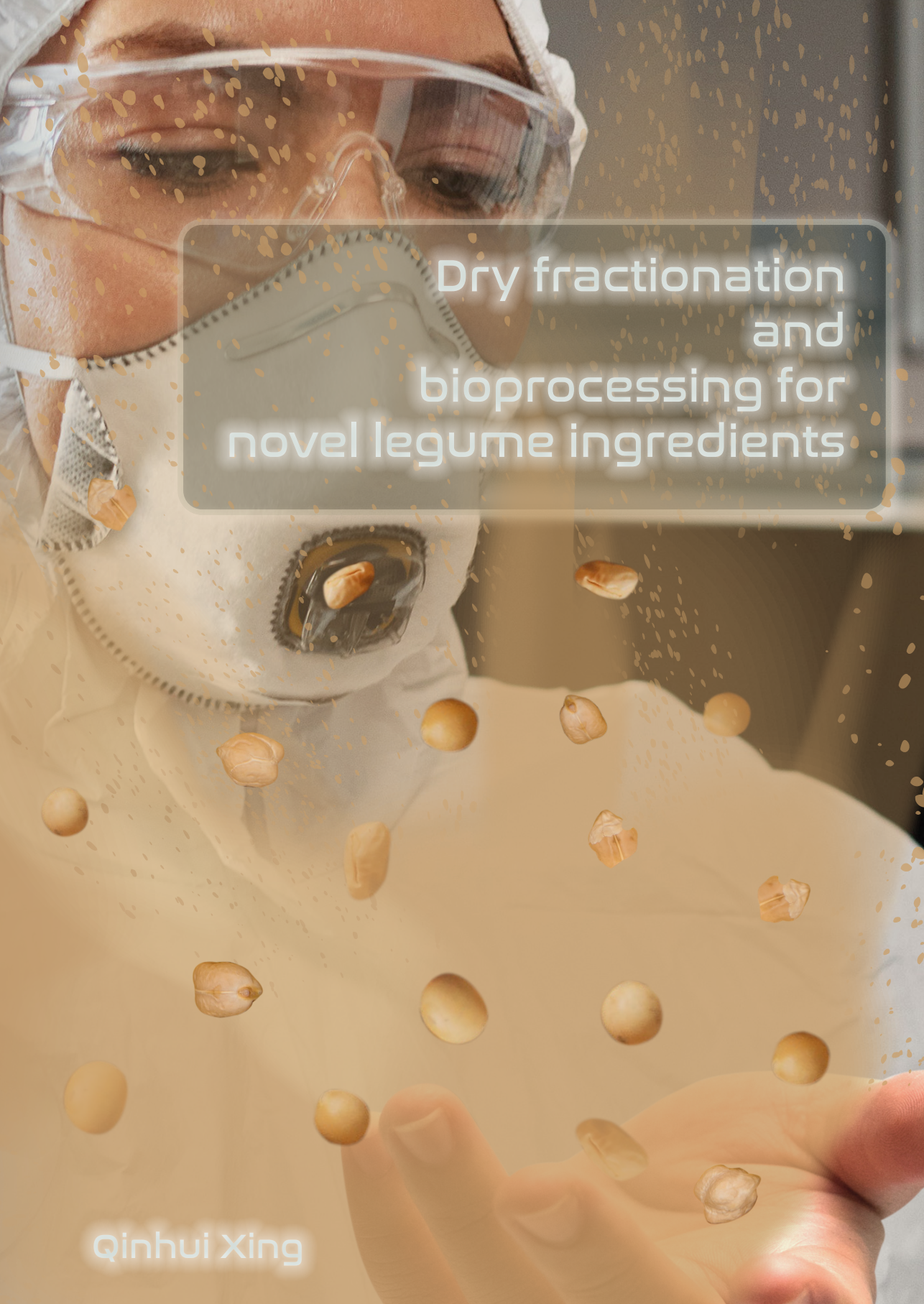

Dry fractionation and bioprocessing for novel legume ingredients

Qinhui Xing

Propositions

1. The best dry separation technique for a legume is determined by its microstructure. (this thesis).

2. Dry fractionation becomes a mature technology combined with solid-state fermentation (this thesis).

3. Modern technology applies traditional methods in a new context.

4. Knowledge evolved to understand our surroundings but also became the basis for creation and destruction.

5. Gluten is to wheat bread, what perfectionism is to writing a PhD thesis.

6. Cultural differences are shown not in how people face life, but in how people face death.

Propositions belonging to the thesis, entitled

Dry fractionation and bioprocessing for novel legume ingredients

Qinhui Xing

Wageningen

09/02/2021

Dry fractionation and bioprocessing for novel legume ingredients

Qinhui Xing

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Dry fractionation and bioprocessing for novel legume ingredients

Qinhui Xing

Thesis

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Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 9 February 2021
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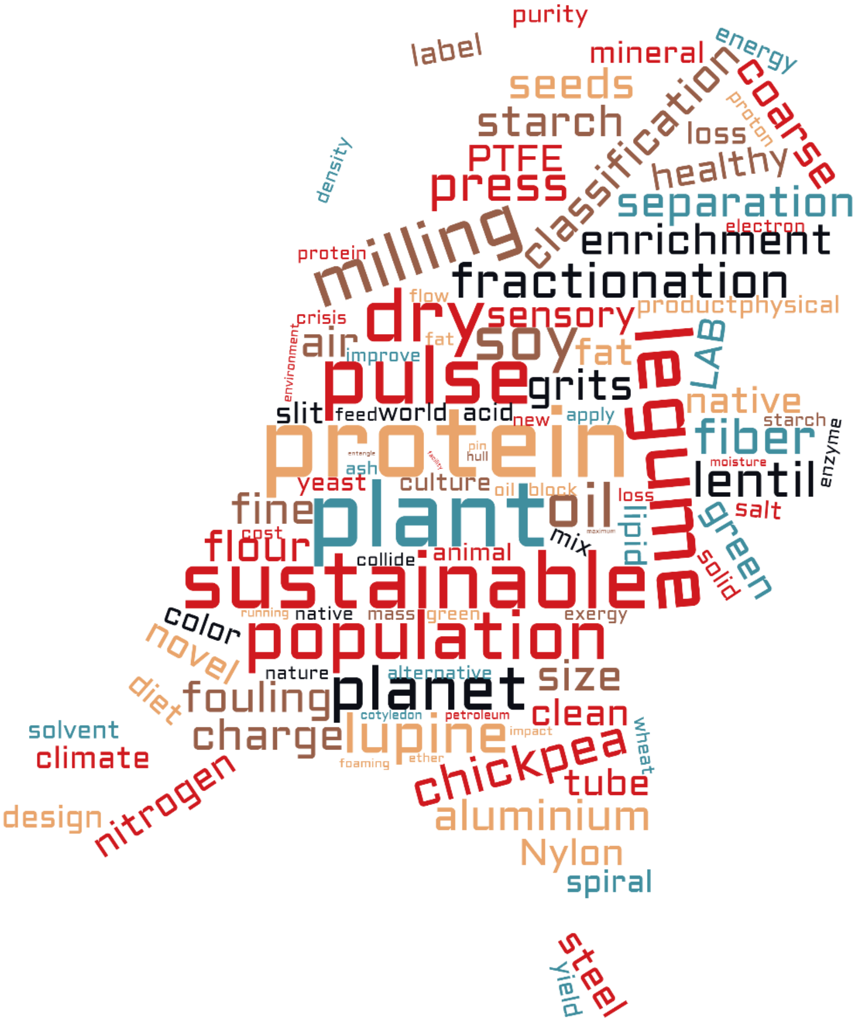
To

MY MOTHER

This dissertation is affectionately dedicated

Contents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label
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spiral
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yield

Chapter 1

General introduction

1.1 Introduction

As the world's population continues to grow and is predicted to reach 9 to 10 billion by 2050, feeding the global population with adequate dietary protein is a challenge (Galli, et al., 2019; Iyer, et al., 2008; Nadathur, Wanasundara, & Scanlin, 2017). Animal protein is an excellent source of nutrients and is well-balanced in essential amino acids (Joshi, et al., 2015; Xing, Liu, Cao, Zhang, & Guanghong, 2019). However, to produce 1 kg of animal protein, 6 kg of plant protein is required on average (Kornelia, et al., 2018; Pimentel & Pimentel, 2003). The related greenhouse gas emissions and the water footprint per kilo of meat are ~7 times higher than for plant protein (Melini, et al., 2017; Nadathur, et al., 2017). An important route to answer the above food challenge is therefore the transition to a more plant protein-based diet. Legumes are an important dietary plant-based protein source, in terms of both quantity and quality, and are traditionally consumed in many cultures and regions (Mariotti & Gardner, 2019; Montemurro, et al., 2019; Nwadi, et al., 2020). They are sometimes referred to as "superfood" or "poor man's meat" and it has been stated that increasing the percentage of legumes in our diet is not a choice but a necessity from nutritional and environmental perspectives (Boukid, Zannini, Carini, & Vittadini, 2019; Shevkani, Singh, Chen, Kaur, & Yu, 2019; Xing, et al., 2020; Yao, et al., 2010).

Although legumes can be consumed as a whole, they typically contain high-calorie components like starch or oil and are often fractionated into ingredients with desired compositional properties (e.g. protein concentrates) and incorporated as such in foods. Plant protein concentrates (>70% protein) and isolates (>90% protein) are conventionally produced by wet fractionation. Protein is typically concentrated by solubilization of some of the carbohydrates (for example by using a mixture of water and ethanol). This yields a protein concentrate that still contains a significant amount of insoluble carbohydrates. Proteins can be isolated into purer ingredients by solubilizing the protein in an alkaline solution and subsequent precipitation of

the proteins at lower pH. The resulting precipitate is then centrifuged, neutralized, and finally dried (Schutyser, Pelgrom, Van der Goot, & Boom, 2015). Although this procedure yields high protein purity, it requires considerable amounts of water and energy. More importantly, native functional properties are lost during processing, while not all protein is recovered and in some cases a significant fraction is lost (Pelgrom, Vissers, Boom, & Schutyser, 2013).

Because foods usually do not contain only protein but consist of a complex mixture of multiple ingredients, high purity ingredients are not always required (van der Goot, et al., 2016). In that respect, we could compromise on purity and enhance functional properties by applying mild fractionation routes, which better retain some of the native protein functionality, and at the same time improve the yield and reduce the use of water and energy. These mild fractionation methods are a promising way forward for the sustainable production of plant-based ingredients (Geerts, 2018).

1.2 Dry fractionation — a promising technique to produce native plant-based protein

Dry fractionation, which combines dry milling and dry separation, is a mild route to produce plant protein concentrates (Pelgrom, 2015). During milling, individual components such as starch granules and protein bodies are detached from the cellular matrix. After fine milling of starch-rich legumes such as pea and chickpea, one can obtain starch granules having a size of 15 to 30 μm (Xing, Hou, Zhang, Han, Yan, & Luo, 2017) and protein-rich fragments with a size less than 5 μm (Thakur, Scanlon, Tyler, Milani, & Paliwal, 2019). The difference in particle size allows the subsequent separation of protein and starch by air classification (Schutyser & Van der Goot, 2011).

However, the difference in size is not always sufficient to allow easy separation of all components by this method. A supplementary dry separation technology is electrostatic separation, which can be applied to separate particles of similar size, but with different compositional properties. This technique generally employs triboelectric charging of materials as a driving force for separation (Mirkowska, Kratzer, Teichert, & Flachberger, 2016). For many legume species, protein bodies (e.g. in soybean and lupine), protein-rich fragments (e.g. in pea, lentil, and chickpea) and starch granules obtain a positive charge after triboelectrification, whereas fibres obtain a negative charge. The charged particles are separated in a transversal electrostatic field, where the protein particles and starch granules are captured by the negative/grounded electrode and the fibres are collected on the positive electrode (Wang, Zhao, De Wit, Boom, & Schutyser, 2016).

Soybean and lupine are oil-containing legumes that are also very rich in protein but comprise practically no starch (Wrigley, 2003). These features make these raw materials interesting to separate with electrostatic separation. Starch-containing legumes such as yellow pea and chickpea first require that starch is removed (Xing, et al., 2017), for example by air classification and thus require two stage separation. Dry separation of both starch and oil-containing legumes are evaluated in this thesis, using air classification and/or electrostatic separation (Figure 1-1).

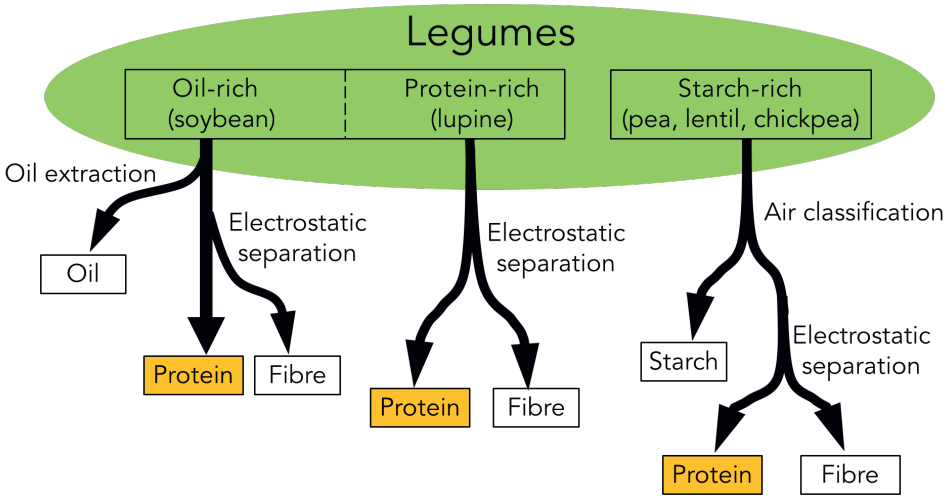


Figure 1-1. Designed technical routes for different varieties of legumes to achieve protein enrichment with dry fractionation.

Electrostatic separation is not a new technology but is widely applied to sort metal/metal, metal/polymer and polymer/polymer mixtures in the mining and recycling industries (Tilmatine, Medles, Bendimerad, Boukholda, & Dascalescu, 2009). However, the use of electrostatics for fractionation of food components is relatively new. Hitherto plant protein enrichment with this technology has been only investigated on small scales and has not been commercialized yet (Table 1-1). The electrostatic separator employed in this dissertation was established on bench-scale based on an existing unit (Wang, de Wit, Boom, & Schutyser, 2015), with modifications. To allow large-scale practical application of electrostatic separation in the food industry, the charging efficiency and subsequent separation performance of legume flours need to be improved. Specifically, we need to better understand how operating conditions influence the separation with different raw materials, giving varying protein purity and yields.

Table 1-1. Overview of dry fractionation of various raw/refined plant materials using electrostatic separation.

Ref.	Tribo-device materials	Feed Material	Charging performance	Enrichment[1]
(Basset, Kedidi, & Barakat, 2016)	Teflon	Rapeseed oil cake	Protein/water soluble carbohydrate (+) Lignin/structural carbohydrates (-)	38.4%
(Sibakov, 2014)	Teflon (PTFE)	Oat bran	Arabinoxylan (+) β-glucan/starch (-)	16.1%
(Hemery, Rouau, Dragan, Bilici, Beleca, & Dascalescu, 2009; Hemery, Anson, Havenaar, Haenen, Noort, & Rouau, 2010)	Teflon	Wheat bran	Aleurone (+) Cell wall (-)/Pericarp (+)	12 - 43.4%
(Chen, Liu, Wang, Li, Wang, & Chen, 2014)	Teflon	Wheat bran	Aleurone (+/-) Pericarp (+)	Not reported
	Nylon	Wheat bran	Aleurone (-) Pericarp (-)	Not reported
	Stainless steel	Wheat bran	Aleurone (+) Pericarp (-)	Not reported
(Tabtabaei, Jafari, Rajabzadeh, & Legge, 2016a, 2016b; Tabtabaei, Vitelli, Rajabzadeh, & Legge, 2017)	PTFE	Navy bean	Protein (+) starch (-)	14.5 - 60.6%
	PVC	Navy bean	Net charge (+)	Not reported
	Copper	Navy bean	Net charge (+)	Not reported
(Landauer, Aigner, Kuhn, & Foerst, 2019;	PTFE	Whey protein/barley starch	Whey protein (+) barley starch (-)	246.7 - 293.3%

[1] Defined as the ratio of the difference between the content of the target component (*e.g.* protein) in its enriched fraction and the original material divided by the content of the original material. For example: (protein content of target fraction-protein content of original material)/protein content of original material.

Ref.	Tribo-device materials	Feed Material	Charging performance	Enrichment[1]
Landauer & Foerst, 2018)	POM	Whey protein/barley starch	Whey protein (+) barley starch (-)	313.3%
	PE	Whey protein/barley starch	Whey protein (+) barley starch (-)	266.7%
	PMMA	Whey protein/barley starch	Whey protein (+) barley starch (-)	280%
(Pelgrom, Wang, Boom, & Schutyser, 2015)	Aluminium	Pea flour	Protein (+) carbohydrate (-)	7.8%
	Aluminium	Lupine flour	Protein (+) carbohydrate (-)	22.1 - 84.1%
(Wang, et al., 2016)	Aluminium	Lupine flour	Protein (+) Carbohydrate (-)	68.1%
(Wang, de Wit, et al., 2015; Wang, Smits, Boom, & Schutyser, 2015)	Aluminium	Gluten/starch	Wheat gluten (+) Wheat starch (-)	6.3 - 56.3%
	Aluminium	Wheat bran	Arabinoxylans (-)	5.9%
(Xing, de Wit, Kyriakopoulou, Boom, & Schutyser, 2018)	Aluminium	Soybean	Protein (+) Carbohydrate (-)	11%
	Stainless steel	Soybean	Protein (+) Carbohydrate (-)	15%
(Bassani, 1988)	Anodized aluminium	Rice	Rice bran (-) Endosperm (+)	Not reported
(Bohm & Kratzer, 2008)	Stainless steel	Wheat grain	Aleurone (-) Shell/bran (+)	Not reported
(Stone & Minifie, 1988)	elutriator column	Wheat bran	Aleurone (+) Pericarp-testa (-)	Not reported

1.3 Solid-state fermentation — to enhance the nutritional value in a sustainable way

Dry-enriched legume ingredients may be applied directly to food or other products. Legume fractions obtained via only dry processing retain their native properties. The protein generally is still well soluble, but all bioactive ingredients are also still

present in the ingredient, including undesired anti-nutritional factors (ANFs) (Assatory, Vitelli, Rajabzadeh, & Legge, 2019). ANFs are normally thermally degraded or washed out during wet isolation of proteins. Phenolic compounds (e.g. tannin, phytic acids, phytates) and protease inhibitors are ubiquitous ANFs in many leguminous crops. They may reduce the bioavailability of minerals or the digestibility of proteins (Ferrando, 1983). Alpha-galactosides (e.g. raffinose, stachyose, verbascose) are responsible for intestinal discomfort (flatulence) (Thirunathan & Manickavasagan, 2019). The presence of ANFs in dry-enriched ingredients may hamper the application of these fractionated legume ingredients if they are not degraded or removed (Schutyser, et al., 2015).

Different treatments such as dehulling, soaking, heating, germination, and fermentation have been used to reduce the amount of ANFs in raw legume flours with different degrees of success (Khattab & Arntfield, 2009). However, some of the methods (soaking or heating) are water and energy intensive. Moreover, the protein functionality or activity of bioactive compounds may also be affected. Solid-state fermentation (SSF) in this respect holds large potential. It is a relatively mild process to remove ANFs, in contrast to especially high-temperature processing (Mubarak, 2005). Compared to conventional submerged fermentation, SSF requires a minimum amount of water compared to submerged fermentation which reduces the energy consumption in a final drying step to obtain a stable ingredient, while fermentation with lactic acid producing bacteria may even result in a stable wet ingredient. Previous research already demonstrated that SSF can be applied to raw legume flours to improve specific nutritional, textural, and functional properties (Galli, et al., 2019). SSF could successfully remove the beany flavour, while interesting, positive aroma compounds were formed, and the shelf-life was extended (Tangyu, Muller, Bolten, & Wittmann, 2019).

During air classification, ANFs may be enriched in the protein-rich fraction, because some ANFs are in fact proteins, or closely linked to proteins, or are embedded in

small fibrous particles (Shevkani, et al., 2019; Tiwari, Gowen, & McKenna, 2011) which end up in the same fraction. This ANF concentration calls for the necessity for a post-treatment to ensure safe and nutritious dry-enriched fractions to be used in foods.

Lactic acid bacteria (LAB) are widely used to remove ANFs from legume flour (Bartkiene, Krungleviciute, Juodeikiene, Vidmantiene, & Maknickiene, 2015). Starter cultures with LAB species were evaluated for fermenting pulse protein-enriched and starch-enriched fractions to obtain a nutritionally improved ingredient (Xing, et al., 2020). The next step to take would be to assess the influence of the ingredient on the properties of the final food product, such as bread. Bread may be enriched with legume protein to increase protein and obtain a more balanced amino acid composition (Millar, Barry-Ryan, Burke, McCarthy, & Gallagher, 2019).

1.4 Objective and outline of the dissertation

This dissertation is based on the hypothesis that dry fractionation based on air classification and/or electrostatic separation in combination with a mild post-treatment such as SSF can be used to create protein intermediates that are both techno-functional and nutritious. The objective of the study is therefore to develop a novel sustainable route for processing legumes into functional protein-enriched ingredients with enhanced nutritional value. The focus is on the use of the combination of dry fractionation and solid-state fermentation. This is divided into the separation as such, and the post-treatment:

a) Separation: optimize the operating conditions of electrostatic separation, such that a good balance between purity and yield is struck. This will expand the range of legumes that can be used with electrostatic separation.

b) Post-treatment: investigate the application window of solid-state fermentation to enhance the functional and nutritional properties of legume

fractions. This will be concretized by an assessment of the application of enriched and bioprocessed fractions in food preparation.

The entire study provides guidelines for food production to move towards better resource efficiency, using legume protein-enriched products. In Figure 1-2 a schematic overview of the chapters and their coherence is provided.

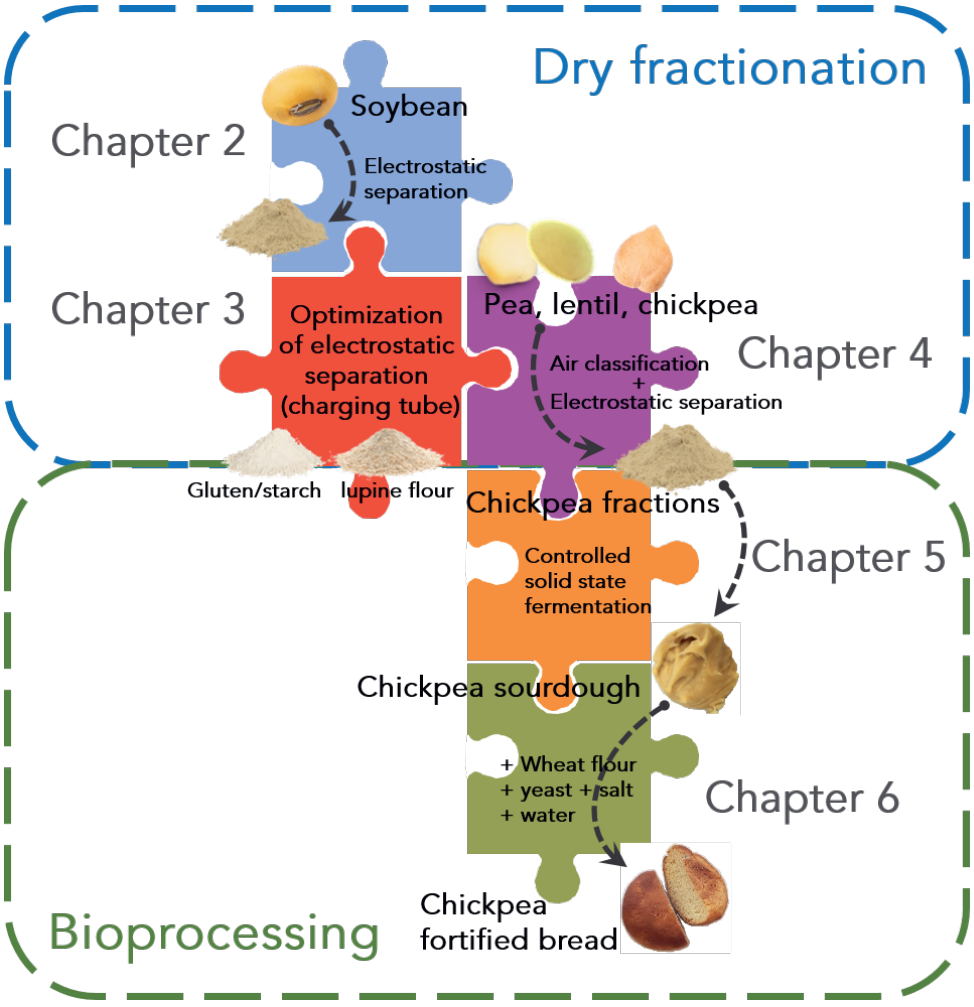


Figure 1-2. Conceptual outline of the dissertation.

Chapter 2 reports on the dry fractionation of soybean seeds by combining defatting, milling, and electrostatic separation. Two different defatting strategies were investigated. The defatted soy flours were obtained by impact milling with different intensities. An electrostatic separator equipped with a slit or a spiral tube was utilized to prepare soy protein-enriched fractions. The optimum conditions for soy protein enrichment were determined by analysing the protein purity, yield, and protein separation efficiency.

In **Chapter 3** the effect of charging tube materials, diameters, and surface properties on the separation performance of refined and raw plant materials are discussed. The chargeability of aluminium, stainless steel, Nylon 6, and PTFE were compared based on the charge-to-mass ratio of pure gluten and starch and milled lupine flour with Faraday cup and electrometer. The protein separation performance of different tubes was then investigated during electrostatic separation with model mixtures and lupine flour.

In **Chapter 4** a two-step procedure combining air classification and electrostatic separation is used to enrich protein from starch-rich legumes. Pea, lentil, and chickpea are selected as model raw materials. Since pulse starch and protein exhibited the same charging polarity, air classification is first applied to remove starch from the fine fraction which contains protein and fibres. The latter two are subsequently fractionated with electrostatic separation based on their opposite charge.

Chapter 5 reports on the development of a processing route combining dry fractionation with solid-state fermentation (SSF) to prepare chickpea concentrates with enhanced nutritional properties. Autochthonous lactic acid bacteria are isolated by a back-slopping procedure and used as a starter culture to investigate the reduction of ANFs and impact on functional properties during fermentation.

Chapter 6 reports on the use of chickpea ingredients obtained from Chapter 5 as an additive in bread. The effects of adding these chickpea fractions on the nutritional, physical, and microbiological properties of wheat bread are investigated.

Chapter 7 concludes the dissertation with a general discussion based on the main findings. The challenges facing the dry fractionation and the future perspectives of bioprocessed legume ingredients towards deployment on larger scales are discussed.


Soy
Protein
Plant
Oil
Air
Dry
Environment
Milling
Defat
Native
Yield
Starch
Energy
Clean
Loss
Green
Electrostatic
Fractionation
Charging
Improve
Sustainable
Particles
Separation
Purity
Classification
Promising
Future

Chapter 2

Protein enrichment of defatted soybean flour by fine milling and electrostatic separation

This chapter has been published as Xing, Q., de Wit, M., Kyriakopoulou, K., Boom, M. R., & Schutyser, M. A. I. (2018). Protein enrichment of defatted soybean flour by fine milling and electrostatic separation. Innovative Food Science and Emerging Technologies, 55, 42–49.

Abstract

Defatting and dry fractionation of soybean flour by the combination of impact milling and tribo-electrostatic separation was investigated to prepare protein–enriched soybean flour. Defatting is crucial to facilitate dry milling required for dry fractionation. Both organic solvent extraction and oil pressing were suitable as defatting methods although oil pressing compacted the tissue structure visually. Moderate impact milling (classifier wheel speed of 3000 rpm) effectively liberated protein bodies while eliminating agglomeration of small particles. Electrostatic separation was conducted with varying charging tube configurations. We found higher yields in the separation with a spiral charging tube design than with a slit design. The soy flour was enriched in protein from 37 g/100 g to 45 g/100 g dry basis by defatting. During electrostatic separation a soy protein enrichment of 15% was achieved and a yield of 62% of the protein was recovered from the defatted soy flour.

2.1. Introduction

Soybean is one of the most important protein sources in the human diet (Hartman, West, & Herman, 2011). Both traditional foods (e.g. tofu, soy milk, natto, tempeh) and recently emerged foods (e.g. snack bars, gluten-free baked products, meat analogues) have soybean protein as important ingredient (Hartman, et al., 2011; Poysa, Woodrow, & Yu, 2006). One of the main advantages of soybean is that it contains more protein than any other crop and that the protein has a well-balanced essential amino acids profile (Bainy, Corredig, Poysa, Woodrow, & Tosh, 2010; Day, 2013; Takamatsu, Tachibana, Matsumoto, & Abe, 2004). Specifically, soy protein has been used as an ingredient for preparation of a wide diversity of foods such as beverages, ice cream, extruded products, dairy, and meat analogues. This is because of the versatile functional properties of the protein that are quantified in terms of for example solubility, water/oil absorption, foaming, emulsification, and gelation properties (Chove, Grandison, & Lewis, 2007; Gularte, Gómez, & Rosell, 2012; Hu, et al., 2017; Maruatona, Duodu, & Minnaar, 2010; Matsumiya & Murray, 2016). Soy protein is produced with different degrees of purities ranging from soybean flour (~40% protein), to soy protein concentrate (SPC, ~70% protein), and soy protein isolate (SPI, >90% protein) (Bonanno, et al., 2012; Chen, Chen, Ren, & Zhao, 2011; Lee, Puddey, & Hodgson, 2008; Medic, Atkinson, & Hurburgh, 2014).

Wet extraction methods have been widely adopted to produce soy protein concentrates and isolates (Betancur-Ancona, Gallegos-Tintoré, & Chel-Guerrero, 2004; Guillon & Champ, 2002), achieving a yield in the range of 50% to 60% (Tenorio, Kyriakopoulou, Suarez-Garcia, van den Berg, & van der Goot, 2018). Generally, soy protein concentrates and soy protein isolates are derived from defatted soy flakes. For soy protein concentrates, soluble carbohydrates, soy whey proteins, and salts are first removed with aqueous alcohol or by hot acid leaching. Subsequently, the pre-treated soy flakes are desolventized and dried. For soy protein isolates, the proteins in the defatted flakes are dissolved, and the protein

solution is separated from the fibre fraction by centrifugation. The protein is precipitated with a pH shift, and finally the protein is centrifuged and dried after several washing steps (Berghout, Boom, & Van der Goot, 2014; Ruiz-Ruiz, Dávila-Ortíz, Chel-Guerrero, & Betancur-Ancona, 2012). The use of an excessive amount of water and subsequent drying make the wet extraction procedure a water and energy intensive process (Trivelato, Mayer, Barakat, Fulcrand, & Aouf, 2016). Moreover, the harsh extraction conditions, especially the high temperatures and extreme pH, affect the native functional soy protein properties (Föste, Elgeti, Brunner, Jekle, & Becker, 2015; Joshi, Adhikari, Aldred, Panozzo, & Kasapis, 2011; Matsumiya, et al., 2016).

Dry fractionation has been proposed as a more sustainable approach to prepare plant protein-enriched ingredients with retained native functional properties (Schutyser, et al., 2015). This method involves the combination of milling and dry separation, such as air classification, to enrich protein from pulses (Vaz Patto, et al., 2015). Dry fractionation with air classification can provide enrichment from 23.8 to 58.5 g protein/100 g dry matter for yellow pea and from 40.4 to 59.4 g protein/100 g dry matter for lupine (Schutyser, et al., 2015).

Alternatively, to air classification which relies on particle size and/or density, electrostatic separation relies on triboelectric charging as a driving force for separation (Hemery, et al., 2011). The advantage of this approach is that mixed particles of similar size but of different composition may be separated. Electrostatic separation was investigated for lupine, for which a protein enrichment up to 65 g protein/100 g dry matter was obtained (Wang, et al., 2016). In addition to lupine, promising results were also obtained for other crops such as wheat bran, rice bran, yellow pea, navy bean, rapeseed, and oat (Basset, et al., 2016; Chuetor, Luque, Barron, Solhy, Rouau, & Barakat, 2015; Schutyser, et al., 2015; Sibakov, Abecassis, Barron, & Poutanen, 2014; Tabtabaei, et al., 2016a, 2016b; Wang, et al., 2016). Because of the similar seed structure of lupine and soybean, which are both oil

containing legumes without starch, we hypothesized that electrostatic separation could be applied to enrich soy flour in protein as well. Since soybean has a much higher oil content, a defatting step cannot be avoided. Two defatting methods were evaluated in this study, namely, organic solvent extraction and oil pressing. The latter can be considered more attractive from a sustainability point of view as no harmful chemicals are used.

A custom-built bench scale electrostatic separator as described by Wang, et al. (2015) was used for soy protein enrichment. This tribo-electrostatic separator consists of several parts: the screw-feeding system, the gas/solids mixing zone, the charging tube, the separation chamber (with the electrodes), and the collecting filter bags. One electrode is grounded, and one is subjected to a positive voltage. Due to the presence of the electrical field between the electrodes, positively charged particles move towards the grounded electrode and the negatively charged particles towards the positive electrode. The soy protein bodies are expected to be positively charged and thus are attracted to the ground electrode. In the current study several modifications were made to the equipment to enhance the charging process (a spiral wound charging tube) and to collect enriched flour from the electrodes (Figure 2-1).

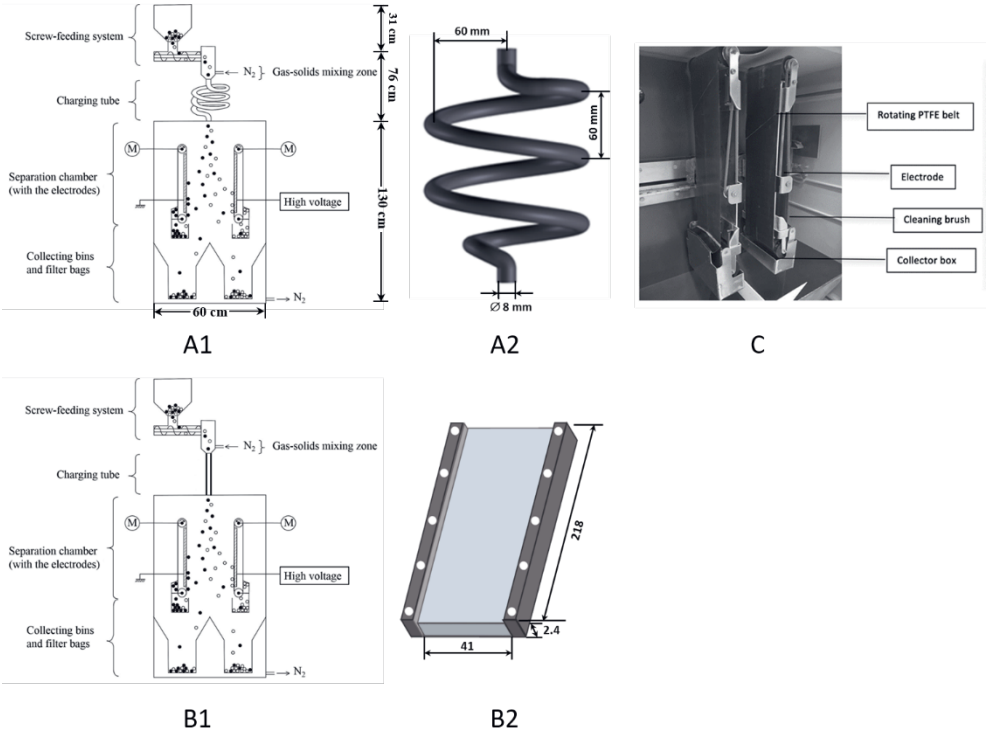


Figure 2-1. Schematic drawing of the custom-built lab-scale electrostatic separator with spiral charging tube (A1) and with charging slit (B1). The detailed configurations of spiral charging tube (A2) and charging slit (B2). And the photo of automated cleaning system (C).

