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Protein extraction from cold-pressed hempseed press cake: From laboratory to pilot scale

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Abstract: During the production of *industrial hempseed* oil, a press cake is formed as a byproduct, which is often used as animal feed although it contains a high amount of protein that could be used for human consumption. Extracting this valuable protein would reduce food waste and increase the availability of plant-based protein. A protein extraction process based on the pH-shift method was adapted to improve the protein extraction yield from industrial hempseed press cake (HPC). Parameters such as alkali extraction pH, time, and temperature, as well as isoelectric precipitation pH, were investigated in laboratory scale and were thereafter carried out in a pilot trial to explore the suitability for future scale up. The phytic acid content of the extracted protein isolate was also analyzed to investigate any potential inhibitory effect on mineral absorption. A final protein yield of 60.6%, with a precipitated protein content of 90.3% (dw), was obtained using a constant alkali extraction pH of 10.5 for 1 h at room temperature, followed by precipitation at pH 5.5. The pilot trial showed promising results for the future production of industrial hemp protein precipitate on a larger scale, showing a protein yield of 57.0% and protein content of 90.8% (dw). The amount of phytic acid in the protein isolate produced in the optimal laboratory experiment and in the pilot trial was 0.595 and 0.557 g phytic acid/100 g dw, respectively, which is 83%–88% less than in the HPC. This is in the range of other plant-based protein sources (tofu, kidney beans, peas, etc.).

KEYWORDS
Hempseed press cake, Plant protein, Protein Extraction

Practical Application: Industrial hempseed press cake is a byproduct in the production of industrial hempseed oil, which is mostly used as animal feed, but has the potential to become an additional source of plant-based protein for human consumption with a suitable protein extraction method. The extracted

hemp protein could be used to develop new plant-based dairy or meat analog products.

1 | INTRODUCTION

Hemp, *Cannabis sativa L.*, is an annual herbaceous plant that has been widely used throughout history for various applications, such as fabric and rope, and has been used as a source of food and medicine in China for over 3000 years (Callaway, 2004a). Due to its content of psycho-active substances, such as δ-9-tetrahydrocannabinol (THC), the cultivation of hemp has been prohibited in many Western countries since the 1930s (Liang et al., 2015). However, some varieties of hemp, referred to as *industrial hemp*, have recently been legalized as they contain less than 0.3% THC. Interest in hemp-based products has thus increased greatly in recent years (Cherney & Small, 2016; Tang et al., 2006), and a steady increase in cultivation has been observed. Industrial hemp is a fast-growing crop that matures in approximately 4 months. It grows well without the application of pesticides, herbicides, or fungicides, and can be cultivated in a variety of climates and soils, even at high latitudes, for example, in Scandinavia, Russia, and Canada (Callaway, 2004b). Its extensive root system makes it efficient in suppressing weeds and increasing soil health (Aluko, 2017). These properties make industrial hemp suitable for organic cultivation, which is important in a sustainable food system. Industrial hemp also fixes carbon dioxide five times more efficiently than a forest of the same area (Aluko, 2017).

The applications for industrial hemp are numerous, as the entire plant can be utilized. The fibers (the outer layer of the stem) are mostly used for lightweight paper, insulation material, and biocomposites, while the shivs (the woody inner core of the stem) are commonly used for animal bedding and in construction materials (Carus & Sarmento, 2017). Research is also ongoing on the development of thermoset and thermoplastic composites using industrial hemp fibers as the raw material (Manaia et al., 2019).

During the production of hempseed oil, 350 g oil and 650 g byproduct, that is, the *press cake*, is generated from 1 kg of cold-pressed industrial hempseed, which is referred to as hempseed press cake (HPC). HPC contains high amounts of protein (30%–50%) (House et al., 2010), and the cold-pressing process maintains the proteins in their native state, thus preserving their functional properties (Östbring et al., 2020). The high protein content of the press cake has made it popular as a source of vegetable protein, and commercially available products such as hemp protein powder and hemp flour have been developed for human consumption (House et al., 2010). Nevertheless, HPC is often used as animal feed (Potin et al., 2019), the reason being that unrefined HPC has a large amount of fiber and a less desirable flavor and appearance. Therefore, isolation and concentration of hemp protein from the press cake is necessary for it to be used as a food ingredient.

Industrial hempseed is considered to be a complete source of protein, providing all the essential amino acids (Callaway, 2004a; Kim & Lee, 2011; Wang et al., 2008), and it is also rich in iron and zinc. These minerals are important for human nutrition; iron deficiency being prevalent in vulnerable groups such as women of fertile age, children, and adolescents (Stoltzfus, 2003). However, like most other plants, industrial hemp contains antinutrients that can impair the uptake of minerals and affect protein digestion.

Phytic acid and polyphenols, which are potent inhibitors of iron absorption, have been detected in industrial HPC, where the phytic acid content can be as high as 22.5 ± 0.07 mg/g (Pojic et al., 2014). The inhibitory effect of phytic acid on iron absorption is dose dependent and is seen even at very low levels of phytic acid (Brune et al., 1992; Hallberg et al., 1989; Hurrell et al., 1992). Therefore, it is necessary to remove as much phytic acid as possible (to levels of ≤30 mg/100 g or ≤0.5 μmol/g) to ensure the bioavailability of iron (Hurrell, 2004).

According to the EAT-Lancet Commission, two of the main strategies for a sustainable global food system are to increase the consumption of plant-based foods and to at least halve food losses and waste (Willett et al., 2019). Food loss could be reduced by extracting the protein in industrial HPC for human consumption, rather than using it as animal feed.

The most frequently reported method of extracting protein from industrial hempseed is to produce a hempseed protein isolate (HPI, at least 90% protein content) (Dapčević-Hadnađev et al., 2019; Tang et al., 2006). Cold-pressed industrial HPC or hemp flour is first defatted using supercritical CO_2 (Pihlanto et al., 2021), Soxhlet extraction (Teh et al., 2014), or hexane and Folch solvent defatting (Shen et al., 2020). The protein is then solubilized at high pH (around 10) and thereafter precipitated close to the isoelectric point (Teh et al., 2014; Wang et al., 2008; Yin et al., 2008). The pH used for precipitation is commonly pH 5 (Hadnađev et al., 2018; Malomo et al., 2014; Teh et al., 2014); however, a recent study revealed that the isoelectric point of HPI is 5.8, and that the lowest solubility was at pH 6 (Shen et al., 2020). Other protein recovery methods

that have been applied to industrial HPC include enzyme treatment (Pihlanto et al., 2021), acid extraction (Teh et al., 2014), and salt extraction (Hadnaďev et al., 2018).

