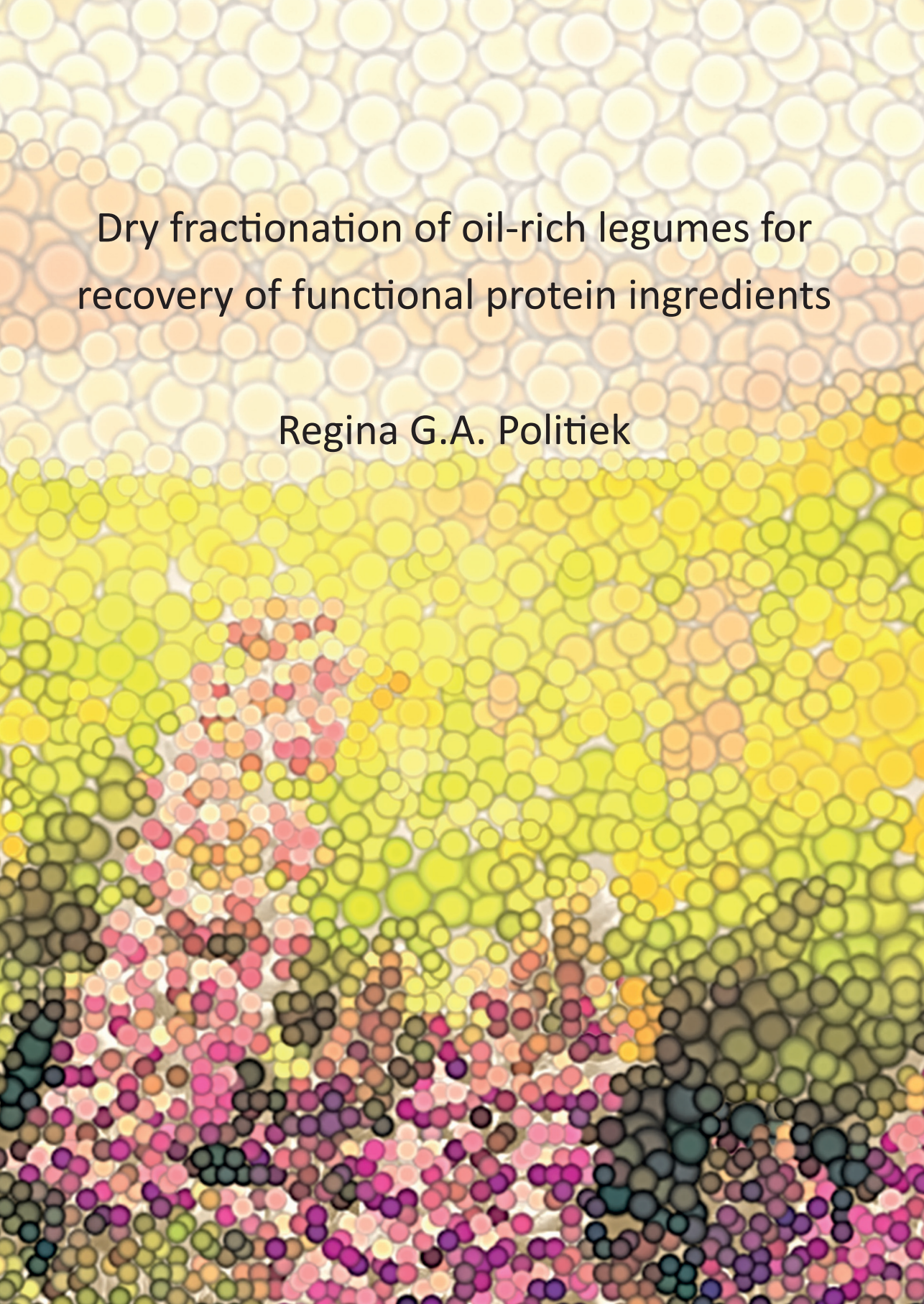
Dry fractionation of oil-rich legumes for recovery of functional protein ingredients

Regina G.A. Politiek



Propositions

1. Removal of oil from seeds enables dry fractionation processes. (this thesis)
2. Powder flowability analysis predicts air classification successfully. (this thesis)
3. Knowing your audience is key for good knowledge transfer.
4. Publishing is the basis for scientific progress.
5. Having a personal workplace at the university improves the work and life balance of a PhD candidate.
6. Science requires volleyball skills; anticipating, aligning and finding the gap to score.

Propositions belonging to the thesis, entitled

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Thesis

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Content

Chapter 1

Introduction

1.1 Sustainable plant-based protein ingredient and food production

Protein is a major nutrient that provides essential amino acids and is found in human diets, for example in whole foods or formulated food products that contain high protein ingredients (Day et al., 2022). These protein ingredients can be produced from animal-based sources such as milk or from plant-based sources like wheat, soy or pea (Day et al., 2022; Lie-Piang et al., 2021). The market and popularity of proteins is expected to grow rapidly in the next decade, as protein is seen as a favourable macronutrient by the consumer. Consumers tend to avoid lipids as a macronutrient, as these have a high calory density and carbohydrates are seen negatively, because sugars can affect the glycaemic index. However, the increased production of food protein, especially high-quality animal protein, presents sustainability challenges as for 1 kg of animal protein 6 kg of plant protein is necessary, which increases the overall pressure on the land and water use (Hertzler et al., 2020). The wider use of plant proteins in the human diet is more sustainable and affordable than an animal-based diet (Onyango, 2022).

Grain legumes such as soybeans, peas, chickpeas and lentils have been part of the human diet for a long time and are an excellent source of protein, among others due to their relatively high protein content and low costs. A unique property of grain legumes is their ability to fixate nitrogen from the atmosphere thanks to the presence of symbiotic nitrogen-fixing bacteria in their root nodules (Onyango, 2022). Nitrogen-fixing legumes are attractive for cultivation as they reduce the need for synthetic fertilizer on the land (Foyer et al., 2016). Legumes may be divided into two types, which are starch-rich legumes (i.e. yellow pea), also referred to pulses, and oil-rich legumes (i.e. soybean). Protein ingredients from these legumes can be divided into three main groups based on the degree of refinement of the ingredients, which are flours, concentrates and isolates. Figure 1.1 represents an example of the production of soy flours, soy protein concentrates and soy protein isolates (Singh et al., 2008). Here, the flours are least refined and isolates are the most refined ingredients with a protein content above 90% per dry matter (Singh et al., 2008). Many recent developed plant-based food products contain high purity protein isolates. (Lie-Piang et al., 2023). However, the production of protein isolates coincides with the use of copious amounts of water and the subsequent use for energy to remove the water again by evaporation during drying, which has a high impact on the sustainability of the produced ingredients (Schutyser et al., 2015).

A more sustainable alternative to produce functional protein-enriched ingredients from legumes is dry fractionation (Lie-Piang et al., 2022). Dry techniques are expected to have

great potential to be commercially applied, given the absence of external heating, co-extraction of carbohydrate fractions and lower energy consumption than conventional wet extraction techniques (Pojić et al., 2018). The main contributor to the lower energy consumption for dry fractionation is that no water is consumed, whereas water must be removed by spray drying upon conventional fractionation (Figure 1.1). To illustrate, the protein delivery efficiency of dry fractionation of pea is 55.8 g protein per MJ, while for wet fractionation of pea this is only 14.6 g protein per MJ (Schutyser et al., 2015).

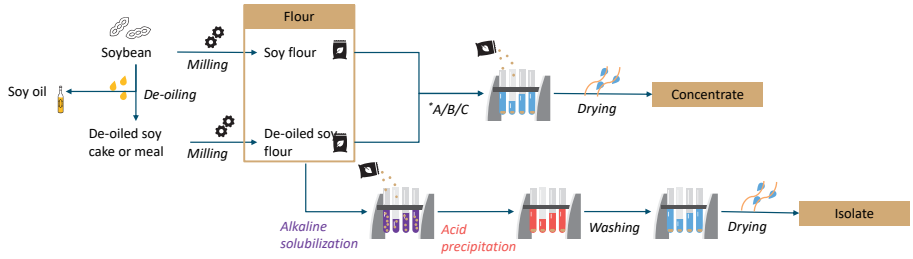

*Figure 1.1: Illustration of current processing scheme to produce soy flours, soy concentrates or soy isolates. *For concentrate production one of the following processes is applied: Aqueous alcohol washing (A), Acid leach at pH 4.5 (B) or moist heat and water washing (C).*

1.2 Development of dry fractionation technologies

Dry fractionation consists of a milling and a separation step to produce protein, starch or fibre enriched ingredients (Assatory et al., 2019). Milling is used to mechanically release different cellular components from legume cotyledon cells. Cotyledon cells of starch rich legumes consist of starch granules and protein bodies, which are surrounded by a fibre-rich cell wall, whereas cotyledon cells of oil-rich legumes consist of oil bodies and protein bodies surrounded by a fibrous matrix. Upon milling legumes, different cellular constituents are released after which they can be separated based on differences in properties. The release of cellular components via milling is necessary to enable consecutive dry separation.

Examples of dry separation techniques are sieving, air classification and electrostatic separation. Figure 1.2A illustrates air classification, where fine milled particles are separated based on a difference in size, density and shape into a fine fraction and a coarse fraction. The classifier wheel speed and airflow rate upon air classification result in a centrifugal force and a drag force, which determine the cut point for separation (Bauder et al., 2004; Guo et al., 2007). Especially air classification is used for starch rich legumes to separate the larger starch granules (~20 µm) from smaller protein bodies (~1-

10 µm), where the size of these cellular components is specific for each material (Schutyser & van der Goot, 2011).

Electrostatic separation is a newer dry separation technique for food materials to enrich components such as protein and fibre from finely milled legume flours (Vitelli et al., 2020). Upon electrostatic separation, the fine milled flour is dispersed in air and the individual particles are charged by particle-particle interactions and particle-wall interactions. Upon these interactions, electrons are exchanged between charge donating and charge accepting particles, which results in respectively positively and negatively charged particles (Figure 1.2C). These charged particles are then subjected to an electric field, which drives the separation of positively and negatively charged particles and results in a protein enriched and a carbohydrate enriched fraction (Figure 1.2B).

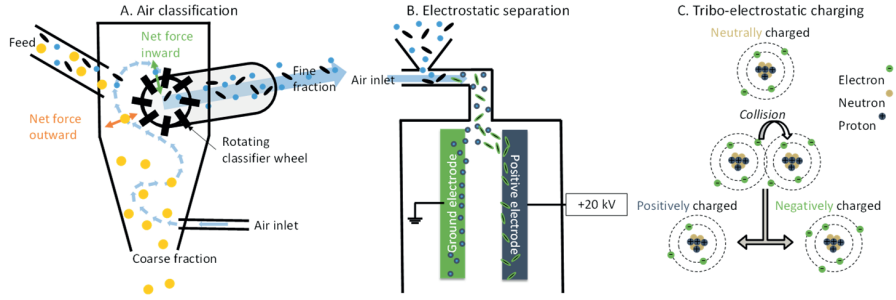

Figure 1.2: Visualisation of air classification (A), electrostatic separation (B), and tribo-electric charging by electron transfer (C).

The charging of particles and subsequent electrostatic separation behaviour is very much composition-dependent, where the presence of a large amount of starch granules can even impair the protein enrichment, as these can obtain similar charges (Pelgrom et al., 2015c; Xing et al., 2020). To overcome this, air classification is used as a first purification step to remove the starch from starch rich legumes, such as yellow pea, prior to electrostatic separation (Pelgrom et al., 2015c; Xing et al., 2020; H.G. Zhu et al., 2021b). In general, dry fractionation of crops with a higher oil content is more challenging than for starch-rich seeds due to the presence of oil, which can result in particle agglomeration (Pojić et al., 2018). Next to that, the absence of starch granules requires that protein bodies need to be separated from fibres, which then should have a large enough difference in size and/or density. For these reasons, air classification is used for lupin and chickpea but not for soy.

1.3 Challenges for dry separation

The production of protein-enriched ingredients via dry fractionation requires a good milling process to produce a finely milled flour that is air dispersible. Next to that, milling should disentangle the cotyledon cells into cellular fragments with different compositions and distinct physical properties such as size or tribo-electric charging (Schutyser & van der Goot, 2011). Protein-enriched ingredients have been produced from starch rich legumes with less than 3% oil, like pea, lentil and navy bean and from legumes with intermediate oil contents (3-10%) like lupin or oat (Pelgrom et al., 2015b; Sibakov et al., 2014; Tabtabaei et al., 2016a; J. Wang et al., 2016b; Xing et al., 2020; H.G. Zhu et al., 2021b). However, legumes with more than 10% oil are expected to be more difficult to mill to a fine particle size, as oil might be released from the cells upon milling, which potentially results in a lower dispersibility and agglomeration of the finely milled particles (chapter 2) and therewith impair further dry separation, such as air classification. Ideally, the processing performance would not vary with changes in environmental conditions upon storage or processing. However, next to oil, humidity might also affect the air classification performance by agglomeration of particles (Armstrong et al., 2014; Dijkink et al., 2007). As both oil and humidity can result in particle agglomeration, we hypothesise that crops with a higher oil content might be affected differently by the relative humidity upon processing (chapter 3).

To overcome existing drawbacks for air classification of oil-rich material research proposed to use electrostatic separation instead of air classification (Pojić et al., 2018). However, oil might also act as an insulator upon electrostatic charging (chapter 4), which can result in insufficient charging to collect the different cellular components, and there with insufficient separation. It is expected that measuring the charge of the material that is deposited on the electrodes helps to evaluate whether the presence of oil indeed results in insufficient charging and therewith gain additional insight in the overall process (Yang, Meda, et al., 2022).

De-oiling is usually applied as a pre-treatment for oil-rich legumes with more than 10% oil, such as soy, rapeseed and sunflower seed. Examples of electrostatically separated legumes with different oil contents are provided in Table 1.1. So far, studies on de-oiling and subsequent electrostatic separation focussed on mechanical de-oiling and solvent de-oiling with petroleum ether or hexane (Table 1.1). However, it is unclear based on the available literature whether the use of a different solvent, or de-hulling could improve

the separation of for example soy and how the oil content affects the charging behaviour of the materials and final functionality of the produced ingredients (chapters 4, 5 and 7).

Table 1.1: Protein enrichment by electrostatic separation of starch-rich crops (upper part) and oil-rich crops (bottom part). Reprinted from Politiek et al. (2023a).

Material	**De-oiling method**	**Oil content [g/100g]**	**Protein content flour [g/100g dry solids]**	**Protein content after separation [g/100 g dry solids]**		**Source**
				Reported	*Corrected to N-factor 5.7*	
Yellow pea[1]	None	1.5	59.7	68.5	*62.5*	(H.G. Zhu et al., 2021b)
Yellow pea[1]	None	1	57.1	67.6	*61.7*	(Xing et al., 2020)
Lentil[1]	None	1	57.8	60.4	*55.1*	(Xing et al., 2020)
Chickpea[1]	None	6	33.8	33.3	*30.4*	(Xing et al., 2020)
Navy bean	None	2.5	25.4	47	*42.9*	(Tabtabaei et al., 2016a)
Lupin	None	N/A	40.5	57.3	*52.3*	(J. Wang, et al., 2016b)
Lupin[2]	None	N/A	51.4	65	*59.3*	(J. Wang, et al., 2016b)
Lupin	None	7.3	35.1	59.3	*54.1*	(Pelgrom et al., 2015c)
Lupin	None	5.6	37.2	65	*59.3*	(Xing et al., 2021)
Soy	Cold pressing	7.4	44.2	48.1	*48.0*	(Xing et al., 2018)
Soy	Petroleum ether (6h)	8.2	45	51.5	*51.4*	(Xing et al., 2018)
Soy	Hexane	3.3	55.3	58.4	*53.3*	(Kuspangaliyeva et al., 2023)
Rapeseed	Cold pressed and dehulled	3.7	37	51	*46.5*	(Basset et al., 2016)
Rapeseed	Cold pressed and dehulled	N/A	35.8	43.8	*N/A*[4]	(Kdidi et al., 2019)
Rapeseed	Cold pressing + hexane	1.7	36.7	59.1	*53.9*	(Laguna et al., 2018)
Sunflower	Solvent or mechanical extraction	N/A	30.8[3]	48.9[3]	*N/A*[4]	(Barakat et al., 2015)
Sunflower	Cold flaked + hexane	2	31.3	61.1	*55.7*	(Laguna et al., 2018)

[1]Fine fraction obtained after air classification subjected to electrostatic separation [2]Recycled fractions for electrostatic separation [3]In % w/w [4]Protein factor was not specified

1.4 Industrial value of enabling dry separation of oil-rich legumes

Even though dry separation of oil-rich legumes might be challenging, it is economically and societally relevant to study these legumes. Currently, the oil seed industry is economically driven by extraction and selling of the oil from the seeds (UDSA, 2023). To illustrate, oilseeds like soybean, rapeseed and sunflower seed cost between 572 – 624 U.S. dollars per metric ton, while the oil is sold for 2-3 times as much (1317 – 1728 U.S. dollars per metric ton) (UDSA, 2023). The production of legume based oil results in the co-production of press-cakes and meals, which are still highly nutritious but have a lower current market value (214 – 510 U.S. dollars per metric ton) (Figure 1.3). Due to a lack of cost-effective and sustainable food processing system, only 2-3% of the cakes and meals are used in food, the other part is used for animal feed purposes, industrial purposes, or in the worst scenario even deposited as waste (Singh & Krishnaswamy, 2022). Dry fractionation of oil-rich legumes and their co-products would enable a cost-effective and sustainable system to produce food ingredients with high nutritional value.

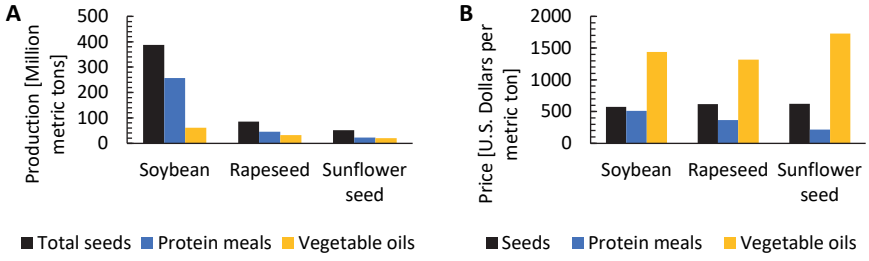

Figure 1.3: Illustration of production (A) and price (B) of soybean, rapeseed and sunflower seed and the protein meals and vegetable oils (UDSA, 2023).

1.5 Objective and thesis outline

The overall objective of this PhD project was to develop knowledge-based processing guidelines for dry fractionation of seeds towards functional protein ingredients for food. The presence of oil or the relative humidity was hypothesized to alter the flour properties in terms of for example particle size, dispersibility, flowability and charging behaviour. Different pre-treatments were studied to alter the composition of the raw materials and understand the influence on flour properties and related dry fractionation performance (purity and yield) during milling and dry separation. We also evaluated the functionality of dry enriched protein fractions to understand how the functionality was affected by applied pre-treatments and dry fractionation. Finally, we reviewed the potential of dry fractionation for completely different raw materials such as animal by-products and

insects and discussed guidelines for dry fractionation of seeds. Figure 1.4 provides an overall chapter overview.

Influence of pre-processing on milling: **Chapter 2** includes a systematic study of the effect of oil content on pin-milling of soybean in terms of milling yield, particle size, energy use and flour dispersibility. It was possible to describe particle size reduction during milling with an adapted Bond's model and milling efficiency could be related to oil content and powder dispersibility.

Influence of pre-conditioning on separation behaviour: **Chapter 3** evaluates the effect of relative humidity on milling, air classification performance and powder properties. Milling and air classification were not affected by an environmental humidity between 30-70%. Storage of the fine milled flours at extremer humidity (70% and 90%) resulted in a reduced air classification performance, which was linked to particle agglomeration, a lower dispersibility and a lower powder flowability. Yellow pea and chickpea were affected differently by the humidity, caused by the difference in oil content.

Pre-processing (de-oiling and re-milling) related to separation efficiency: In **chapter 4** soy and lupin were de-oiled with three different solvents and subjected to electrostatic separation. Electrostatic separation of lupin resulted in higher protein purities than electrostatic separation of soy, caused by the difference in embedment of the protein bodies in the structure. Re-milling after de-oiling was crucial to reach higher protein purities and the use of a different solvent affected the separation efficiency differently for soy and lupin.

Separation efficiency related to functionality: **Chapter 5** evaluates how the functional properties of soy and lupin protein enriched fractions were affected by solvent de-oiling and tribo-electrostatic separation. The protein-enriched fractions had equal or better functionality over the flours, except for emulsification of ethanol de-oiled lupin. No de-oiling was found to be a favourable pre-treatment for emulsion-based applications, while de-oiling resulted in better foaming properties.

Translation to other biomaterials: In **chapter 6** dry fractionation of completely different raw materials is reviewed, including animal by-products and insects. Animal by-products and insects are suitable for dry fractionation. Dry fractionation of animal by-products could be further enhanced by optimising the milling and separation conditions while taking into account the specific material properties, and for insects it is best to focus on the optimisation of the overall processing chain.

Chapter 7 reflects on the findings of all previous chapters. The main findings are summarized and design rules for dry fractionation of oil-rich crops are presented. Suggestions for future development are provided.

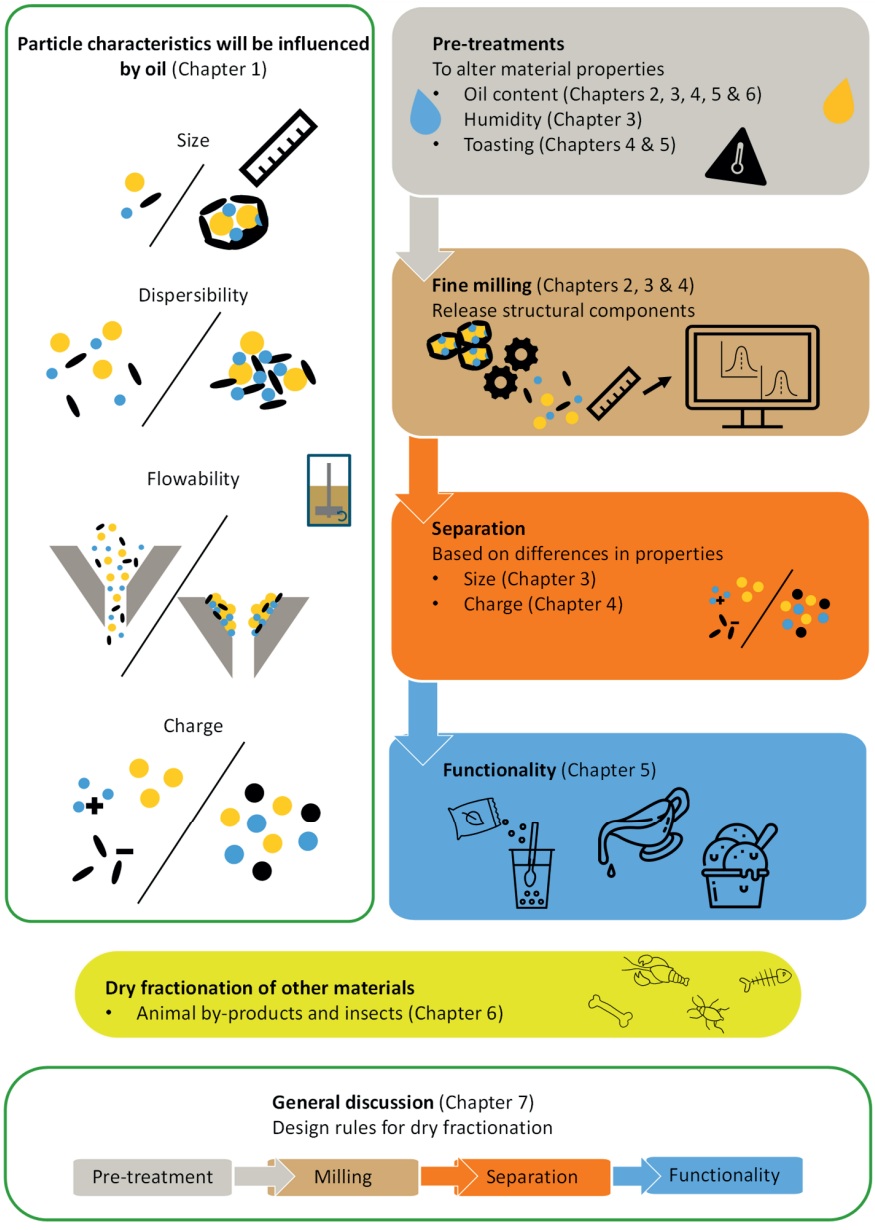

Figure 1.4: Chapter overview of this thesis.

Chapter 2

Effect of oil content on pin-milling of soybean

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Abstract

Milling is a critical step to prepare plant-based food ingredients by dry fractionation. It should provide a dispersible flour of finely milled particles composed of different cellular substructures. Especially, for raw materials with higher oil content such as soybean, this is challenging. We present an investigation on the effect of oil content on milling yield, particle size, energy use and flour dispersibility upon pin-milling of soybean. Soybean (20 g oil/100 g dry solids) and mechanically de-oiled soybeans (9-17 g oil/100 g dry solids) were subjected to pin-milling experiments. Increasing soybean oil content limited milling to smaller particles and lowered the overall milling yield. Particle size reduction can be described with an adapted Bond's model with oil content as an input parameter. The produced soy flours were well dispersible.

2.1 Introduction

The market and popularity of plant proteins is growing rapidly due to an increased awareness of consumers of the health benefits and sustainability of a more plant-based diet (Hertzler et al., 2020). Common plant-based protein sources are grains, pulses and oilseeds (Tabtabaei et al., 2016a). For many food applications it is desired to work with plant protein concentrates or isolates, which can be achieved by wet or dry fractionation. Prior to both processes, usually milling is applied to increase the protein extractability of the raw material. During wet fractionation milling would likely follow oil extraction, alkaline/acid extraction, centrifugation, pH precipitation or ultrafiltration and lastly drying (Chéreau et al., 2016; Schutyser et al., 2015). For dry fractionation, milling is followed by a dry separation step, for example sieving, air classification or electrostatic separation (Schutyser et al., 2015). Milling prior to dry fractionation is a critical step, as it enables liberation of cell structures of different composition into particles of different size, shape and density to aid air classification and/or liberate particles with different tribo-electrostatic charging properties to aid electrostatic separation. Unfortunately, milling of oilseeds such as soybean is very challenging in contrast to starch containing legumes due to the presence of oil. The focus of this study is on the milling behaviour of oilseeds, in particular soybean, which is an oilseed that is also extremely rich in protein (Chéreau et al., 2016).

Native soybean seeds have a particular morphological structure (Figure 2.1), which to great extent determines disclosure of cell components into individual particles during dry milling. Crude soybean seeds consist of two main parts, the hull (seed coat or testa) and the inner cotyledon matrix (Figure 2.1A and 2.1B). Soy hulls are a rich source of carbohydrates (86 g/100 g dry hull) (Medic et al., 2014). Soy cotyledon cells have an average width of 30-50 micron and a length of 70-80 micron, in which protein bodies (8-10 micron) and oil bodies (0.2-2 micron) are embedded as illustrated in Figure 2.1C and 2.1D (Campbell & Glatz, 2009; Rosenthal et al., 1998). During milling, these intact cotyledon cells need to be disrupted to enable protein extraction by either wet or dry fractionation. The protein and oil yields during subsequent separation are found closely related to the low presence of intact cells (Campbell & Glatz, 2009; Russin et al., 2007). Material such as soybean intended for fractionation is usually dehulled prior to milling. In our experiments we use hulled soybeans, as cold-pressing (40 °C) of hulled seeds has resulted in higher oil extraction yields than for de-hulled seeds and a dry separation method like sieving or electrostatic separation can separate the fibrous hull constituents (Koubaa, Mhemdi, & Vorobiev, 2016; J. Wang, Suo, De Wit, Boom, & Schutyser, 2016a).

The presence of hull pieces might cause a scouring effect during milling, especially close to the pins, which could result in less sticking of the flour. However, the presence of the hulls may also negatively affect the milling efficiency and thus result in slightly larger particle sizes (do Carmo et al., 2020). Dehulling will increase the protein content in the starting raw material for dry fractionation but has been found not to influence the protein-enrichment during air classification (do Carmo et al., 2020).

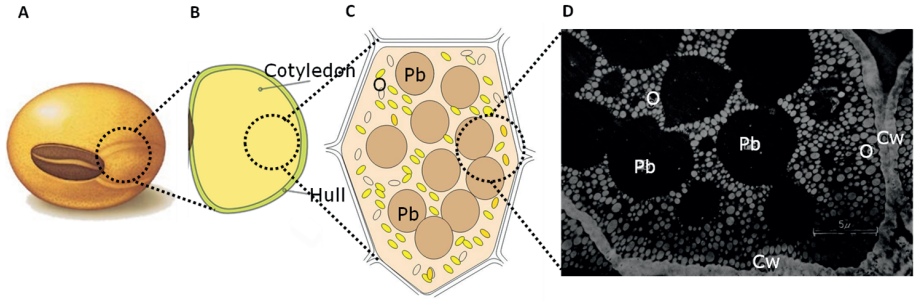

Figure 2.1: Schematic drawing of soybean seed parts: the whole seed (A), the soy cotyledon and hull, (B), a schematic representation of a cotyledon cell (C) and an electron micrograph of soybean cotyledon cells (D). PB indicates protein bodies, CW cell wall and O oil bodies (spherosomes), adopted from Medic et al. (2014) *and Saio and Watanabe* (1968).

Too fine milling can impair the subsequent separation for dry fractionation as was for example found for flours of lupin and de-oiled soy bean (Pelgrom et al., 2014; Xing et al., 2018). If flours contain very small particles, large van der Waals forces between particles contribute to poor dispersibility and thus poor dry separation (Pelgrom et al., 2014). In addition, for oil-rich crops the presence of oil can induce cohesive forces between powder particles via bridging (Pelgrom et al., 2014; Xing et al., 2018). To prevent particle cohesion through oil bridging, often de-oiling, also referred to as defatting, is applied prior to milling (Basset et al., 2016; Chéreau et al., 2016; Laguna et al., 2018; Pelgrom et al., 2014; Xing et al., 2018). Furthermore, oil removal can provide increased oxidative stability of the material, and soybean oil is an important resource by itself (Berghout et al., 2015a). However, the current trend goes towards less processed materials, using the whole flour to reduce material loss and energy use (Berghout et al., 2015a). Partial de-oiling is an interesting approach to retain the relatively stable oil bodies in the final product, as was observed for cold-pressed (<45°C) sunflower cake (Karefyllakis et al., 2019). Partial de-oiling will decrease the extracted oil yield and the remaining oil might still cause oxidation in the flour. However, the presence of oil bodies can be beneficial for the functionality of a product (Berghout et al., 2014, 2015a). For example, the presence of oil bodies in ingredients for meat analogue production did not result in oil

leakage, while addition of oil afterwards did (Peng, 2021). Therefore, it is desirable and relevant to study milling behaviour of both partially de-oiled soy and whole soybeans.

The aim of the current study is to systematically investigate the effect of soy oil content on pin-milling behaviour and flour properties like particle size distribution and flour dispersibility. We specifically investigate how oil content influences powder flow and milling behaviour. Hitherto, the majority of milling studies in scientific literature focussed on milling of inorganic materials and a minority on milling of food materials (i.e. rice grain, dried coriander seeds and soy with different moisture contents) (Lee et al., 2013; Loubes et al., 2022; Shashidhar et al., 2013).

Differences in initial oil content were achieved by mechanical de-oiling of soy and their effect was investigated on fine milling. We investigated particle size reduction of both soy and (partially) de-oiled soy by impact milling with a pin-mill at different rotation speeds. The pin-mill was chosen for practical reasons: easiness to clean, less prone to clogging compared to a classifier impact mill, simultaneous particle size reduction of both the hull and cotyledon to a similar particle size (Maskus et al., 2016), and no recirculation of material through the mill by a classifier wheel. Bond's empirical model was used to describe and compare the grinding kinetics of soy and de-oiled soy. It should be noted that Bond's model is a basic empirical model based on one specific size modulus, which is sufficient for the purpose of this study, but for scale-up procedures more advanced population balance models are recommended (Herbst & Fuerstenau, 1980). We determined flour yield and dispersibility after milling. The results were extensively discussed in relation to their influence on dry separation.

2.2 Materials and methods

2.2.1 Materials

Dry Canadian hulled soybeans (Glycine max, Batch 20-037) were obtained from Frank Food Products (Twello, The Netherlands). The soybeans were stored in vessels closed with a screw cap at 4 °C. Petroleum Ether with a boiling range between 40 and 65 °C was obtained from Avantor Performance Materials B.V. (J.T. Baker, Deventer, The Netherlands). L-Aspartic acid (10.52% Nitrogen determined by Elementar Germany) was obtained from Sigma (Sigma Aldrich, Darmstadt, Germany).

2.2.2 Sample preparation via de-oiling and milling

Oil was removed from part of the soybeans by using a single-screw oil press (KK 20F Universal) from Kern Kraft (Reut, Germany). The oil press was chosen to avoid the use of solvents during de-oiling and to keep intact oil bodies in the product. The temperature was kept constant at 60 °C, to keep the temperature below the denaturation temperature (70 °C) of the major soy storage proteins in soy (Peng, Ren, & Guo, 2016; Xing et al., 2018). A standard screw was used, rotating at 20 rpm. A hard seed extraction unit was mounted around the standard screw followed with a pre-die. Three main die sizes were used with a diameter of 14, 16 and 18 mm. This resulted in material throughput rates of respectively 1.3, 2.5 and 9.4 kg/hr.

Both the soybeans and oil pressed soybeans were pre-milled into grits with a LV 15M pin-mill (Condex-Werk, Wolfgang bei Hanau, Germany). The grits were milled into fine soy flour with a UPZ100 pin-mill with a stationary disk and a rotary disk with each four rings of pins (diameter pins 3 mm, height pins 7 mm) (Hosokawa-Alpine, Augsburg, Germany). The milling speed for soybeans was varied between 8000 rpm and 22000 rpm (838-2304 $rad \cdot s^{-1}$) with an air flow rate of 60 m^3/h and a feed rate of 0.5 kg/h. For de-oiled soybeans three milling speeds of 8000, 15000 and 22000 rpm (838, 1571 and 2304 $rad \cdot s^{-1}$ respectively) were used with an air flow rate of 60 m3/h, and feed rate 0.5 kg/h. The milling yield is defined as the collected mass after milling divided by the feed mass prior to milling.

2.2.3 Sample analysis

Composition

The crude oil content was measured with a Büchi extractor (B-811, Büchi Labortechnik AG Switserland). The samples (2±0.02 g) were exactly weighted and the oil was extracted with an excess amount of solvent (Petroleum Ether 40-65°C), with a sample to solvent ratio between 1:44 and 1:60. The continuous extraction mode was used, which included 2 hours of heating and 30 minutes of rinsing on level 9 followed by 30 minutes of drying with heating level 4. The heating levels were used according to the specifications by the manufacturer (Büchi Labortechnik AG, 2016). After extraction, the samples were dried at room temperature overnight. The moisture content was determined using an Air-Oven Method (105 °C, dried overnight) (AACC Method 44-15.02). The protein content was determined by dumas analysis with a rapid N exceed protein analyser (Elementar, Germany) via high temperature combustion and detection of the released nitrogen. Dry samples were exactly weighted (130-160 mg) in 35x35 mm tin foil for elemental analysis

with a pre-programmed method by the manufacturer for 250 mg analysis. Calibration of a daily factor was done with aspartic acid and for the samples a conversion factor of 5.71 was used.

Particle size

The particle size distributions of the grits and the fine flours were measured with a Mastersizer-3000 equipped with an Aero-S module for dry powder dispersion with a high energy venturi (Malvern Panalytical LTD, United Kingdom). The hopper gap was set to 3 mm and the samples were dispersed in the dry cell with a pressure of 2 bar and a constant feed rate of 60-80%. The average volume-weighted particle size distribution was calculated for non-spherical particles with a general-purpose analysis model.

Flour dispersibility

The dispersibility of the samples was measured by using a pressure titration method. For this the particle size distribution was determined at dispersion pressures of 50, 100, 200 and 400 kPa. It is assumed that the particles are fully dispersed at a high dispersion pressure and remain agglomerated at lower dispersion pressures (Pelgrom et al., 2014). The extent of de-agglomeration (DA) was calculated by the ratio between the particle size (DV_{50}) at full dispersion (4 bar) and the particle size at 0.5 bar (Jaffari et al., 2023; Pelgrom et al., 2014).

$$DA = \frac{DV_{50} \text{ at 4 bar}}{DV_{50} \text{ at 0.5 bar}} \quad (2.1)$$

The dispersive index (DI) for protein particles smaller than 10 µm was calculated by the ratio of the volume percentage of particles smaller than 10 µm at each dispersion pressure of 0.5, 1, 2 and 4 bar (Dijkink, Speranza, Paltsidis, & Vereijken, 2007).

$$DI = \frac{\text{Volume<10 µm at pressure i}}{\text{Volume<10 µm at 4 bar}} \quad (2.2)$$

2.2.4 Mathematical modelling of grinding energy and particle size with empirical models

The rotational energy is described with Equation 2.3, in which $E_{rotational}$ is the rotational kinetic energy (J), I is the moment of inertia of the rotating object (kg·m^2) and ω is the angular frequency (rad·s^{-1}).

$$E_{rotational} = \frac{1}{2} \cdot I \cdot \omega^2 \quad (2.3)$$

For validation, the moment of inertia and the exponent in Equation 2.3 were estimated based on a non-linear least sum of squares method.

The decrease in particle size by the energy input can be described by Bond's empirical model for particles between 10 micron and 5 mm (Sokolowski, 1996). Bond's empirical model is given in Equation 2.4.

$$E_B = \frac{m_p t}{W} = C_B \left(\frac{1}{\sqrt{d_p}} - \frac{1}{\sqrt{d_F}} \right) \quad (2.4)$$

Where E_B is the specific energy (kJ/kg), m_p the power used by the mill (kW) read from the machine, t refers to time (s), W is the sample weight to be milled (kg) and C_B is the milling index or Bond's constant (kJ·mm$^{0.5}$/kg). The d_p and d_F represent the particle size diameter at 80% of the cumulative volume (DV_{80}) of the product and the feed in mm, respectively (Sahay & Singh, 2001). The specific energy was calculated for the different milling speeds, with the average m_p, which was read from the equipment during the experiment and with a throughput of 0.5 kg/h. The milling index was calculated for the soy flours with different oil contents based on the least residual sum of squares method.

Bond's constants reported in literature had varying units and the calculation was not similar across published research, i.e. the use of varying particle size indicators (i.e. DV_{80}, $DV_{3,2}$ or DV_{50}). As Bond's constant is specific for the mill used, combined with the varying calculations and units, broader comparison was not possible (Appendix table 2.1).

2.2.5 Statistical analysis

Data were collected and analysed in R-studio Version 4.0.2 (The R Foundation). A non-linear least squares method was used to estimate the parameters of a non-linear model. Next to this, the 95% confidence interval of the estimated parameters was calculated. The models were compared using the Akaike criterion (AIC):

$$AIC = n \cdot \ln\left(\frac{SS_r}{n}\right) + 2 \cdot (p+1) \quad (2.5a)$$

When the number of experiments was small, n/p<40, Equation 2.5b a corrected version of the AIC (AIC_C) was used:

$$AIC_C = n \cdot \ln\left(\frac{SS_r}{n}\right) + 2 \cdot (p+1) + 2 \cdot (p+1) \cdot \frac{n}{n-p} \quad (2.5b)$$

In which n is the number of data points, p the number of parameters and SS_r the residual sum of squares (Boekel, 2008). The difference between the models was expressed with ΔAIC, in which the model with the lowest AIC value was used as a reference (Equation 2.5c).

$$\Delta AIC = AIC - AIC_{min} \quad (2.5c)$$

As rule of thumb, models with ΔAIC≤ 2-3 are worthwhile to consider, values between 4 and 7 are less supported models and values above 10 indicate models that may be discarded (Boekel, 2008). Data were weighted to obtain an even emphasis on de-oiled and non-de-oiled samples.

2.3 Results and discussion

2.3.1 Composition

The protein and oil contents of non-de-oiled soy flour (milled once or twice) were measured (Table 2.1A) and found comparable to previously reported values, i.e. 32.0%-37.4% and 16.7%-20.5% for protein and oil, respectively (Rotundo et al., 2016). The oil content increased with decreasing particle size (Table 2.1A), which is in line with previous observations and related to an increasing extraction efficiency for smaller particles ((Rosenthal et al., 1998). The protein contents of milled once and twice soy flour (20.32 g oil/100 g dry solids) were similar, which is related to the difference in analysis procedure for protein and oil, where protein analysis relies on combustion rather than on extraction. It is noted that the oil content as determined for the twice milled soy flour (20.32 g/100 g dry solids) is further used as the reference for soy flour as it has a particle size more similar compared to that of the milled de-oiled soy flours (650 – 850 μm). The oil and protein contents of the de-oiled and milled soy flours are given in Table 2.1B. The main die diameter of the oil press was reduced to increase the pressure drop to remove more oil. The oil content was measured after subsequent milling and is assumed not affected by the small differences in particle size. The protein content on dry basis increased with higher degree of de-oiling due to the removed oil.

Table 2.1: Main die diameter, oil content, protein content and average particle size (DV50) of once and twice pre-milled soy (A: above dotted line) and de-oiled soy flours (B: below dotted line).

Sample	Main die diameter [mm]	Oil content [g/100 g dry solids]	Protein content [g/100 g dry solids]	DV_{50} [µm]
Soy	None	17.37±0.08	36.64±0.17	1213±46
Soy twice pre-milled	None	20.32±1.00	36.08±0.71	824±43
Slightly de-oiled soy	18	15.67±0.34	40.84±0.45	739±37
Moderate de-oiled soy	16	10.48±0.05	43.03±0.08	688±41
Highly de-oiled soy	14	8.94±0.04	43.42±0.18	759±32

2.3.2 Milling yield

As might be expected, the milling yield during pin-milling significantly declined with increasing milling speed (Figure 2.2). It was observed that most of the material (>60% of the loss) was lost due to fouling in the milling chamber, whereas the remaining material was lost in piping and corners of other parts of the milling device. Accumulation inside the mill at the stationary disk and the rotating disk are shown in Figure 2.3. The pins of the stationary disk were relatively clean for lower milling speeds, whereas at higher milling speeds more residual material was found on the pins. The rotor disk remained relatively clean during all milling conditions tested.

For de-oiled soy flours milled at 22000 rpm the overall milling yield was significantly higher compared to the soy flour (20.32% oil) (Figure 2.2). In addition, less material was lost in the milling chamber for de-oiled samples (8.94% oil), where both the stator and the chamber of the pin-mill remained cleaner than with soy (20.32% oil) milled at 15000 and 22000 rpm (Figure 2.3). Less material accumulated at the wall, which is likely due to the lower oil content of these samples. Accumulation of material at the wall is expected to be enhanced by the centrifugal force induced upon milling, whereas deposits on the stator are likely formed due to the low shear forces close to the static pins. To reduce the latter, an alternative pin configuration with two rotating disks especially suitable for sticky materials could be evaluated (Furchner, 2009). This will be expected to work well for soy (20.32% oil) as the rotor remained much cleaner than the stator (Figure 2.2).

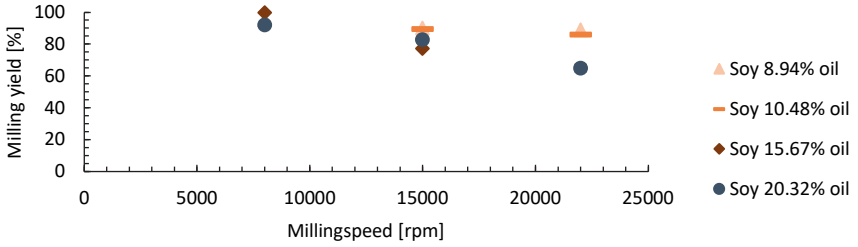

Figure 2.2: Milling yield versus milling speed of fine milled soy flours, the error bars indicate the standard deviation of the yield. The oil percentages are on dry basis.

