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Pilot-Scale Protein Recovery from Cold-Pressed Rapeseed Press Cake: Influence of Solids Recirculation

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Abstract: The agricultural sector is responsible for about 30% of greenhouse gas emissions, and thus there is a need to develop new plant-based proteins with lower climate impact. Rapeseed press cake, a by-product from rapeseed oil production, contains 30% high-quality protein. The purpose of this study was to recover protein from cold-pressed rapeseed press cakes on a pilot scale using a decanter and investigate the effect of recirculation of the spent solids fraction on protein yield. Proteins were extracted under alkaline conditions (pH 10.5) followed by precipitation at pH 3.5. Recirculating the spent solids fraction once increased the accumulated protein yield from 70% to 83%. The efficiency of the recovery process was highest in the first and second cycles. The additional yield after the third and fourth cycles was only 2%. The amino acid composition showed high levels of essential amino acids and was not reduced throughout the recovery process. The glucosinolate and phytate content was reduced in the precipitate after one cycle, although additional process steps are needed to further reduce the phytate content and limit the negative effect on mineral uptake.

Keywords: rapeseed press cake; protein extraction; protein recovery; protein yield; plant protein; pilot-plant scale

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1. Introduction

The agricultural sector is responsible for about 30% of all greenhouse gas emissions [1]. Animal-based foods generate significantly higher emissions than plant-based products, the main reasons being the low protein conversion factor from feed to muscle, and the fact that ruminants generate the potent greenhouse gas methane in the digestive process [2]. There is therefore a need to develop new plant-based protein products to reduce the greenhouse gas emission resulting from the agricultural sector. One strategy could be to recover protein for human consumption from agricultural side streams normally used as animal feed.

Rapeseed (*Brassica napus*, *Brassica rapa*, and *Brassica juncea* rapeseed quality) is the second-largest cultivated oilseed crop in the world [3], with 72 million tons of rapeseed being harvested worldwide in 2020 [4]. Rapeseed is primarily used for edible oil production [3], and the oil is released by the mechanical separation of oil and solids. The processes most commonly used for rapeseed oil production are expeller pressing followed by extraction by solvents and cold-pressing. The expeller pressing process involves a preheating step and a cooking step (80–105 °C), followed by expeller pressing in a series of expellers to extract the oil. The rapeseed meal remaining after this step contains about 16–20% oil, which is usually recovered using solvents [5]. Cold-pressing is performed under milder thermal conditions, and the seeds are pressed in a screw press to extract the oil. The temperature of the oil in cold-pressing does not exceed 35 °C. The press cake has a temperature of about 55–60 °C when exiting the screw press. No solvents are used

during production, and the residual oil (around 20%) remains in the press cake. Studies have shown that the extractability and functionality of protein from rapeseed exposed to heat during processing are lower than when the protein is processed under milder thermal conditions [3,6].

The major components in rapeseed press cake (RPC) are protein (27–30%) and fiber (around 30%). The two main proteins in RPC are the storage protein cruciferin (12S globulin) and napin (2S albumin), which together constitute up to 80% of the total protein content [7,8]. Cruciferin is soluble in salt solutions and has an isoelectric point (pI) at pH 7.2 [7,9]. Napin, on the other hand, is a water-soluble protein and has a pI around pH 11 [7,10]. The remaining fraction is oil body proteins; oleosin being the dominating species [11]. The pI for oil body proteins is reported to be at pH 6.5 [12]. A wide range of PI's have been reported in the literature for the mixture of rapeseed proteins. The most commonly reported isoelectric points are between pH 3.5–6, and botanical differences have been suggested to contribute to the differences [13].

Despite the high protein quality, and the balanced amino acid composition of rapeseed protein [14], RPC is mainly used for animal feed due to its content of antinutritional compounds such as phytate, glucosinolate, and phenolic compounds. During the oil pressing, rapeseed is crushed, and enzymes such as myrosinase are released. Myrosinase hydrolyzes the glucosinolate molecule into glucose as well as potentially toxic compounds, such as thiocyanate and isothiocyanate. The microflora in the gastrointestinal tract can also hydrolyze glucosinolate [3]. The glucosinolate breakdown products have shown a toxic effect in animals including impaired thyroid function, enlargement of the liver and reduced feed intake with growth depression as a consequence [3]. The plant breeding field has been focused on reducing the initial concentrations of glucosinolate at the seed level and has since the 1970s reduced the levels by a factor of ten. However, glucosinolate is a water-soluble molecule and is therefore concentrated in the press cake after oil extraction of the rapeseeds; it is important to reduce the glucosinolate content if the rapeseed protein should be used in food applications [15]. Phytate is known to be a strong chelating agent that can bind metal ions, forming insoluble compounds unavailable for absorption in the human small intestine. The presence of phytate in the diet negatively affects the uptake of minerals such as iron and zinc, for iron is already at very low levels. Iron deficiency is prevalent in vulnerable groups such as women of fertile ages, children, and adolescents [16–20]. Moreover, due to the presence of phenolic compounds, RPC has a bitter off-flavor which would have to be removed for use in human food applications [21,22].

Several processes can be used for the extraction of protein from RPC, but the most common are the pH-shift method and the salt method. In the pH-shift method, the RPC is first milled and soaked in an alkaline solution in which the protein gains a negative surface charge, and is solubilized in the aqueous phase. This is followed by separation into a spent solids fraction (SSF) containing husks, intact cells, and non-solubilized protein, and a light liquid phase (LLP) containing solubilized protein and co-extracted carbohydrates. The pH is then adjusted to the proteins PI, where the net charge of the proteins is zero and hence the proteins are subjected to precipitation. In the following separation step, the precipitated protein is recovered [14,23]. The pH shift method has been used on both defatted [6,23] and non-defatted RPC [6].

Salt extraction is based on the principle of increasing the ionic strength of the extraction media, leading to the breakage of salt bridges, and shielding of the surface charge of the protein. This counteracts protein–protein interactions, which enhances the solubility of the protein [24,25]. Salt extraction can be followed by ultrafiltration to concentrate and purify the protein. Thereafter, the protein solution is diluted with water to reduce the ionic strength, resulting in protein precipitation [14]. The salt extraction method has been used on both defatted [25] and non-defatted RPC [24,26].

Only a limited number of studies have been carried out on rapeseed protein extraction on a pilot scale [3,27–29]. Bérot et al. [27] developed a process to isolate cruciferin and napin on a pilot scale. The rapeseed was first defatted by hexane extraction under mild thermal conditions, then the protein was extracted under mild alkaline conditions (pH 8.5), together with buffer containing sodium chloride, ethylenediaminetetraacetic acid and sodium bisulfite. Combining nanofiltration and chromatographic techniques led to a protein extraction yield of 66%; the recovery of cruciferin being 40% and that of napin being 18%. Chabanon et al. [28] recovered rapeseed protein from industrial defatted rapeseed meal on a pilot scale. The meal was washed four times with ethanol, followed by protein extraction with NaOH. The solids were removed, and the protein was precipitated from the liquid fraction at pH 4.5. Both the supernatant and the precipitate were then purified by dialysis. This resulted in a yield of 30%: the precipitated protein accounting for 20% and the soluble protein for 10%.

