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A water-only process to fractionate yellow peas into its constituents

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ABSTRACT

To meet the consumer demand for minimally processed foods and clean labels, the potential of processes where chemicals are omitted and only water is used needs to be explored. Mild wet fractionation of yellow pea, a water-only process, is investigated on maximum separation and efficient water use. By only using water, starch and protein from pea could be successfully separated, resulting in fractions high in yield and purity. Multiple washing steps of both the starch and the non-soluble protein fraction were performed to enhance separation. As a result of starch- and non-soluble protein pellet washing, the starch fraction was further depleted in protein and the protein solubility in the non-soluble protein fraction decreased. Ultrafiltration of the soluble protein fraction served to concentrate and the extracted water has potential to be reused in the process. Small solutes were concurrently extracted, which resulted in a higher protein purity in the soluble protein concentrate of 75%. The presented method has potential for upscale use in industry to produce protein fractions comparable to protein isolates obtained through conventional fractionation.

1. Introduction

Mild fractionation of crops to produce ingredients aims towards the omittance of chemicals and the reduction of water and energy use in the food production chain. For yellow pea, different mild fractionation routes have been investigated and compared to conventional wet fractionation techniques in regard of their resource use efficiency (Geerts, van Veghel, Zisopoulos, van der Padt, & van der Goot, 2018). From both dry (Pelgrom, Vissers, Boom, & Schutyser, 2013) and mild wet fractionation (Geerts, Mienis, Nikiforidis, van der Padt, & van der Goot, 2017a) of yellow peas, concentrates enriched in protein or starch are obtained. The fractions obtained from dry fractionation yield lower purities (Pelgrom et al., 2013) than those from mild wet fractionation and are both less pure than isolates obtained from conventional fractionation (Geerts, Nikiforidis, van der Goot, & van der Padt, 2017b). In terms of rational exergy efficiency, dry fractionation is most efficient, followed by mild wet fractionation and conventional wet fractionation. The efficiencies are mostly influenced by material loss (Geerts et al., 2018). In a previous study (Möller, van der Padt, & van der Goot, 2021), the potential of mild fractionation processes was studied in more detail through comparing mild wet fractionation and dry fractionation of yellow peas on their purity performance. The results indicated that higher purities in mild wet fractionation are owed to additional disentanglement of the flour particles upon dispersion in water. It was found that the soluble components in the pea flour fragments are solubilized indeed, which explained better separation. This led to the conclusion that water provides a promising additional driving force in mild fractionation. It was further hypothesized that pellet washing in mild wet fractionation could lead to higher purities in the respective fractions (Möller et al., 2021). To further investigate the role of water in mild wet fractionation and to optimize the process in regard of yield and purity, we explore a more efficient use of water in this study. Therefore, the aim of this study is to develop mild wet fractionation further into a method comparable to conventional fractionation regarding purity and yield by a more efficient employment of water and the omittance of chemicals.

2. Materials & methods

Pre-dried yellow peas (*Pisum sativum* L.), were purchased from Alimex (Sint Kruis, The Netherlands).

2.1. Milling

The peas were pre-milled into grits using a pin mill (LV 15 M,

Abbreviations: Soluble protein fraction, SPF; Non-soluble protein fraction, NSPF; Conventional wet fractionation, CWF.

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Condux-Werk, Wolfgang bei Hanau, Germany) at room temperature. The grits were subsequently milled into fine flour using a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany), with an impact mill speed of 8000 rpm, an air flow at 52 m^3/h, a classifier wheel speed of 4000 rpm and a feed rate of 2 rpm (method adopted from (Pelgrom, Boom, & Schutyser, 2014). A thermometer inside the mill was used to monitor temperature.

2.2. Mild wet fractionation

Mild wet fractionation (MWF) was performed following the method of (Geerts, Nikiforidis, van der Goot, & van der Padt, 2017b) with minor modifications. The following water-flour dispersions were prepared: 10 g flour and 20, 50, 80, 90, 100, 110, 120, 150, 200 and 500 g Milli-Q water (resistivity 18.2 MΩ.cm, Merck Millipore, France), respectively. The mixtures were stirred at room temperature for 1 h. Then, the dispersions were submitted to a first centrifugation step at 1500 *g* for 1 s. During this step the insoluble starch granules were separated into the pellet. The supernatant was submitted to a second centrifugation step at 10000 *g* for 30 min. The pellet of this second centrifugation step yielded the so-called non-soluble protein fraction (NSPF) and the supernatant yielded the soluble protein fraction (SPF). The obtained fractions were freeze dried for further analysis using a Pilot freeze dryer (Christ Epsilon 2-6D, Osterode am Harz, Germany). Previous results from mild wet fractionation were performed with the water/flour ratios 10:1, 8:1 (Geerts, Nikiforidis, van der Goot, & van der Padt, 2017b).

2.3. Compositional analysis

The dry matter content was determined using an infrared moisture analyzer (MA35, Sartorius, Germany) at 120 °C, until a constant weight was reached. The protein content of the fractions was determined using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, Interscience, Breda, The Netherlands) using a protein conversion factor of 5.52 (Holt & Sosulski, 1979). The total starch content was determined using the Total Starch Amyloglucosidase/α-Amylase Assay Kit, AOAC Method 996.11 (Megazyme International Ireland Ltd., Bray, Ireland). The protein yield (*Y*) *(%)* based on the total protein present in the flour was calculated with eq. (1).

$$Y = \frac{m_x \cdot x_{p,x}}{m_f \cdot x_{p,f}} \cdot 100\% \quad (1)$$

Here m_x is the mass in (*g*) and $x_{p,\,x}$ the protein content in *(g/g)* of the obtained fraction, m_f is the mass in *(g)* and $x_{p,\,f}$ the protein content in *(g/g)* of the initial flour used for the fractionation. The protein purity (*P*) *(%)* was calculated with (Eqn 2) by dividing the protein mass in the fraction ($m_{p,\,x}$). by the total mass of the fraction (m_x):

