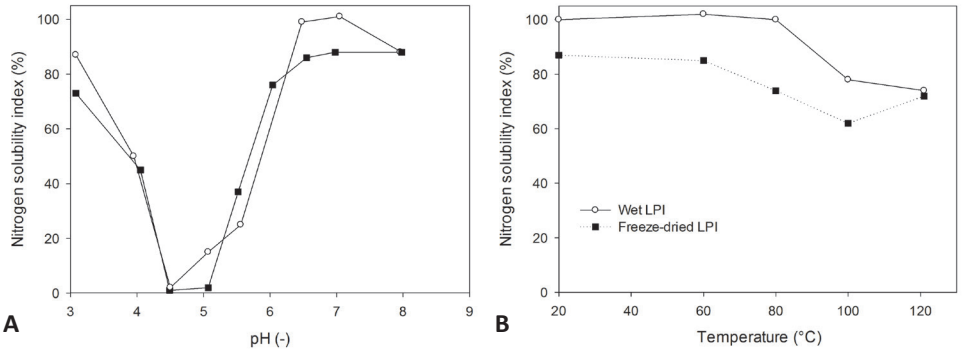


Figure 22 Differences in dispersibility (nitrogen solubility index) of wet and freeze-dried lupin protein isolate at 1% (w/v) as a function of pH (A) and as a function of temperature (B)

was found to be caused by the removal of the protein hydration shell during freeze-drying (Wang, 2000). We found similar behaviour for the NSI of freeze-dried and wet LPI at pH 7.0 as a function of the temperature (Fig 22B).

The NSI of the freeze-dried LPI was lower than the NSI of the wet LPI after heating at 60°C and 80°C. The lower NSI of freeze-dried LPI could be related to the structure of the aggregates as depicted in Fig 21B. Upon centrifugation these aggregates probably end up in the pellet; the pellets obtained after centrifugation were significantly denser for the freeze-dried LPI than for the wet LPI (not shown). The NSI of the freeze-dried and wet LPI decreased with increasing temperature (Fig 22B), indicating that both LPIs still denatured and aggregated upon heating, leading to a reduced dispersibility. For the wet LPI this effect showed at 100°C and for the freeze-dried LPI at 80°C.

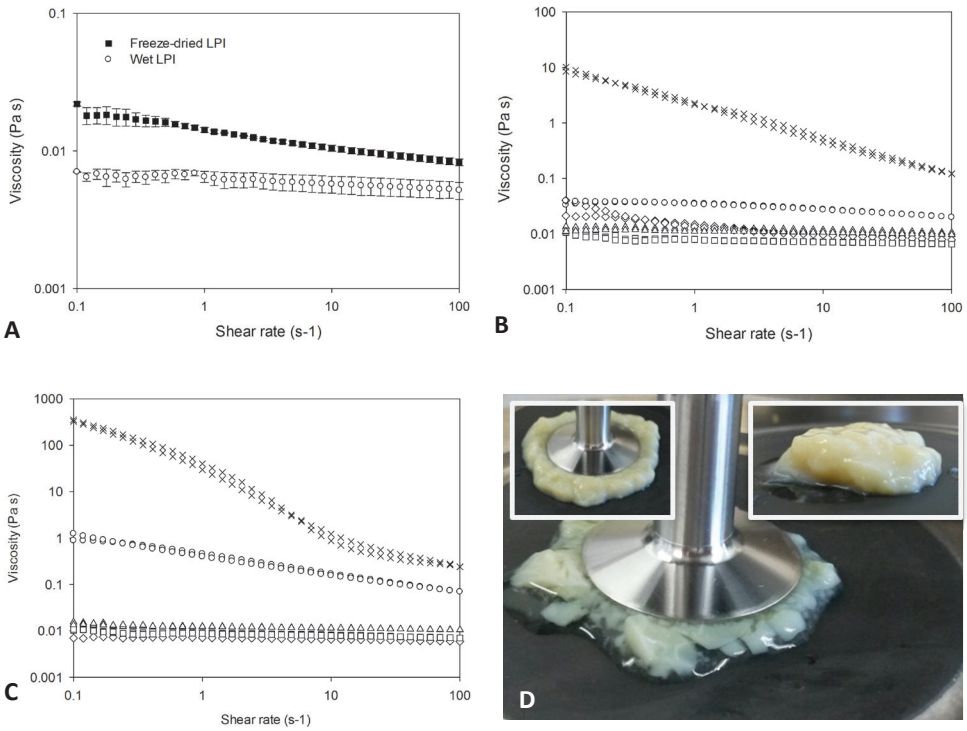


Figure 23 Viscosities of wet and freeze-dried lupin protein isolate (LPI) dispersions at 10% (w/v) before heating (A) freeze-dried LPI before and after heating (B), wet LPI dispersions before and after heating (C), and (D) picture of 10% (w/v) wet LPI gel (heated at 121°C) on the rheometer. Wet LPI dispersions were prepared by concentrating the LPI dispersion to 10% (w/v) after aqueous fractionation with ultrafiltration and freeze-dried LPI dispersions were prepared by dispersing the freeze-dried LPIs at 10% (w/v) in distilled water. For B and C: ◊ is before heating, □ is heat-treated at 60°C, Δ is heat-treated at 80°C, ○ is heat-treated at 100°C and × is heat-treated at 121°C. Symbols of the same kind represent the upward and downward flow curve and overlap in all cases

The viscosity of a 10% (w/v) freeze-dried LPI dispersion was about a factor 2 higher compared with a 10% (w/v) wet LPI dispersion before heat treatment (Fig 23A). The freeze-dried LPI dispersion was more shear thinning compared with the wet LPI dispersion, which is probably due to the alignment of non-spherical aggregates. At higher shear rates (>10 s^{-1}) the viscosity of the freeze-dried LPI dispersion became similar to the viscosity of the wet LPI dispersion. The freeze-dried LPI dispersion became less viscous upon heating at 60°C and 80°C and tended towards Newtonian behaviour (Fig 23B). A possible explanation could be that the protein aggregates folded slightly different upon heating at 60°C and 80°C, which influences the flow behaviour of the dispersion. At low shear rates (0.1 s^{-1}), the viscosity of the freeze-dried LPI increased from 10 mPa·s to 20 mPa·s after heating at 100°C and to 10 Pa·s after heating at 121°C.

The viscosity of the wet LPI in contrast, did not change when heating to 60°C and 80°C, but increased to 1 Pa·s after heating at 100°C and to 100 - 250 Pa·s after heating at 121°C at low shear rates (0.1 s^{-1}). Heating at 100°C and 121°C resulted in shear thinning behaviour of the wet LPI (Fig 23C). The fact that the upward and downward shear sweeps overlapped indicated that no break-up of aggregates occurred during shear. The large increase in viscosity after heat treatment at 121°C can be related to a physical change of the protein but also to chemical reactions that might take place, e.g. Maillard reactions.

The flow curve of the wet LPI that was heated at 121°C showed a steep slope at the beginning of the curve (until 1 s^{-1}), and an even steeper slope between 1 and 10 s^{-1}, suggesting the disruption of a network or wall slip due to syneresis. The gel that formed after heating to 121°C in the autoclave broke into smaller gel fragments when dipping in a spoon but the downward shear sweep showed that the gel reformed quickly. The gel was transferred to the rheometer without breaking it, but moisture was expelled (Fig 23D), thus showing syneresis. To probe the dynamic behaviour of the gel network, a frequency sweep was performed. Fig 24 shows that both moduli of the LPI gel were basically independent of frequency, indicating elastic gel-like properties (Clark and Ross-Murphy, 1987).

5.3.2 Characterisation of wet and freeze-dried lupin protein isolate dispersions

We found that freeze-dried LPI contains irregular, non-spherical protein aggregates (Fig 21B). To determine the change in particle size upon drying and heating, the particle size distributions (PSDs) for the freeze-dried and wet LPI before and after heat treatment were measured (Fig 25). The particles in the freeze-dried LPI had a ten times larger average volume-based size ($d_{0.5}$ = 25.6 ± 1.1 µm) than the aggregates of the wet LPI ($d_{0.5}$ = 2.7 ± 0.1 µm).

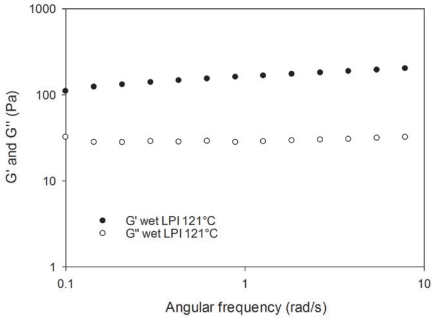
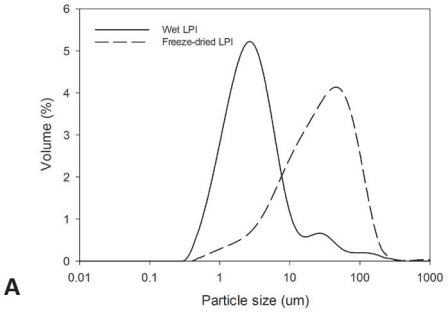
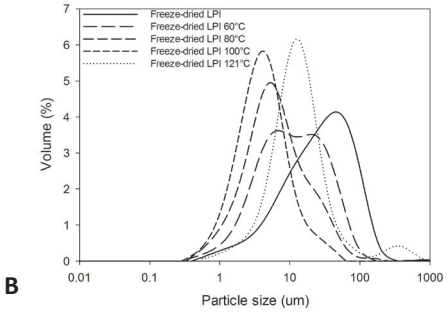


Figure 24 Frequency dependent behaviour of a gel formed by wet LPI at 10% (w/v) that was heated at 121°C



A



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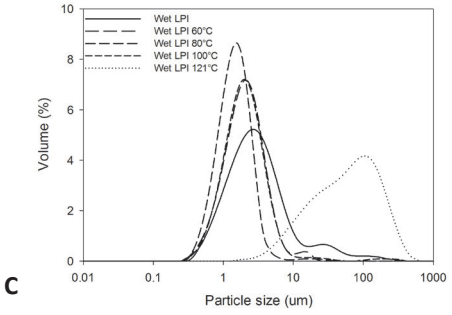

C

Figure 25 Volume-based particle size distributions of freeze-dried and wet lupin protein isolates before heating (A) and of freeze-dried lupin protein isolates after heating (B) and of wet lupin protein isolates after heating (C)

It should be realized that large particles dominate the PSD but that smaller particles are present. Nanosizer experiments showed that particles down to 0.02 μm are present in both LPIs (Appendix B). The wet LPI showed a narrower PSD than the freeze-dried LPI (Fig 25A) hence suggesting that the freeze-dried LPI contains small protein aggregates and large protein aggregates. Drying of the LPI thus changes the morphology of its proteins; probably by aggregation of the initially small aggregates. The aggregates resulting from the drying were irregular, non-spherical in shape, offer a large surface area and are thus prone to aggregate further into a percolating network. Due to aggregation, the viscosity of the protein dispersion increased (Genovese et al., 2007), explaining the higher viscosity measured before heating (Fig 23). The volume-based PSDs of the freeze-dried and wet LPI changed after the heat treatments (Fig 25B & C) where especially the wet LPI at 121°C shows a wider PSD and much bigger particles than before heating.

The flow behaviour of the LPIs suggests that the wet LPIs swell more upon heating, but this was not reflected in the PSDs. We therefore studied the volume fractions occupied by the freeze-dried and the wet LPI upon heating in diluted systems. The volume fraction (ϕ) of proteins is calculated from the initial slope of the concentration dependence of the relative viscosity using Einstein's equation. These slopes were similar for both unheated LPI dispersions but the ϕ of the wet LPI changed after heat treatment at 100°C and 121°C, while the ϕ of the freeze-dried LPI changed only after heat treatment at 121°C (Table 8). At a protein concentration of 30 mg/mL the ϕ of the wet LPI before and after heating at 121°C increased with a factor of 2.1 (from 0.31 to 0.64), while the ϕ of the freeze-dried LPI only increased with a factor of 1.4 (from 0.27 to 0.38) (Table 8).

Table 8 Volume fraction of wet and freeze-dried protein isolates (LPI) before and after heating

Protein concentration (mg/mL)	Volume fraction ϕ					
	Wet LPI	Freeze-dried LPI	Wet LPI 100°C	Freeze-dried LPI 100°C	Wet LPI 121°C	Freeze-dried LPI 121°C
10	0.10	0.09	0.14	0.10	0.21	0.13
20	0.21	0.18	0.28	0.19	0.42	0.25
30	0.31	0.27	0.42	0.29	0.64	0.38

The zeta potential of the particles in a colloidal dispersion is a measure for the stability against aggregation in a colloidal dispersion as caused by electrostatic repulsion. A zeta potential in excess of 25 mV (corresponding with about 1kT) generally leads to an electrostatically stabilized dispersion. Lupin protein has a zeta potential of -35 mV at neutral pH

(7.0), with only minor differences in the zeta potential between the wet and freeze-dried LPI over a pH range from 3.0 -10.0 (Fig 26A). After heating, the zeta potential of the wet LPI becomes less negative (about -28 mV) while the zeta potential of the freeze-dried LPI remains similar (Fig 26B). This difference is really small and since dynamic light scattering assumes that particles are spherical and the protein aggregates were not spherical (Fig 21B), the difference may not be significant.

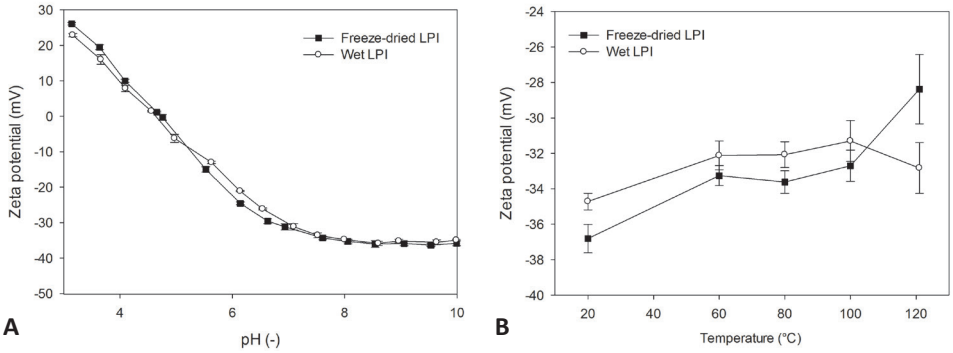

Figure 26 Zeta potential of wet lupin protein isolates and freeze-dried lupin protein isolates as a function of pH (A) and temperature of heat treatment at pH 7.0 (B)

Doxastakis (2000) found spray-dried lupin milk to be unacceptable as a drinkable product because lupin protein was not heat-stable and aggregated upon mild heat treatment (pasteurisation). We here show that freeze-dried LPI is composed of heat-stable particles (up to 100°C) that consist of aggregated protein that hardly swell upon heating to 100°C. Heating at 121°C increased the viscosity of the freeze-dried LPI, caused by heat-induced internal rearrangements where chemical reactions may play a role as well. The wet LPI remained a dispersion of smaller protein aggregates and was heat-stable up to 80°C. Heating to 100°C lead to particle swelling and an increase in the viscosity. At 121°C chemical reactions probably play a role, which may induce stronger binding between the particles.