The objective of this study was therefore to investigate the combination of defatting, milling, and electrostatic separation to obtain soy protein enriched flour. The efficiency of organic solvent extraction and oil pressing was first evaluated and the characteristics of the defatted flours that were generated with the two different defatting procedures were compared. Subsequently, different classifier wheel speeds were investigated during impact milling in order to identify which discloses the protein bodies to the utmost extent and at the same time avoid the aggregation of small particles. The particle morphologies and particle size distributions of milled defatted flour were analysed in order to determine the best milling settings. Finally,

tribo-electrostatic separation was conducted to achieve soy protein enrichment. The yield, protein content, and protein separation efficiency (PSE) were determined to evaluate the separation performance.

2.2. Materials and methods

2.2.1 Material

Dry, unhulled soybean (Glycine max) seeds from Canada, were purchased from Frank Food Products (Twello, The Netherlands). The seeds were stored in tightly screw-capped polyethylene containers at 4 °C in a cooling room. The crude soybeans contained 37.0 ± 1.4% protein, 24.4 ± 0.2% fat, 34.0 ± 1.5% carbohydrates, and 4.7 ± 0.1% ash on dry basis. Pre-drying of the soybean seeds was conducted to have a moisture content in the range of 6.0 - 8.6%.

2.2.2 Preparation of soybean flour

2.2.1.1. Defatting

Organic solvent

To de-fat the soybeans by organic solvent extraction, soybean seeds were first coarsely milled into soy grits with a pin mill (LV 15 M, Condux-Werk, Germany). Then a batch (~500 g) of soy grits was immediately defatted in a custom built Soxhlet extractor using petroleum ether (boiling range 40–60 °C) with a sample-to-solvent ratio of 1: 4 for 6 h (Buck & Barringer, 2007). The defatted soy grits were left in a fume hood overnight to let the residual petroleum ether fully evaporate.

Oil press

Alternatively, whole soybeans were defatted by a single-screw oil press (KK20F Universal, KernKraft, Germany). During pressing, cooling was applied to maintain the temperature at 60 °C and thus avoid possible heat damage. The throughput of

the press was 660 g soybeans per hour. The soybean oil was discarded, and the defatted soy cake was collected for further use.

2.2.1.2. Milling

The defatted soy grits and soy cake were ground with an impact mill (ZPS50, Hosokawa-Alpine, Germany) at ambient room temperature. Classifier wheel speeds of 2000, 3000, 4000, and 6000 rpm were evaluated. The other milling parameters were as follows: impact mill speed of 8000 rpm, gas flow rate of 80 m^3/h, and feed rate of 2 rpm (circa 0.5 kg/h) (in line with (Pelgrom, Berghout, van der Goot, Boom, & Schutyser, 2014)). The milling yield was calculated from the weight of the milled flour over the weight of the feed material. The milled flours were collected and stored in 4 °C before used.

2.2.3 Tribo-electrostatic separator

As mentioned in the introduction, modifications were made to the electrostatic separator (Figure 2-1A). Specifically: 1) the charging tube design was modified; 2) the electrodes in the separation chamber were equipped with a rotating PTFE belt and collector box. The charging tube is a critical part of the electrostatic separation device, as it is the part where the flour particles are tribo-electrically charged due to particle-particle and particle-wall collisions. A spiral charging tube (1226 mm length × 8 mm internal diameter) made of stainless steel and a charging slit (41 mm × 2.4 mm × 218 mm) were compared in this study (Figure 2-1C). The internal surface area for the spiral charging tube (308 cm^2) is nearly 15 times larger than that of the straight tube (21.5 cm^2). By increasing the tube length (1226 mm) and reducing the cross-section area (0.5 cm^2), the particles' residence time and the gas velocity could be independently varied while maintaining the same gas flow rate. Based on the residence time of air in the tubes, a particle is estimated to take approximately 3 times more time to pass through spiral tube compared to the straight tube. An increase in residence time and an increase in gas velocity are both

expected to increase the charging and subsequently enhance the separation performance.

The separation chamber included two electrode plates (12 mm × 37 mm) positioned in a vertical position at a distance of 10 cm, which generates an electrostatic field in between the plates. Both electrodes were equipped with a rotating PTFE conveying belt, driven by an electric motor, and equipped with brushes to allow continuous removal of deposited material from the electrodes. The brushes and small powder collector boxes were placed at the bottom of the electrodes (Figure 2-1B).

The custom-built separator can precisely control the powder dosing rate with a screw feeder. The dosing rate is in the range between 0.5 and 2.5 kg/h.

2.2.4 Separation experiments

For each separation experiment 20 g of defatted soy flour was used as starting material. The feed rate was fixed at 0.5 kg/h and a voltage of 20 kV was applied to the positive electrode. A fixed gas flow rate (50 L/min) of the electrostatic separator was employed for all separation experiments. After one single separation step four fractions were obtained. The flour collected from the grounded electrode was labelled as "GE" and the flour collected from the positive electrode was labelled as "PE". The flours retrieved from the filter bags, installed below the separation chamber, were referred to as "GC" and "PC", respectively. It was assumed from our previous study with lupine that soy protein bodies charge positively and thus move towards the grounded electrode (Pelgrom, Wang, et al., 2015; Wang, et al., 2016).

2.2.5 Analyses

2.2.5.1. Compositional analysis

The moisture, ash, and crude fat contents of flours were determined according to AACC 44–15.02 (1999), AACC 08-01 (1983), and AACC 30-25.01 (1999), respectively. The protein content was determined with a nitrogen analyser (FlashEA

1112 series, Thermo Scientific, Breda, The Netherlands) based on Dumas combustion method. A conversion factor of N × 5.71 (Berghout, Pelgrom, Schutyser, Boom, & Van Der Goot, 2015; Ezeagu, Petzke, Metges, Akinsoyinu, & Ologhobo, 2002) was used for conversion of the nitrogen content to the crude protein content. The carbohydrate content was calculated as the difference.

2.2.5.2. Scanning electron microscopy (SEM)

A scanning electron microscope (Phenom G2 Pure, Eindhoven, the Netherlands) was used to visualize the microstructure of the soybean flours. The flours were observed without pre-treatment or coating. The samples were fixed with double-sided adhesive conductive carbon tabs (JEOL Europe BV, the Netherlands) on 12.7 mm aluminium pin-type stub mounts (JEOL Europe BV, the Netherlands). The accelerating voltage was 5 kV.

2.2.5.3. Particle size distribution (PSD)

The volume-averaged particle size distributions of impact milled soy flour were measured with laser diffraction using a Mastersizer-3000 (Malvern Instrument Ltd., Worcestershire, UK) in combination with the dry module for powders (Aero S). A pressure of 200 kPa was applied and the average particle size in volume Dv(50) was calculated on the basis of the Fraunhofer theory (Ahmed & Al-Attar, 2015).

2.2.5.4. Protein separation efficiency (PSE)

The protein separation efficiency was defined as the ratio of the amount of protein recovered in the target fraction and the amount of protein in the feed (or sum of all collected fractions with a small loss):

$$PSE\ (\%) = \frac{Y_{GE} \cdot P_{GE}}{Y_f \cdot P_f} \times 100 \qquad (Eq\ 2-1)$$

Where Y_{GE} represents the yield of the target fraction (collected from the ground electrode), and P_{GE} is the protein content of the same target fraction. Y_f and P_f stand for the yield and protein content of the feed, respectively.

2.2.5.5. Statistical analysis

Data were collected from duplicate experiments or in specified cases from more experiments. The variances were analysed using SPSS statistics Version 22.0 (IBM, Armonk, NY). Duncan's test was performed to evaluate the statistical significance between samples at a significant level of 95% ($P < 0.05$). All the results were displayed by mean values ± standard deviations.

2.3. Results and discussion

2.3.1. Morphology of soybean seed and milled defatted flours

In order to effectively enrich protein from soybean it is of crucial importance that the protein bodies are physically disentangled from especially the cell wall material that is much lower in protein (Pelgrom, Schutyser, & Boom, 2013). The morphology of the defatted and milled soy flour particles (Figure 2-2A) were observed with scanning electron microscopy. The storage protein bodies are anchored in irregular-rod like cotyledon cells (Figure 2-2B) by a net-like intracellular matrix (Figure 2-2C), which mainly consists of carbohydrates. The average size of nine randomly selected protein bodies is determined to be 10.9 ± 2.2 μm, which is consistent with the size of soy protein bodies reported from Medic, et al. (2014). From previous studies we know that up to 80% of the total protein in soybean is stored in protein bodies (Medic, et al., 2014). The protein purity of the protein bodies is reported to be ~82.5% (Tombs, 1967). This protein content provides an upper limit to soy protein enrichment via dry fractionation.

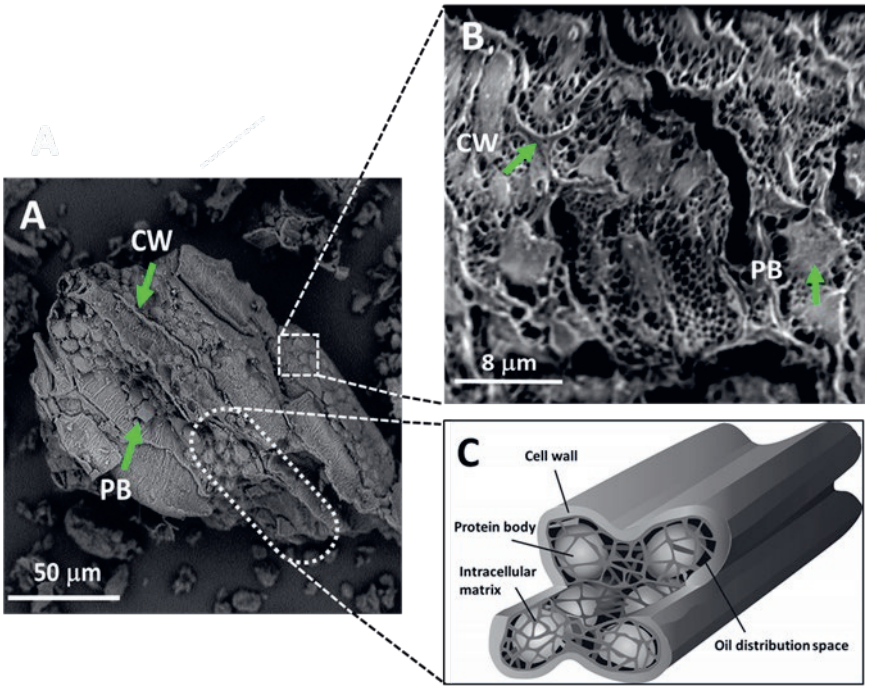


Figure 2-2. Scanning electron microscopy (SEM) images of defatted soybean flour particles (A) and magnified intracellular matrix (B). A schematic drawing of a single soybean cotyledon cell is proposed in C. PB and CW represent protein body and cell wall, respectively.

The soy flours prepared with varying defatting and impact milling treatments are compared in Figure 2-3, after impact milling at different classifier wheel speeds, detached protein bodies can be found in both oil pressed and organic solvent defatted soy flours. The protein bodies and cell walls can be easily distinguished by their shape and surface conditions with the help of SEM images. Pebble-like protein bodies have smooth surfaces, while cell walls can be identified from their amorphous multi-layer folding structure and rough surfaces. For the milled oil-pressed soy cake (Figure 2-3 A–D), single protein bodies were less frequent, possibly because of the high mechanical deformation during oil pressing and as a consequence damage to the protein bodies. The latter is also suggested by the

presence of twisted (Figure 2-3 A) and squeezed (Figure 2-3 B) debris in the milled oil-pressed soy cake. Instead, for the solvent defatted flour (Figure 2-3 E–H), more intact protein bodies can be observed, and the cell wall fragments in the flour that was solvent defatted are still rather porous (Figure 2-3 E and F). Based on these observations we conclude that oil pressing is less preferred over solvent extraction for its negative effect on protein body integrity during milling.

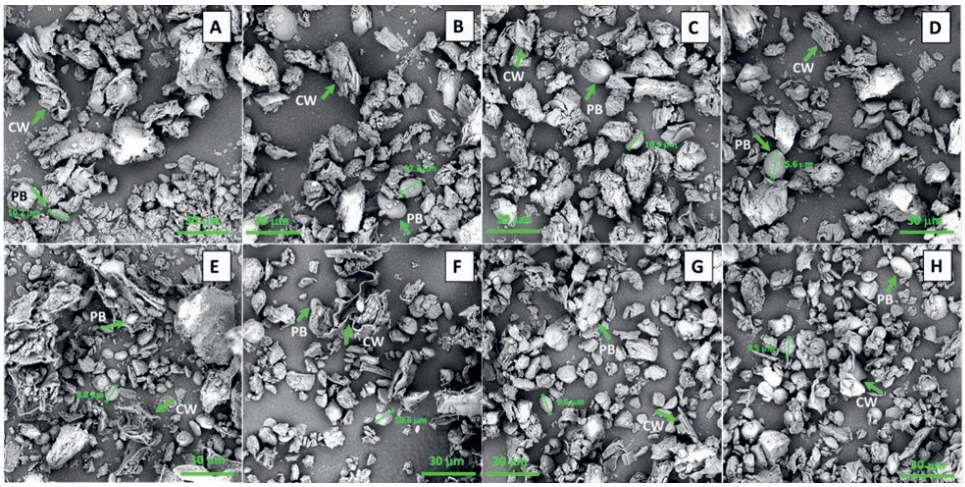


Figure 2-3. SEM images of oil pressed soy flours (A-D) and solvent defatted soy flours (E-H) milled at 2000, 3000, 4000, and 6000 rpm classifier wheel speed, respectively. Arrows indicate protein bodies (PB) and cell wall (CW).

2.3.2. Yield, composition and particle size of milled defatted soybean flours

The characteristics of milled defatted soy flour are summarized in Table 2-1. The amount of residual oil in soy flour defatted with organic solvent and oil press is similar, ranging from 7.4 to 9.3%. The protein content increased after defatting from 37 g/100 g from the soybeans to 46.3 g/100 g dry basis. The lipid and protein content slightly increased with increasing milling speed and thus reducing particle size. The higher fat content may be explained as slightly more fat may be extracted from smaller particles during the measurement of the fat content. A higher protein

content with increasing milling speed may be explained by fouling inside the mill, which contains relatively more fat and fibres, causing a slight improvement in protein content of the flour coming out of the mill (Pelgrom, et al., 2014). This is also obvious from the lower overall yield obtained with the reducing particles sizes obtained by increasing the classifier wheel speed. This can be explained by more significant van der Waals force interactions between smaller particles and between smaller particles and the wall (Pelgrom, Vissers, et al., 2013).

Table 2-1. Characteristics of oil pressed and organic solvent defatted soy flour milled at 2000, 3000, 4000, and 6000 rpm, respectively.

	Classifier wheel speed (rpm)	Yield (g/100 g flour)	Moisture (w/w%)	Protein (g/100 g dry matter)	Lipid (%)	Dv(50)
Oil pressed soy flour	2000	87.2±2.5	8.6±0.6	44.2±0.2	7.6±0.1	84.7±2.6
	3000	80.7±3.3	7.5±0.6	44.2±0.2	7.4±0.1	48.6±0.5
	4000	60.7±7.4	7.6±0.8	43.9±0.0	9.1±0.4	25.3±0.0
	6000	58.3±7.4	8.0±0.0	44.7±0.4	9.3±0.1	17.3±0.4
Organic solvent defatted soy flour	2000	88.5 ±3.5	7.2±1.0	44.1±0.1	8.0±0.9	309±18.2
	3000	80.5±2.6	6.0±1.0	45±0.2	8.2±0.1	48.8±0.2
	4000	65.0±2.0	6.7±0.9	45.8±0.1	8.5±0.4	16.7±0.3
	6000	66.5±6.8	7.3±0.5	46.3±0.0	8.2±0.2	15.7±0.3

A higher classifier wheel speed does not only lead to a smaller average particle size due to the smaller cut-off diameter of particles that can escape via the classifier wheel (Pelgrom, et al., 2014), but it also changes the shapes of the particle size distribution curves. For oil pressed soy flour (Figure 2-4 A), two peaks were observed

at each classifier wheel speed, and the most obvious peak shifting from approximately 110 to 18 µm along with the increasing of classifier wheel speeds. Soy flour that was defatted with oil press has a more compact structure; the protein bodies are pressed together with other components and distinct particle structures seem no longer distinguishable, leading to a single peak. Thus, the separation of protein bodies from fibre and carbohydrate fragments is difficult. In comparison, more peaks are observed in solvent defatted soy flour milled at low classifier wheel speeds and only one peak is observed at high milling speeds (Figure 2-4 B). Three peaks were observed at 3000 rpm, with one at around 10 µm, which indicates the individual protein bodies and two peaks at 110 µm and 756 µm, representing the presence of clustered particles and hull debris. These separate peaks may be explained by the looser structure of solvent defatted soy flour. After relatively coarse milling (3000 rpm), the protein bodies are separated from the cotyledon and retain their integrity. At the same time, other particles like fibres are not milled too fine, avoiding clustering due to Van der Walls forces or other complications during the electrostatic separation afterwards.

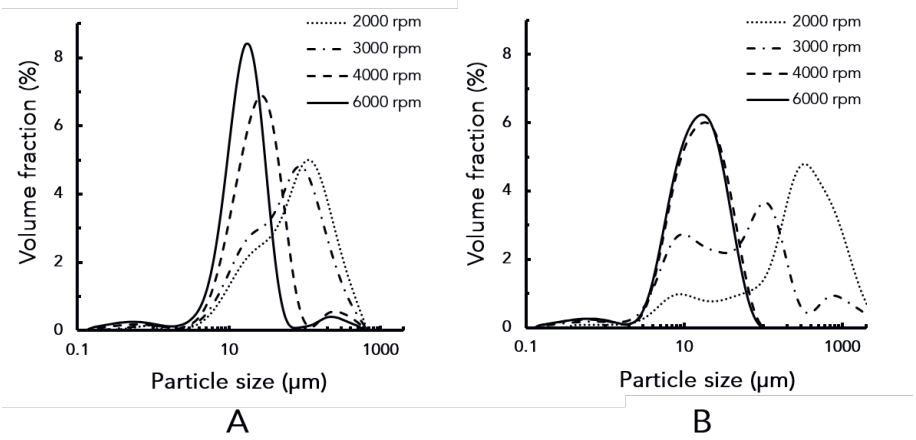
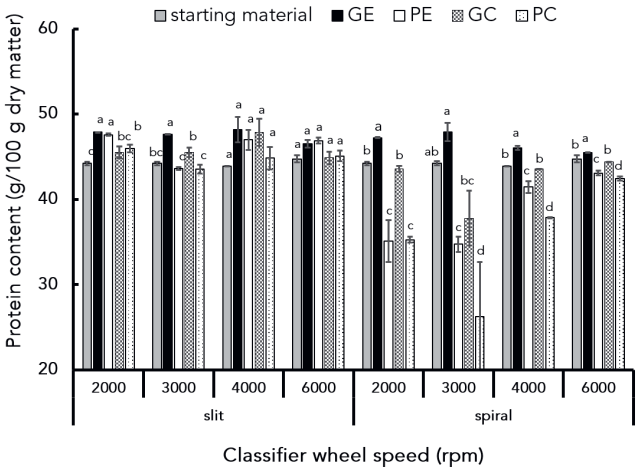


Figure 2-4. Particle size distribution (PSD) curves of soy flour defatted with oil pressing (A) and organic solvent (B) milled at different classifier wheel speed. Each

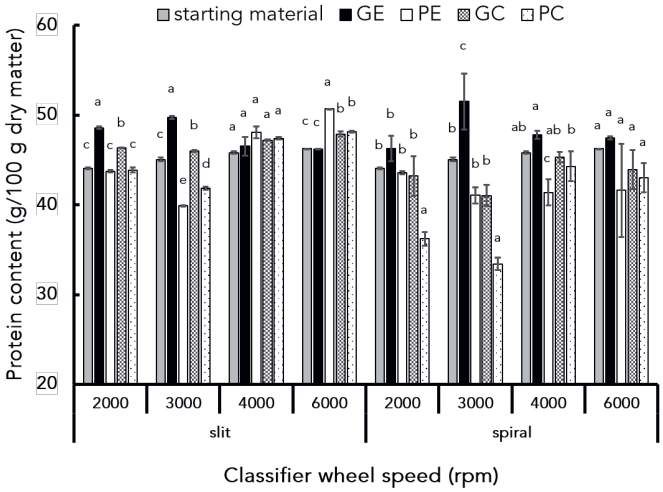
curve is the average of duplicate measurements. The standard deviation was <1.8 and 0.2 v/v%, respectively.

2.3.3. Protein enrichment by electrostatic separation

The flours were subjected to electrostatic separation and the enrichment in protein of the target fraction was evaluated. The protein content of defatted starting material and four electrostatic separated fractions from two defatted flours are plotted in Figure 2-5. Similar to lupine protein bodies, soy protein bodies were positively charged and collected on the ground electrode (GE), while the positive electrode (PE) yielded the protein depleted fraction (Wang, et al., 2016). For solvent defatted soy flour (Figure 2-5 B), maximum 4.7% and 6.5% enrichments were achieved after electrostatic separation with the charging slit and the spiral tube, respectively, while the protein enrichment for oil pressed soy flour was only 4.3% and 3.7% (Figure 2-5 A), respectively. The decreased protein enrichment for oil-pressed flour may be due to the compaction of the material as discussed before. The spiral tube yields larger protein purities than the slit, since it is longer and allows particles of different composition to gain increased opposite charge, leading to better separation.



A



B

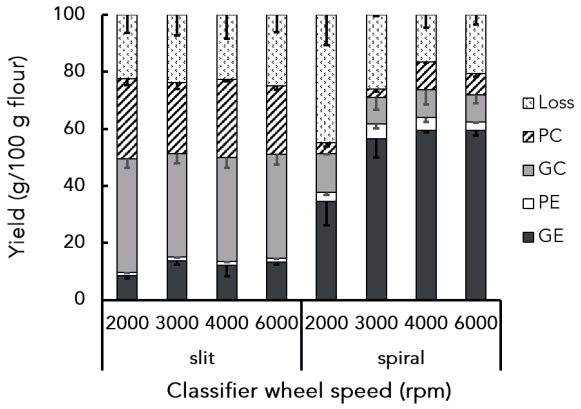
Figure 2-5. One single electrostatic separation of oil pressed (A) and solvent defatted (B) soy flour milled at different classifier wheel speed: protein content of milled defatted starting material and four fractions collected from ground electrode (GE), positive electrode (PE), ground collector (GC), and positive collector (PC), respectively. The error bars indicate standard deviations. Cluster columns in a group of five under the same classifier wheel speed marked with lower case letters means significantly different from each other ($P < 0.05$).

The classifier wheel speed had comparable influence on the protein enrichment for both types of soy flour. Generally, significant ($P < 0.05$) protein enrichment was achieved at relatively coarse milling (3000 rpm). Too coarse milling (2000 rpm) is insufficient to detach protein bodies from other components, while too fine milling (4000 and 6000 rpm) produces more particles of small size. On the one hand, finer powder particles cluster together more, due to more exposed residual lipids and/or the van der Waals force interactions (Wang, et al., 2016). On the other hand, for negatively charged, small fibre and carbohydrate fragments experience more drag from the surrounding gas relative to the electrostatic force, and thus may also migrate to the ground electrode, causing a decrease in the purity of protein

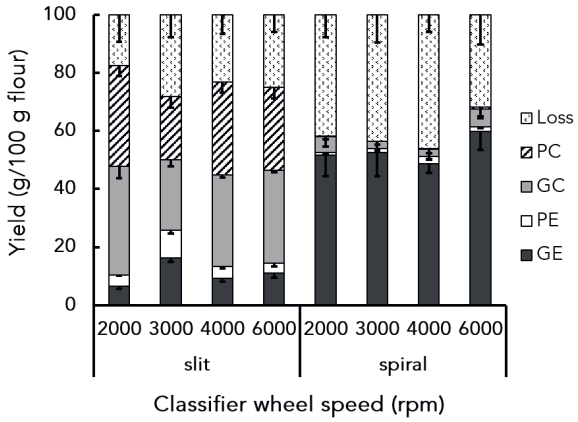
enriched fractions. Relatively coarse milling (3000 rpm) effectively reduces the above-mentioned problems, as long as the detachment of the protein bodies from other components is still achieved. Concluding, with the combination of defatting and dry fractionation, the protein purity of the enriched fraction increased by 15% as compared to the original defatted soybean flour.

2.3.4. Yield of electrostatically separated fractions

The yield is another important parameter evaluating separation performance. For each separation experiment 20 g of defatted starting material was used. The yields of the four fractions were summarized and the weight loss was calculated as the difference with the original amount. As shown in Figure 2-6, with the spiral charging tube, the yields of the protein enriched fractions (GE) were approximately five times higher than with the slit. Such a high yield could previously only be reached after 4 times re-milling and repeated separation with a charging slit (Wang, et al., 2016). The difference may be explained by the increased tube length, which allows particles to pick up more charge more effectively, but also by the curved geometry, inducing many more interactions between the particles and the tube wall. In addition, the conveyor belt and brush remove the attached powder, and thus prevent charge shielding, which, we expect, occurred in the study by Wang, et al. (2016).



A



B

Figure 2-6. One single electrostatic separation of oil pressed (A) and solvent defatted (B) soy flour milled at different classifier wheel speed: yield of four fractions collected from ground electrode (GE), positive electrode (PE), ground collector (GC), and positive collector (PC), respectively. Weight of lost flour was calculated by difference. The error bars indicate standard deviation, only minus direction is shown.

The flours milled at 3000 rpm provided the highest yield. At the lowest classifier wheel speed (2000 rpm), particles may be too large to be captured by the electrodes. However, the yields also decreased for fine milled flours (4000 and 6000 rpm), possibly because of agglomeration as small negatively charged fibres attached to the positively charged protein bodies. This also explains why both yield and protein purity are maximal at 3000 rpm.

The overall loss with the spiral charging tube was higher than with the charging slit. This can be explained by an imperfection in the experimental system. The higher hydrodynamic resistance of the spiral tube that leads to increased pressure before the tube. Since the powder feeding system is not completely airtight, this induces a loss of part of the gas, including entrained particles to the environment, even before it enters the charging tube (Figure 2-1 A1).

2.3.5. Protein separation efficiency

The protein separation efficiency (PSE) combines the protein purity and the yield. It can be concluded from Figure 2-7 that the PSE of the spiral charging tube exceeds that of the charging slit. The longer spiral tube gave much higher yield reaching a maximum of 62% of total protein recovery from the defatted starting material, while for the charging slit only 18% maximum of total protein was recovered on the ground electrode. As for the defatting processes, both oil pressing and organic solvent as defatting pre-treatment suffice for protein enrichment. However, their effect on the PSE was not very different despite the difference in the protein purity and more or less similar yield. More importantly, the PSE showed the highest values for 3000 rpm classifier wheel speed. It shows that moderate milling speed efficiently liberates protein bodies and subsequently facilitates protein enrichment in electrostatic separation.

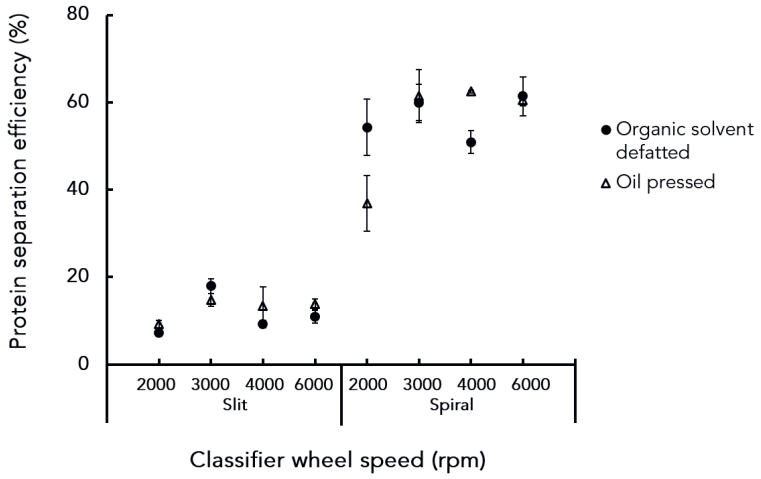


Figure 2-7. One single electrostatic separation of oil pressed and organic solvent defatted soy flour milled at different classifier wheel speed: protein separation efficiency (PSE) of fraction collected from ground electrode. The error bars indicate standard deviations.

2.3.6. Yield vs purity

The yield of protein enriched fractions and their corresponding protein purity is shown in Figure 2-8. Obviously, the spiral charging tube (● and ○) gives higher yields of the target fraction as compared to the charging slit (▲ and △). The purity of the target fractions of higher yields are mostly situated in the range of 46.3–47.8%. However, one outlier, which belongs to milling at 3000 rpm, is more shifted to the right side at 51.5%. A similar, but smaller outlier can be observed when separating with the charging slit. It shows that 3000 rpm is the more optimal milling procedure. Protein bodies liberated from the cellular matrix to larger extent at this milling condition. All spiral charging tube separation experiments showed a yield between 51.6% and 59.9%, except one which belongs to oil pressed soy flour milled at 2000 rpm, which had a yield of only 34.5%. This may be due to the too large size of the particles under coarse milling, while at the same time particles were more compacted due to mechanical pressing. Therefore, those particles were difficult to be captured by the electrodes. Furthermore, for separation with the charging slit, the target fraction from defatted flour milled at 3000 rpm had highest yield (16.3%) as compared to others (6.6 - 13.7%).

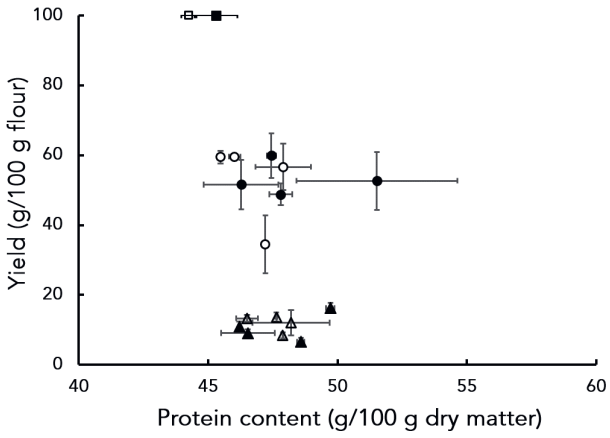


Figure 2-8. The result of electrostatic separation with spiral charging tube and charging slit. The yield of protein enriched fractions from defatted soy flour milled at different classifier wheel speeds as function of their protein content. ■ represents solvent defatted soy flour, □ represents oil pressed soy flour, ● and ▲ is protein enriched fractions separated from solvent defatted soy flour separated with spiral and slit, respectively; ○ and △ is protein enriched fractions separated from oil pressed soy flour with spiral tube and slit, respectively. Error bars represent standard deviations.

2.4. Conclusions

A soy protein concentrate can be prepared by impact milling followed by electrostatic separation. Similar to other legumes like lupine flour, soy protein bodies acquire a positive charged and accumulate on the ground (negative) electrode while fibre and carbohydrate became negatively charged and deposited on the positive electrode.

Oil removal after pre-milling and before final milling is an important prerequisite. Petroleum ether extraction and oil pressing are both effective in defatting and their impact on protein enrichment is similar. However, defatting with organic solvent seems less harmful to the native structure and specifically the protein bodies of the soy than oil pressing.

The optimal classifier wheel speed during impact milling with the mill used, is 3000 rpm. Under these conditions, flour with an original protein content of 37 g/100 g, was enriched to 45 g/100 g. This relatively coarse milling sufficiently detaches the protein bodies from other components, while it still avoids agglomeration of small particles that are generated by intensive milling.

A spiral charging tube was found to be more effective than a straight one. We hypothesise that this is due to the prolonged residence time in the spiral tube as

compared to the charging slit. After electrostatic separation with the spiral charging tube, a maximum protein purity of 52 g/100 g dry basis was obtained for the protein-enriched fraction. Moreover, after one single electrostatic separation, 62% of the total protein present in the original soybean flour was recovered.

Acknowledgements

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Flow
Aluminium
Electrostatic
Tribo
Plant
Stainless
Steel
Fibre
Stainless
Starch
PTFE
Spiral
Protein
Charge
Slit
Bran
Diameter
Electron
PTFE
Charging
Separation
Purity
Tube
Yield
Straight
Nylon
Voltage

Chapter 3

The effect of tube wall material on electrostatic separation of plant raw materials

This chapter has been published as Xing, Q., Kyriakopoulou, K., de Wit, M., Boom, M. R., & Schutyser, M. A. I. (2020). The effect of tube wall material on electrostatic separation of plant raw-materials. Journal of Food Process Engineering, e13575.

Abstract

The influence of charging tube materials and diameter on the separation efficiencies of a gluten-starch model mixture and lupine flour was studied. Offline analysis of tribo-charging with different tube materials showed that gluten takes a positive and starch a negative charge. However, the charge of the mixture was found not equal to the sum of the charge of the individual components and measured charges could not be related to the triboelectric series. During electrostatic separation significant protein enrichment was observed for both plant raw materials. For the model mixture differences in protein enrichment were observed between tube materials, but this was not the case for lupine flour. The lupine protein content increased from 37 to 65 g/100 g dry flour. Concluding, electrostatic separation needs to be evaluated during separation experiments, as particle-particle interactions dominate the charging process and thus separation of mixtures.

3.1. Introduction

The growing world population leads to a rapidly increasing demand for protein, while the potential of our planet to produce foods may well decline due to changes in the global climate (Asseng, et al., 2015). Therefore, the current plant protein production needs to become more efficient. This can be done by shifting to more plant-based diets and by developing more efficient protein isolation routes (Aiking, 2011). Traditional wet protein isolation processes generally aim at high purity (> 90% protein) and are intensive in their use of water and energy. However, the native functional properties of proteins are often lost due to harsh processing conditions. Dry fractionation, which involves the combination of dry milling and dry separation, is proposed as a sustainable and mild route for protein fractionation. Dry fractionation provides less pure but highly functional protein-rich ingredients (Schutyser, et al., 2015), which has been demonstrated for various seeds of cereals and pulses (Schutyser, et al., 2011). The first step of this solid separation process is ultrafine milling of the seeds into a flour (Basset, et al., 2016). In starch-rich legumes the starch granules are liberated from the protein/fibre matrix as it is ground into small powder particles. Subsequent dry separation is often carried out using sieving or air classification depending on the differences in size and density of the particles (Lammi, Barakat, Mayer-Laigle, Djenane, Gontard, & Angellier-Coussy, 2018). A more recent dry separation technique introduced for food ingredients is electrostatic separation, which separates particles on the basis of their triboelectric charging properties (Wang, et al., 2016). Studies demonstrated electrostatic separation for protein enrichment of navy bean, rapeseed, lupine and soybean (Basset, et al., 2016; Tabtabaei, et al., 2017; Wang, et al., 2016; Xing, et al., 2018). A large proportion of protein in lupin and soybeans is stored as protein bodies, which can be enriched during dry separation. Protein bodies were found positively charged and could be collected on the ground electrode (GE) as a protein enriched fraction, while fibres charged negatively and could be collected on the positive

electrode (PE) as a protein depleted fraction (Xing, et al., 2018). Provided that the ideal protein-enriched fraction only contains detached protein bodies, the theoretical limit for protein enrichment with dry fractionation is equal to the protein content of the protein bodies (73~80 g/100 g) (Wang, et al., 2016). Process optimization is required to achieve protein enrichment as close as possible to this limit.