Pilot-scale extraction is often associated with a lower recovery than on a laboratory scale, but improvements in process design could result in more efficient large-scale extraction of rapeseed protein [27]. Single-stage extraction often results in low yields due to slow dissolution once the solution approaches equilibrium. Recirculation of the SSF could therefore provide a means of increasing the protein yield. Recirculation has been used to increase the yield in other applications in the food industry, for example, in the extraction of oleosomes from soybean flour, in the production of soy beverages, and in the extraction of sugar from sugar beet [30–32].

The aim of the study was to increase the protein yield of the recovery process of rapeseed protein by recirculating the SSF. The study was carried out on pilot scale and protein yield and proximate analysis were assessed in the various process streams. Furthermore, the present work aimed to characterize and quantify the effect of recirculation on the levels of the antinutrients glucosinolate and phytate content.

2. Materials and Methods

2.1. Material

Cold-pressed rapeseed press cake (*Brassica napus* L.), a kind gift from Gunnarshögs Jordbruks AB (Hammenhög, Sweden), was used in all the experiments. Two different industrial batches were used and the proximate analysis was the following: 9% moisture, 29% protein, 13% fat, 6% ash, and 43% carbohydrates (calculated by differences.) The screw press at the Gunnarshög production plant yields cold-pressed oil, the temperature of which does not exceed 37 °C, while the press cake has a temperature of about 55–60 °C when exiting the screw press. The press cake was stored at −18 °C prior to the experiments.

Citric acid powder (>95% purity) and NaOH were purchased from Merck (Darmstadt, Germany) and aspartic acid was purchased from Thermo Electron (Milan, Italy).

2.2. Extraction of Protein from Rapeseed Press Cake

The protein was recovered from RPC as described previously by Östbring et al. [33], based on a modification of the method of Wijesundera et al. [12]. Figure 1 shows a flowchart describing the process. Two kilograms of the PRC was ground in a knife mill (R302 v.v. Robot Coupe, Paris, France) for three minutes to obtain a powder. The milled RPC was dispersed in 18 L tap water in a stirred tank, and the pH was adjusted to 10.5 using 2 M NaOH. The pH was re-adjusted to 10.5 after 10 min. The dispersion was stirred for a total of four hours at 200 rpm using a three-bladed propeller stirrer with a diameter of 140 mm (IKA RW 28 digital, Staufen, Germany).

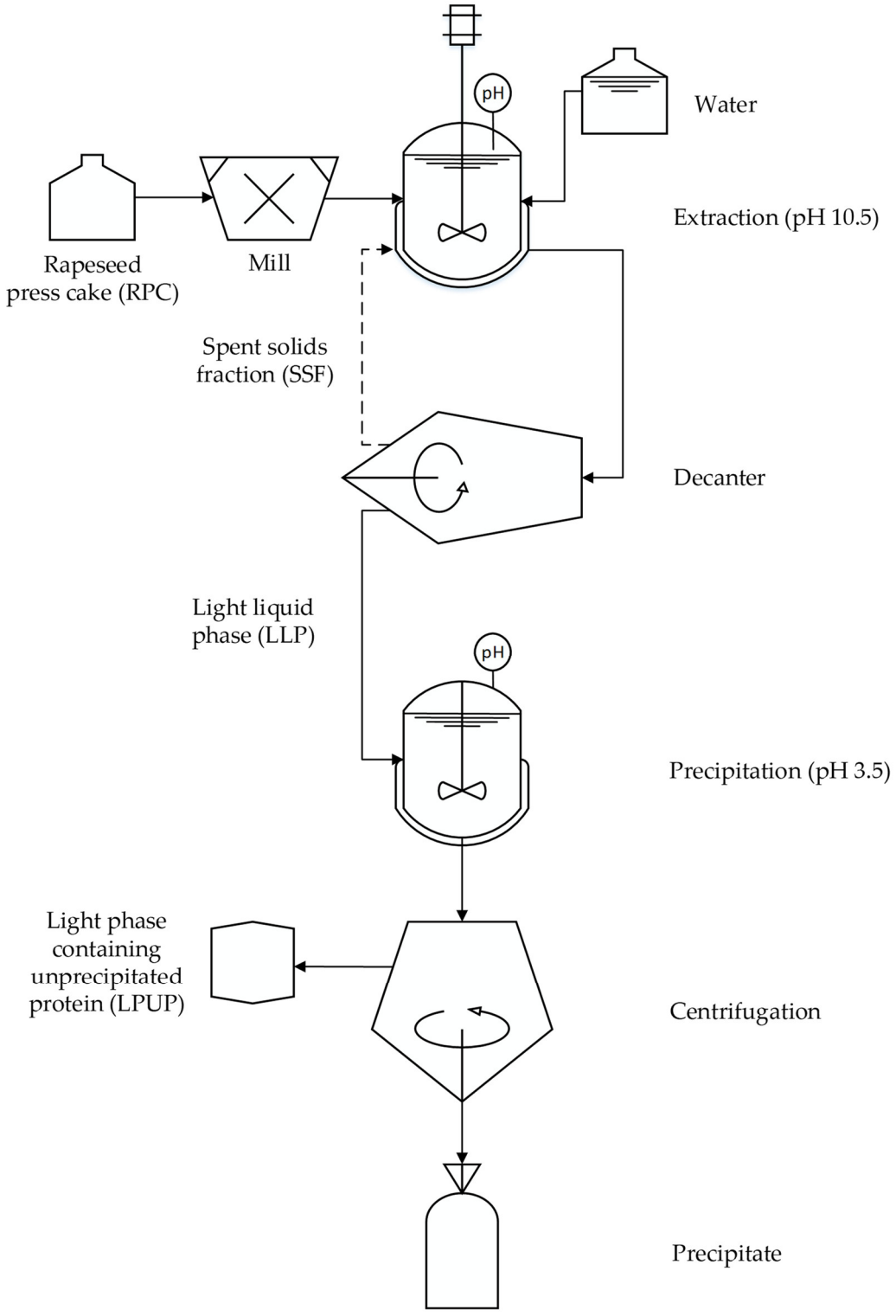


Figure 1. A flowchart of the process used to separate protein from rapeseed press cake. The dashed arrow indicates the recirculation of the spent solids fraction (SSF).

2.3. Decanter Separation

After extraction, the SSF (the raffinate) was separated from the LLP containing the solubilized protein (the extract) using a decanter (Decanter centrifuge MD80, Lemitec, Berlin, Germany) at 6687 rpm (2000× *g*) and a differential speed of 10 rpm. The inner diameter of the weir disc was 56 mm. The flow to the decanter was adjusted to 25 L/h with

a peristaltic pump (Masterflex Easy-load Model 77200-62, Cole-Parmer, Vernon Hills, IL, USA). The SSF was saved for recirculation (see Section 2.5 below) and the LLP was collected.