$$P = \frac{m_{p,x}}{m_x} \cdot 100\% \quad (2)$$

2.4. Protein solubility

Different definitions of protein solubility are described in literature. The measure used in this study is based on the percent protein solubility. It is defined as the percent amount of protein in the supernatant to total amount of protein in the sample before centrifugation (Vojdani, 1996). The determination for protein solubility was partly adopted from (Tanger, Engel, & Kulozik, 2020). Protein solutions of 0.1% were made with both the SPF and NSPF fractions, before washing and after two washing steps. The solutions were rotated using a rotator (Stuart, UK) for 1 h, after which the pH was determined. The samples were then centrifuged again at 6000 *g* at 20 °C for 15 min. The supernatant was separated and used to determine the protein content using a PierceTM Bicinchoninic acid protein assay kit (ThermoFisher Scientific Inc., USA). Bovine serum albumin (BSA) with a known concentration was used to prepare a standard curve (ThermoFisher Scientific Inc., USA). The method was performed according to the standard protocol and the absorbance of the colorimetric reaction was measured 562 nm using a spectrophotometer (Beckman DU720 UV–Vis spectrophotometer (Beckman-Coulter Inc., USA). The protein solubility (*S*) (%) in the fractions was determined by dividing the protein content determined with BCA ($x_{p,\,BCA}$) (g/g) by the initial protein content in the solution (x_s) (g/g) using eq. (3):

$$S = \frac{x_{p,BCA}}{x_s} \cdot 100\% \quad (3)$$

2.5. Pellet washing

Water and flour were mixed in a ratio of 5:1 and MWF was performed, according to the method of (Geerts, Nikiforidis, van der Goot, & van der Padt, 2017b). The pellets were re-dispersed in water to a ratio of 5:1 water to pellet and stirred at room temperature for 1 h. After a second centrifugation was applied, 1500 *g* for 1 s at 20 °C for the starch pellet and 10,000 *g* for 30 min at 20 °C for the NSPF pellet. The pellets were washed twice in triplicates, respectively, (Fig. 1). The obtained SPF and NSPF fractions were combined, their protein yields were summed, and their protein purities were averaged over the combined protein fractions and corrected for their proportion. The combined protein yield *(%)* of the protein rich fractions (Y_{cm}), the normalized protein yield (Y_{Norm}) *(%)*, which is the relation between the protein yield in one fraction and the combined protein yield of the protein rich fractions, and the averaged protein purity (P_{av}) *(%)* of the washed protein rich fractions were calculated with eqs. (4), Eqn 5, (Eqn 6):

$$Y_{cm} = Y_{p,SPF} + Y_{p,NSPF} \quad (4)$$

$$Y_{Norm} = \frac{Y_{p,x}}{Y_{cm}} \cdot 100\% \quad (5)$$

$$P_{av} = \frac{\sum m_{p,x}}{\sum m_x} \cdot 100\% \quad (6)$$

Here $Y_{p,\,SPF}$ and $Y_{p,\,NSPF}$ are the protein yields *(%)* in the soluble and non-soluble protein fraction, respectively and $Y_{p,\,x}$ is either of them according to eq. (1). $m_{p,\,x}$ is the protein mass *(g)* in, and m_x the total mass *(g)* of the respective fraction.

2.6. Ultrafiltration

Approximately 200 mL of the soluble protein fraction was subjected to batch ultrafiltration using an Amicon® stirred cell, 400 mL (Millipore Merck KGaA, Darmstadt, Germany). The smallest protein present in pea is PA2, a dimer of 8 kDa size (Croy, Hoque, Gatehouse, & Boulter, 1984; Higgins et al., 1986). Therefore, a membrane pore size of 3 kDa was chosen to have a sufficiently small cut off size for the ultrafiltration process. The ultrafiltration was performed in single step and repeated in multiple steps with a mass reduction factor *(MRF)* of 1.7 per step. After each step a sample of the permeate and retentate was analyzed for its dry matter and protein content. The mass reduction factor for this batch system is defined by eq. (7):

$$MRF = \frac{m_{fd}}{m_{ret}} \quad (7)$$

Where m_{fd} is the initial feed mass and m_{ret} is the obtained retentate mass both in *(g)*.

2.7. Membrane characterization and modelling

For designing and predicting a continuous ultrafiltration process the retention coefficient (*R*) was calculated from the permeate and retentate concentrations obtained with the batch system ultrafiltration. Eq. (8) defines the retention:

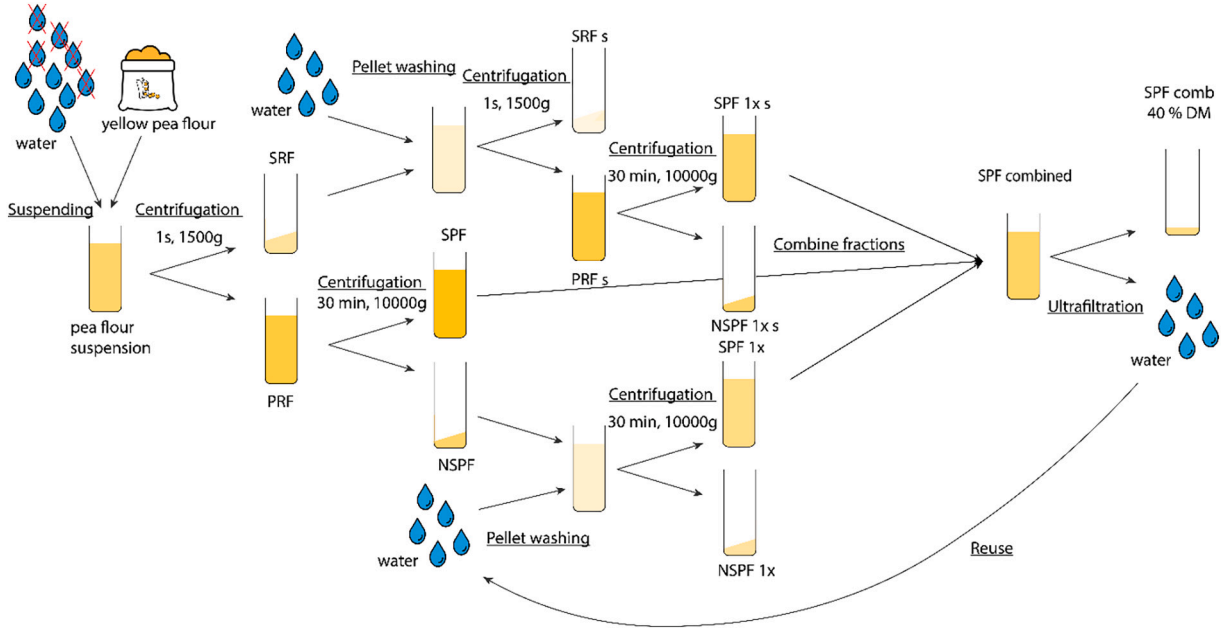

Fig. 1. Graphic representation of the mild wet fractionation process (adopted from (Geerts, Nikiforidis, van der Goot, & van der Padt, 2017b) with adaptations and additions) including one washing step of the starch rich fraction (SRF) and the non-soluble protein fraction (NSPF), and a subsequent ultrafiltration step of the soluble protein fraction (SPF). PRF is the protein rich fraction, 1× indicates obtained after one washing step, the small 's' indicates the fractions' origin from the starch fraction.

$$R = 1 - \frac{x_{per}}{x_{ret}} \tag{8}$$

where x_{per} and x_{ret} are the measured permeate and retentate mass fractions in *(g/g)*, respectively. The retention coefficients for both the protein and non-protein stream were determined with the batch experiments described in *2.6.*

The measured retention coefficient and mass reduction factor from the batch system were used to calculate the yield and purity in a continuous membrane system of 10 stages, eq. (9). Firstly, the component mass fraction $x_{ret,\,c}$ in the retentate can be calculated with the given retention coefficient and mass reduction factor:

$$x_{ret,c} = \frac{MRF \cdot x_{fd,c}}{1 + (MRF - 1)\cdot(1 - R)} \tag{9}$$

The component mass fraction in the permeate can then be calculated using eq. (8).