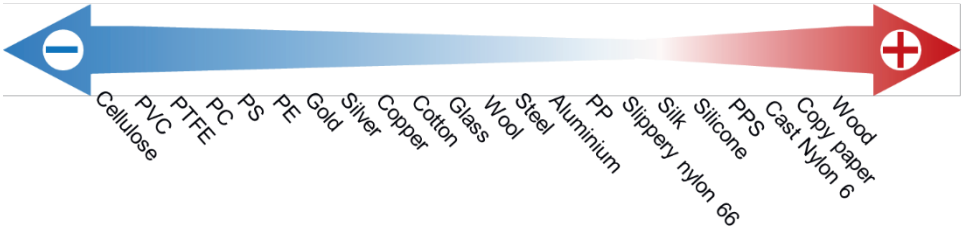

Figure 3-1. Triboelectric series of some common materials (Liu, Zheng, Yang, & Tao, 2018; Zou, et al., 2019).

Triboelectric charging of materials is an often observed, but poorly understood phenomenon (Lacks & Shinbrot, 2019). When two materials are brought into contact, charge transfer induces a positive charge on one material and a negative charge on the other. The transferred charge can be either an electron, an ion or very small material fragments (Lacks, et al., 2019). Different mechanisms are described for charge transfer upon contact between metals, insulators and their combinations. For metal-metal contact the transfer of charge by exchange of electrons has been quantified using material-dependent work functions (Mirkowska, et al., 2016). A metal with a higher work function is closer to the negative end of the so-called triboelectric series (Figure 3-1) and tends to be charged negatively when in friction with another metal with a lower work function (Kwetkus, 1998). For conductor-insulator and insulator-insulator contacts however the exact mechanisms of charge transfer are unknown, although multiple studies have tried to characterise and

develop theories for tribo-charging between these materials (Mirkowska, et al., 2016; Zhang, Chen, Jiang, Lim, & Soh, 2019). In practice, often triboelectric series are reported, but drawback is that the order of materials in the triboelectric series is not always reproducible since many additional factors influence the triboelectrification process. During triboelectric charging of mixed materials both particle-particle and particle-wall may contribute to the overall tribo-charging of the particles.

There are different methods to evaluate charging behaviour of powders (Zafar, Alfano, & Ghadiri, 2018). Often charge is measured of a single material with a Faraday cup, where charging is realized for example with a charging device. Disadvantage is that such a method is less suitable for particle mixtures of different materials, as not only contact occurs between particle and wall, but also between particles. Alternatively, charge may be determined after electrostatic separation of different fractions. Drawback is that these studies are time consuming and more useful for analysing the separation experiment rather than for characterization of the tribo-charging.

Electrostatic separation is already applied on an industrial scale for the beneficiation of minerals, fly ash and recycling of plastics (Chen & Honaker, 2015; Felsing, Kochleus, Buchinger, Brennholt, Stock, & Reifferscheid, 2018; Tabtabaei, et al., 2016a), while not yet for protein fractionation (Tabtabaei, Konakbayeva, Rajabzadeh, & Legge, 2019). Lab-scale tribo-electrostatic separators consist of a dosing system, a charging tube and a separation chamber with an electric field. Materials to be separated are conveyed by air or inert gas via a charging tube and subsequently separated in an electric field (Song & Mehrani, 2017). Very few studies systematically investigated the influence of the tube material choice, diameter and surface properties on the separation of food ingredients. (Tabtabaei, et al., 2016b) compared different charging materials, namely PTFE, PVC, Nylon, and copper, for the enrichment of navy bean flour. The chargeability, in that study, was determined

by measuring the charge of navy bean flour in a Faraday cup acquired after shaking the flour in the different tubes. Based on the results, PTFE was selected as the tribo-charging material. With the PTFE tube, the protein content increased from 25% to 47%. In another study, Chen, et al. (2014) compared different tube walls by dispersing and conveying wheat bran particles in PTFE, Nylon, and steel tubes and collecting those in a Faraday cup. They claimed that insulators (PTFE and Nylon) would be more suitable than stainless steel for separating aleurone from pericarp particles. In our previous study (Xing, et al., 2018), significant legume protein enrichment was achieved by electrostatic separation with the use of aluminium and stainless steel charging materials. From the above we conclude that previous studies came to different conclusions using approaches with tribo-charging measurements and/or electrostatic separation.

This study aims at evaluation of methods to come at best selection of charging tube wall material and studies the effect of tube wall material and tube on electrostatic separation for protein enrichment of flours. A range of charging tube wall materials were investigated, both conductors and insulators. Tribo-charging measurements were carried out with pure wheat gluten, wheat starch and lupine flour. The added value of tribo-charging measurement of pure components and their mixtures was discussed to predict separation of particle mixtures during electrostatic separation. Finally, electrostatic separation experiments were performed on a gluten: starch model mixture and on lupine flour to find out the main contributing factors to protein enrichment.

3.2. Materials and methods

3.2.1 Materials

Wheat gluten and starch were obtained from Roquette (France) and Sigma-Aldrich (USA), respectively, and were stored in tightly screw-capped polyethylene vessels at -20°C. Dry and dehulled lupine seeds were purchased from Frank Food Products (Twello, The Netherlands) and stored in tightly sealed polyethylene containers at 4°C (Table 3-1).

Table 3-1. Compositions of wheat gluten, wheat starch, and lupine flour.

	Protein (g/100 g)	Carbohydrate[2] (g/100 g)	Oil (g/100 g)	Ash (g/100 g)
Gluten	77.9 ± 0.1	19.1 ± 0.0	2.2 ± 0.1	0.8 ± 0.0
Starch	0.8 ± 0.3	98.9 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
Lupine flour	37.2 ± 1.2	55.9 ± 0.3	5.6 ± 0.1	3.0 ± 0.3

3.2.2 Preparation of model mixture

Gluten and wheat starch were mixed at a ratio of 1:1 with a food mixer (Bosch MUM5, Germany). The model mixture was left overnight before using.

3.2.3 Preparation of lupine flour

Lupine flour was prepared by a two-step procedure. First, dry lupine seeds were coarsely milled into lupine grits with a pin mill (LV 15M, Condux-Werk, Germany). Then, the lupine grits were further milled into fine lupine flour with an impact mill (ZPS50, Hosokawa-Alpine, Augsburg, Germany) at ambient temperature. Classifier wheel speed was set at 2500 rpm, impact milling speed was 8000 rpm, and the

[2] Calculated by difference.

airflow was 80 m^3/h (Wang et al., 2016). The prepared lupine flour was stored in sealed plastics bags in the freezer at -20 °C.

3.2.4 Electrostatic separator with varying charging tube configurations

A custom-built electrostatic separator was used for the separation experiments (Figure 3-2). The set-up was previously described in detail (Xing, et al., 2018). The flour was entrained by a nitrogen gas flow which flowed through a charging tube. Upon exiting the charging tube, the entrained particles were exposed to an electric field that was applied between two vertically positioned electrodes at a distance of 10 cm. One electrode was grounded and the other had a positive voltage. Both electrodes were equipped with a PTFE conveying belt and brushes to continuously remove deposited powder from the electrodes. The conveying belts were driven by an electric motor, and brushes and powder collector boxes were placed at the bottom of the electrodes.

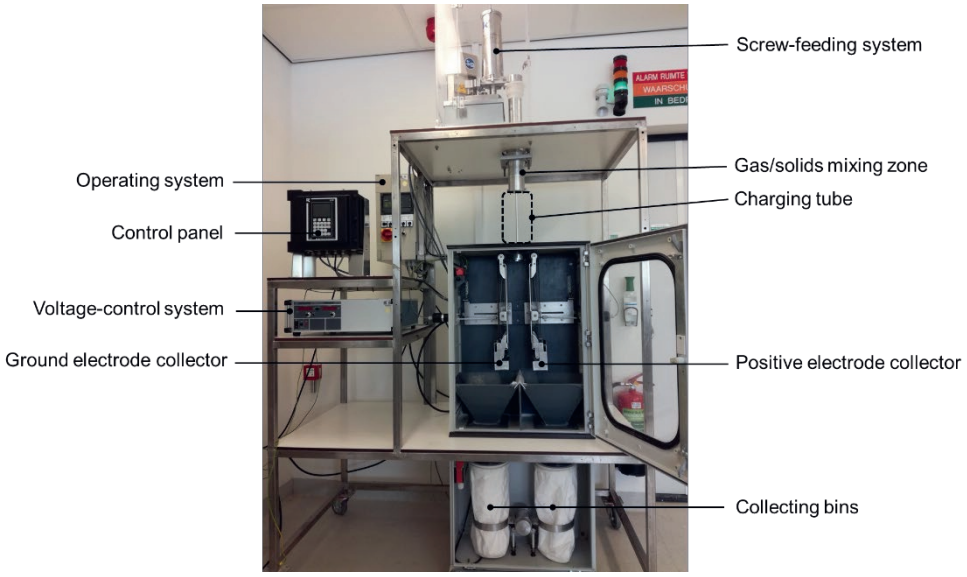

Figure 3-2. The custom-built electrostatic separator. Main parts of the separator are indicated in the picture.

Charging tubes of varying materials (aluminium, stainless steel, Nylon 6, and polytetrafluoroethylene/PTFE), diameters, and surface properties were used as listed in Table 3-2. This choice was based on their use in previous studies, but also for their different positions in the triboelectric series (Chen, et al., 2014; Tabtabaei, et al., 2016b; Wang, de Wit, Schutyser, & Boom, 2014). Different tube diameters were selected to vary the gas flow velocity. A corrugated tube was made to examine the effect of increased convection near the wall: the inner diameter of this tube was 12 mm with 1 mm milled grooves, where the distance between two grooves was 2 mm.

Table 3-2. The configurations of the charging tubes used in this study.

No.	Tube	Material	Shape	Length (mm)	Inner diameter (mm)	Inner surface condition
1		Stainless steel	Straight	296	8	Smooth
2		Stainless steel	Straight	296	13	Smooth
3		Stainless steel	Straight	296	13	Corrugated
4		Aluminium	Straight	296	8	Smooth
5		Nylon 6	Straight	296	8	Smooth
6		PTFE	Straight	296	8	Smooth

For separation experiments with mixtures of wheat and gluten a feed sample of 25 g powder was used. For lupine flour a feed sample of 50 g was used as starting material. The solids feed rate was controlled at 0.5 kg/h by a screw-feeding system. The nitrogen gas flow rate was always fixed at 50 L/min and the voltage applied to the positive electrode was 20,000 V. The corresponding electrical field strength was 200,000 V/m. After each separation run, four fractions were obtained. The fraction

obtained from the ground electrode was labelled "GE" and the fraction collected from the positive electrode was labelled "PE". Fractions collected from the filter bags installed below the separation chamber were referred to as "GC" and "PC", respectively. The separation experiments were carried out in duplicate. Protein enrichment was defined as the ratio of the difference between the protein content of the fraction collected at the ground electrode and the original material divided by the protein content of the original material.

3.2.5 Tribo-charging measurements of particles with varying tube wall materials

Following the method from Tabtabaei, et al. (2016b), the charge of pure gluten and wheat starch upon tribo-charging was measured in a dedicated system with the same tubes (Figure 3-2). This system consisted of: (1) the charging tube (2) a vibrator (HS 250, IKA, Germany), (3) a Faraday cup, and (4) an electrometer (Model 6215, Keithley Instruments, Inc., USA). For each experiment the charging tube was filled with ~0.5 g powder and horizontally fixed on the shaker (Figure 3-3 A). Subsequently, the shaker was activated in the direction parallel to the longitudinal axis of the charging tube, with the highest speed for 1 min. The charged powder was then transferred into a Faraday cup and the charge was measured with the electrometer. The result was expressed as the charge-to-mass ratio (µC/kg). The experiment was repeated for three times and the average values were calculated. Powders tested were gluten, starch, and a 1:1 mixture of both.

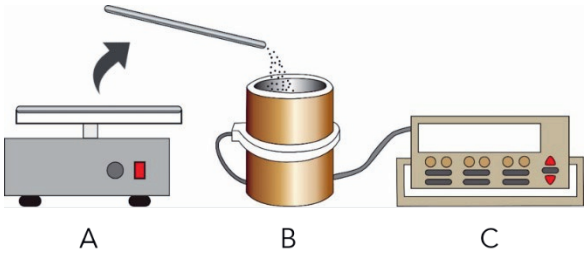

Figure 3-3. The charging measurement system: a vibrator with charging tubes fixed on it (A), a Faraday cup (B) connected with an electrometer (C).

3.2.6 Analyses of plant raw materials

3.2.6.1 Compositional analysis

The oil, ash and moisture contents of gluten, starch and lupine flours were determined by methods AACC 30-25.01 (1999), AACC 08-01 (1983) and AACC 44-15.02 (1999), respectively. The protein content was determined with the Dumas combustion method (FlashEA 1112 series, Thermo Scientific, Breda, The Netherlands). A nitrogen conversion factor of N × 6.25 was used for calculating the protein content (Wang, et al., 2016).

3.2.6.2. Scanning electron microscopy

Scanning electron microscopy (Phenom G2 Pure, Phenom World BV, the Netherlands) was used to visualize the morphology of the wheat starch, gluten and lupine flour particles. All the powder samples were imaged without any pre-treatment. Carbon tabs (SPI Supplies/Structure Probe Inc., West Chester, USA) were used to fix the samples on 12.7 mm aluminium pin mounts (JEOL Europe BV, the Netherlands). The acceleration voltage was set at 5000 V.

3.2.6.3. Particle size distribution

The particle size distribution of wheat gluten, starch and lupine flour was analysed with a Mastersizer-3000 (Malvern Instrument Ltd., Worcestershire, UK) equipped with a module for dry powder dispersion (Aero S, UK). A dispersion pressure of 2 bar was applied and the median for a volume distribution Dv(50) was calculated according to the Fraunhofer light scattering theory.

3.2.7 Statistical analysis

Data were analysed by analysis of variance (One-way ANOVA) using SPSS statistics Version 22.0 (IBM, Armonk, NY). Variances within a group were analysed using least-significant difference multiple comparison analysis (LSD Duncan). Differences at a level of 95% ($P < 0.05$) were considered significant. Average values ± standard deviations are reported for duplicate experiments.

3.3. Results and discussion

3.3.1 Off-line analysis of tribo-charging

In this study, wheat gluten and starch were used as model powders to examine the effect of tube wall material on tribo-charging. Half a gram of pure gluten, pure starch or their 1:1 mixture were loaded and shaken in charging tubes made from stainless steel, aluminium, Nylon, and PTFE, respectively (Table 3-2. No. 1, 4, 5, 6). The tribo-charging in the different tubes was evaluated by analysis of the charge to mass ratio for gluten, starch and the model mixture using a Faraday cup (Figure 3-4).

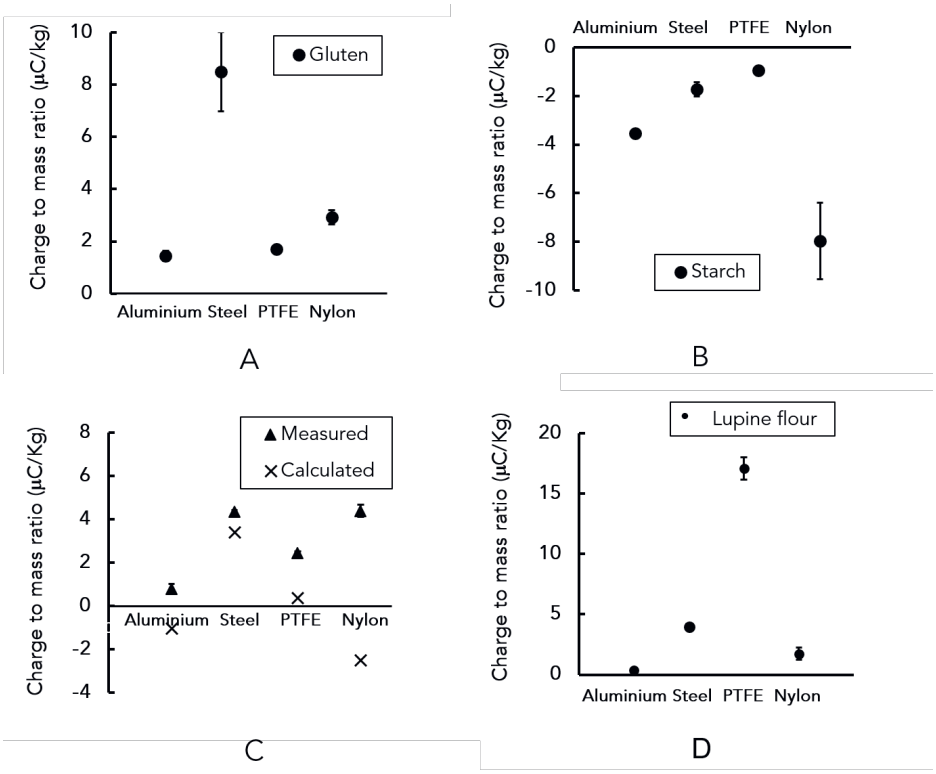

Figure 3-4. Tribo-charging measurements of wheat gluten (A), wheat starch (B) gluten and starch 1:1 model mixture (C) and lupine flour (D) after contact with aluminium (Tube No. 4), stainless steel (Tube No. 1), PTFE (Tube No. 6) and Nylon

(Tube No. 5), respectively. In figure 3-4 C, the calculated charge values of the mixture are calculated on the basis of the charges of the individual components multiplied with their mass fraction, from figures 3-4 A and 3-4 B. Results are expressed as charge to mass ratio (µC/kg). The error bars represent standard deviations.

The results showed that gluten charged positively, and starch charged negatively with all charging materials (Figure 3-4 A and B). The observed charge polarity was expected and has been related earlier to the surface properties of both components (Tabtabaei, et al., 2016b). The measured net charge of the model mixture (Figure 3-4 C) was positive and close to the measured charge of the pure gluten, suggesting that gluten dominates the charging of the mixture despite the 1:1 ratio and the larger particle size of gluten. It can be observed from figure 3-5 A that gluten particles had an irregular shape and rough surface, while starch particles were oval and smooth. The particle size distribution curves (Figure 3-5 C) showed that starch particles were smaller than the gluten particles (65 µm), having an average size of 15 µm.

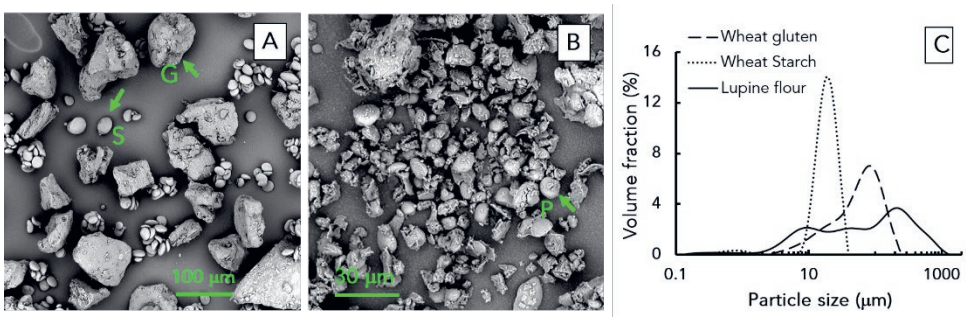

Figure 3-5. Scanning electron microscopy picture of gluten-starch mixture (A) and lupine flour (B). "S", "G" and "P" indicated by arrows represent starch granular, gluten particle and protein body, respectively. The particle size distribution curves of pure gluten, starch and lupine flour are plotted together (C).

By comparing the measured and the calculated charge from the measurements with the pure components, it could be concluded that the charge of the mixture was not simply the sum of the charge of the two different particles (Figure 3-4 C). This finding is in agreement with Wang, et al. (2015) who conducted on-line charging measurements of 1:1 gluten-starch mixture in an aluminium tube. Their measured value was positive, while the calculated value was negative.

The different tube materials are expected to display different chargeability towards the same plant raw materials. However, surprisingly gluten obtained the largest positive charge after contact with stainless steel compared to the other materials (Figure 3-4 A). This was not expected nor in line with the triboelectric series, as materials should exchange more charge and thus become more strongly charged when the distance between two materials in the triboelectric series is larger. This observation demonstrates the limited value of the empirical triboelectric series, as besides the surface properties also the measurement conditions very much determine the charging (Chen, et al., 2014). Specifically, different collision behaviour may explain deviating charging behaviour between off-line measurements (during which particles move due to horizontal vibration) and the on-line measurements (where particles are conveyed by a gas).

Starch obtained its largest negative charge after contact with Nylon compared to other materials (Figure 3-4 B). It may be expected that conductors are more efficient in tribo-charging than insulators, due to their free moving electrons (Wu, Li, & Xu, 2013); with insulators one would expect charge build-up, which influences the charging behaviour (Mirkowska et al., 2016). Both conductor and insulator materials have been applied successfully in electrostatic separation (Tabtabaei, et al., 2016a; Xing, et al., 2018). However, in our study it was observed that Nylon displayed better charging compared to aluminium when contacted with starch or gluten (Figure 3-4 A and B).

Different charging results were obtained for lupine flour (Figure 3-4 D), which may be expected as a result of the different composition of lupin flour, being a mixture of finely milled fibres and protein body fragments (Wang, et al., 2016). The charge of lupine flour obtained after contact with the different tubes was positive. The observation that the net charge of lupine flour with PTFE is higher than with Nylon and with copper is in line with that of Tabtabaei, et al. (2016b) for navy bean flour. However, because the charge of lupine flour is the sum of positively and negatively charged particles, it is impossible to draw conclusions on the chargeability of individual components and thus predict their separation performance.

Overall, the results indicated that it was not possible to directly relate the tribo-charging behaviour of the studied materials to earlier reported triboelectric series. Moreover, one cannot directly predict the overall charge of mixtures of particles from the charge that the individual particles obtained when charged in isolation. Particle-particle interactions in mixtures have large impact on the charging process. This conclusion is also in line with the study of Landauer, et al. (2019) who observed that particle-particle collisions were crucial in separating whey protein-barley starch mixtures. As particle-particle interactions between the two materials to be separated is crucial for subsequent separation in the electric field, these interactions should be optimised in an electrostatic separation device. This may be achieved by increasing residence time, solids concentration or even redesign of the charging part.

3.3.2 Separation performance of varying charging tubes

3.3.2.1 Compositional analysis

The separation performance of four different charging tubes (Table 3-2 No. 1, 4, 5, 6) was first evaluated with the model mixture. The N_2 gas flow rate was 50 L/min, the voltage was 20,000 V and the feeding rate was 0.5 kg/h. The protein content of the starting material was 37.8 g/100 g flour and it can be observed from figure 3-6 that protein enrichment was achieved with all the charging materials, whereas the

protein content of the starch enriched fraction was significantly lower ($P < 0.05$). Significantly higher protein enrichment was observed for aluminium and Nylon tubes ($P < 0.05$), while the fractions obtained from the filter bags of all tubes showed similar compositions. Since the compositions of the latter fractions were not so far from the starting material, in practice they could be recycled. In another study using a mixture of whey protein and barley starch no difference between different tube wall materials (all insulators) were observed on electrostatic separation (Landauer, et al., 2019). The high separation efficiency for Nylon (and also aluminium) can be derived from the increased protein content of the GE fraction (65 g/100 g flour and 60 g/100 g flour for Nylon and aluminium, respectively), which was close to the protein content of gluten. The enrichment decreased from Nylon, aluminium, PTFE to stainless steel. These results are probably related to the measured charge of starch particles (Figure 3-4 B), which was highest for both Nylon and aluminium, but not to that of the gluten (Figure 3-4 A), which obtained a very high charge after contact with steel. However, it seems impossible to select the best charging tube on basis of the tribo-charging measurements only.

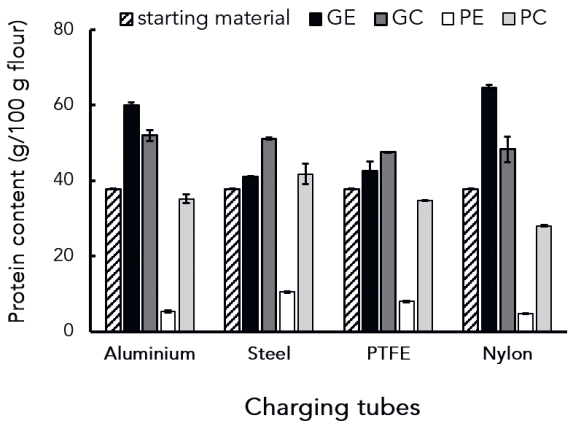

Figure 3-6. Electrostatic separation of gluten and starch mixture (1:1): protein content (g/100 g flour) of starting material and four fractions collected from ground

electrode (GE), positive electrode (PE), ground collector (GC) and positive collector (PC). The error bars represent standard deviations.

The yield of the protein-rich fraction is another important parameter to evaluate the separation performance. The yields of the four fractions are shown in Figure 3-7. The yields of the protein-rich fractions (GE) of the four tube materials were similar ($P > 0.05$), also indicating a limited influence of the charging tube material on the yield. Some very fine material was not captured by either electrodes or filter bags but was dispersed in the relatively large separation chamber. This amount of lost material was relatively large, which was also partly related to the limited sample size used in this study.

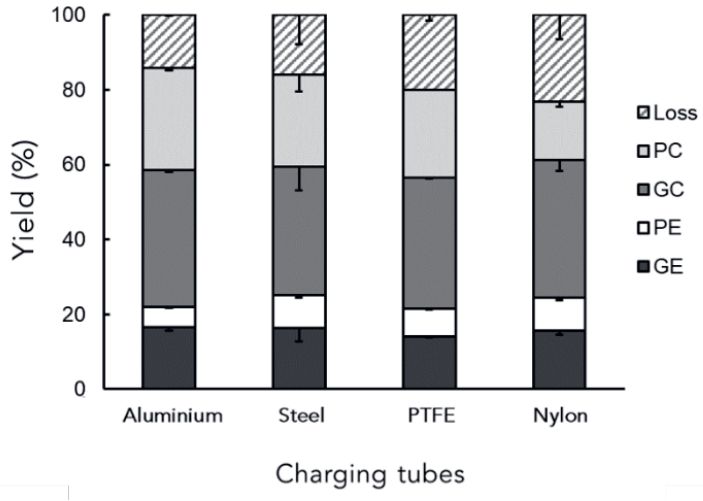

Figure 3-7. Electrostatic separation of model mixture with different charging tubes (8 mm of diameter): yield (g/100 g flour) of four fractions collected from ground electrode (GE), positive electrode (PE), ground collector (GC) and positive collector (PC). Weight of loss was calculated by difference. The error bars represent standard deviations, only minus direction is shown.

3.3.2.2 Lupine protein enrichment

To further investigate the effect of the charging process on the separation performance of plant raw-materials, tribo-electrostatic separation experiments were carried out with lupine flour using charging tubes of different materials and diameters (Table 3-2). The operating conditions were the same as that for separating model material: N_2 gas flow rate was 50 L/min, the voltage was 20,000 V and the feeding rate was 0.5 kg/h. The protein content of lupine flour and those of the different collected fractions are shown in Figure 3-8. The protein content of the GE fractions significantly increased compared to the starting material for all tube materials ($P < 0.05$). However, the PE fractions were not all significantly depleted in protein compared to the starting material ($P > 0.05$), indicating that the overall protein separation, relative to that obtained with the gluten-starch mixture, was much less. The earlier noted observation that the material on the PE electrode was only slightly depleted in protein suggests that the particles on the PE electrode were mostly composite particles containing both fibre and protein.

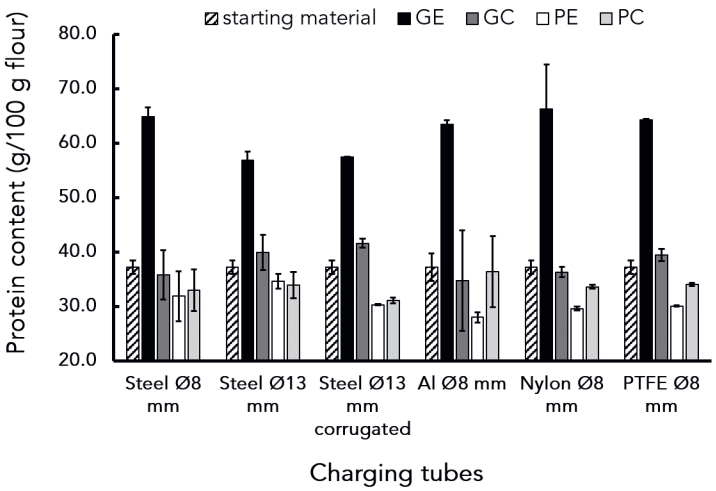

Figure 3-8. Electrostatic separation of lupine flour with different charging tubes: protein content (g/100 g flour) of starting material and four fractions collected from

ground electrode (GE), positive electrode (PE), ground collector (GC) and positive collector (PC). The error bars represent standard deviations.

Despite that tubes were made of different materials, for all tubes with a diameter of 8 mm, the purity of the GE fraction was always in the range of 63~66 g/100 g and no significant differences ($P > 0.05$) were observed. This implies that the choice of tube material did not influence the electrostatic separation of lupine flour. This observation agrees with the conclusions from Landauer, et al. (2019) who reported that the tube wall material had no influence on the tribo-electrostatic separation performance of small particles. This may indicate that inter-particle collisions are more important for the charging than the particle-wall collisions.

In contrast, the diameter of the tube showed significant influence on the separation performance using similar gas flow rates (50 L/min) ($P < 0.05$). The GE fraction obtained with the stainless-steel tube with a diameter of 8 mm showed significantly higher protein purity than that separated with same material but with a diameter of 13 mm ($P < 0.05$). The gas velocities were 4.15 m/s and 1.57 m/s, for the 8- and 13-mm tubes, respectively. Increasing the gas velocity leads to intensified collisions, more charge transfer and thus better separation. A tube with a corrugated inner surface was constructed to enhance convection close to the wall and thus charging. However, after an initial improved separation performance, the improvement quickly diminished, probably due to fouling of the corrugated surface (Figure 3-8).

For each separation experiment a feed sample of 50 g of lupine flour was used as starting material. The yields of four fractions were summarized and the weight of lost flour after each separation was calculated as the difference with the original amount. As shown in figure 3-9, there was no significant difference between tube materials observed in terms of yield for the GE (8.8~12.2%) ($P > 0.05$). The yields of the fractions collected from the positive electrode and its filter bag (PE and PC) were higher than those from the negative electrode and its filter bag (GE and GC). The explanation for this is that the lupine flour has more fibres than protein, and protein

bodies are only liberated to a certain degree from the fibrous matrix. Earlier research showed that collecting the fractions from the filter bag, subsequent milling and a second step of electrostatic separation can improve the purity and yield of the process (Wang, et al., 2016). By calculating the mass balance, the protein content of the loss was in the range of 31.0~43.9%. It is possible to achieve higher protein recovery from the loss after scaling up.

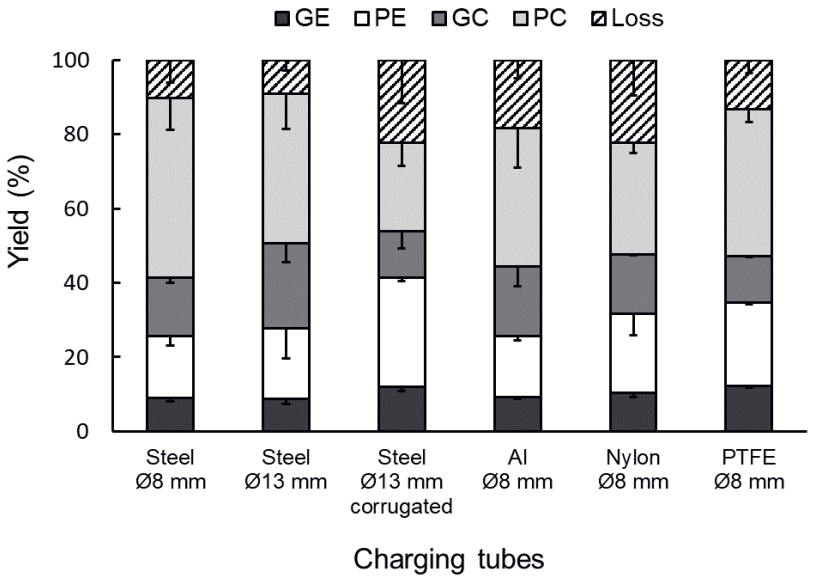

Figure 3-9. Electrostatic separation of lupine flour with different charging tubes: yield (g/100 g flour) of four fractions collected from ground electrode (GE), positive electrode (PE), ground collector (GC) and positive collector (PC). Weight of loss was calculated by difference. The error bars represent standard deviations, only minus direction is shown.

The observation that the charging material does not have a systematic influence on the tribo-charging behaviour, combined with the observation that an increase in flow rate significantly increases the charging efficiency ($P < 0.05$), leads to formulate the hypothesis that particle-particle collisions largely determine the charging. This

would ultimately mean, that the charging system should be optimized to maximize particle-particle collisions by introducing as much mixing as possible.

3.4. Conclusion

Charging tubes made from stainless steel, aluminium, PTFE, and Nylon were used to charge pure gluten, wheat starch, their mixtures and lupine flour. Wheat starch obtained a negative charge whereas gluten particles obtained a positive charge. Even the measured charge of the pure components could not be related to the triboelectric series. The charge of a gluten-starch mixture was also not simply the sum of the charge of the individual components, suggesting that particle-particle interactions have considerable influence on the charge of the mixture.

Electrostatic separation was then carried out using gluten-starch mixtures and lupine flour using again different tube wall materials. The protein enrichment for the model mixture appeared influenced by the wall material and seemed related to the measured starch charge. For lupine flour, the purity of the protein enriched fraction increased from 37 g/100 g flour to 65 g/100 g flour. Interestingly, the separation performance of the lupin flour was not related to the used tube material. Experiments with different tube diameters showed however a large influence of hydrodynamic conditions on the separation.

To conclude, particle-particle collisions are mostly responsible for the charging of mixtures. This conclusion explains why charging experiments with pure components do not predict the separation behaviour during electrostatic separation, but also implies that redesigning the charging system to maximize particle-particle collisions, for example employing a fluidized bed rather than a charging tube, could lead to significantly better charging and thus separation.

Acknowledgements

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SEM
Protein
Surface
Purity
Function
Dry
Dry
Air
Loss
Lentil
SEM
Ash
Classification
Native
Chickpea
Nature
Ingredient
Surface
Fractionation
Negative
Oil
Yield
Separation
Protein
Coarse
Yield
Fraction
Property
Novel
Loss
Healthy
Future
Coarse
Earth
Starch
Density
Fouling
Milling
Extraction
Pea
Oil
Fine
Size
Charge
Improve
Legume
Ash
Morphology
Science
Green
Pea
Air
Air
Use
Ash
Product
Oil
Electrostatic
Milling
Fractionation
SEM
Pea
Energy
Size
Pulse
Dry
Protein
Stream
Lentil
Science
Fraction
Fine
Use
Fine
Positive
Native
Electrostatic
Lentil
Creative
Novel
Particles
Nature
Environment

Chapter 4

A two-step air classification and electrostatic separation process for protein enrichment of starch-containing legumes

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Abstract

A two-step dry fractionation process was investigated that further enriches protein from starch-containing legumes. Legumes (pea, lentil, and chickpea) were subjected to milling, air classification, and subsequent triboelectrostatic separation. The air classification first removes starch, whereas the subsequent electrostatic separation removes fibre from the resulting protein concentrate. Successful enrichment was achieved with pea and lentil, but this was not the case for chickpea due to the smaller starch granules and higher fat content. The best conditions for pea were air classification at an air-classifier wheel speed of 8000 rpm. Subsequently, electrostatic separation was optimized with two passes. With this, a protein purity was obtained of 63.4 g/100 g dry basis and a yield of 15.8 g/100 g dry solids. For the overall two-step dry fractionation process, a protein-enriched fraction with a yield of 4.0 g/100 g pea could be obtained, leading to 7.8% of total protein recovered from yellow pea.