In many studies on the extraction of protein from HPC (described earlier), it is more the rule than exception to use a defatting step or other pretreatment. However, in a larger production scale of hemp protein isolate for human consumption, this would not be optimal based on energy consumption, food safety, and environmental aspects. In the study described by Östbring et al. (2020), a protein extraction on cold-pressed rapeseed press cake, generated the highest protein yield compared to hot-pressed and solvent-extracted (hexane) rapeseed meal. Therefore, the aim of this study was to develop a protein extraction process on cold-pressed HPC based on the pH-shift method described by Östbring et al. (2020) without any defatting or pretreatment step. The optimal process conditions for alkali extraction, that is, pH, time, and temperature were investigated on this raw material, which contributes to the novelty of this study. An applicable protein extraction process on HPC for future commercial production of hemp protein would increase its availability for food applications in plant-based alternatives. The phytic acid levels in the protein precipitates produced were also measured to evaluate possible antinutritional effects.

2 | MATERIALS AND METHODS

2.1 | Materials

Industrial hempseed, hereafter referred to simply as hempseed (harvested in 2016 and 2018 at 55.6361°N, 13.5197°E), and cold-pressed HPC were kind gifts of Mossagården EKO AB (Veberöd, Sweden). The HPC was a byproduct of Mossagården's hempseed oil production. Their hempseed oil is produced at Gunnarshögs Gård AB (Hammenhög, Sweden) by cold pressing the cleaned, dried hempseed without solvents, at an oil temperature not exceeding 35°C, using a screw press. The HPC had a protein content of 28.4% ± 1.31% (ww) (according to the method described in Section 2.4.2) with a water activity of 0.707 ± 0.020 (see Section 2.4.5), and is thus considered to be microbiologically stable (Singh & Heldman, 2013).

Sodium hydroxide (NaOH, CAS 1310-73-2) and citric acid ($C_6H_8O_7$, CAS 77-92-9) were purchased from Merck (Darmstadt, Germany).

2.2 | Lab-scale screening experiments

Lab-scale (LS) screening experiments were performed to determine the conditions that maximized the protein yield (Figure 1), while requiring as small amounts of chemicals as possible (for pH adjustment). Several parameters were investigated, such as alkali extraction pH (LS1), temperature (LS2), constant alkali extraction pH (LS3), alkali extraction time (LS4), and precipitation pH (LS5) (Table 1).

Industrial HPC (50 g, harvested 2016), hereafter referred to as HPC, was milled with a Grindomix GM knife mill 200, 5 periods of 4 s (i.e., 20 s in total) (Retsch, Haan, Germany). The milled HPC was dispersed in tap water (450 g) and the pH was adjusted with 2 M NaOH. Alkali extraction was performed at seven different pH values (8.0, 8.5, 9.0, 10.0, 10.5, 11.0, and 12.0). In one series of experiments, the pH was adjusted at the beginning and after 10 min of alkali extraction (nonconstant pH), and in another, the pH was kept constant throughout the extraction step using a small bioreactor control system (Model CP10/ SARA, Belach Bioteknik AB, Skogås, Sweden). The slurry was stirred at 750 rpm (Microstar 7.5 control, IKA Labortechnik, Staufen, Germany) with a Rushton turbine impeller (30 mm stirrer diameter) in a 1 L plastic beaker. Alkali extraction was performed for 1, 2, 3, and 4 h, and at three temperatures: room temperature (RT, approximately 20°C), 30°C, and 50°C. The dispersion was then centrifuged at 4700 rpm for 20 min at 20°C (Beckman Coulter, Avanti® J-15R Centrifuge, Brea, CA, USA), the top phase was retained (the light liquid phase, LLP), and the spent solids fraction (SSF) was used for later dry matter (DM) and protein analysis. The extracted LLP was precipitated with citric acid in powder form to eight different pH values, from 3 to 6.5, in increments of 0.5. The LLP was again centrifuged at 4700 rpm for 20 min at 20°C, and the protein-rich precipitate was collected, while the supernatant, that is, the light phase containing unprecipitated protein (LPUP) was discarded. Each LS experiment was performed in triplicate, except in LS2 (varying temperature) where only two replicates were performed at 30 and 50°C for practical reasons, and in LS4 (varying alkali extraction time) 2 and 3 h were only measured once, as these were just control samples to not miss a potential optimal time between 1 and 4 h.

2.3 | Pilot trial

The pilot trial was based on the findings of the LS experiments, using a constant alkali extraction pH of 10.5 in RT during 4 h, and precipitation at pH 5.5.

Industrial HPC (2 kg, harvested 2018) was milled with an R302 V.V. knife mill (Robot Coupe, Paris, France) at 3000 rpm for 3 min in 500 g portions. The milled HPC was mixed with 18 L of tap water in a 50-L stainless steel vessel with a cone-shaped bottom. The slurry was mixed at 205 rpm (RW 28 digital, IKA Labortechnik, Staufen, Germany) with a three-bladed propeller stirrer (140 mm