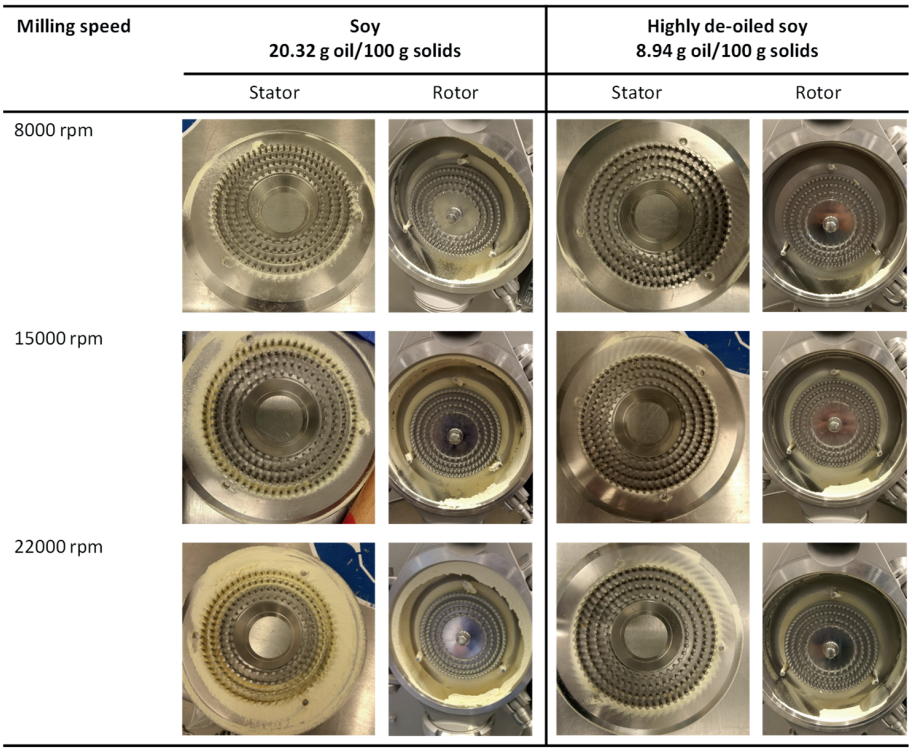

Figure 2.3: Accumulation on the stator and the rotor inside the pin-mill for soy and highly de-oiled soy milled at different milling speeds.

2.3.3 Particle size distribution

The particle size distributions of the milled flours were evaluated and described with Bond's empirical model to relate milling conditions and material properties (especially oil content) to effective size reduction. The particle size distributions were analysed as function of pin-milling conditions and oil content (Figure 2.4). For the highest milling speed (22000 rpm), the average particle size for soy (20.32% oil) was reduced to 122 µm (DV_{50}) and that of highly de-oiled soy was reduced to 51 µm (DV_{50}). The particle sizes were in a similar range as observed in previous studies, which were 90-184 µm for soybean flour and 49 µm for de-oiled soy (Russin et al., 2007; Xing et al., 2018). A lower oil content enabled milling to smaller particle sizes for both pre-milling into grits and fine milling at different rotation speeds.

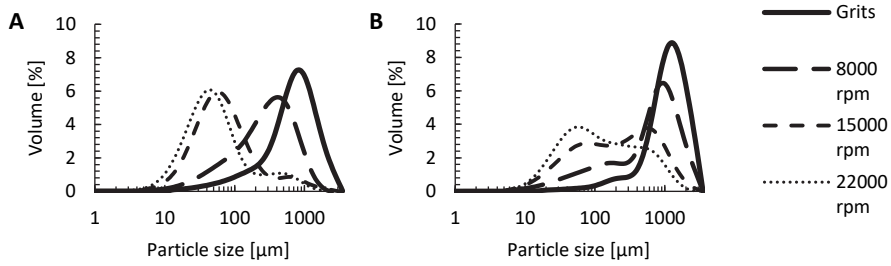

Figure 2.4: Average particle size distribution of milled highly de-oiled soy 8.94% oil (A) and soy 20.32% oil (B). The oil percentages are on dry basis. Grits represent the pre-milled material, whereas the dotted and dashed lines represent the fine milled flours at a defined milling speed, specified in the figure legend.

For both soy (20.32% oil) and highly de-oiled soy the particle size distribution shifted towards smaller particle sizes upon higher pin-milling speeds (Figure 2.4). The single peak with particles up to 100 µm are expected to consist of individual particle structures milled to a similar size, for example cell wall fragments and liberated protein bodies. The peak above 110 µm will likely consist of clustered particles and hull debris (Xing et al., 2018). For soy (20.32% oil) the shift in particle size showed a clear bi-modal distribution, whereas for highly de-oiled soy the shift towards smaller particle sizes occurred faster towards one main peak. Based on the observations for soy (20.32% oil), the particle size of highly de-oiled soy may also have shifted via a bi-modal distribution between 8000 and 15000 rpm.

Differences between milling of whole soybeans and highly de-oiled soy may be explained by the reduction in oil content and/or the degree of compaction of the material during

pressing. The faster shift towards smaller particles with similar milling speed for de-oiled soy flour suggests that after (partial) removal of the oil a more brittle material is obtained that is easier to be fragmented. For soy flour (20.32% oil) one can observe a bimodal distribution, which is different for the de-oiled soy flour. This observation may be related to the compression during de-oiling in the screw press, which compacts different tissue structures (e.g. protein bodies, cell fragments) together. Upon milling such structures may then be less well disentangled and loosened with the consequence that small components will not be released and thus a different particle size distribution is observed.

2.3.4 Modelling of particle size reduction during milling

The particle sizes (DV_{80}) after milling at different speeds (angular frequencies) were determined and correlated to the energy use of the mill (Figure 2.5). The milling speed was strongly correlated to the energy consumption (R^2=0.97) and the rotational energy could be described with Equation 2.6 (R^2=0.998), in which the milling energy (kJ/kg) quadratically increased with the angular frequency ω (rad·s^{-1}).

$$E_B = \frac{1}{2} \cdot 2.5 \cdot 10^{-3} \cdot \omega^{2.0} \qquad (2.6)$$

A summary of the parameter estimation is provided in Appendix tables 2.2 – 2.5. With a 3D model of the UPZ100 pin disk a theoretical moment of inertia of $1.73 \cdot 10^{-3}$ kg·m^2 was calculated (Hosokawa Alpine, 2021). The estimated value in this study ($I=2.5 \cdot 10^{-3}$) was slightly higher than the latter value as the theoretical value does not consider friction forces due to the drive unit or gear related parts.

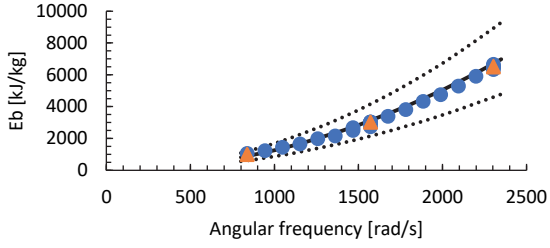

Figure 2.5: Specific energy (EB) during steady state operation against the angular frequency for soy (●) and de-oiled soy (Δ) milling. The solid black line indicates the prediction with Equation 6 (R2=0.998). The upper and lower limit of the 95% confidence interval of the moment of inertia are given with a dotted line (1.73·10-3 and 3.66·10-3 kg·m2). A summary table of the parameter estimates is provided in Appendix table 2.2.

The particle size (DV_{80}) reduced upon an increase in energy consumption (Figure 2.6). The particle size reduction for whole soybeans required significantly more energy than for de-oiled soy, which is related to the presence of more oil leading to increased ductile behaviour (Pelgrom et al., 2014). Similar observations with respect to higher energy consumption have been done for milling at higher moisture contents for soy (8-12%) and wheat (12-18%), where differences in moisture content were obtained via drying, soaking and tempering (Lee et al., 2013; Warechowska et al., 2016).

Bond's empirical model (Equation 2.4) can be used to describe the relationship between the particle size and energy use of a mill. In previous research, Bond's constant is usually determined for each milled sample separately, e.g. by Lee et al. (2013). We followed the latter approach as well, but also used an alternative approach in which we used the oil content and an oil-independent Bond constant to include a clear dependency on oil content:

$$E_B = \frac{m_p t}{W} = Oil\%_{db} \cdot C_{BO} \left(\frac{1}{\sqrt{d_p}} - \frac{1}{\sqrt{d_F}} \right) \quad (2.7)$$

The advantage of the alternative approach is that data from multiple samples can be used together to estimate an oil-independent Bond's constant. Secondly, the explicit inclusion of oil content shows the dependency of the Bond's constant, and thus size reduction by milling, on oil content.

The different approaches were compared using the Akaike criterion (Equation 2.5). The traditional estimation with sample-based Bond's constants is least preferred (ΔAIC = 10.2) (Appendix table 2.6). The estimation using two Bond's constants, one for de-oiled samples and one for non-de-oiled samples appeared better (ΔAIC = 0). However, we hypothesized that decreasing oil contents will gradually affect grinding characteristics and therefore defining these two groups is less desirable. The results of the model with oil content and an oil-independent Bond's constant ($293 \pm 33 \frac{kJ\ mm^{0.5}}{oil\%(db) \cdot kg material\ (wb)}$) as parameters are provided in Figure 2.6 (ΔAIC = 0.8).

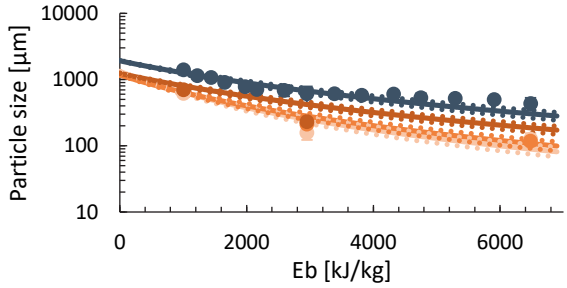

Figure 2.6: Average particle size (DV80) of soy 20.32% oil (●, initial particle size (DF)=1.92 mm), highly de-oiled soy 8.94% oil (●, DF=1.33 mm) moderate de-oiled soy 10.48% oil (●, DF=1.74 mm) and slightly de-oiled soy 15.67% oil (●, DF=1.24 mm) versus the specific energy (EB), error bars indicate the standard deviation. The predicted values with Equation 2.7 are indicated with a solid line and the upper and lower limit of the 95% confidence interval are given with a dotted line.

Prediction of the particle size reduction with Bond's model showed deviations especially for smaller particle sizes (Figure 2.6). To further check the validity of the obtained Bond's model for smaller particle sizes and oil content, the soy flours (with 8.94% oil, 15.67% oil and 20.32% oil and only milled at 8000 rpm) were re-milled at 8000 rpm to generate an additional data set. This resulted for soy with 20.32% oil in a DV_{80} of 1058 ± 34 µm after twice milling at 8000 rpm and 887 ± 24 µm after three times milling at 8000 rpm. Similarly, soy flours (8.94% oil and 20.32% oil) milled at 15000 rpm and 22000 rpm were re-milled. The model with an oil-independent Bond's constant was best able to describe the particle size reduction (ΔAIC = 0; Appendix table 2.7), but also upon re-milling the predicted particle size was smaller than the actual particle size (Figure 2.7). In addition, the higher energy input by re-milling did not result in smaller particle sizes than a similar energy input did for once milling (data labels Figure 2.7; i.e. once milling at 15000 rpm (2952 kJ/kg) resulted in a smaller actual particle size than three times milling at 8000 rpm (3024 kJ/kg)). So, particle size reduction upon milling to smaller and smaller particles becomes increasingly less efficient as the limitations of the mill are reached and the particle size cannot be reduced any further, despite of increased milling speeds. This limitation for soy 20.32% oil was reached for a larger particle size than for soy with 8.94% oil, which is in line with Equation 2.7. However, the presence of a plateau value is not incorporated in Bond's model, which resulted in a deviation from the predicted particle sizes with Bond's model and the actual particle size. For industrial scale-up more detailed population balance models are recommended (Herbst & Fuerstenau, 1980), that consider machine and material parameters separately (Vogel & Peukert, 2005). Such a more advanced mill modelling approach compensates for the mill used and incorporates a lower probability for breaking of smaller particles (Vogel & Peukert, 2005). For

example, single particle breakage tests for zeolite particles were successfully used to develop a population balance model and with that design a pin-milling process (Z. Li et al., 2020). Recently, a multiscale modelling approach for particle breakage in milling has been proposed for quantitative prediction of milling processes (L. G. Wang, Ge, Chen, Zhou, & Chen, 2021).

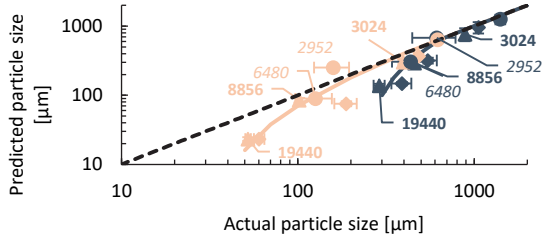

Figure 2.7: Predicted and measured particle size (DV80) of soy 20.32% oil (●) and highly de-oiled soy 8.94% oil (●). Circles (●) represent once milled samples at 8000, 15000 and 22000 rpm (from figure 2.6), diamonds (◊) represent twice milled samples and triangles (Δ) were three times milled. The x error bars represent the particle size standard deviation and the y error bars represent the 95% confidence interval of the prediction with CBO (293 ± 33 "kJ mm0.5" /"oil%" ("db")"·kgmaterial (wb)"). The black dashed line gives y=x other lines are added to guide the eye. The specific energy (kJ/kg) is highlighted for samples milled once (cursive) at 15000 (2952 kJ/kg) and 22000 rpm (6480 kJ/kg) and samples milled three times (bold) at 8000 (3024 kJ/kg), 15000 (8856 kJ/kg) and 22000 rpm (19440 kJ/kg). The reader is referred to the online version for a colour representation.

2.3.5 Particle- and flour dispersibility

An important criterion for dry separation of the finely milled soy flours after pin-milling is a high degree of dispersibility. Therefore, the dispersibility of the milled soy flours was assessed with a pressure titration method, where at low dispersion pressure (0.5 bar) particles may still be agglomerated and at high dispersion pressure (4 bar) primary particles are expected to be fully dispersed. For comparison between different samples the ratio of measured particle sizes at 4 bar and 0.5 bar is calculated to indicate if the particles are dispersible (~1) or poorly dispersible (~0). We compare here two ratios: 1) The extent of de-agglomeration (DA) based on the ratio of the particle size (DV_{50}) as given in Equation 2.1 (Pelgrom et al., 2014), and 2) The dispersive index (DI) defined as the ratio of the volume percentage of (protein) particles smaller than 10 µm as given in Equation 2.2 (Dijkink et al., 2007).

Figure 2.8 represents the cumulative particle size distributions for de-oiled soy (8.94% oil) and soy (20.32% oil). The particle size distribution shifted towards higher particle sizes for lower dispersion pressures, which is in line with findings in previous research for

lupin and starch mixtures (Dijkink et al., 2007). The DV_{50} at high and low dispersion pressure was used to calculate the DA. Perhaps surprisingly, both soy and de-oiled soy were relatively well dispersible with a DA >0.6. This DA is comparable to the dispersibility of lupine flour (Pelgrom et al., 2014). Within the particle size range tested in this research the DA did not change with particle size.

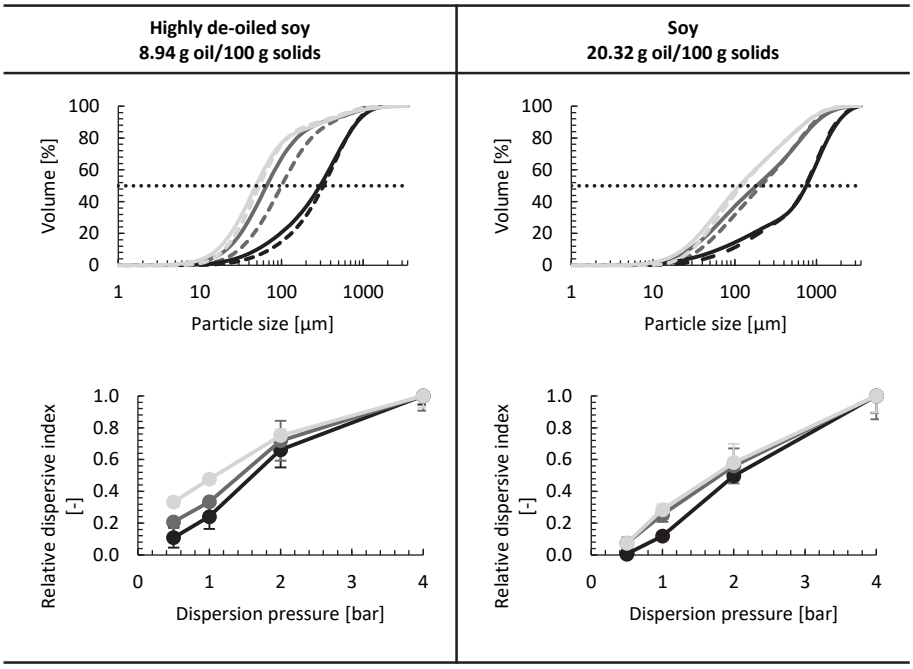

Figure 2.8: Average cumulative particle size distributions measured at low (0.5 bar, dashed lines) and high (4 bar, solid lines) dispersion pressure in which the DV50 at different dispersion pressures (dotted line) is used to calculate the extent of de-agglomeration (DA), and the relative dispersive index (DI) for three milling speeds 8000 rpm (black), 15000 rpm (grey) and 22000 rpm (light grey). Error bars indicate the standard deviation.

For de-oiled soy flour the DI increased with milling speed (Figure 2.8). So, a higher milling speed resulted in both finer particles and more dispersible fine particles, which is favourable for further separation. For soy (20.32% oil) the DI increased slightly from 8000 to 15000 rpm but remained similar for a milling speed of 15000 and 22000 rpm. Here, a higher milling speed did not improve the small particle dispersibility. The higher values of the DI for de-oiled soy (8.94% oil) than for soy (20.32% oil) indicate that the dispersibility was influenced by the presence of oil. In addition, a lower oil content resulted in more dispersible small particles upon re-milling or milling at higher speeds, whereas for higher oil content an optimum in small particle dispersion was observed

depending on specific energy input (Figure 2.9). In comparison, the dispersive index of soy particles <10 µm was lower than the dispersive index of commercial soy protein (DI = 0.83) from literature (Dijkink et al., 2007). This is likely because in literature a protein isolate was used, which contains a larger volume of small particles than whole flour. Lupin flour was found to have a dispersive index of 0.22 (Dijkink et al., 2007), which is more comparable to the measured values for de-oiled soy flour milled at 15000 rpm (Figure 2.8). The poor dispersibility of small particles hampers subsequent dry separation. This effect is thus expected to be more pronounced at higher oil contents (Figure 2.9).

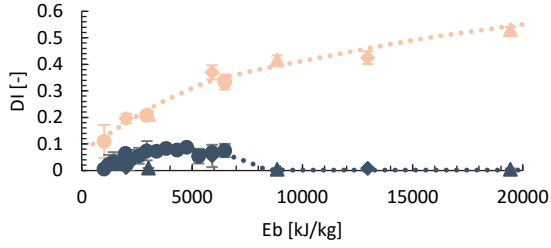

Figure 2.9: Dispersive index (DI) against the specific energy (EB) for soy 20.32% oil (●) and highly de-oiled soy 8.94% oil (●). Circles (●) represent once milled samples, diamonds (◊) represent twice milled samples and triangles (Δ) three times milled samples. Lines are added to guide the eye. The reader is referred to the online version for a colour representation.

2.4 Concluding remarks

Milling is considered a critical step for subsequent dry separation processes as it should achieve sufficient particle size reduction, dispersible particles and physical disentanglement of the plant cell constituents (Schutyser & van der Goot, 2011). In this study it was shown that the dispersive index is a good predictor for the dispersibility of small particles in flours. Although overall all produced soy flours in this study would well disperse (DA>0.6), the higher oil contents showed an optimum in dispersibility for fine particles <10 µm.

Higher oil content in soy limits milling to smaller particles and lowers the overall milling yield. This limit is also determined by the mill device used. The effect of oil content on particle size reduction can be largely described with an adapted Bond's model that used oil content as an input parameter. This approach could be interesting for describing also the effect of milling for other oil-containing crops. However, for crops with a very high oil content (> 35-40 %), like sunflower seeds, de-oiling is inevitable to apply prior to dry milling, as upon milling a paste is obtained rather than a powder.

The limitations in particle size reduction may be overcome by specifically re-milling the coarse fraction to liberate proteins from larger particles and further enhance recovery of the protein (J. Wang, Zhao, De Wit, Boom, & Schutyser, 2016b). Strategies to further optimise dry separation could involve dehulling prior to fine milling and improving the overall milling yield. Soy with a higher oil content resulted in a low milling yield (65%) and deposited material on the stationary disk, which can be optimized on industrial scale by using two rotors, instead of one rotor and one stator, without compromising on the particle size achieved, based on previous research (DV_{97} 150 µm) ((Nieh & Snyder, 1991).

Appendix 2

Appendix table 2.1: Values for Bond's constant and specific energy from literature. The cursive values are recalculated towards units used in this research. Wi stands for Work Index as some researchers only calculated the work index.

Material	Grinder	E_B	C_B	d_p and d_F	Source
Soybeans	220 W dry			$DV_{3,2}$	Lee et al.,
6% moisture	grinder	0.246 kW h/kg (*886 kJ/kg*)	0.133 kW h/kg *(479 kJ/kg)*		2013
8% moisture		0.288 kW h/kg *(1037 kJ/kg)*	0.155 kW h/kg *(558 kJ/kg)*		
12% moisture		0.337 kW h/kg *(1213 kJ/kg)*	0.181 kW h/kg *(652 kJ/kg)*		
Coriander seeds	Hammer mill Sieve opening			DV_{80}	Shashidhar et al., 2013
	3.0 mm	36 kJ/kg	3124		
	1.6 mm	18 kJ/kg	4148		
	0.6 mm	67 kJ/kg	9351		
	0.5 mm	256 kJ/kg	9509		
			No unit indicated		
Coriander seeds	**Pin mill** (160 UPZ) max capacity 100 kg/h	168 kJ/kg	6771 *No unit indicated*	DV_{80}	Shashidhar et al., 2013
Soft Wheat		13 kJ/kg	22 kJ $mm^{0.5}$/kg	Mean	Pujol et al.,
Hard Wheat		18 kJ/kg	37 kJ $mm^{0.5}$/kg	diameter	2000
Durum Wheat		20 kJ/kg	54 kJ $mm^{0.5}$/kg		
Corn	Hammer mill Sieve hole diameter (moisture content%)			DV_{50}	Dabbour, Bahnasawy, Ali, & El-Haddad, 2015
	4 mm (10%)	16 kJ/kg	34 kJ $mm^{0.5}$/kg		
	4 mm (18%)	33 kJ/kg	91 kJ $mm^{0.5}$/kg		
	8 mm (10%)	3 kJ/kg	12 kJ $mm^{0.5}$/kg		
	8 mm (18%)	5 kJ/kg	23 kJ $mm^{0.5}$/kg		
Alfalfa chops	Hammer mill Sieve opening			Geometric mean	Ghorbani, Masoumi, & Hemmat, 2010
	18 mm	7-31 kJ/kg	4.74 kJ/kg		
	15 mm	7-26 kJ/kg	4.30 kJ/kg		
	12 mm	6-21 kJ/kg	3.71 kJ/kg		
Cinnamon	**Pin mill**	486 kJ/kg	**3378** kWh/t (W_i)	DV_{80}	Tangirala, Charithkumar, & Goswami, 2014
	Hammer mill	444 kJ/kg	2787 kWh/t (W_i)		
Coriander	**Pin mill**	488 kJ/kg	**7143** kWh/t (W_i)	DV_{80}	Tangirala et al., 2014
	Hammer mill	483 kJ/kg	4598 kWh/t (W_i)		
Turmeric	**Pin mill**	250 kJ/kg	**1998** kWh/t (W_i)	DV_{80}	Tangirala et al., 2014
	Hammer mill	275 kJ/kg	2691 kWh/t (W_i)		

Appendix table 2.2: Summary of parameter estimation $E_{rotational}=0.5 \cdot I \cdot \omega^{exponent}$

Parameter	Estimate	Std. Error	t value	Pr (>|t|)	Signif. Code	95% Confidence Interval	
						2.5%	97.5%
I	$2.53 \cdot 10^{-3}$	$4.573 \cdot 10^{-4}$	5.527	$5.88 \cdot 10^{-6}$	***	$1.73 \cdot 10^{-3}$	$3.66 \cdot 10^{-3}$
Exponent	2.00	$2.374 \cdot 10^{-2}$	84.055	$<2 \cdot 10^{-16}$	***	1.95	2.04

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Appendix table 2.3: Summary of traditional parameter estimation

Parameter	Estimate	Std. Error	t value	Pr (>|t|)	Signif. Code	95% Confidence Interval	
						2.5%	97.5%
$C_{B8.94\% oil}$	2867	331.7	8.645	$2.93 \cdot 10^{-9}$	***	2186	3547
$C_{B10.48\% oil}$	3009	463.2	6.494	$5.81 \cdot 10^{-7}$	***	2058	3959
$C_{B15.67\% oil}$	2571	899.5	2.858	0.00811	**	725	4416
$C_{B20.32\% oil}$	6441	401.6	16.037	$2.52 \cdot 10^{-15}$	***	5617	7265

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix table 2.4: Summary of parameter estimation separated into de-oiled soy via pressing and soy (Equation 2.4)

Parameter	Estimate	Std. Error	t value	Pr (>|t|)	Signif. Code	95% Confidence Interval	
						2.5%	97.5%
$C_{Bde\text{-}oiled\ soy}$	2887	172.9	16.7	$< 2 \cdot 10^{-16}$	***	2533	3240
C_{Bsoy}	6441	455.8	14.13	$1.55 \cdot 10^{-14}$	***	5509	7373

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix table 2.5: Summary of parameter estimation oil based Bond's constant (Equation 2.7)

Parameter	Estimate	Std. Error	t value	Pr (>|t|)	Signif. Code	95% Confidence Interval	
						2.5%	97.5%
C_{BO}	293	16.11	18.17	$<2 \cdot 10^{-16}$	***	260	326

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix table 2.6: Weighted residuals (w_SS$_r$), AIC and ΔAIC for one throughput calculated with Equation 5b and 5c.

Model parameters	Equation	n	p	w_SS$_r$	AIC	ΔAIC
$C_{B8.94\% oil}$, $C_{B10.48\% oil}$, $C_{B15.67\% oil}$, $C_{B20.32\% oil}$	Equation 4	31	4	0.021	-204.7	10.2
$C_{Bde\text{-}oiled\ soy}$ and C_{Bsoy}	Equation 4	31	2	0.020	-214.9	0.0
C_{BO}	Equation 7	31	1	0.024	-214.1	0.8

Appendix table 2.7: Weighted residuals (w_SSr), AIC and ΔAIC calculated for re-milled samples with Equation 5b and 5c.

Model parameters	Equation	n	p	w_SS$_r$	AIC	ΔAIC
$C_{B8.94\% oil}$, $C_{B10.48\% oil}$, $C_{B15.67\% oil}$, $C_{B20.32\% oil}$	Equation 4	16	4	0.041	-72.2	15.1
$C_{Bde\text{-}oiled\ soy}$ and C_{Bsoy}	Equation 4	16	2	0.039	-83.3	4.0
C_{BO}	Equation 7	16	1	0.041	-87.2	0.0

Chapter 3

Effect of relative humidity on milling and air classification explained by particle dispersion and flowability

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Abstract

Dry fractionation of legumes is used to produce protein and starch-rich fractions with a clean label and lower environmental impact than conventional wet fractionation. Dry fractionation relies on the use of ambient air that varies in humidity. This study assessed the effect of relative humidity (RH) on milling and air classification of yellow pea and chickpea. Particle size analysis and powder rheology were used to assess the particle dispersibility and flowability of the flours. The RH has limited effect on milling and air classification between 30% and 70%. However, upon storage of fine milled chickpea flour at a RH of 70% and storage of fine milled yellow pea flour at a RH of 90% the air classification performance decreased. This was linked to a poorer dispersibility and flowability. Concluding, a relative humidity above 70% should be prevented to perform robust air classification.

3.1 Introduction

Ingredients from pulses, such as yellow pea or chickpea, are of major interest to produce plant-based foods (Grasso et al., 2022). This is due to the fact that pulses grow with a relatively high water use efficiency, have the capability to fix atmospheric nitrogen, which reduces the need for added fertilizers, and their favourable nutritional profile (high in protein and fibre, low in oil) (Gustafson, 2017). Pulses contain about 22% of protein, 43% starch, 19% fibre and 9% moisture, with generally a very low oil content (~2%) except for chickpea (~6%) (S. Wang et al., 2020). However, many plant-based foods require a higher protein content than is naturally present in pulse ingredients, which calls for protein enrichment via processing. The traditional wet fractionation of plant protein requires a copious amount of energy and water, and the harsh conditions can cause protein denaturation, both are usually unfavourable (Assatory et al., 2019). Dry fractionation is considered as a sustainable alternative to produce protein, starch- and fibre concentrates with preserved native properties (Assatory et al., 2019; Lie-Piang et al., 2021; Schutyser & van der Goot, 2011). These pulse ingredients have great commercial potential and can be applied in many food products, such as pasta, noodles, plant-based meat, beverages, soups and sauces (S. Wang et al., 2020). To provide guidelines for industrial dry fractionation, it is important to identify which factors affect the process performance and link these to powder properties.

Dry fractionation includes milling and dry separation by sieving, air classification or electrostatic separation. This study focuses on air classification, which is the most common dry separation process to produce protein and starch concentrates from pulses (S. Wang et al., 2020). Impact milling first fragments the cotyledon into separate starch granules and smaller protein and fibre fragments. Subsequently, air classification is used to separate cellular components based on the difference in particle size and density. This typically results in a protein-rich fine fraction and a starch-rich coarse fraction (Boye et al., 2010). The separation is controlled by the classifier wheel speed and airflow rate, which determine the cut point for separation, where particles have an equal chance to end up in the fine or coarse fraction (Bauder et al., 2004; Guo et al., 2007). The cut point can be estimated by:

$$x = \frac{3}{4} \cdot c_D \cdot \frac{\rho_a}{\rho_p - \rho_a} \cdot \frac{v_r^2}{v_\varphi^2} \cdot r \qquad (3.1)$$

with c_D drag coefficient (-) (a function of Reynolds number which is further related to the air velocity), ρ_a density of airflow (kg/m^3), ρ_p particle density (kg/m^3), v_r radial velocity

(m/s), v_{φ} tangential velocity (m/s), r diameter of the rotor (m) (Bauder et al., 2004; Guo et al., 2007). The radial velocity is the ratio between the airflow (m^3/s) and the total open area of the classifier slits (m^2). For air classification of pulse flours, the cut point should be in between the size of a protein body and a starch granule to facilitate separation as illustrated in Figure 3.1.

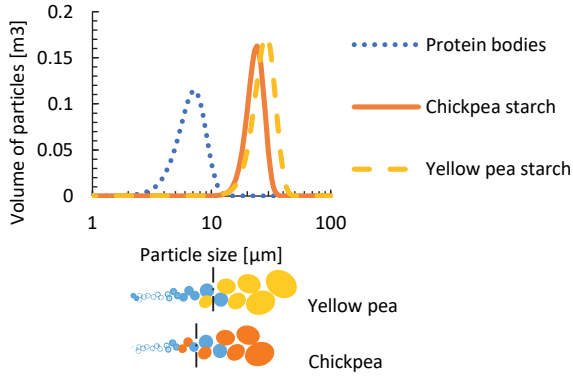


Figure 3.1: Visualized cut point for protein bodies (1-10 µm), yellow pea starch granules (25±6 µm) and chickpea starch granules (22±4 µm) (Schutyser & van der Goot, 2011; Xing et al., 2020).

During milling and air classification ambient air is typically used as processing air. Ideally, the processing performance does not vary with changes in environmental conditions during storage or processing. However, the humidity of the processing air can change with weather conditions. These changes can again affect the milling process, as moisture is known to influence fracture behaviour and energy consumption during milling (Dijkink & Langelaan, 2002b; Pelgrom et al., 2013a). Furthermore, the humidity affects powder flowability and particle dispersibility, where a lower powder flowability and dispersibility have resulted in a lower dry separation efficiency (Armstrong et al., 2014; Dijkink et al., 2007b). A lower flowability can result in fouling by the cohesion of particles between the classifier wheel vanes, or on the classifier chamber walls, which can result in large losses of material and a lower protein recovery (Pelgrom et al., 2013b). Such material losses can pose problems for the application of dry fractionation (Assatory et al., 2019).

An additional effect of humidity is that the air density decreases slightly at higher relative humidity. This can indirectly affect the set cut point during air classification (Equation 3.1). A slightly different cut point can influence the separation, especially when sizes or densities of the different cellular constituents overlap, for example for chickpea (Figure 3.1). A difference in affinity for water can influence the moisture content

equilibration. For example, oil has a lower water affinity than starch or protein, which results in faster equilibration to lower moisture contents for seeds with higher oil contents under the same conditions (Suma et al., 2013). This means that crops with a higher oil content might be affected differently by RH upon processing. In this research we used yellow pea to represent pulses with a low oil content and chickpea to represent pulses with a higher oil content. Till now, research focussed mainly on temperature and humidity upon material storage rather than during processing. Therefore, we want to understand how important it is to control the relative humidity in the processing room during milling and air classification.

This study aims to evaluate the effect of relative humidity on milling and air classification performance of legume flours and powder properties (particle size, dispersibility and flowability). Insight in the powder properties and process performance under different conditions is used to provide guidelines for moisture control during dry fractionation of pulses. Next to that, we evaluate if certain powder properties can be used as qualitative indicators for the process performance. Yellow pea and chickpea were selected as crops to represent pulses with a low oil content and a high oil content, as crops with a higher oil content might be affected differently by RH. The obtained knowledge on the dispersibility of small particles and powder flowability in relation to dry fractionation could accelerate the application of dry fractionation to other, less conventional materials and prevent losses upon dry processing of materials.

3.2 Materials and methods

3.2.1 Materials

Dry yellow pea (Pisum sativum) was obtained from P. van Schelven (Nieuwe-Tonge, the Netherlands) and dry Kabuli chickpea (Cicer arietinum L.) was obtained from Alimex Europe B.V. (Sint-Laureins, Belgium). The pulses were stored at 4°C in closed containers before use.

3.2.2 Control of relative humidity

The RH of the process room was controlled with a condensing dehumidifier Condair DC75 (Condair, Pfäffikon, Switzerland), and the actual real-time RH and temperature were measured with a data logger Testo Savaris 2 (Testo, Lenzkirch, Germany). The low humidity (target RH30) ranged between 28.3% and 43.1%, and the increased humidity (target RH70) ranged between 51.2% and 68.7%. The temperature of the room was between 17°C and 27°C upon milling and between 16°C and 29°C upon air classification.

The materials were equilibrated for 7 days in a climate chamber (Memmert, Schwaback, Germany), as preliminary trials showed that after 7 days the material weight remained constant. Aluminium dishes (8 cm·15 cm) with approximately 100 g grits or 60 g flour were placed in the climate chamber at 19°C at 30% when milled at low humidity and at 70% when milled at high humidity. The humidity conditions used are shown in Figure 3.2. For confirmation purposes, a new batch of yellow pea flour was also equilibrated at RH90.

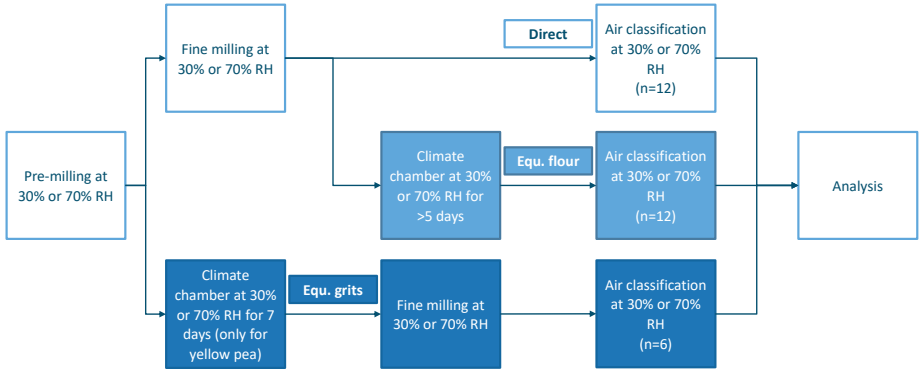


Figure 3.2: Overview of storage conditions before milling and air classification for each crop and humidity. n represents the total number of air classifications (n) carried out for each condition. In figures, direct milled flours are represented with open symbols, and equilibrated flours (Equ. Flour) and equilibrated grits (Equ. Grits) with filled symbols.

3.2.3 Milling and air classification

The hulled pulses were pre-milled into grits (yellow pea DV_{50} 1022 ± 79 µm, chickpea DV_{50} 1213 ± 92 µm) with a pin mill (LV 15 M, Condux-Werk, Germany). The DV_{50} represents the average volume-based particle size. The grits were milled into a fine flour with a ZPS impact mill (Hosokawa-Alpine, Augsburg, Germany) at a milling speed of 8000 rpm and a classifier wheel speed of 4000 rpm and 2900 rpm for yellow pea and chickpea respectively (Pelgrom et al., 2015b; Xing et al., 2020). Two airflows of 40 m^3/h (Xing et al., 2020) and 52 m^3/h (Pelgrom et al., 2015b) were used, where the pressure in the classifier chamber was approximately -10 mbar. Milling experiments were performed with a constant feed rate of 0.5 kg/h and a batch size of 700 g.

The flours were air-classified in an ATP50 classifier (Hosokawa-Alpine, Augsburg, Germany). For yellow pea, the airflow was fixed at 52 m^3/h (Pelgrom et al., 2015b). For chickpea, two airflows of 52 m^3/h (Xing et al., 2020) and 60 m^3/h were used. The higher airflow was used to increase the air classification yield. The classifier wheel speed was

8000 rpm for yellow pea and 10000 rpm for chickpea, with feed rates of respectively 0.5 kg/h and 0.2 kg/h (Pelgrom et al., 2015b; Xing et al., 2020). The batch size was 180-200 g, and one larger batch was used of 750 g. The air classifications for yellow pea milled at 40 m^3/h under different humidity conditions were repeated at least three times to identify the variation, other air classifications were repeated once or twice.

3.2.4 Moisture adsorption isotherms

The water sorption isotherms of the milled flours were determined with the Dynamic Vapour Sorption (DVS) Discovery apparatus (TA Instruments, Allentown, DE, USA) at 20°C and 50°C, to represent the temperature of the milling room and the temperature inside the mill. This because the temperature during milling can rise considerably, due to the heat generated (di Silvestro et al., 2014; Pelgrom et al., 2015a). The temperature inside the mill used in this study was assumed to be in the same range as the temperatures (16-34°C) reported for impact milling of yellow pea, as measured by (Pelgrom et al., 2015a) under the same milling conditions. Approximately 3 mg of sample was loaded in the quartz basket, the RH was increased from 30% to 90% for adsorption and consecutively decreased to 10% for desorption with steps of 10%.

3.2.5 Compositional analysis

The dry matter (DM) content was determined by oven drying (105°C) 1 g of sample for 48 hours.

The protein content was determined by DUMAS analysis (rapid N exceed, Elementar, Langenselbold, Germany) with 6.25 as the nitrogen conversion factor (Pelgrom et al., 2015b). Protein recovery was calculated as the percentage of total grits protein recovered in the fine fraction (Equation 3.2).

$$\text{Protein recovery } (\%) = \frac{\text{Amount of protein in the fine fraction (g)}}{\text{Amount of protein in the grits (g)}} \cdot 100\% \qquad (3.2)$$

The starch content was measured with a Total Starch Amyloglucosidase/a-Amylase Assay Kit (Megazyme International Ireland Ltd, Bray Ireland) based on the use of thermostable α-amylase and amyloglucosidase.

The oil contents of yellow pea and chickpea grits were determined with a Soxtherm SOX416 extractor (Soxtherm, Gerhardt, Germany). The samples (~2 g) were weighted, and the oil was extracted with an excess amount of solvent (Petroleum Ether 40-65°C). First, a hot extraction was executed (25 min), followed by 5 cycles of solvent evaporation.

The extraction time was set at 1 h and 35 min, followed by three evaporation cycles to distil off the bulk of the solvent. Lastly, the samples were dried in the equipment (30 min). The weight was determined after evaporation of residual solvent overnight.

3.2.6 Particle analyses

Particle size distribution and particle dispersibility

The particle size distribution of the flours was determined with laser diffraction in the Mastersizer 3000 with an Aero S dry dispersion unit, equipped with a standard venturi (Malvern Instruments, Worcestershire, UK). The hopper gap was 2.5 mm, and the feed rate was set to 50% to achieve a constant feed flow. Pressures of 4.0 bar and 0.5 bar were used to determine the particle size distribution, the extent of de-agglomeration (DA) and the dispersive index (DI) (Politiek et al., 2022).

Powder flowability

Powder flowability was characterised by an Anton-Paar MC502 rheometer equipped with a powder flow cell having a diameter of 50 mm (Anton Paar, Graz, Austria). A metal impeller ST36-2V-10/PCC (Anton Paar, Graz, Austria) with two rectangular-shaped blades (36 mm x 10 mm) was used to measure the powders' resistance to flow. The measured torque values are used for the semi-quantitative comparison of the powder flowability (Salehi et al., 2017; Schulze, 2008). For each measurement 30 g of flour was added to the powder flow cell and briefly stirred manually to mix the sample, using a spatula.

To assess the behaviour of cohesive powders, we adopted a dynamic measurement sequence that is based on a descending and ascending movement of the blade; a procedure that is also used in Freeman FT4 powder rheometers (Freeman, 2007). First, the metal impeller moves from the default measuring position (50 mm), towards the bottom of the powder cell, whilst rotating at 4 rpm and descending at 0.2 mm/s. When the metal bar reaches a height of 10 mm from the bottom, the descending stops, but it continues to rotate at 4 rpm for 200 s. The impeller then ascends at 0.2 mm/s with a rotation speed of 4 rpm until it is again at 50 mm. The measured torque (mN·m) is plotted against time for the consecutive intervals (Figure 3.3).

The results were used as a semi-quantitative comparison of flow behaviour between the powders. The qualitative comparison of the flowability of powders in the dynamic regime is common and has been successfully applied to numerous materials (Francia et al., 2021).

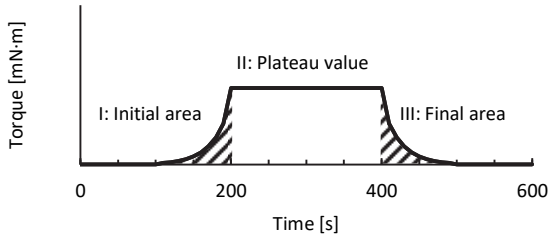


Figure 3.3: Example of three intervals for rheology where I is the interval under the curve between 0 and 200 s, II is the plateau value and III is the area under the curve between 400 and 600 s.

3.2.7 Statistical analysis

Data were collected and analysed in IBM SPSS Statistics 25 (IBM Corporation, Armonk, New York). To compare the means and evaluate the effect of processing conditions on the moisture content and powder flowability a one-way ANOVA test was carried out and a Tukey (homogeneous variance) or Games-Howell (inhomogeneous variance) post hoc test with a significance level of 0.05. For unequal sample sizes Games-Howell was used.

3.3 Results and discussion

3.3.1 The sorption isotherms and moisture content change during milling and air classification

Sorption isotherm curves of yellow pea and chickpea flour were measured to assess the moisture uptake of the two materials under different environmental conditions (Figure 3.4). The moisture content changed with around 7%DM (dry matter) between RH30 and RH70 for all measured samples. The moisture content decreased by around 2%DM for adsorption and by around 3%DM for desorption when the temperature increased from 20˚C to 50˚C. At each RH and temperature, yellow pea holds slightly more moisture than chickpea (0.3% at low humidity up to 1.3% at high humidity). So yellow pea flour is slightly more hygroscopic than chickpea flour, which is in line with previous research (Xu et al., 2019). The difference may be due to the difference in oil content between chickpea (5.2±0.1%DM) and yellow pea (0.8±0.3%DM), as seeds with a higher oil content equilibrate faster under the same conditions than seeds with lower oil contents (Davis, 1939; Suma et al., 2013).

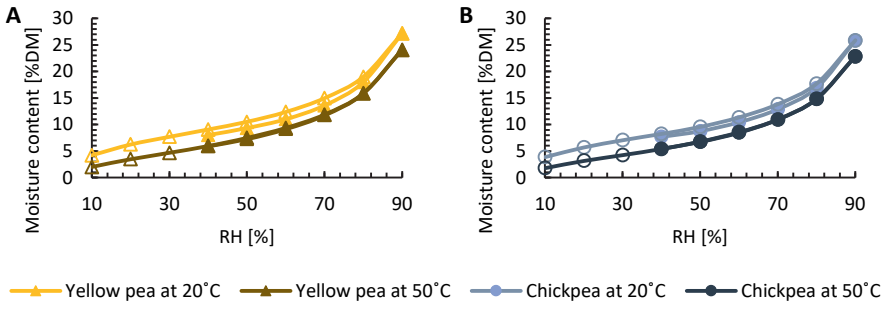


Figure 3.4: Adsorption and desorption curves for yellow pea (A) and chickpea flour (B) at 20 °C and 50 °C. Closed symbols represent moisture adsorption, open symbols represent moisture desorption.