5.3.3 The potential of mild fractionation without drying

Mild fractionation that avoids the use of large amounts of organic solvents, plus avoidance of drying increases the sustainability of fractionation processes (Berghout, Pelgrom, Schutyser et al., 2015). However, higher sustainability should ideally not be developed at the expense of functionality. Kiosseoglou et al. (1999) and Papalamprou et al. (2009) found that a milder treatment, like dialysis or ultrafiltration, resulted in gels at lower protein concentration and with high elasticity. Arogundade et al. (2012) found that potato protein isolate produced by ultra-filtration had a lower viscosity than potato protein isolate produced by iso-electric

precipitation. We therefore investigated the differences in the functionality of wet LPI dispersions obtained with ultrafiltration and freeze-dried LPI dispersions.

The results described above showed that concentration of the wet LPI dispersion with ultrafiltration is a milder fractionation technique than freeze-drying of the LPI dispersion because extended aggregation was absent after ultrafiltration. Ultrafiltration instead of freeze-drying altered the functionality of the LPI dispersion slightly; its NSI was higher compared with a freeze-dried LPI and before heat treatment its viscosity was lower than that of a freeze-dried LPI. These characteristics are often preferred in dairy-like applications. Even though both LPIs were stable to heat treatments at 80°C (pasteurisation temperature), heating to higher temperatures resulted in more viscous dispersions or even mild gelling. After heating at 100°C and 121°C, the viscosity of the wet LPI was much higher than that of the freeze-dried LPI due to stronger swelling of the small protein aggregates. At 121°C, the gelling was probably stronger because of chemical interactions between the aggregates, leading to a stronger network and syneresis. From an application point of view this means that a freeze-dried LPI may be useful for high-protein drinks because of its heat stability, while a wet LPI is more useful for dairy applications with higher viscosity, like pudding.

In the experiments above, the heating time was not varied. In industrial pasteurisation and sterilisation conditions, high temperature is only applied for a short time, and we expect that the isolates will then show an improved heat stability. On the other hand, longer heating could also be used to further alter the gel properties. It would thus be interesting to test the time dependence of the heat stability of the protein isolates at high temperature for short times (seconds) under flow.

5.4 Conclusion

We explored the differences in functionality between freeze-dried lupin protein isolate (LPI) dispersions at 10% (w/v) in water and wet, non-dried LPI dispersions that were concentrated with ultrafiltration to 10% (w/v) in water. The main differences between the LPI dispersions were their particle size distribution, their nitrogen solubility index and their viscosity. Differences in behaviour were ascribed to the fact that a freeze-dried LPI is composed of small and large protein aggregates, while the wet LPI only contains small protein aggregates. The larger protein aggregates in the freeze-dried LPI reduce the solubility of the LPI dispersion relative to the wet LPI dispersion. Freeze-drying thus alters the morphology of protein isolates, which was supported by volume fraction (ϕ) measurements after heat treatment: the ϕ of the wet LPI doubles upon heating, while that of the freeze-dried LPI

increases only slightly upon heating. Before heating, the freeze-dried LPI had a higher viscosity than the wet LPI, but the viscosity of the wet LPI increases upon heating at 100°C and 121°C while the freeze-dried LPI only showed an increase in viscosity at 121°C. The wet LPI can thus still undergo swelling or further aggregation, in contrast to the freeze-dried LPI, in which the proteins already aggregated more extensively during drying. Concluding, freeze-drying induces aggregation in LPIs, creating heat-stable protein aggregates that do not swell extensively. Wet, non-dried LPI is composed of small protein aggregates that are heat-stable up to 80°C but are sensitive for temperatures above 100°C and forms gels at 121°C.

Acknowledgements

The authors thank Steven Cornet for his contribution to the experimental work, Harry Baptist for his help on using the capillary viscometers and the peas foundation and the Institute of Sustainable Process Technology (ISPT) for financial and project support.

The chemical stability of lupin protein isolates (LPIs) obtained through aqueous fractionation (AF, i.e. fractionation without the use of an organic solvent) at 4°C or 20°C was assessed. AF of lupin seeds results in LPIs containing 2 wt% oil. This oil is composed of mono- and poly-unsaturated fatty acids and the isolate may thus be prone to lipid and protein oxidation. Lipid and protein oxidation marker values of LPIs obtained at 4°C and at 20°C were below the acceptability limit for edible vegetable oils and meat tissue protein; the level of lipid oxidation markers was lower at 20°C than at 4°C. The fibre-rich pellet and the protein-rich supernatant obtained after AF also had lower levels of oxidation markers at 20°C than at 4°C. This is probably the result of a higher solubility of oxygen in water at lower temperature, which could promote lipid oxidation. The differences between fractions can be explained by the differences in their composition; the fibre-rich pellet contains polysaccharides that potentially have an anti-oxidative effect, while the protein-rich supernatant is rich in sulphur-rich proteins that may scavenge metal ions and free radicals from the aqueous phase. Additionally, the differences in solubility of metal ions and metal-chelating properties of protein at pH 4.5 and pH 7.0 explain the higher level of oxidation in the LPI at pH 4.5 compared with the LPI at pH 7.0. The application of a heat treatment to reduce oxidation decreased the protein and oil recovery values, and increased oxidation values above the acceptability limit. Therefore, AF at 20°C is the most suitable process to obtain chemically stable LPIs.

Chapter 6

Aqueous fractionation yields chemically stable lupin protein isolates

Highlights:

- Aqueous fractionation at 4°C and 20°C yielded fractions within the quality limits
- Aqueous fractionation at 4°C resulted in higher lipid oxidation values than at 20°C
- Protein and polysaccharides had protective effects against oxidation
- A heat treatment increased lipid oxidation values above the acceptability limit

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Aqueous fractionation yields chemically stable lupin protein isolates

6.1 Introduction

Lupin seeds are rich in protein, which makes this crop a promising candidate for plant-based, high-protein foods. Dehulled lupin seeds of *Lupinus angustifolius* L. contain about 39 - 44 wt% protein, about 7 - 10 wt% oil and are further composed of dietary fibres, sugars, minerals and water (Bähr et al., 2014). *L. angustifolius* L. is a sweet lupin, implying that the alkaloid levels are well below the critical value of 200 mg/kg for lupin-based foodstuffs, and thus not toxic to humans (Small, 2012). Lupin protein isolation is generally performed through wet fractionation processes (Lqari et al., 2002; Wäsche et al., 2001), in which aqueous fractionation is preceded by defatting using an organic solvent. In a previous paper we introduced an alternative fractionation process, referred to as purely aqueous fractionation (AF), which is a sustainable alternative to conventional wet fractionation because the oil extraction step is omitted and thereby the use of organic solvents (Berghout et al., 2014). It is worth mentioning that the solubility and water holding capacity of the lupin protein isolate (LPI) obtained with AF was similar to that obtained with conventional wet fractionation (Berghout et al., 2014). However, a consequence of AF is the presence of some lupin oil during the fractionation process and in the resulting products. The composition of lupin oil has already been studied (Sbihi et al., 2013; Schindler et al., 2011), and includes a substantial amount of polyunsaturated fatty acids (PUFAs), which is an asset from a nutritional point of view, but also makes the oil sensitive to oxidation. Hence, the presence of oil could give rise to increased oxidation of both oil and protein in the obtained fractions. Oil in lupin seeds is present in the form of oil bodies that have a size around 0.1 - 0.5 µm (Tzen, 1992), which are stabilized by phospholipids and a dense protein complex. This outer structure provides physical and chemical protection against environmental stresses, such as moisture variation, temperature fluctuation and the presence of oxidative reagents (Chen et al., 2012; Gray et al., 2010; Karkani et al., 2013).

Lipid oxidation is one of the prime mechanisms of quality deterioration in foods, as it leads to the loss of nutritional value and the formation of unpleasant flavours and odours (Velasco et al., 2010). Mono- and especially poly-unsaturated fatty acids, are related with health benefits (Siriwardhana et al., 2012) and it is thus important that these remain non-oxidized. Due to physical-chemical changes during storage and fractionation (high moisture content) and the presence of oxygen, chemically active compounds can be oxidized, but the stability of lupin flour against oxidation has not yet been the subject of study. The initiation mechanism of lipid oxidation occurs through photo-oxidation, enzymatic oxidation, and auto-oxidation (Berton-Carabin et al., 2014; Kolakowska and Bartosz, 2014; Skibsted, 2010).

While oxidation through direct (sun)light (photo-oxidation) is not of concern for AF of lupin seeds, the other mechanisms are relevant. The enzyme lipoxygenase (LOX) enhances lipid oxidation and is naturally present in lupin seeds. LOX in lupin seeds is reported to have an optimum pH of about 7.5 (Stephany et al., 2014; Yoshie-Stark and Wäsche, 2004) and its activity is suppressed at temperatures below 20°C and above 80°C (Yoshie-Stark et al., 2004). De-hulling, flaking and protein isolation from de-oiled lupin flakes results in about 10 times lower LOX activity (Stephany et al., 2014; Yoshie-Stark and Wäsche, 2004). The activity of LOX in full fat (non-de-oiled) LPIs has not been reported. The free radicals formed through lipid oxidation can oxidize proteins in the aqueous phase as well, leading to the formation of protein carbonyls, peptides, and protein cross-linking, which deteriorates the protein's functional properties (Baron, 2014; Skibsted, 2010). Boatright et al. (1995) studied protein oxidation in different types of soy protein isolates (SPIs) and found that reduced-lipid SPI had significantly lower levels of protein oxidation than full-fat and commercial SPIs.

The aim of the work reported here was to investigate the chemical stability of LPIs containing oil. The level of oxidation of full fat lupin flour was determined at storage temperatures of 4°C and 20°C. Full fat lupin flour was then subjected to AF in various conditions. First, to suppress the potential activity of LOX, we performed the AF process under chilled conditions (4°C). For comparison, the process of AF was also conducted at ambient temperature (20°C). Finally, the influence of a heat treatment (80°C) during AF was evaluated regarding protein and oil recovery, and regarding lipid and protein oxidation.

6.2 Materials and methods

6.2.1 Materials

Untoasted, full fat lupin seeds (*Lupinus angustifolius* L.) were purchased from L.I. Frank (Twello, The Netherlands) in October, 2012 and stored at 4°C in dark, de-aerated containers (8 wt% water). The reagents and chemicals used were of analytical grade and obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA) except for the ethanol, ethyl acetate, n-hexane, petroleum ether that were obtained from Merck (Merck, Germany). The bicinchoninic acid-assay kit was obtained from Sigma Aldrich. Tap water was used throughout, unless stated otherwise.

6.2.2 Processing methods

The seeds were pre-milled to grits with a pin mill (Condux-Werk LV 15 M, Wolfgang bei Hanau, Germany). The grits were then milled into full fat flour with an impact mill ZPS50

(Hosokawa-Alpine, Augsburg, Germany), by setting the classifier wheel at 1000 rpm and the air flow at 80 m^3/h. The screw feeder speed was 2 rpm (0.75 kg/h), the impact mill speed was 8000 rpm and the batch size 1 kg. One-tenth of the full fat flour was stored in six separate containers: three containers were kept at 4°C and three containers were kept at 20°C in the dark for 2 weeks to analyse the oxidation levels. The flour for aqueous fractionation was stored in separate containers; each container had 30 g flour for triplicate experiments.

Aqueous fractionation (AF) was performed as described in Berghout et al. (2014) at 4°C and at 20°C. Additionally, AF was performed with the inclusion of a heat treatment. An overview of the AF processes is shown in Fig 27. The additional heating step was applied after the solubilisation step at pH 9. The flour was solubilized in tap water and adjusted to pH 9.0 with 1 mol/L NaOH. After 1 h, the samples were heated to 80°C in a water bath while shaking. The temperature of the sample reached 80°C after about 40 min and the samples were kept at this temperature for 1 min. Subsequently, the samples were cooled to about 30°C in 20 min and centrifuged at 11,000 x *g* at 4°C for 30 min. The protein-rich supernatant was decanted and adjusted to pH 4.5 with 1 mol/L HCl. After stirring at 4°C for 1 h, the samples were centrifuged at 11,000 x *g* at 20°C for 30 min and the supernatant was discarded. The protein pellet was washed twice with distilled water (Millipore, Merck,

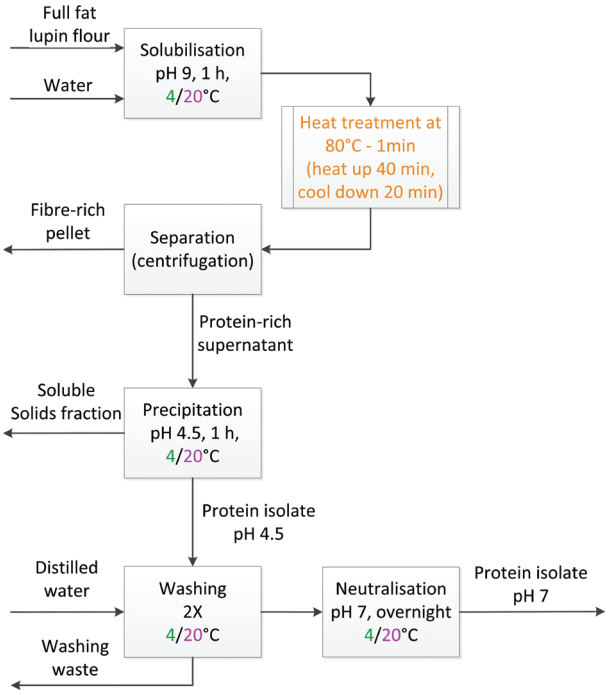


Figure 27 Aqueous fractionation (AF) processes at 4°C (in green), 20°C (in pink) and the inclusion of a heat treatment (in orange) performed during the AF process at 4°C

Germany) and then either kept at pH 4.5 or neutralized to pH 7 with 1 mol/L NaOH. The fibre-rich pellets, protein-rich supernatants and LPIs at pH 4.5 and pH 7 were frozen at -20°C and then freeze-dried. Each AF process was performed in triplicate.

6.2.3 Oil extraction

The oil for oxidation measurements was extracted by performing Standard Soxhlet (Dobarganes Nodar et al., 2002; Özcan and Al Juhaimi, 2014) on a fully automated Büchi B-811 extractor (Flawil, Switzerland) according to AACC method 30-25 (AACC, 1983). Petroleum ether was used as extraction solvent. The extraction was performed for 3 h and the solvent was evaporated on the Büchi extractor until a small layer of solvent remained in the beaker. The beakers were then removed from the equipment, covered in aluminium foil and left in the fume hood so that the solvent could evaporate. Evaporation was considered to be complete when the weight of the beakers remained constant. Longer extraction times (up to 10 h) resulted in 18% higher oil recoveries. Since lipid oxidation values increased with longer waiting times after evaporation of the solvent, it was chosen to have slightly lower oil recoveries to avoid the higher oxidation values.

6.2.4 Chemical composition

The dry matter content of each fraction obtained with AF was determined by weighing every fraction before and after freeze-drying. The moisture contents of the full fat flours stored at 4°C and at 20°C were determined by drying 2 g flour at 105°C in an oven until constant weight. The protein content was determined by measuring the nitrogen content with the Dumas combustion method on a Flash EA 1112 Series NC Analyser (CE Instruments Ltd., Wigan, UK). The protein content was calculated by multiplying the nitrogen content with a nitrogen-to-protein conversion factor of 5.7, a value which is commonly used for seed storage proteins (FAO, 2002). Methionine was used as a standard. Samples of about 10 mg were measured in duplicate. The amount of sulfhydryl groups for each fraction was determined with the sulfhydryl reactivity method as described by Berghout, Boom, & van der Goot (2015). Briefly, protein samples reacted with Ellmann's reagent (DTNB) and the absorbance of the samples was measured at 412 nm with a spectrophotometer UV-vis Beckman Coulter DU-720 (Woerden, The Netherlands), after which the number of sulfhydryl groups was calculated. The iron and copper content of the flour, the fibre-rich pellet and the LPI were determined by the Chemical Biological Laboratory Bodem (soil) (Wageningen University, The Netherlands). The samples (about 300 mg) were dried at 70°C and subsequently destructed using HNO_3-HCl-H_2O_2 in a microwave (MARS-Xpress, CEM,

USA). The iron and copper content were then measured on an Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) (Varian Vista Pro Radial, Varian Inc., USA) according to SWV E1362.