2.4. Precipitation of Protein

Four representative samples of approximately 400 g were collected from the LLP phase to evaluate the recovery. An initial screening study was performed (data not shown) from which it was found that the protein yield was highest at pH 3.5. Precipitation of the protein was therefore carried out after adjusting the pH to 3.5 with citric acid powder. The LLP was stirred continuously for 20 min with a magnetic stirrer, and then centrifuged for 20 min at 20 °C at 5000× g (Beckman Coulter, Allegra® X-15R Centrifuge, Brea, CA, USA). The precipitate was collected as the final product, and the supernatant, hereafter referred to as the light phase containing unprecipitated protein (LPUP), was discarded. Samples of the RPC, SSF, LLP, LPUP, and precipitate were collected throughout the process, and were stored at −18 °C prior to further analysis.

2.5. Recirculation of SSF

The SSF were re-dispersed in tap water to maintain the original dilution factor (1:10 w/w dilution on wet basis), the pH was adjusted to 10.5, and the experiment was repeated as described above. A total of four protein extraction cycles were used, i.e., recirculation of the SSF three times. The extraction experiments were performed in triplicate.

The protein yield was calculated as both the accumulated and relative protein yield. The accumulated protein yield is related to the RPC, while the relative protein yield is related to the starting material of each cycle, i.e., the SSF from the previous cycle. The accumulated protein yield thus reflects how large a proportion of the protein content in the RPC was recovered, while the relative protein yield reflects the protein recovery efficacy for each recirculation.

The accumulated protein yield was calculated as:

$$Accumulated\ protein\ yield = \frac{Accumulated\ mass\ of\ extracted\ protein}{Mass\ of\ protein\ in\ rapeseed\ press\ cake} \times 100 \quad (1)$$

The relative protein yield was calculated as:

$$Relative\ protein\ yield = \frac{Mass\ of\ extracted\ protein}{Mass\ of\ protein\ in\ starting\ material} \times 100 \quad (2)$$

The protein content on dry basis was defined as:

$$Protein\ content = \frac{Mass\ of\ protein}{Mass\ of\ dry\ solids} \times 100 \quad (3)$$

2.6. Proximate Analysis and Water Holding Capacity

The proximate analysis was first performed on the RPC, LLP, LPUP, and the precipitate of all four cycles in terms of dry matter content and protein content. Furthermore, the RPC and the precipitate from the first and second cycle were also analyzed in terms of fat and ash content. The carbohydrate content was calculated by difference.

2.6.1. Dry Matter Content

The dry matter content was determined according to the official method of analysis (AOAC 934.01) [34]. Briefly, solid/semi-solid samples were dried in a convection oven (Termaks, Bergen, Norway) at 103 °C until constant weight (>16 h). The analysis was performed in duplicate. Freeze drying was used to determine the dry matter content of the liquid samples due to the low mass of solids in these samples. The samples were placed in a freeze dryer (Labconco Lyph Lock 18, Kansas City, MO, USA) for four days. To ensure

that all the water had evaporated, the samples were thereafter placed in the oven at 103 °C for a minimum of one day after freeze drying.

2.6.2. Protein Content

The protein content was analyzed using the Dumas method (AOAC 990.03) (Thermo Electron Corp., Flash EA, 1112 Series, Waltham, MA, USA) [35]. Then, 25–50 mg of sample was placed on a 33 mm tin disc for analysis. Air was used as the blank and aspartic acid was used as the reference. A nitrogen-to-protein conversion factor of 6.25 was used. The analysis was performed in duplicate.

2.6.3. Fat Content

The fat content of the RPC and precipitate from the first and second cycle were analyzed using a semiautomatic Soxtec apparatus (Tecator AB, Höganäs, Sweden) according to AOAC 920.39 [36]. The samples were freeze-dried prior to analysis and the fat was extracted using petroleum benzine as a solvent. The analysis was performed in triplicate.

2.6.4. Ash Content

The ash content of the RPC and precipitate from the first and second cycle were analyzed using a muffle furnace (B150, Nabertherm GmbH, Lilienthal, Germany). Freeze-dried samples were placed in the oven at 700 °C for 3 h. The samples cooled down in a desiccator for a minimum of 1 h before being reweighed [37]. The analysis was performed in triplicate.

2.6.5. Water Holding Capacity

The approximate water holding capacity (WHC) of the RPC and precipitate from the first and second cycle were analyzed using the AACC Method 88-04 [38]. Approximately 5 g of freeze-dried sample was mixed with deionized water until all material was completely wet. Thereafter, the sample was centrifuged for 10 min at 2000× g (Beckman Coulter, Avanti®J-15R Centrifuge, Brea, CA, USA). The supernatant was discarded and the WHC (mL/g) was calculated as:

$$WHC = \frac{Mass\ of\ hydrated\ sample - Mass\ of sample}{Mass\ of sample} \quad (4)$$

The analysis was performed in triplicate.

2.7. Amino Acid Profile

The amino acid profile of the RPC and precipitate from the first and second cycle were analyzed at Eurofins Food & Feed Testing Sweden using an amino acid analyzer and the standardized methods ISO 13903:2005, ISO 13904:2016, and ISO 13903:2005. The analysis was performed in triplicate on freeze-dried samples.

2.8. Antinutrients

2.8.1. Phytate Analysis

Phytate, analyzed as inositol hexaphosphate ($InsP_6$), in the RPC and precipitate after the first and second cycle was analyzed by high-performance ion chromatography (HPIC) [39,40]. The samples were freeze-dried prior to analysis. The samples (0.5 g) were extracted with 10 mL of 0.5 mol/L HCl for 3 h using a laboratory shaker (Heidolph Reax 2; Heidolph Instruments GmbH, Schwabach, Germany). Then, 1 mL was removed, centrifuged, and the supernatant was transferred to an HPLC vial. The chromatography setup consisted of an HPLC pump (model PU-4080i; Jasco Inc., Easton, MD, USA) for the eluent and an RHPLC pump (model PU-4180; Jasco) equipped with a PA-100 guard column and a CarboPac PA-100 column. $InsP_6$ was eluted with an isocratic eluent of 80% HCl (1 mol/L) and 20% H_2O at 0.8 mL/min, subjected to a post-column reaction with ferrous nitrate, and detected at 290 nm in a UV–visible HPLC detector (UV-4075; Jasco, Tokyo, Japan).

Each sample had a run time of 7 min, and the $InsP_6$ concentration was calculated using external standards covering the concentration range of 0.1–0.6 µmol/Ml. The analysis was performed in duplicate.

2.8.2. Glucosinolate Analysis

The glucosinolate content in the RPC and the precipitate from the first and second cycle was analyzed by Eurofins USA Food Division (ISO 9167-1:1992). The samples were freeze-dried prior to analysis and analyzed in triplicate.

2.9. Statistical Analysis

Extractions with recirculation were conducted in triplicate and all analyses were carried out in at least duplicate. Statistical analyses were performed using SPSS Statistics 26 (IBM, Armonk, NY, USA). For data sets that were normally distributed, a univariate general model was applied (wet mass, dry solids, mass of protein, protein content, accumulated protein yield of the precipitate, the amino acid profile except the glutamic acid, and the phytate content). Post-hoc analysis was performed using Tukey's test to investigate significant differences. A Kruskal–Wallis one-way ANOVA test with all pairwise comparison was performed to investigate any significant difference in non-parametric data sets (accumulated protein yield of the LPUP, relative protein yield, and glutamic acid). Glucosinolate content was analyzed using Student's t-test. Results were considered significant if p values were <0.05. All results are expressed as the mean and the standard deviation.