The moisture contents of yellow pea and chickpea grits, flour and fractions were measured after milling, equilibration, and air classification at different RH (Table 3.1). Milling grits into flour resulted in a slightly larger decrease in moisture content at lower airflow (40 m^3/h) (Table 3.1). This is due to the combined effect of more moisture that goes through the equipment at higher airflows and a shorter residence time of the powder in the mill (Pelgrom et al., 2014). As expected, fine milled flours obtained or equilibrated at RH70 had a higher moisture content than materials obtained at RH30. Furthermore, the moisture content decreased by the release of water upon milling grits into flour, due to the heat generated and high airflow rates during milling. The increase in temperature results in a decrease in the relative humidity of the air (Singh, 2022). This RH decrease contributes to the drying of the grits by the release of moisture, upon milling. The chamber temperature during air classification was lower than during milling, so the actual relative humidity is expected to be higher upon air classification. This resulted in moisture absorption in the coarse fraction (from 8.0 to 10.4% moisture and from 10.7 to 11.6% moisture for a higher milling airflow) upon direct milling and air classification of yellow pea at RH70.

Table 3.1: Moisture content based on the total mass of yellow pea and chickpea under different equilibration (Equ.) RH and different airflows upon processing (low airflow above the bold line and high airflow below the bold line). Values are presented as mean ± standard deviation between fractionation experiments if applicable. N.A. means "not available". n is the number of repetitions of dry fractionation experiments, which was 3 unless specified otherwise. Bold highlights fractions with a moisture content that was 2.4% higher or lower than the moisture content in the flour.

Crop	Material	Moisture content [%total mass]			
		Grits	Flour	Fine fraction	Coarse fraction
Yellow pea Airflow: Milling 40 m^3/h Air classification 52 m^3/h	Direct RH30	11.6 ± 0.2	7.8 ± 0.7	6.8 ± 0.8	8.7 ± 0.9
	Direct RH70		8.0 ± 0.7	8.2 ± 0.4	**10.4 ± 0.5**
	Equ. flour RH30		8.1 ± 0.4	6.7 ± 0.5	8.0 ± 0.8
	Equ. flour RH70		16.3 ± 0.6	**9.4 ± 0.8**	**12.7 ± 1.2**
	Equ. Grits RH30	8.8 ± 0.3	6.9 ± 0.3	6.8 ± 0.5	8.8 ± 0.8
	Equ. Grits RH70	16.8 ± 0.6	8.0 ± 0.6	7.8 ± 0.3	10.3 ± 0.7
Chickpea Airflow: Milling 40 m^3/h Air classification 52 m^3/h	Direct RH30	12.0 ± 0.2	8.3 ± 0.0*	**5.8 ± 0.3****	7.8 ± 0.2**
	Direct RH70		8.9 ± 0.2*	N.A.	N.A.
	Equ. flour RH30		8.1 ± 0.2**	**5.6 ± 0.1***	7.5 ± 0.1*
	Equ. flour RH70		16.8 ± 0.1*	**6.6 ± 0.1***	**10.6 ± 0.1***
Yellow pea Airflow: Milling 52 m^3/h Air classification 52 m^3/h	Direct RH30	11.6 ± 0.2	7.8 ± 0.2*	7.3 ± 0*	8.2 ± 0.1*
	Direct RH70		10.7 ± 0.1*	9.0 ± 0*	11.6 ± 0*
	Equ. flour RH30		8.8 ± 0*	7.1 ± 0*	8.9 ± 0*
	Equ. flour RH70		17.1 ± 0.1*	**9.8 ± 0.1***	**13.7 ± 0***
Chickpea Airflow: Milling 52 m^3/h Air classification 60 m^3/h	Direct RH30	12.0 ± 0.2	8.3 ± 0*	**5.9 ± 0.1***	8.2 ± 0*
	Direct RH70		10.1 ± 0.1*	7.9 ± 0.8*	11.0 ± 0*
	Equ. flour RH30		7.7 ± 0*	6.1 ± 0.1*	8.0 ± 0*
	Equ. flour RH70		15.6 ± 0.1*	**7.8 ± 0.1***	**10.2 ± 0.2***

*n=1, **n=2

Overall, the effect of the equilibration of flour at RH70 on the moisture content after air classification was more pronounced than that of equilibration of grits, which indicates that moisture control in between fine milling and air classification is more important than moisture control in between pre-milling and fine milling. Furthermore, the coarse fractions had in general higher moisture levels than the fine fractions (Table 3.1). This could be attributed to the higher level of starch and lower level of protein in the coarse fraction (Appendix table 3.1), as starch is more hygroscopic than protein (Pelgrom et al., 2013b). The images and combined size and shape analysis with Morphologi 4 confirmed that the coarse fraction was indeed enriched with starch and the fine fraction was depleted of starch (Appendix figure 3.4; online supplementary material Politiek et al., 2023b).

3.3.2 Particles aggregate upon equilibration at high humidity

This section elaborates on the agglomeration of particles for the different humidity conditions by particle size analysis after milling and equilibration of the flour. Laser diffraction with a venturi set-up was used to measure the particle size distribution of the flour. The venturi tube causes most agglomerates to break, which allows to study the effect of moisture on the primary particles (Schütz et al., 2019). Direct milling and equilibration of grits at RH30 and RH70 resulted in similar overall particle size distributions (Figure 3.5) due to the heat generated, which leads to a decrease in air humidity inside the mill (Singh, 2022). This is in line with previous observations for soaked and untreated pea milled with an impact classifier mill (Pelgrom et al., 2015c). The DV_{50} increased slightly after milling and storing the grits at different humidity conditions (Equ. Grits RH30 13.18 µm, direct milled grits RH30 and RH70 13.56 µm and Equ Grits RH70 14.09 µm), which was likely caused by the difference in moisture content (Table 3.1). The energy consumption in the mill overlapped between 576 – 720 kJ/kg. A reason for overlap in energy consumption is that the moisture content of the pea decreased inside the mill due to heating (towards 6.6 and 10.6 g moisture/100 g material), which was below the critical moisture content of 11% from where changes were previously observed (Dijkink & Langelaan, 2002a, 2002b). As the energy consumption overlapped, some of the milling energy might have been used to evaporate moisture, which slightly affected the DV_{50}. As moisture equilibration of the grits to a humidity of 70% did not result in differences in energy consumption, we further focussed on the effect of moisture equilibration on finely milled flour by storing it at RH70.

Equilibration of flour at RH70 resulted in a particle size distribution shift towards larger particle sizes for both chickpea and yellow pea flour (Figure 3.5). This shift indicates that particles agglomerated upon equilibration. The slight particle size increase was qualitatively confirmed with image analysis with Morphologi 4, by the visual observation of agglomerates and a shift in volume count towards larger particles (Appendix figures 3.2 and 3.3). The agglomeration is caused by the formation of liquid bridges at the contact points of the grains at this high RH, as the powder is in the pendular state (Mitarai & Nori, 2006; Schütz et al., 2019). For chickpea, the shift of equilibrated flour at RH70 was more pronounced than for yellow pea, as also high peaks were observed at ~1600 µm for both airflows. So, it is likely that lipids also play a role in the formation of liquid bridges, which can explain the difference between chickpeas and yellow peas.

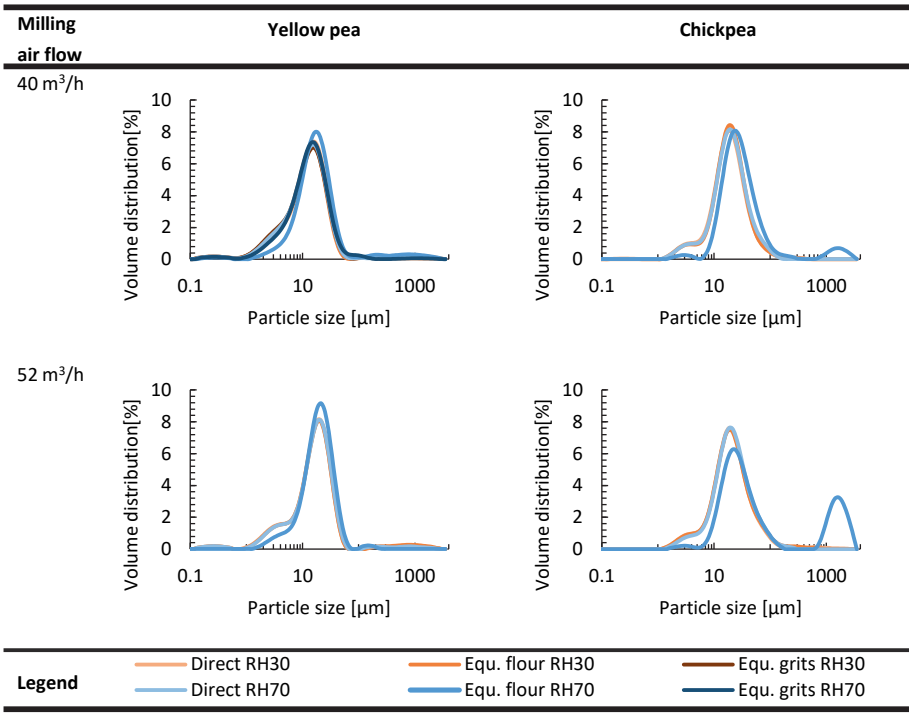


Figure 3.5: Average particle size distributions of yellow pea and chickpea flour milled with airflows of 40 m3/h and 52 m3/h. The equilibration conditions used for the specific samples are shown in the legend.

The degree of de-agglomeration (DA) and the dispersive index of the fine particles (DI) were evaluated for the relative humidity conditions used in this study. Both are a measure of the air classification capability, where a higher DA and DI are favourable for the process performance (Dijkink et al., 2007; Pelgrom et al., 2015b). These two measures for particle agglomeration are based on the DV_{50} (for DA) and the particle size below 10 µm (for DI) at full dispersion (4 bar) and a lower dispersion pressure (0.5 bar). At a low humidity (28-45%) the DA was above 0.78 and not influenced by equilibration at RH30 (Figure 3.6), which was likely because the particles were in a dry state, where there is only weak cohesion between particles (Danov et al., 2018). Equilibration at RH70 of flours milled at a lower airflow (40 m^3/h) slightly decreased the DA, which might result in a slightly decreased air classification performance, while equilibration at RH70 did not influence the DA at higher milling airflow (Figure 3.6A). The DA was still above 0.6 for all samples, which indicates that the overall collected flours were still well dispersible, irrespective of their water content.

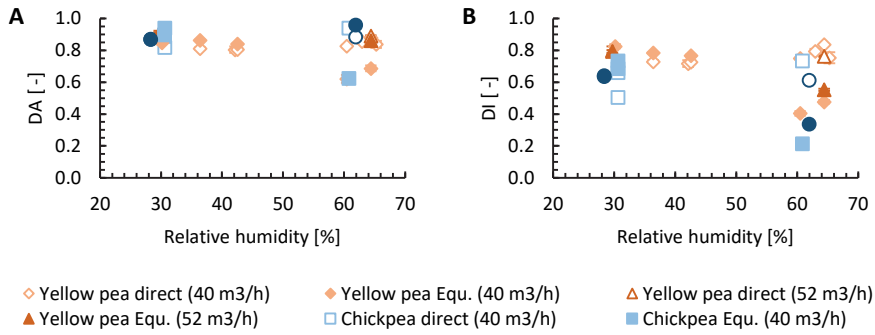


Figure 3.6: Degree of de-agglomeration (DA) and dispersive index (DI) versus the relative humidity upon milling for yellow pea and chickpea at different conditions identified in the figure legend. Open symbols stand for directly milled flours and closed symbols are equilibrated (Equ.) samples. The milling airflows of the samples (40 and 52 m3/h) are shown between brackets. Error bars show the standard deviation in DA and DI.

The dispersive index (DI) of the small particles was similar for equilibration at 30% humidity and decreased upon equilibration of fine milled material at 70% humidity for both airflows and both materials (Figure 3.6B). This was caused by agglomeration between the small particles with larger particles at a higher humidity (Appendix figure 3.3). At a high dispersion pressure, the small particles might still be dispersed from the larger particles. However, at a lower dispersion pressure of 0.5 bar the particles remain attached to the larger particles as the force to disperse the particles from each other is too low to overcome the other forces between the particles. Equilibration at 70% humidity of samples milled with a higher airflow (52 m^3/h) resulted in a better particle dispersion than equilibration of samples milled with a lower airflow (40 m^3/h) (Figure 3.6), which might be due to higher van der Waals forces that act on the smaller particles obtained after milling at 40 m3/h (Pelgrom et al., 2014).

Overall, the small particles were better dispersible in the air when milled at a higher airflow and the dispersive index was more affected by the equilibration conditions than the degree of de-agglomeration, especially at higher humidity (70%). The lower DA and DI for equilibrated samples at RH70 might be indicators for a decrease in air classification performance upon storage at higher humidity, which is discussed in the next section.

3.3.3 Airflow and high storage humidity influence milling and air classification performance

The milling performance is evaluated by the flour yield and composition, and the air classification performance is assessed by the fine and coarse fraction yields and

compositions. The overall process performance is evaluated by the protein recovery in the fine fraction. Here we did not focus on the oil content, as the oil bodies are not selectively separated in air classification processes. First, the effects of humidity and airflow on milling yield, flour- and fraction composition are evaluated. Subsequently, the effect of airflow and humidity on the fine and coarse fraction yields is described and connected to the results from section 3.2 on particle agglomeration and visual observations in the classifier chamber. Lastly, the overall process performance (milling + air classification) is evaluated by the protein content and protein recovery in the fine fraction.

The milling yield was not influenced by the relative humidity (30-70%) during processing (Figure 3.7A). The milling yield of chickpea (75-93%) was comparable to literature (88%) (Xing et al., 2020) and the milling yield of yellow pea milled with an airflow of 52 m^3/h. For yellow pea, the higher airflow (52 m^3/h) doubled the milling yield (~77%) compared to the lower airflow (40 m^3/h) with yields between 20-45% (Figure 3.7A). The low milling yield for yellow pea milled at 40 m^3/h was not expected based on previous research, where milling yields of around 88% were reported for a similar airflow of 40 m^3/h (Xing et al., 2020). The lower yield was caused by insufficient emptying of the milling chamber (Appendix figure 3.1).

The composition of yellow pea flour and the obtained fractions was mainly influenced by the used airflow, rather than the humidity conditions (Appendix table 3.1). A higher airflow (52 m^3/h) resulted in lower protein contents (28.1%DM versus 39.6%DM) and higher starch contents (45.9%DM versus 29.8%DM) in the flour than a lower airflow (40 m^3/h) (Appendix table 3.1). A similar observation was reported in previous research, where the protein content of yellow pea flour milled at 40 m^3/h was 32.4%DM and starch content 36.6%DM, whereas pea flour milled at 52 m^3/h had a protein content of 19.4%DM and starch content of 47.6%DM (Pelgrom et al., 2015a; Xing et al., 2020).

The increase in airflow resulted in a slightly higher starch and lower protein content in the fine fraction of chickpea. This change is due to the shift of the cut-point to larger particle sizes, which allowed more small starch particles to pass the classifier wheel together with the protein (Appendix table 3.1). In general, air classification of chickpea results in lower protein contents in the fine fraction than air classification of yellow pea and other legumes such as fababean (62.6% protein) or lentil (63.0% protein) (Pelgrom et al., 2015b; Sosulski & Youngs, 1979; Xing et al., 2020). The lower protein content in the fine fraction has been attributed to the higher oil content and the smaller starch

granules of chickpea than other starch-rich legumes (Pelgrom et al., 2015b; Sosulski & Youngs, 1979; Xing et al., 2020).

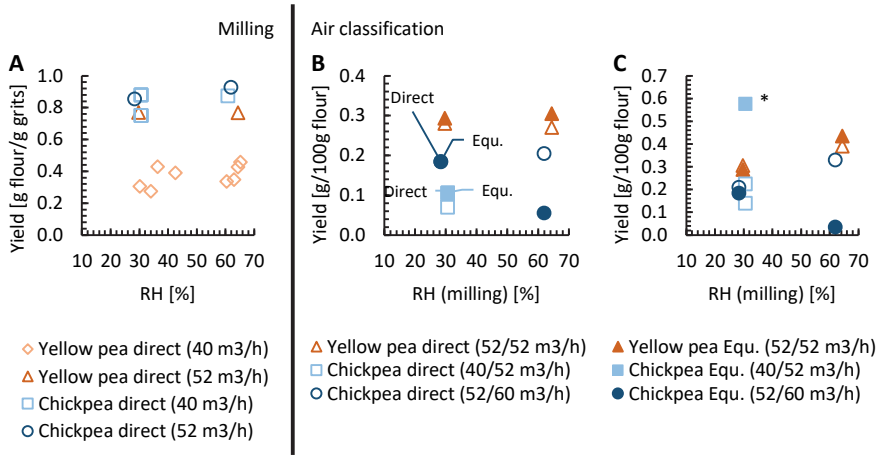


*Figure 3.7: Milling yield (A), fine fraction yield (B) and coarse fraction yield (C) of yellow pea (▲/◆) and chickpea (●/■) against actual average relative humidity (RH) during the milling. The conditions are specified in the figure legend. Moisture loss (Table 3.1) is considered as yield loss. Open symbols are directly milled, and air-classified flours and closed symbols are equilibrated samples between milling and air classification. The milling airflow of the samples (40 and 52 m3/h) is the first value shown between the brackets and the air classification airflow (52 and 60 m3/h) is the second value shown between the brackets. For air classification, samples with a milling yield above 60% are presented, and samples with a milling yield below 60% are included in Appendix figure 3.5. *Indicates that the batch size for air classification was higher (750 g). The points for direct and equilibrated (Equ.) chickpea overlapped at 30% humidity in B.*

The fine and coarse fraction yields in Figures 3.7B and 3.7C represent only samples with higher milling yields (>60%), the other fine and coarse fraction yields of yellow pea are included in Appendix figure 3.5. Direct air classification of chickpea with a higher airflow (60 m^3/h) resulted in an increased fine fraction yield compared to air classification at 52 m^3/h, caused by the increased drag force through the classifier wheel. A higher airflow resulted in similar or higher coarse fraction yields for chickpea, so overall the air classification loss was reduced by increasing the airflow (Figure 3.7).

The fine fraction yield was not influenced by humidity and storage conditions at low humidity upon air classification (Figure 3.7), which was expected as the degree of de-agglomeration and dispersive index at low humidity had similar values with and without equilibration (Figure 3.6). The coarse fraction yield of yellow pea slightly increased with relative humidity, which was observed for both milling airflows of 40 m^3/h and 52 m^3/h

(Figure 3.7B; Appendix figure 3.5). This indicates that, even though the dispersive index of equilibrated material at RH70 was lower for equilibrated yellow pea (0.55) than directly processed yellow pea at RH70 (0.76), it did not negatively affect the overall air classification yields. The slightly higher coarse fraction yield might be caused by a slightly higher gravitational force than the drag force for a larger number of particles at higher humidity (Shapiro & Galperin, 2005). The higher gravitational force was induced by the uptake of water by the particles in equilibrated flour at RH70, which increases the particle density and by a lower drag force i.e. due to a slightly lower air density at higher humidity.

Contrary to yellow pea, equilibration of chickpea flour at RH70 (DI=0.34) was detrimental to the fine and coarse fraction yields. The analysis of the yield was not even possible for air classification of chickpea that was milled at an airflow of 40 m^3/h equilibrated at RH70 (DI=0.21) and air-classified at 52 m^3/h, as the accumulated material completely blocked the inlet of the classifier chamber (Figure 3.8A). So, the lower yields were caused by the higher adhesive (particle-wall) and agglomeration (particle-particle) forces than the gravitational force. Furthermore, a visual inspection of the classifier chamber showed that the amount of material that accumulated at the top of the chamber depended on the material, processing- and equilibration conditions. For example, yellow pea showed much less adhesion and agglomeration at the top of the chamber upon air classification than chickpea (Figures 3.8C and 3.8D). It was observed that the accumulated flour disappeared when a certain mass of powder accumulated and fell due to its weight. This also occurred for equilibrated chickpea flour at RH30, where the use of a larger batch size resulted in an increased coarse fraction yield, while the fine fraction yield remained unaffected (Figure 3.7). For larger scale systems the yield of the coarse fraction is thus expected to be higher.

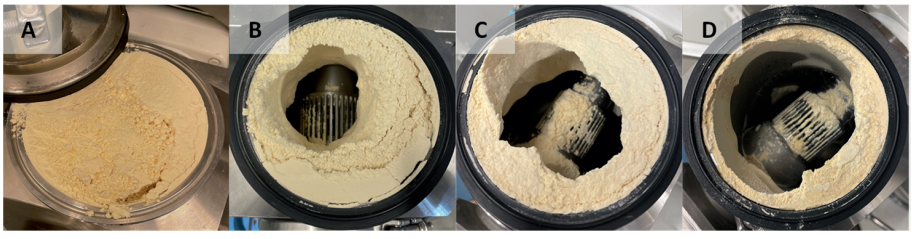


Figure 3.8: Examples of accumulation of flour in the classifier chamber upon air classification for chickpea equilibrated at RH70 milled at 40 m3/h (A) and 52 m3/h (B), direct air-classified chickpea milled at 52 m3/h (C) and yellow pea milled at 52 m3/h (D).

To enable cross-comparison of the overall process in terms of yield and protein content, we consider protein recovery, which is defined as the ratio between the protein in the fine fraction and protein in the grits (Equation 3.2). In the most ideal scenario, one would like to have both high protein content and high protein recovery. The milling yield, fine fraction yield and the protein content of the fine fraction all influence the protein recovery. The protein content was plotted against the protein recovery, where a horizontal black dashed line was used to separate the chickpea results (bottom part, protein content <52.5%DM) and the yellow pea results (upper part, protein content >52.5%DM) (Figure 3.9). The protein recovery of yellow pea increased with a higher airflow during milling, without any significant compromise on the protein content (upper part of Figure 3.9). This higher protein recovery was attributed to the elevated milling yields of yellow pea milled at a higher airflow (52 m^3/h). The protein recovery was also comparable to earlier observations for yellow pea milled at 40 m^3/h (49%) (Xing et al., 2020). No clear impact of relative humidity on protein recovery was seen for yellow pea.

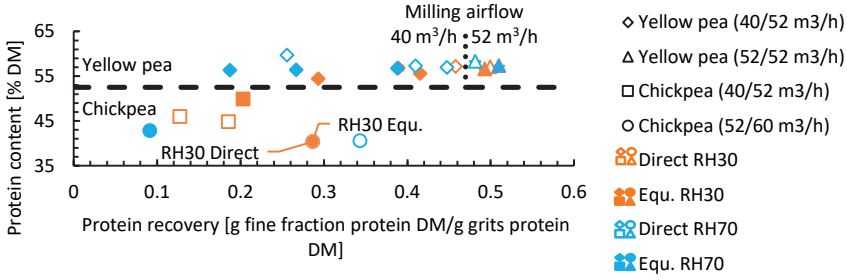


Figure 3.9: Protein content and protein recovery of yellow pea (above black dashed line) and chickpea (below black dashed line). Open symbols represent directly milled and air-classified samples, and closed symbols represent equilibrated samples. Samples with a target RH of 30% are coloured orange and samples with a target RH of 70% are coloured light blue. The shapes in the legend specify the crop and airflow used, which is shown between brackets: the first value gives the milling airflow (40 or 52 m3/h), and the second value gives the air classification airflow (52 or 60 m3/h). The milling airflows of yellow pea are separated with the small dotted line (left 40 m3/h, right 52 m3/h) The error bars represent the protein content standard deviation. Two symbols of chickpea overlapped, which are highlighted with data labels.

A higher milling and air classification airflow improved the protein recovery of chickpea and slightly reduced the protein content (bottom part of Figure 3.9). The protein recovery of chickpea milled (52 m^3/h) and air-classified (60 m^3/h) at RH30 or direct at RH70 was at the higher end of values reported in literature (11-31%) as summarised by (Boukid, 2021). However, the protein recovery of chickpea decreased upon equilibration of the flour at RH70, while the protein content remained similar. In the case of chickpea,

the lower protein recovery was caused by a low fine fraction yield. The fine fraction yield was lower as more mass accumulated in the classifier chamber after equilibration, as illustrated in Figures 3.8B and 3.8C. Thus, the storage of chickpea flour at a high relative humidity negatively affected the overall protein recovery.

3.3.4 Lower flowability of chickpea flour than yellow pea flour

In general, the air classification yields, and protein recoveries were lower for chickpea than for yellow pea, which was consistent with the fouling visually observed after the air classification of chickpea. Based on the locations of the fouled material, it is proposed that when chickpea flour enters the classifier chamber through the feed inlet it accumulates at the inlet at the top of the chamber, instead of being directly dispersed and carried by the airflow for classification (Figure 3.8). In previous research, the accumulation of flour and the impaired separation have been related to a higher cohesion, and consequently poorer flowability of legume powders with higher oil contents (Gueguen, 1983; Pelgrom et al., 2014; Sosulski & Youngs, 1979).

In fluidized systems, such as air classifiers, cohesive powders can accumulate in corners or on surfaces within the equipment (Krantz et al., 2009). Powder rheology was used to describe if chickpea flour indeed has a higher tendency for particle cohesion than yellow pea flour. The torque responses over time for the equilibrated flours at RH70 are shown in Figure 3.10. An overview of all individual measurements, at RH30 and RH70 for both the direct and equilibrated samples, is shown in the Appendix (Appendix figure 3.6). A higher torque response means that the impeller requires more force for the same movement, which indicates a poorer flowability.

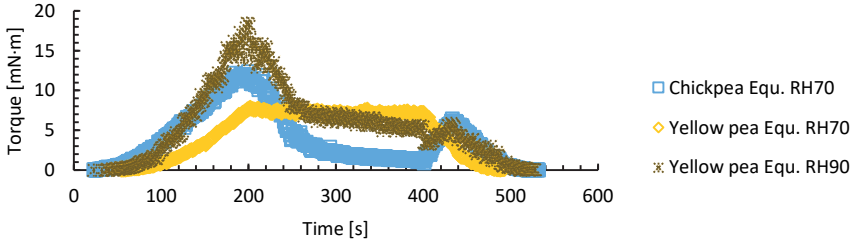


Figure 3.10: Average measurement of powders' resistance to flow with the Anton Paar powder flow cell for yellow pea equilibrated at RH70 (gold), chickpea equilibrated at RH70 (light blue), and yellow pea equilibrated at RH90 (dark gold).

The torque response of the chickpea sample rises more steeply than that of the yellow pea sample under similar conditions. This follows the hypothesis of higher cohesiveness. However, instead of a stable plateau value, the torque declines between 200 and 400 seconds, which coincides with the visual observation of heap formation at the side of the measurement cylinder. This suggests that variations occur in the amount of material that is displaced by the impeller, which directly affects the measurements. The average height of the torque response alone might thus not be sufficient to capture the flow behaviour of cohesive powders. It was, therefore, decided to calculate and compare several characteristic values; a) the area under the curve up to 200 seconds (referred to as Area I), b) the average plateau value between 200 and 400 seconds, c) the difference in torque between the initial average of the plateau (p_i) (200-205 seconds) and the final average of the plateau (p_f) (395-400 seconds), d) the area under the curve between 400 and 600 seconds (referred to as Area III) and e) the difference between Area I and Area III. The determined values for the different samples are summarised in Table 3.2. The values are only used for a semi-quantitative comparison, as they are not related to a physical quantity such as cohesion strength or viscosity. Progress has been made to estimate the shear stress and shear strain, i.e. physical quantities, inside FT4 powder testers by combining DEM simulations and experiments (Hare & Ghadiri, 2017; Khala et al., 2022). However, a qualitative comparison of measured values is still standard for powder rheometers (Francia et al., 2021).

Table 3.2: Averages and standard deviation from rheological cohesion measurements for yellow pea and chickpea at different storage conditions. The dotted lines separate the different storage conditions, an additional sample was added for yellow pea equilibrated at RH90. Different letters indicate significantly different samples. Bold highlights the higher Area I and the difference in plateau value (pi-pf). These samples also showed a decreased air classification performance.

Crop	Condition	Area I [mNm·s]	Average plateau value [mNm]	$p_i - p_f$ [mNm]	Area III [mNm·s]	Area I - Area III [mNm·s]
Yellow pea	Direct RH30	454 ± 16^{a}	10.9 ± 0.1^{de}	0.0 ± 0.3^{a}	182 ± 26^{a}	271 ± 10^{ab}
	Equ. Flour RH30	453 ± 41^{a}	9.6 ± 0.6^{cde}	0.8 ± 0.8^{a}	250 ± 24^{a}	203 ± 17^{a}
	Direct RH70	463 ± 14^{a}	8.55 ± 0.7^{cd}	1.0 ± 1.2^{a}	233 ± 62^{a}	231 ± 58^{a}
	Equ. Flour RH70	389 ± 57^{a}	7.3 ± 0.9^{bc}	0.5 ± 0.3^{a}	214 ± 54^{a}	175 ± 15^{a}
	Equ. Flour RH90	**1014 ± 29^{c}**	7.9 ± 0.1^{bcd}	**10.6 ± 1.3^{b}**	315 ± 58^{a}	699 ± 29^{e}
Chickpea	Direct RH30	652 ± 4^{b}	12.1 ± 0.1^{e}	0.0 ± 0.2^{a}	295 ± 4^{a}	357 ± 1^{bc}
	Equ. Flour RH30	667 ± 10^{b}	11.0 ± 0.3^{de}	-1.1 ± 0.8^{a}	259 ± 11^{a}	409 ± 1^{c}
	Direct RH70	679 ± 36^{b}	11.9 ± 0.1^{e}	0.7 ± 1.8^{a}	323 ± 32^{a}	356 ± 4^{bc}
	Equ. Flour RH70	**907 ± 46^{c}**	3.7 ± 0.4^{a}	**9.9 ± 1.9^{b}**	325 ± 64^{a}	582 ± 18^{d}

The initial torque response (area I) was lower for yellow pea than for chickpea (Table 3.2). For yellow pea, there were no differences between yellow pea samples milled or stored at RH30 and RH70, while for chickpea the sample equilibrated at RH70 showed a higher Area I than the other chickpea samples. The higher torque measured for chickpea equilibrated at RH70 may be related to liquid bridging between the particles. If the particles are in a dry state, there is only weak cohesion between particles via Van der Waals forces. Upon an increased liquid content, the powder will enter the pendular state. In the pendular state particles cluster by directly adhering to each other due to the wetting forces exerted by capillary bridges (Danov et al., 2018; Strauch & Herminghaus, 2012). In the case of equilibrated chickpea flour at RH70, a combination of the oil and elevated moisture content may have encouraged the formation of capillary bridges, which resulted in stronger cohesive forces and thus a higher initial torque response.

In literature, it was observed that storage of a wheat starch protein mixture at RH90 resulted in a lower protein recovery (Dijkink et al., 2007). Therefore, yellow pea flour was also stored at RH90 and subjected to rheological measurements after equilibration at RH90. After equilibration at RH90, the moisture content of the yellow pea flour was 21.3±0.1%, which is comparable to the total liquid content (oil + moisture) of chickpea (21.8±0.3 g/100 g flour). The flowability of yellow pea equilibrated at RH90 (total liquid content 22.3±0.7 g /100 g flour) showed comparable results to chickpea equilibrated at RH70 (Figure 3.10, Table 3.2), which indicates that the total liquid content of the material can affect the powder properties.

The average plateau values were less conclusive between the different flours and storage conditions, apart from the lower value for chickpea flour equilibrated at RH70 (Table 3.2). As previously stated, the impeller dug a hole inside the powder bed, which strongly reduced the torque requirement for continuous stirring at a fixed height. The effect on the torque curve is characterized by $p_i - p_f$, which was, only different for equilibrated chickpea flour at RH70 and yellow pea flour at RH90, which had also a higher Area I. There are no significant differences between yellow pea and chickpea flour samples when comparing Area III, which means that the difference between Area I and Area III does not provide any other benefit over analysing Area I.

Overall, the rheological measurements showed that chickpea flour has a lower flowability and thus higher cohesion than yellow pea flour at similar storage conditions, especially after storage at RH70. To improve the air classification of chickpea flour, one can alter process-related parameters (e.g. airflow, humidity, classifier wheel speed) and

the material properties (e.g. by de-oiling, toasting or addition of flowability aids) (Dijkink et al., 2007; Doğan, Aslan, Gürmeriç, Özgür, & Göksel Saraç, 2019; Kim, Xiao, & Pearce, 2005; Pelgrom et al., 2014; Pelgrom et al., 2015b; Pelgrom et al., 2015c). Toasting or increasing the classifier wheel speed may have the disadvantage that oil is released from within the particles to the surface, which reduces the flowability (Doğan et al., 2019; Kim et al., 2005; Pelgrom et al., 2014). To evaluate if the powder properties improve by a pre-treatment, a comparison of the torque responses in Area I is most useful. However, these results are ideally supplemented with the difference in plateau values ($p_i - p_f$), to provide more information on the powder bed behaviour inside the measurement chamber of the flow cell. This is especially relevant if the influence of heap formation on the torque response in Area I is not known.

3.4 Conclusion

The effect of relative humidity on milling and air classification of yellow pea and chickpea was systematically investigated by varying the humidity. The milling yield was not influenced by the relative humidity in the tested range (30-70%) as drying of the material in the mill overshadowed this possible effect. Particle agglomeration, dispersion and flowability were assessed via particle size analysis and powder rheology, and subsequently linked to the observed air classification performance.

Small particles were better dispersible when milled at a higher airflow (52 m^3/h versus 40 m^3/h), which resulted in higher protein recoveries for both yellow pea and chickpea. The dispersive index (DI) (particles <10 µm) was more affected by the equilibration conditions than the degree of de-agglomeration (based on DV_{50}), especially at higher humidity (70%), where the DI decreased after equilibration. Equilibration at a humidity of 30% did not affect the separation performance in terms of yield, purity, and protein recovery, while equilibration of chickpea flour at RH70 was detrimental for the fraction yields and protein recovery in the fine fraction. Although yellow pea flour equilibrated at RH70 had a slightly lower DI value, the overall air classification performance remained unaffected. Still, a lower DI shows that there is a risk for a decrease in air classification performance. Next to particle dispersion, other factors are still important for the protein recovery upon air classification, such as airflow, classifier wheel speed and difference in size between the materials.

Powder flowability was studied with powder rheology and we evaluated which parameters were most relevant to define differences in flowability. The area of the

torque in the descending time region (Area I) was most useful to conclude on flowability differences. However, if the effect of heap formation on the torque response in this area is unknown, it is relevant to supplement this with the difference in initial- and final plateau value. Overall, chickpea flour had a worse flowability than yellow pea flour under similar conditions, especially after storage at RH70. Equilibration of yellow pea at RH90 resulted in more comparable total liquid contents (oil + moisture) and a similar rheological profile as chickpea equilibration at RH70. The combined oil and elevated moisture content may have encouraged the formation of capillary bridges and the change of a dry powder to a powder in the pendular state, which results in stronger cohesive forces. The flours with lower flowability showed a decrease in protein recovery after air classification.

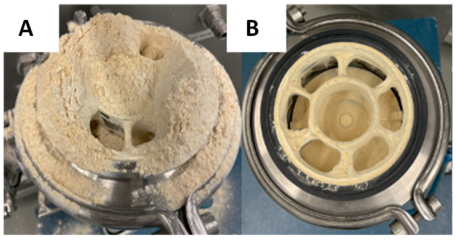
Overall, the dispersive index, particle shape and rheological properties could be used to explain the differences in air classification performance. Using the rheological properties together with particle size and shape analysis as qualitative indicators for the process performance will help industry with the search towards obtaining optimal protein recoveries. Extremer conditions resulted in a decreased dispersive index, agglomerated particles, and a lower powder flowability. A lower flowability and agglomerated particles were detrimental to the air classification process, which results in more fouling and consequently a high loss of material. So, a humidity above 70% should be prevented for robust air classification.

Appendix 3

Appendix table 3.1: Protein and starch contents of yellow pea and chickpea grits, flour and obtained fractions for direct, equilibrated (Equ.) flour and grits at RH30 and RH70. Values are presented as mean ± standard deviation. n represents the number of repetitions of dry fractionation experiments, which was n=3 unless specified otherwise. The settings with the largest difference in moisture content (Direct RH30 and Equ. Flour RH70) are highlighted bold. RH30 and RH70 are separated by a dashed line for each combination of air flows upon milling and air classification (AC).

	Air flow	Direct/Equ. humidity	Protein content (%DM)				Starch content (%DM)		
			Grits	Flour	Fine fraction	Coarse fraction	Flour	Fine fraction	Coarse fraction
Yellow pea	Milling 40 m³/h AC 52 m³/h	**Direct RH30**	25.8± 0.5	**37.9 ± 0.8**	**56.7±0.5**	**19.7±2.2**	**34.0±2.5**	**4.1±0.7**	**57.0±4.7**
		Equ. grits RH30		40.4±5.1	58.2±2.0	30.8±8.8	29.5±7.7	2.6±0.9	50.4±8.6
		Equ. flour RH30		37.9±0.8	56.3±2.4	31.4±9.9	34.0±2.5	3.1±0.7	41.3±17.0
		Direct RH70		40.3±2.3	58.0±1.5	22.8±7.3	27.3±6.3	3.2±1.2	52.6±10.3
		Equ. grits RH70		41.9±1.6	58.9±1.2	30.6±2.0	26.7±1.7	2.0±1.1	44.4±2.9
		Equ. flour RH70		**40.3±2.3**	**56.5±0.2**	**25.5±1.7**	**27.3±6.3**	**3.4±0.9**	**52.1±1.5**
	Milling 52 m³/h AC 52 m³/h	**Direct RH30**		**26.7±0.8***	**57.1±0.1***	**15.0±0.1***	**44.4±0.2***	**2.9±0***	**66.0±1.1***
		Equ. flour RH30		28.2±0.1*	56.6±0.2*	13.9±0.1*	41.0±0.6*	3.2±0*	66.0±0.6*
		Direct RH70		28.2±0*	58.3±0*	15.4±0.1*	49.0±0*	3.5±0*	66.1±0*
		Equ. flour RH70		**29.3±0***	**57.3±0***	**17.4±0.1***	**49.0±0***	**3.5±0***	**66.2±0.4***
Chickpea	Milling 40 m³/h AC 52 m³/h	**Direct RH30**	23.8± 0.5	**22.9±0.1***	**45.4±0.8**	**19.3±0.2**	**47.1±0.6***	**1.9±1.4**	**47.8±3.2**
		Equ. flour RH30		22.5±0*	49.9±0.2*	20.5±0.2*	46.8±0.6*	1.8±0*	N.A.
		Direct RH70		23.2±0*	N.A.	N.A.	38.6±0.1*	N.A.	N.A.
		Equ. flour RH70		**23.2±0***	**38.7±0***	**22.8±0***	**43.5±1.0***	**7.2±0.1***	**45.5±0.2***
	Milling 52 m³/h AC 60 m³/h	**Direct RH30**		**24.0±0.2***	**40.35±0***	**18.9±0.1***	**38.4±0.4***	**11.4±0***	**46.8±0***
		Equ. flour RH30		23.8±0.1*	40.4±0.1*	18.2±0*	32.0±0.2*	10.1±0*	47.1±0.2*
		Direct RH70		24.1±0.1*	40.6±0.1*	18.3±0.1*	36.4±0.4*	10.4±0.1*	53.9±0*
		Equ. flour RH70		**24.2±0.1***	**42.8±0.1***	**23.1±0.1***	**36.4±0.4***	**4.7±0***	**46.3±0.4***

*n=1, **n=2



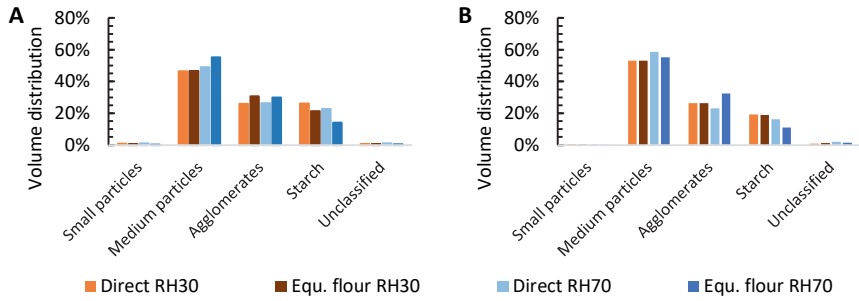
Appendix figure 3.1: Visualisation of insufficient (A) and sufficient emptying of the milling chamber (B).

The particle morphology was measured and analysed by Morphologi 4 (Malvern Instruments, Worcestershire, UK). An extended version of this appendix is provided online with more detailed information on the morphology analysis (Politiek et al., 2023b). The particles were classified into small particles, medium particles, starch, and agglomerates (Appendix table 3.2) by CE diameter, circularity, and convexity.

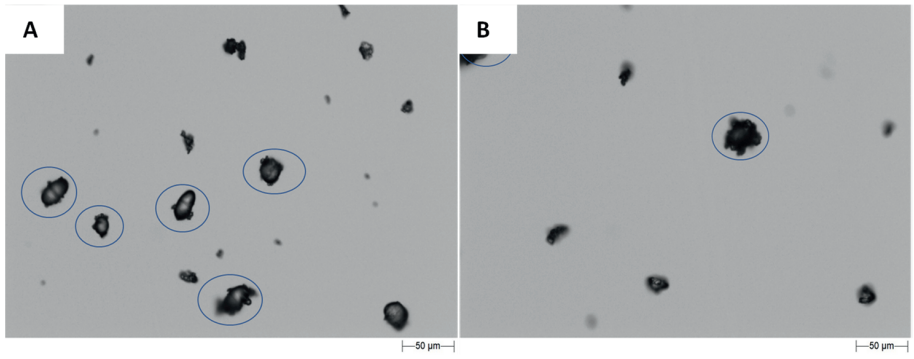
Appendix table 3.2: Classification of particles in morphology analysis by Morphologi 4.

Classification	CE diameter (µm)	Circularity	Convexity
Small particles	<5	-	-
Medium particles	>5	<0.899	>0.853
Agglomerates			<0.853
Starch	5-40	>0.899	-

The slight increase of particle size observed in section 4.3.2 of the manuscript was further confirmed by the particle classification based on morphology where a slight increase of medium particles was observed for yellow pea flour equilibrated at RH70, and an increase in agglomerates was observed for chickpea. The starch classified particles decreased for both yellow pea and chickpea after equilibration of the flour at RH70 (Appendix figure 3.2). The volume of starch classified particles was not consistent with the measured starch contents (Appendix table 3.1), which is likely caused by starch particles that are surrounded by other particles or unreleased starch particles. Visual observation showed that upon equilibration at RH70, an increased amount of starch granules was surrounded by small particles (e.g. protein bodies), which are circled in blue for both yellow pea and chickpea in Appendix figure 3.3, which explains the decrease in particles classified as starch particles in Appendix figure 3.2.

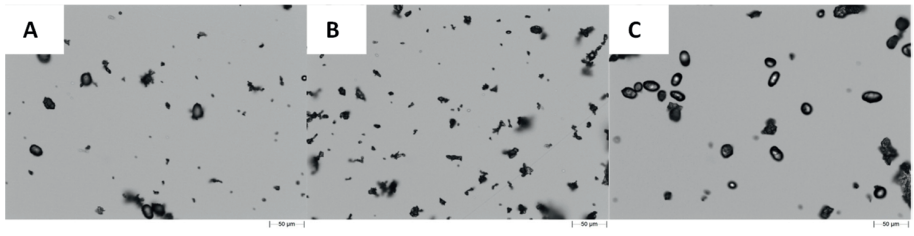


Appendix figure 3.2: Morphology classification based on Appendix table 3.2 of yellow pea and chickpea milled with an airflow of 52 m3/h. The volume distribution is given for small particles, medium particles, agglomerates, starch and unclassified particles.

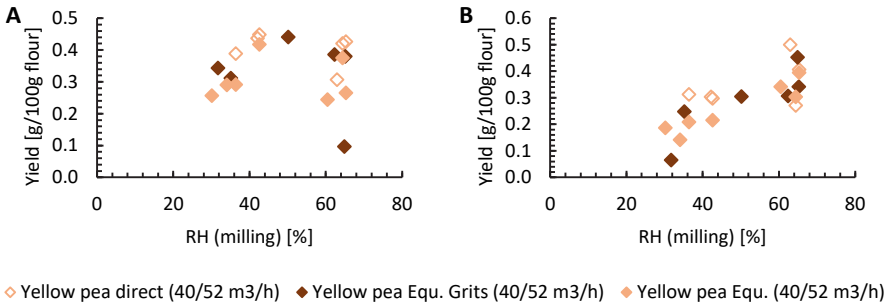


Appendix figure 3.3: Picture of yellow pea (A) and chickpea (B) milled at 52 m3/h and equilibrated flour at RH70. Blue circles indicate starch granules surrounded by small particles.