6.2.5 Water activity measurements

The water activity of the full fat flour that was stored at 4°C and at 20°C was measured with an Aqualab water activity meter (Decagon Devices Inc., Pullman, WA, USA). The water activity was measured in duplicate at 25°C.

6.2.6 Lipid oxidation

6.2.6.1 Peroxide value

Primary lipid oxidation products in the oil extracted from the different samples were determined with the peroxide value (PV), which was determined according to the colorimetric method of Shantha and Decker (1994) with slight modifications for our material. A ferric thiocyanate reagent solution was prepared by mixing 0.132 mol/L barium chloride in 0.4 mol/L HCl with 0.144 mol/L iron sulphate in equal volume, and subsequently mixing this solution with an equal volume of 3.94 mol/L ammonium thiocyanate. The oil was mixed with n-hexane in an oil-to-solvent ratio of 1:60 (w/v). Then, 0.10 mL of the dispersion was added to 1.4 mL methanol/1-butanol (2:1, (v/v)), followed by the addition of 15 µL ferric thiocyanate reagent. After 20 min, the absorbance at 510 nm was measured. A blank of n-hexane was used. Peroxide concentrations of the samples were determined using a standard curve (R^2 is 0.99) prepared with cumene peroxide. Final PV's were calculated in meq oxygen/kg oil according to the equation:

$$PV\left(\frac{meq}{kg\,oil}\right) = \frac{mmol\,peroxide}{2 \cdot m_{oil}}$$

in which 2 is the conversion factor from mmol to meq and m_{oil} is the mass of oil in kg. According to the Codex Alimentarius for vegetable oils, acceptable PV levels for edible vegetable oils are below 10 meq/kg oil (Codex Alimentarius, 2001).

6.2.6.2 Para-anisidine value

The para-anisidine value (pAV) gives an estimation of the amount of secondary oxidation products (2-alkenals and 2-4-alkadienals) (Osborn-Barnes and Akoh, 2003) and was determined according to AOCS's official method Cd 18-90 with some modifications (AOCS, 2004). The oil-to-solvent ratio was 1:60 (w/v) for each sample. First, the absorbance of 1.7

mL sample was measured at 350 nm. Second, 0.3 mL 20.3 mmol/L para-anisidine was added to 1.5 mL oil-hexane sample. After 10 min, the absorbance at 350 nm was measured. A blank of n-hexane was used. The pAV can be calculated with the equation:

$$pAV(-) = \frac{1.2 \cdot As - Ab}{m}$$

in which *Ab* is the absorbance of the blank at 350 nm, *As* is the absorbance of the sample at 350 nm and *m* is mass of substance in test solution in g. The pAV has arbitrary units. The Codex Alimentarius for vegetable oils reports that the pAV for edible vegetable oils should be lower than 10 (Codex Alimentarius, 2001).

6.2.7 Protein oxidation

The reagent used to determine carbonyl groups on proteins is generally 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1990). The protocol by Sante-Lhoutellier et al. (2007) and Levine (1990) for emulsions was adapted for freeze-dried samples. About 4 - 8 mg was diluted with 1.8 mL 1-propanol to solubilize the lipids and precipitate the proteins. The samples were sonicated for 5 min in an ultrasound bath, followed by centrifugation at 15,000 x *g* for 5 min. The supernatant was discarded and the addition of 1-propanol, the sonication, and the centrifugation step were repeated twice more for each tube. Subsequently 500 µL of 10 mmol/L DNPH in 2 mol/L HCl was added to the pellets. The tubes containing the pellets were incubated at room temperature for 1 h in the dark, vortexing every 10 min (Multi Reax vortex Heidolph Instruments GmbH., Schwabach, Germany). Next, protein was precipitated by adding 500 µL trichloracetic acid (20% solution) per tube, followed by keeping the samples on ice for 10 min. The samples were centrifuged (15,000 x *g*, 5 min) and the remaining pellets were washed twice with 1 mL ethanol/ethyl acetate 1:1 (v/v) to remove free reagent. The supernatant was discarded. The precipitated protein was dissolved in 1 mL of a 6 mol/L guanidine hydrochloride solution at 37°C for at least 1 h, vortexing every 10 min (Thermo mixer Eppendorf AG., Hamburg, Germany). The insoluble fractions were removed by centrifugation (15,000 x g, 5 min). The absorbance of the supernatant was measured at 370 nm. The guanidine hydrochloride solution was used as a blank. To calculate the carbonyl content, a molar absorption coefficient (ε) of 22,000 L/mol·cm was used:

$$C_{carbonyls}\left(\frac{mol}{L}\right) = \frac{Abs_{370nm}}{\mu}$$

Due to the centrifugation steps in this protocol, and the possible loss of some insoluble proteins, it was necessary to measure the actual amount of protein dissolved in the final guanidine solution. The bicinchoninic acid (BCA) assay was used for this. The BCA working reagent is prepared by mixing reagent A (BCA, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1 mol/L NaOH) with reagent B (4% w/v copper (II) sulphate pentahydrate) in a ratio of 50:1 (v/v). Subsequently, 2 mL of BCA working reagent was mixed with 0.1 mL protein sample. The guanidine solution (without protein) was used as blank. A standard curve was made with bovine serum albumin (BSA). After 30 min of incubation at 37°C, the samples were cooled to room temperature and the absorbance at 562 nm was measured. The protein concentration (g/L) was determined from the BSA standard curve. The protein-bound carbonyl concentration is presented in mmol/kg protein.

6.2.8 Statistical analysis

All results are presented as means ± standard deviation (n = 3). Student's t-tests were performed to evaluate differences between samples. Differences between samples were considered significant when $p < 0.05$.

6.3 Results and discussion

6.3.1 Oxidation of lupin flour

The results of the peroxide value (PV), the para-anisidine value (pAV), the carbonyl concentration, the water activity measurements and the moisture content of full fat lupin flour that was stored for 2 weeks at 4°C or 20°C, are presented in Table 9.

Table 9 Peroxide values, para-anisidine values, carbonyl concentrations, water activity values and moisture content in full fat lupin flour after 2 weeks of storage at 4°C or 20°C (n=3). For a given column, different superscript letters (a,b) indicate a significant difference ($p<0.05$)

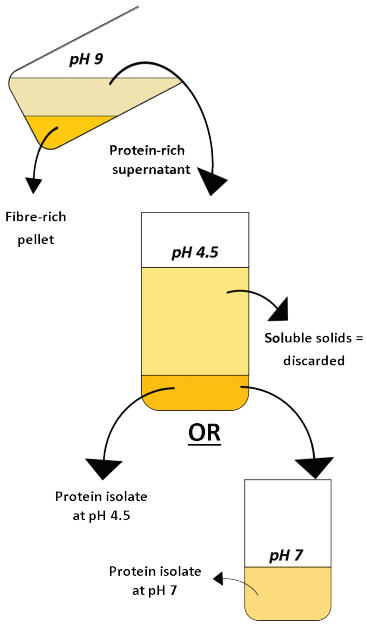
Sample	Peroxide value (meq/kg oil)	Para-anisidine value (-)	Carbonyl concentration (mmol/kg protein)	a_w	Moisture content (%)
Full fat flour at 4°C	1.87±0.04^a	3.04±0.97^a	2.73±0.06^a	0.35±0.00^a	6.7±0.0^a
Full fat flour at 20°C	1.25±0.02^b	1.92±0.03^b	2.79±0.06^a	0.29±0.00^b	5.9±0.0^b

For both flours, the PV and the pAV are clearly below the critical limits as indicated in the Codex Alimentarius (2001). However, the values are slightly higher than the values normally reported for intact seed oil bodies, which are in the range of 0 - 1 meq/kg oil. Those low values can be explained by considering the stabilizing properties of the interfacial membrane and the presence of exogenous proteins in the aqueous extract (natural emulsion) (Gray et al., 2010; Karkani et al., 2013; O'Keefe and Pike, 2010). The Soxhlet method for oil extraction disrupts the structure of the oil bodies and might cause increased lipid oxidation. Hence, the

lipid oxidation values reported in this work are probably a slight overestimation of the actual oxidation values in the flour or aqueous dispersions described in the next sections. Karkani et al. (2013) and Chen et al. (2012) found that oil bodies remained intact after AF of maize germ flour and soybean flour and that they were remarkably stable against oxidation when dispersed in water, and after heat treatment (Chen et al., 2012; Karkani et al., 2013). When the two flours are compared, it can be seen that the PVs and pAVs are significantly higher in the full fat flour that was stored at 4°C. This observation is, at first sight, somewhat surprising, as the rate of lipid oxidation generally increases with increasing temperature (Schaich et al., 2013). A first hypothesis may be that the difference is due to a slight, but significant, higher moisture content in the full fat flour stored at 4°C (Table 9) and subsequently to the resulting higher solubility of oxygen in water at 4°C (13.1 mg O_2/L) than at 20°C (9.15 mg O_2/L) as calculated with Henry's equation for the saturated dissolved oxygen concentration in water (Walstra, 2003). However, estimating the amount of oxygen that can maximally dissolve per g of flour, resulted in 0.88 µg O_2/g flour at 4°C, compared with 0.54 µg O_2/g flour at 20°C. These small amounts of oxygen do probably not explain the higher PV and pAV at 4°C. A second hypothesis is that the difference is due to a higher water activity (a_w) in the flour at 4°C. However, the measured a_w values (Table 9) are in the range in which lipid oxidation is known to have a minimum (a_w 0.25 - 0.35) (Nelson and Labuza, 1994). A clear explanation for the differences in PV and pAV can thus not be found.

On the other hand, the carbonyl concentrations in the flour samples stored at 4°C and 20°C are not significantly different, with a value around 2.8 mmol carbonyl/kg protein. To our knowledge, maximum acceptable carbonyl concentrations for plant-based materials have not been defined yet. Lund (2007) stated that non-oxidized samples of meat tissue have carbonyl concentrations of 1 - 2 mmol/kg protein and acceptable values for fresh meat tissue to be up to 14 mmol/kg protein (Lund, 2007). Finally for comparison, carbonyl concentrations higher than 50 mmol/kg protein were measured for dairy proteins in extensively oxidized emulsion systems (Berton et al., 2012), which indicates that the values that we found (2.7 – 2.8) are quite low.

Based on the lipid and protein oxidation data shown in Table 9, and the fact that the PVs and pAVs for full fat flour stored at 4°C and 20°C were below the acceptability limits for edible vegetable oils (Alimentarius, 2001), we conclude that storing full fat lupin flour under chilled conditions (4°C) does not improve the sample's oxidative stability, compared with storage at 20°C. Therefore, the full fat flour stored at 20°C was chosen as starting material for the AF processes.



	Sample	Dry matter content (g/g)	Protein content (g/g)	Oil content (g/g)
AF 4°C	Fibre-rich pellet	0.19±0.00	0.14±0.01	0.13±0.01
	Protein-rich supernatant	0.04±0.00	0.59±0.02	0.03±0.01
	Lupin protein isolate pH 4.5	0.33±0.00	0.88±0.03	0.02±0.01
	Lupin protein isolate pH 7.0	0.04±0.00	0.88±0.03	0.01±0.00
AF 20°C	Fibre-rich pellet	0.20±0.00	0.15±0.01	0.12±0.00
	Protein-rich supernatant	0.04±0.00	0.61±0.01	0.02±0.00
	Lupin protein isolate pH 4.5	0.37±0.01	0.90±0.02	0.03±0.01
	Lupin protein isolate pH 7.0	0.05±0.00	0.87±0.02	0.01±0.00
AF 4°C + HT	Fibre-rich pellet	0.18±0.00	0.21±0.02	0.16±0.01
	Protein-rich supernatant	0.04±0.00	0.59±0.02	0.01±0.00
	Lupin protein isolate pH 4.5	0.20±0.00	0.84±0.04	0.01±0.00
	Lupin protein isolate pH 7.0	0.03±0.00	0.82±0.01	0.01±0.00

Figure 28 Schematic drawing of separation process and table with chemical compositions of all fractions (dry matter, protein, oil) on a dry weight basis for AF processes at 4°C, at 20°C, and at 4°C including a heat treatment (n=3)

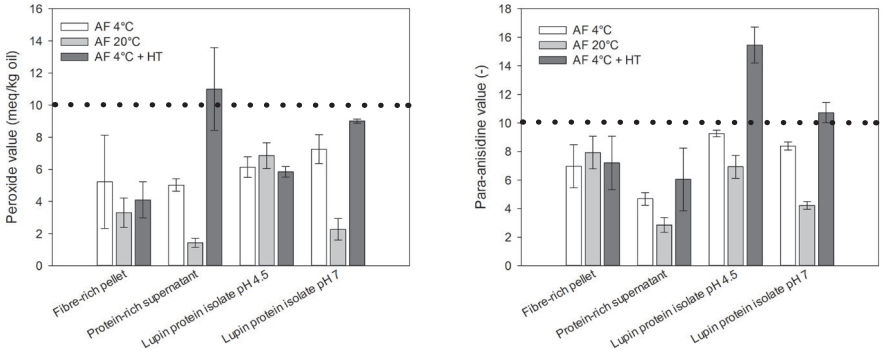


Figure 29 (A) Peroxide values for all fractions of the aqueous fractionation (AF) process at 4°C, 20°C and at 4°C with heat treatment (HT), (B) Para-anisidine values for all fractions of the AF process at 4°C, 20°C and at 4°C with HT. Vertical error bars represent the standard deviation of the means (n=3). The dotted lines represent the maximum values set for edible vegetable oil according to the Codex Alimentarius (2001)

6.3.2 Lipid oxidation during aqueous fractionation

The chemical compositions of all fractions obtained from the AF process, conducted at 4°C or at 20°C, are shown in Fig 28. The lupin protein isolates (LPIs) obtained at 4°C and 20°C contain more than 87 wt% protein and between 1 - 3 wt% oil, which is in line with previous results on AF of lupin seeds (Berghout et al., 2014). Most of the oil was recovered in the fibre-rich pellet (Fig 28), probably because of mechanical reasons. The centrifugal forces pull the fibres down, taking some of the dispersed protein and oil bodies with them. In addition, some of the protein and oil are recovered in this fraction due to the high water holding capacity of the fibres: the aqueous phase held by the fibre-rich pellet contains a significant amount of protein and oil, which is supported by the work of Towa et al. (2011) who showed that oil and protein were trapped in the fibre-rich pellet obtained by aqueous extraction of soybean.

6.3.2.1 The influence of extraction temperature

The PV and pAV of all fractions obtained after AF were found to be lower at 20°C than at 4°C (Fig 29A & B). In this aqueous environment, a possible explanation could be a higher oxygen solubility in water at 4°C (13.1 mg O_2/L) than at 20°C (9.15 mg O_2/L) as was calculated with Henry's equation (Walstra, 2003). A higher oxygen concentration implies that more hydroperoxides can be formed, accounting for higher values of oxidation markers. The PV and pAV values of all fractions after AF at both 4°C and 20°C were, however, below the acceptability limit for edible vegetable oils (Alimentarius, 2001).