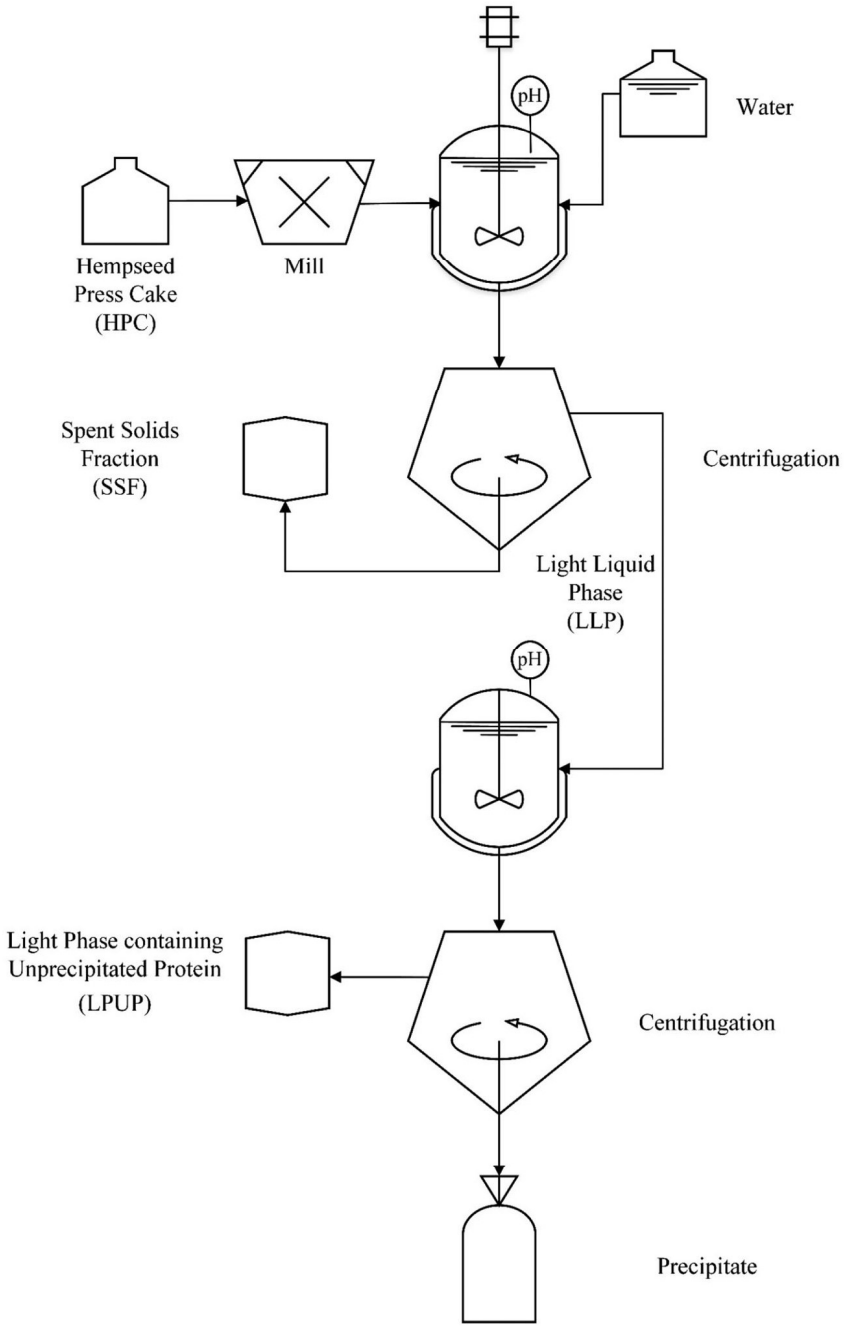

FIGURE 1 The process used for the extraction of protein from cold-pressed HPC on lab-scale

stirrer diameter) at RT, for 4 h at a constant pH of 10.5, adjusted manually with 2 M NaOH. During the first hour of extraction, the pH was adjusted to 10.5 when it had reached pH 10.4, thereafter, the pH was adjusted to 10.5 every 10 min. The first centrifuge separation stage in the LS experiments was replaced by decantation (MD80, Lemitec, Berlin, Germany), where the flow rate was set to 20 L/h with a peristaltic pump (Masterflex Easy-Load 77200–62, Cole-Parmer, Vernon Hills, IL, USA). Decantation was carried out using a decanter bowl revolution speed of 6687 rpm (acceleration 2000 ×g) with a screw differential of 10 rpm. The weir disc height used was 56 mm. The SSF was collected for DM and protein analysis. The extracted LLP was adjusted to pH 5.5 and centrifuged at 4700 rpm for 20 min at 20°C (Beckman Coulter) after which the protein-rich precipitate was collected. The pilot trial was performed once.

2.4 | Analysis methods

The DM and protein content were analyzed in the HPC, SSF, and precipitate. Calculations were performed to determine how much of the total protein content in the HPC was found in the different fractions in the protein extraction process.

TABLE 1 The parameters varied and the values used in the lab-scale screening experiments

	LS1 Varying alkali extraction pH	LS2 Varying alkali extraction temperature	LS3 Constant alkali extraction pH	LS4 Varying alkali extraction time	LS5 Varying precipitation pH
No. of replicates	3	2	3	3 (2 and 3 h were measured once)	3
HPC, amount	50 g				
Water, amount	450 g				
Impeller	Rushton turbine impeller (30 mm stirrer diameter)				
Stirring speed	750 rpm				
Alkali extraction pH	8.0, 8.5, 9.0, 10.0, 10.5, 11.0, 12.0	10.5	10.0, 10.5, 11.0	10.5	10.5
Alkali extraction temperature	RT	30°C, 50°C	RT	RT	RT
Constant/nonconstant alkali extraction pH	Nonconstant	Nonconstant	Constant	Constant	Nonconstant
Alkali extraction time	4 h	4 h	4 h	1 h, 2 h, 3 h	4 h
Precipitation pH	5.5	5.5	5.5	5.5	3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5

2.4.1 | Dry matter analysis

The DM was analyzed according to AACC method 44-15A (AACC, 1995), where weighed samples were dried in an oven at 103°C in metal containers until constant weight. The samples were then placed in a desiccator for at least 20 min to cool before they were reweighed. Precipitate and SSF were collected from each replicate of all LS experiments, including the pilot trial, where each precipitate was analyzed in triplicate and each SSF in duplicate.

2.4.2 | Protein analysis

Protein was quantified using a protein analyzer (Flash EA, 1112 Series, Thermo Electron Corp., Waltham, MA, USA). Each sample was ground by hand using a mortar and pestle, and approximately 25 mg was placed in a tin cylinder (diameter 30 mm) for analysis. Aspartic acid was used as a reference, and the conversion factor used was 6.25. Precipitate and SSF were collected from each replicate of all LS experiments, including the pilot trial, where each precipitate was analyzed in triplicate and each SSF in duplicate.

2.4.3 | Calculations

The LLP extraction coefficient (EC_{LLP}) is a measure of the amount of protein extracted from the HPC into the LLP (Equation 1). The calculations are based on triplicate values from each experiment. P_{HPC} is the protein content in the HPC, and P_{SSF} is the protein content in the SSF.

$$EC_{LLP}\ (\%) = \left(\frac{P_{HPC} \cdot \text{Weight}_{HPC} - P_{SSF} \cdot \text{Weight}_{SSF}}{P_{HPC} \cdot \text{Weight}_{HPC}}\right) \times 100 \quad (1)$$

The precipitation coefficient ($PC_{Precipitate}$) is a measure of the amount of protein in the extracted LLP that is precipitated in the second separation step (Equation 2). The calculations are based on triplicate values obtained from each experiment. $P_{Precipitate}$ is the protein content in the precipitate.

$$PC_{Precipitate}\ (\%) = \left(\frac{P_{Precipitate} \cdot \text{Weight}_{Precipitate}}{P_{LLP} \cdot \text{Weight}_{LLP}}\right) \cdot 100 \quad (2)$$

The yield is a measure of the proportion of protein in the HPC that is found in the precipitate (Equation 3).

$$\text{Yield}\ (\%) = \left(\frac{P_{Precipitate} \cdot \text{Weight}_{Precipitate}}{P_{HPC} \cdot \text{Weight}_{HPC}}\right) \cdot 100 \quad (3)$$

2.4.4 | Phytic acid analysis

Phytic acid, also called inositol hexaphosphoric acid (InsP6), was analyzed using high-performance ion chromatography (HPLC) (Carlsson et al., 2001). InsP6 was measured in HPC harvested in 2016 and 2018, and the precipitates from the pilot trial, LS3 (constant pH 10.5), LS4 (1 h,

constant pH 10.5), and LS5 (all precipitation pH values). InsP6 was extracted from the sample (0.5 g) with 10 ml 0.5 M HCl for 3 h. Then, 1 ml was removed, centrifuged, and the supernatant transferred to a HPLC vial. The chromatography setup consisted of an HPLC pump (model PU-4080i; Jasco Inc., Easton, MD, USA) and an RHPLC pump (model PU-4180; Jasco) equipped with a PA-100 guard column and a CarboPac PA-100 column. InsP6 was eluted with an isocratic eluent of 80% HCl (1 M) and 20% H_2O at 0.8 ml/min, subjected to a postcolumn reaction with ferrous nitrate, and detected at 290 nm with a UV–visible HPLC detector (UV-4075; Jasco). Each sample had a run time of 7 min, and the InsP6 concentration was calculated using external standards covering the concentration range of 0.1–0.6 μM/ml.