With $x_{ret,\,c}$ and $x_{per,\,c}$ the permeate ϕ_{per} and retentate ϕ_{ret} mass flows both in *(g/s)* can be calculated. For each stage the eqs. (10) and (11) hold:

$$\phi_{fd} \cdot x_{fd,c} = \phi_{ret} \cdot x_{ret,c} + \phi_{per} \cdot x_{per,c} \tag{10}$$

$$\phi_{fd} + \phi_d = \phi_{ret} + \phi_{per} \tag{11}$$

Here ϕ_{fd} is the mass flow *(g/s)* and $x_{fd,\,c}$ the component mass fraction *(g/g)* of the feed stream. The equations apply for both the protein and the non-protein components in the streams. Eq. 11 considers the addition of diawater, which is added to dilute the feed stream, avoiding polarization concentration at the membrane surface. Here ϕ_d is the diawater mass flow *(g/s)*.

The protein yield and purity of the streams of each stage were then calculated using eqs. (1) and (2); for the yield, the mass and protein content of the fraction are divided by the mass and protein content of the initial feed stream. For calculations of a batch system, ϕ_{fd} should be replaced by the initial mass (m_{fd}) and ϕ_{ret} *and* ϕ_{per} by the retentate mass (m_{ret}) and the permeate mass (m_{per}), respectively.

2.8. Permeate characterization

High Performance Size Exclusion Chromatography (HPSEC) was carried out in an UltiMate 3000 chromatographer (ThermoFisher Scientific Inc., USA) through a dual column system with TSK gel columns G3000SWXL and G2000SWXL for proteins and peptides. An aqueous solution of 30% acetonitrile (Actu All Chemicals, The Netherlands) and 0.1% trifluoroacetic acid was used as eluent. Signals were measured with UV detector set at 214 nm. Data analysis was performed in Chromeleon 7.2 CDS software (ThermoFisher Scientific Inc., USA). The processing step was adjusted to integrate peaks between molecular weight ranges. A calibration curve of molecular weight on a logarithmic scale against elution time was plotted for thyroglobulin (670 kDa),γ-globulin (158 kDa), ovalbumin (44.3 kDa), α-lactalbumin (14 kDa), aprotinin (6.51 kDa), bacitracin (1.42 kDa) and phenylalanine (165 Da).

3. Results & discussion

The efficiency of mild wet fractionation was investigated in view of the obtained yields and purities in the respective fractions. The mild wet fractionation process was adopted from Geerts, Nikiforidis, van der Goot, & van der Padt, (2017b) with modifications in the employment of water. The method as previously reported included a hydration step of 1 to about 12 h. A two-step centrifugation was used to, separate the suspension into a starch rich, a soluble protein rich and a non-soluble protein rich fraction. In the following sections several process adaptations will be discussed to increase the water use efficiency.

3.1. Optimizing the water use in single step mild wet fractionation

To optimize the water-use for MWF, the effect of water content in the initial hydration step at the minimum constant hydration time of 1 h was studied. Mild wet fractionation was performed using varying water/flour ratios. The process yielded a constant purity of around 52% protein content in the soluble protein fraction (SPF) for all ratios. The purity of the non-soluble protein fraction (NSPF) was constant at around 53% protein content for all ratios except for the lowest at water/flour ratio

2:1, see Fig. 2a.

Differences in outcomes due to a variation in water/flour ratios are more prominent when looking at the protein yield. The combined protein yield depicts the protein yield in both the SPF and NSPF based on the protein initially present in yellow pea flour (Eq. 3, Appendix Fig. A1Figure A1). The increase in combined protein yield indicates that with an increase in water more protein is extracted from the starch fraction. Moreover, the constant purity in both fractions (52 and 53%, respectively) suggests that other components solubilized with protein in a similar manner. A constant protein yield at higher water/flour ratios indicates a critical water-flour ratio, above which no further extraction was achieved. The maximum combined protein yield of SPF and NSPF was determined around 86% at ratio 9:1. Consequently, at least 14% of the initial protein remained in the starch fraction. Further, the cumulative yield of protein was approximately constant above a ratio of 2.

Next, protein yields of SPF and NSPF were normalized to the combined protein yield of the protein rich fractions, for a clearer representation of the protein distribution between both fractions, Fig. 2b. Protein yield in the SPF increased, while protein yield of NSPF decreased with increasing water/flour ratios. However, at a ratio of 20 and above the protein yields of both fractions remain constant at 83% and 17%, respectively. Hence with the employment of more water, no more soluble protein is extracted from the NSPF. This indicates that above a ratio of 20, protein solubility is independent of water ratio, which might imply that all soluble protein is in the SPF and all non-soluble protein in the NSPF. From the results obtained it was concluded that the previously reported water/flour ratios of 8:1 and 10:1 are not needed in regard of water use efficiency. Instead a ratio of 5:1 can be chosen to yield the same purities and the same combined yield, while decreasing the amount of water employed in the fractionation step. To recover the protein remaining in the starch fraction and to investigate the protein distribution between SPF and NSPF, pellet washing steps were introduced to investigate extra protein extraction.