The comparison of primary and secondary lipid oxidation products can provide information about the relative rate and extent of hydroperoxide decomposition, even though direct comparison of primary and secondary lipid oxidation values is irrelevant because the PV and the pAV have different units. The pAV has arbitrary units and, to our knowledge, there

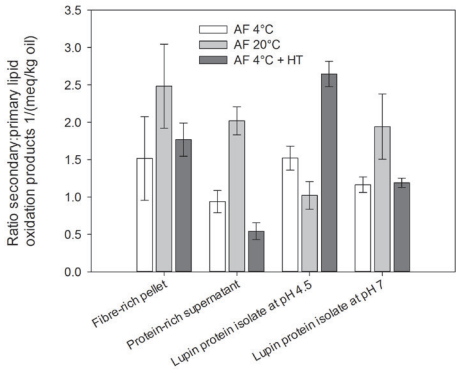


Figure 30 Ratio of secondary-to-primary oxidation products for all fractions of the AF processes at 4°C and 20°C, calculated from the data in Figure 29 (n=3)

is no conversion factor into molar units of secondary oxidation products (aldehydes) per kg of oil. We therefore converted the PV and pAV data of Fig 29 into a ratio of secondary-to-primary oxidation products (Fig 30). This ratio was expected to be higher with treatment at higher temperature, and Fig 30 shows that the protein-rich supernatant and the LPI at pH 7 had indeed a higher ratio at 20°C than at 4°C, except at pH 4.5. The difference in ratio for the fibre-rich pellet was not significant, which may be related to the chemical composition of the fibre-rich pellet. The lower ratio for the LPI at pH 4.5 at 20°C may be related to the physical state of the protein at this pH. At pH 4.5, lupin proteins exhibit zero net charge (Ruiz Jr and Hove, 1976) and are therefore expected to form a denser network (around oil droplets) in the protein pellet, providing a barrier against oxidation.

We therefore conclude that 20°C is still the preferred temperature during AF because AF at 20°C avoids the use of extra energy and water to cool the process, and it gives relatively low values of secondary oxidation products in the protein fractions.

6.3.2.2 The influence of the chemical composition

The extracted fractions have different chemical compositions; the protein isolates had the highest protein content (87 - 90 wt%) and contained 2 – 3 wt% oil, while the protein-rich supernatant contained 60 wt% protein and about 2 wt% oil, while the fibre-rich pellet contained fibres, most of the oil and less protein (14 wt%) than the other fractions (Fig 28). These differences explain part of the differences found in PV and pAV between fractions because proteins and polysaccharides can show anti- or pro-oxidative action (Waraho et al., 2011). The exact anti- or pro-oxidative effect of these components is rather complex because it depends on their concentration, their reactivity, the partitioning between phases, the interactions with other components, and on environmental conditions like temperature, pH and ionic strength (Waraho et al., 2011). The effect of these factors on oxidation markers are often studied for emulsions and even though our system is more diluted than an emulsion, it contains proteins and oil dispersed in an aqueous phase and we therefore compare our results with those reported for emulsion systems.

Proteins can inhibit lipid oxidation by chelation of metal ions and the free radical scavenging properties of their amino acids, and by physically (sterically) hindering the substrate – pro-oxidant interactions (Elias et al., 2008; Karkani et al., 2013; Levine et al., 1999). The free radical scavenging properties depend on the amino acid composition of the protein and are limited by the tertiary structure of the polypeptide because free radicals need to be buried within the core to be inaccessible to oxidants (Elias et al., 2008). The

metal-chelating properties depend on the specific protein, and on the presence of aromatic and sulphur-rich amino acid residues that can chelate the metal ions (Elias et al., 2008; Fuentes et al., 2014). Conglutin-γ and conglutin-δ are sulphur-rich proteins found in lupin at a concentration of about 5 wt% and about 10 wt% respectively, relative to the total amount of protein (Duranti et al., 2008). Conglutin-γ has a net zero charge around pH 7.8 – 8.0, therefore ends up in the protein-rich supernatant and subsequently in the soluble solids fraction (Blagrove et al., 1980). After AF, the sulphur-rich proteins are mainly recovered in the protein-rich supernatant. The sulfhydryl reactivity of the soluble solids fraction that was discarded added to the sulfhydryl reactivity of the protein-rich supernatant, which was 26 µmol/g protein compared with 19.5 µmol/g protein in the LPI, and may have improved the protection against oxidation in the protein-rich supernatant. Conglutin-γ was shown to chelate iron ions and especially copper ions (Duranti et al., 2001). The anti-oxidant capacity of the sulphur-rich proteins thus may have caused the lower levels of oxidation markers in the protein-rich supernatant than in the LPI fractions (Fig 29).

The physical hindrance or barrier effect is due to extraneous protein that is either adsorbed on the oil body surface or present in the continuous phase of an emulsion (Decker and McClements, 2001). The proteins on the interface permit limited access for oxygen or pro-oxidants to the oil body (Fisk et al., 2008; Karkani et al., 2013). Fisk et al. (2008) and Karkani et al. (2013) suggested that sunflower seed protein and maize germ protein in the continuous (aqueous) phase can chelate the metal ions and thus improve the oxidative stability of emulsions. Gray et al. (2010) studied emulsions prepared with oil bodies from Echium plantagineum and states that the protective effect of the protein is caused by interfacial proteins on the oil bodies, forming a barrier to oxygen and hydroperoxides and besides preventing the oil bodies from coalescing. The anti-oxidative mechanism of lupin protein may thus be due to interfacial and continuous phase proteins. Even though higher protein concentrations usually imply better protection against oxidation, the LPIs exhibited higher oxidation marker values than the protein-rich supernatant and thus the presence of sulphur-rich proteins in the protein-rich supernatant may be a more important anti-oxidant than the higher protein concentrations.

Polysaccharides can have anti-oxidative properties as well, as reported for oil-in-water emulsions (Chen et al., 2004; Mateos-Aparicio et al., 2010; Wang et al., 2013; Waraho et al., 2011; Zhou et al., 2008). The anti-oxidative mechanism is due to their metal-chelating activity, free radical scavenging activity, and additionally by the increase in viscosity (Waraho et al., 2011). Mateos-Aparicio et al. (2010) reported that the soluble polysaccharide fraction

of soybean cell wall material shows reductive power towards iron (III) and shows radical scavenging activity. The presence of polysaccharide-protein complexes, e.g. containing glycoproteins, made it difficult to completely eliminate the role of the anti-oxidative properties of protein (13 wt% on dry basis), but the 'fibre-rich pellet' material did show anti-oxidative properties (Mateos-Aparicio et al., 2010). Since lupin has a higher total dietary fibre content and a higher soluble dietary fibre percentage than soybean (Bähr et al., 2014), the fibre-rich pellet may exhibit even higher anti-oxidative activity than the fibre-rich material from soybean. Additionally, uronic acids, present in pectin, are positively related with an improved oxidative stability of polysaccharides in tea and brown algae through radical-scavenging (Chen et al., 2004; Zhou et al., 2008). The cell walls of the lupin cotyledon contain a substantial amount of pectins (Guillon and Champ, 2002), and thus an improved oxidative stability of the fibre-rich pellet was to be expected. Besides, a higher viscosity increases anti-oxidative activity because of reduced oxygen ingestion (Waraho et al., 2011). All these effects may thus have a role in the lower oxidation marker values found for the fibre-rich pellet (Fig 28 and Fig 29).

6.3.2.3 The influence of pH

In general, the PV and the pAV were higher for LPI at pH 4.5 than for LPI at pH 7 (Fig 29). At pH 4.5, lupin proteins were expected to form a denser network around oil droplets providing a barrier against oxidation (see 6.3.2.1 The influence of extraction temperature). This is not in agreement with our results; obviously other mechanisms dominate. One other mechanism could be related to the metal chelation of the proteins in the aqueous dispersions. At higher pH (far from the pI), the protein is anionic and thus able to bind positively charged iron ions (Berton-Carabin et al., 2014). At pH 4.5, the metal-binding capacity of lupin proteins may thus have been reduced. Faraji et al. (2004) showed that whey protein isolate (WPI) and soy protein isolate (SPI) continuous phase proteins inhibited lipid oxidation at pH 7.0. This was related to the metal-chelating properties of free sulfhydryl groups on WPI and SPI (Faraji et al., 2004). Since LPIs also contain free sulfhydryl groups (Berghout et al., 2015), a similar effect may have improved the oxidative stability of lupin oil.

6.3.2.4 The role of other minor components

The differences in PV and pAV at 4°C and 20°C and between fractions are thus a result of oxygen solubility, composition, but may additionally be caused by differences in solubility and the anti- or pro-oxidative activity of minor ingredients, such as phenolic compounds (anti- and pro-oxidative) and trace metals (pro-oxidants). Lupin seeds contain natural anti-

oxidants, such as tocopherols and other phenolic compounds (Sbihi et al., 2013; Siger et al., 2012). The specific effect of these separate compounds on the overall anti-oxidant capacity was outside the scope of this work.

Other potential pro-oxidants in lupin fractions are metal ions, such as iron and copper. The iron and copper content of lupin flour, the fibre-rich pellet and the LPI are shown in Table 10. Iron contents were higher than copper contents in all fractions, while the highest concentrations of iron and copper were found in the LPI. Sørensen et al. (2008) showed that iron increased the PV of an emulsion at low pH (3.0) compared with pH 6.0 because iron has a higher solubility at low pH and might have played a role in the higher lipid oxidation levels in the LPI dispersion at pH 4.5, compared with the LPI dispersion at pH 7.0. Osborn-Barnes and Akoh (2003) showed that copper had a smaller influence on lipid oxidation values at low pH (3.0) and a larger influence on lipid oxidation values at higher pH (7.0). The exact pro- and anti-oxidant effects of metal ions in an aqueous dispersion, or natural emulsion, thus depends on many different factors. A further specification of these factors was outside the scope of this work.

Table 10 Iron and copper content of full fat flour, fibre-rich pellet and lupin protein isolate on a dry basis

	Iron (mg/kg)	Copper (mg/kg)
Full fat flour	56.5±0.5	4.2±0.1
Fibre-rich pellet	76.0±1.0	2.6±0.1
Lupin protein isolate	91.5±0.5	12.7±0.2

6.3.3 Carbonyl formation during aqueous fractionation

A common protein oxidation marker is the carbonyl concentration (Nyström, 2005). The carbonyl concentrations of all the fractions at 4°C and 20°C (Fig 31) were below the acceptability limit of 14 mmol/kg oil for fresh meat tissue (Lund, 2007). The carbonyl concentrations were highest in the fibre-rich pellet even though this fraction had the lowest protein content of all tested fractions (Fig 28). Additionally, the fibre-rich pellet had the lowest solubilized protein content in the final measured solution (see values in the bars in Fig 31). This is in line with results previously reported by Berton et al. (2012) who found the highest carbonyl concentrations at the lowest protein solubilities in the creamed phase of an emulsion.

A possible explanation for the high carbonyl concentration is the presence of other components that are measured as well with the DNPH-assay, such as secondary lipid

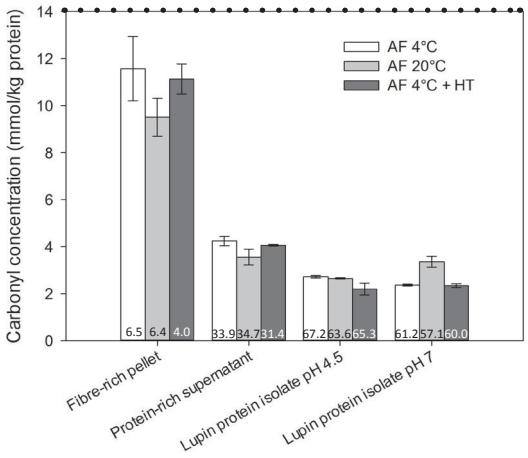


Figure 31 Carbonyl concentrations of all fractions after aqueous fractionation (AF) at 4°C, 20°C and AF at 4°C with heat treatment (HT). The values in the bars represent the percentage of soluble proteins in guanidine chloride for which the carbonyl concentration was measured. The horizontal dotted line indicates the acceptability limit for fresh meat tissue (Lund, 2007)

products that can react with sulfhydryl groups to form stable, covalent thioethers with carbonyl groups (Armenteros et al., 2009). Since the fibre-rich pellet contains most of the oil and relatively high levels of secondary lipid oxidation products (evidenced by the pAV), we hypothesize that the DNPH-assay measures these exogenous derivatives as well. The carbonyl concentration is in fact underestimated because less than half of the total protein amount was solubilized in the guanidine solution. However, the LPIs at pH 4.5 and pH 7 have the highest solubility in the guanidine solution and the lowest carbonyl concentrations, which concurs with the behaviour of the unabsorbed proteins found in the aqueous phase of an emulsion (Berton et al., 2012). This thereby suggests that AF is a suitable process to obtain chemically (oxidatively) stable LPIs.

6.3.4 Oxidation after heat treatment

In some of the experiments, a heat treatment (80°C, 1 min) was performed after the flour was solubilized (Fig 27). This was to inactivate any lipoxygenase (LOX) that may influence the measured oxidation values. Such a heat treatment is known to inactivate LOX (Seth and Nath, 2007). Yet, the heat treatment increased the PV in the protein-rich supernatant and in the LPI at pH 7, and it increased the pAV in the LPI at pH 4.5 and pH 7 (Fig 29). This suggests that either LOX is not strongly involved in the lipid oxidation or that the heat treatment itself induces lipid oxidation. Surprisingly, the PV increased in the protein-rich supernatant but remained similar in the fibre-rich pellet, even though the fractions were obtained from the

same bottle (Fig 28). We suspect that this could be due to a slightly different oil composition in the fibre-rich pellet and the protein-rich supernatant (Fig 29). Hydroperoxides are known to be surface-active (Nuchi et al., 2002) and therefore tend to migrate to the oil-water interface where they are accessible for pro-oxidants in the aqueous phase (protein-rich supernatant) (Decker and McClements, 2001). The higher pAV in the LPIs then are a result of the conversion of the hydroperoxides in the protein-rich supernatant after heat treatment into secondary lipid oxidation products.

Remarkably, none of the fractions had higher protein oxidation levels by heat treatment. Protein oxidation generally increases with increasing processing temperature and time, for example for cooking beef and pig tissue (Gatellier et al., 2010; Traore et al., 2012). It is possible that higher temperatures are needed to oxidize the protein further. For example, Gatellier et al. (2010) showed that a temperature of 65°C did not influence the protein oxidation levels of beef tissue, but a temperature of 96°C and higher increased the protein oxidation levels of beef tissue significantly.

The heat treatment decreased the protein and oil recovery of the AF process as shown by the fact that the amount of protein recovered in the LPIs was only half of the value measured without heat treatment (21 wt% versus 47 wt%, respectively). This can be explained by the formation of local, denser protein networks surrounding the oil bodies, much like oil-filled gels from SPI and WPI (Gu et al., 2009; Sala, 2007). These locally dense networks with oil and high protein contents will have a high density, closer to that of water, and will end up in the fibre-rich pellet as evidenced by its increased protein content (Fig 28).