2.4.5 | Water activity analysis of HPC

The water activity (a_w) of the HPC was analyzed to evaluate its microbial stability. A water activity below 0.7 inhibits microbial growth and the material can be considered storage stable (Singh & Heldman, 2013). A water activity meter (AquaLab Ver 3TE, Decagon Devises, Pullman, WA, USA) was used and was calibrated with standard salt solutions (13.41 M LiCl [0.250 a_w], 8.57 M LiCl [0.500 a_w], and 6 M NaCl [0.760 a_w]). Analysis was performed in triplicate.

2.4.6 | Visual appearance

Extracted precipitates from LS4 (alkali extraction times 1, 2, and 3 h) were photographed with a mobile phone (Samsung Galaxy S9).

2.4.7 | Statistical analysis

The results were analyzed using ANOVA and Student's *t*-test. Results were considered significant when *p*-values were ≤0.05. The GRUBBS outlier detection test was also performed. All results are expressed as means and standard deviations.

3 | RESULTS AND DISCUSSION

3.1 | Extraction coefficient

The extraction coefficient, that is, the proportion of protein, in the LLP increased with increasing alkali extraction pH (LS1) (Figure 2). At pH 8.0, 11.7% of the protein was found in the LLP, whereas at pH 12.0, the value was 76.6% (LS1). The extraction coefficient also increased with increasing alkali extraction temperature (LS2).

When a constant pH was applied (LS3), the extraction coefficient improved at pH 10.0 and 10.5, while no effect was seen on extraction at pH 11 when compared to a nonconstant pH (LS1). At a constant pH of 10.5, the alkali extraction process was as efficient as in the lab experiment with nonconstant pH at pH 10.5 and 50°C (LS2). The alkali extraction time (LS4) did not have any effect on the extraction coefficient when the pH was constant, showing that an extraction time of 1 h was adequate. Variations in the extraction coefficient were observed in LS5 (precipitation pH) despite the fact that the alkali extraction parameters were the same for all samples (nonconstant pH 10.5, RT, 4 h). The variation could be due to the natural variation of biological materials. The extraction coefficient in the pilot trial was the same as its laboratory counterpart in LS3 (constant pH 10.5, RT, 4 h) (Figure 2), which indicates that the alkali extraction process is as efficient on pilot scale as on LS.

3.2 | Dry matter content in the spent solid fraction

The DM in the SSF differed slightly between the different experiments (Table 2). In LS1 (varying alkali extraction pH), the DM decreased as the pH increased, from about 42% at pH 8.0 to 35% at pH 12.0, which indicates that the SSF had a higher water content at higher alkali extraction pH. The same trend was seen in LS3 (constant pH), although the water content was higher in LS3. The DM in SSF increased with increasing temperature (LS2: varying alkali extraction temperature). Varying the alkali extraction time (LS4) appeared to have no significant effects on the DM. The SSF in the pilot trial showed the highest value of DM, which could be due to the use of decantation instead of LS centrifugation in the first separation step.

3.3 | Precipitation coefficient

Although some variations were seen in the extraction coefficient in LS5 (varying precipitation pH, Figure 2), a completely different trend was observed in the precipitation coefficient (Figure 3). As the precipitation coefficient was highest at pH 5.5, it was concluded that a pH of 5.5 would be the most suitable choice for precipitation. A pH of 5.5 was thus applied in all other experiments, leading to the precipitation of ≥80% of the protein from the extracted

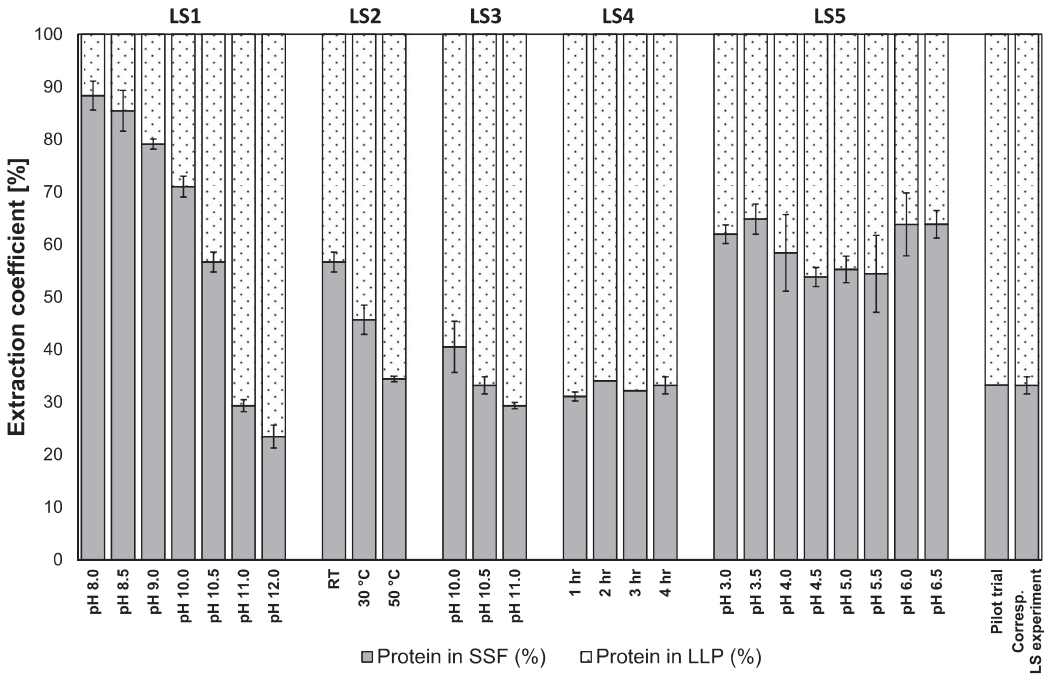

FIGURE 2 The extraction coefficient, that is, the proportion of protein, in SSF and the extracted LLP. LS1, varying alkali extraction pH (8.0–2.0); LS2, varying alkali extraction temperature (RT, 30°C, 50°C); LS3, constant alkali extraction pH (10.0–11.0); LS4, varying alkali extraction time (1–4 h); LS5, varying precipitation pH (3.0–6.5); and pilot trial (constant alkali extraction pH 10.5, RT, 4 h). The error bars represent the standard deviation of *n* replicates