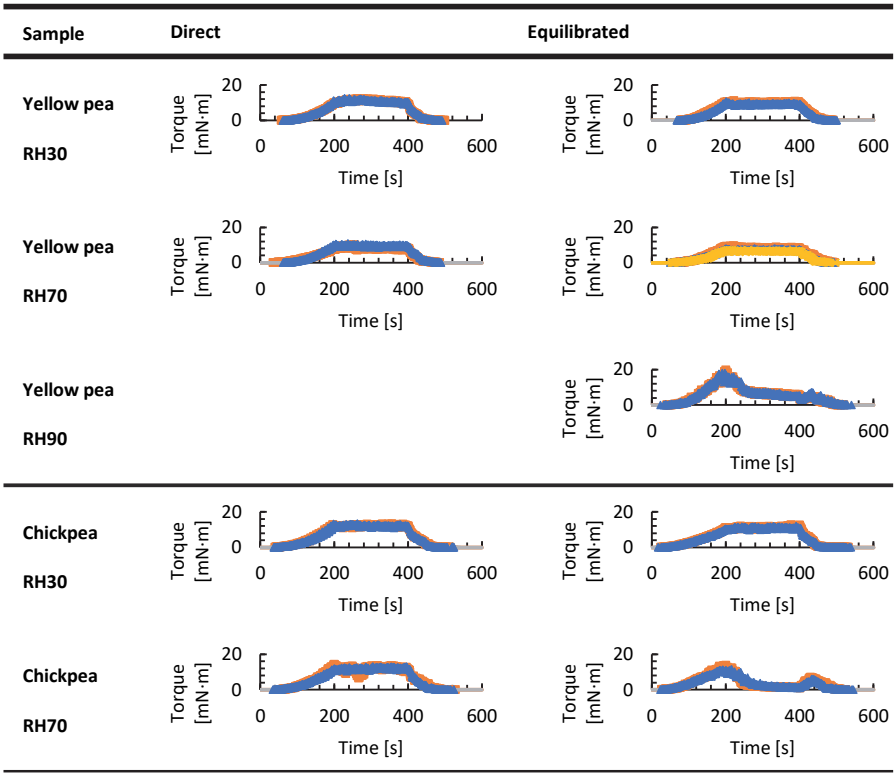
Appendix figure 3.4 shows an example of yellow pea flour milled and air classified at RH30, where the round particles represent starch granules. The images and combined size and shape analysis confirmed that the coarse fraction was enriched with starch and the fine fraction was depleted of starch.



Appendix figure 3.4: Visualisation of yellow pea flour (A) and the fine (B) and coarse fraction (C) after milling and direct air classification at RH30. The scale bar indicates 50 µm.



Appendix figure 3.5: Fine fraction yield (A) and coarse fraction yield (B) of yellow pea (milling air flow 40 m3/h air classification air flow 52 m3/h) against actual average relative humidity (RH) during air classification for different equilibration conditions identified in the legend. Equilibrated yellow pea grits had similar milling yields as yellow pea grits that were directly milled at these settings (milling yields 21-43%).



Appendix figure 3.6: Measurement of powders' resistance to flow with Anton paar powder flow cell for yellow pea and chickpea (milling air flow 52 m3/h) under different humidity conditions. Distinct colours indicate duplicates or triplicates.

Chapter 4

Comparing electrostatic separation of soy and lupin: Effect of de-oiling by solvent extraction

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Abstract

Electrostatic separation is a sustainable dry separation technique based on triboelectric charging of different cellular tissue components (i.e. protein bodies and fibres). This research aimed to determine the mechanism behind the ineffective electrostatic separation of soy and if it could be altered by de-oiling. Several scenarios were compared, which involved electrostatic separation of soy and lupin de-oiled with different solvents (none, acetone, ethanol and hexane). Separation of lupin resulted in a higher true protein content (58.5%DM (dry matter) (Nfactor=5.7)) than separation of soy (45.0%DM protein). Separation was less effective for soy because its protein bodies were still embedded in the cellular structure after impact milling, which was also reflected in the larger particle size and lower small particle dispersibility. De-oiling soy with hexane and extra milling improved the purity of soy protein-enriched fractions (59.6%DM protein) reaching more similar purity as for lupin protein-enriched fractions. The protein purity of lupin fractions could be increased most via the use of polar solvents (acetone and ethanol). Better electrostatic separation after de-oiling and extra milling for soy could be explained by increased liberated protein bodies, which was key towards the improved separation of soy obtaining protein purities closer to that of pure protein bodies.

4.1 Introduction

The production of protein ingredients from pulses, grains and oilseeds is traditionally based on wet fractionation techniques. Wet fractionation processes are applied to produce protein isolates (>90%), but most foods such as emulsions, foams, gels or doughs only require moderate protein concentrations (Tabtabaei et al., 2016a). Dry fractionation is an attractive sustainable alternative to produce ingredient concentrates, as it uses no water, requires less energy and maintains (native) protein structure and protein functionality (Assatory et al., 2019; Schutyser & van der Goot, 2011; Vogelsang-o'Dwyer et al., 2020; H.G. Zhu et al., 2021a). Traditional dry separation techniques are sieving and air classification, which are based on separation by particle size and density. Electrostatic separation is a newer dry separation technique for food materials to enrich components such as protein and fibre from finely milled legume flours (Vitelli et al., 2020). The separation is based on the triboelectric charging properties of the particles. The charge is induced by particle-particle and particle-wall interactions, where particle-particle interactions were found to dominate the charging process in turbulent airflows (Landauer et al., 2019; Xing et al., 2021). The charging of particles present in the milled flour (i.e. starch, fibre or protein) and subsequent separation behaviour is very much composition-dependent (Pelgrom et al., 2015c). The presence of a large amount of starch can impair the protein enrichment via electrostatic separation as starch granules obtain a similar charge as protein bodies (Xing et al., 2020). Oilseeds usually have low starch contents and the present fibres and proteins charge differently. Therefore, electrostatic separation is a promising technique for oilseeds such as lupin, soy, rapeseed and sunflower seeds and their de-oiled cakes and meals to obtain protein-enriched ingredients for the food industry (Barakat et al., 2015; Basset et al., 2016; Kdidi et al., 2019; Kuspangaliyeva et al., 2023; Laguna et al., 2018; Pelgrom et al., 2015c; J. Wang, et al., 2016b; Xing et al., 2018, 2021). This study will focus on two oil-rich seeds, i.e. lupin, being cultivated in more temperate climate, and soy, usually requiring more warm climates for cultivation.

To achieve successful electrostatic separation, it is required to (a) release individual cellular components (i.e., fibre, protein) upon milling, (b) have components that acquire opposite tribo-charges and (c) obtain air-dispersible components. The air dispersibility can be measured by using a pressure titration method (Jaffari et al., 2013). The ratio between the percentage of small particles at a lower pressure and at the highest dispersion pressure results in a relative dispersive index, where materials with a higher relative dispersive index are better air-dispersible. The relative dispersive index of small

particles (i.e., protein bodies) was lower for materials with a higher oil content, caused by stronger cohesive forces due to liquid bridging by oil (Fitzpatrick et al., 2007; Politiek et al., 2022; Politiek et al., 2023b; Rennie et al., 1999). To improve the particle dispersibility, oil can be removed via mechanical de-oiling or solvent extraction prior to electrostatic separation. Examples of solvents that are in principle allowed for food production are hexane, butane, ethyl acetate, ethanol, carbon dioxide and acetone (Carre, 2021; European Parliament & Council of the European Union, 2009). So far, studies on de-oiling and subsequent electrostatic separation focussed on mechanical de-oiling and solvent extraction with petroleum ether or hexane (Table 1.1). However, especially hexane is also listed as an undesirable solvent based on safety and environmental classifications (Alfonsi et al., 2008). Polar solvents may be more preferred, but information on the use of these solvents for de-oiling prior to electrostatic separation is lacking (Alfonsi et al., 2008; Moldoveanu & David, 2015). Therefore, in this study we used two solvents with high polarity (acetone and ethanol) and compared it to hexane. An advantage of using these two more polar solvents is that they are listed as preferred solvents compared to hexane based on safety and environmental classifications (Alfonsi et al., 2008).

Many factors can influence tribo-charging, which include the material properties (e.g. composition, particle size, shape, surface roughness, relative humidity, dispersibility and chargeability) and the collision conditions in the electrostatic separator design (Karner & Anne Urbanetz, 2011; Landauer & Foerst, 2019; H.G. Zhu et al., 2021a). The particle size varies with the milling intensity, where a higher milling intensity results in smaller particles. For choosing the milling settings it is important that the protein particles are disentangled from the other particles present in the cell matrix, as this results in more effective electrostatic separation (Thomas et al., 2023). Too fine milling should be avoided as this can impair protein enrichment by electrostatic separation due to the agglomeration of small particles (J. Wang et al., 2016b). Several studies have used different types of mills and milling intensities for particle size reduction of legumes prior to electrostatic separation, the results from these studies underpin the importance of milling prior to tribo-electrostatic separation and optimising the milling settings for dry separation purposes (Kdidi et al., 2019; Laguna et al., 2018; Thomas et al., 2023; Vitelli et al., 2020; J. Wang et al., 2016b; Xing et al., 2018). In the present study, industrially impact milled flours are used, which are tailored for dry separation purposes.

As many factors can influence tribo-electrostatic separation it is rather difficult to evaluate the electrostatic separation performance of soy and lupin via literature alone,

as different separator designs were used, the materials were pre-treated differently (i.e. de-oiled or not de-oiled), and the particle size could have been too big or too small. Based on the available literature, it is expected that the separation of soy will be less effective based on the lower reported protein enrichments (protein enrichment 5.6% on a dry basis (DM) or 14.4 %DM) than the separation of lupin (protein enrichment of 60.5 %DM) (Kuspangaliyeva et al., 2023; J. Wang et al., 2016b; Xing et al., 2018). However, reason why the separation of soy is less effective and if de-oiling with for example different solvents could improve the separation remains unclear.

Therefore, this research aims to determine the mechanism behind the ineffective separation of soy and if it could be altered by de-oiling. For this, we compare the electrostatic separation of commercially available toasted lupin- and toasted soy flour and the separation of de-oiled lupin and soy flour with acetone, ethanol and hexane. We used two electrostatic separator designs in this study. A simple horizontal electrostatic separator to evaluate the protein purity and charge at the electrodes and a modified vertical electrostatic separator to also evaluate yield. Because the presence of oil can impair the dispersion of particles we used the *venturi* principle to disperse the flours and thus better enable dry separation of materials with higher oil contents. First, we evaluate the electrostatic separation performance of the non-de-oiled lupin and soy flours with both electrostatic separation set-ups and we further investigate the material structure with scanning electron microscopy. Then we evaluate the effect of de-oiling on the material properties (composition, particle size, dispersive index and particle chargeability) and the electrostatic separation performance after de-oiling (yield, protein content and protein enrichment).

4.2 Materials and methods

4.2.1 Materials

Toasted de-hulled impact milled soy flour and toasted de-hulled impact milled lupin flour were kindly provided by Frank Foods (Twello, the Netherlands). These materials were stored in tightly screw-capped polyethylene containers at 4 °C. Acetone and n-hexane were obtained from Actu-all Chemicals (Oss, The Netherlands). Ethanol (96% v/v) was obtained from VWR International (Rosny-sous-Bois, France). Petroleum ether was obtained from Avantor Performance Materials B.V. (J.T. Baker, Deventer, The Netherlands).

4.2.2 De-oiling and milling

A custom-built Soxhlet extractor was used to de-oil 400-500 g of impact milled soy and lupin flours. The materials were de-oiled for 6 h with either hexane, ethanol, or acetone. The de-oiled flours were left in the fume hood overnight to fully evaporate the residual solvent (Xing et al., 2018). After de-oiling, powder lumps were broken up by milling with a Pulverisette 5 bead mill (Fritsch GmbH, Germany). This batchwise operating mill was chosen to minimize losses of material and to keep the material composition as similar as possible before and after milling. Approximately 150 g of material was put into a 500 ml bowl filled with 24 beads with a diameter of 17 mm. The material was milled for a total of 4 minutes at 400 rpm, with one minute on and off to prevent heating of the flour (Palavecino et al., 2019).

4.2.3 Electrostatic separation

Two custom-built electrostatic separators were used, referred to as the horizontal set-up and the vertical setup. The horizontal set-up has been described previously by J. Wang et al. (2015b). The tribo-charging unit used in this study was a round stainless-steel tube with a length of 0.2 m and an inner diameter of 16 mm. The electrodes were mounted next to each other on a horizontal plate (Figure 4.2C) and the distance between the electrodes was 4 cm. A voltage of 5 kV was set on the positive electrode (PE-H) and -5 kV was set on the negative electrode (NE-H), which resulted in an electric field strength of 250 kV/m. The feed rate was 2.9 ± 1.1 kg/h and the flow rate of the carrier nitrogen was 158 L/min. Each time 20 g of material was fed into the system. The charging tube was cleaned between experiments by passing a brush through the tube. Additionally, when switching to new material, the first pass of the new material was discarded. In this study, the current (µA) was measured every second directly at both electrodes with a squirrel data logger. The measured current was positive for the positive electrode and negative for the negative electrode. The specific charge [µC/g] is calculated as the inverse of the area between the base line and the measured current [µC], divided by the amount of product collected at the corresponding electrode [g] (Figure 4.1, Equation 4.1).

$$\text{Specific charge [µC/gram]} = \frac{-\sum_{t=0\,[s]}^{t=n\,[s]} (\text{Measured current [µA]-baseline [µA]})}{\text{Product collected at plate [g]}} \qquad (4.1)$$

Electrostatic separation with the horizontal set-up yields two fractions, one at the negative electrode (NE-H) and one at the positive electrode (PE-H) (Figure 4.2A, 4.2B). The particles that were not collected at either of the electrodes are considered as loss. This loss contains particles that fell to the bottom of the separation chamber due to

gravity before being collected at either of the electrodes and particles that were not charged or did not charge sufficiently to be collected at either of the electrodes. Separation with the horizontal set-up was done in triplicate and fractions were stored in closed containers at 4°C.

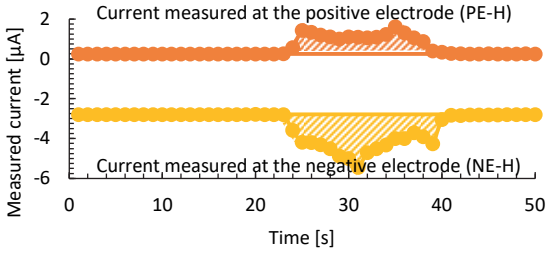

Figure 4.1: Example of a measurement of the current with the horizontal set-up. The total charge [µC] is the inverse of the area between the baseline and the measured current. The charge per gram of product [µC/g] is calculated with Equation 4.1.

For the adapted vertical set-up, the feed supply to the separation chamber was modified from the previous set-up used in our group (J. Wang et al., 2015a; Xing et al., 2018). In the adapted design, a venturi principle is used to disperse the flours. The venturi was placed below the feeder as used before and connected to a nitrogen airflow. During operation, first the separation chamber is filled with nitrogen gas. Subsequently, the material is fed perpendicular to the nitrogen flow and dispersed in the airflow by the generated under pressure (Figure 4.2D). The tribo-charging tube is made of stainless steel and has an inner diameter of 5 mm (Figure 4.2E). The flow rate of the carrier nitrogen was set at 30 L/min.

For the vertical set-up, the voltage on the positive electrode was set to 20 kV and the distance between the electrodes was 10 cm, which resulted in a field strength of 200 kV/m (Xing et al., 2018). For each separation, 100 g of material was dosed (feed rate 0.5 kg/h) by a screw feeder into the system. Between experiments, the funnel and the charging tube were cleaned with a vacuum cleaner and compressed air. In the vertical set-up four fractions are obtained after separation. Material that did not deposit at either of the electrodes was collected in the collector bags at the bottom of the vertical separator (Figure 4.2D, 4.2E). The four fractions were labelled as ground electrode (GE-V), positive electrode (PE-V), ground collector (GC-V) and positive collector (PC-V) (Figure 4.2D, 4.2E). The experiments with the vertical separator were performed in duplicate and the fractions were stored in close containers at 4°C.

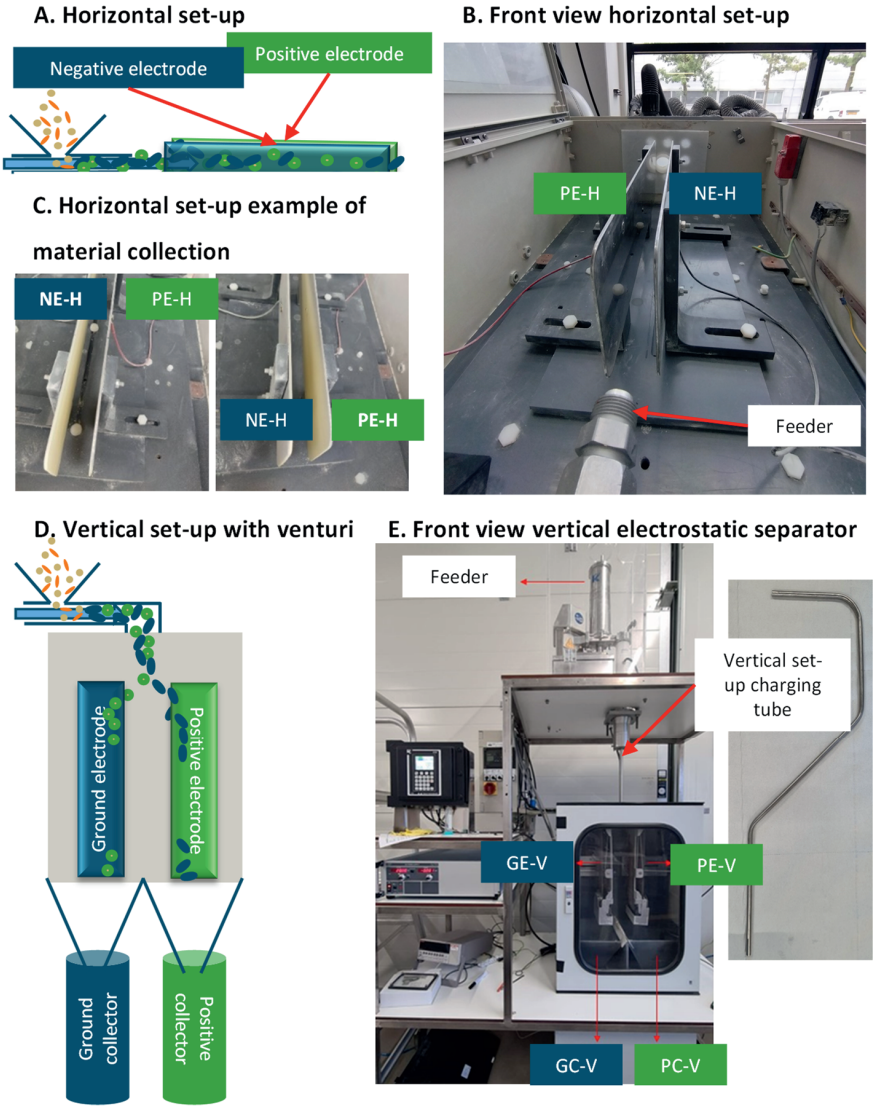

Figure 4.2: Illustration of horizontal set-up (A), the front view (B), an example of material collection from the top view (C). Schematical illustration of the vertical set-up (D) and front view and charging tube that connects the venturi and the separator chamber (E). The light blue arrow indicates the direction of the N-flow through the systems, which was perpendicular (A) or parallel (B) to the material feed flow. The particles are charged in the charging tube and collected at the oppositely charged electrode. Material was collected at the negative electrode (NE-H), ground electrode (GE-V), positive electrode (PE-H/PE-V), ground collector bag (GC-V) and positive collector bag (PC-V).

Advantages of the horizontal set-up over the vertical set-up are the possibility to measure the deposited charge on the electrodes and that the protein purity of the

enriched fraction can be evaluated with a smaller sample size. This set-up delivers the highest purity. A disadvantage of the horizontal set-up is that shielding of the electrodes might occur because the material remains on the electrode, which results in a limit to the amount of material that can be obtained making yield determination difficult. Conveyor belts are installed in the vertical set-up to systematically collect the material from the electrodes. Material that is not deposited at either of the electrodes is collected in collector bags below the electrodes. More feed can be used than with the horizontal set-up and both the purity and yield can be evaluated. Both set-ups were used next to each-other to evaluate the protein purity, charge at the electrodes and fraction yield.

4.2.4 Compositional analysis

The dry matter content was determined by using an air-oven method to dry approximately 1 g of material overnight at 105 °C (AACC Method 44-15.02). The protein content was determined with Dumas analysis with a rapid N exceed protein analyser (Elementar, Germany). The sample weight was 140±10 mg. A protein conversion factor of N x 5.7 was used and protein contents are reported on a dry weight basis (Maclean et al., 2003). Protein enrichment was calculated with Equation 4.2 and protein recovery was calculated with Equation 4.3.

$$\text{Protein enrichment [\%DM]} = \frac{\text{protein fraction [g/100 g DM\%]} - \text{protein flour [g/100 g DM\%]}}{\text{protein content flour [g/100 g DM\%]}} \cdot 100\% \quad (4.2)$$

$$\text{Protein recovery [\%]} = \frac{\text{protein in fraction [g]}}{\text{protein in flour [g]}} \cdot 100\% \quad (4.3)$$

The residual oil content was determined with a continuous Soxhlet extraction method (SOX416, Soxtherm, Germany) with an excess amount of solvent (Petroleum Ether 40-65 °C). Hot extraction (25 min) was followed by solvent evaporation (5 cycles), extraction via refluxing the condensed solvent (1h 35 min), evaporation (3 cycles) and drying (30 min). After extraction, the samples were dried at room temperature overnight and the oil content on a dry basis was calculated as the extracted oil divided by the dry sample weight. The oil contents of lupin and soy were measured in duplicate at the start of the experiments and after one year. The oil content did not change over time (P-value>0.05).

The concentration of small sugars up to a degree of polymerisation (DP) of 5 as determined by using high-performance liquid chromatography (HPLC) (Ultimate 3000 HPLC, ThermoFisher, USA) coupled to a refractive index (RI) detector (Shodex RI-501). Solutions of 50 mg/mL (w/w) were prepared, of which 10 µL was injected into the column

(Shodex KS-802 8.0 × 300 mm, column temperature 50 °C) with deionized water (Milli-Q®) as an eluent with a flow rate of 1 mL/min.

On some occasions, the individual fraction weights were not sufficient (<2 g) for the compositional analysis of the fractions collected with the horizontal set-up. To enable an analysis of these fractions, equivalent fractions were combined. Appendix table 4.1 lists seven fractions that had to be pooled, all other fractions were not pooled and measured in duplicate.

4.2.5 Particle analysis

The particle size distribution of the flours was analysed with a Mastersizer 3000 equipped with an Aero S dry dispersion unit and a standard venturi tube (Malvern Instruments, Worcestershire UK) at different dispersion pressures (400, 200, 50 and 10 kPa). Five measurements were completed per dispersion pressure. The hopper gap was set to 3 mm and the feed rate was set at 30-40% for flowable materials and 80-90% for more cohesive materials to achieve a constant laser obscuration between 0.5 and 20%. It was assumed that full dispersion is achieved at the highest pressure. At lower pressures the particles do not fully disperse and remain agglomerated, which results in a larger measured particle size. The dispersive index (DI) of small particles (below 10 µm) was calculated with Equation 4.4 (Dijkink et al., 2007; Politiek et al., 2022).

$$\text{DI } [-] = \frac{\text{Volume<10µm at pressure i } [\%]}{\text{Volume<10µm at 4 bar } [\%]} \tag{4.4}$$

The flours were also analysed with Scanning Electron Microscopy (SEM). The samples were fixed on the sample holder by using carbon adhesive tabs and coated with gold by a sputter coater (JEOL, Smart-Coater, JEOL USA, Peabody, MA, USA). After coating, SEM images were taken at 10 kV, with a JEOL JCM-7000 (JEOL USA, Peabody, MA, USA). For each sample one montage picture was prepared (field image magnification 300), 3 images at field image magnification 300 and one image at a field image magnification of 100.

4.2.6 Statistical analysis

Data was collected in Excel and statistical analysis of the data was done with SPSS (IBM SPSS Statistics version 28.0.1.1 (15)). Results are displayed as the mean ± standard deviation. Levene's test of equality of error variances was used to test whether the error variance was equal across groups. Then a one-way ANOVA test was carried out. For

groups with homogeneous variance a Tukey post-hoc test was used, and for groups with inhomogeneous variance Games-Howel was used. The significance level used was 0.05.

4.3 Results and Discussion

4.3.1 Electrostatic separation of lupin resulted in a higher protein purity than the separation of soy

Electrostatic separation of non-de-oiled soy and lupin was performed with the two set-ups. A simple horizontal set-up with a negative electrode (NE-H) and positive electrode (PE-H) and a vertical set-up with rotating belts and a brush to collect material from the two electrodes (GE-V and PE-V) and below two collector bags (GC-V and PC-V) to collect non-disposed material. The separation with the horizontal set-up indeed resulted in a higher loss than separation with the vertical set-up, and separation with the horizontal set-up resulted in the collection of protein enriched fractions with a higher protein purity (Figure 4.3A, 4.3B). This can be explained by the design of the separators (H.G. Zhu et al., 2021a), and how the materials are collected (Section 4.2.3). The separation efficiency with the horizontal set-up can be further improved by decreasing the powder loss (Landauer & Foerst, 2018). The vertical set-up allows for a better comparison based on both material yield and purity, which is likely more representative for what can be achieved in continuous industrial systems. With the horizontal set-up it was possible to measure the charge of material deposited on the electrode directly, which is considered as an advantage to gain more insight in the overall process (Yang, Meda, et al., 2022).

The separation performance can be described by fraction yield, protein purity, protein enrichment and protein recovery, which is shown in Figure 4.3 for lupin and soy. The least material was collected at the positive electrode (PE-V and PE-H) for both soy and lupin (Figure 4.3A). Protein was enriched at the ground (GE-V) and negative electrode (NE-H) for both soy and lupin (Figures 4.3B and 4.3C). Most of the protein was recovered in the GE-V, NE-H and GC-V fractions, whereas less protein was recovered at the positive electrodes (PE-V and PE-H) (Figure 4.3D). These results align with the expectation that protein would obtain a positive charge and agglomerate onto the ground and negative electrodes (Basset et al., 2016; Laguna et al., 2018; J. Wang et al., 2016b). The lower yield at the positive electrode can be explained by the larger particle size of the materials collected, where fibres are usually much larger than the protein particles. This aligns with previous research, where the positively charged particles were indeed finer than those of the negatively charged fraction and the yield of negatively charged particles collected at the positive electrode was also lower (Basset et al., 2016; Landauer & Foerst, 2019).

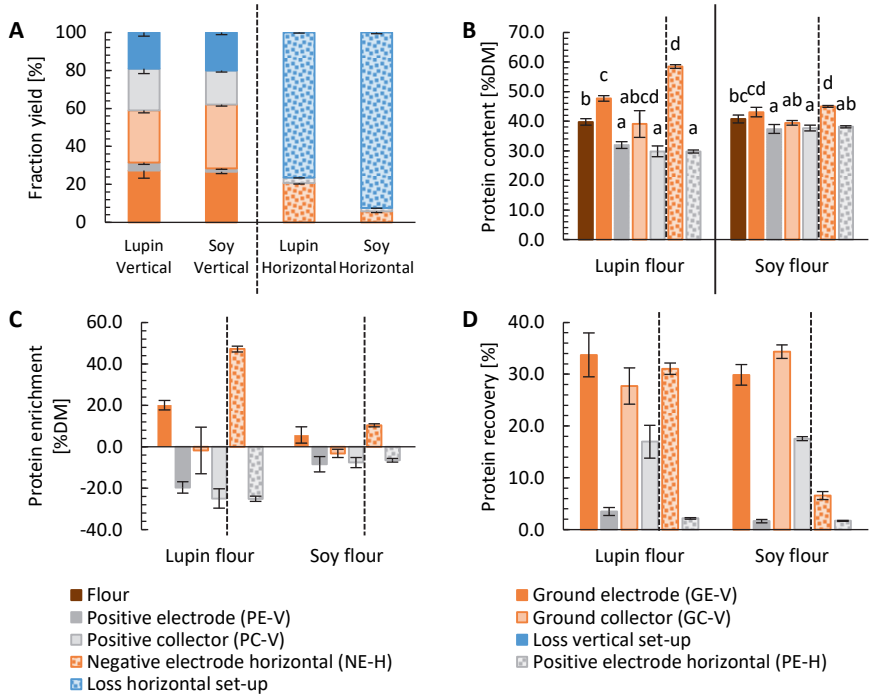

Figure 4.3: Fraction yield (A), protein content (B), where different letters indicate significantly different protein contents (P < 0.05) per material (lupin and soy), protein enrichment (C) and protein recovery (D) of soy and lupin separated with two set-ups (vertical and horizontal) as described in section 4.2.3. Error bars indicate the standard deviation based on three separations, for the fraction yield only the negative error bar is presented.

Electrostatic separation of toasted lupin resulted in a higher protein purity (58.5 %DM) and protein enrichment (47.1 %DM) than electrostatic separation of toasted soy (protein purity 45.0 %DM, protein enrichment 10.3 %DM). The charge measured per gram of product collected at the negative electrode was comparable for both soy (2.2±0.2 µC/g) and lupin (2.4±0.2 µC/g), but the total charge measured was much higher for lupin (9.9±0.8 µC) than for soy (2.9±0.4 µC). This indicates that more lupin particles were charged, which resulted in a higher overall yield, but the charge per collected mass was similar. The lower overall chargeability of soy flour is likely caused by the higher oil content of the material, as oil can have an insulating effect. In addition, the larger particle size of soy (DV_{50}=56.1 µm) than lupin (DV_{50}=40.8 µm) could have contributed to the lower chargeability (i.e., due to a higher gravitational force, lower protein purity or a lower relative charge to mass ratio). Lastly, the small particles in soy flour were less dispersible than the small particles in lupin flour; at 0.5 bar the relative dispersive indices were 0.1%

for soy and 0.5% for lupin, which could be one of the reasons for poorer protein separation of the soy flour.

4.3.2 The soy protein bodies were still trapped in the cellular structure

It is expected that when protein bodies are clearly visible, a protein purity closer to the protein purity of pure protein bodies (70-75 %DM) can be achieved (Plant & Moore, 1983). To visualise the material structures, scanning electron microscopy (SEM) was used. Here, the protein bodies can be distinguished from cell wall material by the shape and surface, where protein bodies are spherical with a smooth surface and cell wall particles have a less defined specific shape with a rough folded surface (Kuspangaliyeva et al., 2023; Xing et al., 2018). The lupin protein bodies were clearly visible as individual particles under the microscope, even though some of the protein bodies were a bit clustered (Figure 4.4A). The protein bodies of soy were less clearly distinguishable and the visible clusters were denser in structure (Figure 4.4B). As the soy protein bodies were less detached and the particles were larger and less dispersible (Section 3.1), it was difficult to achieve effective protein enrichment (Laguna et al., 2018). After de-oiling, the material structure became less densely packed, which made it easier to observe how the protein bodies are present in lupin and soy (Figures 4.4C and 4.4D). For soy, the protein bodies are enclosed inside the matrix of cell wall particles, where some are exposed, a few are detached and a large part is still trapped inside the cell wall material (Figure 4.4D). In comparison, previous research on commercially available hexane de-oiled soybean meal flour showed protein bodies that were mainly detached from the cell walls on SEM images (Kuspangaliyeva et al., 2023). The absence of large amounts of free protein bodies in Figure 4.4D can be explained by the cellular structure of soy as proposed by Xing et al. (2018a), where soy protein bodies are anchored in irregular rod-like cotyledon cells by a net-like intracellular matrix of oil and carbohydrates. If the oil is removed, the protein bodies remain trapped in this intracellular matrix (Figure 4.4D).

Because of the entrapped protein bodies, separation of the de-oiled soy material without re-milling after de-oiling did not result in protein enrichment, while for lupin electrostatic separation without re-milling was already possible (protein enrichment of 8.0±1.1% after de-oiling with hexane and 50.8±2% after de-oiling with acetone). After de-oiling finely milled soy flour with hexane and consecutive mild milling, the protein bodies were well detached from the cellular material (Figure 4.4F), and the SEM images were more comparable to those of commercially hexane de-oiled soybean meal flour

(Kuspangaliyeva et al., 2023). Therefore, it can be concluded that re-milling after de-oiling finely milled soy flour results in detached cellular components (Figure 4.4G), which is expected to favour triboelectric separation. The material properties (composition, particle size, dispersibility and chargeability) of the de-oiled and milled materials are further discussed in the next paragraph followed by the electrostatic separation performance.

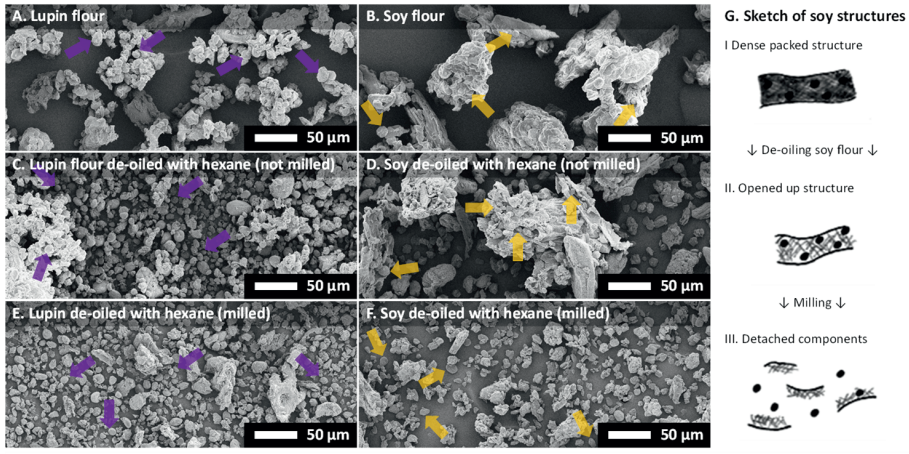

Figure 4.4: Scanning electron microscope (SEM) image of lupin flour (A), soy flour (B), hexane de-oiled lupin flour (C), hexane de-oiled soy flour (D), hexane de-oiled lupin flour after milling (E), hexane de-oiled soy flour after milling (F) and a sketch to visualise the change in material morphology (G). Magnification x300. Lupin protein bodies are indicated with purple arrows and soy protein bodies are indicated with yellow arrows.

4.3.3 The material properties improved by de-oiling with acetone and hexane

This section evaluates the effect of pre-treatment on powder properties, which include the composition, particle size distribution, relative particle dispersibility and particle chargeability. The composition of the flours and volume-based particle sizes (DV_{10}, DV_{50}, DV_{90}) are reported in Table 4.1. De-oiling with acetone and hexane resulted in more efficient oil extraction than de-oiling with ethanol. In addition, the particle size decreased after de-oiling with acetone and hexane, whereas the particle size remained similar upon de-oiling soy and lupin with ethanol (Table 4.1). The more efficient oil extraction was caused by the higher affinity of n-hexane (log $K_{octanol/water}$ 3.13) and acetone (log $K_{octanol/water}$ 0.11) for the hydrophobic oil phase than ethanol (log $K_{octanol/water}$ -0.16) (Moldoveanu & David, 2015). This aligns with previous research, where ultrasound assisted oil extraction of sunflower seeds (Dv_{50} 250 µm) with hexane was also most

efficient followed by acetone and ethanol (Moradi et al., 2018). The decrease in particle size after de-oiling with hexane and acetone was likely caused by the combined effect of de-oiling and re-milling. Materials with a lower oil and/or moisture content are more brittle, which results in a lower energy requirement to mill the material into smaller particles (Lee et al., 2013; Politiek et al., 2022). As the input of milling energy was the same, the particles could become significantly smaller after de-oiling with acetone and hexane (Table 4.1). The average particle size of the ethanol de-oiled material remained more similar to the non-de-oiled material because of the higher residual oil content. Furthermore, upon further milling of the materials oil could be released, which can result in re-agglomeration of particles due to liquid bridging. This was observed for lupin where the DV10 increased after de-oiling with ethanol and consecutive milling (Table 4.1), which implies that smaller particles agglomerated after de-oiling with ethanol and consecutive milling. The particle sizes for the acetone and hexane de-oiled soy flour (Table 4.1) are in between particle sizes reported in previous studies (commercially hexane de-oiled soy DV_{50} 16.7 µm, petroleum ether de-oiled soy DV_{50} 48.8 µm (Kuspangaliyeva et al., 2023; Xing et al., 2018). The slightly larger particle size than 16.7 µm might be beneficial for soy protein enrichment with electrostatic separation as it was hypothesized that slightly larger particle sizes than 16.7 µm might improve enrichment and the electrostatic separation efficiency (Kuspangaliyeva et al., 2023).

Table 4.1: Average composition and volume-based particle sizes of the flours used in this study. The average volume-based particle sizes are measured at a dispersion pressure of 4 bar. Different letters indicate significant differences (P < 0.05) in composition or particle size.

Source	Solvent	Composition			Volume-based particle sizes		
		Moisture content [g/100 g]	Protein content [DM%]	Oil content [DM%]	Dv_{10} [µm]	Dv_{50} [µm]	Dv_{90} [µm]
Lupin	None	10.8±0.1	39.7±1.1^{a}	8.2±0.5^{a}	9.6±0.2^{c}	40.8±1.0^{c}	169±6^{ab}
	Ethanol*	12.1±0.3	43.2±0.3^{c}	4.2±0.1^{b}	11.6±0.4^{b}	42.6±0.7^{c}	169±2^{ab}
	Acetone*	7.5±0.4	41.8±0.5^{bc}	0.4±0.2^{c}	5.5±0.1^{d}	28.9±0.4^{e}	159±3^{b}
	Hexane*	9.9±1.0	42.6±0.2^{c}	0.6±0.2^{c}	5.7±0.3^{d}	34.3±0.6^{d}	168±3^{ab}
Soy	None	10.9±0.9	40.8±1.3^{ab}	24.2±0.3^{d}	19.5±0.2^{a}	56.1±1.6^{a}	181±24^{a}
	Ethanol*	10.6±0.2	48.8±0.6^{d}	14.2±1.6^{e}	19.5±0.6^{a}	50.5±2.4^{b}	166±17^{ab}
	Acetone*	8.0±0.3	50.4±0.7^{de}	2.6±1.6^{bc}	5.5±0.0^{d}	23.1±0.2^{f}	89±3^{c}
	Hexane*	9.7±1.2	51.4±0.9^{e}	0.4±0.4^{c}	4.6±0.1^{e}	20.6±0.9^{g}	182±8^{a}

*The partition coefficient of the solvent in octanol and water is given by the log Koctanol/water (log Ko/w) obtained from (Moldoveanu & David, 2015), which was -0.16 for ethanol, 0.11 for acetone and 3.13 for hexane.

It was expected that the relative dispersive index (DI) of small particles (<10 µm) would increase upon de-oiling (Politiek et al., 2022), as this could reduce agglomeration of

particles caused by liquid bridging (Pelgrom et al., 2014). Furthermore, the presence of spherical particles results in less mechanical interlocking (Fernando & Manthey, 2022). The lower proportion of spherical particles combined with the higher oil content of soy flour resulted in a lower particle dispersibility than lupin flour. The dispersive index increased after de-oiling with acetone and hexane, but de-oiling with ethanol resulted in similar relative dispersive indices as the original flour (Figure 4.5), which was related to the similar particle size at 4 bar and the higher residual oil content of the material.

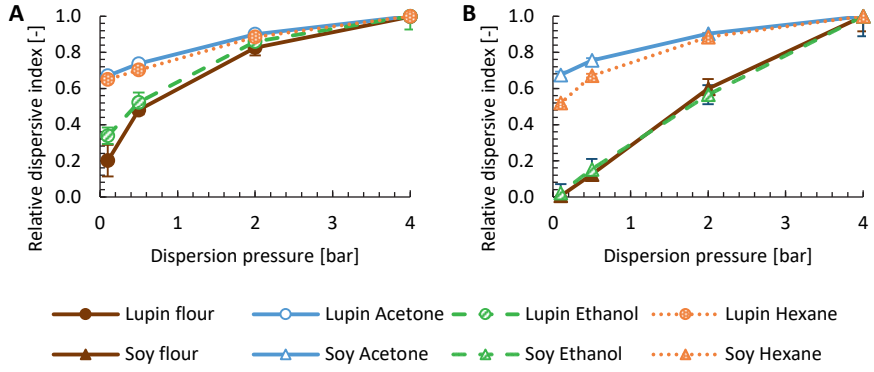

Figure 4.5: Relative dispersive index (DI) versus dispersion pressure for soy and lupin and de-oiled lupin (A) and soy (B) with different solvents indicated in the legend. The error bars represent the standard deviation based on 5 measurements.

It was expected that the removal of oil would increase the particle chargeability as oil has an insulating effect. The removal of oil indeed resulted in a higher specific charge for all de-oiled lupin flours and soy de-oiled with acetone and hexane (Figure 4.6A). However, de-oiling soy with ethanol resulted in a lower specific charge (1.5±0.2 µC/gram). This could be because exposure to solvents could also have (a) extracted ionic segments, (b) caused migration of oppositely charging tribo-ions to form co-polymer chains or (c) neutralised the charge in the case of liquids with acid-base properties (Burgo et al., 2012). Previous research showed that ethanol was indeed more effective than water or n-hexane to remove tribo-charge from polymers (Burgo et al., 2012). Furthermore, tribo-chargeable components such as salts and sugars (Matsusaka et al., 2010; Murtomaa & Laine, 2000), are better soluble in ethanol than in acetone or hexane (Katainen et al., 2005; M. Li et al., 2010). The residual sugar content in the materials de-oiled with ethanol was indeed lower than after de-oiling with hexane or acetone (Figure 4.6B). Here, small DP2 sugars were co-extracted with the oil after treatment with ethanol. As lupin had a lower initial small sugar content, the total sugar content of lupin was less affected than the sugar content of soy after treatment with

ethanol and therewith less tribo-chargeable components were co-extracted with the lupin oil (Figure 4.6B).

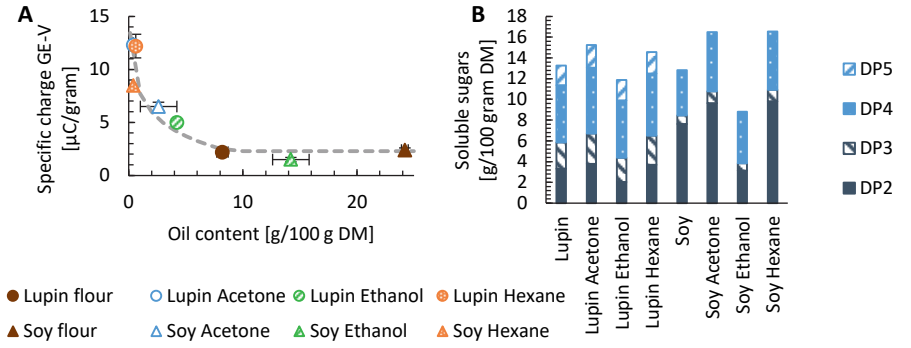

Figure 4.6: Specific charge of the product recovered at the ground electrode (GE-V) of soy and lupin materials against the oil content of the materials (A) and the sugar content of the materials (B). The sugars were subdivided into sugars with a degree of polymerisation (DP) between 2 and 5 as indicated in the legend. The error bars indicate two sided standard deviation.

Overall, the material properties improved upon de-oiling and milling, especially upon de-oiling with acetone and hexane. The improved particle dispersibility and chargeability are expected to be beneficial for further separation.

4.3.4 The solvent used affected protein enrichment depending on the material

Electrostatic separation of the flours resulted in protein enrichment at the ground and negative electrodes and protein depletion at the positive electrode, which is shown by negative values for protein enrichment (referred to as protein depletion) at the positive electrode calculated with Equation 4.2. To evaluate which pre-treatment resulted in the best improvement in terms of protein purification, the protein enrichment at the ground and negative electrode was plotted against the protein depletion at the positive electrode for both electrostatic separation setups. Samples that are located more towards the upper right corner have a better separation based on protein- enrichment and depletion at the oppositely charged electrodes (Figure 4.7). The relative protein depletion at the positive electrode was lower than the relative protein enrichment at the ground and negative electrode (Figure 4.7), because the protein bodies are smaller particles, which are easier caught by the electrodes than the other particles (Basset et al., 2016; Landauer & Foerst, 2019). These larger particles ended up in the collector bags, which resulted in protein depletion in the collector bag below the positive electrode.