The specific involvement of LOX in the oxidation mechanism of lupin oil was not assessed, but given the negative influence of heating we hypothesize that auto-oxidation has a larger influence than enzymatic oxidation. We found that a longer time of evaporation of the extraction solvent is positively correlated with increased oxidation values (data not shown). Besides, LOX is known to have a higher activity at 20°C than at 4°C (Yoshie-Stark and Wäsche, 2004) while we found higher oxidation values at 4°C than at 20°C. This suggests that auto-oxidation, the composition of the fractions, and the solubility of oxygen in water, are more important factors involved in the oxidative destabilisation of oil in our samples before and during AF compared with the activity of LOX.

6.4 Conclusion

We investigated the chemical stability of lupin protein isolates (LPIs) obtained through aqueous fractionation (AF). AF at 4°C and 20°C resulted in LPIs containing 88 wt% protein and 2 wt% oil, with lipid and protein oxidation values well below the acceptability limit for edible vegetable oils and meat tissue protein. The level of lipid oxidation markers was lower when the AF process was conducted at 20°C compared with 4°C, which is attributed to the higher solubility of oxygen in water at lower temperature (4°C). The differences in oxidation values between the different fractions obtained by the AF process were related to their different chemical compositions, with protective effects of the matrix of protein and polysaccharides that surround the oil. Additionally, the metal-chelating properties of protein and the different solubilities of trace metals and proteins at pH 4.5 or 7.0 probably play a role in the oxidation mechanism of lupin oil. The application of a heat treatment to inactivate oxidative enzymes, did not reduce the oxidation marker values but instead increased oxidation marker values above the acceptability limit, and additionally decreased the protein and oil recovery values. Based on the lipid and protein oxidation levels of all fractions obtained through AF, a processing temperature of 20°C is preferable to obtain a chemically stable LPI from lupin seeds.

Acknowledgements

The authors thank Peter Nobels from the Chemical Biological Laboratory Bodem for the ICP-AES determinations and the peas foundation and the Institute for Sustainable Process Technology (ISPT) for financial and project support.

Chapter 7
General discussion

Highlights:

- The functional properties of lupin protein isolates depend mainly on drying
- Before designing fractionation processes, one should have a clear image of the structure and interactions of the components of the seed or bean
- Integration of fractionation and product processing sites is crucial for better sustainability
- Functionality-driven fractionation can be extended to other legumes and pulses

7.1 Outline

The conventional approach to separate a raw food material into its pure ingredients and then blend these to the final composition of the food product is not efficient. Firstly, the separation processes require dilution with water and many chemicals, like organic solvents, alkali and acids and secondly, drying of the ingredients consumes a lot of energy. It is more efficient to prepare fractions with the desired composition and functional properties for the food product; a concept referred to as functionality-driven fractionation. The aim of this thesis was to obtain understanding of the sustainable production of functional, protein-rich materials from lupin seeds. This chapter summarizes the main findings of the preceding chapters, after which the potentials and bottlenecks of simpler fractionation techniques are discussed. The chapter ends with a future outlook on the functionality-driven design of fractionation processes.

7.2 Main findings and conclusions

The design of conventional wet fractionation processes for the production of protein isolates focuses on achieving high purity and high yield, for which organic solvents, chemicals and loads of water are necessary. Since no end product is composed of a single ingredient and lupin seeds have a relative low oil content, a focus on purity and yield may not be necessary. Instead, functionality and sustainability should form the focus of modern fractionation processes. Consequently, we investigated the potential of purely aqueous fractionation (AF) of lupin seeds in **chapter 2**, in which the use of organic solvents was avoided completely and several extraction steps performed in the conventional wet fractionation process were skipped. This functionality-driven approach led to lupin protein isolates (LPIs) that contain about 2 wt% oil and have similar functionality as the conventional, wet-fractionated LPI. The higher extraction temperatures affected the separation efficiency and functionality of the LPI; an altered protein functionality could also be achieved by applying a heat treatment after fractionation.

Common functionalities reported for lupin protein are foaming capacity and stability, emulsifying capacity and stability, and solubility (D'Agostina et al., 2006; Pozani et al., 2002; Wäsche et al., 2001). Other relevant functional properties for legume proteins are the water holding capacity, viscosity and gelling capacity. In the evaluation of the gelling properties of lupin protein, soy protein was taken as benchmark (Banerjee and Bhattacharya, 2012; Day, 2013). In **chapter 3**, we showed that for several protein concentrations, LPI formed weaker

and more deformable gels than soy protein isolate (SPI). Microscopic imaging and static light scattering measurements showed that both protein isolates formed particle gels in which lupin protein particles swelled less upon heating than soy protein particles. The gelling properties of LPI and SPI were related to their sulfhydryl reactivity. The higher sulfhydryl reactivity of LPI and the increasing reactivity upon heating, suggested that LPI forms less or no new disulphide bonds to strengthen the particle network. The opposite behaviour was observed for SPI; a decreasing sulfhydryl reactivity upon heating, indicating the formation of (new) disulphide bonds. LPI may thus be less suitable for semi-solid food products but offers opportunities for high-protein foods that require a low viscosity after heat treatment.

The environmental impact of fractionation processes for lupin seeds is discussed in **chapter 4**, where we reported on a sustainability assessment with the use of mass, energy and exergy balances to pinpoint inefficiencies in the use of raw materials and in processes. Dry fractionation yields protein-enriched flours (54 wt% protein) and consumes least energy because it requires no water at all. For higher protein contents (>85 wt%), wet fractionation processes are needed. As expected, the conventional wet fractionation process requires more water and energy than the AF process. Replacing the drying step with a concentration step (i.e. producing the isolate in a concentrated liquid form) reduces the water and energy consumption of all fractionation processes. Water consumption was further reduced by combining dry and AF processes to obtain LPIs. Finally, the exergetic efficiency of all fractionation processes could be increased by valorising all the by-product fractions obtained, e.g. lupin oil, the soluble solids fractions, the fibre-rich pellet and the coarse fraction.

Replacing drying by concentration, i.e. membrane (ultra)filtration, resulted in 'wet' LPIs with different functional properties than freeze-dried LPIs. In **chapter 5** the differences in the technical functionality of these LPIs was investigated. After heat treatment, the viscosity of the wet LPI increased and eventually resulted in gel formation at 121°C, while the viscosity of the freeze-dried LPI was slightly lower and did not result in gel formation. This difference is caused by a difference in the degree of aggregation, as evidenced by microscopic imaging, static light scattering, and swelling capacity measurements after heat treatment. The freeze-dried LPI was more intensively aggregated due to the drying process (freezing and freeze-drying) while the wet LPI was less aggregated and could swell upon heating and aggregate further after heat treatment above 100°C. We concluded that the extent of concentration is a parameter to control the final product properties. Wet LPIs show potential for yogurt-style alternatives, while the low viscosity of the freeze-dried LPIs show potential for high-protein beverages.

Chapter 6 dealt with the chemical stability of the LPIs obtained with AF. The LPIs contain oil, have a high degree of unsaturated fatty acids and may thus be prone to oil and protein oxidation. We showed that AF at 4°C and 20°C resulted in LPIs for which the oil and protein oxidation marker values were within the quality limits. AF at 20°C is thus a feasible option to obtain chemically stable LPIs that contain oil, which means that cooling of the process is not necessary and thus AF consumes 5.4 MJ/kg LPI less energy (**chapter 4**).

Overall, it can be concluded that the environmental impact of oilseed fractionation can be reduced when the final application is considered as a design parameter for the fractionation process. For most food applications, pure ingredients are not necessary, but the production of pure ingredients requires most resources by far. In case of protein, enrichment is sufficient for application in foods, and dry fractionation might be an interesting option, provided that the by-product stream can be used for food applications as well. For applications in which higher protein contents are required, and the presence of oil is allowed (which will be the case in many food applications), the oil recovery step can be omitted. A detailed analysis showed that oxidation of oil and protein remained within quality limits in the simplified AF process.

7.3 Evaluation of fractionation processes

The separation principle of conventional wet fractionation processes is based on differences in solubility of the different components. Generally, lipids are soluble in organic solvents, simple carbohydrates and minerals are soluble in water and proteins are soluble in dilute-salt solutions and water at a pH above their iso-electric point, while dietary fibres are not soluble in water or most organic solvents. By making use of organic solvents and pH switches, water-soluble and water-insoluble components can be separated. However, the use of organic solvents, water, and alkali and acids is not desirable from an environment and economic point of view. Therefore, the focus of future fractionation techniques should be on reducing the environmental impact of fractionation processes, by simplifying separation processes even further.

7.3.1 Simplification of separation processes

Raw materials are highly complex, structured mixtures of macro- and micro-nutrients. For several of these nutrients, isolation procedures have been developed. These isolation procedures disrupt the naturally present structures of the matrix in which the component of interest is embedded, which requires intensive processing, leading to waste production.

We proposed to focus less on molecular purity and more on the natural functionality of fractions of the raw material. With this we aim to obtain functional fractions that can be produced with a more efficient process, i.e. less intensive, producing less waste. The low alkaloid level of *Lupinus angustifolius* L. allowed us to skip acidic extraction steps before alkaline solubilisation, which simplified the fractionation process. We also performed all extraction steps with fewer repetitions, again simplifying the fractionation process. Further simplification included leaving out the oil extraction step. The presence of oil was not detrimental for the functionality and chemical stability of the LPI (**chapter 2** and **chapter 6**), and significantly reduced the environmental burden of fractionation processes (**chapter 4**), thus enabling environmental benign fractionation processes for legume seeds.

Reducing the environmental burden of fractionation processes even further was achieved by combining dry and AF processes, achieving a reduction of 34 wt% in water use and a similar amount in the use of chemicals for fractionation. This combination showed great potential to obtain LPIs that have functionality interesting for high-protein, plant-based products and that are produced in a more sustainable way (**chapter 4**). AF makes use of water and pH shifts to separate components such as protein, simple carbohydrates and fibres based on differences in solubility. Omitting the pH shifts to separate these components would make the AF process even simpler and more sustainable, making the use of chemicals redundant. This leaves only water and temperature as means for separation. In **chapter 2**, the use of a higher extraction temperature (in combination with pH shifts) was shown to decrease the protein solubility, which may not be desired for many high-protein food products. These temperatures (50°C and 90°C) were rather extreme, and thus we recommend to study the effect of less extreme extraction temperatures on functionality further.

Drying is energy-intensive (**chapter 4**) and influences the functionality of the LPIs significantly (**chapter 5**). Skipping a drying step reduces the energy consumption of the fractionation processes (12.7 MJ/kg LPI) (**chapter 4**). Instead of drying, the use of a concentration step was considered, to obtain a protein concentration that is appropriate for the preparation of high-protein foods (about 10% (w/v)). This concentration was achieved with ultrafiltration and resulted in a 'wet' LPI dispersion (**chapter 5**). Producing 'wet' fractions is however not common in industry because of increased (microbial) spoilage risks, physical and chemical instabilities, a reduction in standardization and costs of transportation. Processing the fractions right after fractionation may overcome some of these problems, but will have big implications for the current logistics in industry. A possible drawback of membrane filtration might be the fouling, which requires the use of chemicals and water for removal, or even

membrane replacement costs and down time (Mondor et al., 2010). The impact of these factors on the sustainability of membrane filtration processes should be evaluated versus the unique functionality of the protein.

7.3.2 Controlling LPI functional properties through drying conditions

Drying strongly influences the functional properties of the LPI. A mild drying technique involves flash-freezing or fast-freezing of the LPIs. We mimicked this by quickly freezing the wet LPIs with liquid nitrogen (N_2) and subsequently freeze-drying the LPIs (Fig 32 and Fig 33). Compared with the freezer-frozen-freeze-dried LPI (referred to as FF LPI), the N_2-frozen-freeze-dried LPI (referred to as N_2F LPI) did not result in a powder, but yielded a fibre-like material with a more porous structure, as evidenced from scanning electron microscope images (Fig 32). After grinding the N_2F LPI to a powder, it was observed that the N_2F LPI shows electrostatic behaviour, which the FF LPI does not show. The particle size distribution (PSD) (Fig 33) shows that the N_2F LPI consists of smaller particles than the FF LPI and has a PSD similar to the wet LPI. Smaller particle sizes can lead to more electrostatic behaviour because of a larger surface-to-volume ratio (Wang et al., 2014). It was also observed that the N_2F LPI dispersed faster in water after freeze-drying than the FF LPI, which may be related to

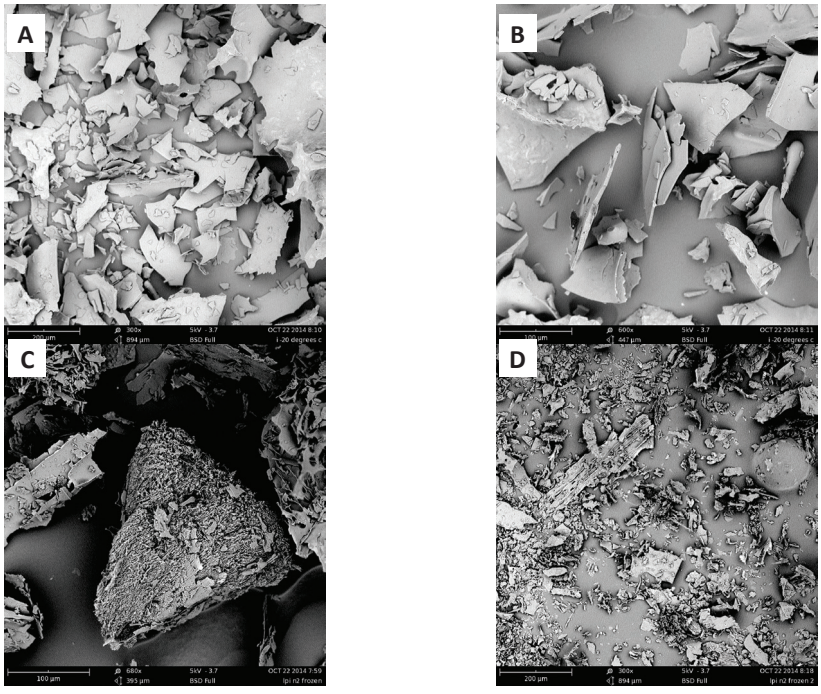


Figure 32 Scanning Electron Microscope images of freezer-frozen-freeze-dried protein isolate (A & B) and N_2-frozen-freeze-dried protein isolate (C & D)

their PSDs and the porous structure of the N_2F LPI. This thus shows that the speed of freezing has a strong influence on the physical state of the LPI. The capital costs for the equipment for immersion freezing in N_2 are relatively low but the liquid N_2 itself is expensive (Smith, 2011). Therefore, cryogenic freezing is mainly used for high-value, specialty ingredients.

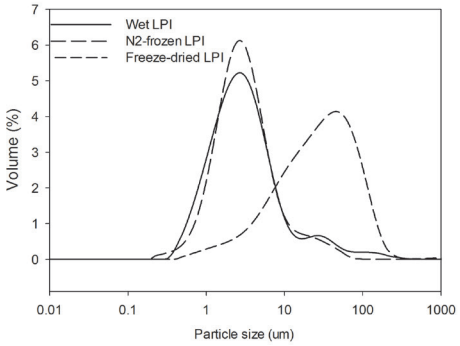


Figure 33 Particle size distribution of wet (—), N_2-frozen-freeze-dried (- - -), and freezer-frozen-freeze-dried (••••) LPIs, measured with static light scattering

Finally, there are options for drying protein isolates with fewer resources, such as supercritical CO_2 drying (SC-CO_2) because SC-CO_2 is non-toxic, inexpensive and it is recyclable (Brown, 2010; Bušić et al., 2014; Nuchuchua et al., 2014). However, the use of SC-CO_2 for drying needs further optimisation before implementation because the necessary equipment is capital intensive. In addition, since water has a limited solubility in SC-CO_2, drying is not fast, and because of the extreme operating pressures that are needed (> 100 MPa) the size of the extraction vessels and thus throughput is limited (Temelli and Ciftci, 2014), while aroma properties and preservation of bioactive compounds in e.g. basil leaves was not as good as with freeze- and air-drying yet (Bušić et al., 2014).