4.1. Introduction

Starch-containing grain legumes such as pea, chickpea, and lentils are a major source of dietary protein for over one billion consumers worldwide (Khazaei, Subedi, Nickerson, Martínez-Villaluenga, Frias, & Vandenberg, 2019). These legumes live in symbiosis with nitrogen binding bacteria in their root nodules, which reduces the need for artificial fertilizers compared to other plant protein sources. Besides, they can grow in temperate climate zones, and therefore in proximity to many of the world's population centres. Therefore, legumes have an advantage in meeting the growing demand for sustainable dietary plant protein (Schutyser, et al., 2011). Proteins from legumes have been extracted as an ingredient (e.g. concentrate or isolate) and are applied in numerous food applications, where functional behaviour such as foaming, gelling and emulsifying properties is critical (Stone, Nosworthy, Chiremba, House, & Nickerson). Traditionally, legume proteins are often extracted via wet extraction methods that involve energy consuming steps such as drying and lead to the loss of native functional properties due to the use of solvents or alkaline conditions during the extraction and the thermal load due to drying (Assatory, et al., 2019).

Dry fractionation by dry milling and dry separation is a more resource-efficient alternative to wet extraction, while the native functional properties of the proteins are better retained (Mayer-Laigle, et al., 2018). During milling, starch granules are disclosed as larger particles; the proteins and fibres are primarily present as smaller fragments. Subsequently, dry separation can be carried out via air classification, using the size or density difference as separation principle, or via electrostatic separation, which uses the different triboelectric charging properties of the materials (Schutyser, et al., 2015). Air classification was successfully applied to separate larger starch granules from smaller protein particles to produce starch and protein-enriched fractions from pea, navy bean, faba bean and lentil (Boye, Zare, & Pletch, 2010; Wang, de Wit, et al., 2015; Wang, et al., 2016; Xing, et al., 2018).

Direct electrostatic separation of starch-containing legume flours was shown to be infeasible (Pelgrom, Boom, & Schutyser, 2015), despite the observation that artificial mixtures of wheat gluten and starch could be separated with this method (Wang, de Wit, et al., 2015). However, further protein enrichment could be obtained by subjecting the protein-rich fine fraction obtained by air classification, to subsequent electrostatic separation (Pelgrom, Boom, et al., 2015). During triboelectric charging, the protein and fibre fragments obtain an opposite charge and thus can be separated in an electrostatic field. However, starch obtains a similar polarity as the protein and thus is attracted to the same electrode as the protein, which impairs their separation. This suggests a two-step approach by combining air classification and electrostatic separation to obtain pea protein concentrates with higher purity. This approach was only demonstrated using a lab-scale electrostatic separator in which yields could not be reported (Pelgrom, Boom, et al., 2015).

The aim of the current study is to further develop the two-step dry separation approach using yellow pea, lentil, and chickpea for protein enrichment. The protein content of these legumes have been reported 21.9 ± 1.5, 20.6 ± 0.4, and 18.5 ± 1.7 g/100 g and the starch content 48.0 ± 1.4, 46.5 ± 0.5, and 44.6 ± 1.7 g/100 g, respectively (Chung, Liu, Hoover, Warkentin, & Vandenberg, 2008; de Almeida Costa, da Silva Queiroz-Monici, Reis, & de Oliveira, 2006). Fine fractions (protein-rich) produced by air classification are further purified with a custom-built bench-scale electrostatic separator and evaluated on purity and yield. Pea was selected to optimize the process parameters for obtaining fractions with the highest purities and yields.

4.2. Materials and methods

4.2.1 Materials

Dry yellow pea (Pisum sativum), lentil (Lens culinaris) and Kabuli chickpea (Cicer arietinum) seeds were purchased from a local market (Alimex, Sint Kruis, The Netherlands). All seeds were stored until use at 4°C in tightly sealed polyethylene containers.

4.2.2 Milling

Legume seeds were pre-milled into grits with a pin mill (LV 15M Condux-Werk, Wolfgang bei Hanau, Germany). Subsequently, the coarse grits were further milled into flour with a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) at ambient temperature. The unrecovered material after this milling step is ~12 g/100 g flour. The classifier wheel speeds during milling for pea, lentil, and chickpea are 4000, 2200, and 2900 rpm respectively, based on our previous study (Pelgrom, Boom, et al., 2015). An airflow rate 40 m^3/h, an impact milling speed of 8000 rpm, and a feed rate of 0.5 kg/h was used for all pulses grits during impact milling.

4.2.3 Air classification

Protein-rich fine fractions of the three pulses were produced using an ATP50 air-classifier (Hosokawa-Alpine, Augsburg, Germany) at ambient temperature. Based on previous experience (Pelgrom, Boom, et al., 2015), the classifier wheel speed was set at 10000 rpm. The airflow was kept constant at 52 m^3/h and the feed rate was ~0.5 kg/h.

For a next series of experiments, pea fine fractions having different starch content were prepared. The classifier wheel speeds for these experiments were 6000, 8000 and 10,000 rpm. The airflow was set at 52 m^3/h and the feed rate was ~0.5 kg/h.

4.2.4 Electrostatic separation

A custom-built bench electrostatic separator was used for protein enrichment. This equipment was extensively described in a previous report (Wang, de Wit, et al., 2015). In the current study, a charging slit made of aluminium and a straight tube made of stainless steel with an internal diameter of 8 mm were used. The height of the charging slit was 21.8 cm, and the cross-section length and width were 4.1 cm and 0.24 cm, respectively (Xing, et al., 2018). The height of the straight tube was 29.6 cm and the inner diameter was 0.8 cm. For each single step electrostatic separation experiment, 25 g raw material was used. The N_2 flow rate was fixed at 50 L/min, the distance between electrodes was 10 cm, the voltage set on the positive electrode was 20 kV and the dosing rates were 0.5 and 1.25 kg/h. After each separation, four fractions labelled as "GE", "PE", "GC" and "PC" were collected from the grounded electrode (protein-enriched), positive electrode (fibre-enriched), ground collector bag and positive collector bag, respectively.

During the two-step electrostatic separation experiments, 300 g raw material was used in the first step. An overview of the two-step electrostatic separation process is shown in Figure 4-1. The protein-enriched fraction (GE1) and the mixture of the fractions obtained from the two collector bags (GC1 + PC1) were used as feed for the second separation step. The former strategy aims to further increase the protein content in fraction GE2E and the latter strategy was used to recover the additional protein from the fractions in the collecting bags (GC1 + PC1) into fraction GE2C.

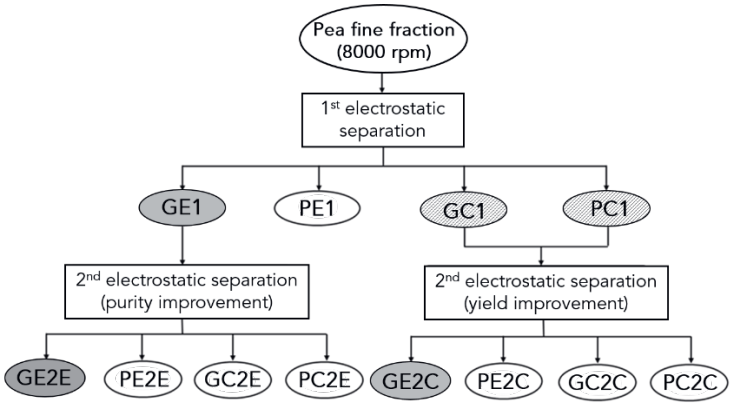


Figure 4-1. The diagram of the two-step electrostatic separation in this study. GE1, PE1, GC1, and PC1 represent fractions collected from the grounded electrode, positive electrode, grounded collector, and positive collector, respectively, after the 1st separation. GE2E and GE2C represent protein-enriched fractions obtained from GE1 and the mixture of GC1 and PC1, respectively.

4.2.5 Analyses

4.2.5.1. Compositional analysis

The protein content of pea, lentil, and chickpea flours and fractions was determined by the Dumas method with a Nitrogen Analyser FlashEA 1112 series (Thermo Scientific, Breda, The Netherlands). To calculate the protein content, a nitrogen conversion factor of N × 6.25 was used. The moisture, oil, and ash contents were determined by methods AACC 44-15.02 (1999), AACC 30-25.01 (1999), and AACC 08-01 (1983), respectively. The starch content was analysed with a Total Starch Assay Kit (Megazyme, Ireland). The content of fibre was approximated by the difference.

4.2.5.2. Protein enrichment

The protein enrichment is defined as the increase in protein purity of the target fraction to the protein purity relative to that of the starting material.

$$\text{Protein enrichment} = \frac{\text{Protein purity}_{\text{target fraction}}\text{-Protein purity}_{\text{starting material}}}{\text{Protein purity}_{\text{starting material}}} \times 100\% \quad (\text{Eq } 4-1)$$

The yield is defined as the mass of the target fraction divided by 100 g of the starting material.

$$\text{Yield} = \frac{\text{Mass}_{\text{target fraction}}(g)}{100\ (g)} \times 100\ (\%) \quad (\text{Eq } 4-2)$$

The protein recovery is defined as the ratio of the protein mass present in the target fraction to the protein mass present in the starting material.

$$Protein\ recovery = \frac{Protein\ mass_{target\ fraction}}{Protein\ mass_{starting\ material}} \times 100\% \quad (\text{Eq } 4-3)$$

4.2.5.3. Scanning electron microscopy

The particles of pea, lentil, and chickpea flours and fractions were visualized using scanning electron microscopy (Phenom G2 Pure, Phenom World BV, the Netherlands). Powder samples without any pre-treatment were sprinkled on 12.7 mm aluminium pin mounts (JEOL Europe BV, the Netherlands) with carbon tabs (SPI Supplies/Structure Probe Inc., West Chester, USA) and placed into the microscope chamber for observation. The acceleration voltage was set at 5 kV.

4.2.5.4. Particle size distributions

The particle size distributions (PSDs) of pea, lentil and chickpea flours and fractions were analysed with a Mastersizer-3000 (Malvern Instrument Ltd., Worcestershire, UK) equipped with a module for dry powder dispersion (Aero S, UK). A dispersion

pressure of 2 bar was applied and the volume-weighted particle size distribution was estimated using the Fraunhofer theory.

4.2.5.5. Statistical analysis

All measurements were carried out in duplicate unless indicated differently. Data were analysed by one-way ANOVA using SPSS statistics Version 25.0 (IBM, Armonk, NY). A p-value < 0.05 meant the difference between data was statistically significant. The results are expressed as average values ± standard deviations.

4.3 Results and discussion

4.3.1 Air classification of starch-containing legumes

After air classification of the three legume flours at 10,000 rpm, the flour and their corresponding fine and coarse fractions were compared on their composition (Figure 4-2). The protein content of pea, lentil, and chickpea fine fractions increased 107%, 129%, and 58% compared to the original flours, respectively. These results are consistent with those from previous research (Pelgrom, Boom, et al., 2015). It is noted however that the initial protein content and thus also the protein content after enrichment for these legumes will vary with season and in general with environmental cultivation conditions (Lascano, Schmidt, & Barahona Rosales, 2001). Starch was depleted in the protein-enriched fraction. Especially in pea and lentil fine fractions, residual starch was only 1.5 and 2.3 g/100 g dry solids, respectively, while the chickpea fine fraction had a starch content of 23.8 g/100 g dry solids (Figure 4-2). The reason for the inefficient separation of starch from chickpea is probably the smaller starch granule size compared to those of pea and lentil. This leads to incomplete separation as the starch granule size is close to the cut point for separating the protein-rich particles (Pelgrom, Wang, et al., 2015). Additionally, the higher oil content of chickpea (6 g/100 g flour compared to 1 g/100 g flour in pea or lentil on dry basis) contributes probably to a higher tendency to agglomeration which negatively affects the separation (Sosulski & Youngs, 1979).

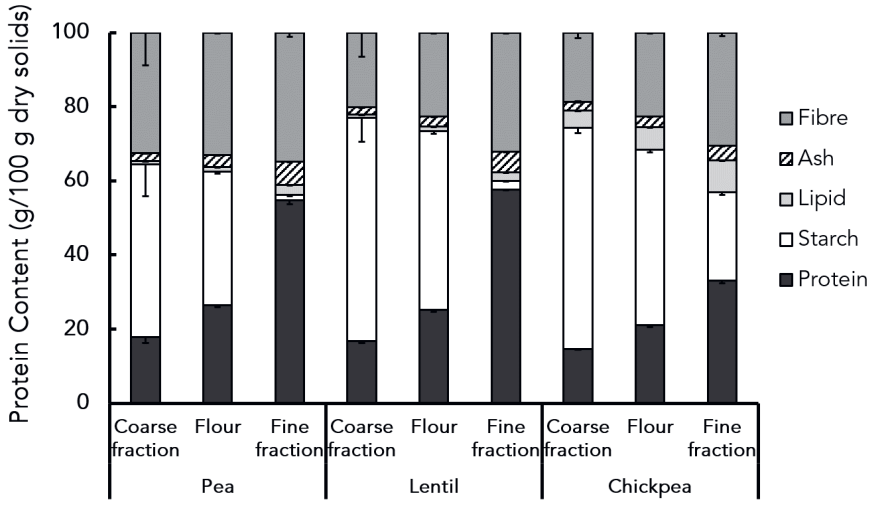


Figure 4-2. Compositions of flour, coarse and fine fraction of pea, lentil, and chickpea. The amount of fibre was calculated by difference. The error bars indicate standard deviation, only minus direction is shown.

The SEM pictures show pea, lentil, and chickpea flours and fractions obtained after milling and air classification. As the cotyledons are ground into powders, starch granules are released from the cellular matrix, which also contains protein-rich particles and fibres. Pea, lentil, and chickpea starch granules can be recognized as smooth spherical or oval particles. The fragments of different sizes and irregular shapes are most probably protein and fibre particles. In pea and lentil fine fractions (Figure 4-3 B and E), starch granules are hardly seen, while for chickpea, starch granules can be observed in the fine fraction (Figure 4-3 H), indicating poorer separation. The size of the starch granules decreases in the order from pea (25 ± 6 μm) > lentil (23 ± 5 μm) > chickpea (22 ± 4 μm), which is in line with another study, which reported sizes of 32 ± 14 μm, 25 ± 13 μm and 22 ± 12 μm, respectively (Chung, et al., 2008).

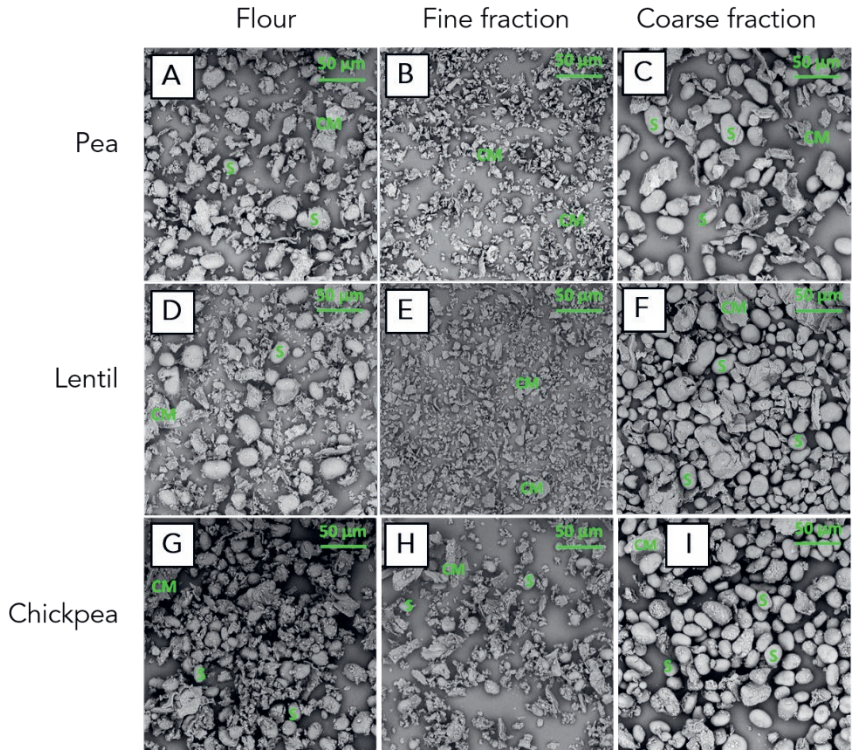


Figure 4-3. Scanning electron microscopic pictures of pea, lentil, chickpea flour, and their air classified fine and coarse fractions, respectively. From A to C: pea flour, pea fine fraction, and pea coarse fraction. From D to F: lentil flour, lentil fine fraction, and lentil coarse fraction. From G to I: chickpea flour, chickpea fine fraction, and chickpea coarse fraction. S: starch granules. CM: cellular material.

The particle size distributions of the chickpea fine and coarse fractions overlap to a larger extent than those of pea and lentil (Figure 4-4). This confirms the more diffuse separation for the finely milled chickpea flour during air classification (Pelgrom, Boom, et al., 2015). To further increase the protein purity of the fine fractions, the fine fractions of the three legumes were subjected to subsequent electrostatic separation. Coarse fractions were not considered further, as the presence of larger amounts of starch content impairs effective separation (Pelgrom, Boom, et al., 2015).

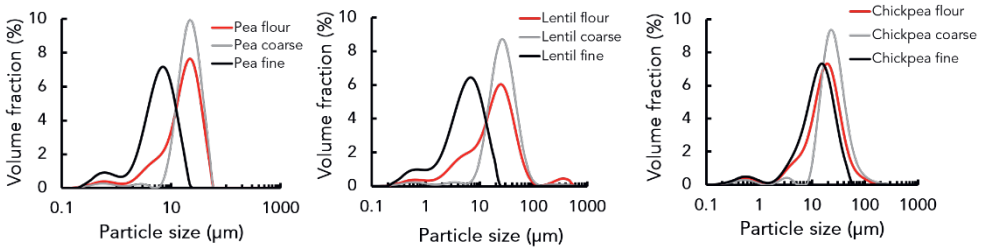


Figure 4-4. Particle size distribution curves of pea, lentil, and chickpea flour compared with those of the fine and coarse fractions.

4.3.2 Electrostatic separation of starch-containing legumes

During the first electrostatic separation experiments, a charging slit was used for separation (Xing, et al., 2018) and the dosing rate was set at 1.25 kg/h. The protein content after separation is shown in figure 4-5 A. For pea and lentil fine fractions, a slight protein enrichment (6% on dry basis) was observed for the GE fractions. This is consistent with our previous research using a laboratory-scale electrostatic separation which showed ~8% protein enrichment (Pelgrom, Boom, et al., 2015). The fractions of pea and lentil collected on the positive electrode (PE) were depleted in protein and thus enriched in fibre as the starch had already been removed during air classification. No protein enrichment was achieved for the chickpea fine fraction, which was expected given the presence of larger amounts of starch granules. During previous research, it was already suggested that starch granules obtain similar charges as the protein-rich particles, which impairs their separation (Pelgrom, Boom, et al., 2015). This explains the better separation for pea and lentil, which is thanks to the effective removal of the starch granules during the air classification step. It was found that the protein content of the ground collector (GC) and the positive collector (PC) are close to that of the starting material. These fractions may be recombined and subjected to a second separation pass for enlarging the overall protein yield.

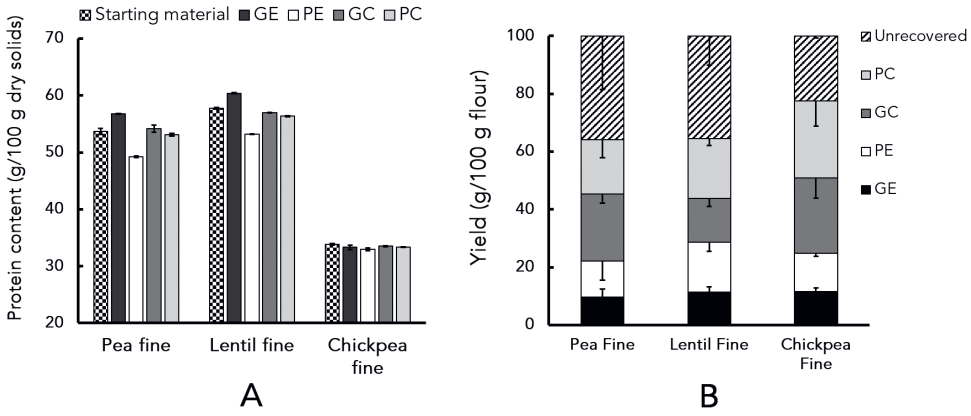


Figure 4-5. One-step electrostatic separations of pea, lentil, and chickpea fine fractions (obtained at an air-classifier wheel speed of 10,000 rpm) with a charging slit. A: protein content of the starting material and four fractions collected from the grounded electrode (GE), ground collector (GC), positive electrode (PE), and positive collector (PC), respectively. B: the yield of four fractions and the weight of unrecovered material which was calculated by difference. The error bars indicate standard deviation, only minus direction is shown.

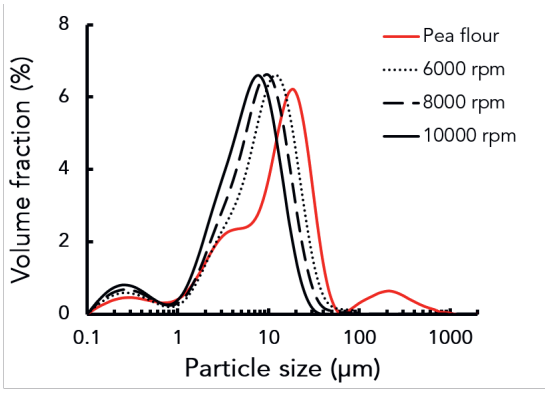
The yields of the protein-enriched fractions (GE) for the three legumes were similar ($P > 0.05$) (Figure 4-5 B). We expected that the yield of chickpea might be lower due to the lower protein content of the fine fraction, but this was not found. The similar yield for chickpea can be also explained by the presence of higher amounts of starch in the chickpea GE fraction. Starch attracted on the grounded electrode resulting in lower protein purity but similar mass yield. A significant amount of powder was not recovered. This is due to the experimental system (fouling inside the equipment) and will need to be reduced by improving the design of the equipment.

The yield of the fractions from the collector bags exceeded that of the electrodes. In the next section, we present results on increasing the recovery of pea protein by

recycling the fractions in the collector bags. Pea was selected to further optimize the dry fractionation process because pea protein is increasingly being used in for example meat substitutes (Rempel, Geng, & Zhang, 2019). Moreover, we have ample prior experience with milling and air classification of yellow pea (Pelgrom, Vissers, et al., 2013; Pelgrom, Wang, et al., 2015). Thus, it is a good start to investigate the effect of air classification on subsequent electrostatic separation.

4.3.3 Dry fractionation of pea protein

Pea fine fractions were prepared by air classification using three different air-classifier wheel speeds (6000, 8000, and 10,000 rpm) providing fractions differing in composition and yield (Figure 4-6). Data of the coarse fractions are not shown. As the classifier wheel speed increased, the particle size shifted to smaller sizes for the fine fraction (Figure 4-6 A).



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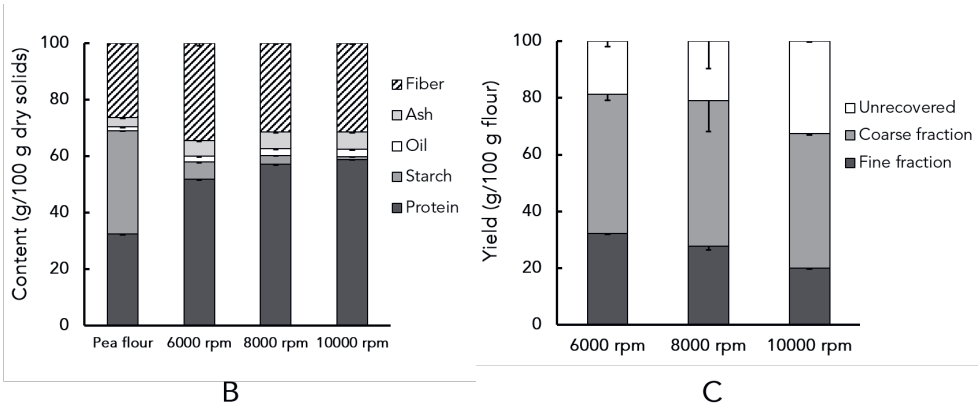

Figure 4-6. A: The particle size distribution curves of pea flour and pea fine fractions obtained at different air-classifier wheel speeds. B: The compositions of pea flour and fine fractions as a function of the air-classifier wheel speed. Fibre content was calculated by difference. C: The yield of pea fine, coarse fractions, and the mass of unrecovered material as a function of the air-classifier wheel speed. The error bars indicate standard deviation, only minus direction is shown.

The compositions of the pea fine fractions are shown in Figure 4-6 B. With increasing classifier wheel speed, the protein content of the fine fractions increased from 51.8 to 58.8 g/100 g dry solids (with 32.4 g/100 g dry solids in pea flour). The fine fractions were depleted of starch, with a negative correlation to the protein content. The fine fractions were also richer in fibre, ash, and fat. This is related to the high purity of the starch, while the protein is more integrated with the other components in the cotyledon tissue structure (Sridharan, Meinders, Bitter, & Nikiforidis, 2020). Although using 10,000 rpm gave the highest protein content (Figure 4-6 B), this also resulted in a decreased yield (Figure 4-6 C) of the fine fraction (from 32.2 g/100 g flour at 6000 rpm to 20.0 g/100 g flour at 10000 rpm) due to the lower cut point, which is in line with a previous study on air classification of pea (do Carmo, et al., 2020). The yield went further down as the classifier wheel speed increased. This is

because small particles are more prone to remain unrecovered by adhering to the inner walls of the equipment. Classifier wheel speeds below 6000 rpm (data not shown) did not lead to protein and starch separation (only one fraction was obtained). With the air-classifier wheel speed at 8000 rpm, a protein recovery of 49.0% from pea flour could be obtained.

Pea fine fractions obtained from air classification at 6000, 8000, and 10,000 rpm were subjected to electrostatic separation with a straight charging tube (Figure 4-7 A). The dosing rate was kept constant at 0.5 kg/h. The separation showed protein enrichment for all the pea fine fractions in the GE fraction (Table 4-1). Pea protein enrichment (14.6%) at 10,000 rpm was larger compared to the electrostatic separation using the charging slit (6%) (Figure 4-5 A). The halved cross-section area of the straight tube (0.5 cm^2 compared to 1.0 cm^2 of the slit), which leads to larger gas velocity and thus increased charging may explain the improved separation performance.

The protein purity of the GE fraction increased for the fine fractions prepared with higher classifier wheel speeds between 6000 and 8000 rpm (Figure 4-7 A) but did not increase further when using a classifier wheel speed of 10,000 rpm. The initial increase in the separation efficiency may be due to the better removal of pea starch granules at higher air-classifier wheel speeds enabling better electrostatic separation. A further increase in air-classifier wheel speed (10,000 rpm) did not remove additional starch granules (Figure 4-6 B) and therefore also subsequent electrostatic separation did not improve further.

Pea fine fractions prepared with air classification at 8000 and 10,000 rpm and subsequently subjected to electrostatic separation yielded the highest protein content. The yield of protein-enriched fractions separated from different pea fine fractions was highest for 8000 rpm (13.1 g/100 g fine fraction), though differences are not very large (Figure 4-7 B). The presence of more residual starch granules affected the yield of the 6000 rpm fine fractions, while the small particle size

reduced the yield of the 10,000-rpm fraction (Wang, Smits, et al., 2015). In summary, air classification at 8000 rpm is preferable to prepare the feed for subsequent electrostatic separation (Table 4-1).

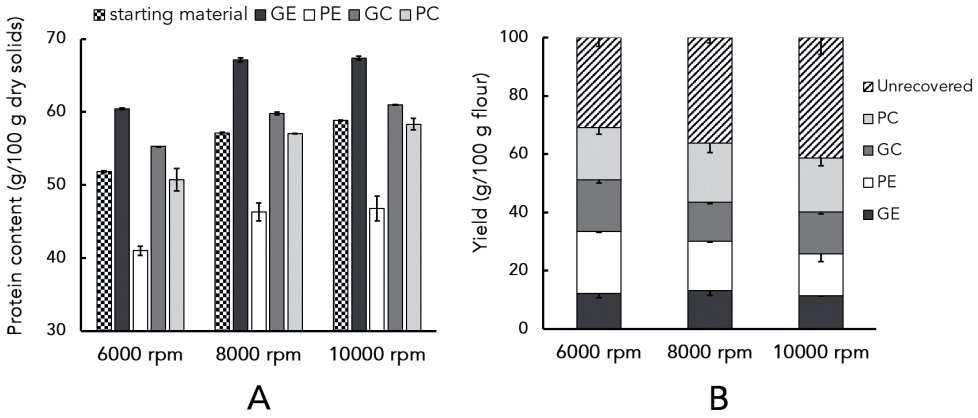

Figure 4-7. One-step electrostatic separation of pea fine fractions obtained at three different air-classifier wheel speeds with the charging tube. A: protein content of starting materials and four fractions collected from the grounded electrode (GE), ground collector (GC), positive electrode (PE), and positive collector (PC), respectively. B: the yield of four fractions and the mass of unrecovered material which was calculated by difference. The error bars indicate standard deviations. Only minus direction is shown in B.

Table 4-1. Summary on protein enrichment and protein recovery achieved by air classification and electrostatic separation (compared to pea flour) as function of classifier wheel speed. Data marked with a different lowercase superscript in the same column indicate significant differences ($P < 0.05$).

Classifier wheel speed (rpm)	Air classification		Electrostatic separation	
	Protein enrichment (%)	Protein recovery (%)	Protein enrichment (%)	Protein recovery (%)
6000	$59.9^{a} \pm 0.9$	$51.4^{b} \pm 0.0$	$86.5^{a} \pm 0.1$	$7.4^{b} \pm 0.8$
8000	$76.1^{b} \pm 1.0$	$49.0^{b} \pm 2.6$	$107.2^{b} \pm 0.2$	$7.5^{b} \pm 0.9$
10000	$81.5^{c} \pm 0.8$	$36.3^{a} \pm 0.8$	$107.9^{b} \pm 0.3$	$4.7^{a} \pm 0.0$

4.3.4 Optimization of protein enrichment by repeated electrostatic separation

4.3.4.1. Purity improvement

To increase the protein purity and yield, fractions collected during a first electrostatic separation were collected and subjected to a second electrostatic separation step. After a 1st electrostatic separation, a protein enrichment of 11.7% was achieved (Figure 4-8). Theoretically, a protein purity of maximally 76 g/100 g dry solids might be achieved, as this has been reported the protein concentration in proteosomes (also known as protein bodies), suggesting room for possible further protein enrichment (Pelgrom, Wang, et al., 2015). Therefore, as described in figure 4-1, a second electrostatic separation was carried out. Figure 4-8 showed that after a 2nd separation, the protein content of the protein-enriched fraction can be further increased from 62.2 to 67.6 g/100 g dry solids, i.e. a protein enrichment of 8.7%, achieving an overall enrichment of 21.4% starting from the fine fraction. However, this higher purity is at the expense of low yield (Figure 4-8). In the first separation, only 12.8% of the total amount of protein in the feed (fine fraction) was recovered in the GE fraction. This was reduced further in the second step.

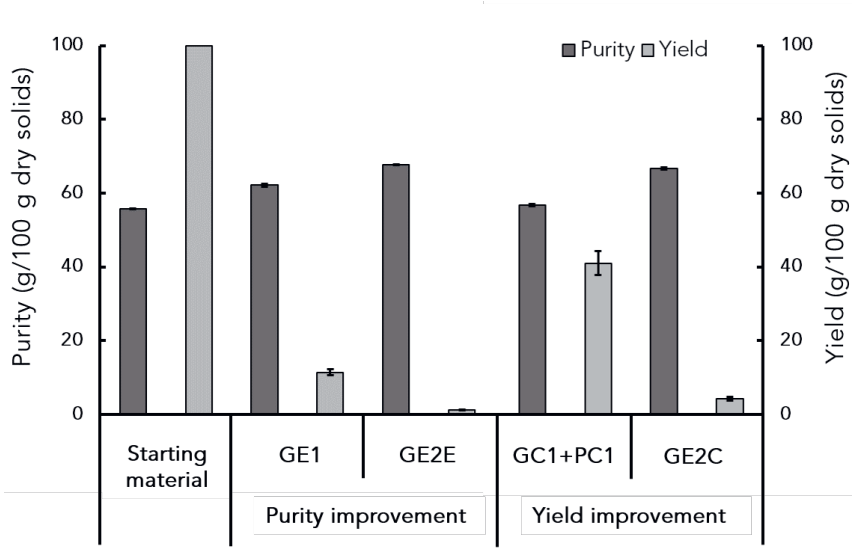

Figure 4-8. Protein content and yield of protein-enriched fractions after the 1st and 2nd electrostatic separation following the purity improvement and the yield improvement strategies, respectively. The starting material was pea fine fraction obtained from air classification at 8000 rpm and the charging tube was used. The error bars represent the standard deviations.

4.3.4.2. Yield improvement

The compositions of the GC and the PC fractions are approximately equal to the starting material (Figure 4-7 A). The fractions collected from the two collecting bags (GC1 + PC1) were therefore mixed and subjected to a 2nd electrostatic separation (Figure 4-1). As shown in Figure 4-8, the protein content of GC1 + PC1 (56.7 g/100 g dry solids) was similar to that of the starting material (55.6 g/100 g dry solids). The protein content of the protein-enriched fraction (GE2C) increased in the second separation up to 66.7 g/100 g dry solids. Interestingly, this protein content is similar to the GE2E fraction obtain in the previous experiment. Apparently, there are still easily separable protein-rich particles present in the collector fractions that were not caught by the grounded electrode during a single pass. This indicates that the electrostatic separator itself can still be improved. The fraction GE2C might be

added to the GE1 fraction to obtain a high purity protein concentrate with a higher yield. This is visualized in Figure 4-9.

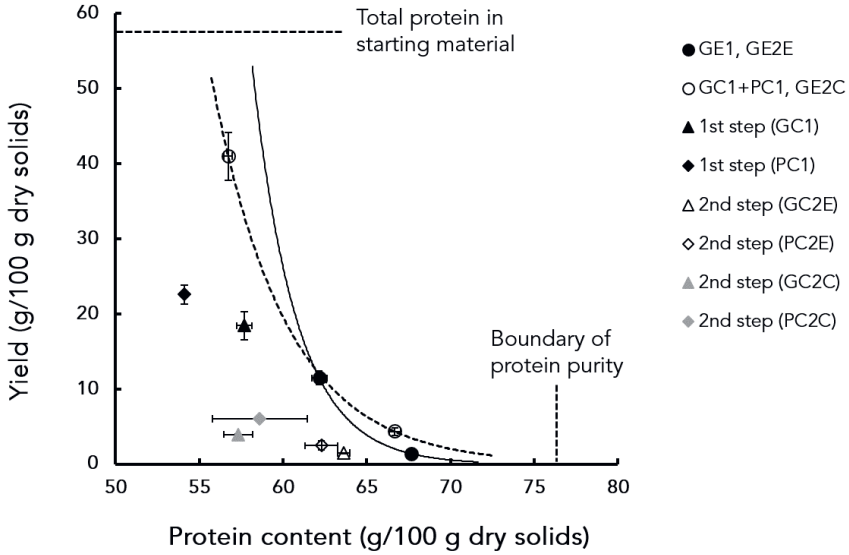

Figure 4-9. The relation between yield and protein purity of fractions by 1st and 2nd electrostatic separation. The protein-depleted fractions were not plotted. The error bars represent the standard deviations. The solid line is drawn to indicate the protein-enriched fractions from 1st (GE1) and 2nd (GE2E) electrostatic separation. The dotted line is drawn to indicate the protein-enriched fraction (GE2C) by recycling of collecting bags (GC1 + PC1) from the 1st electrostatic separation. The dotted straight lines represent the upper limit of yield and protein purity for the protein-enriched fraction, respectively.

