

Optimization of Protein Extraction from The Novel Oil Crop Field Cress Press Cake

Luthfi Fathul Huda and Abdull-Wahidi Hamad Salim

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MASTER THESIS



Optimization of Protein Extraction from The Novel Oil Crop Field Cress Press Cake

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Popular Science Summary

Unlocking the Hidden Gem of Ignored Press Cake

What if the leftovers from biofuel production could help to feed the world? This study shows how a high-protein, but fibrous, side-stream of oil extraction can be turned into valuable plant-based protein.

Every harvest season, large amounts of crop waste are generated, often discarded, burned, or used as animal feed. Field cress, a winter-tolerant crop now being developed in Sweden, is no exception. If you are checking out a flight ticket, and they offer you "green fuel", that fuel can in the future be based on oil from field cress. It is just like rapeseed, while the seeds are pressed for oil, the leftover cake is packed with protein and fibre. Yet until now, humans have ignored this valuable press cake in their food chain.

Our project aimed to change that; we wanted to find out how to extract protein from field cress press cake sustainably and on a scalable basis. However, we have a problem with this press cake having very high viscosity, making it harder for us to extract the protein. We discovered that using an enzyme called cellulase helped to break down the plant's cell wall, liberating the trapped protein inside. This enzyme cuts down the long chain of carbohydrates to smaller parts, making the slurry less viscous and flowing more easily. But here is the interesting part: by using a smaller volume of enzymes rather than a larger volume, we could get more protein out from the press cake. This counterintuitive result may help reduce costs and energy usage in future applications.

How is the magic recipe? Mix the cake with warm water, add the enzyme, adjust the acidity, then increase the alkali to dissolve the protein from the cake. Finally, put more acid again to get the "real" protein that can be collected and dried. And Bum! This simple method gave us nearly 40% of the original protein content.

Why does this matter? Because we are facing global challenges, including food insecurity, rising population, and climate change. By reusing agricultural leftovers into food-grade protein, we can reduce waste, support a circular bioeconomy, and feed more people using fewer resources.

Imagine tomorrow's plant-based burger containing protein from what used to be discarded press cake. That is the kind of future this work makes possible, "one where waste becomes resources, and problems become solutions."

Authors:

Luthfi Fathul Huda & Abdull-Wahidi Hamad Salim

Lund University, Faculty of Engineering (LTH)

Abstract

This study investigated the sustainable extraction of protein from field cress (*Lepidium Campestre*) press cake, an underutilized by-product of oil extraction from a novel oilseed crop, which is currently being domesticated in Sweden. Field cress is recognized for its resilience in Nordic climates and its potential as a source of sustainable aviation fuel (SAF), yet the protein and fibre-rich cake generated during the oil extraction has historically been relegated to low-value uses such as animal feed or discarded as waste.

The pH-shifting method was carried out to establish the baseline of field cress press cake protein extraction. The baseline of protein extraction only yielded approximately 3%. This result led to a further extraction using the enzyme-assisted protein extraction method. The enzyme-assisted protein extraction method was used to optimise the protein recovery yield. The enzymatic hydrolysis facilitated the disruption of the plant cell wall matrix and released the trapped protein. Among the five enzymes used, cellulase (Cellic[®] Ctec 3 HS) was identified as the most effective enzyme, in which its relatively low dosage of 1.2 millilitres per extraction batch significantly improved the protein yield. The dosage not only enhanced the process efficiency but also reduced the operational cost and energy input, making the method suitable for industrial upscaling.

The optimized protocol involved mixing the press cakes with water, enzymatic treatment, and a two-step pH adjustment—first to solubilize protein and then to precipitate and recover it. The extraction pH at 12.0 yielded higher values than lower pH, approximately 40%, while the precipitation pH at 5.0 led to a higher precipitation coefficient, around 80%. When taking all steps into account, the highest protein recovery yield (39%) was achieved at alkali pH 12.0 and precipitation at pH 4.0.

Beyond technical optimization, this study situates its work within the broader context of the global food system and climate change. With agriculture and food processing contributing substantially to greenhouse gas emissions, the valorisation of agricultural by-products like field cress press cake is essential for advancing circular bioeconomy principles and reducing food waste. Moreover, this study aligns with the UN Sustainable Development Goals for zero hunger and responsible production.

The outcomes of this study demonstrate that field cress press cake can be efficiently converted into functional protein ingredients. This study contributes to environmental stewardship and the diversification of protein sources for a growing global population by unlocking the potential of what was considered waste.