7.3.3 Towards total use (of raw materials)

The complete use of a raw material reduces the waste production and thus improves the chemical exergy efficiency and sustainability of fractionation processes (**chapter 4**). This illustrates the general principle that for better sustainability, cycles need to be closed, in which the products and residues from one industry serve as the raw material for the next (Vardanega et al., 2014). By-product streams of the fractionation processes that we discussed were the coarse fraction (after dry fractionation), the fibre-rich pellet and the soluble solids fraction after conventional wet fractionation and AF. Valorisation of by-product streams is dependent on the composition and quality of the components in the stream,

and may be useful for food, chemicals, materials, bio fuels or feed. The extracted oil was not seen as a waste stream, but it should be mentioned that lupin oil is not commercially marketed because of the relative low oil contents in comparison with soybean, rapeseed, sunflower, and peanut oil contents (Doxastakis, 2000; Rodrigues et al., 2012), which makes it unattractive as a primary product. Besides, valorising lupin oil for human consumption will require purification steps, thus increasing the environmental burden of the fractionation process. Therefore, it is attractive to keep the oil inside functional fractions.

The fibre-rich fractions and the coarse fractions contain valuable nutrients, like insoluble dietary fibre (celluloses, hemicelluloses), soluble dietary fibre (pectins), oil and proteins. Some of these fractions may find an application as is, but others require refinement. Lupin cotyledon fibre is rich in soluble fibres (pectin) and has a high water holding capacity (~8.5-11.1 g/g dry solids) (Turnbull et al., 2005), and improves the texture and shelf-life of breads (Güémes-Vera et al., 2008) and bowel health (Fechner et al., 2013). The high water binding capacity may however complicate food production as was shown for expanded extrudates from wheat bran (Santala, 2014). For this, enzymatic hydrolysis may potentially be used to decrease the water holding capacity of the fibres. The fibre-rich pellet is rich in dietary fibre and protein, but also in oil. Since the fibre-rich pellet is in a wet state (17 wt% dry matter), the oil may be separated by adding some water and subsequent centrifugation before hydrolysis with carbohydrases, e.g. pectinases, to reduce the water holding capacity of the fibres. Lupin flour is added to e.g. breads to improve the nutritional value due to the high lysine and low methionine content of lupin protein (Dervas et al., 1999). The addition of lupin flour also improves texture, taste, colour and overall acceptability of bread (Dervas et al., 1999). The coarse fraction is in powder form, contains native protein, oil and is enriched in dietary fibres and may thus be applied as food ingredient for the fibre-enrichment and improved nutritional value of baked goods as is (Turnbull et al., 2005).

The fibre-rich pellet contains a significant amount of protein and to test whether the protein could be extracted from the matrix, we performed repeated extractions with water or a 10% NaCl solution (Fig 34). The 10% NaCl solution was able to extract slightly more protein from the fibre-rich pellet than pure water. Preferably no chemicals should be used because their removal from the fraction requires more processing. The use of water for protein extraction would thus be a good option. The soluble solids fraction also contains (sulphur-rich) protein, as was shown in **chapter 6**. The amount of free sulfhydryl groups in the soluble solids fraction was about 26 µmol/g protein compared with 19.5 µmol/g protein in the LPI. These sulphur-rich proteins may have (unique) interesting functional properties

and can possibly be separated from the simple carbohydrates and minerals with e.g. membrane filtration techniques as was done for liquors from soybean processing (Moure et al., 2005). The refining of protein requires extra water; therefore the added value in terms of their functionality should be evaluated versus resource consumption and waste production.

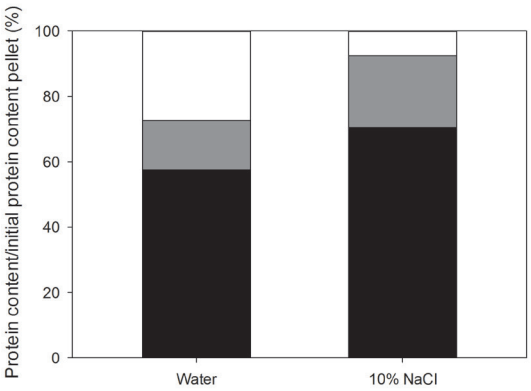


Figure 34 Protein extraction from the fibre-rich pellet obtained after AF with water and a 10% NaCl solution. From bottom to top: black is the protein obtained with the first extraction step, grey is the protein obtained with the second extraction step, and white is the protein in the pellet after extraction

7.3.4 Implications for process chains

The feasibility of this novel approach of simplification depends on the physical distance between the fractionation and food product processing sites. This implies that integration of bio-refinery concepts with other industries is crucial and the de-centralization of local-scale production is necessary (Bruins and Sanders, 2012; Herrero and Ibáñez, 2014; Santos et al., 2014). An example of economically attractive integration of industries was given by Santos et al. (2014), who evaluated the economic feasibility of integrating the supercritical extraction of bioactive compounds from ginseng roots with the bio-refining of sugarcane and found that the integration had a high economic potential because the supercritical extraction process could use the heat, CO_2, ethanol and electricity already available. In the case of the production of the wet, concentrated LPI dispersions by AF and ultrafiltration, the bio-refining site should be close to the point where the end products, e.g. dairy alternatives, are produced. Using local resources (and also local crops) has the benefit of shorter supply chains and less deterioration. Small scale bio-refineries can be successful and economically attractive when capital investments are relatively low and when they are not aimed at full (pure) conversion (Bruins and Sanders, 2012). Additional costs may be covered by reduced

transportation costs and the use of local produce and recycling of water and minerals (Bruins and Sanders, 2012). Integration of fractionation of lupin seeds and product processing may not seem realistic at first, but with research focusing on the integration of bio-refineries and other industries (Bruins and Sanders, 2012; Varbanov and Seferlis, 2014), concepts may become more realistic in the future.

The production of the isolates close to the point of use also implies that the use of the other product streams (coarse fraction, fibre-rich fraction) may not be close to their point of use. While in general this shows the benefit of a larger cluster of related industries, this point may need further attention in future studies.

7.4 Future prospects on the design of fractionation techniques and new scientific challenges

7.4.1 The importance of food structure

An improved understanding of the components and the interactions between components in lupin seeds can provide better leads for the design of (more sustainable) fractionation processes. Lupin cotyledon cells are built of cell walls that are composed of cellulose, hemicellulose, pectin and non-storage proteins. The cells contain protein bodies and oil bodies as separate 'structures' that contain most of the storage proteins and oil (Aguilera, 1989; Rosenthal et al., 1996), and other minor components such as starch and non-storage proteins (Garnczarska et al., 2007; Morkunas et al., 2012).

The protein bodies (PBs) are composed of 70 wt% storage proteins, 12.5 wt% lipids, 4 wt% soluble carbohydrates, 8 wt% mineral residue, and about 5.5 wt% water (Plant and Moore, 1983), and have a size of about 5 – 25 µm (Pelgrom et al., 2014). PBs are dispersable in water at neutral pH and partly disintegrate, as was shown for soybean by microscopic imaging (Preece et al., 2015). Using water for fractionation therefore does not seem a logical option. Dry fractionation by fine milling and air classification is based on differences in particle size. Dry fractionation of lupin seeds yielded max 54 wt% protein in the fine fraction (**chapter 4**). Further protein enrichment may not be possible since the difference in particle size between PBs and milled fibres is too small to be separated with just air classification (Pelgrom et al., 2014). Another dry fractionation method is electrostatic separation. This method is based on the separation of oppositely-charged particles (Wang et al., 2014) and may potentially be used to separate the small fibres from the protein bodies in the fine fraction (Pelgrom et al., 2015). Dry fractionation will however never reach higher protein contents than 70 wt%

because of the intrinsic composition of the PBs. For higher protein contents, the PBs need to be dispersed in aqueous environments to separate the protein from the sugars and minerals. Since food products generally do contain water, it is not a problem to use water for aqueous processing, as long as it is done in concentrated conditions, such that evaporation of water (energy-intensive, **chapter 4**) can be avoided or kept to a minimum. Since dry fractionation can increase the protein concentration of lupin flour, the use of water should be the very last step in the fractionation process to reduce spoilage risks, and omit the need for drying steps before product processing. Ultrafiltration may then be an interesting technique to further purify the protein fraction by removing soluble components < 5 kDa by diafiltration (for example). After the addition of water for further purification in concentrated conditions, heating steps can be applied for preservation, instead of applying drying steps to produce dry ingredients and then mixing these with water for product processing.

Oil bodies have a size of about 0.1 – 0.5 µm and can be separated with organic solvents or via aqueous extraction (Rosenthal et al., 1996). Aqueous extraction makes use of the insolubility of oil in an aqueous environment, thus creating an oil and an aqueous phase (de Moura et al., 2008; Rosenthal et al., 1998). The recovery of intact oil bodies depends on the particle size after milling (not too coarse), interactions between oil and protein, extraction temperature, solid-water ratio, and on the addition of proteases (Campbell, 2010; Rosenthal et al., 1996). Latif et al. (2008 and 2011) and Campbell et al. (2010) reported the combined extraction of protein and oil from sesame seeds, rapeseeds, and soybeans, which required less energy than the separate extractions of oil and protein. Nevertheless, separating the protein from the oil in the emulsion by de-emulsification required the use of enzymes and extra processing steps (Campbell, 2010; Rosenthal et al., 1996). However, complete fractionation might not be necessary for most food applications. For aqueous extraction of oil bodies, no pH switches have to be applied, thus relying more on differences in dispersibility than in solubility. For lupin seeds, oil bodies did not impede the dry fractionation, but higher oil contents in other seeds seem to be a challenge because of stickiness.

In summary, one should have a clear image of the structure of the components, their interactions, and the dispersibility of the components before designing a fractionation process for legumes and pulses. Dry fractionation by fine milling and air classification, followed by electrostatic separation may be an interesting pre-processing route to obtain protein-rich fractions from lupin seeds that contain oil. To reach higher protein contents, aqueous processing is necessary. The addition of water to protein-enriched flours, preferably in concentrated conditions, should be postponed however until the end of the process

just before application/structuring, to reduce the risk of spoilage and omit the need for intermediate drying steps.

7.4.2 Towards the use of functional fractions

Until now, we have been looking for applications for the by-product streams that were created after the main fraction of interest had been extracted; the protein isolate. We concentrated on functional fractions and therefore the functionality, not the purity, of the AF LPI had to be similar to that of a wet-fractionated LPI (**chapter 2**). However, we did not test what concentration of impurities could be tolerated before the functionality is altered or lost. The reduced focus on molecular purity coincides with the current trend that consumers and consequently industry turn towards organic products (Morgera et al., 2012). Organic processing has many restrictions and does not allow the use of organic solvents (hexane), of chemicals like sodium hydroxide (except for specialty bakery products) and hydrochloric acid, but does approve the use of dry fractionation and water (IFOAM, 2012). This is thus another motivation for future research to focus less on molecular purity and more on functionality.

For feeling for the degrees of freedom in producing functional fractions, it would be helpful to design a model or diagram which can be used to predict the technical functionality and nutritional value of e.g. lupin fractions versus molecular purity and nativity. A hypothetical example of such a diagram is depicted in Fig 35. The technical functionality of a protein-

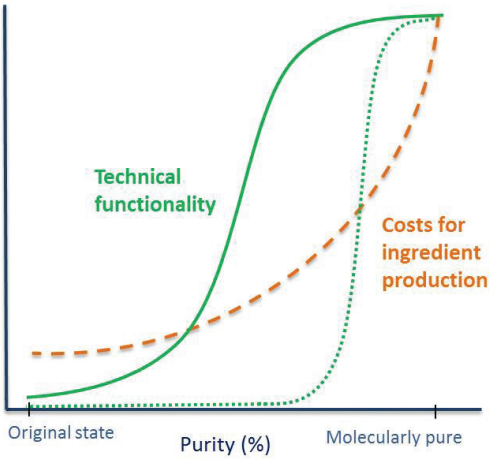


Figure 35 Schematic diagram of technical functionality (green line and green dotted line) and cost for ingredient production (orange dashed line) as a function of purity (%)

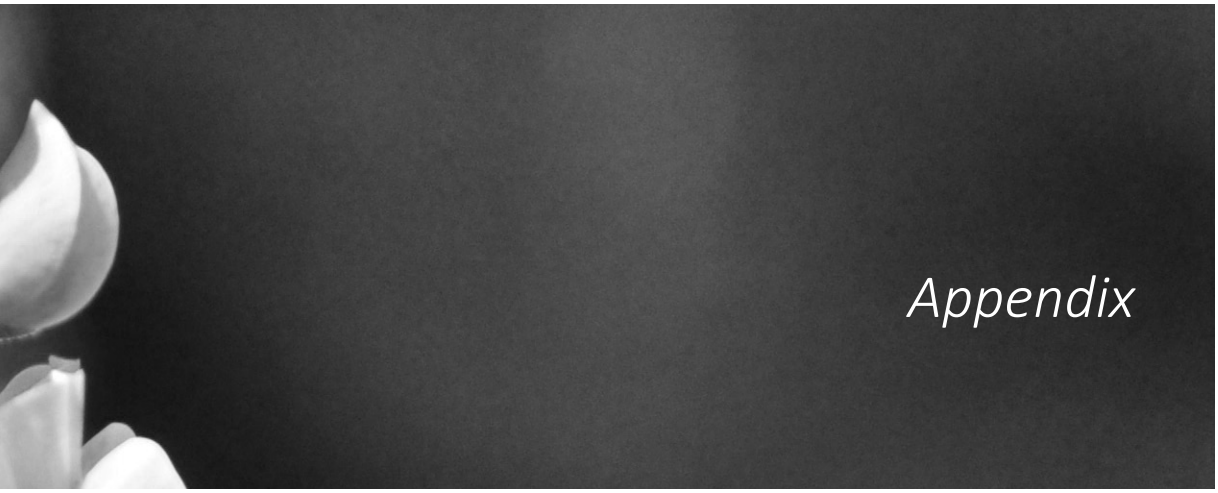
enriched lupin fraction may follow an S-shaped curve (green line), where molecular purity can be greatly reduced without compromising on technical functionality, or a steep descending curve towards reduced molecular purity (dotted green line), which means that technical functionality will suffer a lot from a small decrease in molecular purity. Such a diagram requires experiments that explore the relation between technical functionality and molecular purity, but also nutritional value and nativity.

We showed in **chapter 6** that not extracting the oil did not impose risk from a chemical stability point of view; lipid and protein oxidation marker values of all fractions obtained with the AF process were all below the acceptability levels. Oil bodies are known to be well protected by a dense layer of phospholipids and protein and there are several anti-oxidative components naturally present in lupin seeds, such as phenolic compounds, carotenoids and tocopherols that may protect the oil bodies from oxidation (Sbihi et al., 2013). However, the chemical stability of the fractions over longer times (upon storage) should be investigated, especially when kept in a wet state.