3.2. Multiple pellet washing steps for further purification in mild wet fractionation

Previously, a hypothesis was introduced that additional pellet washing steps could lead to further enrichment in mild wet fractionation (Möller et al., 2021). During centrifugation interstitial water remains in the pellets, in which proteins and other soluble components are solubilized. This hypothesis could explain the presence of residual protein in the starch fraction. Therefore, multiple pellet washing steps were added to the mild wet fractionation process and the effect on yield and purity in the respective fractions was investigated. Multiple SPF and NSPF streams were obtained after two-step washing of both the starch fraction and the NSPF, which were combined into one SPF and one NSPF streams. Fig. 3 depicts a representation of the dry matter mass flow during mild wet fractionation including two pellet washing steps for both the starch fraction and the NSPF. After the first separation step, the main part of the protein is collected in the protein rich fraction, and the main part of the rest in the starch rich fraction. The second centrifugation step separates the protein rich stream into the SPF and NSPF. With each starch pellet washing step, more protein was extracted from the starch fraction into the soluble and non-soluble protein fraction, enriching both streams. NSPF pellet washing resulted in an extraction of protein from the NSPF into a secondary and tertiary SPF. All SPF and NSPF streams obtained from pellet washing can be combined respectively and would result in the depicted streams, Fig. 3. Next to protein also rest is extracted with each washing step. The extraction of the rest explains why the protein purity only slightly increases in the SPF and NSPF, while the protein yield is mainly increasing. It is assumed that the non-protein components extracted from the starch fraction through pellet washing do not contain starch, due to its insolubility at room temperature.

The protein yields and purities of the fractions were determined during fractionation and after each pellet washing step. The results are listed in Table 1. The purities and yields after one and two washes are from the cumulative fractions. In the SPF, the protein yield increased with each washing step. While in the NSPF, the protein yield increased with the first washing step and decreased with the second washing step. In the starch fraction, protein purity and yield decreased with each washing step. Hence, more protein could indeed be extracted from the starch fraction and was distributed over the protein fractions. The average purity of SPF slightly increases with each washing step, while the averaged protein purity of the combined NSPF increases with the first and decreases with the second washing step. For the NSPFs, the purity of the primary pellets increased stepwise; however, the protein purity of the secondary NSPF obtained from starch pellet washing has a lower protein purity due to a higher rest content, decreasing the cumulative purity after two washing steps.

3.3. Protein fractionation into the soluble and non-soluble protein fractions and the effect of pellet washing

The separation of the SPF and NSPF indicates that mild wet fractionation, allows additional fractionation of the proteins based on their solubility characteristics, on top of the separation of protein and starch. This hypothesis was supported by the constant yield in both SPF and NSPF obtained at water/flour ratios above 20 (Appendix Fig. A1),

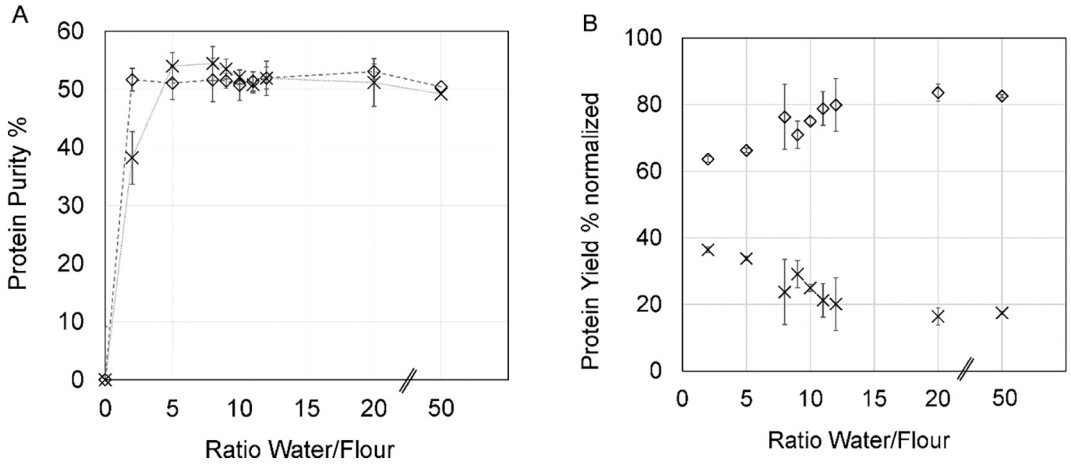

Fig. 2. The protein purity (%) (a) of the SPF ◊ and NSPF X and normalized protein yield % (b) of SPF ◊ and NSPF X (eq. (3)) are depicted at varying water/flour ratios. The lines are meant to guide the eye.