Key words: Field cress press cake, oilseed crop, viscosity, protein extraction, enzymatic hydrolysis, cellulase, pH adjustment, protein yield, sustainable process.

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List of Abbreviations

ANOVA - Analysis of Variance

DSC - Differential Scanning Calorimetry

FCPC - Field Cress Press Cake

GHG - Greenhouse Gases

FAA - Federal Aviation Administration

HPC - Hempseed Press Cake

LGC - Least Gelling Concentration

LLP - Light Liquid Phase

LPUP - Liquid Phase containing Unprecipitated Protein

RISE – The Research Institute of Sweden

SAF - Sustainable Aviation Fuel

SDG - Sustainable Development Goal

SLU - Swedish University of Agricultural Sciences

SSF – Spent Solid Fraction

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1 Background

The food system encompasses all stages of food production, from the farm to the table, and plays a vital role in food security, social welfare, and environmental sustainability (Lawrence and Friel, 2020; Von Braun et al., 2021). However, it evolves due to climate change, population growth, and unsustainable agricultural practices, which contribute to global greenhouse gas emissions up to 34%, with agriculture itself responsible for 73% of it (Crippa et al., 2021; Thakur, 2024). On the other hand, sectors such as aviation are also faced with imminent pressure to reduce their carbon footprint. In 2023, aviation contributed 2.5% of global CO₂ emissions, growing faster than other transportation modes (Lombardo and Gauch, 2025). This has driven interest in sustainable aviation fuels (SAFs), particularly those derived from bio-based sources, such as oilseed crops (IEA, 2024).

Field cress (*Lepidium campestre*), a newly domesticated oilseed crop in Sweden, has emerged as a promising candidate for SAF production due to its winter hardiness and agronomic benefits (Ivarson, 2016). Its seeds contain approximately 20% oil, 20% protein, and 40% dietary fibre (Ivarson, 2016). However, oil extraction leaves behind a protein and fibre-rich press cake that is often underutilised.

This research was conducted as part of a collaborative project between Lund University, the Swedish University of Agricultural Sciences (SLU), and PREEM, which focuses on optimizing the protein extraction from field cress press cake.

2 Aim of the thesis

This research aims to find the optimum condition for protein extraction from field cress press cake (FCPC). The specific goals are to determine the right enzyme type and its optimum conditions. Then, considering the protein's isoelectric point, find the proper pH for protein extraction and recovery to get the optimum yield.

3 Theoretical background

3.1 Food system and climate change

Food systems refer to the whole chain of activities involved in producing, processing, distributing, eating, and handling the waste of food, including their stakeholders (Von Braun et al., 2021). These food systems come from farming, livestock, forestry, fisheries, and the food industry. It also has an important role in social welfare, food security, and environmental sustainability (Lawrence and Friel, 2020). However, it continuously evolves due to industrial trends, geopolitical conflicts, global pandemics, technological advancements, population growth, economic shifts, and climate change.

Climate change both affects and is influenced by agricultural production activities, as traditional agricultural practices contribute to environmental degradation and excessive resource utilization (Thakur, 2024). Globally, food systems are responsible for around 30% of global anthropogenic greenhouse gases (GHG), which includes 9-14% from crop and livestock farming, 5-14% from land use and land change, and 5-10% from food processing and its chain (Jean-

François Soussana et al., 2025). On the other hand, the global population is rapidly growing and will reach 9.7 billion by 2050, posing substantial challenges to ensuring food security and accessibility (Thakur, 2024).

As the global food system continues to contribute to environmental degradation, another sector, like transportation, in 2020 accounted for approximately 21% of GHG emissions globally, which becomes 24% when considering only carbon dioxide-related emissions, with road passengers accounted for 45.1%, 29.4% of freight transport, 11.6% of aviation, 10.6% of shipping, and 1% of rail (Ritchie, 2020). Specifically, the aviation sector faces imminent pressure to minimize its environmental footprint. In 2023, this industry accounted for 2.5% of global CO₂ emissions, which grew faster than other transportation modes (Lombardo and Gauch, 2025). This has accelerated efforts to develop sustainable aviation fuels (SAFs) derived from bio-based sources. Liquid biofuels are one of the SAFs that have risen from almost zero to over 0.8 million barrels of oil equivalent per day (mboe/d), which indicates the increasing demand for biofuels (IEA, 2024).