An important further issue for the use of lupin seeds and functional fractions derived thereof is their allergenic potential. The allergenic lupin protein shows cross-reactivity with peanut protein, which is a serious and frequently occurring allergy (Sirtori et al., 2011). Identifying the allergenic protein and reducing the allergenic potential is a topic of investigation (Alvarez-Alvarez et al., 2005; Sirtori et al., 2011). Alvarez-Alvarez et al. (2005) studied the effect of microwave cooking, extrusion and autoclaving on the allergenic potential and found that the allergenic potential was reduced after an autoclave treatment at 138°C for 20 min. Even though autoclaving was a promising method to reduce the allergenic potential of lupin protein, further investigations are needed, especially on the functionality, nutritional value and protein digestibility after such a treatment.

The concept of functionality-driven fractionation is a useful concept towards full use of raw materials, less water and energy consuming processes, and the use of less chemicals and organic solvents. The concept is not only useful for the fractionation of lupin seeds, but also for other food (oil-rich) legumes and pulses (de Moura et al., 2008; Latif and Anwar, 2011; Latif et al., 2008; Rosenthal et al., 1998), and for non-food crops, like the rubber seed kernel (Widyarani et al., 2014). A combination of dry and AF processes with the use of water and temperature may be very promising to obtain functional fractions. The design of fractionation processes depends on the relation between technical functionality, nutritional value and molecular purity for the main fraction of interest. Lupin seeds are relatively low in anti-nutritional factors because sweet varieties have been developed, while the

presence of phenolic compounds did not trouble either the fractionation processes itself, or the oxidative stability of the products. However, for crops with higher concentrations of phenolics, e.g. glucosinolates and erucic acid in canola, complexes of phenols and proteins may be formed that darken the products and are responsible for undesirable flavours and lower nutritional value (Aider and Barbana, 2011). The fate of these micro-nutrients should thus be taken into account as well.



Appendix

Appendix A

Calculations on cooling duty

We assumed there are three processes that require cooling: (1) mixing flour with water and NaOH, (2) mixing protein-rich supernatant with HCl, and (3) rinsing the protein pellet with water.

Temperature differences assumptions: (1) from 15°C to 4°C, (2) from 5°C to 4°C, and (3) from 5°C to 4°C. The following formula was used to calculate the cooling duty supplied by the cooler per ton protein isolate.

$$Q_{cool} = m \cdot c_p \cdot \Delta T$$

where Q_{cool} is the cooling duty (kJ), m is the mass (kg), c_p the heat capacity (kJ/kg·K) and ΔT the temperature difference (K).

The cooling duty was corrected for the efficiency of the cooler with:

$$Q_{cooler} = \frac{Q_{cool}}{efficiency}$$

where the efficiency of the cooler was assumed to be 0.5.

Table A1 Composition of by-product streams

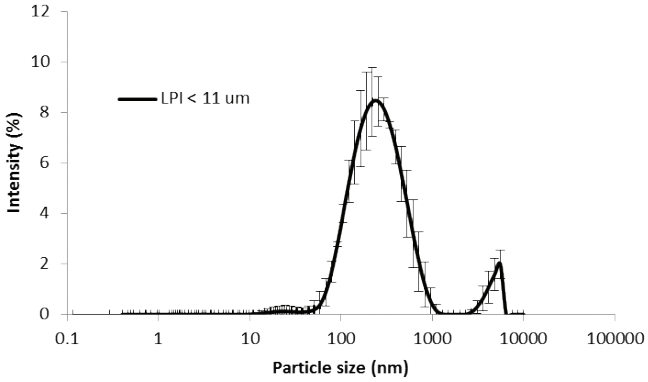
		Mass fraction on dried material (-)				
		Protein	Oil	Carbohydrates	Mineral residue	Water
Conventional wet fractionation	Fibre-rich pellet	0.23	0.00	0.68	0.04	0.05
	Soluble solids fraction	0.28	0.00	0.59	0.08	0.05
	Oil	0.00	1.00	0.00	0.00	0.00
Aqueous fractionation	Fibre-rich pellet	0.12	0.16	0.65	0.02	0.05
	Soluble solids fraction	0.37	0.00	0.50	0.08	0.05
Dry and aqueous combination	Coarse fraction	0.32	0.06	0.51	0.02	0.10
	Fibre-rich pellet	0.20	0.26	0.47	0.02	0.05
	Soluble solids fraction	0.64	0.00	0.22	0.10	0.05

Table A2 Standard chemical exergies

Standard chemical exergy (MJ/kg)	
Water (liquid)	0.0499
Water (vapour)	0.5272
Protein	22.61
Oil	43.09
Carbohydrates	17.64
Mineral residue	0.04
Hexane	47.7
Hydrochloric acid (powder form)	2.3
Sodium hydroxide (powder form)	1.9

Appendix B

Particle size distribution (nm) of a filtered lupin protein isolate dispersion.



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Summary
Samenvatting

The growth in the world population requires an increase in the production of protein-rich foods from plant-based materials. Lupin seeds have potential to become a novel plant protein source for food products because they are rich in protein (about 37 wt%) and they can be grown in moderate temperature climates as in north-western Europe. Besides a high protein content, lupin seeds are rich in dietary fibres (soluble and insoluble), contain about 7-10 wt% oil, and are low in starch. To optimally use the proteins present in lupin, a fractionation process has to be developed. For other legumes, refining of protein is usually performed through wet fractionation techniques. However, wet fractionation techniques are resource-intensive and herewith the sustainability of increasing the use of plant-based materials for foods decreases.

The aim of this thesis is to obtain understanding of the production of functional, protein-rich material from lupin seeds with reduced environmental impact. In this thesis, it is shown that focus on functionality rather than (molecular) purity can lead to simplified fractionation processes, which is a concept referred to as functionality-driven fractionation. The influence of these simplifications on protein functionality and on physical and chemical stability of the protein isolates is explored. Furthermore, we performed a sustainability assessment of fractionation processes.

Conventionally, protein isolates are produced with wet fractionation processes, based on differences in solubility of the components of seeds. Conventional wet fractionation includes an oil extraction step, which requires the use of organic solvents, and repeated pH shifts, leading to chemical and water consumption. **Chapter 2** compares conventional wet fractionation and aqueous fractionation processing (being conventional wet fractionation in which the de-oiling step is excluded) to obtain lupin protein isolates (LPIs). The aqueous fractionation process led to LPIs with slightly altered composition, mainly due to the inclusion of 2 wt% oil. Nevertheless, the functionality of the aqueous fractionated LPI was similar to that of the conventional wet-fractionated LPI. The separation efficiency and protein solubility of the aqueous fractionated LPI were influenced by higher extraction temperatures. A post-process heat treatment was also effective in altering the LPIs protein solubility and water holding capacity, which is interesting for high-protein foods with different functionality requirements.

The gelling properties of the aqueous fractionated LPI were benchmarked against soy protein isolate (SPI) in **chapter 3**. Generally, SPI formed firm gels while LPI formed weak and deformable gels. Microscopic images and static light scattering measurements showed that both protein isolates form particle gels, but LPI particles swelled less upon heating

than SPI particles. Even prolonged heating with the aim to improve unfolding of lupin proteins, resulted in deformable gels. The difference in the gelling properties of LPI and SPI could be related to the higher sulfhydryl reactivity of LPI than of SPI and the increased sulfhydryl reactivity of LPI after heat treatment. The latter indicated that LPI particles were not able to form (new) disulphide bonds to strengthen the particle network. The opposite behaviour was observed for SPI, where the sulfhydryl reactivity decreased upon heating, indicating the formation of new disulphide bonds that strengthened the network. Even though LPI seems less useful for semi-solid food products (as a single ingredient in water), it offers opportunities for high-protein food products that require a low viscosity after heat treatment.

The environmental impact of various lupin fractionation processes is discussed in **chapter 4**. The fractionation processes that were included in the evaluation were dry fractionation, conventional wet fractionation, aqueous fractionation and combinations thereof. We performed a sustainability assessment with the use of mass, energy, and exergy balances. Exergy is a thermodynamic state variable that describes the potential work that can be performed with a specific stream. Indicators such as exergy efficiency and exergy losses were used to calculate and visualize inefficiencies in processes or the conversion of raw materials. Dry fractionation was found to be the most sustainable route to obtain protein-enriched flours. Conventional wet and aqueous fractionation processes were used to further increase the protein content of lupin flour or the protein-enriched flour. Wet fractionation required more energy and resulted in higher exergy losses than aqueous fractionation because of the de-oiling step. Both conventional wet and aqueous fractionation processes involve high water consumption and energy consumption for drying of the fractions, leading to high exergy losses. A route to reduce water consumption and exergy losses is combining dry and aqueous fractionation processes to obtain LPIs. The consumption of energy can be further reduced by concentrating the LPI to higher protein concentrations instead of drying. Finally, a key factor in improving the exergetic efficiency of all fractionation processes was to valorise all the by-products obtained, e.g. lupin oil, the soluble solids fractions, the fibre-rich pellet and the coarse fraction.

Replacing drying steps with concentration steps resulted in ‘wet’ LPI dispersions. The technical functionality of wet LPIs produced with aqueous fractionation was compared with the technical functionality of the freeze-dried LPIs. **Chapter 5** reports on the viscosity, solubility and swelling properties of wet and freeze-dried LPIs. Wet LPIs had a higher solubility than freeze-dried LPIs, which was attributed to protein aggregation leading to the

large protein particles found in the freeze-dried LPI as evidenced by microscopic images and static light scattering measurements. The viscosity and swelling capacity of wet LPI dispersions increased after heat treatment at 100°C while the viscosity and swelling capacity of the freeze-dried LPI dispersion remained similar. The freeze-drying process thus induced protein aggregation, creating heat-stable particles that did not swell extensively after heat treatment. Freeze-dried LPIs may be interesting for high-protein beverages that require low viscosity after heat treatment, while wet LPIs may be interesting for higher viscosity dairy alternatives.

The LPI prepared with aqueous fractionation contains about 2 wt% oil, with a high level of poly-unsaturated fatty acids, which may thus be prone to lipid oxidation and consequently protein oxidation. If LPIs are to be produced with simplified fractionation processes that omit the extraction of oil, the chemical stability of the LPIs needs to be assured. Lipid and protein oxidation marker levels were determined for fractions obtained with aqueous fractionation at two extraction temperatures (4°C and 20°C), as is described in **Chapter 6**. Primary and secondary lipid oxidation marker values and protein oxidation values were found to be below the acceptable values for both extraction temperatures. A heat treatment that was applied to reduce the activity of lipoxygenase, decreased oil and protein recovery and increased lipid oxidation marker values. Therefore, cooling of the process and an intermediate heat treatment are not necessary, herewith reducing the environmental impact of the aqueous fractionation process that can be performed at 20°C.

Chapter 7 concludes with a general discussion of all results presented in the thesis. It starts with summarizing the main findings, after which potentials and bottlenecks of the novel fractionation processes are discussed. The chapter ends with a future outlook on further scientific research on functionality-driven fractionation processes and possible applications.

The results presented in this thesis provide steps towards more sustainable production of functional fractions for food applications obtained with simplified fractionation processes. This work provides future perspectives for functionality-driven fractionation processes that may be extended to other legumes and pulses as well. This approach leads to the development of ingredients and fractions of seeds and legumes that can be used for plant-based food products.

De groeiende wereldbevolking vereist een toename in de productie van eiwitrijke levensmiddelen. Een groot gedeelte van de eiwitten in het humane dieet zijn afkomstig van dieren en zuivel, zoals vlees, kaas en melk. Voor de productie van dierlijk eiwit zijn veel meer water, grondstoffen en land nodig dan voor de productie van plantaardige eiwitten. Dit is niet efficiënt en daarom zouden er meer plantaardige eiwitten in het humane dieet moeten zitten. Een goede kandidaat voor een nieuwe plantaardige eiwitbron is lupine. De zaden van lupine zijn peulvruchten met een relatief hoog eiwitgehalte van 37g/100g. Lupinezaden bevatten ook oplosbare en onoplosbare vezels, 7-10g olie /100g, en nauwelijks zetmeel. Een extra voordeel is dat lupines kunnen groeien in een gematigd klimaat zoals in Noordwest Europa. Om de eiwitten uit lupinezaden goed te benutten, zijn er efficiënte fractioneringsprocessen nodig. Voor andere peulvruchten, zoals soja, worden de eiwitten vaak gefractioneerd met behulp van natte scheidingstechnieken. Deze natte scheidingstechnieken verbruiken veel chemicaliën, organische oplosmiddelen, water, en energie en hierdoor worden de milieuvoordelen van het gebruik van plantaardige materialen voor levensmiddelen teniet gedaan.

Het doel van het in dit proefschrift beschreven onderzoek is om functionele, eiwitrijke fracties uit lupinezaden te verkrijgen met processen die een lagere impact op het milieu hebben. In dit proefschrift wordt aangetoond dat focus op functionaliteit in plaats van moleculaire zuiverheid kan leiden tot simpelere fractioneringsprocessen, of functionaliteits-gedreven fractionering. De invloed van simpelere fractioneringsprocessen op eiwitfunctionaliteit en op fysische en chemische stabiliteit van de eiwitisolaten is onderzocht. Daarnaast hebben we een duurzaamheidsanalyse uitgevoerd voor de verschillende fractioneringsprocessen.

Normaliter worden eiwitisolaten geproduceerd met klassieke natte scheidingsprocessen die zijn gebaseerd op oplosbaarheidsverschillen van de componenten van zaden of bonen. Klassieke natte scheidingsprocessen voor oliehoudende zaden bestaan uit een olie-extractiestap, waarvoor een organische oplosmiddel nodig is, en herhaalde pH-verschuivingsstappen, waarvoor chemicaliën en water benodigd zijn. **Hoofdstuk 2** vergelijkt het klassieke natte scheidingsproces met een waterige scheidingsproces (gedefinieerd als de klassieke methode, maar zonder een olie-extractiestap) om lupine-eiwitisolaten te maken. Het waterige scheidingsproces resulteerde in eiwitisolaten met 2g olie /100g. De functionaliteit van deze eiwitisolaten was vergelijkbaar met die van het klassieke eiwitisolaat. De scheidingsefficiëntie en de oplosbaarheid van de eiwitisolaten werd beïnvloed door hogere extractietemperaturen. Een verhittingsstap als nabehandeling veranderde de oplosbaarheid en het waterbindend vermogen van de eiwitisolaten, waardoor de mogelijkheden voor

toepassing van dit lupine-eiwitisolaat in tal van levensmiddelen verder vergroot is.

De geleringseigenschappen van de eiwitisolaten verkregen met waterige scheiding werden vergeleken met die van soja-eiwitisolaten in **hoofdstuk 3**. Over het algemeen vormen soja-eiwitisolaten sterke gelen terwijl lupine-eiwitisolaten zwakke en vervormbare gelen vormen. Beide eiwitisolaten vormen deeltjesgelen, waarin de lupine-eiwitdeeltjes minder zwellen dan de soja-eiwitdeeltjes. Zelfs bij een langer durende verhittingsstap met als doel om de lupine-eiwitten meer te laten ontvouwen, resulteerde in vervormbare gels. Het verschil in geleringseigenschappen kon worden gerelateerd aan de hogere reactiviteit van de zwavelgroepen van lupine-eiwitisolaat in vergelijking met soja-eiwitisolaat; de activiteit van de zwavelgroepen van lupine-eiwitisolaat nam toe tijdens verhitting en dat van soja-eiwitisolaat nam af. Dit kan erop wijzen dat lupine-eiwitisolaat geen nieuwe zwavelbruggen kan vormen om het gelnetwerk te versterken, terwijl soja-eiwitisolaat wel nieuwe zwavelbruggen vormt om het gelnetwerk te versterken. Hierdoor lijkt lupine-eiwitisolaat minder geschikt voor semi-vaste levensmiddelenproducten (als enig ingrediënt in water), maar het biedt wel mogelijkheden voor eiwitrijke levensmiddelen waarbij een lage viscositeit na verhitting gewenst is.