After two separation steps, a protein-enriched fraction (GE2E + GE2C) with a purity of 66.9 g/100 g dry solids and a yield of 5.6 g/100 g dry solids was obtained. This is 6.7% of the protein in the starting raw material. However, combining GE1 and GE2C fractions shows a better balance between protein purity and yield. A product with a protein content of 63.4 g/100 g dry solids and a yield of 15.8 g/100 g dry

solids was obtained, recovering 18.0% of the original protein in the starting material (pea fine fraction). Comparison of the two lines drawn in figure 4-9 shows that it is useful to further fractionate the collector fractions by subsequent steps to achieve higher yield while maintaining the protein purity.

In summary, the obtained protein purity by combining air classification and electrostatic separation is higher (at reasonable yields) compared to that from only air classification, which indicates that electrostatic separation is a valuable additional processing step. With the optimized dry fractionation process, a protein-enriched fraction with a yield of 4.0 g/100 g pea was obtained, leading to 7.8% of total protein recovered from yellow pea (Table 4-2). The mass balance for the entire dry fractionation process is visualized in a Sankey diagram (Figure 4-10). The yield in protein reported in this study may be further optimized by improved equipment electrostatic separator design. It was estimated that the yield for the optimized dry fractionation process may then be more than doubled to 10.9 g/100 g pea with 22.7% protein recovery (Table 4-2). However, of course, improving design and scale-up is still a major challenge, where ideally electrostatic separation should become a more continuous multi-stage process that enables separation to high purity and optimum yields.

Table 4-2. Comparison of purity, yield and protein recovery for protein-enriched fractions (compared to yellow pea with a protein purity of 32 g/100 g dry basis) obtained from three different dry fractionation processes on basis of measurements (with unrecovered material) and calculated potential assuming full recovery (e.g. for improved design). Different scenarios of electrostatic separation are included as well as air classification only. Data marked with a different lowercase superscript in the same column indicate significant differences ($P < 0.05$).

	Protein purity (g/100 g dry basis)	From current study		Calculated with fully recovered material	
		Yield (g/100 g pea)	Protein recovery (%)	Yield (g/100 g pea)	Protein recovery (%)
Air classification only	57.1^{a} ± 0.2	24.5^{c} ± 1.1	49.0^{c} ± 2.6	36.3^{d} ± 2.0	70.5^{d} ± 2.3
Air classification + electrostatic separation	67.1^{c} ± 0.3	3.2^{b} ± 0.2	6.6^{b} ± 0.4	7.1^{b} ± 1.2	13.3^{b} ± 1.7
Air classification + 2-step electrostatic separation (GE2E)	67.6^{c} ± 0.1	0.3^{a} ± 0.0	0.7^{a} ± 0.0	1.4^{a} ± 0.3	2.9^{a} ± 0.5
Air classification + 2-step electrostatic separation (GE1+GE2C)	63.4^{b} ± 0.5	4.0^{b} ± 0.3	7.8^{b} ± 0.6	10.9^{c} ± 0.5	22.7^{c} ± 1.0

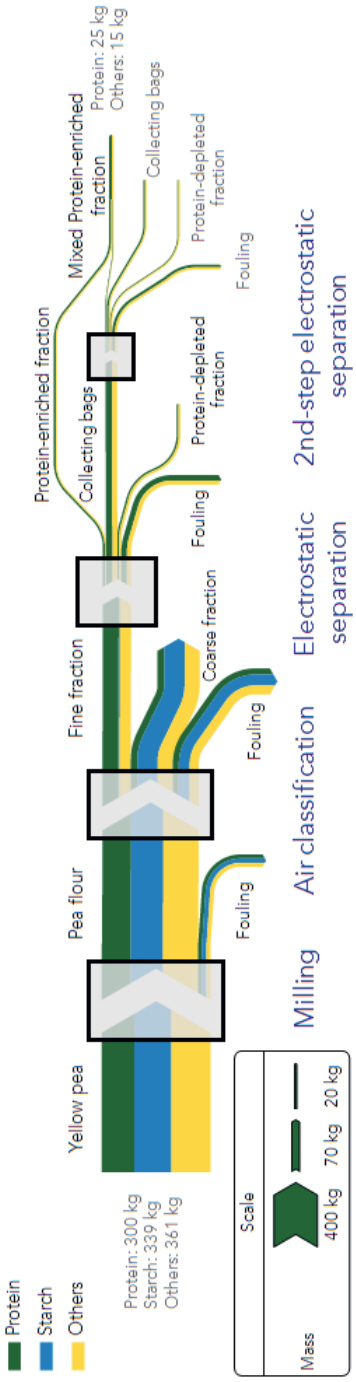

Figure 4-10. Sankey diagram of the mass flow of the dry fractionation process on the yellow pea (starting with 1000 kg pea). The distribution of colour indicates the dry mass of each component in each stream. For easy visualization, the " Starch" is merged into the category of " Others" after electrostatic separation.

4.4. Conclusion

Dry fractionation of three starch-containing legumes was achieved by combining air classification and electrostatic separation. By fine milling flours consisting of starch granules and smaller protein-rich fragments were prepared that could be used for subsequent air classification and electrostatic separation. Specifically, the fine fractions were subjected to electrostatic separation as it was known from a previous study that the presence of large amounts of starch impaired the electrostatic separation performance. Modest protein enrichment (4.6 - 5.8%) was achieved for the pea and lentil fine fractions, whereas no protein enrichment was observed for chickpea fine fraction.

Further optimization of the electrostatic separation was carried out using pea fine fraction. An optimum balance between protein purity and yield was achieved by adjusting the classifier wheel speed to 8000 rpm, where a pea fine fraction with a protein purity of 57.1 g/100 g dry solids was obtained and 49.0% of the protein was recovered from pea flour. After a single-step electrostatic separation, a protein-enriched fraction with a protein purity of 67.1 g/100 g dry solids and a yield of 13.1 g/100 g fine fraction was obtained, recovering 15.4% of the total protein in the pea fine fraction.

The protein purity and yield of the protein-enriched fraction was further improved by applying a second electrostatic separation. In the first strategy, protein-enriched fraction obtained from 1st separation was subjected to a 2nd separation. By doing so, a protein-enriched fraction with a protein purity of 67.6 g/100 g dry solids was obtained while only 1.6% protein was recovered from the starting material (fine fraction). In the second strategy, fractions obtained from the two collecting bags in the 1st separation were mixed and used for a 2nd separation. The optimum combination of protein-enriched fractions from two separation steps yielded a protein purity of 63.4 g/100 g dry solids with a yield of 15.8 g/100 g fine fraction. It means 18.0% of the protein was recovered from the pea fine fraction.

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Chapter 5

Enhanced nutritional value of chickpea protein concentrate by dry separation and solid-state fermentation

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Abstract

A sustainable dry processing method to obtain nutritional and functional chickpea products was developed, yielding protein concentrates suited to prepare products without additives. Chickpeas were milled and air-classified into protein and starch-enriched concentrates. Subsequently, spontaneous solid-state fermentation (SSF) with daily back-slopping was performed at 37 °C. The dominant autochthonous lactic acid bacteria (LAB) strains in chickpea flour and enriched fractions were *Pediococcus pentosaceus* and *Pediococcus acidilactici*. Strains were selected on their ability to metabolise flatulence-causing α-galactosides. SSF reduced the pH of the doughs in 24 h from 6.6 to 4.2. After 72 h, concentrations of raffinose and stachyose were reduced by 88.3 – 99.1%, while verbascose became undetectable. Moreover, phytic acid reduced with 17% while total phenolic contents increased with 119%. Besides the observed differences in smell, texture and colour, the sourdoughs showed 67% higher water-holding capacity. This natural route to produce chickpea concentrates thus increases both the nutritive and technical functionality.

Abbreviations

ANFs	anti-nutritional factors
CF	chickpea flour
CPF	chickpea protein-enriched fraction
CSF	chickpea starch-enriched fraction
cf-CF (P.p.3)	controlled fermented chickpea flour (inoculate with strain P.p.3)
cf-CPF	controlled fermented chickpea protein-enriched fraction
cf-CSF	controlled fermented chickpea starch-enriched fraction
sf-CF	spontaneously fermented chickpea flour
sf-CPF	spontaneously fermented chickpea protein-enriched fraction
sf-CSF	spontaneously fermented chickpea starch-enriched fraction
SSF	solid state fermentation
TPC	total phenolic compounds
WHC	water-holding capacity

5.1. Introduction

The global production of chickpeas (Cicer arietinum L.) has been growing steadily in the past decade, now constituting the third largest grain pulse after dry beans (27 million tons) and dry peas (14million tons) (Faostat & Production, 2016). Chickpea is a highly nutritious crop which contains good quality protein (13 – 31%), adequate carbohydrate (54 - 71%) and physiologically active ingredients (polyphenols, γ-aminobutyric acid, etc.) (Frias, Vidal-Valverde, Sotomayor, Diaz-Pollan, & Urbano, 2000). Allergenicity for chickpea is observed less frequently than for soybeans (Bar-El Dadon, Abbo, & Reifen, 2017). Chickpea therefore is a promising protein source (Bar-El Dadon, et al., 2017; Roland, Pouvreau, Curran, van de Velde, & de Kok, 2017), both for new products such as meat analogues, and for supplementation of traditional foods, such as chickpea-fortified bread (Coda, et al., 2015).

Milling and air classification have been used successfully to fractionate chickpea into protein and starch-enriched fractions. This dry fractionation route is energy-efficient and retains the native functional properties of proteins (Schutyser, et al., 2015). (Pelgrom, Boom, et al., 2015) showed that the chickpea protein content could be increased from 21.6 g/100 g to 45.3 g/100 g in the protein-enriched fraction. A disadvantage of dry fractionation is that anti-nutritional factors (ANFs) like phytic acid, tannins, trypsin inhibitors, and α-galactosides (raffinose, stachyose and verbascose) are not removed (in contrast to wet protein isolation processes) and thus remain in the dry-enriched fractions (Hall, Hillen, & Garden Robinson, 2017; Sozer, Holopainen‐Mantila, & Poutanen, 2017). Indigestible α-galactosides are not taken up by the human small intestine and therefore are fermented in the large intestine by the colon microbiota (Martínez-Villaluenga, Frías, & Vidal-Valverde, 2006), causing flatulence. At the same time α-galactosides have been suggested to increase susceptibility to bowel diseases and negatively affect the symptoms for

patients with irritable bowel syndrome (Thirunathan, et al., 2019). Reducing the presence of α-galactosides via breeding is difficult as these components help plant seeds to protect them against periods of severe abiotic and biotic stress. In a study by Frias, et al. (2000) the total α-galactosides content in unprocessed chickpea was found to be 4.84% (w/w), consisting of 0.46% raffinose, 1.68% stachyose, and 2.70% ciceritol.

Anti-nutritional factors can be reduced by dehulling, soaking, heat treatment or biochemical conversion (e.g., through germination, fermentation or enzymatic treatment) (Coda, et al., 2015). Solid state fermentation (SSF) is perceived natural and avoids addition of large amounts of water (Sadh, Duhan, & Duhan, 2018). SSF may reduce the ANF content for various legumes. In chickpea, the trypsin inhibitor activity was reduced by fermentation with a lyophilised yoghurt culture (Chandra-Hioe, Wong, & Arcot, 2016). In kidney bean, chickpea, pea, lentil, and grass bean, the raffinose and condensed tannin levels were decreased after fermentation with selected starters consisting of Lactobacillus plantarum and Lactobacillus brevis (Curiel, Coda, Centomani, Summo, Gobbetti, & Rizzello, 2015). With common beans, a fungal fermentation with *Rhizopus oligosporus* was used to reduce the ANFs (Valdez - González, et al., 2017). However, studies on fermenting chickpea ingredients with selected autochthonous microorganisms isolated via a back-slopping procedure have not yet been reported hitherto. We therefore followed this route.

Since the chickpea protein-enriched fraction (CPF) and chickpea starch-enriched fraction (CSF) differ in their compositions by definition, we evaluated the presence of dominating microorganisms in the fractions and flour. The use of autochthonous microorganisms as a starter culture is preferable as these are already well-adapted to the raw material and are expected to grow fast on the substrate and thereby inhibiting spontaneous growth of other (undesirable) microorganisms (Chen, et al., 2015).

In this study we aimed to establish a sustainable processing method, which involves the combination of dry fractionation and solid-state fermentation to produce novel, protein-enriched chickpea ingredients. Dry-enriched chickpea fractions were obtained by milling and air classification. The autochthonous microorganisms enriched on spontaneously fermented chickpea flour and fractions after several back-slop cycles were identified. These species were then further selected on their ability to digest the α-galactosides. Controlled SSF was performed and the nutritional and techno-functional properties of the chickpea ingredients were evaluated.

5.2. Materials and methods

5.2.1 Materials

Chickpea (Cicer arietinum) seeds (Kabuli type) were purchased from a local supermarket. The seeds were stored in a tightly screwed polyethylene container at 4 °C.

5.2.2 Dry fractionation

5.2.2.1. Preparation of chickpea flour

To obtain chickpea flour, a batch of 2 kg of whole chickpea seeds were coarsely milled into grits with a pin mill (LV 15 M, Condux-Werk, Germany). The grits were then further milled into flour with a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) at ambient temperature at a feed rate of 2 - 5 rpm (circa 0.5 kg/h). The classifier wheel speed was fixed at 2900 rpm, the air flow rate was at 40 m^3/h (Pelgrom, Boom, et al., 2015).

5.2.2.2. Preparation of chickpea protein-enriched and starch-enriched fractions

Chickpea protein-enriched fraction (CPF) and starch-enriched fraction (CSF) were obtained by air classification (Pelgrom, Boom, et al., 2015) with an ATP50 classifier (Hosokawa-Alpine, Augsburg, Germany) at ambient temperature. A batch of 900 g

of chickpea flour was classified at a classifier wheel speed of 10,000 rpm, air flow rate of 52 m^3/h and a feed rate of 2 - 5 rpm (circa 0.5 kg/h). Approximately 280 g of protein-enriched fraction and 460 g of starch-enriched fraction were collected. The remaining 160 g of flour remained stuck to the lining of the mill, which is due to the small size of the batch and the relatively large milling machine.

5.2.3 Selection of starter culture

5.2.3.1. Spontaneous fermentation

The most dominant autochthonous microorganisms were identified by following a back-slopping fermentation procedure starting with spontaneous fermentation. Six grams of sterilized water were added into 12 g of (1) chickpea flour (CF), (2) protein-enriched and (3) starch-enriched fractions, respectively. After the doughs were well mixed in screw-tight 50-mL containers, they were put in an incubator (IKS International, The Netherlands) at 37 °C for 24 h. Subsequently, the next day 2.7 g (15%, w/w) of each of the incubated doughs were taken and supplemented with 10.2 g of flour and 5.1 g of sterilized water and again incubated at the same conditions after manual mixing with a spoon. This procedure with back-slopping was performed daily for 10 days.

5.2.3.2. Classification of autochthonous microorganisms

One gram of each fermented doughs made from chickpea flour (sf-CF), protein-enriched (sf-CPF) and starch-enriched fractions (sf-CSF) was suspended in 9 mL peptone physiological salt solution (PPS), and ten-fold series dilutions were made. Fifty milliliters of the suspension at the respective dilution were spread out on selective media using a spiral plater (Neutec Group Inc., USA). For isolation of the microorganisms we applied: Plate Count Agar (Oxoid Ltd., UK) incubates at 30 °C for 2 days in aerobiosis; Yeast and Mould Growth Medium (DRBC agar, Oxoid Ltd., UK) incubated at 25 °C for 3 - 5 days in aerobiosis; de Man, Rogosa and Sharpe (MRS) agar (Merck KGaA, Germany) with 0.2% of natamycin (Delvocid) incubated under microaerobic conditions at 30 °C for 3 - 5 days. Microaerobic conditions were

established by gas purging the incubation chamber using an Anoxomat Anaerobic Chamber System under microaerobic mode before incubation. The colonies less than 200 CFU per plate were counted and the result was expressed as a log10 CFU per g of dough.

5.2.3.3. Strain identification of isolated bacteria

Autochthonous LAB from three types of chickpea doughs were obtained from MRS agar plates. From each MRS plate, 20 colonies (in total 60 colonies) were picked up randomly with an inoculating loop and cultivated on fresh MRS agar plates using a triplet streak method. After incubation for 2 days, each single colony was picked up carefully from the MRS plates with an inoculating loop and inoculated into 10 mL fresh MRS broth media. The inoculated MRS broth was incubated under static conditions at 30 °C overnight. After vortexing, 300 mL of the LAB suspension was transferred into a sterilized cryotube containing 700 mL glycerol and five glass beads and preserved at -80 °C after mixing.

The genomic DNA of the isolated LAB strains was extracted using a Wizard® Genomic DNA Purification Kit (Promega, USA). The 16S rRNA genes were amplified by PCR (Veriti 96 Well Thermal Cycler, Applied Biosystems, USA) using the forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-AAGGAGGTGATCCAGCCGCA-3'). The PCR reaction conditions were: preliminary denaturation at 94 °C for 5 min; amplification in 35 cycles: denaturation at 94 °C for 30 s, annealing 20 s at 56 °C and elongation at 72 °C for 1 min; final elongation at 72 °C for 7 min. The amplification of the target DNA fragment was confirmed by agarose gel electrophoresis. The procedure was as follows: 1.0% (w/v) of agarose (Lonza, USA) gel was prepared with 50-mL TAE buffer (Bio-Rad, Germany) and 1.25 μL SYBR Safe DNA gel stain (Invitrogen, USA) was added before the gel was solidified; 6-μL GeneRuler DNA Ladder Mix (Thermo Scientific, Lithuania) and 5 μL DNA samples premixed with 1 μL 6 × TriTrack DNA loading dyes (Thermo Scientific, Lithuania) were loaded into wells; electrophoresis was performed in TAE buffer at

100 V for 30 min and the DNA fragments were visualised under UV light (UVITEC, Cambridge, UK).

The purity of the DNA fragments and the concentration of nucleic acids were checked using a NanoDrop spectrophotometer (Marshall Scientific, USA) before sequencing. A volume of 2 µL of DNA rehydration solution was exposed to the NanoDrop sensor as the blank solution. Then the purity of the DNA was evaluated via measurement of the absorbance at 260 and 280 nm. If the 260/280 ratio was ~1.8 (the DNA is generally accepted pure) and the concentration of nucleic acids was in the range of 10 – 50 ng/µL then the sample was used as is; else further dilution was performed. Subsequently, the 16S rRNA gene sequencing by Sanger sequencing was conducted (Eurofins Genomics, Germany) and identification query was fulfilled on Web BLAST (https://blast-ncbi-nlm-nih-gov.ezproxy.library.wur.nl/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

5.2.3.4. α-Galactoside digestion

An API® 50 CHL (BioMérieux SA, France) test was performed according to the manufacturer's instruction. Subsequently, the raffinose positive LAB strains screened from the API test were inoculated in 2.5% (w/v) of d-raffinose (≥ 98%, Sigma-Aldrich, Germany), stachyose (≥ 98%, Sigma-Aldrich, Germany) and verbascose (≥ 95%, Megazyme, Ireland) solutions (because the API test kit does not evaluate stachyose or verbascose). The optical density (OD value) of the inoculated spent medium (MRS broth) was monitored with a Bioscreen C MBR system (Thermo Fisher Scientific, USA) at 600 nm with intervals of 30 min over 72 h.

5.2.4 Solid state fermentation with starter culture

Selected LAB strains were inoculated into 10-mL MRS broth and incubated at 30 °C overnight. One millilitre of the cell suspension was centrifuged at 10,000 rpm for 5 min. The supernatant was discarded, and the pellet was washed once with 1-mL PPS solution once. The suspension was centrifuged at the same condition and the

pellet was re-suspended in 1-mL PPS. From each of the LAB suspensions, 300 µL was added into 20 g sterilized demineralised water and well mixed with 40 g of chickpea flour, starch-enriched and protein-enriched fractions, respectively. The inoculated doughs were incubated at 37 °C for 72 h.

5.2.5 Characterization of chickpea doughs

5.2.5.1. Compositional analysis

High performance anion exchange chromatography (HPAEC) was used to evaluate the concentration of α-galactosides. A Dionex ICS-5000 system (Thermo Fisher Scientific Inc., USA) was used with a CarboPac PA1 (2 mm × 250 mm) guard column (Thermo Fisher Scientific Inc., USA) and a pulsed amperometric detector (HPAEC-PAD). The α-galactosides were first extracted by dispersing 1 g of dough in 30 mL of demineralised water and stirring for 30 min. Subsequently, the extract was transferred to a tube and centrifuged at 10,000 rpm for 5 min. A 500 µL aliquot of the supernatant was transferred into a 2 mL centrifuge tube and Carrez reagent (250 µL Carrez A followed by 250 µL Carrez B) was added to precipitate proteins. After centrifugation at 10,000 rpm for 5 min, 200 µL of the supernatant was transferred into a vial (0.3 mL PP Short Thread Vial 32 × 11.6 mm clear, BGB, Switzerland) for further analysis. Standard solutions in the range of 1 - 20 mg/L were prepared with raffinose, stachyose, and verbascose for identification and quantification. For the measurement, 10 µL of sample solution was injected and the eluent flow rate was set at 0.3 mL/min. Chromeleon 7.0 was used for numerical integration of the peak surface. All HPAEC analyses were carried out in duplicate.

Samples of the doughs were frozen at -20 °C overnight. Subsequently, the samples were freeze-dried for 48 h using a standard program with stepwise changing temperatures and pressures (Christ Epsilon 2-6D, Germany). The resulting powders were ground and stored at 4 °C for further analysis.

The protein content was determined with a nitrogen analyser (FlashEA 1112 series, Thermo Scientific, The Netherlands) based on the Dumas combustion method. A conversion factor of N × 5.71 (Berghout, et al., 2015) was used for the conversion of the nitrogen content into the crude protein content. The starch content was assayed with a Total Starch Assay Kit (Megazyme, Ireland). The oil, ash, and moisture contents were determined according to AACC 30-25.01 (1999), AACC 08-01 (1983), and AACC 44–15.02 (1999) procedures, respectively. The content of total fibre was calculated as remaining material. The total phenolic content (TPC) was measured with the method of (Xu & Chang, 2007). The content of phytic acid was measured with an assay kit (Megazyme, Ireland). The pH during fermentation was measured by dispersing 1 g of the sourdough in 9 mL PPS.

Soluble protein hydrolysis was evaluated according to the method from (Nielsen, Petersen, & Dambmann, 2001). Freeze-dried powders of cf-CF, cf-CPF, and cf-CSF were weighted; solutions were prepared with a protein concentration of ~0.4 mg/mL in a 50 mM Tris/HCl buffer solution (pH 8.5). The soluble proteins were digested by trypsin (Sigma-Aldrich, Germany). O-phthaldialdehyde (OPA reagent) was added to react with the present free amino groups. The increase in absorbance was measured with a spectrophotometer (Beckman DU 720, Beckman Coulter, USA) at 340 nm. The concentration of free amino groups was determined using a calibration solution of l-serine (Alfa Aesar, The Netherlands). The results were expressed as the degree of protein hydrolysis (DH), as defined in Eq 5-1. All analyses were carried out in duplicate.

$$DH\ (\%) = \frac{N\ groups_{after} - N\ group_{before}}{h_{tot}} \times 100 \qquad (Eq\ 5-1)$$

$N\ groups_{before}$: the amount of free amino groups in protein before digestion. Determined by the number of arginine and lysine in chickpea protein (refer to UniProt); $N\ groups_{after}$: the amount of free amino groups in protein after digestion. Determined by absorbance of samples, molecular weight of protein (56,251 Da),

and soluble protein content (data not shown); h_{tot} is the total amount of amino groups in chickpea protein (refer to UniProt).

5.2.5.2. Microbiological analysis

One gram of chickpea sourdough was serially diluted in 9-mL PPS. A 50 µL of diluted sample was spread out on PCA and MRS agar plates with a spiral plater (Eddy Jet IUL, Neutec Group Inc., USA) and incubated at 30 °C for 48 – 72 h. After incubation the colonies were counted, and the results were expressed as colony forming units per gram ($CFU \cdot g^{-1}$). This analysis was carried out in duplicate.

5.2.5.3. Techno-functional analysis

Five grams of freeze-dried powder were weighed to analyse the water-holding capacity (WHC) according to the method used by (Wang, et al., 2017). The foaming ability was determined with 2 g of freeze-dried powder based on the method from (Narayana & Narasinga Rao, 1982) with some minor modifications. All these analyses were carried out in duplicate.

5.2.6 Statistical analysis

The experimental data were analysed using SPSS statistics Version 22.0 (IBM, Armonk, NY). The statistical difference ($P < 0.05$) between results was evaluated with Duncan's test.

5.3. Results and discussion

5.3.1 Air classification of chickpea flour

Chickpea seeds were milled into a batch of chickpea flour to detach starch granules from their surrounding matrix. Subsequently, the chickpea flour was classified with air into a chickpea protein-enriched fraction (CPF) and a chickpea starch-enriched fraction (CSF). The yields of the two fractions were 28.4% and 47.7%, respectively. The rest 23.9% of material remained stuck to the lining of the mill because of the experiment configuration (the small size of the batch and the relatively large milling

equipment). The compositional differences between the original chickpea flour and the two fractions are reported in figure 5-1. The composition of the fractions is in agreement with the earlier study by Pelgrom, Boom, et al. (2015) who reported 45.3 g/100 g chickpea protein in the fine fraction, albeit with a lower yield of 11%. The oil and total fibre were also enriched in the protein-enriched fraction CPF (Figure 5-1), which is consistent with other studies (Schutyser, et al., 2015). The starch granules can be separated relatively well into the starch fraction CSF, which pushes the other components towards the protein-enriched fraction CPF. In addition, the lipids are known to associate with the proteins as well (Rempel, et al., 2019; Song, Wang, & Rose, 2017).

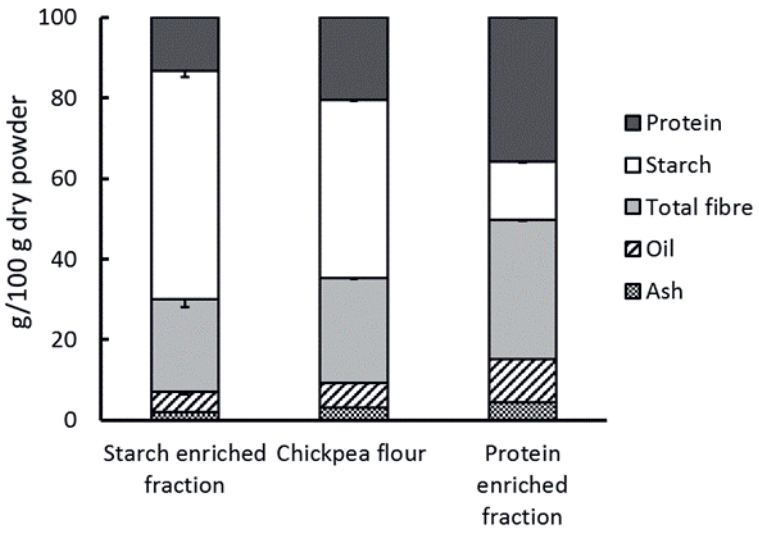


Figure 5-1. Compositions of chickpea protein enriched fraction, whole chickpea flour and starch enriched fractions. Results are expressed in g/100 g powder, on dry basis. The error bars represent standard deviations, only minus direction is shown.

5.3.2 Identification of autochthonous microbes in chickpea flour and dry-enriched fractions

Spontaneous fermentation with back-slopping was used to isolate the most dominant microorganisms of the autochthonous species in the different doughs. Since the three types of doughs differed in carbohydrate (C source) and protein (N source) content, the microbial community that would be isolated from the different fractions could potentially be different qualitatively and quantitatively.

After 10-fold back-slopping, the naturally presented microbes in those doughs were pre-classified using DRBC (for yeast and mould) and MRS agar (for LAB) plates. Neither yeast nor moulds were present in the doughs. As is shown in Figure 5-2, *Pediococcus pentosaceus* and *Pediococcus acidilactici* were the two major species of microorganisms found in the chickpea dough prepared from the flour and the fractions. These lactic acid bacteria (LAB) have been previously observed in chickpea by Sáez, Saavedra, Hebert, & Zárate (2018). The bacterial species found in the different doughs were partly overlapping with only small variations in relative abundance. Sourdough from chickpea flour (sf-CF), was dominated by *P. pentosaceus*, while the sourdoughs from the enriched fractions were dominated by *P. acidilactici*. Furthermore, a small population (10%) of *Pediococcus lolii* was found in sf-CSF. These observations are consistent with those of Katsaboxakis & Mallidis, (1996) and Thirunathan, et al. (2019), who reported that LAB are frequently observed during natural fermentation of chickpea and many other legumes. Another study also showed that yeasts can play a role in the spontaneous fermentation of chickpea (Sayaslan & Şahin, 2018). However, the exact back-slopping procedure (e.g., number and duration) can influence the relative abundance of yeast and LAB (Pétel, Onno, & Prost, 2017).

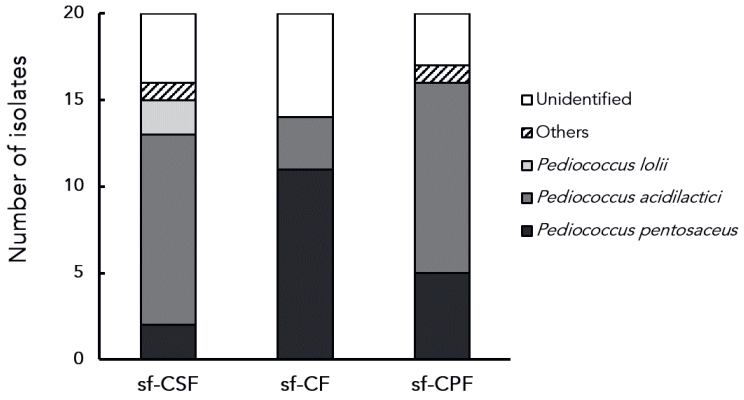


Figure 5-2. Distribution of LAB species in chickpea sourdoughs prepared from whole flour (sf-CF), protein enriched fraction (sf-CPF) and starch enriched fraction (sf-CSF).

5.3.3 Selection of the starter cultures

Based on the 16S rRNA sequencing results, all 44 identified LAB strains were given a new identifier name (Appendix A). According to their similarity, 22 of them were selected for subsequent API testing of which the results are shown in figure 5-3. All strains could metabolize small sugars like glucose, fructose, and galactose as a carbon source. Surprisingly, none of the 22 LAB strains metabolized starch, indicating that autochthonous LAB species in chickpea sourdoughs including *P. pentosaceus* and *P. acidilactici* are not able to hydrolyze and utilize chickpea starch as a carbon source (Sáez, et al., 2018). This is in line with the observations of Juodeikiene, et al. (2016) who reported that many LAB species cannot digest starch. One reason could be the absence of the enzyme amylase; another explanation could be that the affinity of the bacterial amylase, if present, is poor for raw starch (Rodriguez-Sanoja, Ruiz, Guyot, & Sanchez, 2005). In addition, legume starch has a high amylose content, which is more resistant against digestion compared to amylopectin (Guillon, et al., 2002). Instead of metabolizing starch, *P. pentosaceus* and *P. acidilactici* hydrolyze non-starch polysaccharides (Juodeikiene, et al., 2016).

Among the tested LAB strains, thirteen were capable of utilizing D-raffinose of which almost two-third belong to *P. pentosaceus*. The result is verified by previous research that showed that the capability of utilizing raffinose is a plasmid encoded trait in *P. pentosaceus* (Rizzello, et al., 2019). This gives *P. pentosaceus* outstanding ability to degrade raffinose compared to many other LAB strains.

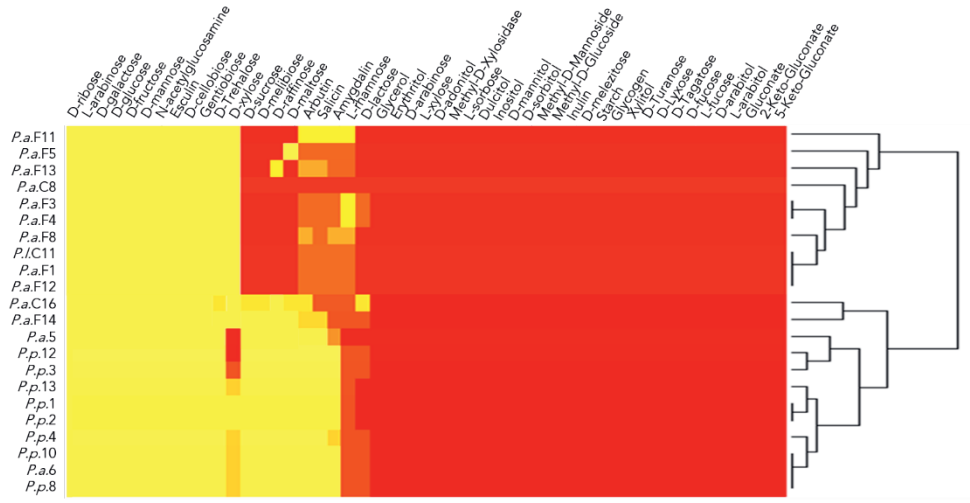
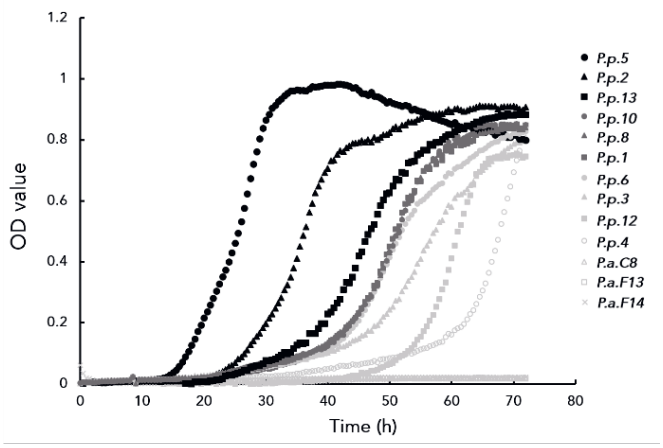


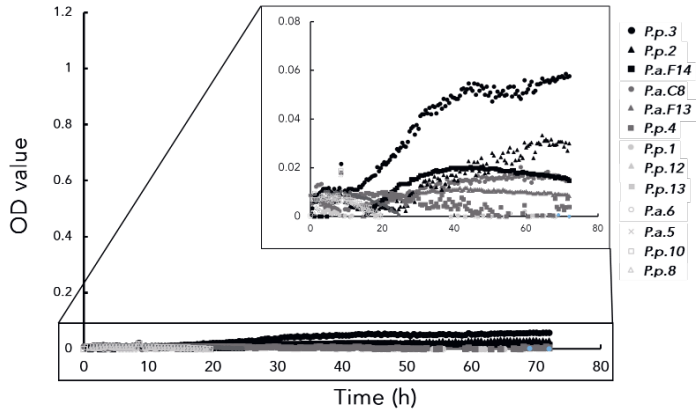
Figure 5-3. Heatmap of the results from the API tests on selected *Pediococcus* strains (vertical axis). The colours represent the ability of utilizing 50 different types of carbohydrates (horizontal axis). The yellower the colour is, the better the utilization, while redder indicates the opposite. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Because other α-galactosides in chickpea seeds, like stachyose and verbascose, were not included in the API test, more screening experiments were conducted to narrow the selection of starter cultures. The optical density (OD values) increase due to bacterial growth on a medium containing the different α-galactosides (stachyose, verbascose, and raffinose) as sole carbon and energy source is plotted against the

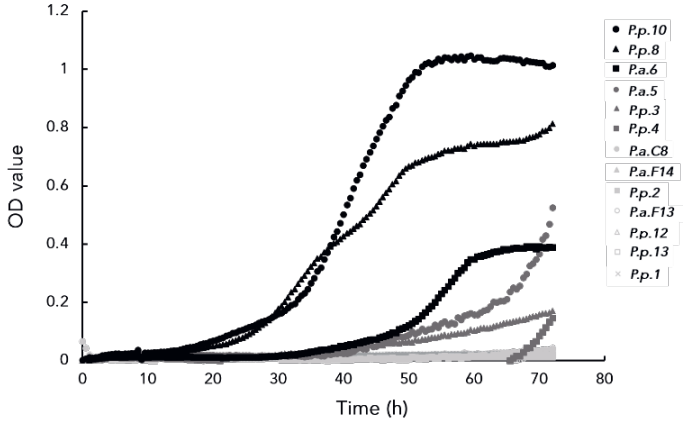
incubation time (72 h). In each graph of figure 5-4, the three fastest growing LAB strains of each type of microorganism were plotted. Growth on raffinose and verbascose supplemented medium was substantial within the studied incubation time, where much less growth was observed on the stachyose solution. The three strains (P.p.3, P.a.5, and P.p.10) that exhibited the highest optical density increase in the respective α-galactosides supplemented media were selected as starter culture for further study.