Agriculture plays a significant role in this transition by providing various oil crops that can be extracted into biofuel with minimum environmental impact (Pari, 2021). Biofuel can be derived from every part of oil crops, mainly from the extraction process of their seeds. However, this alternative biofuel cannot be strictly classified as environmentally friendly, as agricultural activities themselves constitute a large proportion of food system emissions. Moreover, oil extraction from oil crops often leaves residual products that might end up as waste or feed. Those food residuals, including those from oilseed extraction, if unmanaged sustainably, contribute significantly to food waste emissions, representing approximately 5-10% of total food system GHG emissions (Jean-François Soussana et al., 2025).

To meet the objective of the FAO, which is to minimize agricultural waste while reducing environmental damage, those side-stream products should be reutilized or recovered (FAO, 2022). Developing innovative solutions to food production that reduce environmental impact and achieve the United Nations' Sustainable Development Goal (SDG) of "zero hunger" is crucial.

Given climate change and agriculture's interlinked impacts, shifting towards a sustainable food system is very important. One key strategy for sustainability is the valorization of agricultural by-products, such as press cakes from oilseed crops, converting potential waste into valuable food resources. Additionally, developing resilient crops adapted to the climate further supports sustainable agriculture practices.

3.2 Field cress (*Lepidium campestre*)

Field cress (*Lepidium campestre*) is a novel oil crop that has recently been domesticated in Sweden by SLU. It is a wild species within the *Brassicaceae* family known for its exceptional winter hardiness, which allows it to grow even in Nordic climates. These characteristics attracted attention as a promising candidate for a novel oilseed and cover crop during winter (Ivarson, 2016; Sandgrind et al., 2023). Beyond its agronomic benefit, field cress is gaining much attention as a sustainable source for biofuel production. With the increasing demand for SAFs to minimize carbon emissions, field cress is a promising alternative source. Traditionally, SAFs are produced from wood biomass, converting alcohol from sugar, forest wastes, and fat oil greases ("Alternative Fuels Data Center," n.d.; Federal Aviation Administration (FAA) et al., 2021). However, research and development to find alternative resources of SAFs is conducted in many countries (Federal Aviation Administration (FAA) et al., 2021). Regarding the

potential of field cress as an alternative SAFs source, the wild *Lepidium* seeds contain 20% oil, 20% protein, and 40% dietary fibre, making it not only a viable source of biofuel but also a valuable byproduct for food application (Ivarson, 2016).

The oil content from *L. campestre* seeds is lower than that of conventional oilseed crops, such as rapeseed. The wild-type of field cress oil profile is dominated by polyunsaturated fatty acids (PUFAs), with 40% of linolenic acid (C18:3), 20% erucic acid (C22:1), and 11% oleic acid (C18:1) (Sandgrind et al., 2023). A high content of PUFAs makes the oil prone to oxidation due to their multiple double bonds, which can lead to off-flavors, rancidity, and a short shelf life (Swetha and Mathanghi, 2024).

The presence of erucic acid is potentially disadvantageous to humans due to its association with cardiotoxic effects. These effects have been linked to fat accumulation in the heart muscle, called myocardial lipidosis, which impairs heart function (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2016; Galanty et al., 2023). Historically, the original rapeseed oil also exhibited similarly high erucic acid content during the early 20th century (Russo et al., 2021). Through extensive breeding and genetic modification, commercial rapeseed has now been developed to contain primarily beneficial PUFAs such as oleic and linoleic acids, with very low erucic acid levels (Sandelius, 2017).

Currently, the oil profile of field cress is unsuitable for food but potentially viable for biofuel and industrial applications. However, like what has been done in rapeseed, genetic engineering has been employed by using CRISPR/Cas9 and RNAi techniques to reduce erucic acid and total PUFAs levels, while increasing the oleic acid content, which is making the oil more suitable for food and industrial uses (Ivarson, 2016; Sandgrind et al., 2023).

After the oil has been extracted from the field cress seeds, the press cake is left and called field cress press cake (FCPC). The recent trend of sustainability, the reutilization of by-products, is one way to reduce food waste and obtain valuable food ingredients (Manneteg, 2017). In addition, the press cake was shown forming a gel when soaked with water. This gel did not have any smell or taste, and comprises 80% dietary fibre, mainly pectin (Manneteg, 2017).