3. Results and Discussion

The study was performed in two parts, with the first one focusing on how recirculation affected the protein extraction, precipitation, and consequent yields. The second part investigated any potential differences in the quality of the rapeseed protein after multiple pH shifts.

3.1. The Effect of Recirculation on Protein Extraction, Precipitation and Recovery Yield

The wet mass of the starting material of the first cycle was 2000 ± 0 g RPC, containing 565 ± 12 g protein (Table 1). The protein that remained non-solubilized in the SSF after separation in the decanter was recirculated as starting material in the following cycle. The starting material for the second cycle contained significantly more water than the RPC used in the first cycle, leading to an increase in the wet mass of the starting material in the second cycle, 3100 ± 50 g. The wet mass of the starting material decreased in each successive cycle. The significantly highest mass of dry solids was found in the starting material in the first cycle, and the lowest mass in the fourth cycle. The starting material for the second cycle contained 147 ± 12 g protein, which decreased to 56.4 ± 14 g in the third cycle and to 30.5 ± 6.8 g in the fourth cycle. The majority of the protein in the press cake was successfully extracted to the LLP and the extraction yield was 70% in the first cycle. Ma et al. [41] reported an extraction yield of 84% when rapeseed protein was extracted from a defatted meal. This was higher than the yield in the present study. They used pH 12 in the extraction phase, as well as elevated temperature (48 °C) together with ultrasonic treatment, which together increased the extractability of rapeseed protein. Fetzer et al. [24] reported an extraction yield of 58% for defatted cold-pressed rapeseed protein. Fetzer and colleagues used defatted cold-pressed rapeseed press cakes and used pH 11 together with 0.25 M NaCl during extraction.

After pH precipitation at pH 3.5 and the second separation step, the precipitate was separated from the LPUP. The wet mass of the precipitate decreased with each cycle. In the first cycle, 1590 ± 130 g of precipitate was recovered, compared with 104 ± 40 g in the fourth cycle (Table 1). The dry solids content of the precipitates decreased with each cycle; being 470 ± 20 g in the first cycle and 4.2 ± 0.8 g in the fourth cycle. The protein that did not precipitate at pH 3.5 remained solubilized in the LPUP. The mass of dry solids in this

fraction decreased with successive cycles, from 440 ± 40 g in the first cycle to 7.0 ± 4 g in the fourth cycle.

Table 1. Wet mass, dry solids, and protein in the starting material, precipitate, and LPUP for all four cycles. All data are expressed as the mean ± standard deviation. Different letters in each row indicate significant differences, $p \leq 0.05$.

	Cycle	1st	2nd	3rd	4th
Starting material		RPC = Rapeseed Press Cake	SSF = Spent Solids Fraction		
	Wet mass (g)	2000 ± 0 ^{b}	3100 ± 50 ^{a}	2100 ± 200 ^{b}	1300 ± 200 ^{c}
	Dry solids (g)	1820 ± 15 ^{a}	723 ± 28 ^{b}	389 ± 43 ^{c}	256 ± 74 ^{d}
	Protein (g)	565 ± 12 ^{a}	147 ± 12 ^{b}	56.4 ± 14 ^{c}	30.5 ± 6.8 ^{c}
LLP = light liquid phase		Precipitate			
	Wet mass (g)	1590 ± 130 ^{a}	633 ± 34 ^{b}	246 ± 15 ^{c}	104 ± 40 ^{c}
	Dry solids (g)	470 ± 20 ^{a}	120 ± 2 ^{b}	19 ± 1 ^{c}	4.2 ± 0.8 ^{c}
	Protein (g)	290 ± 20 ^{a}	52 ± 1 ^{b}	5.7 ± 0.3 ^{c}	1.0 ± 0.2 ^{c}
		LPUP = light phase containing unprecipitated protein			
	Wet mass (g)	12,700 ± 930 ^{c}	24,500 ± 660 ^{a}	16,100 ± 930 ^{b}	8900 ± 1500 ^{d}
	Dry solids (g)	440 ± 40 ^{a}	91 ± 3 ^{b}	17 ± 2 ^{c}	7.0 ± 4 ^{c}
	Protein (g)	100 ± 5 ^{a}	8.6 ± 2 ^{b}	0.61 ± 0.0 ^{c}	0.10 ± 0.0 ^{c}

A significantly larger mass of protein was recovered in the precipitate in the first two cycles, and only small masses were detected in the third and fourth cycles. In the first cycle, 290 ± 20 g of protein was successfully recovered in the precipitate, and in the second cycle, an additional 52 ± 1 g of protein was recovered. Only 5.7 ± 0.3 g and 1.0 ± 0.2 g protein were detected in the precipitate from the third and fourth cycles, respectively. The mass of protein in the LPUP followed a similar trend as the precipitate: 100 ± 5 g protein in the first cycle, and 8.6 ± 2 g protein in the second cycle. Only small amounts of protein were detected in the third and fourth cycles; 0.61 ± 0.0 g and 0.10 ± 0.0 g, respectively.

The protein content (dry basis) of both the precipitate and the LPUP varied with the recirculation cycle (Figure 2). The protein content was overall higher in the precipitate than in the LPUP, regardless of the recirculation cycle. The precipitate from the first cycle had a protein content of 63%, compared with 23% in the corresponding LPUP. The protein content decreased in the precipitate with successive recirculation cycles, indicating increased co-precipitation of non-nitrogenous compounds. Moreover, the protein content decreased in the LPUP. After the fourth cycle, the protein content had decreased to 24% in the precipitate and 2% in the LPUP. The protein content of the precipitate in the first cycle was 63%, which is slightly lower than protein content of 71% in the precipitate reported by Yoshie-Stark et al. [29]. However, they used rapeseed press cake that had been defatted with hexane, which reduced the oil content and thereby increased the relative proportion of protein in the starting material compared to the present study, where an industrial side stream was used.

In the first cycle, 70% of the protein in the RPC was successfully extracted and separated in the decanter (Figure 3a). This is in line with the finding of Bérot et al. [27], who reported a protein yield of 66% from RPC on a pilot scale. Furthermore, the protein yield in the present study is higher than the yield in a study by Fetzer et al. [24], who reported an extraction yield of 60% protein from cold-pressed RPC on a laboratory scale.