A



B

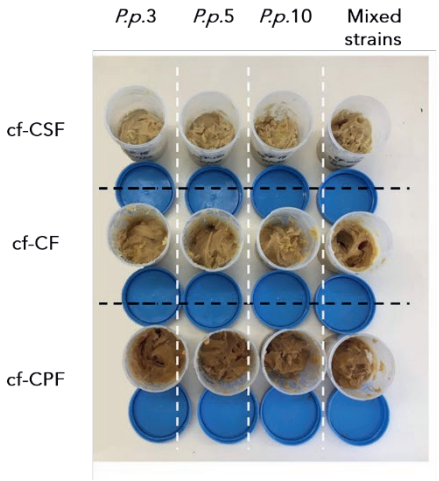


C

Figure 5-4. Growth of single strain cultures of thirteen D-raffinose degrading *Pediococcus* strains selected by API tests on MRS broth spent medium supplemented with respectively. A: raffinose, B: stachyose, C: verbascose. Bacterial growth was measured as optical density of the cell suspension at 600 nm.

5.3.4 Properties of chickpea sourdoughs

Chickpea sourdoughs were prepared from whole flour (cf-CF), protein-enriched fraction (cf-CPF), and starch-enriched fraction (cf-CSF) inoculated with the individual *Pediococcus* strains (P.p.3, P.a.5, and P.p.10) and with a mixture of all three strains (Figure 5-5). The smell of the fermented sourdoughs was completely different compared to that of the non-fermented doughs (Table 5-1). While the non-fermented doughs had a beany smell, the fermented doughs had a more acidic/mild odour. Specifically, the protein-enriched sourdough had a sweeter, nutty smell. Therefore, SSF mitigates the specific (off-)flavour characteristics of chickpea. It may also indicate that the protein and fatty acids in CPF might be critical substrates in forming flavour compounds (Kaczmarska, Chandra-Hioe, Frank, & Arcot, 2018).



	P.p.3	P.a.5	P.p.10	Mixed strains
L*	63.68 ± 0.6	62.67 ± 0.4	63.38 ± 0.4	62.96 ± 0.4
a*	1.17 ± 0.6	1.14	1.61	1.24 ± 0.2
b*	21.99 ± 1.4	22.55 ± 0.2	21.34 ± 0.5	21.53 ± 0.5
L*	61.04 ± 0.1	61.31 ± 0.1	61.51	61.22
a*	2.79 ± 0.1	1.27	1.28	1.41
b*	25.46 ± 0.6	28.62 ± 0.1	33.25	32.3
L*	46.38 ± 0.5	45.51 ± 0.3	47.17 ± 1.2	47.65 ± 0.5
a*	5.40 ± 0.6	4.59 ± 0.4	4.39 ± 0.8	5.14 ± 0.2
b*	23.31 ± 2.4	23.13 ± 0.9	25.34 ± 0.6	23.93 ± 0.7

Figure 5-5. Picture of controlled fermented chickpea sourdoughs (inoculated with either a single LAB strains or their mixture) and the colour measurement results.

Table 5-1. The qualitative sensory properties of the chickpea doughs.

	Non-fermented	Cf-CSF	Cf-CF	Cf-CPF
Odour	Beany	Acidic/mild	Acidic/mild	Acidic/mild, relatively sweet, nutty
Appearance	Less sticky	Non-sticky	Less sticky	Sticky

Sourdoughs with a higher protein content were stickier and more difficult to handle and mix. This may indicate the presence of more soluble protein and less insoluble starch in the protein-enriched fractions, whereas the starch-enriched fractions of course contained relatively more starch which was not metabolized.

The starch-enriched sourdough had a lighter colour due to the starch, whereas the protein-enriched sourdoughs were darker brown as this fraction was richer in more fibre and phenolics. Differences in colour were quantified with colour measurements: with increasing protein content, the brightness (L*) decreased and the redness (a*) increased (Yu, Ramaswamy, & Boye, 2013), reflecting the brownish colour of protein-enriched sourdoughs (Figure 5-5). It is noted that non-fermented chickpea doughs in time became slightly darker in colour, probably due to the change in moisture content, pH, the polyphenol content, and degree of oxidation during fermentation (data not shown) (Cuellar-Álvarez, Cuellar-Álvarez, Galeano-García, & Suárez-Salazar, 2017).

5.3.4.1 Microbiological properties

During all sourdough fermentations, the pH decreased from 6.7 to approximately 4.5 within 24 h and then stabilized at 4.0 - 4.2 after 48 h (Figure 5-6). This shows that the selected *Pediococcus* strains acidify the chickpea dough by producing lactic acids and have high tolerance to acidity (Wang, Dong, Li, Chen, & Shao, 2019). Table 5-2 shows that the number of LAB increased by 0.4 - 1.4 log CFU/g over 72 h in the cf-CPF and only 0.1 - 0.3 log CFU/g in the cf-CSF: the acidification stabilizes the population. The protein-enriched fraction shows the largest increase in LAB counts. This can be explained because CPF contains the highest concentration of fermentable sugars i.e., raffinose, stachyose, and verbascose. For the sourdough made from the flour the increase was in between (0.5 - 1.1 log CFU/g). This observation suggests that only very moderate growth occurs in all sourdoughs. This observation may be related to the choice of the plates (MRS agar), which does not support growth of a large proportion of the microbes.

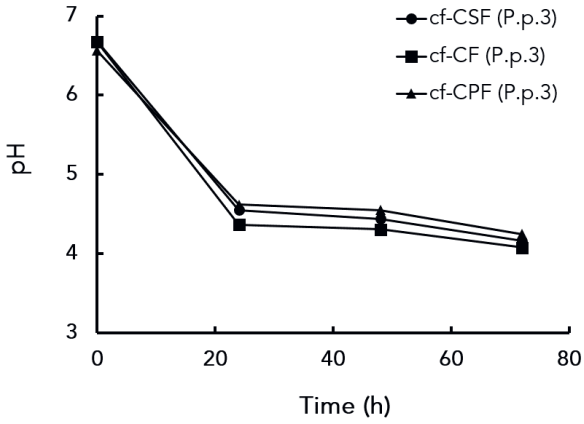


Figure 5-6. The pH values of sourdoughs inoculated with P.p. 3 made from chickpea flour (cf-CF), chickpea protein-rich fraction (cf-CPF), and chickpea starch-rich fraction (cf-CSF) as function of incubation time. Sourdoughs inoculated with P.a.5, P.p.10, and mixed strains showed similar pH profiles and therefore not plotted.

Table 5-2. Characterization of chickpea flour, protein-enriched, and starch-enriched fractions fermented with individual and mixed starter cultures as compared to unfermented samples. Data marked with different lowercase superscript indicate significant differences ($P < 0.05$).

		Starch-enriched fraction (CSF)					Chickpea flour (CF)					Protein-enriched fraction (CPF)				
		Non-fermented	*P.p.*3	*P.a.*5	*P.p.*10	Mixed strains	Non-fermented	*P.p.*3	*P.a.*5	*P.p.*10	Mixed strains	Non-fermented	*P.p.*3	*P.a.*5	*P.p.*10	Mixed strains
LAB (log CFU/g dough)	72h	6.0^{a}	6.3^{bc}	6.1^{a}	6.1^{a}	6.3^{b}	6.0^{a}	$7.0^{e}\pm 0.1$	7.0^{e}	$6.5^{d}\pm 0.1$	7.0^{e}	6.0^{a}	6.4^{bcd}	7.4^{f}	6.3^{b}	6.4^{cd}
TPC (mg/GAE g)		4.3^{a}	10.0^{d}	9.8^{d}	9.9^{d}	9.7^{d}	5.1^{b}	11.8^{e}	11.9^{e}	$12.3^{f}\pm 0.1$	12.1^{f}	6.9^{c}	15.0^{g}	15.0^{g}	15.3^{h}	15.0^{g}
Phytic acid (g/100 g dry flour)		0.45^{b}	$0.43^{b}\pm 0.1$	$0.43^{b}\pm 0.1$	$0.26^{a}\pm 0.1$	0.45^{b}	0.58^{a}	$0.51^{a}\pm 0.1$	0.52 ± 0.1^{a}	0.55^{a}	$0.52^{a}\pm 0.1$	1.07^{b}	$0.91^{a\,b}$	$0.88^{a\,b}$	$0.95^{a\,b}$	$0.81^{a}\pm 0.1$
Degree of protein hydrolysis (%)		$13.5^{cd}\pm 0.7$	$25.7^{g}\pm 0.9$	$20.0^{f}\pm 0.8$	$20.0^{f}\pm 0.8$	$15.3^{d}\pm 0.7$	$14.4^{cd}\pm 0.8$	$17.2^{e}\pm 0.7$	$15.1^{cd}\pm 0.7$	$18.7^{ef}\pm 0.9$	$11.6^{b}\pm 0.7$	$7.8^{a}\pm 0.5$	$9.1^{a}\pm 0.6$	$13.4^{c}\pm 0.7$	$13.7^{c}\pm 0.8$	$13.4^{c}\pm 0.7$
WHC (g water/g dry powder)		$0.8^{a}\pm 0.1$	1.4^{c}	1.4^{c}	$1.4^{c}\pm 0.1$	1.5^{cd}	$1.1^{b}\pm 0.1$	1.7^{ef}	$1.7^{ef}\pm 0.1$	1.6^{de}	1.7^{f}	1.2^{b}	$2.0^{g}\pm 0.1$	2.0^{g}	2.0^{g}	$2.0^{g}\pm 0.1$
Foaming capacity (mL)	0 s	$30.5^{a}\pm 7.8$	0	0	0	0	$34.0^{a}\pm 5.7$	0	0	0	0	$52.0^{b}\pm 5.7$	0	0	0	0
	60 s	$13.0^{a}\pm 1.4$	0	0	0	0	$18.0^{b}\pm 2.8$	0	0	0	0	$25.0^{c}\pm 8.5$	0	0	0	0

5.3.4.2 Nutritional properties

The α-galactosides content of the doughs before and after fermentation are shown in figure 5-7. The air classification enriches the CPF with the α-galactosides, which is in agreement with previous observations (Elkowicz & Sosulski, 1982) and may indicate the spatial proximity of the α-galactosides to the proteins in the cell. Stachyose was most abundant, whereas only trace amounts of verbascose were detected in the CPF. The HPAEC results reveal that fermentation with the selected starter cultures, consisting of either a single LAB strain or their mixture, significantly ($P < 0.05$) reduces the α-galactoside concentrations. The content of raffinose in sourdough was reduced by 88.3 – 92.3% and the content of stachyose decreased by 97.7 – 99.1%. Selected LAB strains did not show significant growth in stachyose-supplemented medium (Figure 5-4B), which may suggest that other resident microbiota are associated with the reduction of stachyose. Verbascose concentrations were even below the detection limit in the fermented doughs. The degree of reduction of α-galactosides was similar for all chickpea sourdoughs. After 72 h solid-state fermentation with autochthonous LAB strains hardly any α-galactosides is left in all the doughs.

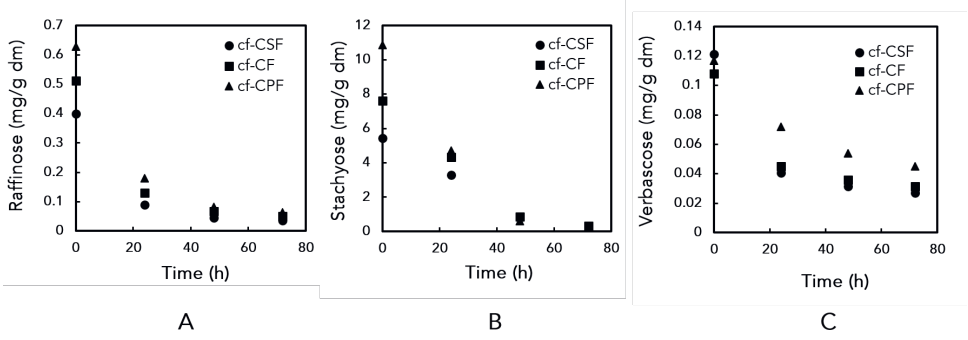


Figure 5-7. The content of α-galactosides (A: raffinose; B: stachyose; C: verbascose) in sourdoughs made from chickpea flour (cf-CF), chickpea protein-rich fraction (cf-CPF), and chickpea starch-rich fraction (cf-CSF) as function of the incubation time.

The total phenolic content (TPC) was highest in the protein rich fraction (Table 5-2), which can be related to its higher fibre content and thus higher TPC as phenolics are present in the cell walls (Sreerama, Neelam, Sashikala, & Pratape, 2010). After fermentation, all fractions showed an increase in TPC ($P < 0.05$). Similar observations were done during sourdough fermentation with chickpea flour-enriched dough by *Lactobacillus plantarum* C48 and *Lactococcus lactis* subsp. lactis PU1 (Coda, Rizzello, & Gobbetti, 2010). The increase in the TPC may be related to release of phenolics from the plant cells due to structural degradation (Adetuyi & Ibrahim, 2014; Đorđević, Šiler-Marinković, & Dimitrijević-Branković, 2010) or due to microbial or enzymatic conversions during fermentation (Dey, Chakraborty, Jain, Sharma, & Kuhad, 2016). It is most probably that the phenolics profile changes during fermentation as it is known that plant phenolics vary in molecular structure such as free or conjugated structures (Wang, Wu, & Shyu, 2014).

The presence of phytic acid in legumes is associated with decreased protein and mineral bioavailability by forming complexes (Coda, et al., 2015). Fermentation can reduce phytic acid contents due to enzymatic activity of microbial phytases (Hashemi, Gholamhosseinpour, & Mousavi Khaneghah, 2019). However, reduction may also be the results of endogenous phytase activity, where phytase is primarily localized in the protein bodies (Jonathan, 1991). The reduction of phytic acid after fermentation was observed minor in the CF and CSF fractions (Table 5-2), which is consistent with the observations from Coda, et al. (2015). However, a larger decrease in phytic acid was observed in the chickpea protein-enriched fraction after fermentation.

The degree of soluble protein hydrolysis (DH) increased ($P < 0.05$) upon fermentation due to proteolytic activity. An increase of approx. 59%, 50.4%, and 11.8% was observed for cf-CPF, cf-CSF, and cf-CF, respectively. Bacterial fermentation may lead to structural protein modifications and as a result better accessibility of digestive enzymes to the substrate. Moreover, bacteria can partially

break down intact proteins, which leads to an increase in the concentration of free amino groups (Coda, et al., 2015; Yadav & Khetarpaul, 1994). Interestingly, samples fermented with the mixed culture did not always yield the highest DH. A possible explanation could be that the presence of non-proteolytic bacteria competes with proteolytic bacteria, shifting the balance towards lower proteolytic activity in the dough. The degree of protein hydrolysis of cf-CSF was observed higher compared to that of the cf-CPF. A similar trend was also reported by Coda, et al. (2015), claiming that the in vitro protein digestibility of protein-enriched sourdough from faba bean was lower than that of starch-enriched sourdough. A higher concentration of components like ANFs and phytic acids that affect the protein digestibility in the protein rich fraction may possibly explain the lower degree of hydrolysis in this fraction (Rosa-Sibakov, Re, Karsma, Laitila, & Nordlund, 2018). Besides, the quick acidification impedes extensive bacterial growth and thus possibly synthesis of microbial protease.

5.3.4.3 Techno-functional properties

The techno-functional properties (WHC and foaming capacity) of the fractions and fermented fractions were evaluated to analyse the effect of the fermentation on their application in food products. The results are summarized in Table 5-2.

The highest WHC was observed for the CPF, whereas the CSF had the lowest value, which is related to the higher content of fibre and protein in the protein-enriched fraction (Figure 5-1) (Du, et al., 2018; Sosulski & McCurdy, 1987). Fermentation increased the WHC for all chickpea fractions, by protein hydrolysis which exposes more hydrophilic groups (Chandra-Hioe, et al., 2016; Idowu, Benjakul, Sinthusamran, Sookchoo, & Kishimura, 2019). Another possible reason may be the modification of the dietary fibres (Jin, et al., 2018). Based on the API results, the ability of the *Pediococcus* strains to utilize D-cellobiose indicates that the strains can metabolize non-starch polysaccharides, and thus may slightly hydrolyse the fibre, which opens and hydrophilizes the fibres.

The foaming capacity was evaluated by the change in foaming volume over 60 s after whipping the suspended flours or powders. The foaming capacity increased with increasing protein content for the original chickpea fractions. After fermentation the foaming capacity decreased by ~50% for all fractions. This is caused by partial hydrolysis of the proteins during fermentation (Chandra-Hioe, et al., 2016; Mustafa, He, Shim, & Reaney, 2018). Of course, one needs to bear in mind that the foaming capacity is also related to the pH and the fermentation time (Çabuk, Stone, Korber, Tanaka, & Nickerson, 2018).

5.4. Conclusion

Chickpea protein-enriched fractions were obtained by dry fractionation and then subjected to solid-state fermentation with autochthonous *Pediococcus spp.*, obtained by a back-slopping procedure starting with a spontaneous fermentation. During fermentation the amounts of indigestible α-galactosides and phytic acid reduced with 90% and 17%, respectively. The water-holding capacity of the freeze-dried sourdough was larger compared to the original flour, whereas the foam capacity was reduced, due to partial proteolysis during fermentation.

The combination of dry separation and subsequent solid-state fermentation provided sourdoughs with improved nutritional value and reduced anti-nutritional factors, but also enhanced techno-functionality. The chickpea sourdoughs can be directly applied in bakery but may also be used as a supplement to other foods. The combination of dry fractionation and solid-state fermentation is sustainable, and no additives are used, which provides a 'clean label' final product.

Acknowledgements

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Appendix A

Table A-1. Sequencing results of selected LAB from natural fermented chickpea flour, fine and coarse fraction.

Selected from	Name	Similarity rate (%)	Source of the most similar sequence	Accession No.
Chickpea flour	*P.p.*1	95	*Pediococcus pentosaceus* strain HT-Z40-B1	KJ526944.1
	*P.p.*2	94	*Pediococcus pentosaceus* strain CE0.5	MH899228.1
	*P.p.*3	96	*Pediococcus pentosaceus* strain MPL25	KF697620.1
	*P.p.*4	96	*Pediococcus pentosaceus* strain JT3	KT719224.1
	*P.a.*5	97	*Pediococcus acidilactici* strain AA106	KY940561.1
	*P.a.*6	93	*P. acidilactici* strain AA106	
	*P.p.*7	95	*Pediococcus pentosaceus* strain CE7.20	MH899296.1
	*P.p.*8	94	*Pediococcus pentosaceus* strain SA8	KX017196.1
	*P.p.*9	96	*Pediococcus pentosaceus* strain HT-Z40-B1	KJ526944.1
	*P.p.*10	96	*Pediococcus pentosaceus* strain VMCU76F	LC035126.1
	*P.p.*11	93	*Pediococcus pentosaceus* strain MPL25	KF697620.1
	*P.p.*12	93	*Pediococcus pentosaceus* strain LD7	KX017195.1
	*P.p.*13	93	*Pediococcus pentosaceus* strain FB058	MF436194.1
Fine fraction	*P.a.*F1	96	*Pediococcus acidilactici* strain B_27LAB	MF480433.1
	*P.a.*F2	98	*Pediococcus acidilactici* strain HBUAS54160	MH701949.1
	*P.a.*F3	100	*Pediococcus acidilactici* strain P12 16S	MH512904.1
	*P.a.*F4	97	*Pediococcus acidilactici* strain KP10	JN592051.1
	*P.a.*F5	85	*Pediococcus acidilactici* strain KLB69-2	DQ294959.1

Selected from	Name	Similarity rate (%)	Source of the most similar sequence	Accession No.
	*P.p.*F6	98	*Pediococcus pentosaceus* strain NCIM2295	KY129623.1
	*P.p.*F7	97	*P. pentosaceus* strain NCIM2295	
	*P.p.*C1	96	*P. pentosaceus* strain NCIM2295	
	*P.a.*F8	99	*Pediococcus acidilactici* strain PB22	CP025471.1
	*P.a.*F9	98	*P. acidilactici* strain PB22	
	*P.a.*F10	98	*P. acidilactici* strain PB22	
	*P.a.*C2	96	*P. acidilactici* strain PB22	
	*P.a.*C3	97	*P. acidilactici* strain PB22	
	*P.a.*F11	97	*Pediococcus acidilactici* strain FM_24LAB	MF480434.1
	*P.a.*C4	93	*P. acidilactici* strain FM_24LAB	
	*P.a.*F12	95	*Pediococcus acidilactici* strain BP110	LC274607.1
	*P.a.*C5	96	*P. acidilactici* strain BP110	
	*P.a.*F13	98	*Pediococcus acidilactici* strain SK2A32	KY433797.1
	*P.a.*C6	94	*P. acidilactici* strain SK2A32	
	*P.a.*C7	84	*P. acidilactici* strain SK2A32	
	*P.a.*C8	95	*P. acidilactici* strain SK2A32	
	*P.a.*F14	84	*Pediococcus acidilactici* strain USIMHCMa	KX346613.1
	*P.a.*F15	92	*P. acidilactici* strain USIMHCMa	
	*P.a.*C9	83	*P. acidilactici* strain USIMHCMa	
Coarse fraction	*P.a.*C10	95	*Pediococcus acidilactici* strain SK2A32	KY433797.1
	*P.l.*C11	96	*Pediococcus lolii* strain CFR2298	KT315923.1
	*P.l.*C12	92	*P. lolii* strain CFR2298	
	*P.a.*C13	89	*Pediococcus acidilactici* strain SMVDUDB2	MK280750.1
	*P.a.*C14	81	*Pediococcus acidilactici* strain AZZ5	KY584255.1
	*P.sp.*C15	96	*Pediococcus sp.* strain L2	MG591705.1
	*P.a.*C16	78	*Pediococcus acidilactici* strain FMAC31	KF060262.1


Products
Plant
Protein
Chickpea
Flour
Fermentation
LAB
Fine
Bread
Salt
Strains
Yeast
Fraction
Nature
Clean
Starch
Sour
Native
Verbascose
Ash
Dough
Mild
Wheat
Water
Raffinose
Milling
Solid
Coarse
pH
Flatulence
Stachyose
Structure
Time
Dry
RNA
DNA
Fibre
Life
Texture
Shelf
Oligosaccharides
Green
Fat
Oil
Flavour
Function
Taste
Sensory
Nutrition
Energy
Potential
Stability
Healthy
Culture
Property
Possibility
Application
Strains

Chapter 6

Protein fortification of wheat bread using dry fractionated and fermented chickpea

This chapter has been submitted as Xing. Q., Kyriakopoulou, K., Zhang. L., Boom, R.M., & Schutyser, M. A. I. (2020). Protein fortification of wheat bread using dry fractionated and fermented chickpea for publishing.

Abstract

Chickpea protein-enriched ingredients were prepared by combining dry milling, air classification, and optionally solid-state fermentation. The fermentation was carried out with the autochthonous LAB strain *Pediococcus acidilactici* to reduce the level of antinutritional factors. A protein-enriched chickpea fraction and its sourdough were used to partially replace wheat flour with 20% to 30% w/w in wheat bread. The protein content of bread increased by 38.5% on dry basis with a 30% w/w replacement. As the substitution level increased from 0% to 20% and 30%, a longer dough mixing time was required, the specific volume of the bread decreased, and the crumb structure became denser. The levels of raffinose, stachyose, and verbascose in the sourdough bread were reduced by 75.4%, 97.6%, and 90.0% compared to the unfermented bread, respectively. With sourdough addition the crust showed less browning and exhibited a longer shelf life compared to that of the other breads.

6.1. Introduction

The growing world population and increasing prosperity require an increasing supply of dietary protein (Schutyser, et al., 2015). The use of more plant protein in our diet will reduce the use of primary agricultural resources and lead to lower greenhouse gas emissions compared to animal protein (Aiking, 2014; Mattila, et al., 2018). In this respect, legumes are an excellent source of dietary protein. The protein content of pulses like pea, bean, chickpea, lupine, and lentil is between 17 and 46%, while cereals such as wheat, maize, and sorghum only have a protein content of 8 to 13% (Foschia, Horstmann, Arendt, & Zannini, 2017; Nkhabutlane, du Rand, & de Kock, 2014). Moreover, regular consumption of pulses has been advised to mitigate the risk of cardiovascular diseases, diabetes, and high serum cholesterol (Asif, Rooney, Ali, & Riaz, 2013; Sokolowski, Higgins, Vishwanathan, & Evans, 2020). Bakery products made with wheat are amongst the most consumed staple foods for many ethnic groups. The incorporation of pulse flours in wheat bread can produce protein-enriched bread with increased nutritional value thanks to the well-balanced amino acid profile and high fibre content of pulses (Boukid, et al., 2019).

Chickpeas have been applied in bakery products for its moderate calories, high protein (17 - 22%), complex carbohydrates, dietary fibres, vitamin, and less beany flavour (Asif, et al., 2013). Previous studies reported that wheat dough that was fortified with up to 10% chickpea flour still had a non-sticky surface and yielded a bread crust with similar colour as plain wheat bread (Boukid, et al., 2019). However, higher levels of replacement lead to decreased dough stability and resistance, resulting in a stiffer bread with smaller volume. These effects may be explained by the dilution of the gluten content and interactions among fibre components, water, and gluten. Overall, studies show that breads can only be reasonably prepared with low levels of replacement, even though fermentation and bread improvers (e.g. xanthan gum) could somewhat mitigate the effects (Shrivastava & Chakraborty, 2018). To further increase the chickpea protein level in bread without impacting the

quality too much, some studies reported the addition of wet-isolated protein (Boukid, et al., 2019). However, wet isolation of proteins from legumes is resource intensive and creates significant waste streams.

In this study, we therefore propose the incorporation of protein-enriched chickpea fractions obtained via dry fractionation, which involves the combination of dry milling and air classification. Dry fractionation requires much less energy, produces less waste and retains the native protein functionality better than conventional wet extraction methods (Xing, et al., 2020). However, the presence of anti-nutritional factors (ANFs) such as flatulence-causing oligosaccharides (raffinose, stachyose, and verbascose), phytic acid, and trypsin inhibitors in air-classified raw protein fractions is undesired for food application (Khattab, et al., 2009). Therefore, solid-state fermentation (SSF) is used as a cost-effective approach to eliminate these ANFs in the chickpea fraction (Shrivastava, et al., 2018). In our previous study, autochthonous lactic acid producing bacteria (LAB) present in chickpea protein-enriched (fine) fractions were identified. A strain that belongs to the *Pediococcus acidilactici* was selected as starter culture based on its ability to selectively metabolize raffinose oligosaccharides (Xing, et al., 2020).

A dry-enriched chickpea protein-enriched (fine) fraction was first produced by milling and air-classification. Subsequently, an autochthonous *P. acidilactici* strain was inoculated to that fraction which was then fermented in solid-state to obtain chickpea sourdough. Finally, wheat flour was partially replaced by protein-enriched chickpea or its sourdough during bread making, and the quality of the obtained breads was evaluated on properties in terms of protein content, ANFs content, colour, specific volume, texture, and shelf-life, etc.

6.2 Materials and methods

6.2.1 Materials

Chickpea (Cicer arietinum) seeds (Kabuli) were purchased from a retailer (Biologische Toko, The Netherlands). The protein, carbohydrate, fat, and ash contents were 20.6 g/100 g, 70.2 g/100 g, 6.0 g/100 g, and 3.2 g/100 g on dry basis, respectively. The seeds were stored in a tightly screwed polyethylene container at 4°C. Wheat flour (Jumbo, The Netherlands), salt (JOZO, The Netherlands), and yeast (Dr. Oetker, The Netherlands) were bought from a local supermarket. The protein, carbohydrate, fat, and ash contents of the wheat flour were 12.6 g/100 g, 79.9 g/100 g, 1.3 g/100 g, and 4.0 g/100 g on dry basis, respectively. The flour was stored in a dry cabinet at room temperature.

6.2.2 Preparation of chickpea protein-enriched (fine) fraction

Whole chickpea seeds were coarsely ground into chickpea grits with a pin mill (LV 15M, Condux-Werk, Germany). The chickpea grits were then milled into chickpea flour with a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany) at ambient temperature. The feed rate was 0.5 kg/h, the air flow rate was 40 m^3/h, and the air classifier wheel speed was fixed at 2900 rpm (Xing, et al., 2020). A chickpea protein-enriched (fine) fraction was obtained by air classification with an ATP50 classifier (Hosokawa-Alpine, Augsburg, Germany) at ambient temperature. The feed rate was 0.2 kg/h, the air flow rate was 52 m^3/h, and the speed for the air classifier wheel was adjusted to 10,000 rpm (Xing, et al., 2020).

6.2.3 Optimization of bread formulation

The bread was prepared by the straight dough method (de Oliveira, da Silva Lucas, Cadaval, & Mellado, 2017). Wheat bread with the recipe that is reported in Table 6-1 was used as a standard. The chickpea protein-enriched fraction was blended with wheat flour in different ratios (20:80 and 30:70). The addition of water was optimized for each formulation with a farinograph (Brabender GmbH & Co. KG,

Duisburg, Germany) based on the AACC method 54-21. For each measurement, 50 g of mixed flour was loaded in the farinograph mixer chamber. With continuous mixing at a speed of 63 rpm for 0.5 min, a certain amount of water was added. A water bath was connected to keep the farinograph at 30°C during the measurement (Zhang, et al., 2019). Each farinograph experiment was recorded using WINMIX software for 15 min. The water absorption (the amount of water taken up by flour to yield the desired dough resistance), the dough development time (the time required from the moment the water is added to the maximum torque value is reached), and the dough stability (the time that dough consistency is kept at 0.98 Nm/500 Brabender Units) of the different formulations were determined. Duplicate measurements were performed for each formulation.

6.2.4 Preparation of chickpea sourdough

Based on previous work, the *Pediococcus acidilactici* strain AA106 (Accession number: KY940561) was selected to ferment chickpea protein-enriched sourdough (Xing, et al., 2020). To be specific, a loop of bacteria taken from a frozen stock (-80°C) was inoculated into 10 ml De Man, Rogosa and Sharpe (MRS) broth (Merck, Germany) tube and incubated for 24 h at 30°C. One milliliter of MRS broth cell suspension was transferred to a new 9 ml MRS broth tube and incubated at 30°C for another 24 h. The cells were then harvested by centrifuging 1 ml MRS broth cell suspension at 10,000 rpm for 5 min. After discarding the supernatant, the pellet was re-suspended in 1 ml peptone physiological salt solution (PPS) (Tritium Microbiologie B.V., The Netherlands) to wash the cells. The centrifugation step was repeated, and the starter culture was prepared by suspending the cell pellet in 1 ml PPS. For 100 g of chickpea protein-enriched fraction, 50 ml of Milli-Q water and 0.5 ml of starter culture solution was added and well mixed manually. The dough was anaerobically incubated at 37°C for 72 h.

6.2.5 Bread making

The wheat flour, chickpea protein-enriched fraction, and yeast were first blended in a bowl, followed by adding 30°C water with salt dissolved. The mixture was kneaded for 9 min by hand until a consistent dough was formed. Proofing was performed in a 30°C electric oven (Memmert GmbH, Germany) for two times and each proofing step took one hour. After the first proofing, the dough was taken out once for shaping to expel extra air. The dough was baked at 200°C. To follow the baking process as function of time, multiple breads were baked for 30 min and breads were taken out with a 5-min time interval for evaluation. The correct baking time was established by evaluating the crust colour.

6.2.6 Evaluation of chickpea sourdough bread

6.2.6.1. Compositional analysis

The nitrogen content of the breads was determined using the Dumas method (FlashEA 1112 series, Thermo Scientific, The Netherlands). A nitrogen conversion factor of N × 5.71 was used for the calculation of the protein content. The oil, ash, and moisture contents of bread were determined by methods AACC 30-25.01 (1999), AACC 08-01 (1983), and AACC 44-15.02 (1999), respectively. The content of carbohydrate was calculated by the difference.

6.2.6.2. Raffinose family oligosaccharides (RFOs)

High-performance anion exchange chromatography (HPAEC) was used to analyse the content of raffinose, stachyose, and verbascose in bread. A Dionex ICS-5000 system (Thermo Fisher Scientific Inc., USA) was used with a CarboPac PA1 (2 mm × 250 mm) guard column (Thermo Fisher Scientific Inc., USA) and a pulsed amperometric detector (HPAEC-PAD). The RFOs were extracted by dispersing 1 g of bread crumb in 30 ml of demi-water and stirring for 30 min. Subsequently, the extract was transferred to a tube and centrifuged at 10,000 rpm for 5 min. 500 µL of the supernatant was transferred into a 2 ml centrifuge tube and the proteins were precipitated by adding 500 µl Carrez reagent (250 µl Carrez A followed by 250 µl

Carrez B). After centrifugation at 10000 rpm for 5 min, 200 µl of the supernatant was transferred into a vial (0.3 ml PP Short Thread Vial 32 × 11.6 mm clear, BGB, Switzerland) for analysis. Standard solutions in the range of 1 - 20 mg/L were prepared for sugar identification and quantification. Other parameters setpoints were: injection volume was 10 µL and the eluent flow rate was 0.3 ml/min. Chromeleon 7.0 software was used for numerical integration of the peak surface. All HPAEC analyses were carried out in duplicate.

6.2.6.3. Compositional analysis

The colour of the crumb and crust of the bread was analysed with a CR-400 colorimeter (Konica Minolta Inc., Japan). Results were expressed as CIE L^*, a^*, and b^* value, in which L^* represents lightness component, a^* represents green (-) and red (+) while b^* value represents blue (-) and yellow (+) (Zhang, et al., 2019). The colorimeter was corrected with a calibration plate before measuring. Each sample was measured at three different spots on crust and crumb, respectively.

6.2.6.4. Specific volume

The specific volume of bread was determined by AACC International Approved Methods 10-05.01 with some modifications. Couscous was used in the displacement method. The bread was weighed 1 h after baking. The specific volume of the bread was calculated and expressed as mL/g.

6.2.6.5. Texture profile analysis

A texture analyser (TA.XTplusC, Stable Micro Systems Ltd., UK) equipped with a cylindrical probe of 40 mm was used to analyse the textural properties of bread. A crumb cube (1 × 1 × 1 cm) cut from the bread centre was compressed to up to 40% strain with a speed of 2 mm/s. Other parameters were set as: pre-speed 1 mm/s, post-speed 2 mm/s, trigger force 5 g, and a delay of 30 s between two compressions. The hardness, springiness, cohesiveness, chewiness, and resilience were determined. Measurements were conducted in duplicate for each bread.

6.2.6.6. C-Cell analysis

The cellular structure of bread was analysed with a C-Cell imaging system (Calibre Control International Ltd., Warrington, UK). The analysis of the bread samples was performed immediately after slicing with a rotary disc blade cutter (Sroan, Bean, & MacRitchie, 2009). Parameters determined include the surface area, cell diameter, and cell wall thickness.