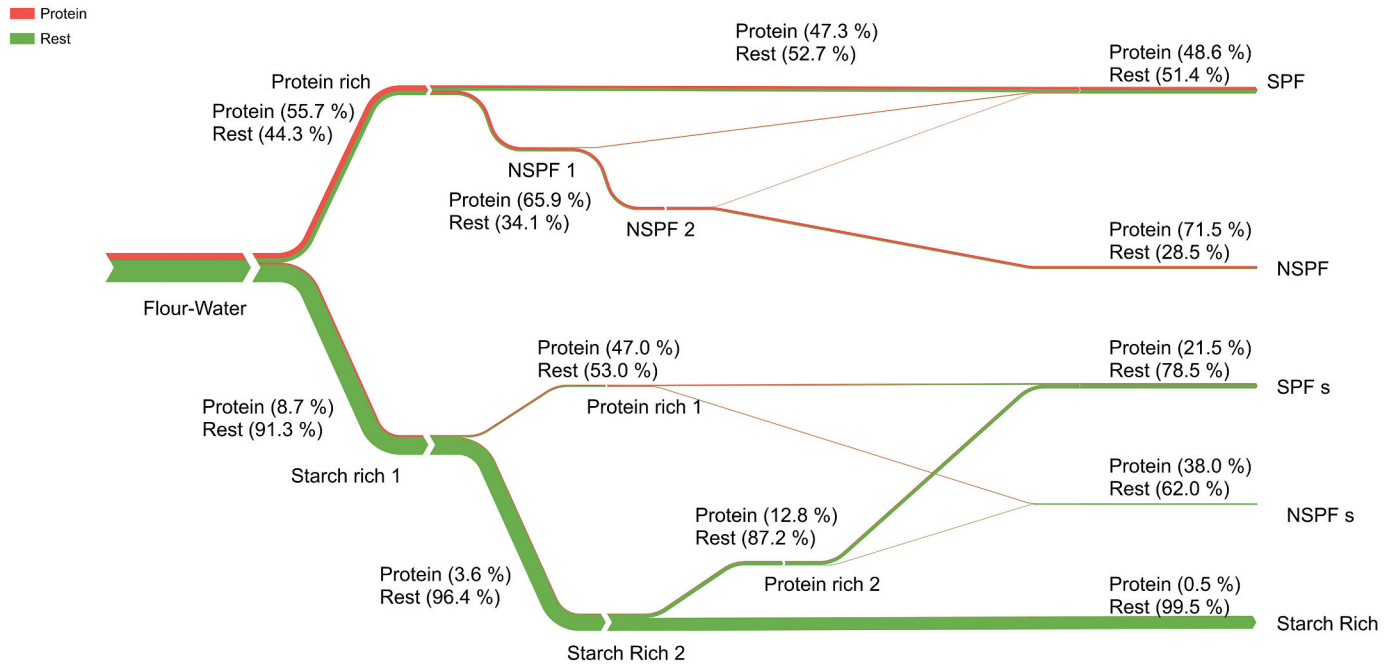

Fig. 3. Mass representation of the dry matter streams during mild wet fractionation and combination of the according fractions into a SPF-, NSPF- and a starch fraction stream. The red stream represents the protein content in dry matter, the green stream represents the rest content in dry matter. Percentages of the main streams indicate purities of the streams and were added to depict the effect of pellet washing. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Protein purity and protein yield of the three fractions obtained through mild wet fractionation of yellow pea flour. The purities of 1 wash and 2 washes are the averaged protein purities (aver.) as calculated in eq. (4), the protein yields of 1 wash and 2 wash are added up from the respective fractions to represent the combined streams (comb.).

Fraction	No wash		1 wash		2 washes	
	Yield [%]	Purity [%]	Yield [%] comb.	Purity [%] aver.	Yield [%] comb.	Purity [%] aver.
SPF	44.5	47.0	60.5	47.5	64.7	47.6
NSPF	33.2	66.7	34.6	68.5	31.9	66.1
Starch	22.3	8.7	4.9	3.7	3.4	2.1

indicating that around 17% of the protein is indeed insoluble. It was further hypothesized that pellet washing of the NSPF would extract soluble protein from the NSPF, which otherwise remained in the fraction with the interstitial water. To further investigate the solubility characteristics of SPF and NSPF, the fractions were diluted to 0.1% protein solutions and the solubility of the proteins in the fractions was determined. The solubility characteristics of SPF and NSPF obtained after one and two pellet washes was accordingly determined. Due to the dependency of protein solubility on pH (Zayas, 1997), the pH was measured for all protein solutions. The pH was between pH 6.5–7 for all samples. Fig. 4 depicts the relative amounts of soluble and non-soluble protein to total protein in the respective SPF and NSPF streams, after the first centrifugation step, and the two pellet washing steps. Neither the SPF nor the NSPF consists of pure soluble or non-soluble protein,

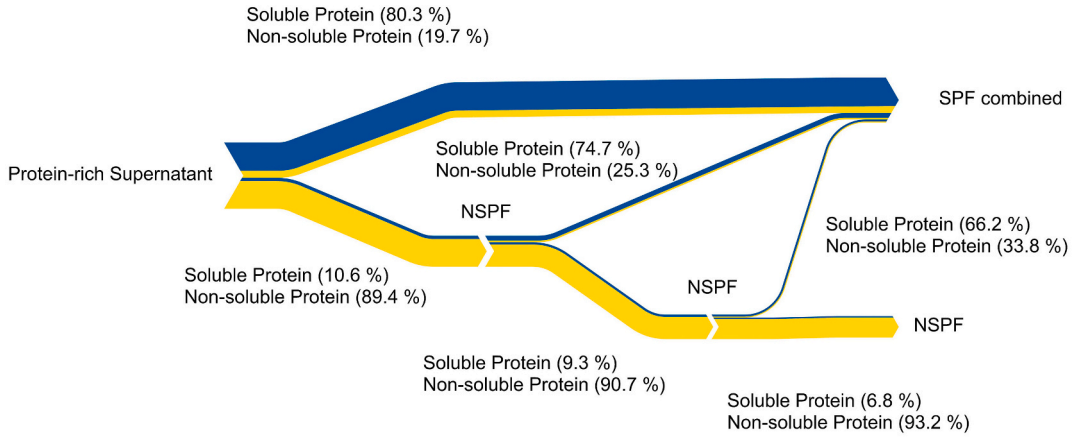

Fig. 4. Graphical representation of the proportion of soluble and non-soluble protein in the protein rich stream after the first centrifugation step. The blue stream represents the relative amount of soluble protein and the yellow stream the non-soluble protein. Two pellet washing steps were performed on the NSPF stream and the obtained supernatants were combined to the SPF stream. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively. With pellet washing, mainly soluble protein is extracted from the NSPF. However, with each washing step, a smaller proportion of non-soluble protein is extracted as well. Yet, using mild wet fractionation the majority of soluble and non-soluble protein was successfully separated. Solubility is one of the main functionalities often analyzed for plant-proteins (Lam, Can Karaca, Tyler, & Nickerson, 2018). Therefore, including fractionation into soluble and non-soluble protein, next to component fractionation, highlights the potential of mild wet fractionation for industrial application.

3.4. Concentration of SPF with ultrafiltration

With the addition of the pellet washing steps to the mild wet fractionation method, more water was introduced into the process. The NSPF and the starch fraction before and after pellet washing have relatively constant dry matters of around 20 and 30%, respectively. The additional water introduced during pellet washing therefore ended up in the SPF mainly, decreasing the cumulative dry matter content of that fraction with every washing step. Ultrafiltration is commonly used in dairy industry for whey protein separation and concentration. Further, ultrafiltration was previously used to produce pea protein isolates, with enhanced functionality, higher protein yields (Boye et al., 2010) and lower amounts of antinutritional factors (Mondor, Tuyishime, & Drolet, 2012). In these studies, ultrafiltration was used subsequent to alkaline extraction, to substitute isoelectric precipitation. Here, ultrafiltration of SPF was performed to extract the excess water from the fraction. Ultrafiltration increased the dry matter content of SPF from approximately 4% to 38%. Moreover, the protein purity increased to 75%. However, the permeate after ultrafiltration to such high dry matter contents had a dry matter content of around 2% of which 19% was protein. Additionally, filtration to such high dry matter contents of around 40% leads to a gel layer formation on the ultrafiltration membrane and the process becomes ineffective. Therefore, the ultrafiltration process was repeated in three steps, using a mass reduction factor of approximately 1.7. The protein retention coefficient was for all samples around 0.95–0.97. During step wise ultrafiltration, it was observed that the purity of SPF already increased to 63% when the dry matter content was only increased to around 7%, (Appendix Table A1Table A1). To check whether the retention could be considered constant, the three-step ultrafiltration was additionally calculated using the equations introduced in section 2.6 and 2.7. The same mass reduction factor was chosen for the continuous membrane system of three stages. A constant protein retention coefficient was chosen at 0.97, which resulted in a non-protein retention coefficient of 0.48 in order to achieve similar filtration results as measured with the batch system. With an initial dry matter content in SPF of 2.3% and a protein content of 46% per dm the dry matter and protein purity increased with each step (Appendix Table A2Table A2). The predicted and measured protein purity are plotted in Fig. 5. The protein purity in step 2 and 3 is slightly overpredicted using the ultrafiltration calculations; however, the prediction is quite close to the measured purities. To elucidate the potential of a continuous ultrafiltration system further, a calculation is added to predict the dry matter content and protein purity in a ten-stage ultrafiltration process.