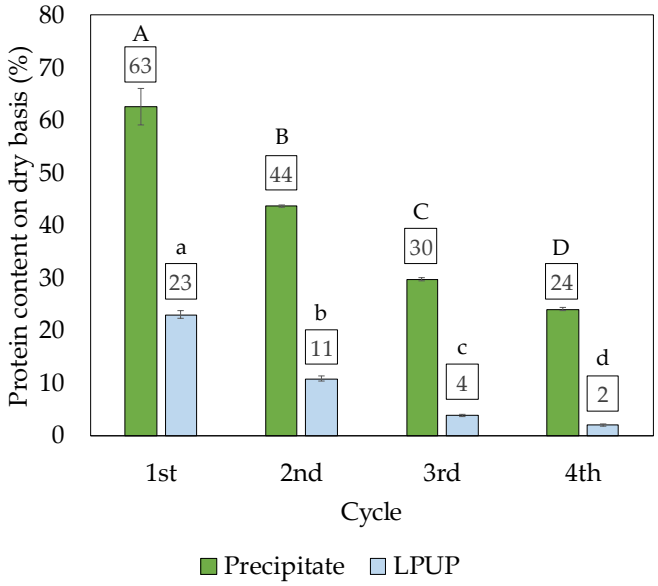
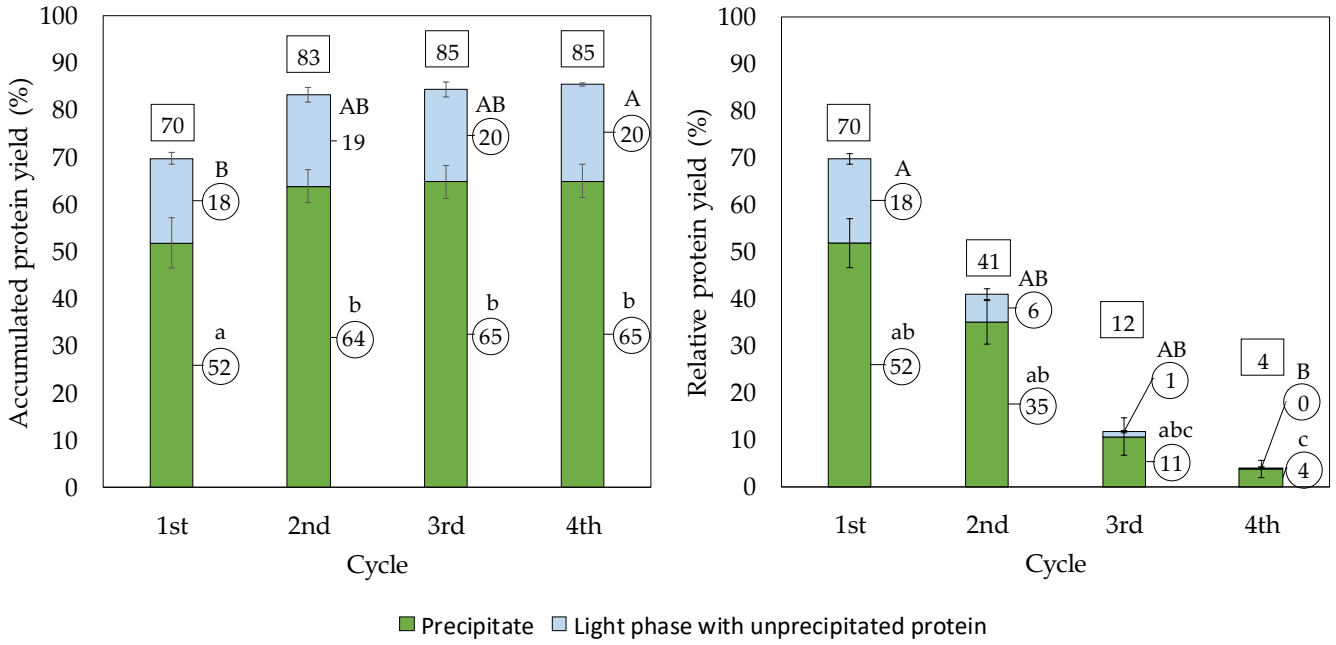


Figure 2. Protein content on dry basis in the precipitate (green) and the LPUP (blue) for the four recirculation cycles. LPUP = light phase with unprecipitated protein. Different uppercase letters indicate significant differences between precipitates and different lowercase letters indicate significant differences between LPUP, $p \leq 0.05$.



(**a**) (**b**)

Figure 3. (**a**) Accumulated protein yield in the precipitate (green) and in the light phase with unprecipitated protein (LPUP) marked in blue over four recirculation cycles of the SSF. (**b**) Relative protein yield showing how large a proportion of the protein in the starting material in each recirculation run was measured in the precipitate (green) and in the LPUP (blue). Different uppercase letters indicate significant differences between LPUP and different lowercase letters indicate significant differences between precipitates, $p \leq 0.05$.

After precipitation at pH 3.5, 52% of the protein from the RPC was recovered in the precipitate, while 18% was soluble in the LPUP (Figure 3a). Chabanon et al. [28] recovered rapeseed proteins on a pilot scale and reported a recovery of 20% of the protein in the

precipitate and 10% in the LPUP. Östbring et al. [6] reported the effect of different oil extraction processes on protein yield on a laboratory scale. They used the same extraction pH (10.5) as in the present study, and pH 5.0 was used in the precipitation step. The highest protein yield was found with cold-pressed RPC; 45% of the protein being found in the precipitate. Based on the results of the present study, a precipitation pH of 3.5 appears to be more favorable in terms of protein yield, as 52% of the protein was recovered at pH 3.5, compared to 45% at pH 5.

As expected, the fraction of protein remaining was successfully reduced in each subsequent recirculation cycle (Table 1). After the first cycle, 70% of all the protein in the RPC was solubilized in the LLP (Figure 3a). After the second cycle, 83% of the protein was solubilized, and only an additional 2% was extracted in the third and fourth cycles, resulting in an accumulated protein yield of 85% (Figure 3a).

The protein yield determined in the present study is slightly higher than that in a study performed by Pedroche et al. [42], where proteins from *Brassica carinata* (a close botanical relative to rapeseed) were recovered at different pHs and the effect of one cycle of recirculation was investigated on laboratory scale. At an extraction pH of 10, the protein yield was 55%; in comparison, 83% was extracted after one recirculation at pH 10.5 in the present study.

As the relative protein yield is based on the starting material in each cycle, the result for the first cycle is identical to the protein yield (Figure 3b). In the second cycle, 41% of the protein from the starting material (SSF from the first cycle) was successfully extracted and separated in the decanter (Figure 3b). Most of the protein was precipitated (35%), and only 6% remained solubilized in the LPUP. In the third and fourth cycles, an additional 12% and 4% protein were extracted from the starting material in each cycle (Figure 3b). Thus, the efficiency of the extraction process was higher in the first and second cycles than in the third and fourth.

3.2. Protein Distribution

The protein distribution in the various process streams of the recovery process was evaluated. The input to the first cycle was 565 g protein, of which 391 g was successfully extracted into the LLP (combination of the blue and green shaded values in Figure 4a), while 147 g remained non-solubilized in the SSF. Furthermore, 290 g of the protein from the RPC was precipitated at pH 3.5, and 101 g remained non-solubilized in the LPUP.