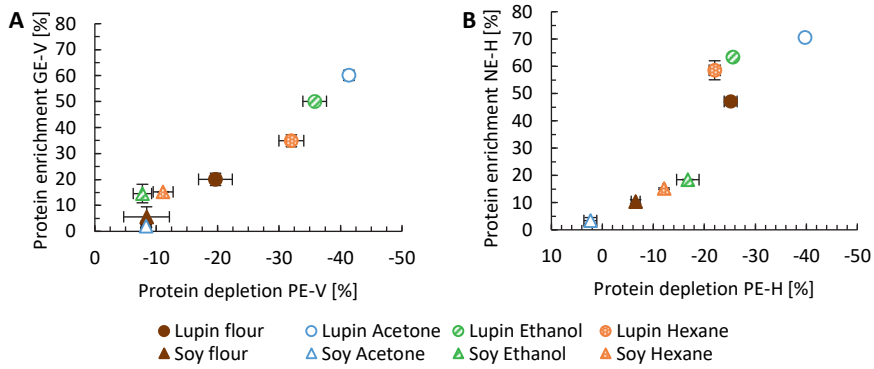

Figure 4.7: Protein enrichment at the ground electrode (GE-V) or negative electrode (NE-H) against protein depletion at the positive electrode (PE) (minus values indicate depletion, the x-axis is reversed) of lupin- (circles) and soy (triangles) flour with a vertical (A) and horizontal (B) electrostatic separation setup. The colours indicate the pre-treatment applied which was the original flour (brown) and flour de-oiled with acetone (light blue), ethanol (green) and hexane (orange). Positive values indicate protein enrichment and negative values indicate protein depletion calculated with Equation (4.2). The error bars indicate two sided standard deviation.

De-oiling and milling of lupin resulted in a more effective protein separation and the use of polar solvents was most beneficial for protein- enrichment and depletion of lupin materials (Figure 4.7). The underlying reason for the different effect of polar solvents might be related to the higher fibre solubility for lupin or the higher solubility of lignin in polar solvents (acetone and ethanol) than in less polar solvents (diethyl ether and hexane) (Bähr et al., 2014; Ponnuchamy et al., 2021). The solubilization of fibres or partial solubilization, might result in more free pure protein bodies for lupin and therewith the possibility to reach a protein concentration that is closer to the protein concentration of pure protein bodies (70-75 %DM) (Plant & Moore, 1983). For soy only de-oiling with hexane and ethanol resulted in a slightly higher protein enrichment at the ground electrode (Figure 4.7A). However, the separation of soy de-oiled with acetone did not result in a higher enriched fraction than the separation of non-treated soy, so the higher protein purity of acetone de-oiled soy was just caused by the removal of oil. This was unexpected based on the similar material properties (average particle size DV_{50}, dispersibility and chargeability) of soy de-oiled with hexane and acetone. The lower protein enrichment of soy after treatment with acetone can be caused by agglomeration of oppositely charged particles via particle-particle interactions, which results in a partly neutralized charge (J. Wang et al., 2015a). Fine particles are more prone to electrostatic agglomeration of oppositely charged particles, caused by the large surface area and small mass of the particles (Deng et al., 2023). Electrostatic agglomerates of just a few particles

larger than about 10 µm can be broken up in the electrostatic field if the field strength is high enough (Schönert et al., 1996). However, if the field strength is below the driving force needed for de-agglomeration, the particles will remain agglomerated, which can be visualised by for example scanning electron microscopy.

A protein fraction that contains almost only pure protein bodies will visually contain mainly smooth and round particles with a low proportion of irregular impurities. To visualise the particles before and after separation scanning electron microscope images were prepared of the flour (first column) and the fractions collected at the ground electrode (GE-V) (second column) and positive electrode (PE-V) (third column) (Figure 4.8). Here, protein bodies can be recognized as smooth and round particles, whereas the cell wall material particles have a more rough and folded structure. The GE-V fraction of acetone de-oiled lupin (Figure 4.8C) contains visually less impurities than the ethanol and hexane de-oiled GE-V fractions (Figures 4.8A, 4.8E), which aligns with the higher protein enrichment for the acetone de-oiled lupin (Figure 4.7).

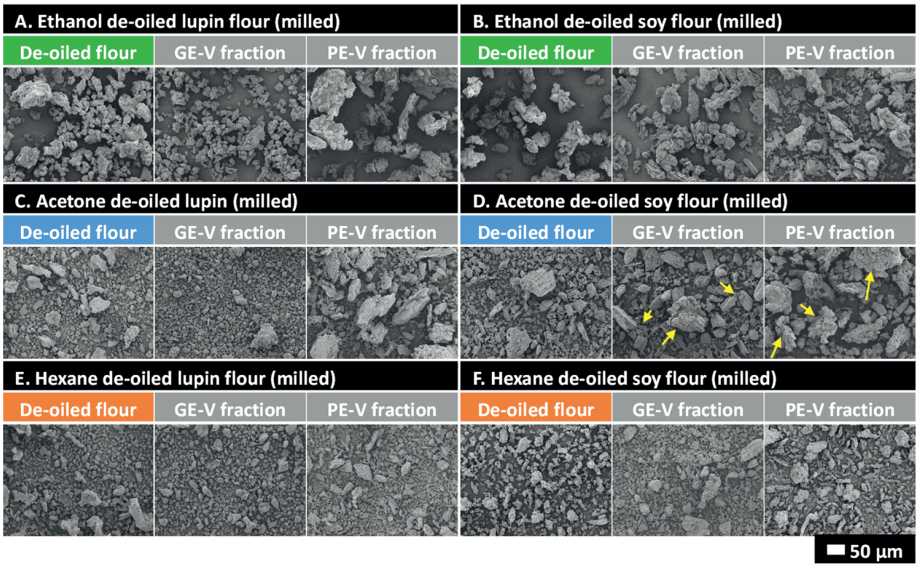

Figure 4.8: Scanning electron microscopy of de-oiled flours and the ground electrode (GE-V) and positive electrode (PE-V) fractions after electrostatic separation with the vertical set-up of ethanol de-oiled lupin (A), ethanol de-oiled soy (B), acetone de-oiled lupin (C), acetone de-oiled soy (D), hexane de-oiled lupin (E) and hexane de-oiled soy (F). The scale bar represents a size of 50 µm for all images. The yellow arrows point at agglomerated particles.

Upon comparing the ground electrode fraction (second column) with the positive electrode fraction (third column) it is evident that the ground electrode fraction

contained smaller particles than the positive electrode fraction (Figure 4.8), which aligns with previous research (Basset et al., 2016; Landauer & Foerst, 2019). Both the GE-V and PE-V fraction of acetone de-oiled soy contain visually larger irregular particles, which supports the hypothesis that the poorer separation of acetone de-oiled soy was caused by electrostatic agglomeration of oppositely charged particles.

To evaluate the separations based on both purity and yield, the protein purity is usually plotted against the fraction yield of the ground electrode (Figure 4.9A). For lupin, de-oiling resulted in a higher protein enrichment and protein purity at the cost of the fraction yield (Figure 4.9). Based on the protein purity and yield, de-oiling lupin with ethanol before electrostatic separation seems slightly more favourable than de-oiling lupin with hexane or acetone, despite the lower specific charge for the protein fraction from lupin de-oiled with ethanol (Appendix table 4.3). Overall, the decision for one or the other pre-treatment for lupin depends on the product application (i.e., protein purity requirements, functionality) and the trade-off between purity and yield. De-oiling soy with hexane resulted in a similar protein enrichment but a higher fraction yield and purity than soy de-oiled with ethanol (Figure 4.9). Here, the higher fraction yield can be explained by the smaller particle size, the better particle dispersibility and lastly the higher chargeability of soy de-oiled with hexane (8.5±0.1 µC/gram) than soy de-oiled with ethanol (1.5±0.2 µC/gram).

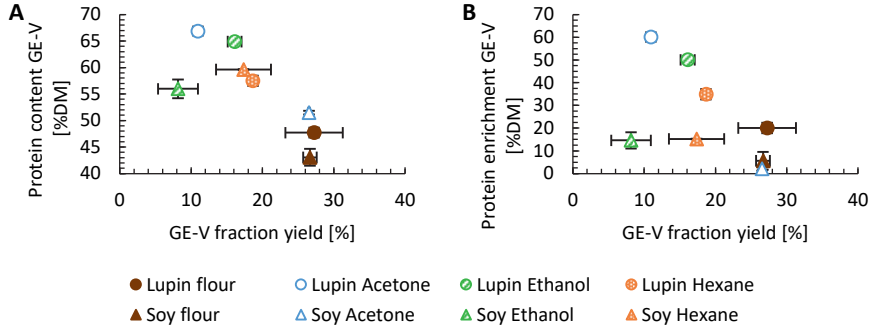

Figure 4.9: Protein purity (A) and protein enrichment (B) against the ground electrode (GE-V) fraction yield for electrostatically separated lupin- (circles) and soy (triangles) flour. The colours indicate the pre-treatment applied which was the original flour (brown) and flour de-oiled with acetone (light blue), ethanol (green) and hexane (orange). The error bars indicate two sided standard deviation.

Overall, it is not completely understood why the solvents affect lupin and soy differently. It might be related to material composition as discussed before but the material

structure prior to de-oiling was also already different in terms of exposure of the protein bodies (Figure 4.4). Next to that the state of the material (i.e. glassy or amorphous) might change upon de-oiling with acetone, ethanol or hexane. Especially for soy this might have a big influence as the protein bodies are still enclosed inside the cellular matrix. For example, if the structure becomes denser or even glassy after extraction with the solvent, as was observed upon aqueous ethanol washing of soy- and sunflower press cakes towards higher ethanol concentrations (Jia et al., 2021; Peng et al., 2021), milling after de-oiling might not liberate the protein bodies, or even result in broken protein bodies if the material is in a glassy state, which impairs electrostatic separation. The lower moisture content in acetone de-oiled material and the smaller DV_{90} for soy de-oiled with acetone after re-milling might also be a hint that the material was more brittle after de-oiling with acetone (Lee et al., 2013). However, further research should be done to provide a full understanding of this effect, for example by using different extraction times to check how the structure changes over time, or different milling times or speeds to evaluate the breakage behaviour further.

Electrostatic separation of hexane de-oiled soy resulted in a higher true protein content (59.6±0.1 %DM) than previous studies after correction to a protein factor of 5.7 (53.5 %DM and 51.4 %DM; Table 1.1) (Kuspangaliyeva et al., 2023; Xing et al., 2018). This might have been caused by a combination of (a) the higher driving force to disperse particles by the venturi used, (b) the slightly larger particle size, as discussed in section 3.1, (c) the lower oil content of the material used in this study or (d) de-oiling of finely milled flour followed by a mild milling step. After correction for the oil content, the relative protein enrichment was still slightly higher (15.2%) than the protein enrichments reported in literature (5.6% soy hexane, 14.4% soy petroleum-ether). The advantage of de-oiling the finely milled flour instead of larger grits is that the oil is more available for extraction, which results in higher oil extraction yields. As the particle size of the de-oiled material is then already quite small, it allows for a mild re-milling step just to liberate the protein bodies from the soy structure.

Overall, the protein enrichment was higher for the lupin samples than for the soy samples, where it was possible to go from a protein purity of 40% towards a purity of ~70% for lupin, while for soy the maximum protein purity was ~60% after de-oiling, re-milling and electrostatic separation.

4.4 Conclusion

This research aimed to determine the mechanism behind the ineffective separation of soy and if it could be altered by de-oiling. Electrostatic separation of full-fat lupin resulted in a higher protein purity (58.5 %DM, N_{factor} = 5.7) and protein enrichment (47.1 %DM) than electrostatic separation of full-fat soy (protein purity 45.0 %DM, protein enrichment 10.3 %DM). So, electrostatic separation of soy was possible but not as effective as separation of lupin. The main reason for this was that the soy protein bodies were still embedded in the cellular structure after milling, while lupin protein bodies were visually disclosed from the cellular matrix. This translated into a smaller measured particle size, better small particle dispersibility and a protein purity closer to the purity of pure protein bodies (70-75 %DM).

It was crucial to combine the de-oiling of soy with re-milling the material to liberate the protein bodies from the cellular material. The specific charge of the materials increased upon the removal of oil, except for soy de-oiled with ethanol, which could be caused by a combined effect of a high residual oil content, the coextraction of ionic segments and tribo-charge by ethanol. Overall, the material properties (particle size, dispersibility and chargeability) improved most upon de-oiling with acetone and hexane. Electrostatic separation of hexane de-oiled soy resulted in soy protein-enriched fractions with a high purity of 59.6 %DM and a protein enrichment of 15.2 %DM. Electrostatic separation of acetone de-oiled soy did not result in protein enrichment. Contrarily, for lupin, the protein purity could be increased to the highest extent (up to 71.3 %DM) by using polar solvents (acetone or ethanol). As the use of the highly polar acetone and ethanol did not favour electrostatic separation of soy, less polar solvents that are in the list of preferred greener solvents and that are compatible with food might be an alternative to the use of hexane, for example ethyl acetate.

This study used a horizontal and vertical electrostatic separator set-up. With the horizontal electrostatic separation set-up purer fractions were collected, but due to the design of this set-up, the potential yield in continuous systems could not be evaluated. Overall, electrostatic separation of lupin resulted in higher protein purities than electrostatic separation of soy, caused by the way that the protein bodies were embedded in the structure. Re-milling after de-oiling was found to be crucial and the use of a different solvent affected the separation efficiency differently for soy and lupin.

Appendix 4

Appendix table 4.1: Combined fractions for compositional analysis.

Material, set-up and fraction	Compositional analyses
Soy horizontal set-up 3 GE fractions	Dry matter content[1]
Soy horizontal set-up 3 NE fractions	Dry matter content[2]
Soy de-oiled with ethanol horizontal set-up 3 GE fractions	Dry matter content[1]
Soy de-oiled with ethanol horizontal set-up 3 NE fractions	Dry matter content[1]
Lupin horizontal set-up 3 NE fractions	Dry matter content[2]
Lupin de-oiled with hexane horizontal set-up 3 NE fractions	Dry matter content[1]
Lupin de-oiled with ethanol horizontal set-up 3 NE fractions	Dry matter content[2]

[1]Indicates that each individual fraction was measured once and that the leftovers of the three fractions were combined for the duplicate measurement.[2]Indicates that all three fractions were combined for both measurements.

Appendix table 4.2: Protein content

Sample	Protein content [%DM]						
	Flour	Vertical set-up				Horizontal set-up	
		GE	PE	GC	PC	GE	PE
Lupin	39.7±1.1	47.7±0.9	31.9±1.1	39±4.5	29.8±1.9	58.5±0.6	29.7±0.5
Lupin acetone	41.8±0.5	66.9±0.8	24.5±0.3	52.1±0.1	32.5±0.7	71.3±0.3	25.2±0.3
Lupin ethanol	43.2±0.3	64.9±0.2	27.8±0.8	45.7±1.7	32.2±0	70.7±0.5	32.2±0.1
Lupin hexane	42.6±0.2	57.5±1	29±0.9	40.9±0.9	31.4±1.1	67.6±1.5	33.2±0.5
Soy	40.8±1.3	43.1±1.6	37.3±1.5	39.4±0.8	37.7±1	45±0.3	38.1±0.4
Soy acetone	50.5±0.6	51.5±0.3	46.2±0.4	49.4±0.1	46.2±0.2	52.2±0.5	51.6±0.6
Soy ethanol	48.8±0.6	56±1.8	45.1±0.7	50.1±0.5	47.7±0.4	57.9±0.1	40.7±1.1
Soy hexane	51.8±1	59.6±0.1	46±0.8	52.5±2.2	50.6±1.1	59.6±0.2	45.5±0.1

Appendix table 4.3: Specific charge

Sample	Specific charge [µC/g]	
	Horizontal set-up	
	GE	PE
Lupin	2.2±0.2	-5.3±0.9
Lupin acetone	12.3±0.3	-8.1±0.4
Lupin ethanol	5±0.3	-8.7±2.7
Lupin hexane	12.2±1.1	-20.1±3.4
Soy	2.4±0.2	-4.3±0.5
Soy acetone	6.5±0.4	-7±0.4
Soy ethanol	1.5±0.2	-2.3±0.9
Soy hexane	8.5±0.1	-10.6±0.4

An extended version of this appendix including fraction yield, protein enrichment and protein recovery is provided online (Politiek et al., 2023a).

Chapter 5

Functional and flavour properties of de-oiled flours and dry-enriched protein concentrates of lupin and soy

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Abstract

Dry fractionation is low in energy and water use and thus a sustainable option to obtain protein-rich ingredients. Air-classification is used to remove starch from legumes, and electrostatic separation can be used to remove fibres from other starting materials like oilseeds. Flour from oil-rich crops needs to be de-oiled to facilitate dry fractionation, which involves the use of organic solvents or mechanical pressing and might affect the ingredient functionality. This research evaluated if the functionality of soy and lupin is affected by solvent de-oiling and electrostatic separation. Industrially toasted soy and lupin flour contained native protein, which was preserved upon electrostatic separation, de-oiling with hexane and de-oiling with acetone, but ethanol de-oiling resulted in protein denaturation. De-oiling with ethanol or hexane is preferred over de-oiling with acetone based on the flavour profile after de-oiling. The use of different solvents affected the solubility to a different extent and electrostatic separation resulted in a similar or higher solubility of the ingredients (22% - 49%), dependent on the solvent, crop and pH condition used. Overall, electrostatic separation resulted in protein-enriched ingredients (43%DM - 67%DM N_{factor}=5.7) that show potential for application as stabilizers in emulsion-based systems like dressings or frozen foam-based systems like ice-cream.

5.1 Introduction

For many years, foods have been created by combining ingredients that are neutral in taste and constant in quality, which resulted in the development of highly refined ingredients such as protein isolates (Lie-Piang et al., 2023). Producing protein isolates from plant-based seeds is conventionally done by wet fractionation. However, wet fractionation has a significant impact on the environment in terms of water usage, energy usage and raw material losses, which amplifies the need for more sustainable ingredients (Henchion et al., 2017; Lie-Piang et al., 2023). Dry fractionation can be used as an alternative to produce functional protein-enriched ingredients for the food industry (Schutyser et al., 2015). Here, air-classification is used to remove starch from legumes, and electrostatic separation can be used to remove fibres from other starting materials like oilseeds.

Electrostatic separation is a new dry separation technique for food ingredients, which is amongst others used to enrich components such as protein and fibre from fine milled legume flours (Vitelli et al., 2020). The separation is based on the tribo-electric charging properties of the particles, where the charge is induced by particle-particle interactions and particle-wall interactions (Landauer et al., 2019; Xing et al., 2021). Electrostatic separation has been successfully applied to oil-rich seeds (i.e. lupin), de-oiled cakes (mechanically de-oiled, ~6-7% oil) and de-oiled meals (solvent de-oiled ~<1% oil), from for example soybean, rapeseed and sunflower seeds (Ancuţa & Sonia, 2020; Basset et al., 2016; Kdidi et al., 2019; Laguna et al., 2018; Xing et al., 2018). Studies on the functionality of protein-enriched fractions after tribo-electrostatic separation are scarce compared to studies on air classification or sieving but show promising results. For example, the order of combined separation steps (air classification and electrostatic separation) did not significantly affect the protein solubility of CO_2 extracted rapeseed press-cake enriched ingredients (Wockenfuss et al., 2023). Furthermore, tribo-electrostatic separation of navy bean (starch-rich crop) resulted in protein-enriched fractions with excellent emulsifying and foaming properties and showed that the native protein functionality could be preserved upon electrostatic separation (Tabtabaei et al., 2019).

We therefore hypothesize that electrostatically separated fractions would similarly result in higher functionality than soy and lupin flours. However, the fractionation performance, flavour and protein nativity of the fractions and thus their functionality is likely further affected by different pre-treatment steps applied. To illustrate, de-oiling is often a prerequisite for effective electrostatic separation of oil-rich seeds, but the choice

of solvents affects the separation performance and the composition of the produced ingredients (Politiek et al., 2023a). Furthermore, toasting and dehulling are generally applied as a pre-treatment steps to reduce the antinutritional factors, which can also affect the protein nativity and functionality of the fractions (Bader et al., 2011; Lawal et al., 2021).

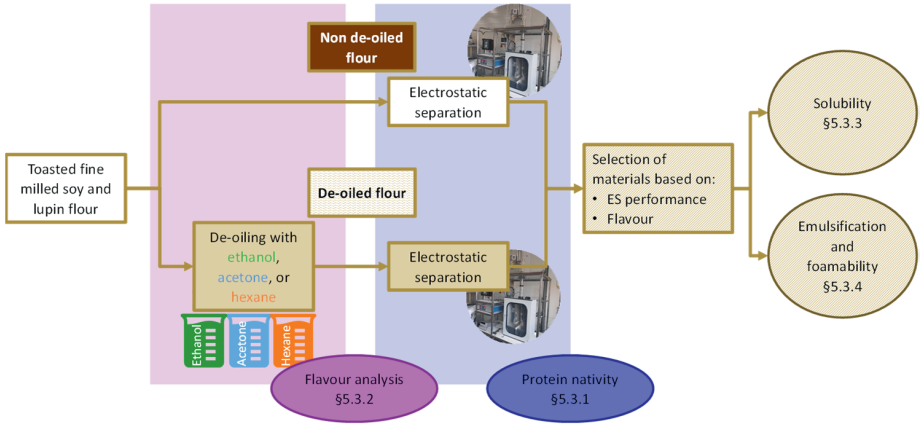


Figure 5.1: Schematic overview of the overall process and functionality analysis. Electrostatic separation (ES) was done for both the non-de-oiled and the de-oiled flours. The numbers refer to the paragraphs (§) where the results are discussed.

This research aimed to evaluate if the functionality of soy and lupin is affected by solvent de-oiling and electrostatic separation. This was done by systematically studying the protein nativity and flavour of the samples after pre-processing (no de-oiling and de-oiling with acetone, ethanol and hexane) and tribo-electrostatic separation (protein-enriched fractions). Based on the flavour profiles of the samples and the electrostatic separation performance (protein purity and fraction yield) four protein-enriched ingredients were selected. The solubility of the selected ingredients was evaluated at different pH (3, 6 and 7). These pH values were chosen to represent products with lower pH like dressings (pH 3) and products with more neutral pH like ice-cream (pH 6 - pH 7). Furthermore, the emulsifying properties of the selected ingredients were assessed at pH 3 (creaming index and freeze-thaw stability) and the foamability of the ingredients was assessed at pH 6. A schematic overview of the different aspects discussed in this research is presented in Figure 5.1. Lastly, an outlook is provided towards potential product application of the protein-enriched ingredients produced by electrostatic separation. Here it is expected that samples with good emulsifying properties at pH 3 will be well applicable as stabilizers in dressings, whereas samples with good foamability at

pH 6 can be used as stabilizers in foam-based systems with more neutral pH like ice-creams.

5.2 Materials and methods

5.2.1 Materials

Fine impact milled toasted de-hulled soy flour (average particle size DV50=56±2 µm) and toasted de-hulled lupin flour (DV50=41±1 µm) were kindly provided by Frank Foods (Twello, the Netherlands). The materials were kept in screw-capped polyethylene containers at 4 °C. The toasted lupin and soy flours (400–500 g) were de-oiled for 6 h with ethanol (96 v/v%, VWR International, Rosny-sous Bois, France), acetone (Actu-all Chemicals, Oss, The Netherlands) and hexane (Actu-all Chemicals, Oss, The Netherlands) by using a custom-built Soxhlet extractor. The de-oiled flours were left in the fume hood overnight (20 °C) to evaporate the residual solvent and consecutively, powder lumps were broken up by milling approximately 150 g of material in 500 ml bowls with a Pulverisette 5 bead mill (Fritsch GmbH, Germany), operated at 400 rpm for a total of 4 min with one min on and off as described in detail in our previous study (Politiek et al., 2023a). After preparation, the samples were stored at 4°C.

5.2.2 Electrostatic separation to obtain protein-enriched fractions

A custom-build electrostatic separator with a venturi tube was used to prepare the protein-enriched fractions as described by Politiek et al. (2023a). For each separation approximately 100 gram was fed into the vertical electrostatic separator and separated into four fractions: ground electrode (GE-V), positive electrode (PE-V), ground collector (GC-V) and positive collector (PC-V). The protein enriched fraction was obtained at the ground electrode and the depleted fraction at the positive electrode (Politiek et al., 2023a). This study focussed on the functionality of the protein-enriched fractions obtained at the ground electrode (referred to as enriched fraction).

5.2.3 Protein denaturation

Denaturation of the samples was determined by measuring a thermogram of each sample with differential scanning calorimetry (DSC) (DSC-250, TA instruments, Newcastle, USA). 20 w/w% protein dispersions of each de-oiled flour and the enriched fraction were prepared and stirred at 1400 rpm for 2 h at 22°C in an Eppendorf thermomixer. A large volume pan was filled with 53±5 mg of protein dispersion (containing ~10 mg sample) and then well sealed. An empty pan was used as a reference. The samples were heated from 20°C to 140°C at a heating rate of 2°C/min and then

cooled to 20°C. A second heating cycle was used to investigate the reversibility of the transition. The peak denaturation temperature (Td) and the enthalpy of denaturation were determined with TRIOS software (TA Instruments).

5.2.4 Headspace analysis of volatile compounds with SPME GC-(O)-MS

To trap and analyse the volatile compounds, solid phase microextraction gas chromatography mass spectrometry (SPME GC-MS) was used as described previously (Pegiou et al., 2021). From each sample, 0.5 g powder was transferred to 10-ml ND18 headspace glass vials with magnetic screw caps (8 mm opening) with silicone/PTFE septa (BGB Analtik®, Germany) and mixed with 1.5 mL NaCl-H2O (26.3 w/w%) to release the volatiles to the headspace. The samples were preconditioned for 10 min at 50°C agitating at 350 rpm. Volatiles in the headspace were afterwards trapped by inserting the SPME fibre (PDMS/DVB/CAR 50/30 µm diameter, 1 cm length, Supelco, PA, USA) to the headspace of the vial for 15 min at 50°C without agitation (Pegiou et al., 2021). The volatiles were desorbed from the SPME fibre in split-less mode by heating the fibre at 250°C for 2 min onto the GC column (Zebron ZB-WAX 30 m x 0.25 mm x 1.00 µm, Phenomenex, the Netherlands) via a cooled injector system (CIS4, Gerstel, Germany) with a constant helium flow of 1 ml/min) (Pegiou et al., 2021). The GC oven temperature was programmed according to the settings used by Siccama et al. (2021): 2 min at 45°C followed by a temperature increase with 8°C/min to 200°C, and a final rate of 15°C/min to 250°C, which was maintained for 3 min.

The lupin samples (none, acetone, ethanol and hexane de-oiled) were selected for evaluation with gas chromatography-olfactometry (GC-O) coupled to an MS system, to determine which peaks were odour active. For analysis with GC-O-MS double the amount of sample (1 g + 3 mL of NaCl water 26.3 w/w%) was used as the signal was split with a GC column splitter to split the signal 1:1 towards the MS and the Olfactory detection port (ODP2, Gerstel, The Netherlands). Three assessors performed sniffing and assigned the peaks of the GC-O-MS profile to specific aroma attributes and noted down the perceived aroma per compound at the given retention times, without knowing which compound corresponds to which peak as described by (Pegiou et al., 2023). The sample order was randomised. Other conditions were the same as with SPME GC-MS.

5.2.5 Material solubility and protein solubility

Both the solubility and the protein solubility of the samples were accessed with adapted methods from Peng et al. (2021), Sweers et al. (2023) and Morr et al. (1985). For this, a 2 w/v% dispersion was prepared in a 15 mL centrifuge tube with a final volume of 10 mL. After adding 9 mL of Milli-Q water, the pH of the dispersion was determined and adjusted to pH 3, 6 and 7 until stable with 5 M HCl (Actu-All Chemicals, the Netherlands) or 5 M NaOH (Sigma-Aldrich U.S.). The volume added to adjust the pH was tracked, and the total volume of the dispersion was adjusted to reach a final volume of 10 mL. The samples were moderately shaken for 2 h at ambient temperature and subsequently centrifuged with a Sorvall Legend XFR centrifuge (Thermo ScientificTM, Waltham, U.S.) at 4500 g for 20 min at 4°C to obtain a supernatant and pellet. The pellets and supernatants were oven-dried at 105°C and weighted (Peng et al., 2021). The initial material was also oven dried over-night to determine the dry matter content (DM) of the initial material. The overall solubility was then calculated with Equation 5.1.

$$\text{Solubility [\%]} = \frac{\text{Dry weight supernatant [g DM]}}{\text{Initial material [g DM]}} \cdot 100\% \quad (5.1)$$

The protein content of the oven-dried samples was determined by Dumas analysis with a rapid N exceed protein analyser (Elementar, Germany). The sample weight was 50±10 mg. A protein conversion factor of N x 5.7 was used and protein contents are reported on a dry weight basis (Maclean et al., 2003). The nitrogen solubility index was calculated with Equation 5.2.

$$\text{Nitrogen solubility index [\%]} = \frac{\text{Soluble protein [g/100 g DM]}}{\text{Initial protein content [g/100 g DM]}} \cdot 100\% \quad (5.2)$$

5.2.6 Emulsion preparation

Protein dispersions were prepared by mixing 1.0 w/w% protein in ~250 g phosphate buffer (10 mM, pH 3.0). A protein concentration of 1 w/w% was chosen as previous research found that this concentration could already result in narrow particle size distributions in emulsions prepared with native soybean proteins (Palazolo et al., 2011). Additionally, 1.0 w/w% salt (NaCl) was added to the dispersions to represent salt concentrations in dressings. The dispersion was gently mixed with a magnetic stirrer at 350 rpm at room temperature for 3 h (Sridharan et al., 2020). Then 30 w/w% canola oil was slowly added to the dispersions and homogenised for 1 min at 11,000 rpm with a rotor-stator homogeniser (Ultra-Turrzx IKA T18 digital, Germany). The pH was adjusted

to pH 3 with 0.1 M HCl (Ray and Rousseau, 2013). The pH of the coarse emulsions was checked and adjusted if necessary. The coarse emulsions were further homogenised to reduce the droplet size and polydispersity using a colloid mill (IKA Magic Lab, Staufen, Germany) with a gap width of 0.16 mm for 2 min at a speed of 15,000 rpm. The cooling element temperature was set at 20 °C to avoid potential protein denaturation upon emulsification, this was well below the denaturation temperatures measured with DSC (Section 3.2). The emulsions were stirred with a magnetic stirrer at 250 rpm for another 30 min, and then transferred to 50 mL and 15 mL tubes. Half of the tubes were stored at 4 °C for a minimum of 3 h before further emulsion stability analysis (Sridharan et al., 2020). The other half was stored at -18 °C for at least 22 h before further freeze-thaw treatments and analysis (Zhang et al., 2017).

5.2.7 Emulsion stability

The emulsion stability was monitored over a period of seven days. At least four pictures were taken during the storage time, with fixed measurements on day one and day seven. The unfrozen emulsions were used as such, whereas the frozen emulsions were thawed after one day by placing them in a water bath for 2 hours at 20°C (Zhang et al., 2017). The creaming index (CI) of the emulsions and thawed emulsions was calculated with Equation 5.3.

$$\text{Creaming index (CI) [\%]} = \frac{H_S}{H_T} \cdot 100\% \qquad (5.3)$$

Where H_S is the height of the serum layer at the bottom of the tube, H_T the total height of the emulsion (Zhang et al., 2017). H_S and H_T were measured using a ruler and Image J (version 1.52, National Institute of Health, USA). The morphology of the emulsion droplets was visualised by using light microscopy. Pictures were taken at day 1 and day 7 at 20x magnification, with a dilution of 10 times using a phosphate buffer (10 mM, pH 3.0) to visualise individual droplets.

5.2.8 Foamability

The foamability of the flours and protein-enriched fractions were determined by using an adapted protocol from (Yang, Kornet, et al., 2022). Dispersions of 1.0 w/w% protein were prepared in phosphate buffer (10 mM, pH 6.0). The dispersions were stirred on a magnetic stirrer plate for 60 minutes at room temperature. Afterwards, 10 mL of the solution was transferred into a cylindrical tube with a diameter of 3.33 cm. The solution was foamed for 2 minutes at 1000 rpm using an overhead stirrer. The cylindrical tube

was transferred to a foaming set-up, where the foam height was monitored with a camera every 5 min. The samples were monitored in triplicate for a minimum of 30 minutes. The foam height was analysed with Image J (version 1.52, National Institute of Health, USA). The foam overrun was calculated with Equation 5.4 (Yang, Kornet, et al., 2022).

$$\text{Foam overrun [\%]} = \frac{\text{Foam volume [mL]}}{\text{Initial liquid volume [mL]}} \cdot 100\% \quad (5.4)$$

5.2.9 Data analysis

The statistical analysis of the data was performed by using IBM SPSS statistics version 29. Tukey post-hoc analysis was used if the variation was homogeneous based on Levene's test for homogeneity, and Games-Howel was used when the data was not homogeneous. To test for correlation, the Pearson correlation test was performed. Here, significance was found at $p < 0.05$ (2-tailed).

5.3 Results and discussion

5.3.1 Fraction composition and protein nativity

The protein nativity of the flours and the protein fractions was examined with DSC (Figure 5.2). The toasted lupin and soy showed a typical DSC profile with two peaks, which correspond to 7S and 11S protein (Devkota et al., 2023; Zhong & Sun, 2000). Both lupin and soy still contained native 7S protein (first peak) and 11S protein (second peak) (Figures 5.2A; 5.2B). This transition was not reversible, as during the 2nd heating no further transitions occurred, showing that the toasted lupin and soy still contain native protein. The moisture content of lupin and soy used in this study was 11% (Politiek et al., 2023a). The retention of native protein is consistent with expectations since heating at low moisture contents usually results in limited protein denaturation, even at high temperatures (up till at least 150°C for soy) (Geerts et al., 2018). At low moisture content, the temperature at which proteins denature increases significantly (Bühler et al., 2022). The denaturation peak temperatures and enthalpies measured with DSC vary with moisture content, but also with sample weight and scanning rate, where variation in the sample weight (2.5 mg – 10 mg) and scanning rate (0.5°C/min to 5°C/min) can already result in a 3°C peak temperature difference (Morales & Kokini, 1997; Saeed et al., 2016; Zhong & Sun, 2000). Based on that, the peak denaturation temperature values measured for soy and lupin flour are in a similar range as previously reported values (Devkota et al., 2023; Peng et al., 2021, 2023). The differences in measured peak temperatures were

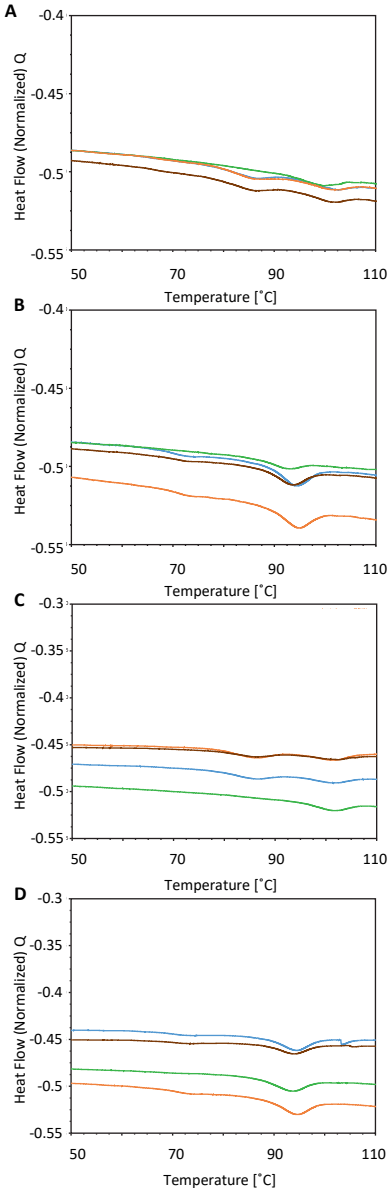
smaller than 3°C for the 7S and 11S protein for lupin and soy under different conditions (Figure 5.2). So de-oiling and electrostatic separation did not result in a significant change in peak temperature.

The retention of protein nativity after solvent de-oiling depended on the solvent used and the solvents showed the same effects for lupin and soy (Figures 5.2A; 5.2B). De-oiling with acetone and hexane had low to no influence on the enthalpy measured, whereas the 7S protein peak disappeared upon de-oiling lupin and soy with ethanol (Figures 5.2A; 5.2B). So de-oiling with ethanol resulted in complete protein denaturation of the 7S protein for both lupin and soy. The difference in retention of protein nativity can be explained by the different hydrophobicity of the solvents, where more hydrophilic solvents have a higher denaturing power towards than more hydrophobic solvents (Fukushima, 1969).

Electrostatic separation did not result in further protein denaturation for both lupin and soy, as after electrostatic separation of lupin and soy two peaks were still visible in the DSC thermograms and these had a similar peak denaturation temperature as the non-separated material (Figure 5.2). The enthalpy of the 11S peak was even higher after electrostatic separation of ethanol de-oiled soy than before separation, which suggests that from the proportion of proteins present in the material relatively more native protein was collected in the enriched fraction. The preservation of native protein upon electrostatic separation is a unique property for soy protein products, as commercial produced soy flours show endothermic peaks, and thus contain native protein, but most commercially produced soy protein isolates and concentrates were completely denatured (no detectable peaks in the DSC thermogram), or had a lower enthalpy per g protein (3.1 – 3.5 Joule/g protein) (Lee et al., 2003), than the soy protein concentrates produced in this study (Figure 5.2).

In our previous research, we evaluated the effect of solvent (none, acetone, ethanol, and hexane) on the electrostatic separation performance of soy and lupin (Politiek et al., 2023a). Electrostatic separation of toasted dehulled lupin- and soy flour resulted in protein enriched fractions with 48 %DM protein and 43 %DM protein respectively with a fraction yield of 27% (Politiek et al., 2023a). Polar solvents acetone and ethanol appeared favourable for dry fractionation of lupin based on composition (protein content 65-67% DM), whereas hexane was more favourable for soy (protein content 60% DM) in terms of protein purity and fraction yield (Politiek et al., 2023a). Protein denaturation was

found as an undesired side effect for ethanol de-oiling. So, next to composition the impact of solvent choice on protein nativity needs to be considered.



Material	Solvent	Peak 1: 7S protein		Peak 2: 11S protein	
		Enthalpy [Joule/g protein]	Peak temp T_d [°C]	Enthalpy [Joule/g protein]	Peak temp T_d [°C]
Lupin Flour	None	2.1	85.8	2.3	100.9
	Ethanol	*ND*	*ND*	2.5	99.7
	Acetone	1.9	86.1	2	101.9
	Hexane	1.7	85.8	2.1	101.4

Material	Solvent	Peak 1: 7S protein		Peak 2: 11S protein	
		Enthalpy [Joule/g protein]	Peak temp T_d [°C]	Enthalpy [Joule/g protein]	Peak temp T_d [°C]
Soy Flour	None	0.5	72.6	4.8	93.6
	Ethanol	*ND*	*ND*	1.9	92.6
	Acetone	0.7	72.5	5	94.3
	Hexane	0.5	73.3	4.8	94.7

Material	Solvent	Peak 1: 7S protein		Peak 2: 11S protein	
		Enthalpy [Joule/g protein]	Peak temp T_d [°C]	Enthalpy [Joule/g protein]	Peak temp T_d [°C]
Lupin GE fraction	None	2±0.1	85.8±0.3	2.3±0.1	101.7±1.1
	Ethanol	*ND*	*ND*	2.9±0.1	101.6±0.4
	Acetone	2.1±0.5	85.5±0.7	2.1±0.6	101.2±0.3
	Hexane	2.6±0.1	86.1±0.1	2.3±0	101.5±0.2

Material	Solvent	Peak 1: 7S protein		Peak 2: 11S protein	
		Enthalpy [Joule/g protein]	Peak temp T_d [°C]	Enthalpy [Joule/g protein]	Peak temp T_d [°C]
Soy GE fraction	None	0.8±0	73.2±0.3	4.9±0.3	93.7±0.2
	Ethanol	*ND*	*ND*	5.6±0.2	95.2±2.3
	Acetone	0.7±0.2	73.3±1.5	5.3±0.1	95.3±1.2
	Hexane	0.7±0.1	74.8±2.6	5.5±0.6	95.5±1.4

Figure 5.2: DSC thermograms of lupin flour (A), soy flour (B), lupin GE fraction (C) and soy GE fraction (D) for non-de-oiled material, ethanol de-oiled material, acetone de-oiled material and hexane de-oiled material. The tables show the enthalpy and peak denaturation temperatures (T_d) of the samples. ND indicates that no peak was detected.

5.3.2 Acetone extraction, where does the smell come from?

The decision for one or the other pre-treatment may not only per se depend on the protein purity and fraction yield and protein nativity, but also on other properties of the ingredients. It was noticed upon performing experiments in the lab that the flavour changed after de-oiling of the flours, where some of the samples had a different or even an unpleasant off-flavour. This could be due to the formation of new volatile compounds, removal or suppression of odour active compounds or the release of volatile compounds upon de-oiling (Bader et al., 2011). The aroma of the flours de oiled with acetone was perceived as sour and fermented, whereas the flours de-oiled with hexane had a very mild legume like aroma. The non-de-oiled material had no specific aroma and the material de-oiled with ethanol had a very mild yeast-type of aroma, which was associated with bread dough.

Aroma properties are mainly reflected by the composition of volatile organic compounds. To better understand the flavour composition of the samples and try to explain the different aromas as smelled in the lab and described above, , a selection of samples was analysed with GC-MS and GC-O-MS. For GC-MS, this selection included lupin and lupin de-oiled with acetone, ethanol and hexane, and soy de-oiled with acetone and hexane. For soy, the flour de-oiled with acetone was selected to verify the results for lupin, as de-oiling with acetone resulted in unfavourable off-flavours. Soy de-oiled with hexane was selected for GC-MS because this process gave the best electrostatic separation performance for soy. As lupin and soy showed similar GC-MS spectra after de-oiling (Appendix figure 5.1), only lupin was selected for GC-O analysis. Clear differences were observed in the volatile profiles of the various samples after de-oiling (Appendix figure 5.1). To annotate the differential peaks, the mass spectra of lupin, lupin de-oiled with acetone, ethanol and hexane and soy de-oiled with acetone and hexane were compared to those present in the NIST17 Mass Spectral Library and in-house databases. Hexanal (base peak 51 at 9.31 min) was present in the de-oiled lupin and soy flours (Appendix figure 5.1). The hexanal peak was odour active and perceived as a grassy, fresh aroma by at least two accessors upon GC-O analysis (Table 5.1). This is in agreement with other literature where hexanal was identified as having a green, beany, grass or fresh aroma (Kaczmarska et al., 2018; Pegiou et al., 2020; Schindler et al., 2011). A $C_6H_{12}O$ ketone (base peak 43 at 10.31 min) was present in both acetone de-oiled soy and lupin flour and this compound was annotated as 4-hydroxy-4-methyl-2-pentanone (Appendix figure 5.1). This ketone may have been formed by aldol condensation of two acetone molecules (McGorrin, 2007), which explains why the peak was specifically observed with

GC-(O)-MS after acetone extraction and not after ethanol or hexane extraction. In addition, the aroma perceived around 10.30 min was described as mouldy/spicy in the GC-O-MS analysis. Therefore, this compound is suggested to highly contribute to the strong off-flavour of the acetone extracted lupin and soy. Focusing on the retention time (RT) window between 10 and 21 min, more volatile compounds were detected for lupin de-oiled with acetone (Appendix figure 5.2). Some of these compounds were also found to be odour-active when GC-O-MS was performed. For example, the peak annotated as 2-octenal/nonenal (RT 15.10 min), 1-octen-3-ol (RT 15.16 min) or the mouldy aromatic compound (RT 21.49 min). Additional to the ketone (RT 10.30 min), these components might have been responsible for the sour and strong off-flavour that was perceived in the lab when smelling the lupin de-oiled with acetone (Table 5.1).

Table 5.1: Summary of GC-O-MS results of lupin de-oiled with acetone, ethanol and hexane. Compounds were annotated by comparing their mass spectra to those in the NIST17 mass spectral library and in-house databases. When the annotation is not reliable, compounds are listed as "unknown" and as "<LOD" when the signal was below the detection limit. The compounds that could have been responsible for the mild bread dough like flavour in ethanol is highlighted in green and the compounds that could have been responsible for the sour fermented smell after de-oiling with acetone are highlighted in blue.