3.5. Preliminary design of a ten-stage continuous ultrafiltration system

Therefore, the calculations were used to further extend and optimize the ultrafiltration prediction to a ten-step continuous ultrafiltration process (Fig. 6). To obtain a dry matter content of 30% with a retention coefficient of 0.97 for protein, the mass reduction factor was lowered to 1.4 in a ten-stage system. The employment of stepwise ultrafiltration could allow to increase the protein purity in the SPF to more than 80% and a dry matter content of >30%. It was however observed that at a dry matter content above 10%, the purity gain reduced. In dairy industry, diawater is often added in ultrafiltration processes to obtain high protein purities and to reduce concentration polarization, protein gel layer

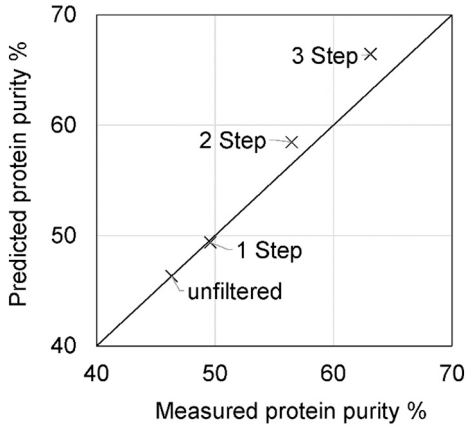

Fig. 5. Prediction of protein purity in retentate over measured protein purity in retentate after 3 step ultrafiltration with an average concentration factor of 1.7, and a protein retention coefficient of 0.97. The line is added to guide the eye.

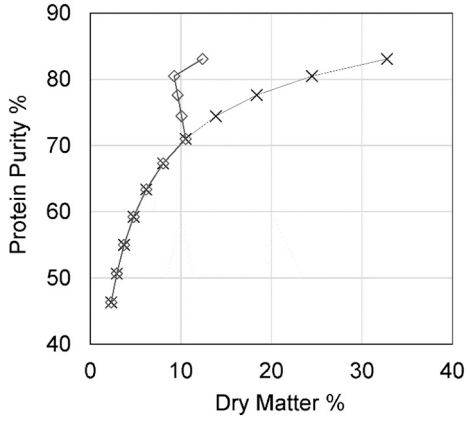

Fig. 6. Predicted protein purity in retentate over predicted dry matter content in retentate after 10 step ultrafiltration with a concentration factor of 1.4, and a retention coefficient of 0.97 for protein. **x** indicates ultrafiltration without addition of diawater, ◊ indicate the addition of diawater in step, 7, 8 and 9 to avoid concentration polarization.

formation, on the membrane surface. Such a combination can yield subsequently dried protein powders of about 85% protein purity (Henning, Baer, Hassan, & Dave, 2006). Therefore, the ultrafiltration was predicted a second time with an addition of diawater after a dry matter content of ≥10% was reached. The addition of diawater had no influence on the protein purity, on dry matter bases, but lead to a constant dry matter content around 10%.

3.6. Quantification of the permeate stream

The permeate samples were additionally subjected to size exclusion chromatography, to determine the approximate size of the proteins (Fig. 7, Permeate SPF). More than 95% of the peptides present in the UF permeate are smaller than 0.5 kDa. To understand the nature of the presence of peptides in the protein fraction, pea flour was diluted in water and size exclusion chromatography was performed right after solubilization (<10 min in solution) (Fig. 7, Pea flour). Around 15% of the proteins and peptides present in pea flour have a size of less than 1 kDa. The protein yield of the UF permeate, based on the initial protein present in pea flour, was around 14%. Hence, the amount of small peptides present in the SPF approximates the amount of small peptides initially present in the pea flour. Indicating that the protein loss during ultrafiltration into the permeate is actually a loss of small peptides. Due

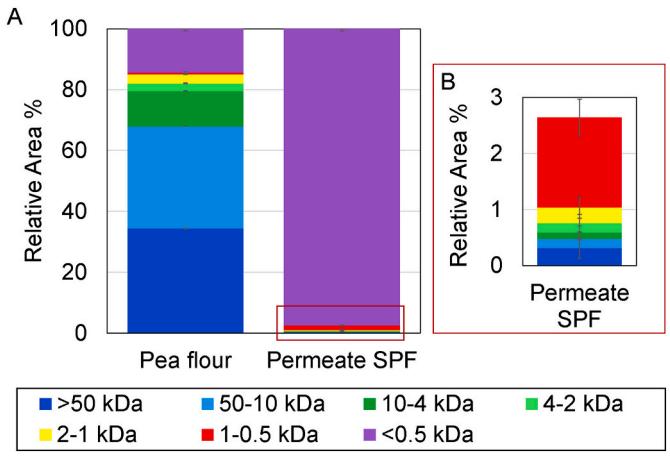

Fig. 7. (A) Peptide profile integrated from size exclusion chromatograms of Pea flour, and SPF permeates obtained from step wise ultrafiltration. The pea flour profile was determined in triplicate. The SPF Permeate includes duplicate ultrafiltration runs determined in triplicates. The bars depict the relative amount of peptides/proteins in the respective size range. The error bars depict a confidence interval of 0.95. (B) Zoom in of the permeate SPF relative area profile for molecules >0.5 kDa, red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to a difference in functional properties and taste of peptides and proteins such a separation could be desired. Furthermore, a separation could enable the analysis of the peptides in regard of their bioactivity and a coordinated employment of the peptides in foods depending on their various functional properties (Karami & Akbari-adergani, 2019).