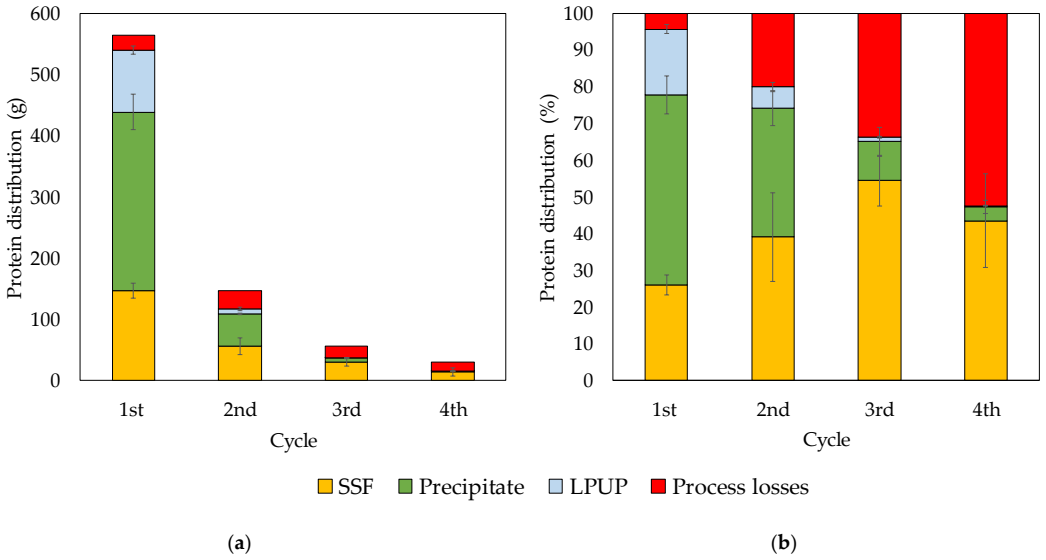


Figure 4. Protein distribution in the various process streams for each recirculation cycle. (**a**) Protein expressed on mass basis. (**b**) Protein expressed as % within each recirculation cycle. SSF= spent solids fraction and LPUP= light phase with unprecipitated protein.

Recirculation of the SSF as starting material in the second cycle resulted in the extraction of an additional 61 g of protein into the LLP (combination of the blue and green shaded values in Figure 4a). However, 56 g of protein, almost 40% of the protein in the starting material of the second cycle, remained non-solubilized in the SSF. Furthermore, in contrast to the first cycle, the protein that was solubilized in the LLP was precipitated to a greater extent. This could be related to the fact that the RPC contains a variety of proteins with different properties. Not all proteins can be solubilized during the extraction step. It is possible that some of the cruciferin remained non-solubilized due to the low salt concentration in the extraction medium, and therefore remained in the SSF regardless of repeated extraction cycles. Furthermore, the protein on the surface of the RPC expeller pellet could have been exposed to temperatures exceeding 60 °C during oil extraction, and could therefore have been subjected to denaturation [3]. Denatured proteins cannot be completely extracted during the extraction step due to changes in their biochemical structure, and would probably therefore remain in the SSF during recirculation [43,44].

Furthermore, only a small amount of protein was extracted in the third and fourth cycles. This could be due to protein remaining non-solubilized in the SSF, or process losses (Figure 4b). However, most of the solubilized protein was precipitated at pH 3.5 in both the third and fourth cycles.

Process losses were calculated from mass balances, and were very similar in all the cycles (Figure 4a). These losses were probably due to the design of the process equipment, for example, material remaining attached to the screw inside the decanter, or in the bottom of the cone-shaped extraction tank. As the mass of protein in the starting material decreased after each cycle, the proportion of process losses increased (Figure 4b). In the first cycle, the process losses were only 4% of the protein, compared to 55% in the fourth cycle.

3.3. Comparison of the Rapeseed Press Cake and Precipitate from the First and Second Cycle

The efficiency of the extraction process was significantly higher in the first and second cycles compared to the third and fourth cycles, where only an additional 2% was extracted. Therefore, the effects of one recirculation were investigated further with an overall proximate analysis. Before preparing material for additional analysis, an initial screening was performed (data not shown) from where it was found that four hours' extraction did not result in a higher extraction coefficient compared to one hour. Therefore, the following recovery process used one hour of extraction. An additional batch of precipitate of the first and second cycle was prepared on a pilot scale. The RPC was ground in a knife mill (R 5 Plus, Robot Coupe, Paris, France) for three minutes to obtain a powder. All precipitate was recovered from the LPP using a benchtop centrifuge (Beckman Coulter, Avanti®J-15R Centrifuge, Brea, CA, USA). The following analysis steps focused on the starting material RPC and the precipitate from the first and second cycle. The amino acid profile was analyzed, the antinutrient levels were determined, and the WHC was investigated to evaluate the effect of one recirculation on the recovery process

3.3.1. Proximate Analysis

The protein content in the starting material, the RPC, was 32 ± 0% (Table 2). The significantly highest protein content was located in the precipitate from the first cycle, 51 ± 2%. The protein content was thereafter reduced to 47 ± 1% in the precipitate from the second cycle. The amount of fat relative to the amount of solids increased along the recovery process. The lowest fat content was located in the RPC (14 ± 1%) and the fat content increased significantly to 39 ± 1% in the precipitate from the second cycle. The fat and protein seem to interact with each other and form a water–soluble complex that has a higher affinity for the aqueous phase rather than the SSF. In the precipitation step of the process, the proteins and fat complex are precipitated and enriched in the sediment. Therefore, the fraction of fat is increased in each extraction and precipitation cycle. The ash content was similar in the RPC and the precipitate from the second cycle, at 6 ± 0% and 5 ± 1%. Moreover, the ash content was significantly increased to 9 ± 1% in the precipitate

from the first cycle. The highest amount of carbohydrates was located in the RPC (48 ± 0%) and the carbohydrates content decreased throughout the recovery process to 9 ± 0% in the precipitate from the second cycle.

Table 2. Proximate analysis on dry basis of the rapeseed press cake and the precipitate from the first and second cycle. Different letters indicate significant differences, $p \leq 0.05$.

	RPC = Rapeseed Press Cake	Precipitate First Cycle	Precipitate Second Cycle
Protein (%)	32 ± 0 ^{c}	51 ± 2 ^{a}	47 ± 1 ^{b}
Fat (%)	14 ± 1 ^{c}	24 ± 1 ^{b}	39 ± 1 ^{a}
Ash (%)	6 ± 0 ^{b}	9 ± 1 ^{a}	5 ± 1 ^{b}
Carbohydrates (%)	48 ± 0 ^{a}	16 ± 1 ^{b}	9 ± 0 ^{c}

3.3.2. Water Holding Capacity

The WHC of a sample is a measurement of how much water a sample can hold against gravity. Prior to the recovery process, the WHC of the RPC was 2.00 ± 0.1 g/g. The WHC was increased to 2.97 ± 0.5 g/g in the precipitate from the first cycle. After one recirculation, the WHC was reduced to 1.76 ± 0.2 g/g in the precipitate from the second cycle. The increased WHC for the precipitate from the first cycle might be due to the increased protein content compared to the RPC (Table 2). Furthermore, partial denaturing of protein as a result of the recovery process with a high alkali pH can also increase the WHC, due to additional formation of hydrogen bonding which increases the water uptake [45]. Additionally, the fat content was increased after the second cycle with reduced protein content as a consequence, which might be related to the reduced WHC for the second precipitate (Table 2). The results for the precipitate from the first cycle are slightly lower than the results in a study performed by Bühler et al. who reported a WHC of 3.53 g/g for soy protein concentrate and 3.10 g/g for faba bean concentrate, although no protein extraction details were provided [46]. Hadnađev et al. investigated the WHC for hemp protein extracted in one cycle at pH 10 and recovered at pH 5.0 [45]. They reported a WHC of 1.59 g/g, which is lower than the precipitate from the first cycle in the present study and the same range as the precipitate from the second cycle.