Average RT (min)	Aroma attribute	Annotated compound	Smelled by at least 2 after de-oiling lupin with
7.08	alcohol, bitter, yeast	C5 alcohol or ketone	Ethanol
7.80	buttery, yeast	pentanal	Ethanol, Hexane
9.35	grassy, fresh	hexanal	Acetone, Ethanol, Hexane
10.30	mouldy, spicy	C6H12O ketone	Acetone
12.05	fishy, acidic	C5 alcohol	Ethanol, Hexane
12.90	citrus, tropical, fruity	C7 ketone or C8 alcohol	Acetone, Ethanol, Hexane
13.09	metallic, fishy	<LOD	Acetone, Ethanol
14.00	beany	diacetone alcohol	Acetone
14.40	sweaty, yeast	unknown	Acetone, Ethanol
14.55	citrus, tropical	nonanal	Acetone, Hexane
15.10	sour	2-octenal or nonenal	Acetone
15.16	off-flavour, earthy	1-octen-3-ol	Acetone, Ethanol
15.70	beany, buttery, yeast	? (base peak 68)	Acetone, Ethanol
16.17	sweet, buttery	decanal	Acetone, Ethanol
16.90	sweet, paint	C8 or C9 alcohol	Acetone
17.65	fruity, flowery	Isophorone	Acetone, Ethanol, Hexane
18.79	yeast, sweet	unknown	Acetone
18.91	off-flavour, earthy	unknown	Ethanol
19.75	plastic, rubbery	unknown	Acetone, Hexane
21.49	mouldy	aromatic compound	Acetone

Concluding, the lupin and soy flours de-oiled with acetone had a different volatile profile compared to the other flours. Moreover, the compounds detected after de-oiling with acetone were odour-active and assigned to strong off-flavours (Table 5.1; Appendix figure 5.1). This aligns with the findings found for lentil protein isolate, where treatment with acetone resulted in the accumulation of more volatile compounds, while treatment with isopropanol and ethanol resulted in samples with lower contents of volatile compounds (Chang et al., 2019). The accumulation of volatile compounds upon de-oiling with acetone is considered as to have a negative impact on the sensory profile as it results in a lower consumer acceptance (Bader et al., 2011). Consumer acceptance is mainly based on sensory properties such as texture, flavour and taste (Lesme et al., 2020). Therefore, the flours de-oiled with ethanol or hexane are expected to have a higher consumer acceptance based on their flavour profiles and these are thus considered as favourable solvents to de-oil soy and lupin flour with prior to electrostatic separation. As the polar solvents were more favourable for electrostatic separation of lupin (Politiek et al., 2023a) and ethanol de-oiled lupin had a better flavour profile than acetone de-oiled lupin, ethanol was chosen to de-oil lupin and hexane was chosen to de-oil soy.

5.3.3 Solubility of enriched fractions

The solubility and the nitrogen solubility index of the flours and fractions were determined at pH 3, pH 6 and pH 7. This section focusses on the results of the selected samples, which are lupin, lupin de-oiled with ethanol, soy and soy de-oiled with hexane and the corresponding protein enriched fractions. The solubilities of acetone de-oiled lupin and soy, hexane de-oiled lupin and ethanol de-oiled soy are included in the solubility analysis to allow further generalisation of the results.

The solubility of lupin was significantly higher at pH 3 and pH 7 than pH 6 (Figure 5.3A), whereas for soy the solubility was not significantly different at pH 3, 6 and 7 (Figure 5.3B). To evaluate whether the solubility of soy could be further increased upon extremer pH, the solubility of soy was also measured at pH 8 (33%), which was significantly higher (P-value=0.006) than the solubilities at pH 3, 6 and 7. The low solubility of both legume flours at pH 6 can be explained by typical protein solubility curves, which show a high solubility at acidic pH, low solubility (<20%) around the isoelectric point (pH 4 – pH 6) and again a high solubility at basic pH (Ma et al., 2022; Shrestha et al., 2021). The higher solubility at extremer pH conditions is caused by the positive charge of the proteins at acidic pH and negative charge of the proteins at basic pH, which results in weak hydrogen

bonds between protein and water. These protein-water interactions are essential for the protein solubility (Torres et al., 2007). To confirm that the increase in solubility was caused by an increase in protein solubility, a correlation test was executed. Here, the solubility and nitrogen solubility index were positively correlated (P-value <0.001) (Appendix figure 5.3).

The solubility of lupin and soy was affected upon de-oiling, where the effect (increased, similar or decreased solubility) depended on the solvent used. Upon de-oiling lupin with ethanol the solubility decreased (Figure 5.3A). The lower solubility after ethanol de-oiling is caused by the denaturation of native protein compared to the non-de-oiled flour as also evident by DSC (Figure 5.2) and in accordance with others (Bader et al., 2011; Ma et al., 2022). De-oiling soy with ethanol also resulted in a lower solubility than the non-de-oiled flour (Figure 5.3B), so the lower solubility observed after de-oiling with ethanol was independent of the legume used and caused by the decreased protein nativity. De-oiling soy with hexane increased the solubility at pH 3 and pH 7 (Figure 5.3B). The higher solubility after de-oiling with hexane might be attributed to the lower oil content or distinctions of protein subunits as was found by Yue et al. (2021) upon de-oiling oat with hexane. However, the higher solubility after de-oiling with hexane is legume dependent as hexane de-oiled lupin (Figure 5.3A), or hexane de-oiled lupin flakes (Bader et al., 2011), resulted in a similar solubility as the non-de-oiled lupin flour. Acetone de-oiling resulted in similar solubilities for lupin and similar or higher (at pH 3) solubilities for soy, which was comparable to the observations for hexane de-oiling of both legumes (Figure 5.3). So, hexane and acetone de-oiling do not negatively affect the material solubility, whereas ethanol de-oiling decreases the solubility.

Electrostatic separation did not negatively affect the solubility of the protein enriched fractions (Figure 5.3). The lupin protein-enriched fractions had a similar or slightly higher (not significantly) solubility, whereas the soy protein-enriched fractions had a significantly higher solubility at pH 3 (Figure 5.3). The solubility of ethanol, acetone and hexane de-oiled lupin increased significantly at pH 3 and pH 7 after electrostatic separation (Figure 5.3A), and electrostatic separation of hexane de-oiled soy also resulted in a significantly higher solubility at pH 3 (Figure 5.3B). This was caused by a higher protein solubility, so the results point towards a higher ratio of soluble proteins in the protein-enriched fractions than in the flour. The ratio of soluble proteins versus insoluble proteins might have changed after electrostatic separation because smaller particles (i.e. protein bodies) are easier caught by the electrodes than the larger poorly

soluble aggregated particles, due to the higher charging capacity of small particles (Basset et al., 2016; Tang et al., 2009). The solubility of electrostatic separated hexane de-oiled soy was not significantly higher at pH 7, as was observed for hexane de-oiled lupin. This might be attributed to the generally lower solubility of soy than the solubility of lupin at pH 3 and pH 7, which is caused by a difference in nitrogen solubility curves. The nitrogen solubility curve was steeper for lupin than for soy when moving away from the iso-electric point (Appendix figure 5.4). So, at extremer pH the solubility might also be significantly higher after electrostatic separation of hexane de-oiled soy.

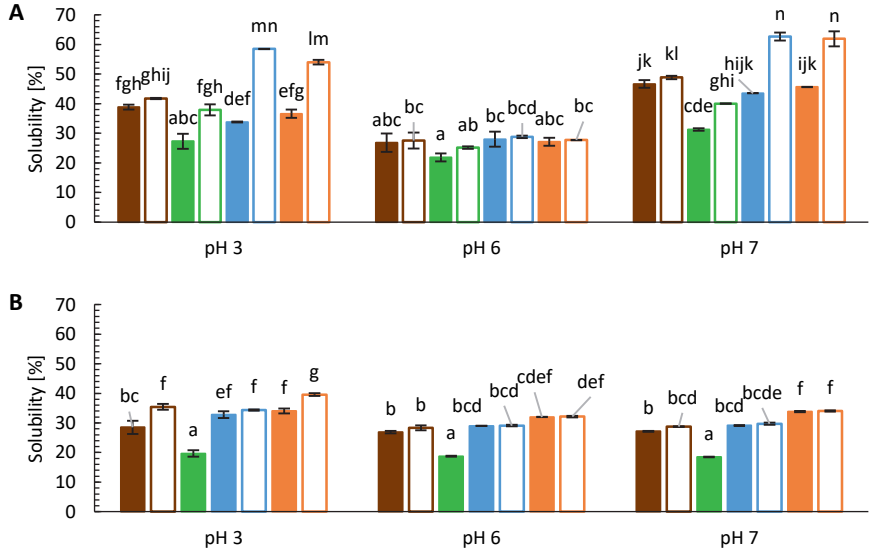


Figure 5.3: Solubility of lupin samples (A) and soy samples (B) at pH 3, pH 6 and pH 7. The solid bars indicate the non-de-oiled flour, ethanol de-oiled flour, acetone de-oiled flour and hexane de-oiled flour. The white-filled bars represent the enriched fractions of the corresponding flours. The error bars represent the standard deviation and different letters indicate a significantly different solubility (P<0.05).

The nitrogen solubility of soy flour (4% - 10%, Appendix figure 5.4) was in a similar range as reported for commercially produced soy flours and showed a similar solubility pattern as flours with a reported solubility below 20% around pH 3 (Lee et al., 2003). The nitrogen solubilities of toasted lupin flour (64 ± 1%) and the protein-enriched fraction from toasted lupin flour (65 ± 1%) at pH 7 were similar to the nitrogen solubility of lupin protein isolate (64 ± 3%) previously reported, which was de-oiled with n-hexane prior to the wet fractionation (Schlegel et al., 2019). However, the harsher conditions (i.e. longer temperature exposures or higher acidic environment) upon wet fractionation at larger

commercial scale likely will result in lower solubility (Ma et al., 2022). Next to that, even without de-oiling, the lupin samples already showed high solubility at pH3 and pH7 (Figure 5.3). So, producing protein concentrates with similar solubility as de-oiled wet fractionated lupin protein isolates via electrostatic separation, is a huge advantage as there is less risk of a reduction in protein solubility upon industrially processing and the protein-enriched fractions might even have superior functionality over the wet fractionated material as was found for electrostatically separated navy bean (Tabtabaei et al., 2019).

5.3.4 Emulsification and foamability of lupin and soy enriched fractions

Emulsions with non-de-oiled lupin, ethanol de-oiled lupin, non-de-oiled soy and hexane de-oiled soy and the corresponding protein enriched fractions were prepared at pH 3 to screen whether they could be applied in dressing like emulsions (pH 3, 30% oil) and the creaming index was monitored over time. The emulsions prepared without de-oiling lupin had a lower creaming index than the emulsions prepared with non-de-oiled soy (Figure 5.4). This was likely caused by the higher solubility of lupin at pH3 than soy at pH3 (Figure 5.3). A positive correlation between solubility and emulsifying properties has also been confirmed by many other studies (Burger & Zhang, 2019). De-oiling lupin with ethanol resulted in an increased creaming index (Figure 5.4A; 5.4D; 5.4E), which might be caused by the denatured protein and less soluble material after ethanol de-oiling. For soy, de-oiling with hexane resulted in a slightly lower creaming index and also colour wise a more milk like emulsion (Figure 5.4B; 5.4I; 5.4J). The lower creaming index after hexane de-oiling might also have been caused by the increased solubility after hexane de-oiling (Figure 5.3B). The more milk-like colour is caused by a smaller droplet size in the emulsion with the hexane de-oiled soy than toasted soy (Appendix figure 5.5), which leads to a stronger light scattering and therefore to a whiter, milk-like appearance of the emulsion (Fan et al., 2023).

After electrostatic separation the creaming index remained similar for the non-de-oiled lupin protein-enriched fractions, but was even worse for the protein-enriched fractions after electrostatic separation of ethanol de-oiled lupin (Figure 5.4A; 5.4C; 5.4F), even though the solubility of the enriched fraction was higher (Figure 5.3). The reason behind the poorer emulsification might be that there is less dry matter added in the emulsion with the enriched fraction and that the other components present in the ethanol de-oiled lupin, such as carbohydrates, contributed to the emulsion stability by increasing the

viscosity of the continuous phase (Funke et al., 2022; Zhou et al., 2020). For soy, the enriched fractions had a similar creaming index for soy de-oiled with hexane and for non-de-oiled soy the creaming index decreased the most after electrostatic separation and the colour of the emulsion was also more milk like after separation (Figure 5.4B; 5.4G). This follows a similar pattern as was observed for the solubility of soy, which improved after de-oiling with hexane and consecutive electrostatic separation. The non-de-oiled lupin protein-enriched fraction was most promising for (non-frozen) dressing like emulsions. The other protein-enriched fractions also show potential for dressing like applications, but this would likely require a higher mass fraction to form emulsions with a lower creaming index than presented in Figure 5.4.

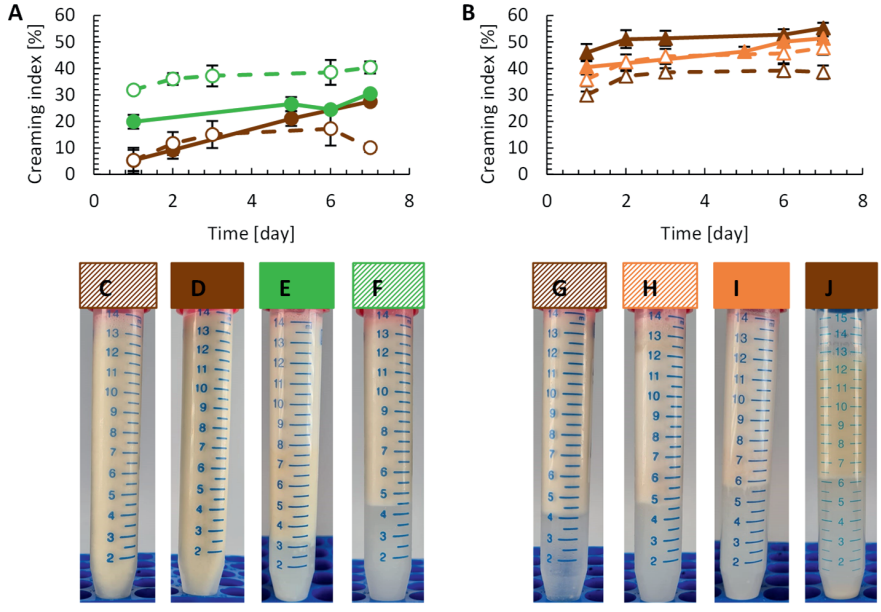


Figure 5.4: Creaming index of lupin (A) and soy (B) at pH3 (1 w/w% protein). The solid lines and symbols represent the non-de-oiled flour (brown), ethanol de-oiled flour (green) and hexane de-oiled flour (orange). The dashed lines and open symbols represent the enriched fractions of the corresponding flours. The error bars represent the standard deviation. Pictures of the emulsions after 1 day are provided from best to worst for lupin (C-F) and for soy (G-J), which were lupin enriched fraction (C), lupin flour (D), lupin ethanol (E), lupin ethanol enriched fraction (F), soy enriched fraction (G), soy hexane enriched fraction (H), soy hexane (I) and soy flour (J).

Next to the creaming index, the freeze thaw stability was evaluated for potential frozen applications. The creaming indices measured after thawing the frozen emulsions in our study can be divided into two groups; one with a creaming index between 25-35% and one with a creaming index above 50% after thawing (Figure 5.5). The hexane de-oiled soy

and the corresponding enriched fraction were not destabilized after freeze-thaw treatment as the creaming indices with and without freezing were the same (Figure 5.5). This is a unique property as frozen emulsions are usually significantly destabilized after thawing (Ghosh & Coupland, 2008; Zhang et al., 2017). To illustrate, an emulsion prepared with soy protein isolate at extremer pH value (pH 8) with less oil (20%) and more protein (1.5%) destabilised with 45% relative to the initial emulsion (Zhang et al., 2017), while the conditions used were more favourable (extremer pH, less oil and more protein) than the emulsification conditions used in the present study. So, the hexane de-oiled soy flour and the enriched fraction produced after electrostatic separation of hexane-de-oiled soy are most promising for frozen applications. The lupin, lupin enriched fraction and the ethanol de-oiled lupin showed a similar creaming index (25-35%), so the samples were not completely destabilized (<30% relative to the initial emulsion, dashed line Figure 5.5) after freezing and might also be promising for frozen applications. However, relative to the initial material the creaming index was higher (Figure 5.5). The frozen emulsions with creaming indices above 50% are considered to be unfavourable for frozen applications, as these were most destabilised.

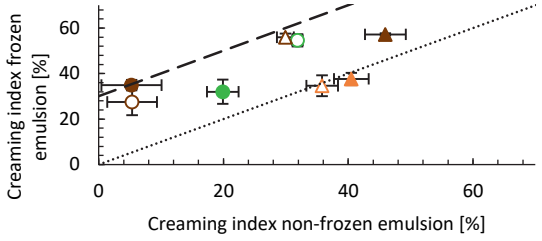


Figure 5.5: Creaming index after freeze-thaw treatment against creaming index without freeze thaw treatment of lupin samples (circles) and soy samples (triangles). The solid symbols represent the non-de-oiled flour (brown), ethanol de-oiled flour (green) and hexane de-oiled flour (orange). The open symbols represent the enriched fractions of the corresponding flours. The error bars represent the standard deviation and the lines are added to guide the eye. The dotted line represents 0% destabilisation and the dashed line represents 30% destabilisation relative to the initial emulsion. The closer to the y=x line the better the freeze-thaw stability.

To evaluate whether the de-oiled flours and enriched fractions would be applicable in a foam-based systems like ice-creams, they were foamed at pH6 and the foam volume was measured over time. The foam overrun was used as a measure of the relative foam volume to the initial liquid volume (Equation 5.4). The foam abilities (foam overrun at t=0) of soy and lupin were similar (27-28%) and de-oiling resulted in a higher foam overrun for both lupin and soy (Figure 5.6). This can be explained by the reduction of the

oil concentration, as at high oil concentration, the oil leads to coalescence of the foam bubbles during the foam generation process by disrupting the foam films (Arnaudov et al., 2001). Next to that, the hexane de-oiled soy had a higher solubility than the soy flour and a smaller particle size (Politiek et al., 2023a), which can also explain the better foamability (Moll et al., 2022). The foam overruns of the non-de-oiled lupin and soy flours were not significantly different from the foam overruns of the corresponding protein-enriched fractions, which is likely due to the low initial foamability caused by the higher initial oil content of the samples. The foam overruns of the protein enriched fractions of lupin de-oiled with ethanol and soy de-oiled with hexane were similar or slightly lower than the unseparated material, which might be because less dry matter is added to the foams prepared from the enriched fractions. As hexane de-oiling improved the foamability more than ethanol de-oiling the solvent used likely affected the foamability of the materials, next to the oil content. Overall, as hexane de-oiling combined with electrostatic separation also resulted in a good freeze-thaw stability next to the high foamability, the enriched fractions after electrostatic separation of hexane de-oiled soy have most potential for frozen applications like ice-cream.

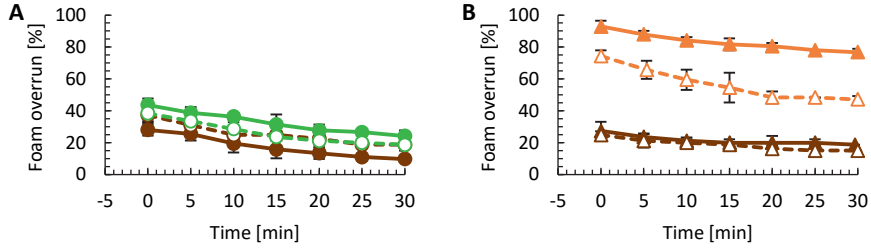


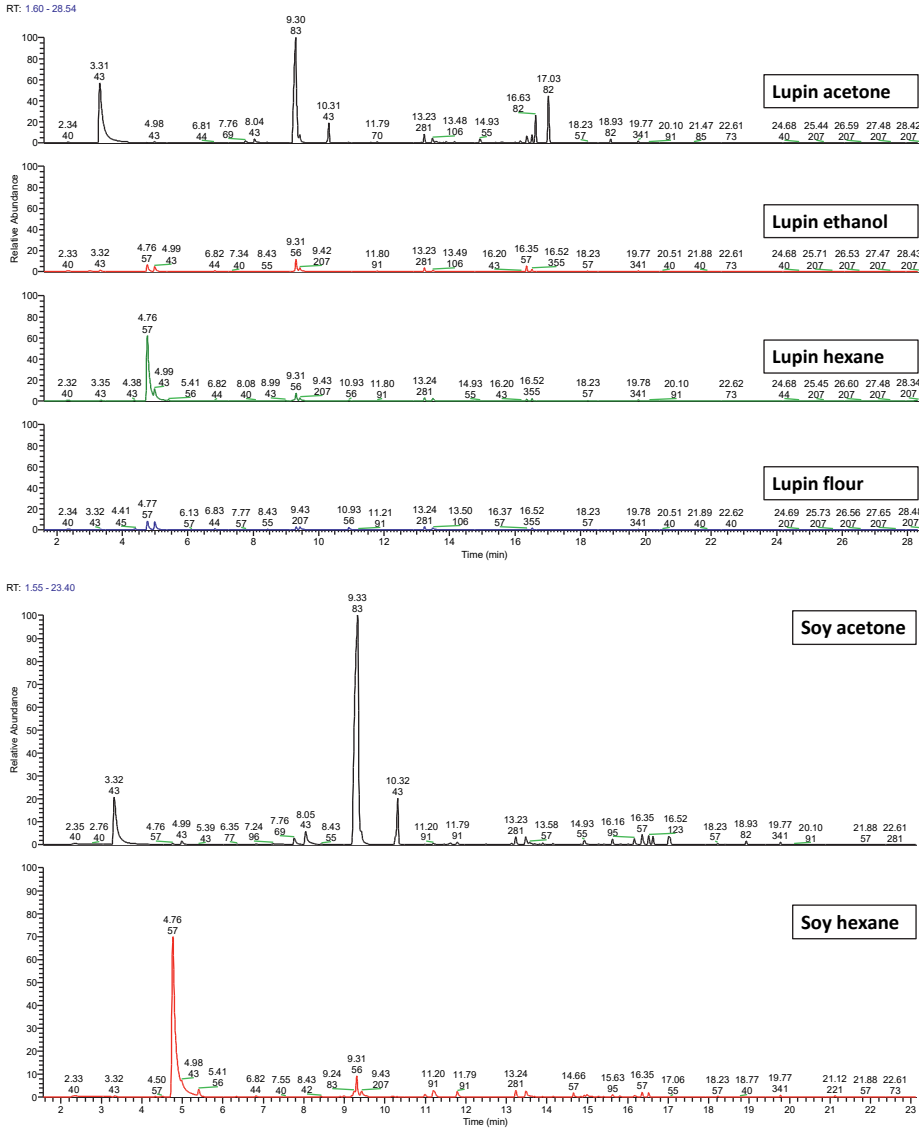
Figure 5.6: Foam overrun against time for lupin (A) and soy (B) at pH6, 1 w/w% protein. The solid lines and symbols represent the non-de-oiled flour (brown), ethanol de-oiled flour (green) and hexane de-oiled flour (orange). The dashed lines and open symbols represent the enriched fractions of the corresponding flours. The error bars represent the standard deviation, lines are added to guide the eye.

5.4 Conclusion

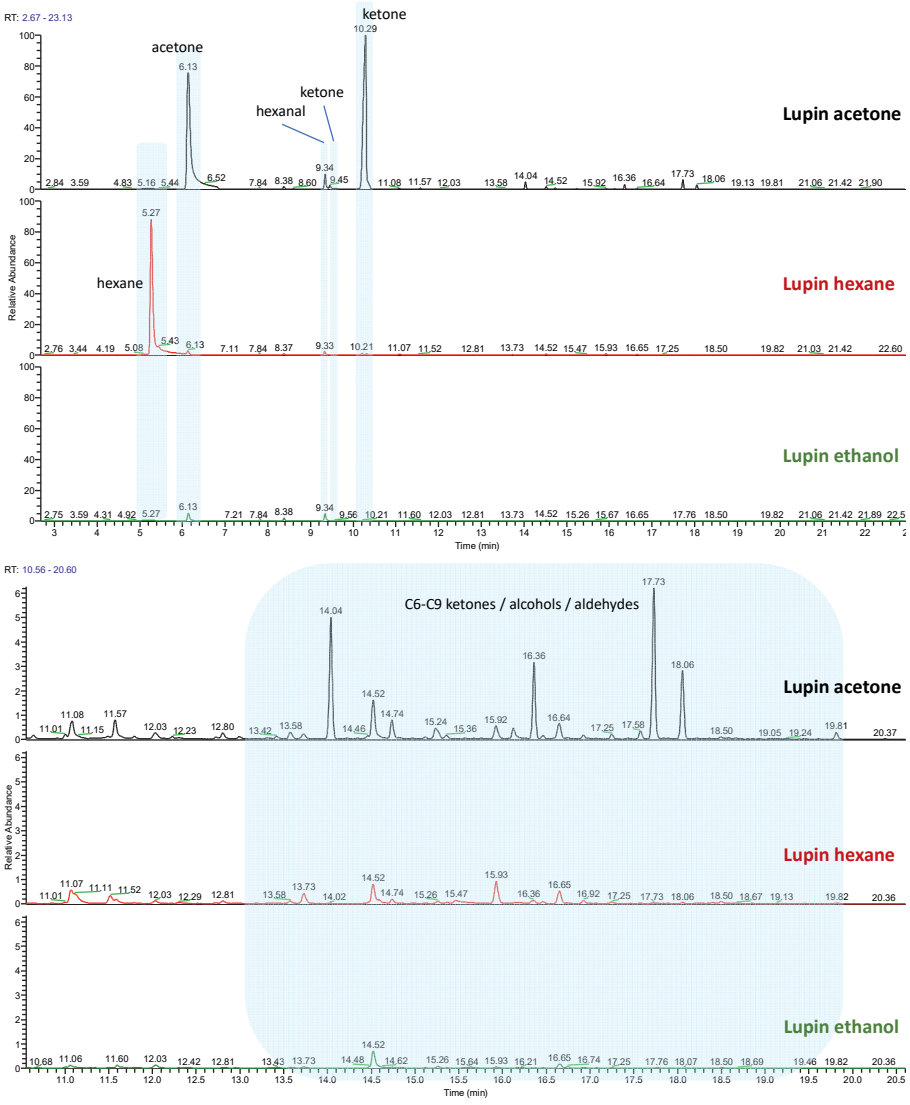
The functionalities and flavour profile of the soy and lupin protein-enriched fractions obtained after electrostatic separation were affected by the de-oiling method. Industrially toasted and milled lupin and soy flours could be used as a starting material to produce functional protein-enriched ingredients with electrostatic separation. No de-oiling, hexane de-oiling and acetone de-oiling were favourable pre-treatments to preserve native protein, whereas ethanol and hexane de-oiling were preferred solvents over acetone for a better flavour profile of the produced ingredients. Electrostatic separation was favourable for the solubility of lupin and de-oiled lupin, where pH 7 showed the highest solubility for the lupin-based ingredients and for soy electrostatically separated hexane de-oiled soy resulted in the highest solubility at pH 3. The protein-enriched fractions had equal or better functionality than the flours, except for emulsification of ethanol de-oiled lupin.

For emulsion-based applications at pH 3, no de-oiling appears necessary prior to electrostatic separation of lupin. For soy, either no de-oiling or hexane de-oiling could be used as a pre-treatment to obtain protein-enriched ingredients for emulsion-based applications like dressings, but a higher amount of the ingredient should be added to produce emulsions with more similar properties as the lupin protein-enriched ingredient. For foam-based applications de-oiling seems necessary as the presence of oil negatively affected foam stability. Overall, the results in this study can be used as a starting point to further optimize lupin and soy protein-enriched ingredients for food applications.

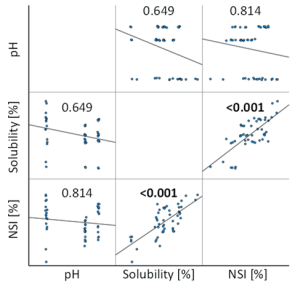
Appendix 5



Appendix figure 5.1: GC chromatograms of lupin flour and lupin de-oiled with acetone, ethanol and hexane. The relative abundance was plotted against the retention time. The labels above the peaks indicate the retention time (min) and the base peak (mass of the highest intensity).



Appendix figure 5.2: GC chromatograms of lupin de-oiled with acetone, hexane and ethanol. The peaks that correspond to hexane, acetone, hexanal and ketones and C6-C9 ketones/alcohols/aldehydes are highlighted. A zoom in is provided at the bottom between the retention times (RT) of 10.56 and 20.60.

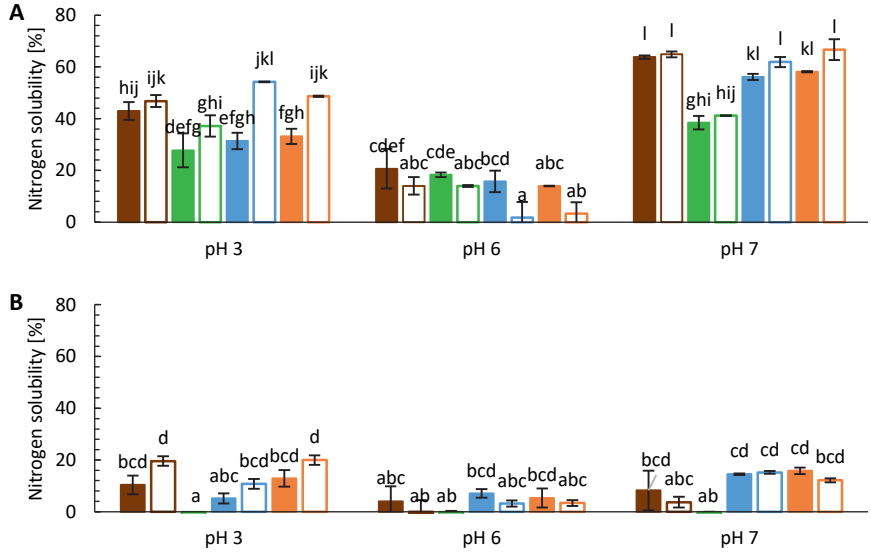


Correlations

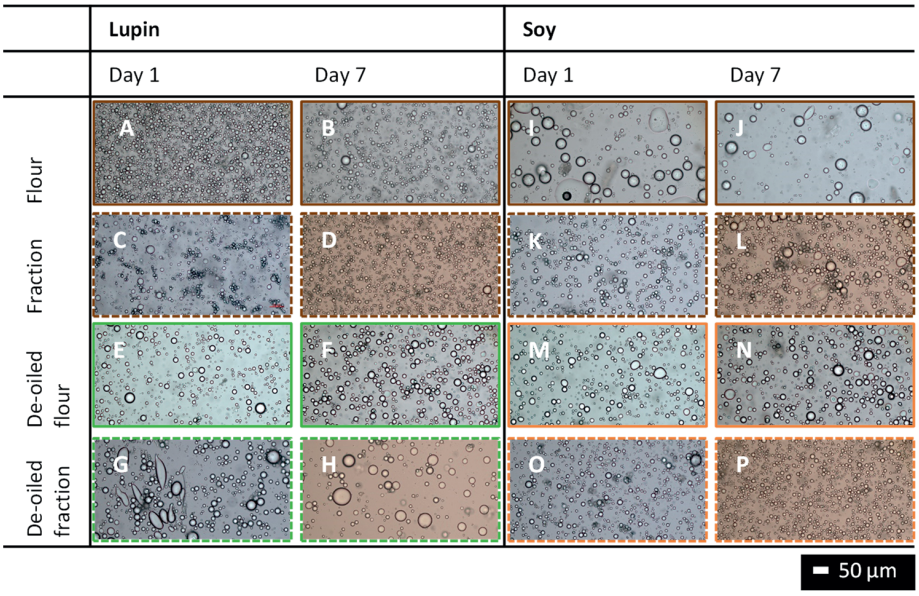
		pH final	Solubility [%]	Nitrogen solubility index [%]
pH final	Pearson Correlation	1	-.044	.023
	Sig. (2-tailed)		.649	.814
	N	107	107	107
Solubility [%]	Pearson Correlation	-.044	1	.853**
	Sig. (2-tailed)	.649		<.001
	N	107	107	107
Nitrogen solubility index [%]	Pearson Correlation	.023	.853**	1
	Sig. (2-tailed)	.814	<.001	
	N	107	107	107

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix figure 5.3: Pearson correlation between pH, solubility and nitrogen solubility index (NSI) with p-values for each comparison (left) and statistical output for the Pearson correlation test (right). A P-value below 0.05 is considered significant.



Appendix figure 5.4: Nitrogen solubility of lupin samples (A) and soy samples (B) at pH 3, pH 6 and pH 7. The solid bars indicate the non-de-oiled flour, ethanol de-oiled flour, acetone de-oiled flour and hexane de-oiled flour and The white-filled bars the enriched fractions of the corresponding flours. The error bars represent the standard deviation and different letters indicate a significantly different solubility (P<0.05).



Appendix figure 5.5: Droplet morphology of emulsion droplets at 20x magnification for lupin and soy samples (pH3, 1 w/w% protein) at day 1 and day 7 of lupin flour (A, B), lupin enriched fraction (C, D), ethanol de-oiled lupin (E, F), ethanol de-oiled lupin enriched fraction (G, H), soy flour (I, J), soy enriched fraction (K, L), hexane de-oiled soy (M, N), hexane de-oiled soy enriched fraction (O, P).

Chapter 6

Dry fractionation for protein enrichment of animal by-products and insects: A review

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[1]Shared first authorship

Abstract

Due to the global need for sustainably produced protein, optimal usage of animal by-product proteins and novel protein sources like insects are being explored. Dry fractionation, an emerging technology, offers significantly lower energy consumptions and no use of chemicals compared to conventional fractionation technologies. This review evaluates the current state and potential of dry fractionation for animal by-products and insects, with respect to characteristics of raw materials, pre-processing methods, milling, and product-oriented process optimisation.

The reviewed studies focussed on compound enrichment or fractions with distinct functionalities, rather than in depth product and process optimisation linked to composition and functionality. For animal by-products, optimisation should focus on milling and separation, whereas for insects optimisation should concern the entire process chain. A product portfolio and insight in compositional and functional properties after dry fractionation would allow more efficient use of animal by-product and insect fractions, thereby supporting the protein transition.

6.1 Introduction

Due to the expected growth in world population, 26% more people must be fed in 2050 (Shepard, 2019). Moreover, the average worldwide meat consumption per capita is expected to increase by 29% as people are becoming more affluent (Wu et al., 2014). The demand for protein thus grows while the availability of agricultural land decreases due to climate change and over-use of the agricultural fields. We therefore need a transition towards different production and use of novel sources of proteins. While meat and seafood are two important traditional protein sources, 32-57% (wet basis) of these sources end up as by-products or waste (Jedrejek et al., 2016; Meeker & Hamilton, 2006). These animal by-products are rich in high value nutrients such as proteins. In addition to by-products, novel protein-rich food sources like insects, require less water and land, emit less greenhouse gases, and have higher feed conversion efficiencies compared to conventional meat sources. In addition, insects can be grown on side streams, effectively upgrading these streams (van Huis & Oonincx, 2017). Although the crude protein content of insect species varies greatly, it is similar to that of cattle, poultry, and fish (Akhtar & Isman, 2018). Overall, both animal by-products and insects may well give us additional proteins with high nutritional and technical functionality, if we find ways to concentrate the proteins without large impact on the environment.

Direct use of animal by-products and insects as food is often disliked by consumers and is hindered by the high level of non-protein components such as ash and chitin, which has a negative impact on the digestibility, taste and technical functionality (Moutinho et al., 2017; Tan et al., 2016). To increase the protein content and digestibility of animal by-products and insects, both dry and wet fractionation strategies may be employed. Wet fractionation generally involves selective solubilisation of proteins or other components at elevated pH, low salt concentration (i.e. salting in), or using a mixture of ethanol and water. This is then followed by precipitation at the isoelectric point, salting out, or use of solvents. Such procedures require the use of water and chemicals. If not all proteins dissolve or precipitate a significant part of the proteins may be lost. Generally, the proteins then need to be dried. Dry fractionation also involves drying but does not require any additional water or chemicals for the separation. The dried material is fragmented by milling, and subsequent mechanical separation of the fragments using air classification or other methods, such as tribo-electrostatic separation.

As compared to wet fractionation methods, dry fractionation methods have lower energy consumption and use neither water, nor chemicals (Jonkman et al., 2020;

Schutyser et al., 2015). For example, the production of fish protein isolates by wet fractionation requires 5-9 parts of water per part of fish, which must then be removed by drying to obtain a protein powder (Shaviklo & Etemadian, 2019). Although animal products contain water that needs to be removed before any dry processing, the amount of water and thus the energy required in drying is much lower than in wet fractionation. As drying is the most energy intensive process of the processes involved in fractionation, a decrease in water that needs to be dried reduces the energy requirements of the whole process considerably (Lie-Piang, 2021). Furthermore, dry fractionation techniques induce less structural changes in proteins, as was demonstrated for plant seed proteins (Assatory et al., 2019). However, wet fractionation can yield fractions with a higher purity than dry fractionation, since the dissolution effectively detaches individual molecules from each other, while with dry fractionation the composition of the individual fragments dictates the maximum enrichment that can be obtained. The fractions obtained after dry fractionation typically differ in composition and functionality from wet enriched fractions (Schutyser & van der Goot, 2011). Dry enriched fractions thus fit better in products that do not require pure ingredients and where a cleaner production approach is targeted. All this implies that the decision between dry and wet fractionation techniques should be based on the product requirements and the nature of the raw materials.

Hitherto, dry fractionation of plant seeds, beans, and pulses has gained considerably more attention than dry fractionation of animal by-products or insects, possibly because animal sources must first be dried before they can be subjected to dry separation processes. The presence of distinct components (i.e. protein, ash, and chitin) could make animal by-products and insects suitable for dry fractionation. However, there is currently not much known about the transferability of the existing dry fractionation techniques, developed for plants proteins, to animal- and insect products. Furthermore, pre-processing steps, such as drying, largely affect the yield and efficiency of such a process (Assatory et al., 2019). Therefore, it is crucial to choose the right pre-processing, milling, and separation conditions to obtain the desired fractions.

This review evaluates the current state and potential of dry fractionation techniques for processing animal by-products and insects. Animal by-products include meat and bone meal (MBM), fish meal, and shellfish waste. The entire process chain employing dry fractionation, as illustrated in Figure 6.1, is described in the present review, starting with the raw materials (§6.2). Here, the differences between animal by-products and insects in comparison to plants are highlighted. After the raw materials section, pre-processing steps and methods are described (§6.3). Pre-processing may include deshelling,

rendering, biological decontamination (e.g. blanching), drying, and defatting. After pre-processing, the products must be milled (§6.4) into a flour. It is described why milling is performed and what effects milling will have on the dry fractionation performance. The flour is consecutively separated via sieving, air classification or electrostatic separation (§6.5), which is indicated as "dry fractionation" in Figure 6.1. Reported work on dry fractionation of animal by-products and insects is elaborated in section 5. Lastly, optimal usage of dry enriched fractions is explored via product-oriented process optimisation (§6.6), to enable the most efficient use of insect- and animal by-product fractions. This section will discuss the possible optimisation approaches of the entire dry fractionation process in industry and indicate routes to maximise fraction usage.

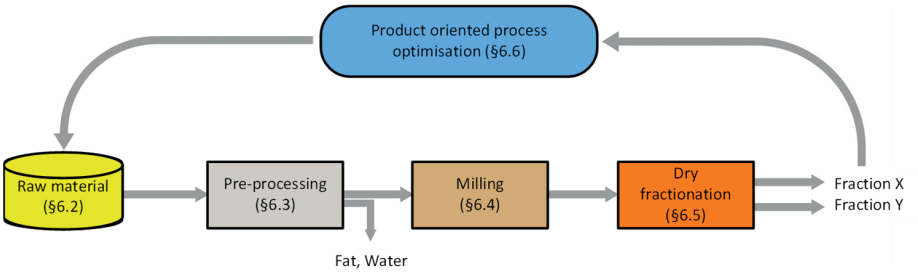

Figure 6.1: General visualisation of processing the raw materials (i.e. animal by-products and insects) into the final fractions. The different sections of the current review are indicated by the section signs (§).

6.2 Raw Materials

To perform dry fractionation on animal by-products and insects, knowledge on the raw materials is crucial. The compositions of various animal by-products and insects are discussed in the following paragraphs and are shown in Table 6.1. Lupin beans are added as reference, as among the plant products studied in literature for dry fractionation, lupin is the most similar crop based on composition (lipid and protein content; absence of starch) and cell size. As meat and bone meal (MBM) and fish meal may come from different animal species, typical values are shown (Garcia et al., 2006). In the present review, a distinction was made between the animal by-products and the insects. In contrast to the by-products, insects are often grown specifically for production of protein, and their processing can therefore be better tailored towards the dry separation procedures. Furthermore, insects are processed as a whole, while animal by-products are specific parts of the animal, such as bones. This implies potentially different dry fractionation strategies.

Since dry fractionation is currently mostly applied for plant materials, it is important to consider the similarities and differences between plant protein sources and animal products. The structure of animal tissue is not the same as the structure of plant tissue. On average, plant cells (10-100 µm) are larger than animal cells (10-30 µm) but have higher structural rigidity due to their cell walls. Another important difference is that plant cells contain specific storage bodies, such as protein or starch bodies, which facilitates separation by mechanical means (Pelgrom et al., 2014). Dry fractionation has been extensively studied for pulses like yellow field pea and navy bean. However, the compositions of yellow field pea and navy bean are very different from that of animal by-products and insects, mainly due to their high starch contents. In contrast, lupin has similar sized cells (30-35 µm) without large starch granules and has a fat content approximately similar to that of the animal products reviewed in this study, as can also be seen in Table 6.1 (Aguilera & Garcia, 1989). The outcomes of studies on lupin will be used as a reference throughout the manuscript. The findings of the studies on starch rich pulses will merely be used to explain the principle of the dry fractionation process and highlight optimisation possibilities and functionalities achieved.

6.2.1 Animal by-products

Slaughtering creates two main types of product streams: whole meat and by-products that are further processed into for example tallow (i.e. extracted fat), degreased bones (for gelatine production) and protein meals (e.g. fish-, meat- and bone meal) (Hicks & Verbeek, 2016). From the whole animals that enter the slaughterhouse, 32-57% (wet basis) end up as by-product or waste (Jedrejek et al., 2016; Meeker & Hamilton, 2006). When considering that in 2019 over 68 million tonnes of cattle meat alone was produced, one can imagine the potential to extract components of value, such as proteins, out of this side stream (FAO, 2020). The edibility of an animal by-product stream depends on the category of the starting material and the hygienic conditions during processing. Furthermore, legislation determines whether animal by-products can be fit for human consumption. According to European law, animal products and animal by-products are classified into three different categories before slaughtering. Both category 1 and 2 comprise material that is not fit for human consumption (e.g. infected material, spinal cord and brain), while category 3 material is fit for human consumption (EFPRA, 2020b; Jedrejek et al., 2016). Meat and bone meals from category 3 material are also known as processed animal protein (PAP) in Europe, if processed according to strict regulations (EFPRA, 2020a). This is due to safety considerations that are a consequence of the BSE outbreak in the 1990s. However, the regulations might soften in the near future, enabling

wider use of these by-products (Ricci et al., 2018). This review will focus on category 3 material.

Table 6.1: Proximate composition based on dry weight of animal by-products, insects, and lupin.