Therefore, the relatively low oil content and high levels of undesirable fatty acids remain challenges for commercial adoption, but ongoing domestication can improve oil quality and yield further.

3.3 Oil crops press cake

After oil extraction, the remaining press cake from oil crops is primarily utilized as animal feed, soil amendments, or plant growth enhancers. These press cakes are notable for their high content of protein, fats, ash, fibre, and carbohydrates (Arrutia et al., 2020a). The chemical composition and thus the nutritive value of oilseed depend on the following factors: oilseed genotype, soil type, climatic conditions, growing area, agricultural practices, and processing conditions (Arrutia et al., 2020b). Considering the exponentially increasing human population and escalating food prices, there is growing interest in shifting towards more sustainable plant-based alternatives (Hadidi et al., 2020). The quality and functional properties of press cake proteins supplement the existing protein sources and solve the problem of oil crop press cake disposal, along with the additional income to the oilseed crop producers and processors. The major oil crops are soybeans, rapeseed, sunflower, hempseed, and peanuts (Pojić et al., 2018).

Soybeans are the most widely produced oilseeds globally, followed by rapeseed and sunflower (Gai et al., 2025). Commercial soybeans are considered the world's most significant oilseed crop. Soybeans contain approximately 40% protein and 20% oil on an average dry basis. Major proteins of soy are globulin (90%): B-conglycinin and glycinin (Gai et al., 2025). Soy protein is a well-balanced protein with excellent processing ability, such as gelling, emulsifying ability, and water-in-oil-holding capacity (Nishinari et al., 2014).

Rapeseed is the second-largest oilseed produced in the world after soybean (USDA, 2025). It contains protein contents of approximately 25-30 % (Chéreau et al., 2016). Native rapeseed contains over 40 distinct protein fractions, with three major proteins that have been studied: cruciferin, napin, and oleosin. Cruciferin is valued for its excellent emulsifying and gelling ability, making it useful in the creation of plant-based films. Napins, on the other hand, are highly soluble and possess outstanding foaming characteristics, which makes them ideal for application in food and beverage sectors, such as the production of plant-based milk alternatives. (Rahman et al., 2021). Oleosin is an oil body protein present in rapeseed, known for its emulsifying properties (Östbring et al., 2020)

Sunflower is the third most-produced oilseed worldwide (USDA, 2025). The sunflower seeds contain about 10-20% protein, depending on the variety and processing method. (Aishwarya and Anisha, 2014). There are three primary types of cultivated sunflower seeds: oilseed types, which account for about 90% of production, confectionary types used for human and pet consumption, and ornamental types. The predominant protein fraction in sunflower seeds is globulins, which can make up to 40-90% of the total protein content. Albumin fraction accounts for 10-30 % of the total sunflower protein. Sunflower protein has good solubility and emulsifying properties (Kaur and Ghoshal, 2022).

Hempseed 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 (Callaway, 2004). The applications of hemp are numerous, as the entire plant can be utilized. Hempseed press cake (HPC) contains high amounts of protein, 30% (Banskota et al., 2022). The high protein content of the press cake has made it popular as a source of vegetable protein, and commercially available products such as hempseed protein powder and hempseed flour have been developed for human consumption (House et al., 2010). Nevertheless, HPC is often used as animal feed, due to its large amount of fibre and a less desirable flavor and appearance (Potin et al., 2019).

3.4 Protein extraction method

Protein extraction is often conducted to concentrate and purify the protein fraction by separating it from other compounds such as fats, carbohydrates, and fibre, thereby enhancing the nutritional and functional quality of the final product. Additionally, the extraction process aims to remove anti-nutritional factors that can interfere with the utilization of nutrients. Achieving these requires careful balance to ensure high protein extraction yield, minimize purification steps and associated costs, preserve protein functionality and nutritional value, and reduce environmental and health impacts (Arrutia et al., 2020b). Recently, many techniques for protein extraction that are mostly used in oilseeds have been explored and divided into conventional and innovative methods.

Among the techniques used in conventional extraction are alkaline extraction-isoelectric precipitation (pH shift method), dry fractionation, and inorganic and organic solvents. pH shift

process refers to the method that proteins are extracted under alkaline conditions, followed by precipitating the solubilised protein by adjusting pH to the isoelectric point (Tang et al., 2006). This process can significantly improve the protein yield and functional characteristics of proteins, such as emulsifying activities, foaming properties, solubility, and water/ oil adsorption ability. Also, pH shift can be due to its non-thermal nature, sustainability, and cost-effectiveness (Jiang et al., 2018). However, certain limitations of the pH shift method were notable, such as requiring a large quantity of chemicals that may impose the issue of safety issues, safe disposal, and compromise environmental sustainability (Tang et al., 2006).