De milieueffecten van verschillende fractioneringsprocessen voor lupine zaden is bediscussieerd in **hoofdstuk 4**. De geëvalueerde fractioneringsprocessen zijn: droog fractioneren, klassieke natte scheiding, waterige scheiding en combinaties hiervan. Een duurzaamheidsanalyse is uitgevoerd met behulp van massa-, energie- en exergiebalansen. Exergie is een thermodynamische toestandsvariabele die het potentiele werk dat een productstroom kan geven beschrijft. Indicatoren zoals de exergetische efficiëntie en het exergieverlies zijn gebruikt om inefficiënties in processen te berekenen en visualiseren. Droog fractioneren was de meest duurzame route om eiwit-verrijkte bloemen te verkrijgen. Klassieke natte en waterige scheidingsprocessen waren nodig om de eiwitconcentratie van lupine bloem of de eiwit-verrijkte bloem verder te verhogen. De olie-extractiestap in klassieke natte scheiding verhoogde het energieverbruik en resulteerde in hogere exergieverliezen dan waterige scheiding. Het watergebruik en de energieconsumptie van beide natte scheidingsmethoden zijn hoog, omdat de fracties na fractionering gedroogd moeten worden. Dit leidt tot hoge exergieverliezen. De waterconsumptie en exergieverliezen kunnen worden gereduceerd door droge en waterige scheidingsprocessen te combineren voor de productie van eiwitisolaten. De energieconsumptie kan verder worden verlaagd door de eiwitisolaten niet te drogen, maar te concentreren naar een eiwitconcentratie relevant voor eiwitrijke levensmiddelen. Tenslotte, bleek dat alle bijproducten van de processen moeten

worden gevaloriseerd, zoals de lupine olie, de oplosbare eiwitten en suikers die niet in het eiwitisolaat terechtkomen, het vezelrijke pellet en de grove fractie (verkregen door droge fractionering) om de exergetische efficiëntie van het scheidingsproces te optimaliseren.

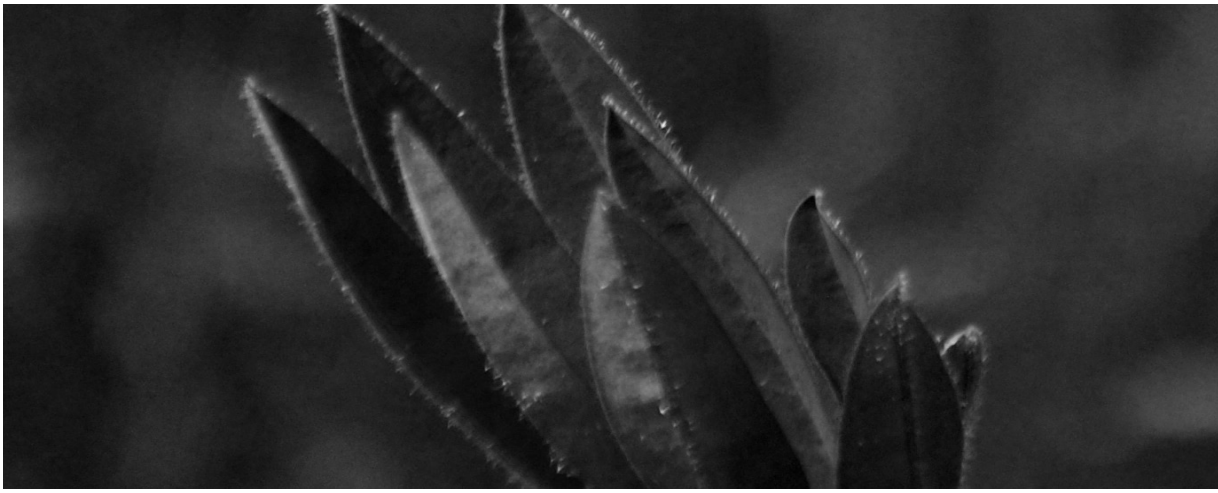
Het vervangen van een droogstap door een concentratiestap resulteert in een 'natte' eiwitisolaat-dispersie. De technische functionaliteit van de natte eiwitisolaten verkregen met waterige scheiding is vergeleken met die van gevriesdroogde eiwitisolaten. **Hoofdstuk 5** beschrijft de viscositeit, de oplosbaarheid en het zwellingsgedrag van de natte en gevriesdroogde eiwitisolaten. Natte eiwitisolaten hadden een hogere oplosbaarheid dan gevriesdroogde eiwitisolaten, wat was toegekend aan een verhoogde eiwitaggregatie door drogen dat resulteerde in grote eiwitdeeltjes in de gevriesdroogde eiwitisolaten. De viscositeit en het zwelgedrag van natte eiwitdispersies nam toe na verhitting bij 100°C terwijl de viscositeit en het zwelgedrag van gevriesdroogde eiwitisolaten gelijk bleef na verhitting. Het vriesdrogen veroorzaakte dus eiwitaggregatie, wat resulteerde in hittestabiele eiwitdeeltjes die niet veel zwollen bij verhitting. Gevriesdroogde eiwitisolaten kunnen interessant zijn voor eiwitrijke drankjes die een lage viscositeit na verhitting dienen te hebben, terwijl natte eiwitisolaten interessant zijn voor zuivelalternatieven waarbij een hogere viscositeit nodig is .

Het met waterige scheiding verkregen eiwitisolaat bevat ongeveer 2g olie /100g. De olie bevat een hoge concentratie meervoudig onverzadigde vetzuren, die de olie gevoelig maakt voor olieoxidatie en verder eiwitoxidatie kan initiëren. Echter voor toepassing dient de chemische stabiliteit van de eiwitisolaten gegarandeerd kunnen worden. Daarom zijn de olie- en eiwitoxidatiemarkers voor de fracties verkregen met waterige scheiding op twee extractietemperaturen (4°C en 20°C) bepaald in **hoofdstuk 6**. Primaire en secundaire olie-oxidatiemarker waarden en eiwitoxidatiemarker waarden waren voor beide extractietemperaturen binnen de toegestane waarden. De toepassing van een verhittingsstap om de activiteit van het van nature in lupinezaden aanwezige enzym lipoxygenase te reduceren verlaagde de olie- en eiwitopbrengst en verhoogde de olie-oxidatiemarker waarden. Daarom lijken een gekoeld proces (4°C) en een verhittingsstap niet nodig voor het verkrijgen van stabiele eiwitisolaten met behulp van waterige scheiding. Hiermee worden de milieueffecten van een waterig fractioneringsproces verder verlaagd.

Hoofdstuk 7 sluit af met een algemene discussie over de in dit proefschrift gepresenteerde resultaten. Het begint met het samenvatten van de belangrijkste resultaten, waarna de mogelijkheden en knelpunten van de nieuwe fractioneringsprocessen worden bediscussieerd. Het hoofdstuk eindigt met een toekomstvisie voor verder wetenschappelijk

onderzoek naar functionaliteits-gedreven fractioneringsprocessen en mogelijke applicaties.

De resultaten van dit proefschrift verschaffen inzichten voor duurzamere productie van functionele fracties voor levensmiddelen verkregen met versimpelde fractioneringsprocessen. Dit werk geeft toekomstperspectieven voor functionaliteits-gedreven fractioneringsprocessen die mogelijk kunnen worden uitgebreid naar andere peulvruchten. Op deze wijze kunnen ingrediënten of fracties van zaden en peulvruchten worden ontwikkeld die een vervanging van dierlijke componenten door plantaardige alternatieven verder mogelijk maakt.



Acknowledgements
About the author
List of publications
Overview of completed training activities

Abstract

Tadaa! Four years of work written down. Actually, this thesis is only a part of all the work. It does not include all the research performed during those four years, it does not include the television performances, and it does not cover all of the people that directly or indirectly contributed to this work. Here, the importance of having great people around you is emphasized.

Introduction

During a PhD trajectory, you learn about yourself, you are handed tools to develop yourself, you learn to become an independent researcher, and you learn to not do everything by yourself. This requires people around you that support you, coach you, advise you, people you look up to, people that remind you there is more to life than work, and people that simply make you feel happy. This part of the thesis focuses on the ingredients that were needed to successfully perform my research, write my thesis (ok yes, it is my writing), and made me smile and laugh.

Materials and methods
(not in chronological order)

My supervisors: Atze Jan van der Goot and Remko Boom. The technicians: Jos, Maurice, Jarno, and Martin (and Harry Baptist). The APPI-team (Kasia, Pascalle, George, Maarten and Atze Jan), ISPT, the peas foundation, de vegetarische slager, the co-authors of the papers in this thesis (Paul, Claire, Costas), my Bachelor and Master thesis students (Robbin, Esther, Stefano, Steven, Marlous, Evelien, Cynthia, Suzanne). The opponents who approved my thesis. All FPE-colleagues. Karin Schroën, Lena, Francisco, my former housemate, colleague and friend Nicolas, Marta, Angélica, Jue, Laura, Ekaraj, Sami, Jorien, Rianne, Filippos. The BPE-colleagues, especially Lenneke, Anne, Lenny, Jeroen, Guido, Ward, Kim. My Food Technology friends (Marieke, Thomas, Milou, Nicole, Pascalle, Jacob, Ids), my former housemates (Vera, Marjolein, Leo, Lies, Kwallie aka Pascalle), Karsten, Karin, my dance-colleagues, my family and last but definitely not least Jurian!

Results and discussion

Motivation, hard work, inspiring meetings, crazy ideas (that turned out just fine and lead to new results), good ideas, more work, new perspectives, nice collaborations, dancing, discussing research (especially in lekentaal), good food!, coffee, more coffee, lovely drinks, fun borrels, and lots of laughter! Oh and a written thesis of course.

Conclusion

A PhD-thesis requires many people and above all, people that inspire you, motivate you, and laugh with you. Thank you all!

Recommendations

Uit onderzoek is gebleken dat onderzoeken veel leuker is dan antwoorden vinden - Loesje

Als de moed je in de schoenen is gezakt, ga dan eens op je kop staan - Loesje

About the author

Jacqueline Alida Maria Berghout was born in Alkmaar, The Netherlands, on May 8 1987. She went to GSg Schagen, where she obtained her VWO diploma in 2005, with a major in Natuur en Gezondheid (Nature and Health).

In 2005, she started her study Food Technology at Wageningen University. In her minor thesis project at the laboratory of Food Quality and Design of Wageningen University she worked on the effect of domestic cooking methods on bioactive compounds in broccoli and carrot. Jacqueline completed her major thesis at the Food Process Engineering group of Wageningen University, working on the visualisation of gluten aggregation in the shear-induced separation of wheat flour. During her internship at the Bioprocess department of CSIRO in Werribee, Australia, she worked on the development of stimuli-responsive resins for molecule separation.

After completing her MSc studies, she continued working as a PhD at the Food Process Engineering group of Wageningen University on the project *Functionality-driven fractionation of lupin seeds*.

List of publications

Van der Zalm, E. E. J., **Berghout, J. A. M.**, van der Goot, A. J., & Boom, R. M. (2012). Starch–gluten separation by shearing: Influence of the device geometry. Chemical Engineering Science, 73, 421–430.

Berghout, J. A. M., Boom, R. M., & van der Goot, A. J. (2014). The potential of aqueous fractionation of lupin seeds for high-protein foods. Food Chemistry, 159, 64–70.

Pelgrom, P. J. M., **Berghout, J. A. M.**, van der Goot, A. J., Boom, R. M., & Schutyser, M. A. I. (2014). Preparation of functional lupine protein fractions by dry separation. LWT - Food Science and Technology, 1–9.

Berghout, J. A. M., Boom, R. M., & van der Goot, A. J. (2015). Understanding the differences in gelling properties between lupin protein isolate and soy protein isolate. Food Hydrocolloids, 43, 465–472.

Berghout, J. A. M., Pelgrom, P. J. M., Schutyser, M. A. I., Boom, R. M., & van der Goot, A. J. (2014). Sustainability assessment of oilseed fractionation processes: a case study on lupin seeds. Journal of Food Engineering, 150, 117-124.

van der Goot, AJ., Pelgrom, P.J.M., **Berghout, J.A.M.**, Geerts, M., Jankowiak, L., Hardt, N.A., Schutyser, M.A.I., Nikiforidis. C.V., Boom, R.M., Concepts for further sustainable production of foods. Submitted for publication.

Berghout, J.A.M., Venema, P., Boom, R.M., & van der Goot, A.J., Freeze-drying induces aggregation in lupin protein isolates. Submitted for publication.

Berghout, J.A.M., Marmolejo-Garcia, C., Berton-Carabin, C.C., Nikiforidis, C.V., Boom, R.M., & van der Goot, A.J., Aqueous fractionation yields chemically stable lupin protein isolates. Submitted for publication.

Overview of completed training activities

Discipline specific activities

Courses

Sensory Perception and Food Preference in Wageningen (NL) 2011

Sustainability Analysis in Food Production in Wageningen (NL) 2011

Food and Biorefinery Enzymology in Wageningen (NL) 2011[a]

Biorefinery for Biomolecules in Wageningen (NL) 2012

Food Structure and Rheology in Wageningen (NL) 2012

Industrial Proteins in Wageningen (NL) 2013[a]

Conferences

NPS (Annual Dutch Process Technology symposium) in Papendal (NL) 2011[a]

NPS in Utrecht (NL) 2014[a]

International Symposium on Food Rheology and Structure in Zurich (CH) 2012[a]

FoodBalt 2012 in Kaunas (LT) 2012

EFFoST in Montpellier (FR) 2012[b]

ECCE in Den Haag (NL) 2013[b]

General courses

VLAG PhD Week in Baarlo (NL) 2011

Teaching and Supervising Thesis Students in Wageningen (NL) 2011

Effective Behaviour in your Professional Surroundings in Wageningen (NL) 2012

Competence Assessment in Wageningen (NL) 2013

Scientific Writing in Wageningen (NL) 2013

Techniques for Writing and Presenting a Scientific Paper in Wageningen (NL) 2014

Career Orientation in Wageningen (NL) 2014

Optionals

ISPT team meetings in Amersfoort, Delft or Wageningen (NL) 2011-2014[b]

Working in Projects (by ISPT) in Amersfoort (NL) 2011

Workshop Visuals in Wageningen (NL) 2011

Scientific PhD excursion to the Baltic States and Finland in 2012[a,b]

Scientific PhD excursion to Chile and Brazil in 2014[b]

Food Process Engineering Brainstorm Days in Wageningen (NL) 2011-2014[b]

[a] *Poster*

[b] *Presentation*

The work presented in this thesis has been carried out in the framework of the Institute for Sustainable Process Technology (ISPT) under the project “PI-00-03 IPS: Intensified Protein Structuring for More Sustainable Food.

This thesis was printed by Ridderprint, Ridderkerk, The Netherlands.

Edition: 200 copies

Photos on the cover and chapter pages were kindly provided by Karin Schröen.