6.2.6.7. Confocal laser scanning microscopy

Dough samples were cut into 60 µm thick slices with a cryo-microtome (CR 50-H Bio-med, Heidelberg) and placed on a glass slide. An aqueous solution of rhodamine B (0.002%, w/v) and fluorescein isothiocyanate (FITC) (0.05%, w/v) was used for staining the gluten and starch, respectively. A cover slip was dropped carefully to avoid air bubbles. After staining in dark conditions for 30 min, an LSM 510-META confocal microscopy (Carl ZEISS, Germany) was used for observation. The λ_{exc} and λ_{emi} of FITC were 488 nm and 525 nm. The λ_{exc} and λ_{emi} of rhodamine B were 543 nm and 627 nm.

6.2.6.8. Microbiological properties

The microbiological stability of wheat bread and chickpea fortified bread was evaluated following (Belz, et al., 2019) with some modifications. Bread samples were sliced, and 5 pieces of each bread were exposed to air for 10 min. Each of the slices was then packaged in ziplocked polyethylene bags and stored at 30°C for 5 days for the spoilage experiment, and slices were sampled every 24 h. One gram of sample was serially diluted in 9 ml PPS. Subsequently, 50 µl of dilution was spread out on plate count agar (PCA) (Merck, Germany) plates using a spiral plater (Eddy Jet IUL, Neutec Group Inc., USA). The plates were incubated at 30°C for 24 h.

6.2.7 Statistics analysis

Means and standard deviations were calculated using SPSS (Version 22.0, IBM, USA) statistical software. One-way ANOVA was performed to evaluate the effect of

chickpea sourdough addition on properties of bread. Duncan's test at a 95% confidence level was applied to verify the differences between groups.

6.3 Results and discussion

Partial replacement of the wheat flour by a chickpea protein-enriched fraction enhances the protein content in final bread products, but since chickpea protein has different properties compared to wheat gluten such as solubility and water holding capacity (Jagannadham, Parimalavalli, Babu, & Rao, 2014), the addition will influence the dough properties. Therefore, the amount of water in the recipe of chickpea-fortified bread is optimised. Farinograph measurements were applied to establish the appropriate amount of water at substitution levels of 20% and 30%. Subsequently, the browning of bread was characterized as a function of baking time to determine the baking condition to obtain an acceptable bread quality. Finally, with the optimized recipe and baking conditions, the nutritional, physical, microstructural, and microbiological properties of the composite chickpea-wheat bread were evaluated.

6.3.1 Recipe development

The dough development of composite chickpea-wheat flour mixtures is affected due to the addition of the chickpea fraction, which is high in protein and non-starch polysaccharides (e.g. pentosan). The wheat flour is diluted by the introduction of the fraction, while adding the right amount of water is crucial for the development of the gluten network (Mohammed, Ahmed, & Senge, 2014). A farinograph was used to record the resistance to deformation of the dough during mixing. This was done to establish the optimal ratio of water to flour (leading to a dough resistance of 500 BU) and record the dough development time and dough stability. The water sorption decreased with more chickpea from 61.9% to 52.9% (Table 6-2), which means less water was needed in the dough to achieve the same dough consistency during mixing/kneading with chickpea fraction added. If the water addition was kept

the same as for wheat dough, the doughs with chickpea fraction yielded a very sticky dough that was difficult to knead. The high-water sorption of gluten compared to other proteins explains why less water is needed with high-protein chickpea dough (Yousseff, Salem, & Abdel-Rahman, 1976). Earlier, a decrease in water sorption was also observed for wheat flour that was partially substituted with lentil flour (Portman, et al., 2018). The dough-development time gradually increased as wheat flour was replaced by the chickpea protein-enriched fraction, indicating the dough took more time to reach its maximum consistency. This is due to a weakening of the gluten network due to dilution and hydration. At the same time, the dough stability time increased after the addition of chickpea fraction. According to Zafar et al. (2020), the glycoproteins (lectin, protease inhibitor) presented in chickpea fine fraction are responsible for the improved dough stability time. Based on the farinograph results, the adjusted recipes for bread with 20% and 30% chickpea protein-enriched fraction were determined (Table 6-1). The recipe for sourdough chickpea bread is similar to the normal one, except that less water is added to compensate for water added for the sourdough fermentation at a later stage.

Table 6-1. Formulations of wheat bread (control) and bread prepared with fermented and unfermented chickpea protein-enriched fraction.

Ingredients	Wheat bread (control)	Bread prepared with chickpea protein-enriched fraction		Bread prepared with fermented chickpea protein-enriched fraction	
		20%	30%	20%	30%
Wheat flour (g)	60.2	50.0	45.7	50.0	45.7
Chickpea protein-enriched fraction (g)	—	12.5	18.7	—	—
Sourdough (g)	—	—	—	18.8	29.4
Water (g)	37.3	35.0	32.2	28.7	22.4
Yeast (g)	1.5	1.5	1.5	1.5	1.5
Salt (g)	1.0	1.0	1.0	1.0	1.0

Table 6-2. Farinograph data of wheat dough and doughs with added chickpea protein-enriched fraction. Data marked with different lowercase superscript in the same column indicate significant differences ($P < 0.05$).

	Water absorption (ml/100 g)	Dough-development time (min)	Dough stability (min)
Wheat dough	$61.9^{b} \pm 0.3$	$1.5^{a} \pm 0.1$	$4.2^{a} \pm 0.1$
Dough with 20% chickpea protein-enriched fraction	$57.2^{ab} \pm 1.2$	$3.0^{ab} \pm 0.5$	$14.0^{b} \pm 0.5$
Dough with 30% chickpea protein-enriched fraction	$52.9^{a} \pm 2.9$	$3.6^{b} \pm 0.6$	$12.9^{b} \pm 0.7$

6.3.2 Browning as function of baking time

Surface browning of bread is caused by non-enzymatic browning including Maillard reaction, which is an important indicator of the bread quality. To follow the browning of the breads as function of the baking time, breads were baked at 200°C. As shown in figure 6-1, the colour of the crust became browner with longer baking times. Bread prepared with chickpea had a significantly darker colour than pure wheat bread (control) and the colour deepened as the level of substitution increased. This is consistent with previous findings, where researchers attributed the colour change to the larger lysine content of chickpea. Lysine is the most active essential amino acids to react with reducing sugar in the Maillard reaction (Mohammed, et al., 2014). Moreover, fructose, glucose, and sucrose can caramelize as the temperature of the crust approaches the oven temperature, adding to the colouration (Ajandouz, Tchiakpe, Ore, Benajiba, & Puigserver, 2001; Zhang, Taal, Boom, Chen, & Schutyser, 2018). The formation of brown pigments gave the breads a yellow-brown colour. Interestingly, it was observed that with the same substitution level, the breads prepared with fermented chickpea protein-enriched fraction had a somewhat lighter colour than the unfermented ones, with the differences becoming larger as the baking time increased (Figure 6-1). This can be explained by the metabolization of reducing sugars like glucose and fructose by the LAB during fermentation (Hatzikamari, Kyriakidis, Tzanetakis, Biliaderis, & Litopoulou-tzanetaki, 2007; Xing, et al., 2020), and thus a lower availability of reactants for the Maillard reaction. Besides, a lower pH can slow the Maillard reaction and the citric acid that is produced during fermentation may de-colour phenolic compounds like catechins and tannins which are naturally present in chickpea, and that are responsible for the characteristic yellow colour (Ajandouz, et al., 2001; Güémes-Vera, Peña-Bautista, Jiménez-Martínez, Dávila-Ortiz, & Calderón-Domínguez, 2008). In further experiments, a baking time of 20 minutes was selected. Baking for longer than 20 minutes caused too extensive browning of the crust, which decreases the

acceptability of the chickpea-enriched bread (Castro, Oblitas, Chuquizuta, & Avila-George, 2017).

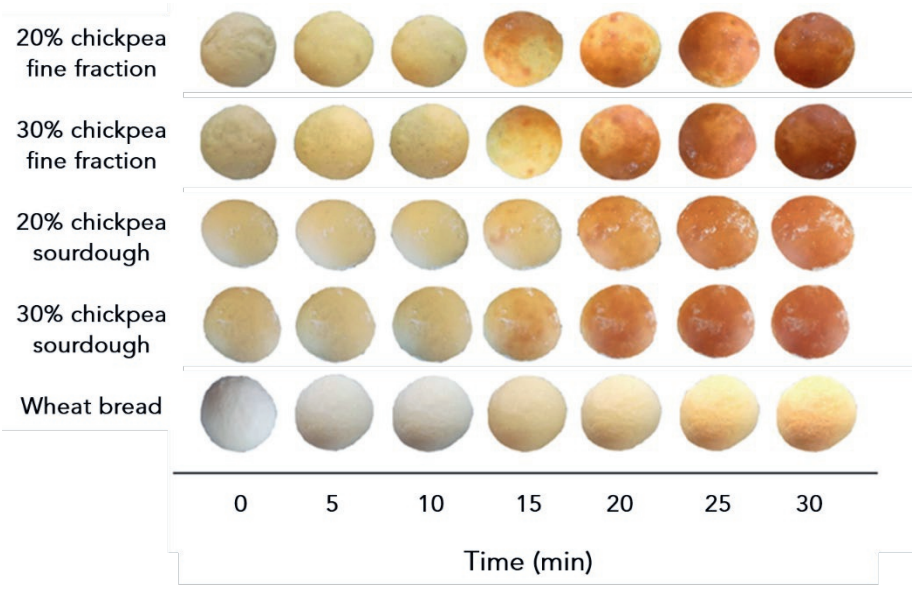


Figure 6-1. The colour change of bread baked over time (up to 30 min) at 200°C.

6.3.3 Nutritional properties

6.3.3.1. Protein content

A chickpea protein-enriched fraction produced by air classification had a protein content of 31.4 g/100 g on dry basis, which is 57% higher than that of chickpea flour. Therefore, wheat flour replacement by chickpea protein-enriched fraction is more efficient compared to chickpea flour in terms of protein improvement. In comparison to wheat bread, the protein content of bread prepared with 20% and 30% of chickpea protein-enriched fraction was increased by 26.5% and 38.5%, respectively (Figure 6-2). The chickpea breads have improved protein content compared to wheat bread and may be classified as "source of protein" (requiring

that at least 12% of the energy value of food is provided by protein) according to Regulation (EC) No 1924/2006. Bread supplemented with fermented chickpea protein-enriched fraction showed similar protein content as found with the unfermented ones at the same substitution level.

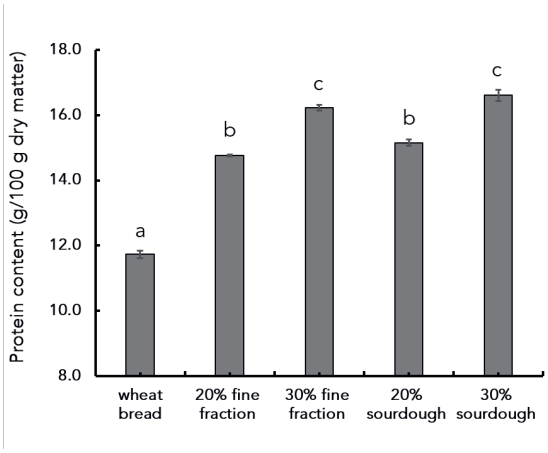


Figure 6-2. The protein content of wheat bread (control) and bread prepared with fermented and unfermented chickpea protein-enrich fraction. Data marked with a different lowercase superscript in the same column indicate significant differences ($P < 0.05$).

6.3.3.2. Content of oligosaccharides

The presence of flatulence-causing oligosaccharides reduces the acceptability of chickpea fortified bread (Veenstra, et al., 2010). Solid-state fermentation can reduce the content of oligosaccharides in chickpea fraction (Galli, et al., 2019). Besides, oligosaccharides are not very heat-stable and start decomposing above 200°C (Forgo, Kiss, Korózs, & Rapi, 2013), combining fermentation and heat treatment can contribute to a large reduction of the oligosaccharides (Khattab, et al., 2009). Figure 6-3 shows that with the use of fermented chickpea protein-enriched fraction instead of the same unfermented fraction, the content of raffinose, stachyose, and verbascose decreased by 75.4 ± 0.3%, 97.6 ± 0.8%, and 90.0 ± 2.1%, respectively.

This means that a much larger intake will be needed to cause flatus. Earlier studies reported a reduction of 63.2% for oligosaccharides of the raffinose family in bread prepared with fermented chickpea flour in which a foreign *Lactobacillus plantarum* strain was used (Galli, et al., 2019). In our study the reduction was much higher because *Pediococcus acidilactici* were isolated from chickpea itself (Xing, et al., 2020), thus are best adapted to the substrate. The levels of oligosaccharides in chickpea sourdough bread in fact were close to that of wheat bread, indicating that the fermentation effectively removes these anti-nutritional factors in legume fortified bread to acceptable levels.

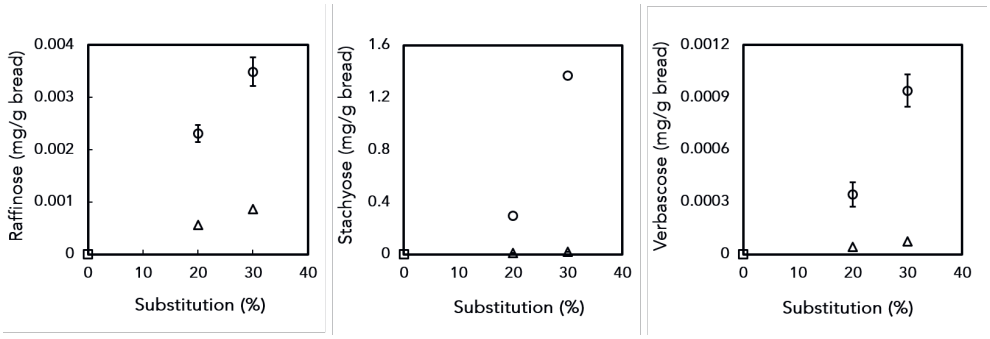


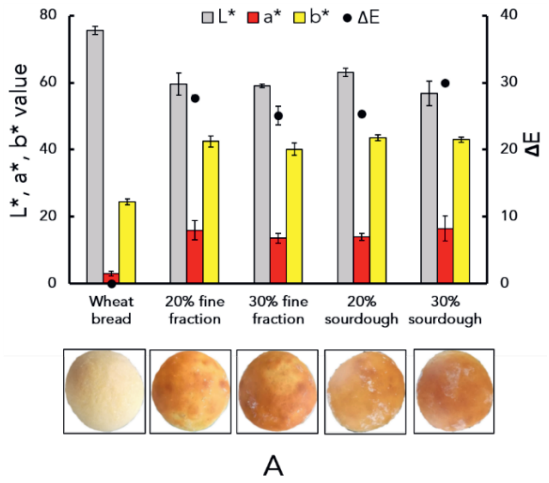
Figure 6-3. The content of oligosaccharides (raffinose, stachyose, and verbascose) in wheat bread (□), bread prepared with unfermented (○), and fermented (△) chickpea protein-enriched fraction. The error bars represent the standard deviation. Some error bars are not visible due to the small standard deviation values.

6.3.4 Physical properties

6.3.4.1. Colour

The colour of the bread crust and crumb was expressed in CIE *L**, *a**, and *b** values, corresponding to brightness, green (-)/red (+), and blue (-)/yellow (+), respectively. The colour difference (Δ E) of the bread was calculated with the standard being the wheat bread. The *L** value of the crust decreased and the *a** and *b** parameters increased, as wheat flour replaced by chickpea ingredients (Figure 6-4 A), indicating

that a redder and yellower crust was obtained for chickpea fortified bread. The darker crust colour was attributed to increased Maillard reaction due to more reducing sugar and higher lysine content (Mohammed, Ahmed, & Senge, 2012; Yousseff, et al., 1976). However, the colour difference caused by different substitution levels was not obvious until after 20 minutes baking time. As is also demonstrated in figure 6-1, the effect of the fermentation on browning was more significant when baked at a longer time (30 min). The crumb was less red and yellow compared to the crust, while differences between wheat bread and chickpea enriched breads were small (Figure 6-4 B). In summary, the substitution of chickpea protein-enriched fraction had a larger impact on the crust colour than on the crumb, and fermentation did not significantly affect the colour compared to that of the unfermented ones.



A

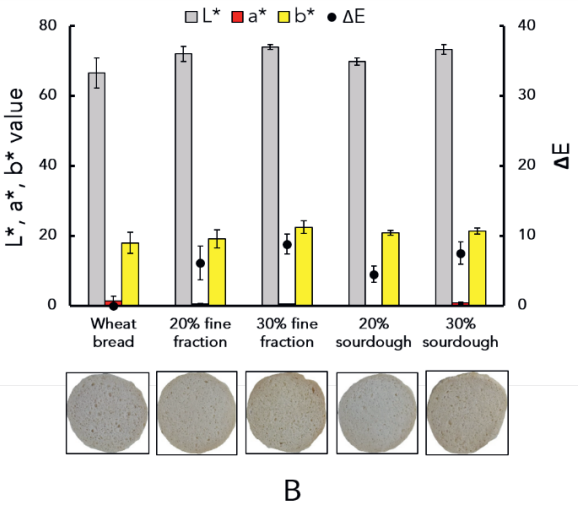


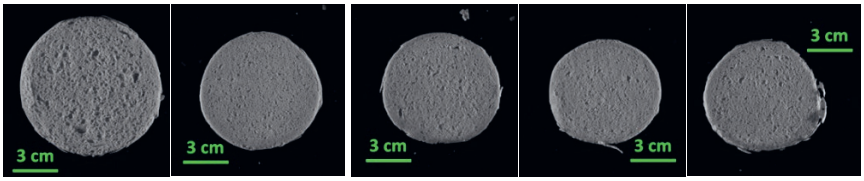
Figure 6-4. CIE *L**, *a**, *b** of bread crust (A) and crumb (B), and the colour difference (Δ E) between the control (wheat bread) and different substitutions. The error bar represents the standard deviation. Photos of corresponding crust and crumb are shown beneath the bar charts.

6.3.4.2. Other physical properties

An overview of the measured physical properties of the breads is provided in table 6-3. The volume, cellular structure, and texture of bread changed with the addition of chickpea ingredients. In general, chickpea fortified bread had a significantly ($P < 0.05$) smaller specific volume than that of pure wheat bread. This can be explained by the weakened gluten structure and the decreased dough elasticity (Mohammed, et al., 2012). The structure of the breads was visualized by the C-Cell imaging system, since the cellular structure contributes to bread appearance and texture (Millar, et al., 2019). Pure wheat bread showed a large average cell diameter, suggesting a soft and fluffy crumb, while the chickpea fortified bread exhibited a smaller cell diameter and a higher total cell number per cm^2 indicating a denser crumb (Millar, et al., 2019). The cell wall thickness of the cells in the crumb was found to decrease upon the addition of chickpea, which coincided with a decrease in specific bread

volume (Table 6-3). The weakened gluten network due to the introduction of the chickpea fractions led to thinner cell walls, lower gas retention and thus smaller breads (Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2015). Bread with chickpea addition showed significantly ($P < 0.05$) higher hardness and chewiness, and the values increased as the substitution level increased. Finally, the sourdough bread had similar hardness and chewiness compared to the bread with non-fermented chickpea fraction.

Table 6-3. Cellular structure and texture of chickpea fortified bread compared to the wheat bread (control). Data in the same row marked with different lower-case letters indicate significant differences ($P < 0.05$).

	Wheat bread	20% chickpea fine fraction	30% chickpea fine fraction	20% chickpea sourdough	30% chickpea sourdough
Cross-section image					
Surface area (cm^2)	$66.88^{c} \pm 0.83$	$49.53^{b} \pm 0.29$	$44.03^{ab} \pm 1.65$	$42.30^{a} \pm 1.40$	$42.68^{a} \pm 2.98$
Cell diameter (mm)	$1.56^{b} \pm 0.00$	$0.96^{a} \pm 0.03$	$1.08^{a} \pm 0.06$	$1.07^{a} \pm 0.02$	$1.07^{a} \pm 0.09$
Cell wall thickness (mm)	$0.43^{a} \pm 0.01$	$0.37^{b} \pm 0.00$	$0.38^{b} \pm 0.01$	$0.37^{b} \pm 0.00$	$0.39^{b} \pm 0.00$
Total cell per (cm^2)	$79.9^{a} \pm 0.1$	$122.6^{c} \pm 3.6$	$110.8^{bc} \pm 4.8$	$121.3^{c} \pm 4.3$	$108.2^{b} \pm 2.2$
Specific volume (ml/g)	$2.44^{b} \pm 0.21$	$2.10^{ab} \pm 0.05$	$1.84^{a} \pm 0.03$	$1.95^{a} \pm 0.11$	$1.78^{a} \pm 0.04$
Hardness (N)	$6.01^{a} \pm 0.02$	$10.04^{b} \pm 1.50$	$13.86^{c} \pm 0.15$	$11.95^{bc} \pm 0.29$	$16.92^{d} \pm 1.63$
Chewiness (N)	$4.21^{a} \pm 0.01$	$6.06^{ab} \pm 0.58$	$7.62^{ab} \pm 0.37$	$10.50^{b} \pm 2.52$	$10.68^{b} \pm 1.55$
Cohesiveness (-)	$0.73^{b} \pm 0.00$	$0.68^{ab} \pm 0.03$	$0.64^{a} \pm 0.03$	$0.67^{ab} \pm 0.02$	$0.66^{ab} \pm 0.01$

6.3.4.3. Micro-structure of doughs and breads

CLSM was used to visualize the distribution of protein and starch in the dough matrix, which helps also to understand the effect of chickpea ingredients on the microstructure of the final bread. Starch was stained green by FITC and protein was stained red by rhodamine B. Since FITC is also reactive towards amino groups on proteins (Mariotti, Lucisano, Pagani, & Ng, 2009), proteins stain orange instead of red when the two dyes are used. In figure 6-5, the strand-like gluten structure was not observed directly (McCann & Day, 2013), yet the elongated protein clusters in the pure wheat dough (Figure 6-5a) are representative of it, and are more prominent than in the other doughs. The microstructure of the doughs prepared with different levels of chickpea ingredients is similar, with protein aggregates distributed evenly and a large number of small air bubbles. Replacing the chickpea protein-enriched fraction with the sourdough did not lead to any clear changes in the microstructure of the doughs. Air is included during dough kneading and yielded a microstructure similar to that in a previous study (Bousquieres, Deligny, Riaublanc, & Lucas, 2014). All breads prepared with chickpea had a significantly smaller crumb gas cell diameter and a higher cell number density (Table 6-3). Because the doughs were pictured by CLSM before proofing, we expect that the difference in structure between wheat and chickpea enriched bread emerges during proofing.

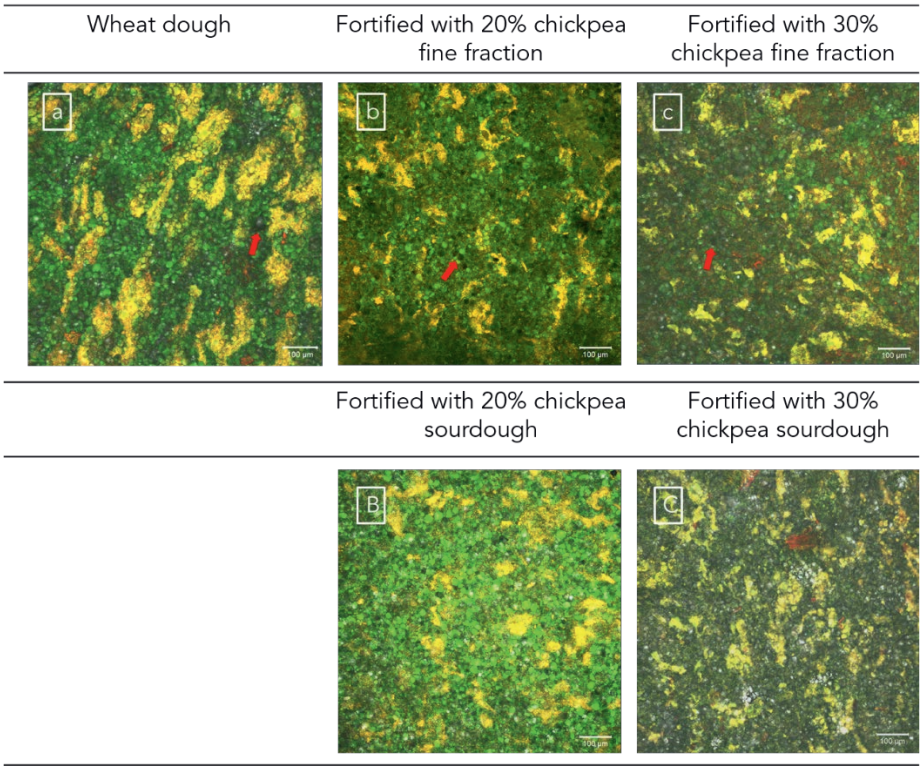


Figure 6-5. CLSM pictures of wheat dough (a), wheat dough added with 20% (b) and 30% (c) chickpea protein-enriched fraction, and dough added with 20% (B) and 30% (C) sourdough. Proteins are stained red/orange, starch granules are stained green, and air bubbles are in black. The white bar represents 100 µm. Red arrows indicate air bubbles.

6.3.5 Microbiological properties

In figure 6-6, the microbiological properties of bread with added fermented chickpea protein-enriched fraction were compared with that of unfermented chickpea protein-enriched bread and wheat bread (control). Plate count agar was used in this study for the enumeration of spoilage bacteria of bread during a period of 5 days. The sourdough bread displayed the lowest colony number among all five samples during 5 days of storage (Figure 6-6). Also, no mold growth was observed

(results not shown). This is in contrast to the other breads where the total number of colonies dramatically increased to 5.3 - 5.7 log CFU/g bread within 24 h and reached 7.0 - 8.4 log CFU/g bread over five days.

The lower total colony count for sourdough bread is due to the accumulation of organic acids during LAB fermentation hampering the growth of spoilage microorganisms. The pH value of the dough with fermented chickpea protein-enriched fraction was 4.5 - 4.6, while the pH of the other doughs was 5.4 - 5.5. Moreover, LAB strains produce antimicrobial substances like hydrogen peroxide and bacteriocin, inhibiting both Gram-positive and Gram-negative pathogens (Olatunde, Obadina, Omemu, Oyewole, Olugbile, & Olukomaiya, 2018). The substitution amount of the sourdoughs did not influence the total colony count within five days of storage, even though at higher sourdough replacements one may expect a stronger inhibition on microbial growth (Hendek Ertop & Coşkun, 2018).

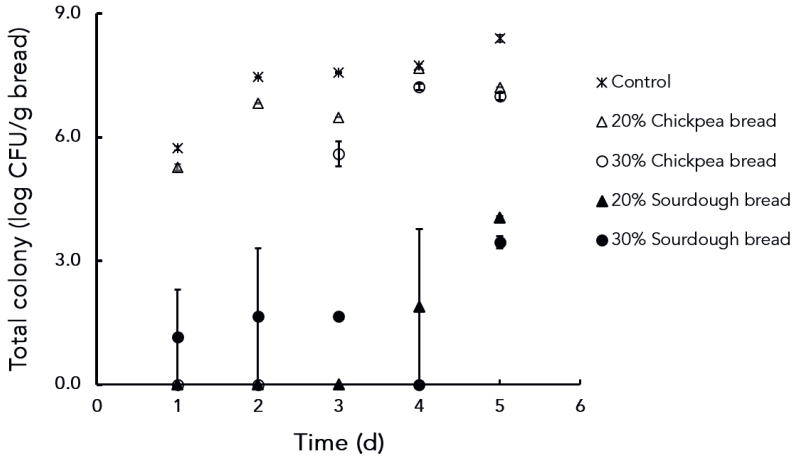


Figure 6-6. Total colony count of wheat bread (control), bread fortified with 20% and 30% of chickpea protein-enriched fraction or chickpea sourdough for a period of 5 days when stored at 30°C. The error bars represent standard deviation.

6.4 Conclusions

Wheat bread could be fortified by using dry fractionated and fermented chickpea protein-enriched (fine) fraction. The level of flatulence-causing anti-nutritional factors was strongly reduced by solid-state fermentation. Like the unfermented fraction, the addition of fermented chickpea protein-enriched fraction decreases the specific volume and increases the crumb hardness and firmness. Depending on the baking time, the sourdough yields less crust browning during baking, and less microbial spoilage over storage. Based on these results, the dry fractionated, protein-enriched and fermented chickpea ingredient have potential as ingredient for developing protein-enriched bakery products, while keeping the low environmental footprint of the ingredients obtained with dry separation.

Acknowledgements

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Chapter 7

General discussion

7.1. Introduction

Feeding the increasing world population will require a sustainable and economic way to produce dietary protein. In terms of water, chemical, energy, and raw material use, dry fractionation has major advantages compared to conventional wet extraction in the production of protein concentrates. Moreover, thanks to the mild processing conditions, the native protein functionalities of the concentrate are better preserved. However, the purity of the dry protein-enriched fraction is lower compared to that of wet extracted isolates, while often a significant amount of proteins is lost in the separation. Another issue is that anti-nutritional factors like α-galactosides and phytic acid are retained and concentrated together with the protein during dry fractionation and not washed out (Vogelsang-O'Dwyer, et al., 2020). Therefore, in the research reported in this dissertation, we investigated solid-state fermentation as an additional processing step to eliminate these undesired chemical compounds before the raw legume ingredients are applied in food. The study aimed to develop a sustainable processing route to convert legumes into functional protein-enriched ingredients with enhanced nutritional value. In this chapter, the main findings of the dissertation are discussed and processing schemes for sustainable protein enrichment from different types of legumes are visualized and discussed. Finally, the chapter ends with recommendations and an outlook for future research.

7.2 Discussion of the main findings

Dry fractionation has demonstrated its effectiveness on protein enrichment from legumes such as pea, lentil, chickpea, lupine, and navy bean (Pelgrom, Boom, et al., 2015; Tabtabaei, et al., 2016b; Wang, et al., 2016). Dry fractionation usually combines dry milling with air classification or electrostatic separation; the specific processing route depends on the type of legume. It was hypothesized that the

electrostatic separation of defatted soybean could be effective as it contains a mixture of especially protein and fibres. **Chapter 2** discussed the use of defatting before milling and electrostatic separation to dry-enrich the protein from soybean. As soybeans have a much higher fat content than lupin, a defatting step was proposed to be carried out before impact milling. Both organic solvent extraction and oil pressing could effectively remove the oil. The latter method compressed the microstructure of the soy, although it was observed that this had little influence on protein enrichment. Moderate milling speed (3000 rpm) effectively liberated the protein bodies whilst minimizing the agglomeration of small fragments. Tribo-electrostatic separation was conducted with different charging tubes. The highest yield was observed with a longer spiral charging tube thanks to prolonged residence time. After a single-step electrostatic separation with a spiral tube, a protein-enriched fraction with a purity of 52 g/100 g dry matter basis was obtained with a recovery of 62% of the defatted soy flour.

Based on these findings, the effect of the charging tube materials and diameter on the separation performance of a gluten-starch model mixture and of lupine flour was studied in **Chapter 3**. Off-line charging did not reveal any relation to the tribo-electrostatic series. Moreover, the overall charge of the gluten-starch mixture was not the same as the sum of the charge of the individual components, suggesting that particle-particle interactions are important. During electrostatic separation with the gluten-starch mixture, differences in protein enrichment were observed between tube materials. In contrast, for lupine flour, the separation performance was not related to the used tube materials. With all selected tube materials, the lupine protein content increased from 37 to ~65 g/100 g dry weight. A charging tube having a smaller inner diameter was more efficient in separation. The study showed that the separation performance cannot be simply predicted by off-line charging experiments, as the particle-particle interactions are mostly responsible for the charging of mixtures. This means that replacing the charging system with a fluidized bed could facilitate the charging and separation processes.

A two-step dry separation process was then applied to achieve protein enrichment from starch-rich legumes in **Chapter 4**. The hypothesis for this two-step process was that starch granules are more effectively separated from proteins with an air classification step, whereas proteins and fibres are more effectively separated during electrostatic separation. Pea, lentil, and chickpea were subjected to impact milling at optimized settings obtained from a previous study (Pelgrom, Boom, et al., 2015). Starch granules were effectively removed upon air classification. Subsequently, electrostatic separation was applied to remove fibres from protein-enriched fractions. A further protein enrichment (4.6 - 5.8%) was achieved for pea and lentil fine fractions. No protein enrichment was observed in the chickpea fine fraction due to the higher oil content and the smaller starch granules. An optimization of the entire process was conducted with pea. The balance between protein purity and yield obtained with an air-classifier wheel speed at 8000 rpm was found optimal. The electrostatic separation step was further optimized by recycling the fractions collected in the bags for a second separation pass. The protein-enriched fractions obtained from the optimized electrostatic separation resulted in a protein purity of 63.4% dry basis and a yield of 15.8 g/100 g, where 18.0% of protein from the pea was recovered from the fine fraction. Although these purities are sufficient, the recovery is still low due to significant powder losses in the current small-scale electrostatic separation device. In the future, this needs to be further addressed.

During air classification, anti-nutritional factors (ANFs) such as oligosaccharides and phytic acid are concentrated in the protein-enriched fraction as these are usually associated with fibres and/or proteins rather than with starch granules (Shevkani, et al., 2019; Tiwari, Gowen, & McKenna, 2011). The effect of solid-state fermentation on the presence of several ANFs in the flour, fine, and coarse fractions of chickpea was evaluated in **Chapter 5**. Additionally, the techno-functional and microbiological properties of chickpea sourdoughs obtained in this way were analysed and compared. Air classification resulted in a fine fraction with a protein content of 36%

(compared to 21% in chickpea flour) and the coarse fraction with a starch content of 57% (44% in chickpea flour). Via a back-slopping procedure, we found autochthonous lactic acid bacteria from *Pediococcus spp.* dominating in these chickpea flour and fractions. Specific strains were selected based on their ability to metabolize α-galactosides. Solid-state fermentation by selected LAB decreased the pH from 6.6 to 4.2 in 48 h. The content of stachyose and raffinose decreased by 88 to 99% after 72 h, while verbascose became undetectable. The content of the total phenolic compounds increased and the phytic acid content was reduced. The chickpea sourdough showed higher water holding capacity but a reduced foaming ability. Differences in smell, texture, and colour were also observed.

Chapter 6 presents an investigation for partial substitution of wheat flour by (fermented) chickpea fine fraction to enhance nutritional value of wheat bread. The impact of this substitution on the physical, nutritional, and microbiological properties of the bread was evaluated. The addition of 20% and 30% chickpea fine fraction could increase the protein content with 27% and 39%, respectively, but the fraction introduced flatulence-causing anti-nutritional factors (raffinose, stachyose, and verbascose). By using the solid-state fermentation step developed in our previous study the amount of these anti-nutritional factors could be lowered with 75 to 98%. Chickpea addition resulted in increased browning of the crust during baking, which was less with fermented chickpea probably because reduced sugars were digested by LAB during fermentation. As a result of chickpea addition, dough development time and stability time increased, indicating that a longer mixing time is required. Moreover, the specific volume of the bread decreased because the addition of chickpea interferes with the formation of the gluten network. In summary, with up to 30% substitution level, the physical properties of sourdough breads are similar to that of unfermented chickpea breads, while the content of ANFs decreased. Besides, the fermented chickpea bread had longer shelf-life than wheat bread and non-fermented chickpea bread because the low pH and generated organic acids retard microbial growth.

7.3 Conceptual processing routes for novel legumes ingredients

The learnings from the previous chapters are here compiled into a conceptual design of processing routes to create novel legume-based ingredients, using the potential of milling, air classification and electrostatic separation.

Legumes can be roughly divided into protein-rich (soybean and lupine), starch-rich (pea, chickpea, faba bean, etc.), and oil-rich (peanut) based on their compositions (Table 7-1). The differences in composition impact the selection and configuration of processing routes. A process scheme of dry fractionation for protein enrichment of legumes is shown in figure 7-1. The process starts with whole seeds or dehulled seeds. Dehulling is usually applied before milling to remove crude fibres, but starting with whole seeds is also practical as it has been proven that the dehulling process leads to only limited improvement on protein content (do Carmo, et al., 2020).