Innovative protein extraction techniques have gained significant attention in recent years due to the growing demand for sustainable protein sources. As mentioned, conventional methods have several drawbacks, so to overcome these limitations, various innovative techniques have been developed, e.g., electrostatic extraction, ultrasound-assisted extraction, and enzyme-assisted extraction (Pojić et al., 2018). These techniques can enhance protein solubility, reduce processing time, and improve protein extraction yield while maintaining the bioactivity of the extracted proteins (Tiwari, 2015).

Electrostatic separation is a novel, environmentally friendly technology that involves sieving and air classification techniques for the fractionation of protein-rich and fibre-rich fractions and starch. (Zhu et al., 2021). This process yields two distinct fractions, with one fraction exhibiting a higher protein concentration than the original material. Key benefits of this technique include separation without the use of water solvents, reduced energy requirements and operational costs, and preservation of protein functionality. However, they have some disadvantages, e.g., impurity of the resulting fractions and particle agglomeration, particularly when used with oilseeds due to their residual lipid content (Laguna et al., 2018).

Ultrasound technology involves the use of sound waves with a frequency of 20 kHz to induce the cavitation phenomenon, which increases the porosity of the matrix by forming micro-fissures and channels, enhancing solvent penetration. The application of ultrasound in extraction processes offers several benefits, e.g., more efficient mixing, rapid energy transfer, selective extraction, reduced thermal gradients and extraction temperature, smaller equipment size, faster response to process extraction control, faster start-up, and higher production (Hashemi Moosavi et al., 2021; Orellana-Palacios et al., 2022). Although ultrasound extraction has several advantages for protein extraction, it can also cause changes in protein structure. The use of high-power and prolonged sonication can lead to protein denaturation. (Ampofo and Ngadi, 2022)

Enzyme-assisted protein extraction utilizes targeted enzymes to break down key constituents of plant cell walls, including cellulose, hemicellulose, and pectin (Hernández-Álvarez et al., 2023). The efficiency of this technique is influenced by several factors, such as the type and amount of enzyme used, pH level, temperature, duration of incubation, and the particle size (surface area) of the plant material (González-Serrano et al., 2022). Carbohydrases are commonly used to disintegrate the cell walls into plant tissues and facilitate protein release. These enzymes enhance protein extraction by releasing the proteins from the surrounding polysaccharide matrix (Potkule et al., 2024). Enzyme-assisted protein extraction tends to involve higher operational expenses and significant energy use. Additionally, this method causes permanent breakdown of the carbohydrate-protein matrix and requires precise optimization conditions, such as pH and temperature, to achieve the result. However, it is deemed to be a gentler extraction method with a lower environmental footprint as compared to other extraction methods (Pojić et al., 2018).

4 Materials and Methods

4.1 Materials

Field cress press cakes, FCPC (*Lepidium campestre*), obtained from industrial production of cold-pressed field cress oil (Skeby Gårdar AB, Källby, Sweden). Some of those press cakes were sent to the Research Institute of Sweden (RISE) for defatting. Then, both non-defatted and defatted FCPC were utilized as the raw material for protein extraction. The industrial FCPC has an exit temperature range of 70- 80 °C for one minute, then cooled down to 60 °C, while the FCPC from RISE was boiled in hexane at no more than 69 °C and then filtered and washed with fresh hexane at room temperature to remove the fat from the FCPC. The hexane was then evaporated at temperatures not exceeding 69 °C. Protein extraction from field cress press cake was performed at Lund University on the Food and Bioprocessing Laboratory according to Helstad et al. (2022). The method was then modified by performing the enzymatic treatment of the sample, which included adjusting the pH and temperature of the slurry before extraction. All the raw materials were ground into fine powder using the Grindomix GM 200 knife mill (Retsch, Germany) prior to the analyses.