Raw material		Protein (%)	Lipid (%)	Carbohydrates/ fibre/ chitin (%)	Ash (%)	References
Animal by-products	Pure meat meal	75-87	ND	ND	8-14	Garcia & Phillips (2009)
	Pure bone meal	29-32	ND	ND	62-66	Garcia & Phillips (2009)
	Meat and bone meal	43-63	8-16	ND	16-40	Adedokun et al. (2014) Garcia et al. (2006) Garcia & Piazza (2015) Parsons et al. (1996) Shirley & Parsons (2001)
	Fish meal	56-82	5-16	ND	10-33	FAO (1986) Flynn et al. (2020) Hansen et al. (2010) de Lima et al. (2014)
	Shrimp waste	10-40	0-14	15-46 (chitin)	30-60	Nirmal et al. (2020) Tan et al. (2020)
	Crab waste	10-35	1-2	13-29 (chitin)	38-58	Antunes-Valcareggi et al. (2017) Hamdi et al. (2017) Jung et al. (2007) Muralidhara & Maggin (1985)
Insects	Yellow mealworm larvae	45-65 (crude)	13-35	4-15 (fibre)	3-7	Boulos et al. (2020) Purschke et al. (2018) Roos (2018) Rumbos et al. (2019)
	Adult house crickets	55-75 (crude)	7-23	3-23 (fibre)	2-14	Boulos et al. (2020) Kulma et al. (2019) Roos (2018) Rumbos et al. (2019) Rumpold & Schlüter (2013)
Plant seeds	Lupin	33-37	6-8	52-57	2-3	Erbersdobler et al. (2018) Pelgrom et al. (2015)

Pure meat meal, made exclusively from soft tissue particles like muscle cells, has consistent crude protein (75-87%) and ash (8-14%) contents regardless of the species, which also holds true for the protein (29-33%) and ash (62-66%) contents of pure bones

(Table 6.1). Bone tissue is mainly composed of collagen, crystalline minerals and other minor proteins, aggregated into fibrillar structures (~500 nm in diameter). The fibrils are stacked and mineralised to form larger fibres (micron to millimetre scale), to form either the inner light porous bone or the outer dense and protective bone (Kane & Ma, 2013). As explained previously, meat and bone meals (MBMs) are often mixtures of dried soft tissue and bones. Therefore, various compositions were reported for meat and bone meal, as seen in Table 6.1. For similar reasons, fish meal compositions are also reported with varying protein (56-82%), fat (5-16%), ash (10-33%) and dry matter content (90-98%), as is summarised in Table 6.1. MBM and fish meal usually have a high protein quality. The protein quality can be assessed via several methods, for example the protein digestibility-corrected amino acid score (PDCAAS). The PDCAAS can range between 0 and 1, in which 1 is the best score, indicating a high-quality protein. Seafood and animal proteins have in general a high PDCAAS of 1 or close to 1 (Huang et al., 2018; Tan et al., 2018). Specific proteins might have a less favourable amino acid profile. Collagen for example is deficient in some essential amino acids resulting in a lower PDCAAS of 0.52 for young children (2-5) and 0.94 for adults (Dong et al., 2014). This does not implicate that these proteins are not of use, for example collagen has very high technical functionality as a gelling agent (after hydrolysis into gelatine). They can also be combined with other protein sources to obtain an overall better nutritional balance.

Next to mammals and fish, animal by-products also originate from shellfish, that are aquatic invertebrates with an exoskeleton. These include amongst others crustaceans like shrimps, crabs, and lobsters, which are genetically related to insects (Mishyna & Glumac, 2021). The shellfish processing industries produce a significant amount of by-products. Depending on the species, 50-75% of the total weight of shellfish ends up as waste (Saima et al., 2013). It is estimated that in 2012 the major lobster processing countries (Canada, United States of America and Australia) produced over 50,000 tons of lobster by-products (Nguyen et al., 2017). The crustacean exoskeleton or shell is an important part of these by-products. This exoskeleton is made of a cuticle consisting of four different layers (Nagasawa, 2012). In general, shellfish by-products contain, as seen in Table 6.1, 10-40% proteins, 30-60% ash (mainly calcium carbonate), and 13-46% chitin along with other compounds like pigments and lipids. Shellfish also include mussels and other molluscs. Mussel by-products are not included in this literature review, as to our knowledge no research has been done towards dry fractionation of mussel by-products, although this could potentially be of interest. Dry fractionation could be used to separate

protein, calcium carbonate, and/or chitin, which are the main constituents of mussel shells (Naik & Hayes, 2019; Varma & Vasudevan, 2020).

6.2.2 Insects

In contrast to animal by-products, for insect processing typically the whole insect is used. Currently, over 2000 edible insect species are known (Jongema, 2017). Although insects are already consumed in many tropical countries for millennia, Western countries currently have strict regulations on insects for food and feed consumption. However, the legislation with respect to insects for food and feed, amongst others in the European Union, is easing, which increases the possibilities for the food and feed industry to include insects in their products (Belluco et al., 2017; Meijer & van der Fels-Klerx, 2017). Currently, the insect industry is growing rapidly, and this growth is predicted to continue for the coming years (Wade & Hoelle, 2020).

Insects are in general a good protein source with a protein content and quality similar to that of cattle, poultry, and fish (Churchward-Venne et al., 2017). Insects contain different types of proteins, including cuticular proteins in the exoskeleton, muscle proteins, and haemolymph (Yi et al., 2013). Furthermore, insects contain more polyunsaturated fatty acids, iron and zinc than conventional meat sources (Rumpold & Schlüter, 2013). Insects are also a source of fibre, which is mainly found in the exoskeleton in the form of chitin, together with the cuticular proteins (Finke & Oonincx, 2017; Yi et al., 2013). These compositions vary largely between insect species and within individual insect species caused by factors like the feed and the developmental stage of the insect. In the developmental stage of insects, one of the important aspects is the difference between larvae and adults. Larvae and adults have different amino acid profiles, yellow mealworm (Tenebrio molitor) adults contain for example more protein and chitin, while the larvae contain more lipids (Finke & Oonincx, 2017; Nowak et al., 2016). Table 6.1 shows the composition of yellow mealworm larvae (T. molitor) and adult house crickets (Acheta domesticus). Here, the crude protein contents are given, which also include nitrogen originating from chitin, based on dry weight (Churchward-Venne et al., 2017; Roos, 2018).

The composition and structure of animal by-products and insects are different from plant sources commonly used for dry fractionation. Furthermore, there are large distinctions in the composition of various animal by-products and insects. However, based on the composition and protein quality of the animal by-products and insects there is a high potential to upgrade these products into safer and more versatile ingredients, with

increased sustainable and nutritional aspects. The presence of different compounds, i.e. protein and non-protein components like ash and chitin, suggest that the materials are suitable for dry fractionation.

6.3 Pre-Processing

Raw materials (i.e. animal by-products and insects) must be pre-processed and milled before dry fractionation. For animal by-products and insects, pre-processing steps may include deshelling, rendering, biological decontamination defatting, and drying. The purpose of these pre-processing steps is to minimise safety concerns and to prepare the material for the dry fractionation process. The pre-processing steps chosen depend on the raw materials and will affect the properties of the products. Deshelling and rendering are usually already carried out with current by-products processing but need to be addressed here as these processes affect the product properties. After pre-processing, milling is a necessary step to fragment the material into a flour to enable subsequent dry fractionation (Schutyser & van der Goot, 2011).

6.3.1 Deshelling

Of all the materials of interest for the present review, both insects and shellfish have a shell, i.e. exoskeleton. Shellfish are deshelled, while insects are generally too small and too soft for deshelling. However, the chitin from the insect shells can later be removed via dry fractionation. Deshelling is done to separate the meat from the shells, which is the by-product. There is great potential to extract the residual proteins in the by-products, but also to extract the chitin. Chitin could be used in, amongst others, food applications and agriculture (Barikani et al., 2014). Deshelling is either done manually or mechanically. Before the deshelling process, shell loosening is often necessary. For shrimps, this is traditionally done by letting the shrimps mature in ice or brine for several days. To shorten this process, alternative techniques for deshelling are now emerging, like high pressure, ultrasound and enzyme treatments. In addition to efficient loosening of shells, these techniques allow the preservation of food quality and ensure food safety. For example, instead of boiling lobsters and crabs to loosen the shell and reduce the number of microorganisms, high pressure can be used (Dang et al., 2018).

6.3.2 Rendering

After cleaning and slaughtering of vertebrates, by-products are rendered, such as cattle skeletal bones. Rendering plants must strictly follow approved methods with specified processing conditions (i.e. time, temperature and pressure applied) and meet standards

for the final product quality, such as microbial counts (Ricci et al., 2018). In general, all rendering processes consist of heating for decontamination, moisture removal and consecutive separation of fat and other solids, yielding crude animal fat and protein meal (Hicks & Verbeek, 2016; Meeker & Hamilton, 2006). Moisture is removed to improve the product stability, the animal fat is then further cleaned in a separate step and, depending on the risk category of the starting material, used as edible fat in food or as inedible fat for other applications (Alm, 2020). Water and/or steam is used in wet rendering, but dry rendering is also performed. In this method, the raw material is cooked to melt the fat and to condition the animal fibrous tissue. The cooked material is then drained and pressed to separate the fat from the protein material (Prokop, 1985). During the heating of the materials, the proteins are most likely denatured. Therefore, further biorefining to separate those streams into materials of value should be driven by sustainability instead of by preserving native functionality.

6.3.3 Microbiological and enzymatic stabilisation

Microbiological stabilisation extends the shelf-life and minimises safety concerns related to the further use of the raw materials. Various methods can be applied to decontaminate the raw materials, but the most common methods to inactivate micro-organisms and enzymes involve a heat treatment, for example blanching for insects. Similarly, vertebrate by-products are rendered to amongst others provide biological stabilisation, which was discussed section 3.2. While a heat treatment is effective, it also denatures proteins and thereby degrades the quality in terms of native functionality, as can result in for example a reduced solubility. On the other hand, a heat treatment can, depending on the type of material, improve both the digestibility and taste of the product. Thermal inactivation of enzymes as proteases and lipases, prevents or minimises amongst others enzymatic browning in insects, which is undesired for food products with lighter colours (Janssen et al., 2019; Purschke et al., 2018). When heat treatments are undesired, milder processes that apply milder temperatures (<40 °C) may be used for microbiological and enzymatic stabilisation. Examples of relatively mild processes include high pressure processing and atmospheric cold plasma (Barba et al., 2017). High pressure processing and cold plasma treatments have been performed with yellow mealworm (T. molitor) larvae (Rumpold et al., 2014). Drawbacks of milder processing include their higher cost and their limited applicability, e.g. only surface treatment by cold plasma is possible.

6.3.4 Defatting

Depending on the fat content of the raw material, fat may be released upon milling, which may impede the milling process. A defatting step before milling may prevent agglomeration during milling and dry fractionation. The extracted animal fat can be used as an ingredient in food and feed. Meat and bone meals are already defatted by rendering. However, additional defatting is sometimes performed to further enhance the flowability. Nevertheless, thorough defatting can result in increased dust release during dry fractionation and thus again lead to larger product losses. Therefore, chilling below the melting trajectory of the fat, or the addition of anticaking agents may be alternatives to traditional defatting (Garcia et al., 2007; Garcia & Piazza, 2015). Defatting has been used more frequently with plant seed fractionation. For instance, Xing et al. (2018) achieved successful soybean protein enrichment of 15% by dry fractionation after defatting. Protein enrichment was also reported for rapeseeds, sunflower seeds, and lupin (Basset et al., 2016; Laguna et al., 2018; Pelgrom et al., 2014).

Mechanical pressing and organic solvent extraction are widely used methods to remove oil. Both processes may lead to protein degradation. Solvents such as ethanol and hexane tend to denature proteins, but also the force and temperature applied during mechanical pressing may affect the protein nativity in the obtained cake. Furthermore, application of organic solvents (e.g. hexane) has downsides concerning sustainability, cost, and safety restrictions. Alternatively, milder and more sustainable extraction techniques can be used. For example, cold pressing better preserves the product quality, albeit at the cost of some oil yield (Nde & Foncha, 2020). Cold pressing has been used for fat extraction of black soldier fly (Hermetia illucens) larvae (Matthäus et al., 2019). Supercritical carbon dioxide (SC-CO2) extraction causes minimal protein denaturation and may retard lipid oxidation, while the oil yield is similar to the oil yield after pure hexane extraction (Russin et al., 2011). The effectiveness of SC-CO2 was for example demonstrated for defatting of yellow mealworms (Purschke et al., 2017). However, the use of SC-CO2 also has some disadvantages, including high capital and operational costs (Russin et al., 2011). To conclude, defatting is applied prior to dry fractionation to extract oil as a fraction from the raw materials, to reduce agglomeration and to increase powder flowability. The chosen method impacts the final product quality in terms of sustainability, composition, and protein functionality after dry fractionation.

6.3.5 Drying

For dry fractionation, the feed material must be dry. In some cases, the material is already dry enough (i.e. rendered animal protein meals), while in other cases an additional drying step needs to be performed. The moisture content must be low enough to allow the creation of a free-flowing flour that is sufficiently fine for resolution between the different constituents, but not yet giving rise to clumping and aggregation due to interparticle interactions. There is no golden standard for the moisture content needed for dry fraction, as this depends on the raw material and the exact process to be applied. For example, the optimum moisture content for air classification of legumes was between 7-9%, while for other applications, like debranning of wheat grains, higher moisture contents are allowed (Owusu-Ansah et al., 1991; Schutyser & van der Goot, 2011). Drying methods for animal by-products and insects include contact-, oven- or microwave drying. These thermal methods will likely induce similar protein denaturation as in the stabilisation step. If this is undesired, alternative drying methods can be used that involve temperatures below the denaturation temperature. An example of such a method is dehumidified air drying, in which relatively low temperatures of below 40 °C can be used (Djaeni et al., 2018). Another example of a lower-temperature drying method is freeze-drying. An additional advantage of freeze-drying in case of insects is that the matrix becomes porous, which will ease the subsequent milling (Purschke et al., 2018). However, disadvantages are that freeze drying is quite energy intensive, expensive and time consuming, and it has been reported to reduce the solubility of proteins as well (Berghout et al., 2015b; Ratti, 2001).

In summary, before dry fractionation of animal by-products and insects, pre-processing steps are applied dependent on the raw material. Deshelling of shellfish and rendering of animal by-products are usually already performed. These processes are important to take into account as they strongly affect the final product properties. After deshelling and rendering, additional pre-processes can be applied to alter the powder properties before milling and separation. As insects are mainly processed whole, there is more freedom to apply the desired pre-processing steps for the purpose of dry fractionation. Within the pre-processing steps described, a wide range of methods is available. These methods can have different effects on the final quality of the materials, depending on the method and the raw material. When selecting pre-processing methods, the quality needs for the final product application must be considered.

6.4 Milling

Milling is considered perhaps the most critical step to enable subsequent dry fractionation. During milling, small fragments with different composition are created. This is critical as without physical detachment, dry mechanical separation would be impossible (Schutyser & van der Goot, 2011). Milling is often done in two consecutive steps. First rough milling is performed to increase the surface area, to allow for defatting and for the second fine milling. This fine milling is performed to give the material the desired particle size distribution for the dry fractionation process, and to release cellular components. Different mill types can be used. One distinction can be made between classifier mills and other mills. In a classifier mill, particles larger than the desired particle size are recirculated until they have reduced to the desired size. When materials are temperature sensitive, contain volatile components, or are too elastic or soft, cryogenic milling can be used, in which materials are milled while immersed in a cryogen, usually liquid nitrogen. The type of mill used might also affect the final separation efficiency, as was observed by Vitelli et al. (2020). The milling method used has to be tuned to the characteristics of the raw materials and the desired particle size reduction. A classification of the main milling methods is given by Gao et al. (2020).

6.4.1 Consequences of ineffective milling

There are only limited studies on the effects of milling on the dry fractionation efficiency of animal by-products and insects; most studies have been performed on the optimal milling settings for dry fractionation of plant products. During fine milling of starch-containing pulses such as yellow field peas, large starch granules (~22 µm) and small protein bodies (1-3 µm) in the cotyledon are released (Pelgrom et al., 2013b). It is critical for the subsequent separation that these components are physically detached. Both too coarse and too fine milling can lead to unsuccessful dry separation. Too coarse milling leads to poor cell breakage and insufficient release of individual components, which results in lower yields of the components and lower purities of the fractions. Too fine milling on its turn causes damage of larger cellular materials, like starch granules, resulting in similarly sized starch granule fragments and protein-rich particles. Too fine milling also causes poor flowability and the formation of aggregates due to increasing van der Waals forces, ultimately leading to poor dry fractionation results (Pelgrom et al., 2013b). Even though animal products do not contain starch granules, milling into an optimal particle size is still essential, as agglomeration will take place upon too fine milling and microstructures will not be detached upon too coarse milling.

6.4.2 Effective milling for dry fractionation

To discuss effective milling conditions to be used for dry fractionation, we use three examples from various plant materials that were milled to different degrees. It must be noted that the relation between the wheel speed and the obtained particle size distribution is machine specific and depends on factors such as the air speed and the wheel type and size, so the specific settings give only an indication for comparison purposes. In the first example, for air classification of lupin an optimal wheel speed of 1000 rpm was found during impact milling (ZPS50 impact mill Hosokawa Alpine) and for electrostatic separation an optimal wheel speed of 2500 rpm was found. In the second example, impact milling (ZPS50 impact mill Hosokawa Alpine) of different plant seeds showed optimal classifier wheel speeds of 2200 (lentil), 2900 (chickpea), and 4000 (yellow pea) rpm (Pelgrom et al., 2015b). In the third example, impact and shear milling (UPZ100 impact and shear mill Hosokawa Alpine) of rapeseed meal and sunflower meal also resulted in different ideal grid sizes for electrostatic separation of respectively 0.1 mm (average particle size of 23.7 µm) and 0.5 mm (average particle size 105 µm) (Laguna et al., 2018). Therefore, these three examples indicate that the optimal particle size and best milling settings are dependent on both the fractionation method applied and the raw material used. Typically, one has to find the balance between good separation and minimising losses in the system due to clumping, as discussed above.

For plant materials, the required milling intensity increases with a higher hardness, a lower brittleness, and/or a higher crude fibre content (Assatory et al., 2019). When two substructures (e.g. protein bodies vs. starch granules in pulses) differ in brittleness (e.g. one in the rubbery state and one in the glassy state), this will benefit disclosure during milling and thus favour separation (van Donkelaar et al., 2015). Furthermore, pre-treatments influence the final particle size after milling, as was observed for lupin (Pelgrom et al., 2015b). In general, animal cells (10-30 µm) are smaller than plant cells (10-100 µm), which may require higher milling speeds than for plant material. However, it is not clear whether results from dry fractionation of plant materials can be translated to animal tissue, as plants have for example rigid cell walls, which are absent in animal cells. Furthermore, components in animal by-products and insects are organised in larger structures such as the exoskeleton, which may require milling strategies targeted at larger particle sizes. In summary, the degree of milling critically affects the efficiency of the dry fractionation process. An optimal particle size distribution should be found for each raw material. The best settings for animal by-products and insects, are yet to be identified.

6.5 Dry Fractionation of Finely Milled Material

After milling, dry fractionation can be achieved using different techniques, such as sieving, air classification and (tribo)electrostatic separation. Sieving is directly based on the size of the particles, where larger particles remain on top of the sieve and smaller particles pass through. Air classification is based on the combination of the particle size and density (Boye et al., 2010). Particles with smaller size and lower density are separated from larger and more dense particles by an upward airstream. In electrostatic separation, particles are separated based on their tribo-electric charging properties. Tribo-electric charging of particles is induced by particle-particle interactions and particle-wall interactions (Hemery et al., 2011). From these interactions, proteins are expected to gain a positive charge where most carbohydrates are expected to gain a slightly negative charge (Tabtabaei et al., 2016a). When subjected to a transversal electrostatic field, the fractions can be collected at respectively the ground and the positive electrode.

The three dry separation methods require different pre-processing and milling. Table 6.2 summarises the driving forces for separation, the effects of milling and defatting and general examples of usage of the three separation techniques. The impacts of milling and defatting are divided into "large", "medium" and "small". The effect of milling and defatting is smaller for sieving purposes as sieving is suitable for separation of larger particles. A medium to large effect of defatting was indicated for air classification and electrostatic separation as the effect varies between materials. For example, defatting had no impact on dry fractionation of yellow pea, whereas the protein separation efficiency increased for defatted lupin (Pelgrom et al., 2015c). The degree of milling is expected to have a larger influence on the separation efficiency of air classification and electrostatic separation than defatting. This because the separation is not effective when milling is not performed correctly, independent of the material.

Table 6.2: Three dry fractionation methods, their corresponding driving forces, the effect of milling and defatting on their separation efficiency, general examples of usage, and their specific advantages and disadvantages (Assatory et al., 2019; Schutyser and van der Goot, 2011; Xing et al., 2020; H.G. Zhu et al., 2021a).

	Sieving	**Air Classification**	**Electrostatic Separation**
Driving force(s)	Particle size	Particle size and density	Tribo-electric charging properties
Effect of milling	Medium	Large	Large
Effect of defatting	Small to medium	Medium to large	Medium to large
General example(s) of usage	Particles with larger sizes	Separation of protein and ash Separation of protein and starch	Separation of proteins and fibres
Advantage(s)	Mature technology with well understood separation mechanism Well suitable for multicomponent separation Low capital investment	Relatively mature technology with well understood separation mechanism	Translation to food materials is relatively recent Combination with air classification is straightforward and can lead to superior separation
Disadvantage(s)	Sieve blinding and bridging Only suitable for powders with larger particles Scalability is limited	Large gas volumes needed Finely milled particles may aggregate due to electrostatic- and Van der Waals forces Prior defatting necessary to avoid capillary bridging Strongly dependent on the humidity of materials and gas Risk of powder explosion if run with air	Separation mechanism not yet completely understood

As already mentioned in the introduction, the advantages of dry fractionation in comparison with wet fractionation include that no chemicals and water are required, the process is more energy efficient, and the process preserves the native structure and thus functionality of proteins (Assatory et al., 2019; Jonkman et al., 2020; Schutyser et al., 2015; H.G. Zhu et al., 2021a). Disadvantages include a lower obtained protein purity, and the need of a difference in triboelectric or other physical properties for separation purposes (Schutyser & van der Goot, 2011; Tabtabaei et al., 2016a). Next to general

advantages and disadvantages of dry fractionation, each of the three different separation methods also have their specific advantages and disadvantages, which are listed in Table 6.2. While specific for the separation method, these advantages and disadvantages are valid for most materials, including animal products. To mitigate the risk of powder explosion as indicated in Table 6.2, many studies use an inert gas (e.g. nitrogen) to convey the powder during electrostatic separation (H.G. Zhu et al., 2021a). When more than two fractions with different particle sizes are required for optimal separation, sieving may be a suitable method, as an additional sieve can easily be added. For air classification an additional air classifier unit behind the first one should be added or a multi-product air classification system (e.g. elbow jet air classifier) should be used (Furchner & Zampini, 2009; Yuan et al., 2013). For electrostatic separation more than two fractions can be obtained by adding collection bins and/or areas, the use of multiple electrodes, or the addition of a second electrostatic separator (Tabtabaei et al., 2016b; J. Wang et al., 2016b; Xing et al., 2018).

The dry separation methods applied can be cascaded or combined to alter the product properties in terms of composition and the component yields (Assatory et al., 2019). For example, sieving can be a pre-screening technique to eliminate larger fragments like the exoskeleton of insects. This can then be followed by fine milling and air classification or electrostatic separation. The advantage of this is that the purity can be improved and that more than two components (e.g. protein, chitin, and ash) can be separated by combining sieving, air classification and/or electrostatic separation. Next to this, repeated separations and intermediate re-milling can be used to improve the purity or yield (J. Wang et al., 2016b; Xing et al., 2020).

6.5.1 Meat and bone meal (MBM)

Meat and bone meal can be fractionated into protein rich fractions and ash/mineral rich fractions. Figure 6.2 is a visualisation of the dry fractionation results of different raw materials. The first 3 blocks, indicated with A, B and C, show the compositions and yields before (indicated as initial) and after three different dry fractionation processes of MBM (indicated as low and high ash fractions). For example, sieving MBM (47.7% protein, 27.9% ash) resulted in a high ash fraction (yield 13.3%, 34.0% crude protein and 40.8% ash) and a low ash fraction (yield 86.7%, crude protein 50.0% and ash 26.0%), as can be seen in Figure 6.2A (Garcia & Piazza, 2015).

Research on air classified meat and bone meal mainly focused on the functionalities of the air classified fractions rather than the process itself. The few studies that did focus

on the fractionation will be discussed first. In a study on air classification of meat and bone meal by Garcia et al. (2005), protein and ash were separated using an aspirator. An aspirator is a specific air classification system where the particles are separated based on the terminal velocity of particles in an air stream. For this study meat and bone meal (55.8% crude protein, 34.5% ash, 9.7% fat) was obtained commercially; the particle size was not reported. A slow feed rate and a low negative pressure yielded the highest protein content (60.9%) while the ash content dropped (24.8%), as illustrated in Figure 6.2B. Garcia & Piazza (2015) combined sieving and subsequent air classification. The separation of commercially obtained meat and bone meal with this process resulted in ash and protein enriched fractions. The tested meat and bone meal had a broad particle size distribution with a mean particle size of 343 µm. Even though the particles were quite large, the protein content in the protein-rich fraction increased from 47.7% to 54.8% and the ash content of the high ash fraction increased from 26.1% to 34.4% (Figure 6.2C). Moreover, the combined process provided a better separation (i.e. higher ash/protein shift) of particle types with different compositions than sieving or air classification alone.

Other studies on air classified meat and bone meal focussed on improving the digestibility by reducing ash levels. Bureau et al. (1999) found that low ash air classified MBM had an apparent crude protein digestibility that increased from 83% to 87%. Shirley & Parsons (2001) found a higher PER (protein efficiency ratio), a measure for protein quality, for 16.5% ash MBM than for 35.2% ash MBM. The authors suggested that the higher protein digestibility in low ash MBM was caused by a lower levels of collagen. These studies show that air classification may lead to a better functionality, in this case a higher digestibility of animal by-products, even if there is no overall increase in the protein content as such.

Air classification of meat and bone meal has been reported in literature and is already applied in industry, but electrostatic separation of meat and bone meal has, to our knowledge, not yet been reported in scientific literature. However, Industrial trials with electrostatic separation were carried out for meat and bone meal: in one patented industrial trial, oven dried and sieved bovine bone meal (41% protein, 50.5% ash) was fractionated into a protein enriched fraction (65.5% protein and 25.1% ash) and an ash enriched fraction (38.7% protein and 54.4% ash) (Flynn et al., 2020). This shows the potential of electrostatic separation of meat and bone meal. In conclusion, sieving, air classification and electrostatic separation can be used separately or in combination, to separate meat and bone meal into fractions that are enriched in specific components, or

have better functionality, but the available studies are still quite sparse and more work is needed to explore the potential more fully.

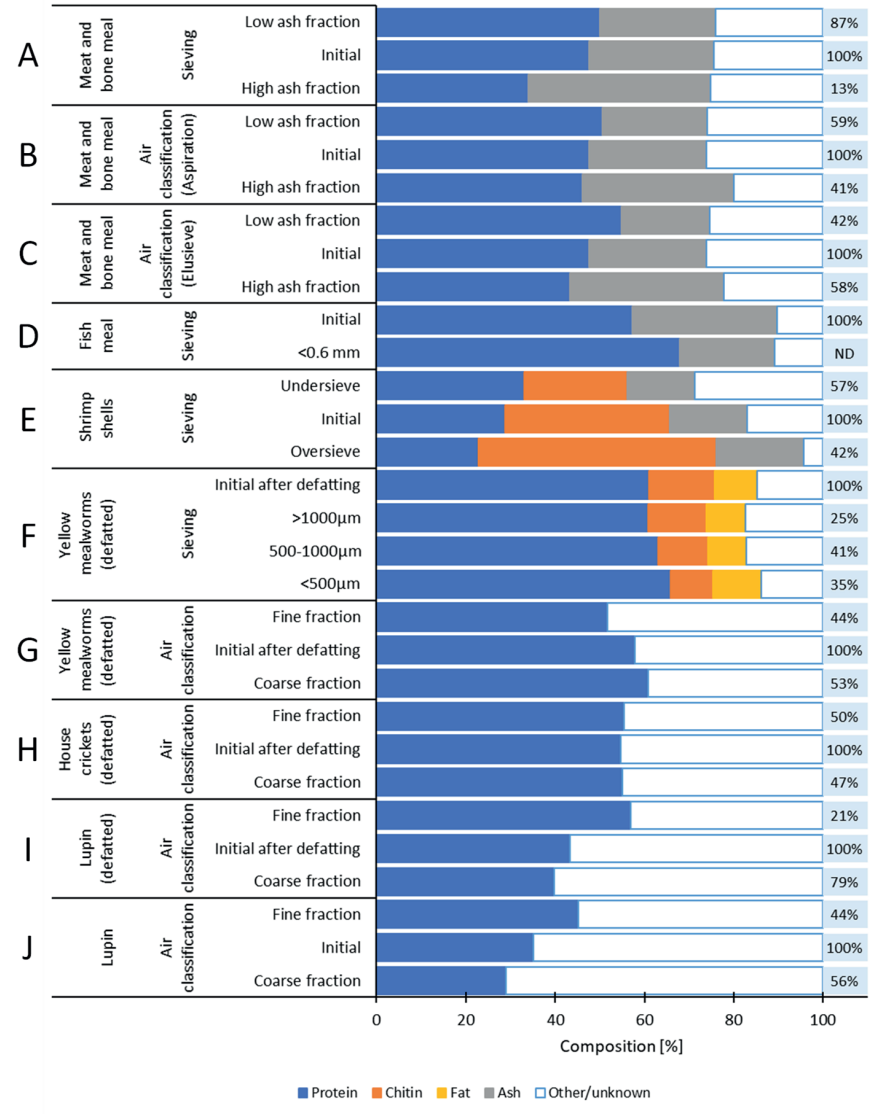

Figure 6.2: Composition of the fractions before and after dry fractionation. The different studies are separated into blocks indicated with A-J. The percentages on the right indicate the mass percentage compared to the initial mass. ND indicates that no mass data was given (A–C: Garcia and Piazza, 2015; D: de Lima et al., 2014; E: Aye and Stevens, 2004; F: Purschke et al., 2018; G–H: Sipponen et al., 2018; I–J: Pelgrom et al., 2015c).

6.5.2 Fish meal

Fish meal can be further fractionated into a protein rich fraction and an ash rich fraction. Sieving through a 0.6 mm sieve increased the protein content to 68% (initially 57%) and decreased the ash content to 21% (initially 32%), although fraction yields were not reported (Figure 6.2D; de Lima et al., 2014). Air classification and electrostatic separation of fish meal have not been reported.

Some industries claim successful ash reduction with air classification, but no sufficient data on the extent of the reduction is shown. In one example the initial ash content (21%) was reduced to 14-18%, where the initial protein content (62%) was increased up to 64-68% (Hannibal Solutions International, n.d.). Flynn et al. (2020) achieved protein enrichment from fish meal (average particle size of 81 µm) using an industrial patented electrostatic separation process from 73.4% to 80.4% protein with a yield of 81.3%. Combination of different methods can result in a better functionality or higher purities, as was observed for pea by Xing et al. (2020b). In conclusion, the current data on sieving, air classification and electrostatic separation of fish meal is very limited, but some successes have been claimed, and there seems to be substantial potential.

6.5.3 Shellfish by-products

Two studies reported on dry fractionation of shellfish by-products, aimed at protein and chitin. Aye & Stevens (2004) studied dry sieving of black tiger shrimp (Penaeus monodon) shells. The dried shells (55 °C for 24 hours, moisture content 3-5%) were ground into pieces with a diameter of 2-5 mm. Sieving was performed with a 0.85 mm screen to separate the proteins and the chitin. Of the ground shrimp shells, 57.1% were able to pass the sieve (i.e. undersieve), and the protein content increased from 28.7% to 33.0% (Figure 6.2E). The undersieve fraction still contained some chitin (~36% of the total chitin). The oversieve fraction was decalcified and deproteinated to produce chitin, as chitin is cross-linked with protein, possibly reinforced with calcium carbonate (Calvert, 1987). Even though about one third of the chitin was lost in the fine fraction, the authors conclude that this loss is easily compensated by the financial value of the protein powder.

Muralidhara & Maggin (1985) separated crab picking waste into a chitin-rich and a protein-rich fraction. Blue crab waste was dried and crushed to pass a 6.4 mm hardware cloth. Subsequent air classification yields a fraction (65%) that had approximately the same composition as the feedstock (13-15% chitin, 30-35% protein, and 50% $CaCO_3$), and a second one (35%) that was sieved. The authors could achieve up to 58% protein in a fraction representing 18% of the dried starting material. It is likely that the process may

be further improved to obtain a higher yield and/or purity, as the separation efficiency depends on the settings.

6.5.4 Insects

As of today, there are two articles to our knowledge about dry fractionation of insects to obtain protein-rich fractions. Purschke et al. (2018) used various pre-processing methods (blanching, drying, and defatting) on the dry fractionation behaviour and the physico-chemical properties of yellow mealworm (T. molitor) larvae. Sieve classification with various mesh sizes was used as a separation step. Both freeze drying and partial defatting by supercritical CO2 extraction resulted in a significantly higher proportion of smaller particles (<500 µm) as compared to coarse particles (>1000 µm), due to the lower mechanical hardness of the material before milling. The highest proportion of smaller particles was found in the partially defatted powder, which was most likely caused by a reduced stickiness, preventing agglomeration of the fine particles. The chitin content was lower in the fine fractions, except for non-defatted blanched oven-dried powders. The lower chitin content in the fine fractions was probably caused by the larger chitin-protein complexes in the insect exoskeleton ending up in the coarse fraction. Logically, the highest crude proteins contents (up to 66%) were found in the partially defatted powders due to the lower fat content, while the fat containing powders consisted of up to 58% crude protein. The resulting protein enrichment of 5.4% is thus limited (Figure 6.2F). The size of the particles in the smallest fraction (<355 µm) is still large compared to the typical size of animal cells, and we thus expect that the fragments are not broken down enough. However, the authors do suggest that chitin can be separated at these larger particle sizes, as it will end up in the coarser fractions. Enrichment of the proteins then may require re-milling of the finest fractions.

Sipponen et al. (2018) used supercritical carbon dioxide (SC-CO2) extraction and pin milling, followed by air classification on house crickets (A. domesticus) and yellow mealworm larvae (T. molitor). At a rotor speed of 6000 rpm (Minisplit Classifier British Rema), a minor protein enrichment was observed for defatted yellow mealworms and no large differences in overall protein content were observed for defatted house crickets (Figures 6.2G and 6.2H). However, the fractions differed in amino acid compositions, solubility, and sensory characteristics. Contrary to animal by-products, protein was here enriched in the coarse fraction, which contained more chitin, as assessed with fluorescence microscopy. The coarse fraction will therefore be enriched with protein originating from the exoskeleton. This coarse fraction had a lower protein solubility than

the fine fraction. In terms of sensory profiling, the fine fraction was rated significantly powderier and had significantly higher meat-like flavour ratings, but there were no significant differences in flavour intensity and saltiness. The particle size and size distribution (2-350 µm) of the milled particles was comparable to the particle size and size distribution of milled lupin (Pelgrom et al., 2014), for which an optimal rotor speed of 8000 rpm was found in a tested range between 6000 and 10000 rpm (50 ATP classifier Hosokawa Alpine) (Xing et al., 2020). Silventoinen et al. (2018) found that for barley the protein content increased when increasing the classifier wheel speed from 4000 rpm to 10000 rpm (50 ATP classifier Hosokawa Alpine), remained constant between 10000 rpm – 18000 rpm, and then increased again from 18000 rpm – 21500 rpm. Although Sipponen et al. (2018) tested a rotor speed of 21000 rpm, though at a lower air flow (50 m3/h instead of 120 m3/h) and a different classifier (50 ATP classifier Hosokawa Alpine), no rotor speeds between 6000 and 21000 rpm were investigated, which may indicate that the ideal settings have yet to be found, and the yield and/or purity may still be improved.

6.5.5 Dry fractionation optimisation for animal by-products and insects

Dry fractionation on animal by-products and insects until now is still in the phase of proving the principle. Further process optimisation is rarely performed, so the full potential to obtain higher yields and/or purities of the desired fraction(s) may not yet have been reached. For one, the relation between the milling intensity and good detachment of fragments for air classification and sieving is still lacking. In several of the discussed articles on air classification, the particle size was significantly larger than the average size of animal cells, which cannot result in optimal detachment of components (Aye & Stevens, 2004; Garcia & Piazza, 2015; Muralidhara & Maggin, 1985; Purschke et al., 2018). This is in agreement with the relatively small increases in protein content that were found.

Secondly, the protein content is negatively correlated to the protein yield, with a higher protein purity resulting in a lower protein yield. It is known from plant materials such as lupin, that insight in the relation between protein purity and yield in dry fractionation is obtained by tuning the degree of milling and the separation settings (Pelgrom et al., 2014; Pelgrom et al., 2015c). An example of protein enriched full-fat lupin and defatted lupin is given in Figures 6.2I and 6.2J. The separation settings, such as the rotor speed and air flow, were also varied in some studies on air classification of animal by-products and insects (Garcia & Piazza, 2015; Sipponen et al., 2018). However, compared to the

fine tuning and the insight gained that took place on literature for plant seeds, the knowledge gained on insects and animal by products is only minor in this respect. Since the milling intensity was not systematically varied in the articles on dry fractionation of animal by-products and insects, there might be an opportunity in optimisation of air classification of animal by-products.

Very limited research has been done on electrostatic separation in animal products and no scientific data have been published. Still, electrostatic separation brings interesting possibilities as it allows separation of particles of similar size, but different in composition. When two materials are brought into contact via triboelectric charging, a positive charge will be induced to one material and a negative charge to the other (Xing et al., 2021). This charge can be induced by the material of the charging tube but also by particle-particle interactions. Based on the data for meat and bone meal, fish meal and plant seeds, it may be expected that the ash present in shellfish and insects will most likely gather a negative charge. Chitin and protein may both obtain a positive charge, due to the presence of amino groups in both chitin and protein. The intensity of the positive charge of chitin and the proteins can differ, and this may still allow separation, for example by using a plate-type tribo-electrostatic separator with separate collection sections (Tabtabaei et al., 2016a). A theoretical example of an industrial fractionation scheme including electrostatic separation of black soldier fly (H. illucens) larvae is given by Ravi et al. (2020). After selecting appropriate electrostatic separation equipment, separation can be further optimised by, for example, varying the particle size, air flow rate, voltage, angle of the charging plate(s), charging tube size, shape, length and material, as was also done for lupin and navy bean (Xing et al., 2020; Xing et al., 2021; Tabtabaei et al., 2016b; J. Wang et al., 2014). Cascading the milling and/or the separation of a certain fraction (i.e. recycling), can result in both higher protein yield and higher purity (J. Wang et al., 2016b; Tabtabaei et al., 2017). Combinations of different dry fractionation methods can also be used to improve the product yield and purity (Assatory et al., 2019). In a few articles on dry fractionation of animal by-products and insects, combined techniques were already used (Garcia & Piazza, 2015; Muralidhara & Maggin, 1985).

6.6 Product-oriented process optimisation

Dry fractionation results in multiple fractions with different compositions (e.g. rich in protein or chitin) and physicochemical and functional properties. These fractions can either be used as they are, or they can be further purified using consecutive wet

fractionation. The combined process of dry and wet fractionation is also referred to as hybrid fractionation. Hybrid fractionation might be desirable when a protein with a high purity is needed. Hybrid fractionation will be more sustainable than only wet fractionation, as there is less material at the start of the wet fractionation step, so less water and energy are involved (Lie-Piang et al., 2021).

There are several aspects on which the optimisation can be based, such as the composition and/or the required functionality of the final product. The essential processing steps and key optimisation strategies for dry fractionation are shown in Figure 6.3. For optimisation of the fractionation parameters based on composition, the raw material source and processing method should be considered. It is expected that optimisation of dry fractionation of insects will differ from optimisation of animal by-products. Insects are bred specifically for food consumption, so the entire process chain can be altered specifically for the purpose of dry fractionation. In case of animal by-products, the pre-processing of the raw materials are given, and the available of animal by-products and their composition determine the feedstock for dry fractionation. Therefore, optimisation possibilities for insects can be found in the entire process chain, while for animal by-products optimisation can mainly be performed by altering the milling intensity and the settings during the separation step.

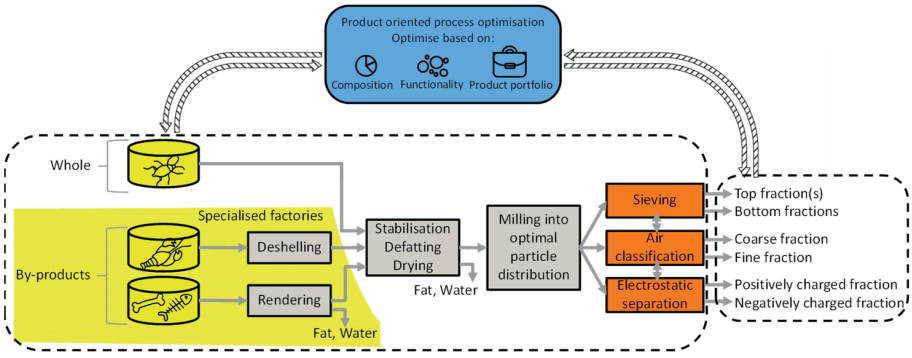

Figure 6.3: Visualisation of processing the animal by-products and insects into the final fractions, including key optimisation strategies. The grey arrows indicate process streams, double headed arrows indicate possibilities for combined dry-fractionation routes. The striped arrows and boxes indicate feedback routes for product oriented process optimisation. The transparent grey area indicates that the products are already pre-processed in specialised factories. Note that rendering already includes decontamination, defatting and drying and the resulting by-products can be directly milled.

Compositional differences between tissues of the same species are important to consider. For example, tuna frames and tuna trimmings are both by-products from the tuna industry, where frames include the bones with residual meat, while the trimmings include the waste from cutting the fish. Tuna frames with 28.7% protein and 44.1% ash may yield larger compositional improvements than dried milled tuna trimmings with 80.7% protein and 3.4% ash (Abbey et al., 2017). We expect that air classification could be suitable to obtain a protein-rich fraction with reduced ash contents. This will likely cause larger improvements in the tuna frames than in the tuna trimmings. Dry fractionation of insects can result in protein rich and chitin rich fractions. Also, here the initial composition varies widely and might for example result in larger improvements in purity for higher chitin rich insect species. Recycling of fractions can be used as a method to optimise the composition and increase yields.

It is interesting that a number of studies showed that the processes could achieve different functionality, even when there was no big change in the overall protein content. This was for example shown in section 5.4: the fine and coarse fractions of air-classified insects did not differ greatly in protein content but did have different solubility and sensory properties (Sipponen et al., 2018). The exploration of these differences in functionality instead of just the protein content could well be an important route to take. This will require better insight into the interaction between different types of functionality, the composition, and the chosen processing conditions. For this, the industry that processes animal by-products and insects may follow the lead from recent innovations in the plant protein processing field. Jonkman et al. (2020) showed that it is possible to substantially reduce the use of water and energy by usage of a portfolio of food products with not only purified fractions, but by using mildly modified fractions in the product portfolio: the same product compositions can be achieved while saving significantly on the separation intensity. In case of animal by-products and insects, examples of products that might be included in a product portfolio are collagen drinks, food packaging, and insect burgers. Different dry fractionation methods may be combined or compared to obtain the fractions that combine in the best possible way into a product portfolio.

6.7 Conclusion

Research on dry fractionation has significantly advanced over the last decade but has so far mainly focused on plant materials. In general, little research has been performed on dry fractionation of animal by-products and insects, and on many insect species and animal by-products (e.g. mussel by-products), dry fractionation has not even been performed at all. The research that has been performed primarily concerned its feasibility instead of process optimisation. However, first results for fractionation of animal by-products and insects were promising. In some cases, dry fractionation could yield significant increases in protein (or other) content. In other cases, the separations yielded different functional properties such as solubility and sensory properties, without a substantial change in overall protein content. This may give rise to different dry fractionation strategies that are not solely based on yield and purity but also on functionality. Research that takes the effect of particle size for dry fractionation of animal products into account is clearly needed, as it is crucial to achieve required purities, yields and functionality. Different optimisation strategies will be required for different raw materials. For insects all pre-processing steps can be specifically aimed towards dry fractionation, but for animal by-products the origins of the materials and the established rendering and deshelling steps narrow the possibilities to tune the materials for the separation. Therefore, especially for insects development of the pre-processing steps and methods for dry fractionation is important.

While pre-processing and milling are crucially important, the settings during dry fractionation, such as the air classifier wheel speed during air classification, are also of great importance. Cascading the separation steps, for example by re-milling and combined or repeated dry fractionation steps, can further improve the material yield and purity. Furthermore, a structured and systematic approach about the effects on physicochemical and functional properties is currently also lacking. Insight in these properties and the use of a product portfolio would enable more efficient use of animal by-product and insect fractions. This can reduce the environmental impact and be essential in the protein transition to reach the sustainability goals.

Chapter 7

General discussion

7.1 Introduction

The objective of this thesis was to provide knowledge-based processing guidelines for dry fractionation of seeds towards functional protein ingredients for food. Therefore, the overall process was evaluated, which includes pre-treatment of the materials, fine milling, dry separation and functionality of the produced ingredient fractions. This chapter presents the main findings in this thesis. Subsequently, the effect of pre-treatments on milling and consecutive dry separation is discussed. Based on these insights, guidelines are provided to improve dry fractionation of oil-rich legumes. Next to that, powder flowability is determined for differently pre-treated samples to evaluate its influence on dry fractionation performance (purity and yield). In order to generalize the results, the rheological profiles from various different crops are compared, namely chickpea and yellow pea (**chapter 3**) and lupin and soy (**chapter 4**). Next, the addition of the flow aid silica is discussed as a pre-treatment to improve electrostatic separation. Because functional behaviour of protein ingredients is key for final food application, the pre- and post-processing of dry-enriched ingredients is discussed with respect to their effect on the physico-chemical and functional properties of various fractions. Processing suggestions are provided to obtain fractions with promising functionalities for future food applications. Finally, we discuss the scaling up of dry fractionation and how the obtained insights can be used to guide further research and industrial implementation of dry fractionation.