TABLE 2 DM content (%) of the SSF in the various experiments

Alkali extraction pH	LS1 Varying alkali extraction pH	LS2 Varying alkali extraction temperature			LS3 Constant alkali extraction pH	LS4 Varying alkali extraction time				Pilot trial (constant pH 10.5, RT, 4h)
		RT	*30°C*	*50°C*		*1 h*	*2 h*	*3 h*	*4 h*	
pH 8.0	41.6^{a}									
pH 8.5	40.7^{a}									
pH 9.0	38.5^{b}									
pH 10.0	37.9^{cu}		34.1^{av}							
pH 10.5	35.2^{dv}	35.2^{v}	34.4^{x}	33.7^{xyz}	33.0^{bz}	35.0^{vxy}	32.9^{yz}	32.9^{yz}	33.0^{yz}	41.8^{u}
pH 11.0	35.7^{du}		32.7^{bv}							
pH 12.0	34.9^{d}									

Mean values within a column (a–d) or a row (u–z) not containing the same letter are significantly different ($p < 0.05$).

LLP in almost all experiments (Figure 3). Using a pH of 5.5 for precipitation also meant that the amount of citric acid required to reduce the pH for precipitation was minimized.

The protein content in the precipitate increased with increasing alkali extraction pH (LS1) and increasing temperature (LS2) (Figure 4). The protein content was higher in the experiments with constant extraction pH (LS3) than in their counterparts in LS1, while no difference was seen between the different extraction times (LS4). In LS5 (varying precipitation pH), the highest protein content in the precipitate was obtained at pH 5.5 and pH 6.5, both of which are close to the isoelectric pH of HPI of 5.8 (Shen et al., 2020). The highest protein content in the precipitate, >90% (dw), was obtained in the pilot trial and in LS4 (constant pH 10.5, RT, 1h), thus fulfilling the EU requirement for a protein isolate in food (European Commission, 2018) (Figure 4). The protein content in the precipitates obtained in this study are comparable to the protein contents of 85.9%–91.4% in HPI reported in other studies in which similar pH-shift methods were used (Hadnađev et al., 2018; Mamone et al., 2019).

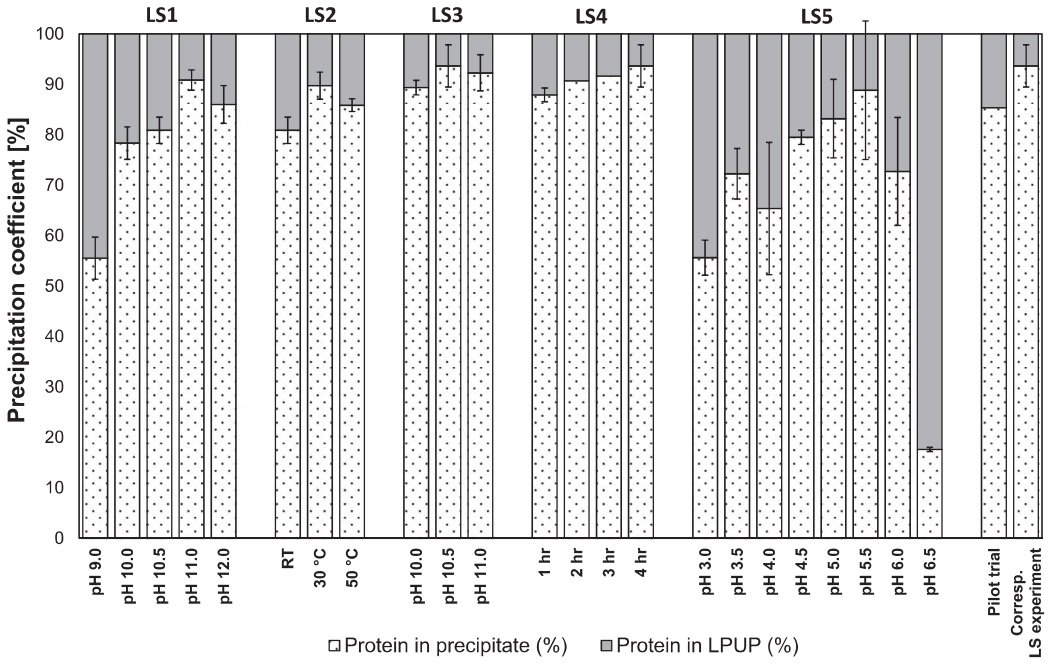

FIGURE 3 The precipitation coefficient, that is, the proportion of protein in the precipitate and the LPUP. LS1, varying alkali extraction pH (9.0–12.0); LS2, varying alkali extraction temperature (RT, 30°C, 50°C); LS3, constant alkali extraction pH (10.0–11.0); LS4, varying alkali extraction time (1–4 h); Trial 5, varying precipitation pH (3.0–6.5); and pilot trial (constant alkali extraction pH 10.5, RT, 4 h). The error bars represent the standard deviation of *n* replicates