3.7. Comparison to conventional wet fractionation

The adapted mild wet fractionation process offers potential to replace the conventional wet fractionation method. Omitting chemicals throughout the process allows to recover and reuse the water in the process. The addition of the ultrafiltration step of SPF to the fractionation process increases the protein purity to more than 75% in the SPF (see Fig. 8). Hence, the process becomes more comparable to CWF, where protein purities of around 75% (Nx5.52) are obtained (Roquette-Frères, 2021). The cumulative protein yield of the SPF and NSPF, subtracting the 14% peptides lost during ultrafiltration, is approximately 83%. CWF can yield up to approximately 76% (Geerts et al., 2018) to 82% (Lie-Piang, Braconi, Boom, & van der Padt, 2021) of the initial protein in the protein isolate (protein in pea flour 21.4%). To obtain these high purities and yields in CWF alkaline extraction and isoelectric precipitation are used, by changing the pH of the solution, to first solubilize all proteins and separate them from non-protein components and secondly precipitate proteins for further purification. However, the use of chemicals affects the structure and functionality of pea proteins. Moreover, alkaline extraction is responsible for adverse chemical reactions of the amino acid side chains (Lam et al., 2018). Isoelectric precipitation also influences the protein conformation and functionality (Boye et al., 2010). Hence, omitting the use of chemicals can not only yield comparable purities and yields but additionally limits the modification of the protein nativity.

With the method presented above, we therefore show possibilities to mildly fractionate proteins from yellow pea, while omitting chemicals. The protein concentrates and isolates can be obtained with different protein purities and yields, according to the process intensity. Besides, the method enables the separation of proteins into soluble and non-soluble protein, potentially offering a wider functional spectrum of the ingredients. Mild wet fractionation introduces the possibility to adjust and alter the composition and functionality of the ingredients based on the application. Performing less, or more processing steps, the protein purity and separation can be controlled, which in turn has an influence on the resource use efficiency and functionality of the final ingredient.

For the fractionation process, including pellet washing step and ultrafiltration, without the addition of diawater, about 23 kg water are necessary to fractionate 1 kg of pea flour. Due to these large amounts of water, the recovery and reuse of water in the process becomes increasingly important. Moreover, as the use of chemicals was fully omitted in this processing technology, the reuse and recovery of water is possible and has no influence on the following fractionation processes. In Fig. 9 the mild wet fractionation process including two pellet washing steps is depicted with subsequent steps to recover the water using ultrafiltration followed by a nanofiltration water purification step. Per kg of pea flour, around 20 kg of water can be recirculated with the proposed process. The recovered water could be reused in the fractionation process, which notably decreases the amount of fresh water necessary for fractionation. Fig. 9 depicts the potential of upscaling the mild fractionation process showing a concept, how mild wet fractionation could be applied to produce protein and starch isolates without the use of chemicals.

In the presented process the centrifugation and pellet washing steps could be replaced with two decanters to separate starch and protein. This would on top decrease the water consumption in the initial process phase. Ultrafiltration of protein solutions is frequently applied in diary industry, e.g. for the production of protein concentrates from whey and for the pre-concentrations of milk, both processes of cheese manufacture (Berk, 2013). The calculated water usage for the presented pea fractionation upscale is around 100 kg water/ kg pea protein (i.e. see Fig. 8 and Fig. 9, 20 kg water/ 0.2 kg protein). To provide a reference, the diawater consumption for whey protein isolate (WPI) production from thick whey (35% DM) was calculated using the same methodology. A

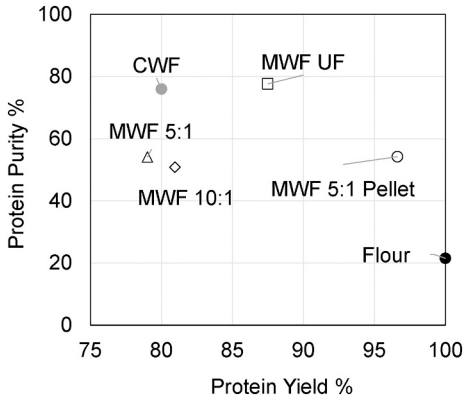

Fig. 8. Yield-purity curve of fractionation methods discussed in this research. CWF, MWF 10:1 (mild wet fractionation as performed by (M.E.J. Geerts, Nikiforidis, van der Goot, & van der Padt, 2017b)), MWF 5:1 (mild wet fractionation at ratio 5:1), MWF 5:1 washed (mild wet fractionation with additional pellet washing steps), MWF 5:1 UF (mild wet fractionation at ratio 5:1 with pellet washing and ultrafiltration) and pea flour. The purities of SPF and NSPF were averaged per proportion, the yields were added up.