7.2 Main findings of this thesis

Dry fractionation consists of one or multiple pre-treatments followed by milling and dry separation. As oil may be expected to have a large impact on the milling behaviour and particle dispersibility, we evaluated pin-milling of soy with different oil contents in **chapter 2**. We found that an increasing oil content limited the milling performance towards smaller particles. Moreover, for full-fat soy the overall milling yield declined more upon increasing the milling speed than for de-oiled soy. Particle size reduction during milling was tested against Bond's empirical model, where the particle size reduction could be largely described with an adapted Bond's model with oil content as an input parameter. The milled flours were well dispersible, but the dispersibility of small particles was influenced by the presence of oil, where small particles (<10 µm) of de-oiled and milled soy were better dispersible than the small particles of non-de-oiled and milled soy. For non-de-oiled soy, optimal milling is necessary to reach the smallest particle size, best small particle dispersibility and highest material yield.

We hypothesized that the effect of humidity on air classification would be more pronounced for legumes with a higher oil content. Therefore, the effect of relative humidity (RH) on milling and air classification performance of yellow pea (0.8 g oil/100 g dry matter) and chickpea (5.2 g oil/100 g dry matter) was studied in **chapter 3**. The milling yield was not influenced by the relative humidity between 30-70% for both yellow pea and chickpea, as drying of the material inside the mill could have overshadowed this possible effect. The air classification performance of chickpea was already affected at a relative humidity of 70%, whereas the air classification performance of yellow pea only declined after equilibration at 90% relative humidity. So, the effect of humidity upon storage prior to air classification was indeed more pronounced for legumes with a higher oil content, which was linked to a lower small particle (<10 µm) dispersibility and an inferior flowability profile relative to materials conditioned at low humidity (30%). Overall, a maximum RH was found for optimum milling and air classification of legumes.

In **chapter 4**, several scenarios were compared, which involved electrostatic separation of soy and lupin de-oiled with different solvents (none, acetone, ethanol and hexane). Electrostatic separation of soy was less effective than electrostatic separation of lupin. The soy protein bodies were still embedded in the cellular matrix after impact milling, which was reflected in a larger particle size and lower small particle dispersibility of soy than lupin flour. It was crucial to re-mill soy after de-oiling the flour with hexane to break up powder lumps and release the protein bodies from the cellular matrix to obtain protein enrichment after electrostatic separation towards a protein content of 59.6 gram/100 gram dry matter (N_{factor} = 5.7). The protein purity of lupin protein-enriched fractions increased most by using polar solvents (acetone and ethanol) instead of hexane.

In **chapter 5** the flavour profile and functionality of toasted soy and lupin flours and their protein enriched fractions by electrostatic separation were evaluated to provide directions for the pre-treatment. Acetone de-oiling resulted in most odour active components, which is unfavourable for foam-based or emulsion-based applications. Furthermore, ethanol de-oiling resulted in protein denaturation and a lower solubility of the materials. For emulsion-based applications no de-oiling pre-treatment was necessary for the lupin based ingredients, whereas for soy no de-oiling or hexane de-oiling could be applied prior to electrostatic separation to produce functional fractions for emulsion-based applications. For foams, de-oiling was necessary, as the presence of high levels of oil negatively affected the foam stability. Concluding, appropriate pre-treatment and electrostatic separation provided protein-enriched ingredients suitable for application as

stabilizers in emulsion-based systems like dressings or frozen foam-based systems like ice-cream.

In **chapter 6** the applicability of dry fractionation to animal by-products and insects was reviewed, which included the characteristics of the raw materials, pre-processing, milling, separation, and final fraction functionality. To further enable dry fractionation of animal by-products research should focus on optimising milling and separation to produce functional ingredient fractions, whereas for insects research should focus on optimisation of the entire process chain. Insight in the compositional and functional properties after dry fractionation of animal by-products and insects would allow more efficient use of these ingredient fractions.

7.3 Pre-treatment affecting milling and subsequent dry separation

This section discusses the influence of de-oiling, dehulling, and toasting on milling and subsequent dry separation. The choice of pre-treatment is an important decision for dry fractionation with consequences for all consecutive steps. The obtained knowledge on pre-treatments is used to discuss how to further improve dry fractionation of chickpea as an example.

De-oiling of legumes and legume flours enables milling towards smaller particles than would be the case for untreated material under similar conditions. This effect is more pronounced the more oil is removed by the de-oiling step (chapters 2 and 4). De-oiling is inevitable for materials with a very high oil content (>30%), for example sunflower seeds or rapeseed. To illustrate this, in a preliminary study it was found that ambient milling of sunflower seeds appeared not possible as caking occurred already upon pre-milling of the seeds. Cryogenic milling of sunflower seeds at -80 °C appeared also not possible as this resulted in caking and blockage of the equipment as well. Ambient milling of full fat rapeseed was possible, but resulted in larger particle sizes and material losses in the mill compared to milling results of soy under similar conditions. Furthermore, milling of full-fat rapeseed did not provide disclosure of dispersible protein bodies and subsequent electrostatic separation resulted in blockage of the venturi. Thus, also for rapeseed de-oiling is strongly advised to improve the milling yield, reach sufficient disclosure of protein bodies from the cells and to enable consecutive electrostatic separation. Furthermore, it is recommended to aim for ambient milling conditions unless the lower temperature is desired to preserve volatile compounds (Pulivarthi et al., 2023).

Previous research hypothesized that the presence of hulls will impair milling towards smaller particle sizes (do Carmo et al., 2020). Furthermore, toasting can result in a more brittle material and therewith also smaller particle sizes (W.J. Wang et al., 2022). To evaluate this, the volume of particles was plotted against the particle size for non-treated and toasted and dehulled soy and lupin (Figure 7.1). Dehulling and toasting of lupin and soy prior to milling indeed resulted in narrower particle size distributions for both lupin and a larger volume of smaller particles (Figure 7.1).

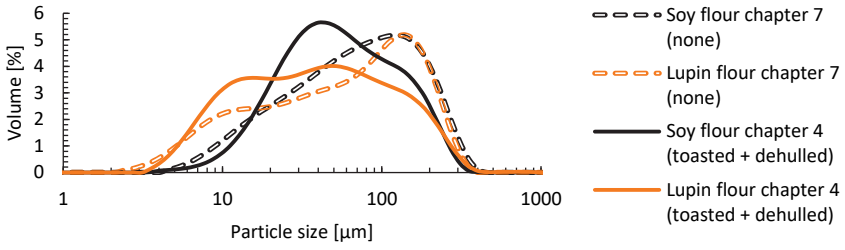

Figure 7.1: Particle size distributions of hulled (dashed lines) and toasted and de-hulled (solid lines) soy (black) and lupin (orange).

The effect of toasting and dehulling on the electrostatic separation performance was found to depend on the legume (chapter 5). For soy, toasting and dehulling were necessary for protein enrichment, as electrostatic separation of the non-pre-treated soy flour did not result in protein enrichment. For lupin, both no pre-treatment and application of toasting and dehulling prior to milling resulted in protein enrichment. For milling and air classification of yellow pea and faba bean the effect of dehulling was also found to be legume dependent (do Carmo et al., 2020). Even though, for lupin no pre-treatment also resulted in protein enrichment, it is still strongly advised to use toasting and dehulling as pre-treatments prior to milling and electrostatic separation to reduce the anti-nutritional factors.

Chickpea had a poorer fine particle dispersibility and flowability than yellow pea after milling. To improve milling and air classification of chickpea, de-oiling and/or dehulling and toasting can be used as pre-treatments to enable milling to smaller particles under similar conditions. De-oiling will improve the relative dispersive index of the material (chapters 2 and 4), and de-oiling will also improve the powder flowability (Section 7.4), which is expected to improve the protein recovery in the fine fraction. De-oiling and dehulling will increase the initial protein and starch content of the materials, which might contribute to higher fraction purities (do Carmo et al., 2020). Next to de-oiling and

dehulling, the addition of flowability aids to chickpea flour is also expected to improve the yield of the fine fraction (Pelgrom et al., 2014). The choice of flow aid type is also relevant. For example, addition of hydrophobic silica as flow aid for corn starch was found more effective to improve flowability than the untreated hydrophilic silica (Yang, Sliva et al., 2005).

7.4 Powder rheology profiles as a tool to guide dry processing

This study evaluated several powder characteristics, which included the particle size of the materials discussed in section 7.3, the particle dispersibility, the powder flowability and the chargeability. These powder properties can be used to verify whether a pre-treatment improved the powder properties for dry separation purposes. In this section, powder rheology is proposed as a qualitative indicator for powder flowability during dry fractionation. For this, we compare the powder rheology profiles of chickpea and yellow pea from chapter 3 with the powder rheology profiles of lupin and soy before and after de-oiling in chapter 4. Consecutively, we evaluate what the rheological profile means for the dry fractionation performance (purity and yield). As silica is often used to improve the flowability, we did a small preliminary study to evaluate if adding silica would affect the electrostatic separation performance in terms of yield and purity.

The flowability of soy and lupin was measured according to the powder rheology method described in chapter 3.2.6 to evaluate whether soy and lupin had similar or worse flowability than chickpea and yellow pea flour milled at 30% humidity (data replotted from chapter 3). The initial area was lowest for yellow pea, followed by chickpea, lupin and soy. Soy had thus inferior flowability compared to the other materials based on the instable plateau value and higher initial area (Figure 7.2A). De-oiling of lupin significantly improved the flowability (Figure 7.2B) and de-oiling soy with hexane or acetone also significantly improved the flowability (Figure 7.2C). However, soy de-oiled with ethanol still resulted in an unstable plateau, which is likely caused by the higher residual oil content in the material. Based on the rheological profiles, ethanol de-oiling of soy was not sufficient to improve its powder properties.

The measured flowability is indicative for appropriate emptying of the feeder to the electrostatic separator and subsequent fraction recovery, but does not relate to the performance of electrostatic separation in terms of protein purity. To illustrate, soy de-oiled with acetone showed a good rheological profile, but electrostatic separation did not result in protein enrichment (Figure 7.2B; chapter 5). Significant losses of material in

the feeder (21%-31%) (soy and soy de-oiled with ethanol) correlated to poor flowability (Politiek et al., 2023a). The material loss occurred during the rotation of the feeder screws, which induced particle agglomeration and sticking of material to the walls (visual observation). During powder rheology this translates to heap formation and thus no stable plateau is obtained (Figure 7.2). Overall, the rheological profile can therefore be used as a semi-quantitative guideline for material recovery. A rheological profile with a stable plateau and a lower initial area will result in higher material recoveries upon air classification and better emptying of the feeder and thus recovery upon electrostatic separation.

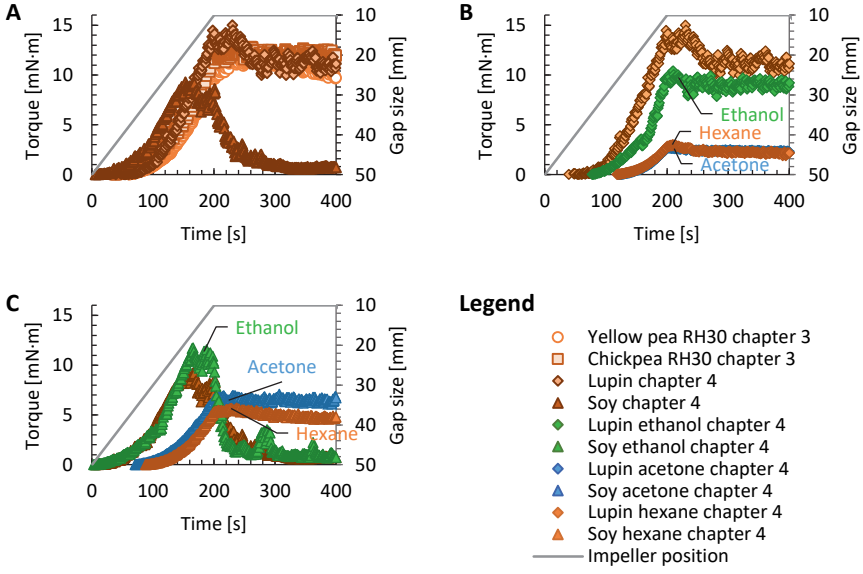

Figure 7.2: Average measurement of powders' resistance to flow with the Anton Paar powder flow cell for yellow pea (relative humidity 30%), chickpea (relative humidity 30%), lupin and soy (A), and for lupin and lupin de-oiled with ethanol, acetone and hexane (B), and for soy, soy de-oiled with ethanol, acetone and hexane (C). The grey line represents the impeller position at each time on the secondary y-axis.

Addition of flow aids to steer tribo-electrostatic separation

Next to de-oiling to improve the flowability, it is interesting to add anticaking agents or flowability aids, such as fumed silica particles, to improve the flowability and processing performance of the legume flours. Of course, it should always be considered if addition of food grade flow-aids is acceptable from a clean label ingredient perspective. If acceptable, it is expected that there will be an optimum concentration of flow aid for maximising the flowability (Fulchini et al., 2017). However, the addition of silica particles

might not only affect the flowability but also the tribo-electrostatic charging as in the field of mineral separation fumed silica is applied as 'charge control agent'. A charge control agent modifies the charging behaviour through changed properties of the particle surface by coating (Mirkowska et al., 2016). To the best of our knowledge, aerosil particles have not been added to legume flours prior to electrostatic separation. Therefore, we ran some preliminary tests on electrostatic separation of lupin and soy flour with different concentrations of food grade 200F (hydrophilic) silica and R812 (hydrophobic) silica.

Surprisingly, the fraction yield decreased at the ground electrode side and increased at the positive electrode side of the electrostatic separator upon the addition of fumed silica (Figure 7.3A). Furthermore, protein enriched at the positive electrode instead of the ground electrode upon the addition of silica to lupin flour (Figure 7.3B). A similar trend was observed upon adding hydrophobic silica (R812, data not shown). The change in fraction yield and switch in protein content suggest that the addition of silica results in charge inversion of the protein bodies. Based on the triboelectric series, silica tends to donate electrons, so the small silica particles (~23 nm) might acquire a positive charge (Cardenas et al., 2018). If these electrons are donated to the protein bodies, these will acquire a negative charge, which resulted in a higher protein content and fraction yield at the positive electrode in case of lupin. Next to silica, other charge control agents might be tested, as enhanced charging might benefit the separation process, which is relevant for industrial application.

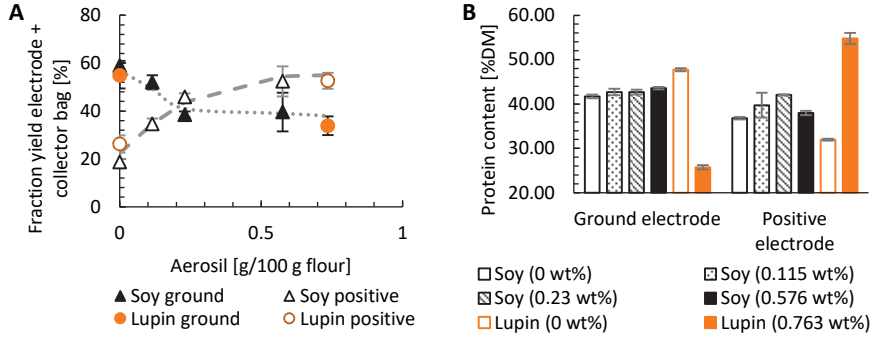

Figure 7.3: Fraction yield of the ground electrode and ground collector bag (closed symbols) and fraction yield of the positive electrode and positive collector bag (open symbols) (A) and protein content at the ground and positive electrodes (B) for soy (black) and lupin (orange) with different concentrations of aerosil 200F (g/100 g flour wt%). Lines are added to guide the eye.

7.5 Guidelines to tailor the functionality of the produced ingredients

Next to separation performance, the functionality of the produced ingredients is important for industrial application in foods. This section evaluates how the emulsification and foaming behaviour can be improved and applications where the presence of insoluble components might be favourable. Next to ingredient functionality to improve the structural properties of food, ingredients also contribute to the nutritional properties of the produced food. For this, we give an example how a pre-treatment can affect the nutritional quality, where we focus on small sugars.

Dry fractionation results in ingredients of varying purities, which greatly differs from the commercial isolates or concentrates. Thus, they allow a different range of functionalities, which needs to be evaluated and ideally coupled with the fractionation process to produce fractions for specific applications: For so-called semi-dilute emulsions (<30 w/w% oil) (Y. Zhu et al., 2020), prepared in chapter 5, the produced fractions showed to result in only partially stable emulsions. Stability can be improved by adding a higher concentration of the ingredient to increase the protein content for better emulsification. The presence of insoluble particles such as insoluble fibres might however also negatively have affected the emulsion stability in the semi-dilute emulsions. Another study found that emulsification of air classified and consecutive aqueous extracted soluble pea protein (having less fibre) was comparable to commercial pea protein isolate and exhibited better freeze-thaw stability (Geerts et al., 2017). Thus, primarily soluble protein fractions without insoluble fibres seem to be promising for dilute emulsions with good freeze-thaw stability. The protein-enriched lupin and soy ingredients produced via electrostatic separation in chapter 5 also had a better freeze-thaw stability than commercial protein isolates, so these can be used to produce foods with improved freeze-thaw stability. The fractions might benefit from application of an additional aqueous fractionation step to extract the soluble proteins and remove the insoluble fibres to improve their emulsification behaviour in semi-dilute oil-in-water emulsions (10-30%).

For other applications than dilute emulsions, the presence of insoluble components after dry fractionation might be beneficial, for which we give three examples: 1. as stabilizers for highly concentrated oil-in-water emulsions (60 w/w% oil), which was found for lentil protein enriched fractions (Funke et al., 2022) or 2. as gelling agents where dry enriched fractions exhibited improved gelation behaviour compared to highly refined protein isolates (do Carmo et al., 2020; Kornet et al., 2021; Vogelsang-o'Dwyer et al., 2020) or 3.

as foaming agents as was found for navy bean protein enriched fractions, which exhibited higher foamability than the isolates and soy protein concentrate (Tabtabaei et al., 2019). However, the presence of oil as water insoluble component is generally not advantageous for foaming, and thus de-oiling is applied, which might also be beneficial for the foamability of other substrates like insects (Gravel et al., 2021). Next to emulsification, foaming and gelation, the solubility, viscosity, water holding capacity and oil holding capacity are physico-chemical properties that are often studied because of their high correlation to specific functionalities (Ma et al., 2022). The presence of multiple components such as proteins and fibres in the dry enriched fraction will also affect these properties; The presence of fibres can for example increase the water holding capacity (do Carmo et al., 2020) and the viscosity (Pelgrom et al., 2014), which makes fractions that are high in fibres promising as thickening and/or gelling agent. However, it is important to note that the observed functionality change in less purified fractions (i.e. water holding capacity) will be dependent on the material source (Schlangen et al., 2022), because different plant proteins and fibres can have different properties. Furthermore, the pre-processing history (e.g. toasting or de-oiling) will also strongly affect the final functionality of the fractions.

Overall, less refined protein ingredient fractions have a different application range and more research is needed to identify their potential application. It should also be kept in mind that the composition of less purified fractions will affect the nutritional properties of foods: The small sugars present in legumes and their enriched fractions consist for example partially of indigestible α-galactosides (raffinose, stachyose and verbascose), which can give rise to flatulence. Moreover, small sugars have high glycaemic index, which means that they act as substrates for the energy metabolism, and with that they can also increase the blood glucose levels (Cummings & Stephen, 2007). Stronger glycaemic responses are undesirable for diet-induced diseases like diabetes type 2. Less refined fractions still contain these sugars although we found that by de-oiling with ethanol instead of hexane a high proportion of these sugars is removed. Thus, the de-oiling step can at the same time be used to remove these components, albeit with consequences on the flavour profile and nativity of the resulting fractions.

However, the presence of fibre and protein in less purified fractions also counteracts the presence of small sugars in plant-based materials. For example, previous research found that the addition of fibre and protein from lupin and soy to carbohydrate-rich beverages reduced the glycaemic response compared to the control (Dove et al., 2011), which

shows potential for application of the fibre and protein enriched fractions to enhance the nutritional properties of the food product.

As de-oiling with ethanol resulted in a too high residual oil content to sufficiently charge soy flour (chapter 5), mechanical de-oiling followed by ethanol de-oiling might be studied to produce ingredients with a lower residual sugar content. To avoid protein denaturation due to ethanol extraction, fermentation with lactic acid bacteria might be applied instead. Fermentation will reduce the residual small polysaccharides, such as raffinose, stachyose and verbascose, and therewith also alter the flavour of the produced sourdough (Xing et al., 2019).

Overall, the protein fraction functionality and nutritional quality can be tailored by the application of different pre-treatments (i.e. dehulling, toasting and de-oiling) or post-treatments (i.e. aqueous fractionation to obtain the soluble protein fraction, fermentation) to dry fractionation.

7.6 Outlook to optimise and scale-up dry fractionation of oil-rich crops

Electrostatic separation increased the protein content of lupin from 39.7%DM to 58.5%DM. For soy the protein content increased from 40.8% to 45% and electrostatic separation of hexane de-oiled soy increased the protein content further from 51.8%DM to 59.6%DM. This section evaluates the industrial applicability of de-oiling, milling and tribo-electrostatic separation and provides an outlook for future research for each of these processing steps. For tribo-electrostatic separation we first discuss the design of an electrostatic separator for oil-rich legumes and we hypothesize how the dispersibility and chargeability might be used in this system. Then two examples are provided of electrostatic separation on a larger scale for food materials. Finally, we provide an outlook for the translation to other bio-materials.

De-oiling is already industrially applied to extract oil from plant seeds for the food industry. In chapter 2 we applied mechanical de-oiling at 60°C and the oil content of soy was reduced to 9% oil. However, for efficient charging a lower oil content might be preferred (Figure 5.6). The oil content can be further reduced by combing mechanical oil extraction with solvent extraction (i.e. hexane or ethanol), or without the use of solvents by using a higher temperature upon pressing. However, too high temperatures upon oil pressing should be avoided to prevent potential protein denaturation and maintain the functionality of the produced ingredients. Research might evaluate the effect of pressing

temperature on the composition, milling and electrostatic separation performance and final functionality of the ingredients.

Also for milling, upscaling from pilot scale (0.5 kg/h) to industrial scale (140-150 kg/h or more) is well possible as the input parameters for milling are well defined (Hosokawa Alpine, 2023). The industrially toasted, dehulled and impact milled soy flour could already well be used for consecutive electrostatic separation (chapters 4 and 5). It might be expected that milling at larger scale will improve as the mill chamber is relatively larger, which is especially beneficial for milling of more adhesive products. When materials are de-oiled and toasted prior to milling, the generation of dust during milling potentially increases. High amounts of dust increase the risk of dust explosion and inhalation, which has adverse effect to human health. Generally, industrial milling systems are closed and cyclones are used to recover dust (<5 µm) from the air via agglomeration (Jo et al., 2000; Paiva et al., 2010; Advanced Cyclone Systems, 2023). An alternative to complete de-oiling is partial de-oiling, which limits the formation of dust, but still has the benefit of increased chargeability of cellular components for electrostatic separation after milling.

Upon scaling up the electrostatic separation for oil-rich legumes, the free dispersion of particles is crucial, especially due to the presence of (residual) oil. A better choice is here to apply a venturi dispersion system for electrostatic separation of oil-rich legumes, as this is an efficient way to break up agglomerated particles. Here, research should evaluate if the dispersibility curve of the materials can be used to set the air dispersion pressure of the venturi, where we hypothesize that higher dispersion pressures are required for materials with a lower relative dispersive index. However, in some of our preliminary trials with the vertical electrostatic separator with venturi dispersion it was observed that some of the pilot-scale fine milled materials contained particles that were too large to fit through the small gap of the venturi. This can be solved by using a venturi system with a bigger gap. Next to the dispersion of the powder, the way fractions are subsequently separated in the electric field and collected is important to obtain a high purity and yield. In the horizontal separator set-up higher purities but lower yields were obtained compared to the vertical set up (chapter 4). Collecting the materials directly on the electrode plates is advantageous for the purity but decreases the yield. Different electrostatic separator designs should be evaluated on their performance for food materials, where both yield and purity are relevant.

Electrostatic separation of food ingredients is not applied on industrial scale yet, but recently larger electrostatic separation devices have been successfully demonstrated. A first example comes from a manufacturer (ST Equipment & Technology, USA) that has been active in the mineral industry since 1995 and entered the food and feed industry in 2018. They separated de-oiled roller milled sunflower meal (2.7% moisture, throughput 12000 kg/h/meter of electrode width), successfully into a protein enriched (51.7% protein) and protein depleted fraction (17.6% protein) (Gupta et al., 2021). Their commercial electrostatic separator can handle throughputs between 40 – 17000 kg/hour/meter of electrode width and has a low energy consumption (1-2 kWh/ton feed). The separator works slightly different from the separators used in this thesis, as the positive and negative electrode are located above each other, the applied voltage is in a similar range (8-20 kV), and a rotating belt is used to collect the material (Gupta et al., 2022). A second example is derived from a patented scalable electrostatic separator design equipped with rotating plate electrodes, where at least three parallel plates that can be charged in a pattern (Dijkink & Schroot, 2021). This device showed promising results for lupin and soy: The protein content of electrostatically separated lupin flour was 63%, 51% for toasted soy flour and 58% for de-oiled soy flour (Dijkink & Schroot, 2021).

Overall, dry fractionation of oil-rich seeds and de-oiled cakes and meals at industrial scale seems technically feasible. Air classification is already applied for plant-based materials, i.e. starch rich legumes and although electrostatic separation is not yet applied on industrial scale, its potential is demonstrated on somewhat larger scale. However, dry fractionation is not limited to plant-based materials (chapter 6), but the potential to apply air classification or electrostatic separation to other materials like animal by-products and insects is hardly researched. Future research should focus on valorising all kinds of bio-materials. For the implementation of dry fractionation to other biomass, it is important to evaluate the effect of the pre-treatment, as this will affect all consecutive steps, including final ingredient functionality. Furthermore, milling should liberate particles with different compositions with physical differences (i.e. size or charge), to enable dry separation via air classification or electrostatic separation. Enabling dry fractionation for different kinds of biomass, will result in a more effective use of the available sources.

7.7 Conclusion

The aim of the presented thesis was to develop guidelines for dry fractionation of oil-rich legumes by evaluating the whole processing chain. Dry fractionation of oil-rich legumes is possible by using milling and tribo-electrostatic separation. Here, pre-treatments were found to be key to tailor the process performance by conducting dehulling, toasting and de-oiling, which had various consequences along the processing line up to ingredient flavour perception, sugar content and ingredient functionality. Above all, it was highlighted that scaling towards industrial size seems well possible, but here more insights in the functionality of the produced ingredients are necessary in the future. Furthermore, research should focus on elucidating if the tribo-charging of the materials can indeed be steered by the addition of other chargeable particles, as this would open opportunities to alter the tribo-charging behaviour of the materials and therewith the protein purity and fraction yield of the collected materials. For translation towards other bio-materials it is necessary to evaluate whether the combination of pre-treatments and milling resulted in the release of cellular components with different composition and distinctly different physical properties (i.e. size or tribo-charging behaviour) and secondly if the powders have favourable bulk properties for dry separation (i.e. well dispersible small particles and flowable flour).

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Summary

Summary

Many modern food products contain purified ingredients such as protein, starch and oil. Protein ingredients from grain legumes are often prepared by wet extracting the proteins followed by drying. To produce protein ingredients with much less water and energy use, dry fractionation is an attractive sustainable alternative. This method consists of a milling and a dry separation step such as air classification or electrostatic separation. Air classification is based on a difference in size, density or shape, whereas electrostatic separation is based on a difference in tribo-charging behaviour. Tribo-charge is induced by friction, where charge transfers from one particle to another particle resulting in opposite charges. While for starch-rich legumes dry fractionation is a highly promising alternative, for oil-rich legumes it is unclear whether dry fractionation is possible. Therefore, the objective of this thesis was to provide knowledge-based processing guidelines for dry fractionation of oil-rich seeds towards functional protein ingredients. For this, we considered the effect of oil content on fine milling, dry separation and functionality of the ingredients after different pre-treatments.

Chapter 2 shows that the presence of oil limited milling to smaller particles and resulted in a lower dispersibility of the small particles. The oil content directly related to size reduction during milling via the Bond's model. Thus mechanical de-oiling can improve the powder properties for dry fractionation purposes. **Chapter 3**, demonstrates that relative humidity does not have a major effect on milling and air classification of yellow pea and chickpea up until 70 % relative humidity. The protein recovery upon air classification of chickpea (6% oil), was negatively affected by equilibration at a relative humidity of 70%, whereas air classification of yellow pea was negatively affected upon equilibration at 90% relative humidity. The poorer air classification performance was linked to a lower small particle (<10 µm) dispersibility and an inferior flowability profile relative to materials conditioned at low humidity (30%).

Chapter 4 showed that electrostatic separation of soy was less effective than electrostatic separation of lupin, caused by the soy protein bodies that were still embedded in the cellular structure after milling. A higher purity could be reached by de-oiling soy with hexane followed by re-milling to release the protein bodies from the cellular matrix. For lupin, de-oiling with polar solvents resulted in the highest protein purities. Furthermore, removal of oil resulted in a higher specific charge.

Chapter 5 gave evident that the choice of solvents for de-oiling had a pronounce effect on functionality and flavour. Acetone de-oiling resulted in odour active components,

while ethanol de-oiling led to protein denaturation and reduced solubility. Thus, both solvents can be unfavourable for certain product applications and hexane is the most advantageous choice in both aspects Functional protein ingredients were derived even without de-oiling from lupin and soy, demonstrating that de-oiling is not strictly necessary for emulsion formation. However, for foam-based applications, de-oiling was necessary, demonstrating that pre-processing should be adapted based on the target application.

Chapter 6 translated the findings to other materials like animal by-products and insects. For animal by-products, research should focus on optimising milling and separation to produce functional ingredient fractions, whereas for insects, research should focus on the optimisation of the entire process chain. Insight in the compositional and functional properties after dry fractionation of animal by-products and insects would allow more efficient use of these ingredient fractions.

Chapter 7 summarises the main findings of this thesis and evaluates different aspects of the overall process, including pre-treatments, milling, dry separation, functionality and scalability of the overall process towards industrial application. Dry fractionation of oil-rich legumes is possible by using milling and electrostatic separation, but pre-treatments were found to be key to tailor the process performance. These pre-treatments had various consequences along the processing line up to ingredient flavour perception and functionality. Although scaling towards larger size seems technically feasible, electrostatic separation devices for food ingredients should still be improved and proven on industrial scale. Future research might further focus on the ingredient functionality and on elucidating the effect of adding other chargeable components. For translation towards other bio-mass materials it is key that the combination of pre-treatments and milling results in the release of cellular components with different composition and distinctly different physical properties (i.e. size or tribo-charging behaviour), with favourable bulk properties for dry separation (i.e. dispersible small particles and flowable flour).

Samenvatting

Levensmiddelen bevatten vaak opgezuiverde ingrediënten zoals eiwitten, zetmeel en olie. Traditioneel worden plantaardige eiwit ingrediënten vaak gemaakt door de eiwitten eerst op te lossen in water om vervolgens het water weer te verdampen. Het drogen kost veel energie en daarom hebben we binnen dit proefschrift gekeken naar droog fractioneren. Droog fractioneren is een duurzamer alternatief om eiwitrijke ingrediënten te maken omdat er geen water toegevoegd wordt in het proces, wat energie en water bespaart ten opzichte van het natte proces. Droog fractioneren bestaat uit malen en een droge scheidingsstap. Voorbeelden van droge scheidingen zijn windziften en elektrostatische scheiding. Windziften is gebaseerd op een verschil in deeltjesgrootte en elektrostatische scheiding op een verschil in statische oplading. Statische oplading vindt plaats door wrijving waardoor lading tussen deeltjes wordt uitgewisseld. Echter is het voor olie rijke onduidelijk of droog fractioneren mogelijk is. Daarom was het doel van dit proefschrift om kennis gedreven richtlijnen te ontwikkelen voor het droog fractioneren van olierijke zaden om functionele eiwitrijke ingrediënten te produceren voor in levensmiddelen. Hiervoor hebben we gekeken naar het effect van olie op het malen, het scheiden en de functionaliteit na verschillende voorbewerkingsstappen.

In **hoofdstuk 2** is het effect van olie op malen systematisch bestudeerd. De aanwezigheid van meer olie beperkt het malen tot kleinere deeltjes en leidde tot een verminderde dispergeerbaarheid van de deeltjes. Dus mechanisch ontvetten kan gebruikt worden om de poedereigenschappen van olie rijke gewassen te verbeteren voor het droog fractioneren. In **hoofdstuk 3** is gekeken naar het effect van luchtvochtigheid op droog fractioneren. De luchtvochtigheid tijdens het fractioneren zelf heeft geen grote invloed op het malen en windziften van gele erwten en kikker erwten. Echter heeft het bewaren van het fijn vermalen materiaal bij hogere luchtvochtigheid wel een groot effect op de hoeveelheid eiwit die gewonnen kan worden. Voor kikkererwten (6% olie) verminderde de eiwit opbrengst bij een relatieve luchtvochtigheid van 70% en voor gele erwten (1% olie) verminderde de opbrengst na het conditioneren bij 90% luchtvochtigheid. Deze observaties konden we ook relateren aan een lagere dispergeerbaarheid van de kleine deeltjes (<10 µm) en een verminderde poeder stroombaarheid ten opzichte van dezelfde poeders die geconditioneerd waren bij een lagere luchtvochtigheid van 30%.

Hoofdstuk 4 laat zien dat het droog fractioneren van vol vet soja minder goed gaat dan lupine wat samenhangt met de meer samengepakte cel structuur van soja na het malen vergeleken met lupine. Bij lupine waren de individuele eiwitbolletjes goed zichtbaar,

terwijl dit niet het geval was bij soja. Om een hogere zuiverheid te bereiken is het nodig om soja te ontvetten met hexaan en het ontvette materiaal nogmaals te vermalen om meer vrije eiwitbolletjes te verkrijgen. Voor lupine resulteert het ontvetten met polaire oplosmiddelen (aceton en ethanol) in hogere eiwit gehaltes waar voor soja het apolaire hexaan gunstiger is voor een hoger eiwit gehalte. Ontvetten is gunstig voor de statische oplading van de deeltjes, waarbij lagere oliegehaltes samen gaan met hogere ladingen.

Hoofdstuk 5 laat zien dat de keuze voor bepaalde oplosmiddelen een groot effect heeft op de functionaliteit en geur van de ingrediënten. Zo leidt het ontvetten met aceton tot de vorming van onaangename geuren, en het ontvetten met ethanol tot het ontvouwen van de natieve eiwitstructuur waardoor de oplosbaarheid van de eiwitten verloren gaat. Dus deze twee oplosmiddelen kunnen onvoordelig zijn voor de toepasbaarheid in bepaalde levensmiddelen en hexaan is als oplosmiddel een betere keuze met betrekking tot zowel geur en het behouden van de natieve eiwit structuur. Voor het vormen van emulsies was het niet strikt noodzakelijk om lupine en soja te ontvetten. Echter was ontvetten wel nodig voor het vormen van schuim. Het voorbewerkingsproces moet daarom gekozen worden op basis van de uiteindelijke product applicaties.

Hoofdstuk 6 vertaalt de bevindingen naar nadere materialen zoals dierlijke bijproducten en insecten. Verder onderzoek naar dierlijke bijproducten kan de focus leggen op het optimaliseren van het malen en scheiden, terwijl voor onderzoek naar droog scheiden van insecten ook de voorbewerking mee kan nemen. Inzicht in de samenstelling en functionaliteit van deze dierlijke producten is nodig om de beschikbare materiaal stromen efficiënt te kunnen gebruiken.

In **hoofdstuk 7** worden de bevindingen van dit proefschrift bediscussieerd. Het is mogelijk om eiwitrijke ingrediënten te bereiden uit olierijke zaden met droge scheiding. Hierbij is de voorbewerking de sleutel tot succes, omdat dit consequenties heeft voor het hele proces. Verder lijkt het goed haalbaar te zijn om droog fractioneren op te schalen. Toekomstig onderzoek kan zich verder richten op evaluatie van de functionaliteit van de ingrediënten en daarnaast is het een interessante optie om de elektrostatische oplading te sturen door toevoeging van andere oplaadbare hulpstoffen. Voor de vertaling naar andere gewassen of materialen is het belangrijk dat de combinatie van voorbewerkingsstappen en het malen resulteert in vrije cellulaire componenten die een verschillende samenstelling en duidelijk verschillende eigenschappen hebben (grootte en/of statische oplading) met gunstige poeder eigenschappen voor droge scheiding (dispergeerbare deeltjes en goede poeder stroombaarheid).

Appendices

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If you asked me about my plans after work you would have discovered that I enjoy playing volleyball with my team-mates from **Nuovo DS1** and a lot of weekend trips to Friesland to visit **friends** and **family** there. Ik wil mijn teamgenoten van **Nuovo DS1** bedanken, ik heb veel van jullie mogen leren op volleybal gebied en het was fijn om op vaste tijden de Wageningen bubbel even te verlaten om vrij te volleyballen. **Laura**, bedankt dat ik in mijn eerste jaar bij Nuovo altijd met jou mee mocht rijden, ik heb genoten van de gezellige autoritten en het samen eten voordat we weg moesten. **Véronique**, we hebben lange tijd samen gereden naar de volleybal, verder was het fijn om met jou te sparren gezien we in dezelfde fase van onze PhD zaten, daar heb ik veel aan gehad. **Marna** en **Nicole,** bedankt voor het managen en zorgen voor ons team. **Olga,** je was er altijd voor iedereen in het team, heel lief! **Jullianne,** ik heb veel van jou mogen leren op volleybalgebied en ook voor mijn onderzoek had je goede adviezen. **Esmer**, het was gezellig om de afgelopen twee jaar samen te rijden naar de volleybal en te kunnen sparren over van alles en nog wat. **Marit**, jij kent zo ongeveer de halve regio aan volleyballers wat maakt dat we goed op de hoogte blijven van hoe andere teams spelen onderweg.

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Regina

About the author

Regina Politiek was born on 23 September 1996 in Wons, the Netherlands. She obtained her VWO diploma in 2014 at Bornego College in Heerenveen, with a double major in Natuur en Gezondheid (Nature and Health) and Natuur en Techniek (Nature and Technology). Regina studied the BSc Food Technology from 2014 till 2017 at Wageningen University. In 2016 she did a minor in Chemical Sciences at Wageningen University and she finalised her studies cum-laude in 2017 with a thesis at the Laboratory of Food Chemistry about the influence of protein structure degradation on enzymatic hydrolysis.

Regina started her master Food Technology in 2017 at Wageningen University with a specialisation in Sustainable Food Process Engineering. During her master thesis at the Laboratory of Food Process Engineering she studied particle morphology development of maltodextrin formulations during drying of micron sized droplets. Regina continued her internship at Jacobs Douwe Egberts, to study the main driving parameters for coffee crema. She obtained her master's degree in 2019.

Next to her studies, Regina was active as a board member (Treasurer) of the student association WSSFS from September 2016 – March 2018 and she played in regional volleyball competition teams of WaHo from 2015-2019 followed by Nuovo from 2019 onwards. Furthermore, she worked together with Joep Spaen on a fermented non-alcoholic beverage "TWIST" for the Food Innovation challenge by Wageningen University. They won the third prize in the first edition of the Wageningen University Award of Food Innovation 2019 and the prize for best video advertisement.

Regina started as a PhD candidate at the Laboratory of Food Process Engineering in collaboration with Wageningen Food & Biobased research in October 2019. She worked on dry fractionation of oil-rich legumes for the recovery of functional protein ingredients. The results of her PhD research are described in this thesis. During her PhD, Regina presented her work at five conferences and she received the second prize for the Young scientist award at the NIZO plant protein functionality conference in 2022.

Publications

Alpiger, S.B., **Politiek, R.G.A.**, Corredig, M., Schutyser M.A.I., & van der Goot, A.J. (Manuscript in preparation). Importance of pressing temperature on protein enrichment by electrostatic separation of rapeseed press cakes.

Politiek, R.G.A., Pegiou, E., Balfoort, L. L., Bruins, M.E., Schutyser, M.A.I., & Keppler, J.K. (2023). Functional and flavour properties of de-oiled flours and dry-enriched protein concentrates of lupin and soy. *Future Foods*, *8*(12), 100274.

Politiek, R.G.A., Dijkink, B.H., Boogaard, L.M. van den, Keppler, J.K., Schutyser, M.A.I., & Bruins, M.E. (2023). Comparing electrostatic separation of soy and lupin: Effect of de-oiling by solvent extraction. *LWT, 187*(9)*,* 115290.

Politiek, R.G.A., He, S., Wilms, P.F.C., Keppler, J.K., Bruins, M.E., & Schutyser, M.A.I. (2023). Effect of relative humidity on milling and air classification explained by particle dispersion and flowability. *Journal of Food Engineering, 358*(12)*,* 111663*.*

Politiek, R.G.A., Bruins, M.E., Keppler, J.K., & Schutyser, M.A.I. (2022). Effect of oil content on pin-milling of soybean. *Journal of Food Engineering, 334*(12)*,* 111149.

Sweers, L.J.H.[1], **Politiek, R.G.A.[1]**, Lakemond, C.M.M., Bruins, M.E., Boom, R.M., Fogliano, V., Mishyna, M., Keppler, J.K. & Schutyser, M.A.I. (2022). Dry fractionation for protein enrichment of animal by-products and insects: A review. *Journal of Food Engineering*, *313*(1), 110759.

Siemons, I., **Politiek, R.G.A.**, Boom, R.M., Van der Sman, R.G.M., & Schutyser, M.A.I. (2020). Dextrose equivalence of maltodextrins determines particle morphology development during single sessile droplet drying. *Food Research International*, *131*(5), 108988.

[1]Shared first authorship

Overview of completed training activities

Discipline specific activities

33rd EFFoST International Conference *(volunteered)* (*EFFoST*)	2019
Introduction to cryogenic milling (*Hosokawa*)	2019
Protein transition community day (*Wageningen Dialogue*)	2020
Virtual 15th Plant-Based Foods & Proteins Summit North-America (*Bridge2Food*)	2020
VLAG online lectures (*VLAG*)	2020-2021
Virtual 1st Global Plant-Based Foods & Proteins Research Conference (*Bridge2Food*)	2020
Food structure & Functionality Online Mini Symposium (*Elsevier*)	2020
NIZO Plant Protein Functionality Conference (*NIZO*)	2020
FOOD PROTEINS: Significance, Reactions and Modifications (*Department of Food Science UCPH and FCH WUR*)	2020
AOCS Annual Meeting & Expo[a] (*AOCS*)	2021
Rheology: The do's and don'ts (*VLAG*)	2021
5th Food structure and functionality symposium[b] (*Food structure and functionality forum*)	2022
2nd NIZO Plant Protein Functionality Conference[ab*] (*NIZO*)	2022
18th Netherlands Process Technology Symposium[ab] (*NPS*)	2023
37th EFFoST International Conference[a] (*EFFoST*)	2023

General courses

VLAG PhD week (*VLAG*)	2019
Introduction to R (*VLAG*)	2020
Brain friendly working and writing (*WGS*)	2020
Critical thinking and argumentation (*WGS*)	2020
Presenting with Impact (*WGS*)	2020
Applied statistics (*VLAG*)	2020
Supervising BSc & MSc thesis students (*WGS*)	2020
Chemometrics (*VLAG*)	2020
Scientific writing (*WGS*)	2020
Certified peer reviewer course (*Elsevier Researcher Academy*)	2021
Career Perspectives (*WGS*)	2023

Assisting in teaching and supervision activities

Supervision of 2 BSc- and 5 MSc- students (*FPE*)	2020-2023
Practicum assistance FPE-21306 Food Production and Preservation (*FPE*)	2020-2022
Practicum assistance FPH-10306 Introduction to Food Technology (*FPE*)	2021-2022

Other activities

Preparation of research proposal (*FPE*)	2019
Weekly group meetings (*FPE/WFBR*)	2019-2023
PhD study tour to Singapore[ab] (*FPE*)	2022
Podcast: Fractionation of oil-rich seed through dry fractionation (*Wageningen alternative protein project*)	2021
Reviewing scientific articles (*FPE*)	2021-2022

[a]Oral presentation, [b]Poster presentation, **Second price Young scientist award*

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Cover design by Regina Politiek.

The design of the cover was inspired by a lupin field with purple blooming flowers. The green bubbles refer to sustainable processing as grass in between the pink and purple lupin flowers. The yellow bubbles refer to the oil as “the gold” obtained from oil rich legumes. The pink and white bubbles at the top refer to the protein bodies and protein enrichment (colour difference from left to right), which was the main focus of this thesis.

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