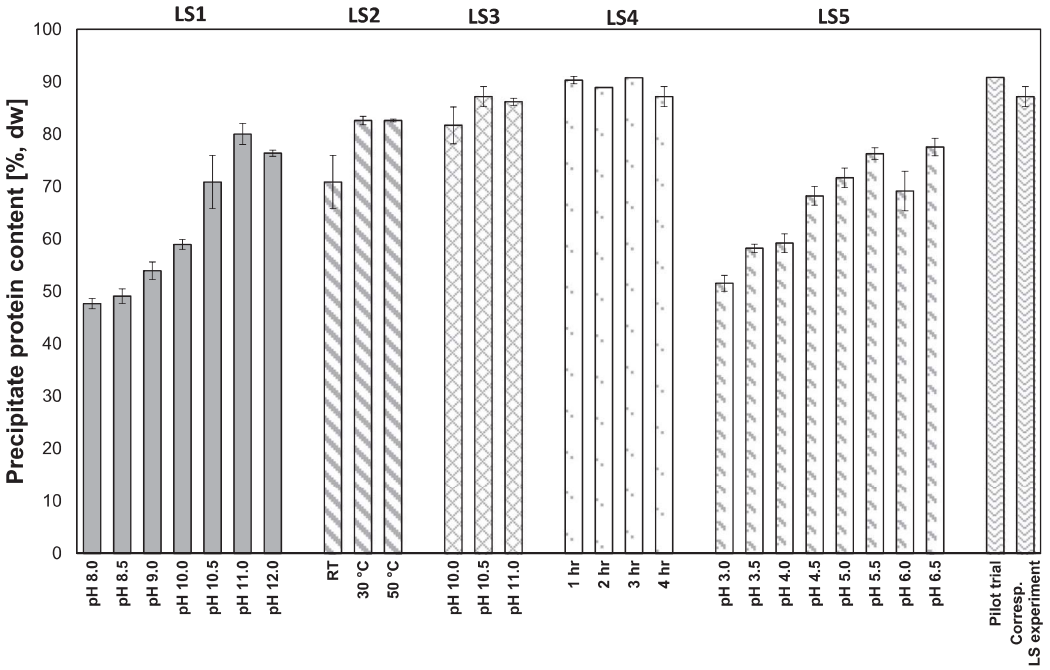

FIGURE 4 Protein content in the precipitate (%, dw) in the different experiments. LS1, varying alkali extraction pH (8.0–12.0); LS2, varying alkali extraction temperature (RT, 30°C, 50°C); LS3, constant alkali extraction pH (10.0–11.0); LS4, varying alkali extraction time (1–4 h); LS5, varying precipitation pH (3.0–6.5); and pilot trial (constant alkali extraction pH 10.5, RT, 4 h). The error bars represent the standard deviation of *n* replicates

TABLE 3 DM content (%) of the precipitates obtained in the various experiments

Alkali extraction pH	LS1 Varying alkali extraction pH	LS2 Varying alkali extraction temperature			LS3 Constant alkali extraction pH	LS4 Varying alkali extraction time				Pilot trial (constant pH 10.5, RT, 4 h)
		RT	*30°C*	*50°C*		*1 h*	*2 h*	*3 h*	*4 h*	
pH 9.0	27.1^{b}									
pH 10.0	26.3^{cv}		24.4^{bx}							
pH 10.5	24.6^{dyz}	24.6^{yz}	23.7^{z}	28.4^{v}	25.6^{by}	24.9^{y}	24.1^{yz}	24.9^{yz}	25.6^{v}	27.3^{x}
pH 11.0	26.1^{bcx}		28.1^{av}							

Mean values within a column (a–d) or a row (u–z) not containing the same letter are significantly different ($p < 0.05$).

A high DM is advantageous if the resulting precipitate is to be dried, for example, in applications in extruded products. Among the different values of alkali extraction pH investigated in LS1, the lowest DM was obtained at pH 10.5, and was higher at both higher and lower alkali extraction pH (Table 3). When using a constant pH (LS3), the DM increased with increasing alkali extraction pH, while no significant differences were seen when varying the alkali extraction time (LS4). The sample with the highest DM was obtained when using an alkali extraction temperature of 50°C and the next highest in the pilot trial.

3.4 | Yield

The yields obtained are shown in Figure 5. The results of LS5 (varying precipitation pH) indicated that the optimal precipitation pH was 5.5, which is consistent with the results for the precipitation coefficient and protein content. The highest yields were around 65% for nonconstant extraction pH of 11 and 12 (LS1) and for constant pH of 10.5 and 11 (LS3); however, there was no significant difference between these conditions. Therefore, a constant pH at pH 10.5 were chosen since higher pHs increases the risk for protein denaturation and chemical usage, both for the alkali extraction and the subsequent neutralization using citric acid. The yield in the pilot trial was 57.0%, which is similar to the value obtained in the comparable LS experiment of 62.5% (LS3, constant pH 10.5, 4 h).

3.5 | Optimal process conditions

A summary of the conditions investigated are presented in Figure 6, where the best process conditions are highlighted. The best alkali extraction pH was determined to be a constant pH of 10.5, as it is important to use as low a pH as possible in alkali extraction to reduce the requirement of chemicals (both NaOH and citric acid), which is advantageous in both the environmental and safety perspectives, as well as to lessen corrosion of the equipment. It is also important to avoid possible protein denaturation and oxidation under highly alkaline conditions. Denaturation should be avoided not only to maintain protein functionality (Hadnađev et al., 2018), but also as this would allow for extrusion, for example, to produce meat analogs (Riaz, 2011). The temperature was set to room temperature for most experiments for convenience, but room temperature would also make a future larger production cheaper, as less energy would be required. However, higher temperatures should be considered for further improvement of the process as the yield is higher at higher temperatures. A temperature of 30°C would not be appropriate due to food hygiene considerations, but 50°C, which is a common temperature for processing food materials (such as milk), should be considered to increase the separation efficiency. It was found that varying the extraction time from 1 to 4 h had no effect on the yield when the pH was kept constant; therefore, 1 h was used in the optimal process. A precipitation pH of 5.5 was found to be best as this gave a higher precipitation coefficient and yield than other pH values.

In the study by Östbring et al. (2020), the protein yield from cold-pressed rapeseed press cake was reported to be 45% under alkali extraction conditions with a nonconstant pH of 10.5 at RT for 4 h. When using the same conditions in the current study, the yield from cold-pressed HPC was only 35.0%. However, the yield from HPC could be increased to 60.6% by optimizing the process conditions.

3.6 | Visual evaluation

The fresh precipitate from all trials had a smooth consistency, similar to Turkish yoghurt, with a beige-green color. A longer extraction time resulted in slightly darker green precipitates (Figure 7).