Table 7-1. Overview on the compositions of several commonly cultivated legumes (Elkowicz, et al., 1982; Ghavidel & Prakash, 2007; Piecyk, Wołosiak, Drużynska, & Worobiej, 2012; Rui, Boye, Ribereau, Simpson, & Prasher, 2011; Song, et al., 2016; Sosulski, et al., 1979; Davis & Dean, 2016; Settaluri, Kandala, Puppala, & Sundaram, 2012).

		Protein (g/100 g dry matter)	Starch (g/100 g dry matter)	Oil (g/100 g dry matter)
Protein-rich	Soybean	31.2 - 58.5	—	14.2 - 24.0
	Lupine	36.4 - 39.3	—	7.6 - 12.9
Starch-rich	Field pea	21.5 - 25.3	45.9 - 50.2	1.0 - 1.1
	Chickpea	22.1 - 23.1	42.6 - 50.0	5.5 - 7.4
	Lentil	19.5 - 26.5	47.8 - 52.8	0.7 - 0.9
	Dry bean	22.4 - 28.5	44.2 - 50.0	1.2 - 1.8
Oil-rich	Peanut	25.8 - 31.6	16.1 - 21.5	47.0 - 50.1

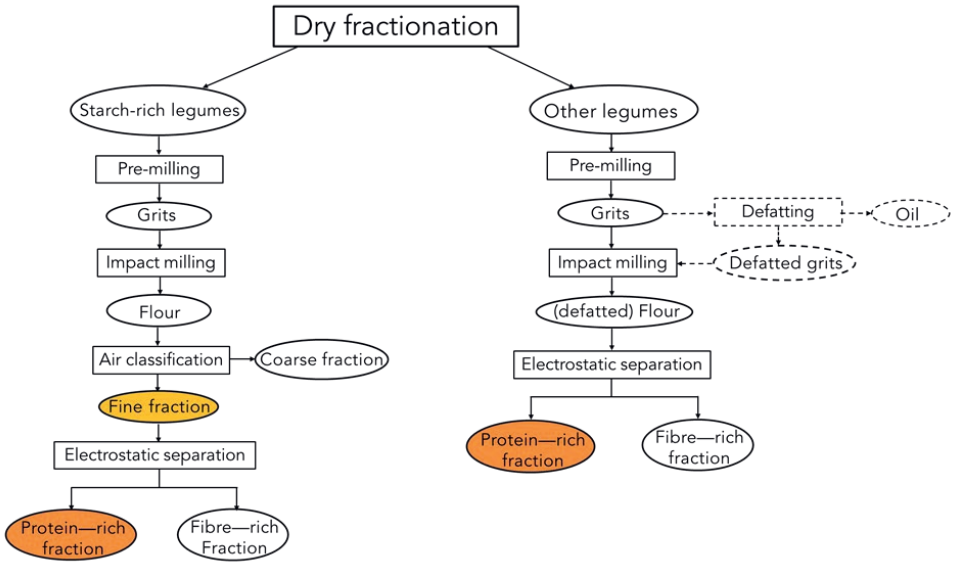

Figure 7-1. Conceptual design of dry fractionation for protein enrichment from all types of legumes. Defatting is an optional procedure for oil-rich legumes, indicated by dashed lines.

For all legumes, proper milling is the first step, in which the starch granules or in case of non-starchy legumes, the protein bodies are released from the cellular matrix. Either too little or too intensive milling will lead to insufficient protein separation by not dissociating components or inducing agglomeration of fine particles, which impairs the subsequent separation. For soybean seeds that contain more than a certain amount of oil, a defatting step before fine milling is necessary. The oil forms capillary bridges, aggregating particles, which will render the milling process more difficult and will inhibit the separation. After milling, for legumes such as lupine and soybean that do not contain starch granules, the flour is directly subjected to electrostatic separation. For starch-rich pulses like pea and lentil, air classification is conducted before electrostatic separation. This is because the starch

in pulses is charged the same as the protein and thus cannot be separated with this method. Air classification first separates protein and starch based on their different size and density. Subsequently, electrostatic separation is applied to separate protein and fibres according to their opposite charging polarity. In summary, the dry fractionation process is determined by the compositional and structural characteristics of the legume.

To achieve higher protein purity and yield, electrostatic separation with multiple passes can be applied. The middle fraction (collected from the two bags) obtained from the electrostatic separation can be collected for a second electrostatic separation step. The protein-rich fractions obtained from two electrostatic separation passes can be combined as a final product (Chapter 4). This will improve the balance between protein purity and yield. The multiple-step electrostatic separation can be applied to all types of legumes.

7.3.1 Dry fractionation of pea

To show the results of dry fractionation of legumes more intuitively, the mass flows of pea protein in each step during dry fractionation are visualized in a Sankey diagram. Chapter 4 provided the data used for figure 7-2 (upper graph). A total of 1,000 kg of dry yellow peas (dehulled) are used as the starting material. Pea flour is obtained after milling with a yield of 88 g/100 g pea on a dry basis and then subjected to air classification. Pea fine and coarse fractions are obtained with a yield of 28 and 51 g/100 g pea flour, respectively. The fine fraction is enriched in protein (green) and the coarse fraction is enriched in starch (blue). The fine fraction is subsequently subjected to electrostatic separation to obtain higher protein content. During this step, protein bodies are separated from other components (yellow) which are predominantly fibres. The final protein-enriched fraction has a mass yield of 13 g/100 g fine fraction, and a protein purity of 67 g/100 g. Additional protein can be recovered by applying a second-step electrostatic separation pass using the material that was collected in the collector bags. By mixing protein-enriched

fractions obtained from a two-step electrostatic separation, a product with a protein purity of 63.4 g/100 g dry basis was obtained (compared to ~32.4 g/100 g in pea). The combined protein-enriched fraction has a mass yield of 4.0 g/100 g pea, leading to a protein yield of 7.8% of total protein recovered from the yellow pea. The design and small size of the equipment used partially explains the high losses of material in the system. Specifically, the powder is lost in the relatively large separation chamber of the current bench-scale electrostatic separation equipment. In figure 7-2 (lower graph) an ideal picture is drawn of the electrostatic separation process assuming no material losses, which shows the potential gain if powder loss could be avoided. It would almost triple the yield of the final protein-enriched fraction. Better design, especially of the electrostatic separation equipment is required to improve these yields.

The coarse fraction (enriched in starch) produced from air classification and the protein-depleted fraction (enriched in fibre) from electrostatic separation may well be used for other purposes such as a thickening or dietary fibre supplementation. However, this discussion is beyond the scope of this dissertation.

7.3.2 Dry fractionation and bioprocessing of chickpea

To process the dry-enriched fractions into functional and healthy legume ingredients, bioprocessing of chickpea was studied. Dry fractionation of chickpea only consists of milling and air classification. The chickpea fine fraction (protein-rich) is subjected to solid-state fermentation with a small amount of water (fine fraction : water = 2 : 1). During a 72-h fermentation period, the amounts of α-galactosides reduced to acceptable levels. With a batch of 1,000 kg chickpea, a yield of up to 558 kg chickpea protein-enriched sourdough is expected (Chapter 5). The sourdough can be directly used as a food ingredient in products like fortified bread without the need of an additional drying step in between or dried and stored for later usage.

This combined technique is energy efficient since no drying is necessary. Of course, there should also be a useful purpose for the coarse starch-rich fraction. Following the proposed dry processing route, it is estimated that ~4 MJ energy is needed to produce one kg of protein on a dry basis. On the contrary, with wet fractionation, one requires approximately ~60 MJ to produce one kg chickpea protein isolates on a dry basis (Berghout, et al., 2015). This shows that the production of novel legume ingredients by dry fractionation is much more sustainable. Therefore, delivery of 1 kg of protein with reduced ANFs by dry fractionation and fermentation is more efficient than by using wet fractionation. The most effective use of resources results from applying the combination of processes that uses the least amount of energy in producing the products (Satō, 2004). Without considering the loss of materials during processing, the energy consumption from the processing can be reduced by optimizing the process efficiency of every unit. This can be achieved by optimizing the operation settings of milling and air classification.

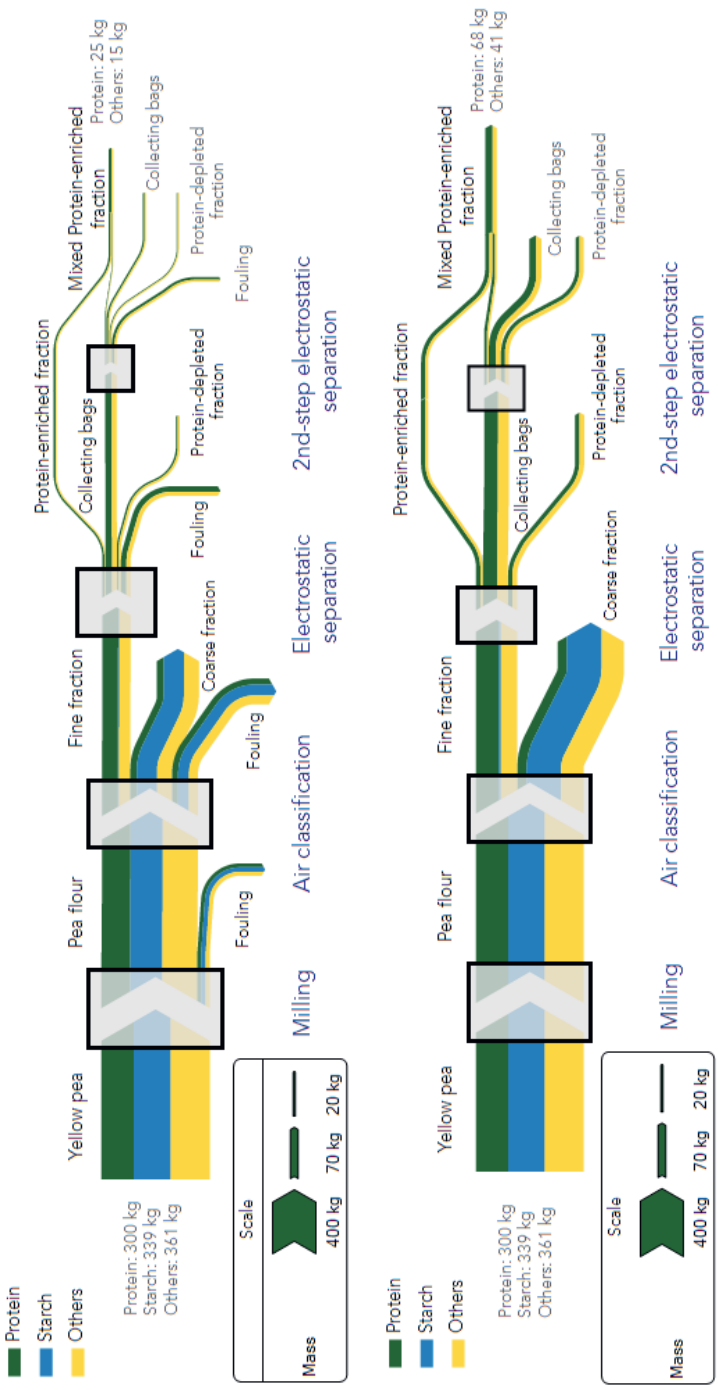

Figure 7-2. Sankey diagram of the mass flow (upper: with losses; lower: without losses) of the dry fractionation process on the yellow pea. Data referred to Chapter 4. The distribution of colour indicates the dry mass of each component in each stream. For easy distinction, the " Starch" is merged into the category of " Others" after electrostatic separation.

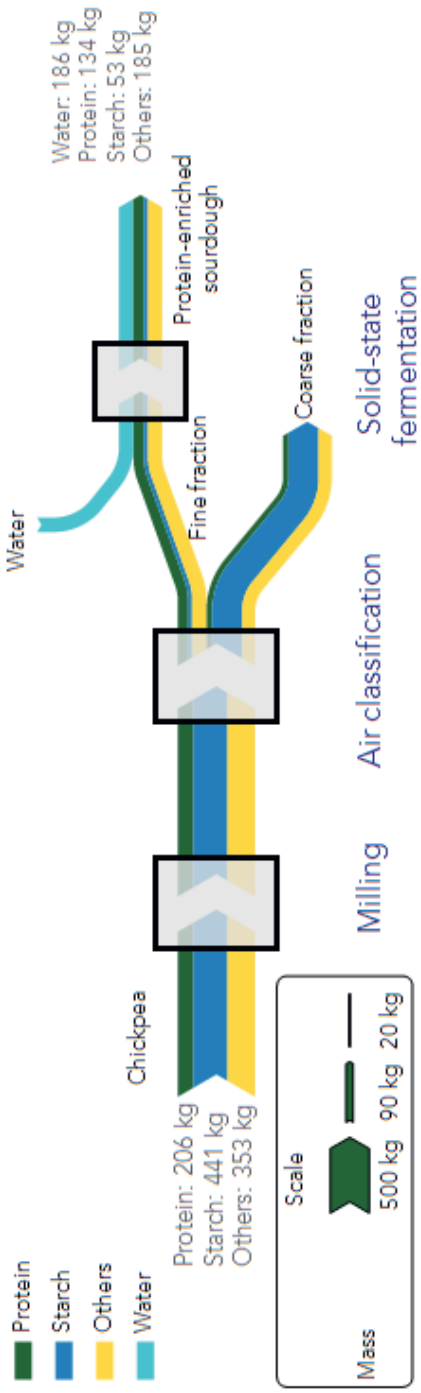

Figure 7-3. Diagram of mass flow (starting from 1000 kg chickpea) and in chickpea dry fractionation and bioprocessing without considering material loss. The actual loss during milling and air classification was 29.8% of chickpea and 23.9% of chickpea flour, respectively (Xing, Dekker, Kyriakopoulou, Boom, Smid, & Schutyser, 2020).

7.4 Outlook for application

These days, more and more foods emerge based on legume-derived ingredients (Curtain & Grafenauer, 2019). To name a few, soy-based dairy and soy-based burger meat are popular with consumers. Pea is considered to have large potential for plant-based meat replacers, as it is blander in taste and less allergenic than soy. Chickpea is non-allergic as well, is important in many traditional products, has become more popular in other products and is also considered as meat and egg replacer (Curtain & Grafenauer, 2019). Often, in these foods, legume protein concentrates instead of whole legume flour are used to further improve the nutritional values or techno-functional properties of products. However, the processes to concentrate or isolate plant proteins are not very efficient and use large quantities of water and energy. At the same time, the addition of legume ingredients introduces anti-nutritional factors, brings technological challenges, and reduces the acceptability of the products (Sozer, et al., 2017). Therefore, the use of fermented legume ingredients has been favoured by many manufacturers. As shown in figure 7-4, products incorporated with fermented legume ingredients have emerged. This dissertation takes another step forward in using this ancient processing technique.

The protein-enriched legume sourdough prepared by dry fractionation and solid-state fermentation can bring an improvement in nutritional properties compared to its normal sourdough counterpart. Firstly, products containing the protein-enriched sourdough have a better amino acid profile and are higher in protein and antioxidants, relative to the conventional protein isolates or concentrates. This is especially beneficial to people who take whole cereal products as a daily staple food. The chickpea protein-enriched sourdough is a food matrix that contains live microorganisms which may exert benefits in the human gastrointestinal tract (Holzapfel, Haberer, Geisen, Björkroth, & Schillinger, 2001).

Secondly, the protein-enriched sourdough is prepared in a very mild fractionation process that retains the native functional properties, fibres, and other micronutrients. The techno-functional properties of the protein-enriched sourdough are important to consider when developing new foods. The chickpea protein-enriched sourdough showed increased water holding capacity and gelling property compared to the fermented original flour. The water holding capacity is useful to produce foods like gluten-free products (Melini, et al., 2017) and plant-based meat, while the gelling can favor curd-like foods such as cheese analogs (Boye, et al., 2010). Other functional properties of the sourdough such as emulsion stability and foaming ability will be influenced by the pH and the course of the fermentation (Çabuk, et al., 2018). These properties are critical for beverages, spreads, ice cream, egg imitation products, etc.

Thirdly, the higher protein content of the ingredient increases the yellowness of products. This is a good attribute for products that need an alluring colour as a selling point such as pasta, pancake, and ravioli (Day & Swanson, 2013). Meanwhile, the fermented ingredient has a less bitter taste and beany flavour but an increase in acidic flavour, and is dominant in a roasted, sweet, and nutty aroma (Ben-Harb, et al., 2019; Xing, et al., 2020). The fact that it is more or less free of off-flavours is relevant for many novel plant-based protein products. Finally, the ingredient maintains an acidic environment and contains natural bacteriocins. While this has consequences for the formulations and types of products that it can be applied in, the products could be exempt from preservatives while having an extended shelf-life. Therefore, they could be suitable for products that will be marketed as clean-labeled foods.

Although legumes are consumed around the world, they are still undervalued as a source of nutrients and underdeveloped as food ingredients (Ebert, 2014). To increase the consumption of legumes, the development of legume-fortified food products is a good strategy. The legume fractions and ingredients may meet several

requirements for pursuing a healthy diet as well as for a lower environmental footprint, specifically:

1) As a nutritional supplement, especially protein.
2) As a substitute for ingredients that cause allergic or otherwise adverse reactions in the digestive system, e.g., celiac disease (caused by gluten).
3) As a replacement for animal protein to reduce the overall footprint of our diets.
4) Increasing the choice for those who have demands for a special diet, e.g. vegetarian, pescatarian, ascetics.

The protein-enriched legume sourdough developed by this dissertation may be a good starting point and inspire the development of more legume-fortified food products.

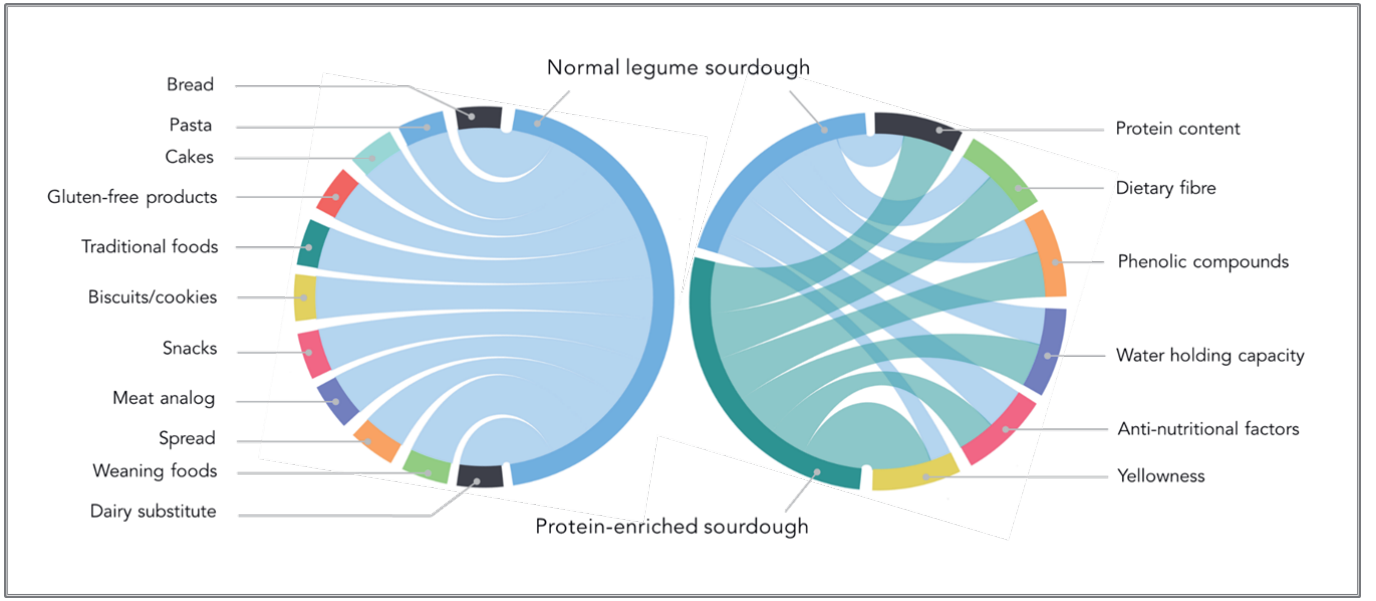

Figure 7-4. Conceptual diagram describing the applications of legume sourdoughs and the comparisons on properties between normal and protein-enriched legume sourdough. The wider the connection in the right wheel, the greater the value according to the literature. For each criterion the sum of the values for the ingredients is a 100%. (Adebayo-Oyetoro, Adeyeye, Olatidoye, Ogundipe, & Adenekan, 2019; de Oliveira Silva, Lemos, Sandôra, Monteiro, & Perrone, 2020; Galli, Venturi, Pini, Guerrini, & Granchi, 2019; Iyer & Ananthanarayan, 2008; Joshi & Kumar, 2015; Kornelia, Chandra-Hioe, Frank, & Arcot, 2018; Melini, Melini, Luziatelli, & Ruzzi, 2017; Montemurro, Coda, & Rizzello, 2019; Nwadi, Uchegbu, & Oyeyinka, 2020; Xing, Dekker, Kyriakopoulou, Boom, Smid, & Schutyser, 2020; Yao, Pan, Wang, & Xu, 2010).

7.5 Recommendations for future research

7.5.1 Improved milling procedures

Proper milling and optimized operation settings are prerequisites for sufficient disentanglement of components and thus successful dry separation. Therefore, further study should be conducted on the optimization of the milling conditions (pin/impact/jet/vortex milling) for specific types of legumes. The use of advanced microscopy and particle size and shape analysis may help to evaluate and optimize milling processes. It would be valuable to micro-locate the anti-nutritional factors in the seed or bean, such that better strategies can be devised to removed them more effectively.

For oil-rich seeds, it is not yet clear what the effect is of specific defatting methods on the functionalities of the dry-enriched fraction. By using cryogenic conditions, one might avoid the capillary bridging by oils, as they would remain solid. This could perhaps enable the separation of not just proteins and fibres, but also of oils, by using dry separation. This would however require the full process; milling, air classification and electrostatic separations to be run at cryogenic conditions (Chapter 2).

7.5.2 Better dry separation

A good separation determines the quality of the protein-enriched fraction. Further improvement in protein purity and yield of the protein-enriched fraction remains a primary goal. Air classification and electrostatic separation rely on particle size and density and triboelectric charging as driving forces for separation, respectively. Unlike air classification, electrostatic separation has not yet been applied in the food industry on large scales because the tribo-charging process has until now limited the processing capacity. Therefore, electrostatic charging needs to be improved for upscaling. The finding that the charging is not predominantly determined by the system wall, but by inter-particle charging is very important, as it implies that for

example a fluidized bed could be used for charging, which is already applied on very large scales in the food industry (Chapter 3). The yield of protein-enriched fractions can be improved by increasing the effective area of the electrodes. Moreover, the speed of the conveyor belts on the electrodes can be optimized so that particles are attracted as much as possible without being affected by electric shielding. Besides, an innovative gas recycling system can be designed to save energy and cost.

7.5.3 Towards different foods

The goal of the study on dry-enriched and fermented legume ingredients is to develop sustainable, healthy, nutritious, and tasty food products. Better resource efficiency is achieved by dry fractionation. Solid-state fermentation makes the protein-enriched ingredients better digestible and nutritious by reducing or eliminating the anti-nutritional factors.

More work can be done on the improvement of the nutritional value, functional properties, and overall acceptability of the legumes' ingredients by screening for other micro-organisms. For example, one could employ a mix of micro-organisms that produce vitamin B12, release exopolysaccharides, or eliminate the undesired beany flavour (Chapter 5).

This thesis was focused on the protein fractions; but the other fractions produced should bear value as well. Therefore, more attention should be paid to the non-protein fractions like starch-enriched fraction. Both for economic and for sustainability reasons, it is important that as much as possible of the raw material will be used for high-value products (Chapter 6). Given the residual protein, the absence of heating and the mild process conditions, we do expect that the other fractions have potential for that.

7.6 Conclusions

This dissertation has outlined a novel and sustainable processing route for the food industry to produce nutritious, functional, healthy, and tasty protein-enriched legume ingredients. Dry fractionation combining dry milling, air classification, and/or electrostatic separation was applied to produce protein-enriched fractions from different types of legumes. This approach needs far less energy than the wet extraction method and proteins retain the native functionalities. The optimization of dry fractionation aimed to seek a good balance between protein purity and yield.

Controlled solid-state fermentation was conducted on the dry-enriched legume ingredients to reduce or eliminate anti-nutritional factors. The techno-functional and organoleptic properties also improved after fermentation. The combination of dry fractionation and solid-state fermentation is sustainable, and no additives are required for achieving shelf life. This may give potential for foods that aspire to 'clean labelling'.

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Summary

Summary

Dry fractionation can enrich legume components such as protein through dry milling and dry separation. Depending on the structure and composition of the legume, air classification or electrostatic separation or their combination can be employed. During dry fractionation especially the protein fraction is enriched in antinutritional factors (ANFs), which requires further mild processing to reduce their concentrations. Therefore, the aim of this dissertation was to develop a sustainable processing route combining dry fractionation and solid-state fermentation to process different legumes into functional protein-enriched ingredients with enhanced nutritional value. The focus is to seek an optimum balance between purity and yield by optimizing the operating conditions of electrostatic separation and explore the potential of dry fractionation for selected legume varieties. Subsequently, the effect of solid-state fermentation on improving the nutritional and functional properties of the dry-enriched fractions is evaluated. Finally, the potential use of the enriched and fermented ingredients for bread making is demonstrated.

In Chapter 2, soy protein enrichment was achieved by defatting, milling, and electrostatic separation. Both oil pressing and organic solvent effectively removed the oil from soybean, although oil pressing compacted the microstructure of soy meal visually. Moderate impact milling speed (3000 rpm) was observed to effectively liberate protein bodies from the cellular matrix whilst preventing agglomeration of small fragments. Electrostatic separation was evaluated using two different charging tube designs. A higher yield was found after separation with a spiral tube compared to that obtained with a charging slit. The spiral tube provides a longer residence time improving the charging and subsequent separation process. A maximum of 15% of protein enrichment was achieved during electrostatic separation (from 45 to 52 g protein per 100 g dry basis) having recovered 62% of total protein from the defatted soy flour.

Since the charging tube design is crucial for the separation process, in Chapter 3 the impact of charging tube materials and diameter on the separation performances of a gluten-starch model mixture and lupine flour is discussed. The results showed that gluten takes a positive charge and wheat starch takes a negative charge after contact with all the charging materials (aluminium, stainless steel, PTFE, Nylon). The charge of the gluten-starch mixture was not however the same as the sum of the charge of individual components. Additionally, the charge magnitude of pure materials did not reveal any relation to the literature reported triboelectric series, which is probably related to different charging conditions. For the gluten-starch mixture, different protein enrichment was achieved with different charging materials. For lupine flour, the protein purity increased from 37 to ~65 g/100 g dry matter basis for all used tube materials. Tubes with different diameters showed the largest influence on the separation performance. Overall, the results suggested that particle-particle collisions may be responsible for much of the charging of particles. This explains why charging experiments with pure components do not predict the separation behaviour during electrostatic separation, but it also implies that redesigning the charging system to maximize particle-particle collisions could lead to significantly better charging and thus separation.

In Chapter 4, protein enrichment by a two-step dry separation process combining air classification and electrostatic separation was investigated for starch-rich legumes. Yellow pea, lentil, and chickpea were subjected to impact milling at optimized settings. Subsequently, starch granules and fibres were removed from proteins during air classification and electrostatic separation, respectively. This two-step process showed enrichment for pea and lentil but not for chickpea due to the smaller starch granules and higher fat content. Process optimization for pea showed that the pea fine fraction had an optimum balance between protein purity and yield when the air-classifier wheel speed was set at 8000 rpm. The subsequent electrostatic separation was optimized with two passes for pea fine fraction. By recycling fractions from collector bags for a second separation pass, we obtained a

protein purity of 63.4% dry basis with a yield of 15.8 g/100 g, leading to a protein recovery of 18.0% from pea.

A sustainable dry processing method to obtain nutritional and functional chickpea products was developed in Chapter 5. Chickpea was subjected to air classification and spontaneous solid-state fermentation (SSF). By daily back-slopping at 37 °C dominant autochthonous lactic acid bacteria (LAB) in chickpea flour and dry-enriched fractions were isolated, which included *Pediococcus pentosaceus* and *Pediococcus acidilactici*. LAB strains were selected based on their ability to metabolize α-galactosides (raffinose, stachyose, and verbascose). In the first 24 h during SSF, the pH of chickpea doughs decreased from 6.6 to 4.2. After 72 h, the amount of raffinose and stachyose decreased by 88 and 99%, respectively. The content of phytic acid reduced by 17% and the total phenolic contents increased by 119%. The chickpea sourdoughs showed higher water holding capacity but a decreased foaming ability. Changes in smell, texture, and colour were also observed.

The chickpea ingredients described in chapter 5 were applied to partially replace wheat flour in bread and thus fortify wheat bread with protein. Chapter 6 discussed the impact of the chickpea substitution on the physical, nutritional, and microbiological properties of wheat bread. In line with our previous study, we found the contents of raffinose, stachyose, and verbascose were reduced in bread with fermented chickpea. Bread with substitution had a redder and yellower crust, but fermentation slightly reduced browning during baking probably due to the conversion of reducing sugars during fermentation, and due to the lower pH. Crumb hardness increased as the substitution level increased. Bread crumb with chickpea fractions had a denser structure. The chickpea weakened the gluten network, which led to poorer gas retention and the formation of smaller gas cells in the crumb. No difference between fermented and non-fermented chickpea ingredients was

observed there. The sourdough bread exhibited better microbiological stability compared to that of unfermented chickpea bread and wheat bread.

In Chapter 7 the dissertation is concluded with a general discussion. Key factors for producing healthy and functional legume ingredients by dry fractionation and bioprocessing are summarized. The differences in compositions between protein-rich, starch-rich, or oil-rich legumes and the impacts on the selection and configuration of dry separation were discussed. Sustainability analysis showed that the production of 1 kg of protein with reduced ANFs by dry fractionation and fermentation is clearly more efficient than delivered by using wet fractionation. Subsequently, the potential applications of the novel legume ingredients are discussed. The changes in the nutritional, organoleptic, and techno-functional properties and their contributions to developing novel bakery products, ethnic foods, imitation products are assessed. Finally, challenges and recommendations for future research are provided.

The dissertation shows the potential for producing protein concentrates from different legumes by dry fractionation. Additionally, solid-state fermentation was applied to enhance the use of dry-enriched legume ingredients. Based on the findings of this study, further technology development and scaling-up of these processes are expected in the future.


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Qinhui

Wageningen

31-10-2020

II

庚子鼠年菊秋，博士论文搁笔。异域求学四余载，虽时有度日如年之感，终察乌飞兔走时多。所幸余未蹉跎时光，力学笃行，尽心竭力，不敢懈怠。自觉上不负椿萱，下无愧初心。今感不日业之将成，心绪难平，千言万语难抒胸意，唯以拙句四十行尽拳拳之情。

毕业感怀

年少心高何所忧，立志扬帆出九州。
怀藏素愿勤躬行，四载耕耘又三秋。
可怜百转无佳讯，秋蝉惊梦望梁空。
忽如一朝喜信到，得见云开长安笑。
倒履急行报父母，择日谨向先人告。
家父默默打行装，家母依依与儿叨。
不觉临别日催急，欲破无言仅归期。
整装负笈万里行，翻山逐日北海迎。
红瓦星罗穿玉带，绿原棋布枕莱茵。
山鹛啼息鸥声起，唯闻鸿鹄久长鸣。
长安一去两万里，和日戏雨卷云低。
薄礼谒师晤同业，同胞异貌俱相惜。
烹茶点灯重开卷，笔走神游梦似现。
春梦戏人逐遗香，欢愉未央隐有伤。
邀与踏巷尝五味，藉口营营实米贵。
闲游四方争豁目，耳闻省却舟车费。
前尘时扰使人愁，空廊独灯惬忘忧。
同砚争相学长技，汉儿为强习胡语。
初安三餐犹兼味，日久埋卷渐食陈。
谈天阅人识见长，磨心劳形功俞深。

夏雨秋雾冬踏雪，卯起四闻子规啼。
求学中道把家还，入门相拥谓儿长。
花信已去何故长，再思已是泪两行。
高堂银丝痛入目，味咸食减忧转肠。
初见小侄坐犹难，今闻侄儿唤姑响。
谈笑漫心辞舅父，抱憾回乡祭已亡。
故友多烦儿溺疾，陋室孤音被衾凉。
流年催人倍勤勉，春去方觉紫述香。
学业经岁近煞尾，戏球不慎竟折踝。
卧榻三月忆刻骨，忧愤如鬼彻夜来。
挚友携鲜纷造访，浣洗炊爨俱不烦。
疾去痛消复能行，四海大疫事皆乱。
固步方寸且远朋，借数归期盼速眠。
今为游子方知难，内忧外扰何曾断。
终识前路阻且远，仍需吾辈勇与坚。
虫书鸟篆用虽广，犹感神鬼惧仓颉。
不才天资乏异禀，廿载出师方立业。
今宵莫问凌云志，邀影共庆荫椿萱。
关中麦陇复新绿，八水绕城又迎冬。
压枝火晶染骊山，滋味可与去时同？

邢沁浈
于 瓦赫宁根
2020 年 10 月 31 日

About the author

Name: Qinhui Xing

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Place of birth: Xi'an, Shaanxi, China.

Educational background:

Wageningen University & Research (The Netherlands) *Food process engineering*	Ph.D.	2016-2020
Northwest A&F University (China) *Processing and storage of agricultural products*	MSc.	2013-2016
Tianjin University of Science & Technology (China) *Food biotechnology*	BSc.	2009-2013

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List of publications

This dissertation

Qinhui Xing, Martin de Wit, Konstantina Kyriakopoulou, Remko M. Boom, Maarten A. I. Schutyser*. 2018. Protein Enrichment of Defatted Soybean Flour by Fine Milling and Electrostatic Separation, *Innovative Food Science & Emerging Technologies*. 50: 42~49.

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Qinhui Xing, Zhengmao Zhang*, Chuanli Hou, Ke Han, Qiaozhen Yan, Jia Luo. 2017. Comparative study on the physicochemical properties of pea, chickpea, and wheat starch gels in the presence of sweeteners, *Starch/Stärke*. 69: n9-10.

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Zhang Z, **Xing Q**, Liu Y. 2018. Chapter 9: Chinese steamed bread and novel wheat products. In Flour: Production, Varieties and Nutrition. Nova Science Publishers. p. 175-184.

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Overview of completed training activities

Discipline Courses

Advanced Food Analysis (Wageningen, NL)	2017
Dry biorefinery of agro-resources (Montpellier, FR)	2017
6th Food and biorefinery enzymology (Wageningen, NL)	2017
1st Healthy food design (Wageningen, NL)	2018

Conferences

NWGD symposium (Wageningen, NL)	2018-2019
5th PhD Symposium (Wageningen, NL)	2018
32nd EFFoST (Nantes, FR)	2018
33rd EFFoST (Roterdam, NL)	2019
1st Global Plant-Based Foods & Proteins (virtual)	2020
Nizo Plant Protein Functionality (virtual)	2020

General courses

VLAG PhD week (Baarlo, NL)	2017
Scientific writing (Wageningen, NL)	2017
PhD workshop carousel (Wageningen, NL)	2017
Supervising BSc and MSc thesis students (Wageningen, NL)	2017
Philosophy and Ethics of Food Science and Technology (Wageningen, NL)	2018
Introduction to R (Wageningen, NL)	2018
Career assessment (Wageningen, NL)	2019
Working on your PhD research in times of crisis (Wageningen, NL)	2020

Other activities

Preparation of research proposal	2016

Scientific PhD excursion (CA)	2018
Group days of Food Process Engineering (Wageningen, NL)	2017-2019
Weekly group meeting Food Process Engineering (Wageningen, NL)	2016-2020
Teaching assistant: Food fermentation	2019

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