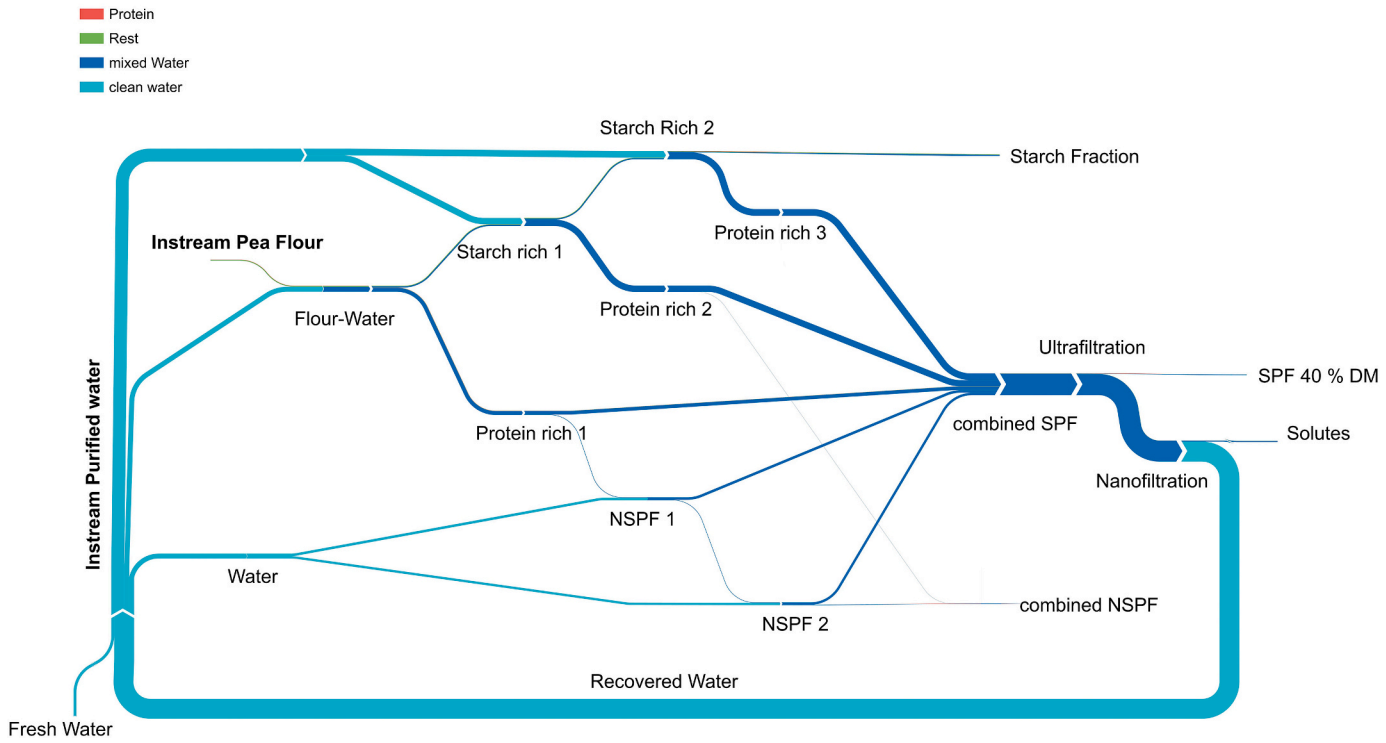

Fig. 9. Graphic representation of mass flow during mild wet fractionation including pellet washings and the potential of recovery and reuse of water after ultrafiltration and water purification. Fresh water is light blue, dispersions are dark blue. The flow quantities are summarized in the Appendix Table A3Table A3 as kg stream/ kg pea flour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

water usage of 62 kg water/ kg whey protein was estimated to produce WPI. The water consumption of both processes was evaluated to be around the same order of magnitude, as a proper diafiltration design will reduce the water usage of the pea fractionation process, which was demonstrated by (Gavazzi-April, Benoit, Doyen, Britten, & Pouliot, 2018; Lipnizki, Boelsmand, & Madsen, 2002). Moreover, so far the presented values are based on lab scale experiments. Therefore, this paper could form the basis to set-up pilot scale experiments to arrive at a conceptual design for industrial pea protein isolation using only water.

4. Conclusions

With the well-directed employment of water in mild wet fractionation we present a promising method for the extraction of high purity and yield protein and starch fractions. The protein purity in the SPF can be increased to around 75% according to our experiments, and potentially even to around 85% comparable to purities obtained in dairy industry, when using a combination of pellet washing with ultrafiltration. Pellet washing of the starch fraction increased the cumulative yield in the protein fractions. While pellet washing of the NSPF led to a further separation of soluble and non-soluble protein in the respective fractions. Ultrafiltration of the SPF extracted small soluble non-protein components from the fractions, together with small peptides, resulting in increased protein purity in the SPF, despite the extraction of the peptides. The mild wet fractionation process offers the potential to produce similar fractions compared to conventional wet fractionation, with the advantage to omit chemicals, recirculate water and limit changes in techno-functional properties. Furthermore, the introduced process shows the potential to add or skip processing steps depending on the desired final ingredient composition and functionality. The presented research highlights that the extensive knowhow of whey protein isolation applied in plant protein purification could provide new routes to more effective plant ingredient production.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix

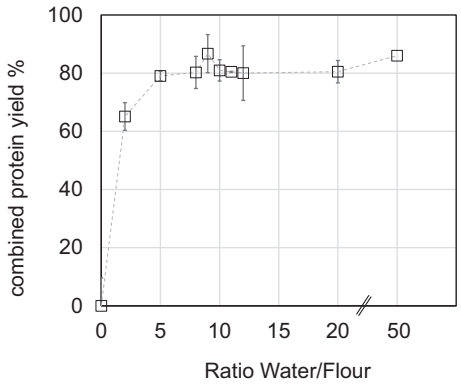

Fig. A1. The combined protein yield % (Eq. 2) of the sum of SPF and NSPF are depicted at varying water/flour ratios. The line is meant to guide the eye.

Table A1
Measured results of 3 step batch ultrafiltration. After each filtration a sample for the compositional analysis was taken, resulting in the mass difference in output and input retentate. Results are depicted in Fig. 5.

Filtration		Sample	Total [g]	Total Dry Matter [g]	Total protein [g]	Dry Matter %	Protein/ DM %	Retention coefficient DM [g/g]	Retention coefficient Protein [g/g]	MRF
	Feed	SPF	200.41	4.66	2.16	2.33	46.33			
1.	Out	Retentate 1	157.52	4.26	2.11	2.70	49.61	0.91	0.98	1.27
	Out	Permeate 1	42.89	0.36	0.05	0.84	13.09			
	Feed	Retentate 1	132.87	3.59	1.78	2.70	49.61			
2.	Out	Retentate 2	68.06	2.92	1.65	4.29	56.47	0.81	0.93	1.96
	Out	Permeate 2	64.81	0.66	0.08	1.02	12.20			
	Feed	Retentate 2	50.17	2.12	1.20	4.29	56.47			
3.	Out	Retentate 3	26.8	1.82	1.15	6.80	63.13	0.86	0.96	1.87
	Out	Permeate 3	23.37	0.30	0.04	1.29	11.87			

Table A2
Calculated 3 step ultrafiltration for designing a continuous ultrafiltration system with a constant protein retention coefficient of 0.97. Results are depicted in Fig. 5.

Filtration		Sample	Total [g]	Total Dry Matter [g]	Total Protein [g]	Dry Matter %	Protein/DM %	Retention coefficient DM [g/g]	MRF
	In	SPF	200	4.66	2.16	2.33	46.33		
1.	Out	Retentate 1	157	4.33	2.14	2.75	49.37	0.75	1.27
	Out	Permeate 1	43	0.33	0.02	0.77	6.09		
	In	Retentate 1	157	4.33	2.14	2.75	49.37		
2.	Out	Retentate 2	81	3.54	2.07	4.40	58.47	0.75	1.96
	Out	Permeate 2	77	0.79	0.07	1.03	8.55		
	In	Retentate 2	81	3.54	2.07	4.40	58.47		
3.	Out	Retentate 3	43	3.02	2.01	7.03	66.46	0.75	1.87
	Out	Permeate 3	38	0.52	0.06	1.38	11.63		

Table A3
Mass flows during mild wet fractionation from Fig. 9. Presented as kg stream/ kg pea flour to be fractionated.

Stream name	Stream quantity [kg/kg pea flour]
Flour-water	6.00
Starch rich 1	1.32
Protein rich 1	4.68
Water to SR 1	6.25
Starch rich 2	1.06
Protein rich 2	6.51
Water to SR 2	6.25
Water to NSPF 1	2.50
NSPF 1	0.46
Water to NSPF 2	2.50
NSPF 2	0.38
Starch fraction	0.97
Comb. SPF	22.07
Comb. NSPF	0.46
SPF 40% DM	0.62
Solutes	1.20
Recovered water	20.25
Fresh water	2.25

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