3.3.3. Amino Acid Profile

The amino acid profile of the RPC and precipitate from cycle 1 and 2 is shown in Table 3, together with the adult daily requirements from Food and Agriculture Organization, World Health Organization and United Nations University (FAO/WHO/UNU) [47]. FAO/WHO/UNU recommends the indispensable protein requirement of an adult to be 0.66 g protein/kg body weight. Furthermore, the recommendation also states that for each g of indispensable amino acids consumed, 277 mg should come from essential amino acids. The RPC contained high levels of essential amino acids (389 ± 1 mg/g) and thereby exceeds the suggested recommendations. The amino acid composition was not significantly affected by the recovery process and the total essential amino acid content was still higher than the recommendations in the precipitate after the first and second cycle (413 ± 4 mg/g and 418 ± 3 mg/g).

The amount of total non-essential amino acids was in the same range for all the samples. Therefore, the proportions of essential amino acids to the total amino acids (E/T) were unaltered throughout the recovery process (40–42%).

Lysine is known to be heat sensitive and negatively affected by commercial oil pressing under hot conditions. Therefore, the reported lysine content is usually 9–10% lower in RPC compared to the seed [3]. The lysine content in the present study was determined to be 64 ± 1 mg/g in the RPC. Newkirk et al. [48] reported a lysine content of 55.6 mg/g in the

RPC which had been exposed to temperatures up to 110 °C. The difference in the lysine content might be due to the milder pressing condition of the RPC in the present study.

Table 3. The amino acid profile is expressed as mg indispensable amino acid per g protein. The amino acid profile of the rapeseed press cake and the precipitate from the first and second cycle are shown in the table. Additionally, the daily requirements (mg/g) for an adult according to FAO/WHO/UNU (2007) [47] are presented in the table. Dash (-) indicates that the amino acid concentration was below the detection level of the method used. Different letters indicate significant differences, $p \leq 0.05$.

Amino Acid	RPC = Rapeseed Press Cake	Precipitate Cycle 1	Precipitate Cycle 2	Adult Daily Requirement
Essential				
Cysteine [a]	25 ± 1 [a]	14 ± 1 [c]	17 ± 1 [b]	
Histidine	29 ± 0 [b]	28 ± 0 [b]	30 ± 0 [a]	15
Isoleucine	40 ± 1 [b]	47 ± 0 [a]	46 ± 0 [a]	30
Leucine	74 ± 1 [b]	87 ± 0 [a]	85 ± 1 [a]	59
Lysine	64 ± 1 [a]	55 ± 2 [c]	60 ± 0 [b]	45
Methionine	21 ± 1 [b]	21 ± 0 [b]	23 ± 1 [a]	
Phenylalanine	42 ± 0 [c]	49 ± 1 [a]	48 ± 1 [b]	
Threonine	50 ± 1 [a]	52 ± 0 [a]	52 ± 2 [a]	23
Tryptophan	16 ± 1 [a]	17 ± 0 [a]	16 ± 1 [a]	6
Tyrosine	32 ± 1 [b]	38 ± 1 [a]	39 ± 1 [a]	
Valine	54 ± 2 [b]	58 ± 1 [a]	58 ± 1 [a]	39
Non-essential				
Alanine	48 ± 0 [b]	51 ± 0 [a]	51 ± 1 [a]	
Arginine	64 ± 1 [b]	70 ± 1 [a]	71 ± 1 [a]	
Aspartic acid	80 ± 1 [c]	96 ± 1 [a]	83 ± 1 [b]	
Glutamic acid	175 ± 2 [a]	160 ± 3 [a]	157 ± 6 [a]	
Glycine	56 ± 0 [b]	58 ± 0 [a]	56 ± 1 [b]	
Hydroxyproline	17 ± 3	-	-	
Proline	64 ± 1 [a]	50 ± 2 [c]	55 ± 2 [b]	
Serine	49 ± 2 [a]	50 ± 1 [a]	51 ± 1 [a]	
Ornitin	-	-	-	
Total sulfur-containing amino acids (Met and Cys)	46 ± 2 [a]	35 ± 1 [c]	39 ± 0 [b]	22
Total aromatic amino acids (Phe and Tyr)	74 ± 1 [b]	87 ± 2 [a]	87 ± 1 [a]	38
Total essential amino acids	389 ± 1 [b]	413 ± 4 [a]	418 ± 3 [a]	277
Total non-essential amino acids	594 ± 3 [a]	587 ± 4 [ab]	582 ± 3 [b]	
E/T (%) [a]	40 ± 0 [b]	41 ± 0 [a]	42 ± 0 [a]	

[a] Cysteine and cystine combined. [b] The proportion of essential amino acids to the total amino acids.

Methionine is converted to cysteine during metabolism and is therefore presented separately and combined in Table 3. Furthermore, phenylalanine is converted to tyrosine during metabolism and is presented in the same way [49].

The highest content of cysteine was located in the RPC (25 ± 1 mg/g) and was thereafter reduced in the recovery process. The lowest content was located in the precipitate from

the first cycle, 14 ± 1 mg/g. The two main proteins in rapeseed, cruciferin and napin differ in amino acid composition. The recovery process might have favored the extraction of one of the proteins, which explains the difference in cysteine content. Wanasundara et al. [3] reports different amino acid composition of two rapeseed protein samples, one containing a 2S isolate (napin) and one 11S concentrate (cruciferin). The 2S concentrate contained a high concentration of cysteine (81 mg/g) compared to the 11S isolate (14 mg/g). The present study might therefore have favored the recovery of cruciferin in the recovery process.

3.3.4. Antinutrients

The RPC contained 16 µmol/g glucosinolate on dry basis prior to the recovery process; after extraction and precipitation in the first cycle, the glucosinolate concentration was reduced to 0.12 µmol/g on dry basis in the precipitate (Table 4). After the second cycle, the glucosinolate concentration in the precipitate was under the detection limit. The reduction is probably due to the high solubility in aqueous solutions [50]. The present study shows that glucosinolate in the RPC was not concentrated in the protein precipitate fraction, but is probably found in the LPUP. The glucosinolate content in the precipitate is in line with a study performed by Von Der Haar et al. [51], who reported a glucosinolate content of 0.3 µmol/g on dry basis in the precipitate after pH precipitation. Furthermore, the glucosinolate content in the present study is below the content in commercial rapeseed protein isolates, which are reported to have a glucosinolate content of up to 2.53 µmol/g on dry basis [3].