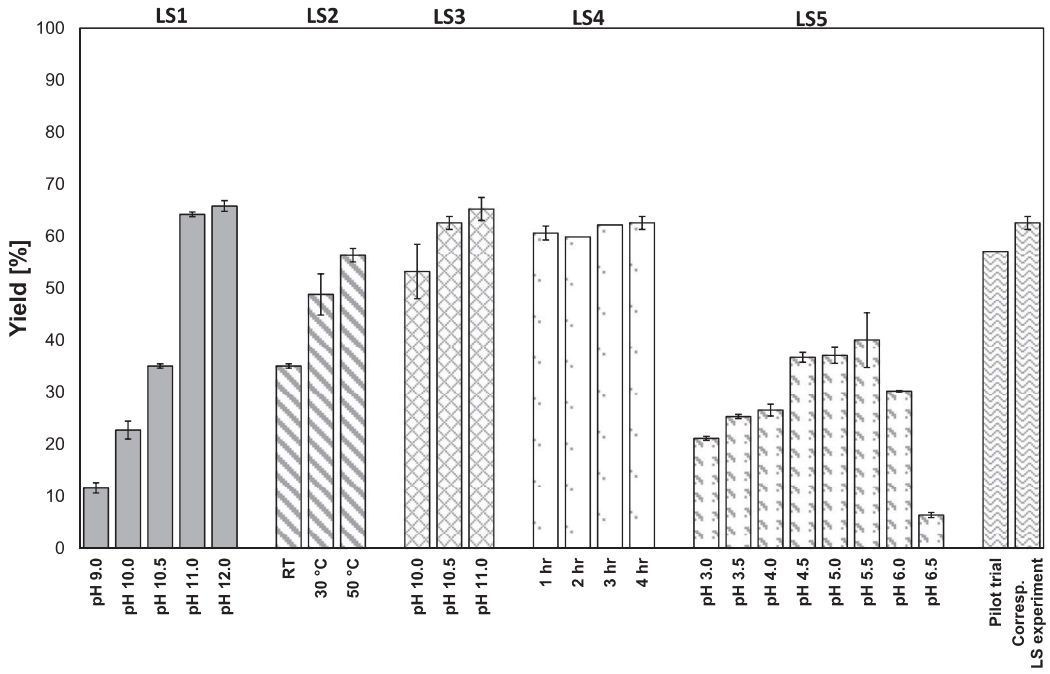

FIGURE 5 The calculated yield in all experiments. LS1, varying alkali extraction pH (9.0–12.0); LS2, varying alkali extraction temperature (RT, 30°C, 50°C); LS3, constant alkali extraction pH (10.0–11.0); LS4, varying alkali extraction time (1–4 h); LS5, varying precipitation pH (3.0–6.5); and pilot trial (constant alkali extraction pH 10.5, RT, 4 h). The error bars represent the standard deviation of *n* replicates

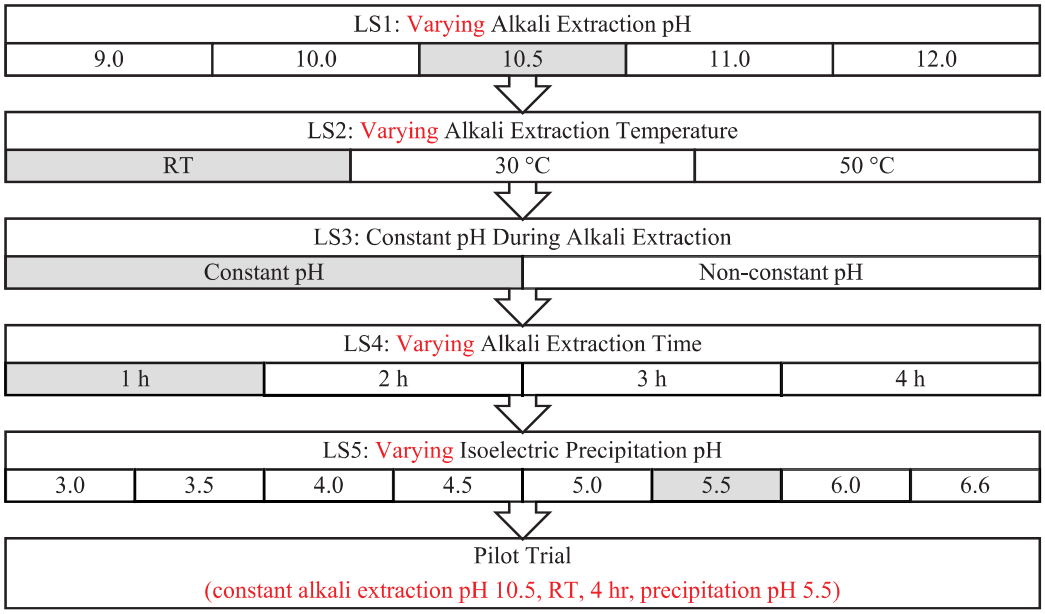

FIGURE 6 Summary of the conditions investigated in chronological order. The values highlighted in gray were found to be the best for protein extraction from cold-pressed HPC

3.7 | Phytic acid content in starting materials and precipitates

The content of phytic acid in the different precipitates from LS3 (at constant pH 10.5), LS4 (1 h), LS5 (varying precipitation pH), and the pilot trial were all lower than in the HPC from 2016 and 2018 (Figure 8). The removal of fibers and shells after the first separation was assumed to explain the significant decrease in phytic acid in the precipitates compared to the starting material. However,

FIGURE 7 Fresh precipitate from LS4 in which the alkali extraction time was 1 h (left), 2 h (middle), and 3 h (right)

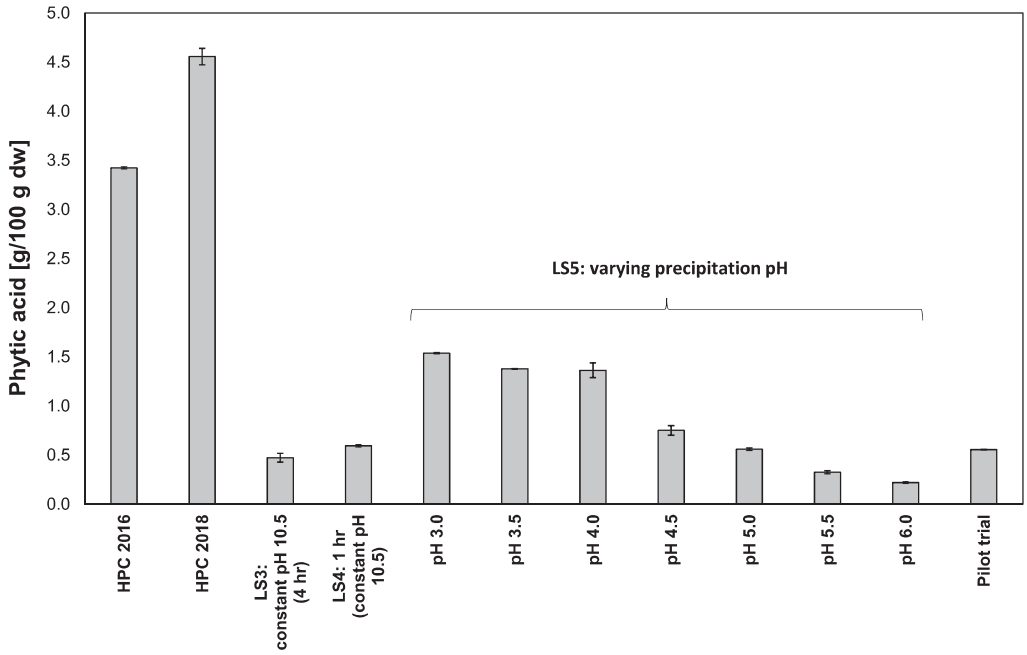

FIGURE 8 Phytic acid levels in cold-pressed HPC harvested in 2016 and 2018, chosen precipitates from LS3 (varying constant alkali extraction pH) and LS4 (varying time), all precipitates from LS5 (varying precipitation pH), and pilot trial precipitate. All precipitates were extracted from HPC 2016, except in the pilot trial, where the precipitate was extracted from HPC 2018. The error bars represent the standard deviation of duplicate values

the content of phytic acid decreased further following the precipitation step and was correlated to increasing precipitation pH. The greatest reductions in phytic acid were observed at a precipitation pH of 6.0 (93.6% reduction) and pH 5.5 (90.5% reduction) (Table 4). Plant phytases, which hydrolyze phytic acid, have an activity optima between pH 4 and 6 (Pallauf & Rimbach, 1997), which could explain the decreasing content of phytic acid with increasing precipitation pH.

There are no regulations in Sweden regarding acceptable levels of phytic acid in foods in general; however, there are EU regulations on the acceptable amount of phytic acid in protein isolates from rapeseed. According to EC2014/424EU, it should not exceed 1.5% (1.5 g phytic acid/100 g dw). This decision was made in conjunction with the authorization by EFSA for a rapeseed protein product as a novel food ingredient (Regulation (EC) No 258/97). In the present study, the precipitates from LS3 (constant pH 10.5), LS4 (1 h), and the pilot trial had phytic acid contents of 0.56 (87.8% reduction), 0.47 (86.2% reduction), and 0.59 (82.6% reduction) g phytic acid/100 g dw, respectively (Table 4), which is below the limit of 1.5 g phytic acid/100 g dw. In comparison to rapeseed protein concentrate (5.3–7.5 g/100 g dw), soy concentrate (10.7 g/100 g dw), kidney beans (0.61–2.38 g/100 g dw), oat (0.42–1.16 g/100 g dw), and many other plant-based foods (Schlemmer et al., 2009), the phytic acid in the produced precipitates in this study is not higher than any other plant-based source. If the pilot trial precipitate was to replace a portion of chicken (100 g) with a 30 g protein content, 33.3 g of precipitate would be required, containing a total of 0.2 g of phytic acid. In Sweden, the aver-

TABLE 4 Average values of phytic acid content in HPC harvested in 2016 and 2018, chosen precipitates from LS3 (varying constant alkali extraction pH) and LS4 (varying time), all precipitates from LS5 (varying precipitation pH), and pilot trial precipitate

Material	Phytic acid (μmol/g dw)	Phytic acid (mg/100 g dw)	Percentage reduction (%)
HPC 2016	52 ± 0.7	3424 ± 45	
HPC 2018	69 ± 0.2	4556 ± 10	
LS3: constant pH 10.5 (4 h)	7.2 ± 0.0	473 ± 3	86[a]
LS4: 1 h (constant pH 10.5)	9.0 ± 0.1	595 ± 3	83[a]
LS5: precipitation pH 3.0	23 ± 1.1	1536 ± 75	55[a]
LS5: precipitation pH 3.5	21 ± 0.8	1378 ± 50	60[a]
LS5: precipitation pH 4.0	21 ± 0.2	1362 ± 13	60[a]
LS5: precipitation pH 4.5	11 ± 0.2	751 ± 15	78[a]
LS5: precipitation pH 5.0	8.5 ± 0.1	560 ± 8	84[a]
LS5: precipitation pH 5.5	4.9 ± 0.0	326 ± 0	90[a]
LS5: precipitation pH 6.0	3.3 ± 0.1	220 ± 3	94[a]
Pilot trial (4 h)	8.4 ± 0.1	557 ± 6	88[b]

[a]Percentage reduction based on HPC 2018.
[b]Percentage reduction based on HPC 2016.

age daily intake of phytic acid for a person with a Western type diet is 0.37 g and for a person with a vegetarian diet, it is 1.15 g (Schlemmer et al., 2009). This concludes that a food product based on the pilot trial precipitate would not increase the daily intake of phytic acid more than usual.

However, small amounts of phytic acid can still inhibit iron absorption. In a study by Hurrell (2004), the phytic acid content should be less than 0.5 μmol/g (0.03 g/100 g), or <10 mg phytic acid per meal, to markedly improve iron absorption. The inhibitory effects of phytic acid can be counteracted to a certain degree by additives such as ascorbic acid, the meat factor (found in meat and fish), and lactic-acid-fermented vegetables, as these stimulate iron absorption (Scheers et al., 2016; Tuntawiroon et al., 1990). Another solution could be to add an extra process step, for example, hydrothermal treatment, fermentation, the addition of phytase (Fredlund et al., 1997; Hussin et al., 2010), or ultrafiltration/diafiltration (Lai et al., 2013) to reduce the level of phytic acid.

4 | CONCLUSIONS

The protein extraction process used by Östbring et al. (2019) for rapeseed press cake was successfully adapted for the extraction of proteins from HPC. The optimal conditions were found to be alkali extraction with at constant pH of 10.5 for 1 h at RT, and precipitation at pH 5.5. Under these conditions, it was possible to achieve a protein yield of 60.6%, with a 90.3% (dw) protein content in the precipitate, despite the fact that no defatting step or enzymatic pretreatment was used. The pilot trial had a yield of 57.0%, which was almost as high as that in the comparable LS experiment, which showed a yield of 62.5%. This indicates the potential to scale up the process for commercial production.

When a constant pH of 10.5 was used for *alkali extraction*, the protein yield increased from 35.0% to 62.5%, which is almost as high as the yield under nonconstant pH 12, which gave the highest yield in all the LS experiments (65.8%). A constant pH of 10.5 was therefore deemed to be suitable, as a lower pH reduces the risk of protein denaturation and is also better from both safety and environmental perspectives. Increasing the temperature during alkali extraction improved the yield; so 50°C should be considered for future large-scale production.

The phytic acid content was reduced under all conditions, but the greatest decrease in the precipitates was seen at precipitation pH 5.5 and 6.0 (LS5). A plant-based meat or dairy analog based on the pilot trial precipitate would not increase the daily intake of phytic acid for a person living in Sweden. However, the inhibitory effects should be considered in future food products, where the addition of an iron absorption enhancer or an additional process step could be applicable.

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AUTHOR CONTRIBUTIONS

Amanda Helstad: Data curation-Equal, Formal analysis-Equal, Investigation-Equal, Methodology-Equal, Validation-Equal, Visualization-Equal, Writing-original draft-Lead. **Erica Forsén**: Formal analysis-Equal, Investigation-Equal, Methodology-Equal, Writing-original draft-Equal. **Cecilia Ahlström**: Methodology-Equal, Supervision-Equal, Writing-review & editing-Supporting. **Inger-Cecilia Mayer Labba**: Formal analysis-Equal, Methodology-Equal, Validation-Equal, Writing-review & editing-Equal. **Ann-Sofie Sandberg**: Validation-Equal, Writing-review & editing-Equal. **Marilyn Rayner**: Conceptualization-Equal, Funding acquisition-Supporting, Resources-Equal, Supervision-Supporting, Writing-review & editing-Equal. **Jeanette K. Purhagen**: Conceptualization-Equal, Data curation-Equal, Funding acquisition-Lead, Project administration-Lead, Resources-Equal, Supervision-Lead, Writing-original draft-Equal, Writing-review & editing-Equal.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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