Table 4. The concentration of the glucosinolates in the rapeseed press cake and the precipitate from the first and second cycle. The concentration is expressed as µmol/g on dry basis and different letters indicate significant differences. Dash (-) indicates that the glucosinolate concentration was below the detection level.

Glucosinolate Type	RPC = Rapeseed Press Cake	Precipitate First Cycle	Precipitate Second Cycle
Glucoiberin	-	-	-
Progoitrin	6.56 ± 0.1 [a]	0.06 ± 0.0 [b]	-
Epiprogoitrin	0.14 ± 0.0	-	-
Glucorafanin	-	-	-
Gluconapoleiferin	0.39 ± 0.0	-	-
Glucoalyssin	0.30 ± 0.0	-	-
Sinalbin	-	-	-
Glukonapin	3.73 ± 0.0 [a]	0.06 ± 0.0 [b]	-
4-Hydroksyglukobrassicin	3.53 ± 0.0	-	-
Glucobrassicanapin	1.03 ± 0.0	-	-
Glucotropaeolin	-	-	-
Glucoerucin	0.05 ± 0.0	-	-
Glucobrassicin	0.18 ± 0.0	-	-
Gluconasturtiin	0.11 ± 0.0	-	-
Neoglucobrassicin	0.10 ± 0.0	-	-
Total glucosinolates	16.12 ± 0.2 [a]	0.12 ± 0.0 [b]	-

The phytate content in the RPC was 3.16 g/100 g on dry basis; in the precipitate from the first cycle, the phytate content was reduced to 2.11 g/100 g on dry basis (Figure 5). After the first recirculation, the phytate content had increased to 2.79 g/100 g on dry basis. These levels of phytate cause a low bioavailability of iron. The concentration of RPC and

the precipitate from the first and second cycle are higher than other commercial food products such as soy isolate (0.82 g/100 g on dry basis), soy concentrate (1.0 g/100 g on dry basis), and lupin seed protein isolate (1.4 g/100 g on dry basis) [52]. A suggested point of intersection for phytate to markedly improve the bioavailability of iron is 0.033 g/100 g on dry basis, indicating that the bioavailability of iron from the RPC as well as the precipitate is very low. The negative effects of phytate can, to a certain degree, be counteracted by the addition of enhancing food components such as ascorbic acid, the meat factor (found in fish and meat) [18], and lactic acid fermented vegetables [53]. The effects on iron absorption from a very high phytate content are difficult to counteract, which is why adding an extra processing step such as hydrothermal treatment, fermentation, or addition of phytase could be considered to improve the bioavailability of iron [54]. The phytate content is to some extent pH-dependent, as shown by Helstad et al. [55] in the extraction of protein from hempseed press cake. It should be investigated if the same applies to rapeseed protein and whether it is possible to balance a high protein recovery yield with a reduced phytate content.

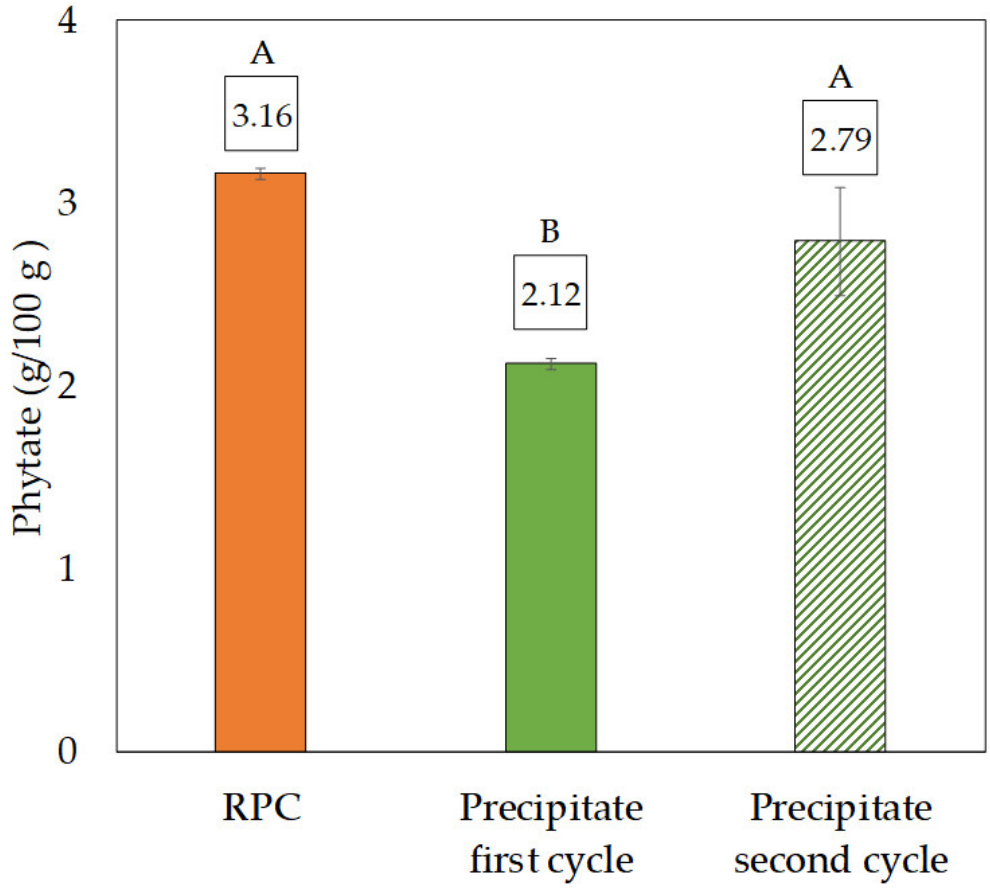


Figure 5. Phytate content (g/100 g on dry basis) in RPC (rapeseed press cake) and the precipitate after the first and second cycle. Different letters indicate significant differences, $p \leq 0.05$.

4. Conclusions

It was possible to increase the recovery of rapeseed protein on a pilot scale by recirculation of the SSF. One cycle of recirculation of the SSF increased the protein yield from 70% to 83%. The efficiency of the extraction process was significantly higher in the first and second cycles than in the third and fourth cycles, where only an additional 2% was extracted. Therefore, one recirculation cycle is recommended to improve the accumulated protein yield in the recovery of rapeseed protein from rapeseed press cake. A mass balance was calculated over the entire process to determine the protein distribution in the various process streams.

It was found that the fraction of non-solubilized protein remaining in the SSF was successfully reduced in each recirculation cycle, and that the process losses (on mass basis) were constant due to equipment design. The protein content was higher in the precipitate than in the LPUP, regardless of the recirculation cycle. The highest protein content was obtained in the first cycle for both the precipitate (63%) and the LPUP (23%). The amino acid profile of both the RPC and the precipitate from the first and second cycle contained high levels of essential amino acid that exceeds the recommendations from FAO/WHO/UNU. The glucosinolate content was successfully reduced in the precipitate after one cycle and was in the same range as commercial plant-based protein ingredients. The phytate content was reduced in the recovery process. However, the phytate content in the precipitate from the first and second cycle was still higher than other commercial plant-based products, which corresponds to a very low bioavailability of iron. An additional processing step could be considered for improved iron bioavailability.

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