Enzymes used- AMG 300L, Fungamyl® 800L, BAN® 480L, and Cellic® CTec 3 HS- were purchased from Novozymes (Bagsværd, Denmark), while Pectinex® Ultra SPL was purchased from Sigma-Aldrich (Sobørg, Denmark). Sodium hydroxide and citric acid were obtained from VWR International (Fontenay-sous-Bois, France, and Leuven, Belgium, respectively), along with petroleum ether and aspartic acid (Stockholm, Sweden). The other samples for reference, rapeseed press cake was obtained from Gunnarshögs Gård AB, and flax seed (Le Bonta, Italy) was purchased at a local supermarket (ICA Malmborgs, Lund, Sweden).

4.2 Characterisation of field cress press cake

4.2.1 Proximate analysis

Proximate analysis was performed on the raw materials, including non-defatted FCPC, defatted FCPC, and rapeseed press cake. Moisture content was determined by oven-drying at 105°C for 16 hours, following the standard protocol in AOAC 934.01 (AOAC Official Method 934.01, 2002). Crude protein content was analysed using the Dumas method with a Flash EA 1112 Series N/protein analyser (Thermo Scientific, Waltham, MA, USA), according to AOAC 968.06 (Latimer, 2019). A protein conversion factor of 6.25 was applied as a universal nitrogen-to-protein conversion factor. Fat content was quantified using semi-automated Soxtec (Tecator AB, Höganäs, Sweden), following AOAC 2003.05 (Latimer, 2019). Then, fat extraction was slightly modified by replacing the diethyl ether with petroleum ether. Ash content was measured by incinerating the samples in a muffle furnace (N150, Nabertherm GmbH, Lilienthal, Germany) at 700 °C for 3 hours, following AOAC 942.05 with a slight modification of the original temperature of 500 °C (Latimer, 2019). All analyses were conducted in triplicate.

The non-defatted field cress was sent to an external laboratory for analyses of dietary fibre, starch, and free glucose, cellulose, acid detergent fibre, and lignin (Eurofins Food & Feed Testing, Lidköping, Sweden) and analysed in duplicate. The dietary fibre, including soluble and insoluble fibre, was analyzed using an enzymatic gravimetry method according to AOAC 991.43 (AOAC, 2023). Starch and glucose were analyzed using enzymatic spectrophotometry according to the internal standard method from Eurofins (Sweden). While acid detergent fibre (ADF) was digested with acid detergent, the weight of the residues, except the ash, determines

the amount of ADF. ADF consists of cellulose and lignin; therefore, the lignin fraction was determined by digesting the ADF fraction with 72% sulfuric acid and corrected for ash, while total ADF was calculated as cellulose and lignin fractions.

4.2.2 Thermal properties

The protein denaturation temperatures of the raw materials (non-defatted FCPC, defatted FCPC, and rapeseed press cake) were evaluated using Differential Scanning Calorimetry (DSC) (EXSTAR6000 DSC, Seiko Instruments Inc., Shizuoka, Japan). The instrument was calibrated with indium, and an empty pan was used as the reference. Approximately 2-2.5mg of grounded FCPC was weighed and placed into a coated aluminum pan. MilliQ water was then added in an amount equivalent to three times the sample weight. The pans were hermetically sealed and heated from 25 °C to 140 °C at a constant rate of 10 °C/min, slightly modifying the method from Zahari et al. (2020). Each sample was analyzed in triplicate. Thermograms were processed using DSC Software (SII EXSTAR6000 Muse, Shizuoka, Japan), and the enthalpy values were determined based on the sample's dry weight.

4.2.3 Least gelling concentration (LGC)

The least gelling concentration (LGC) of non-defatted FCPC, defatted FCPC, flax seeds, and rapeseed press cake was determined following Coffmann & Garciaj, (1977) with minor modifications. Dispersions were prepared at 5%, 6%, 7%, 8%, and 9% (w/v) of dry matter, mixed with water. For flax seeds, the dispersion was made based on their mass, instead of their dry matter. The mixtures were then mixed on the magnetic stirrer for 10 minutes to blend the solids and the continuous phase. After that, 10 mL of each dispersion was later transferred into the test tubes and heated in a water bath (Lauda E100, Lauda-Brinkman, Delran, NJ, USA) at 90 °C for 1 hour. A thermocouple data logger (PICO Logger TC08, Pico Technology, Eaton Socon, UK) was used to monitor the temperature of the water bath. After heating, the samples were cooled in a room-temperature water bath for 5 minutes and placed in cold storage at 4 °C for 2 hours. After refrigeration, the tubes were inverted, and the least gelling concentration was identified as the lowest concentration when the sample did not flow or slip down the tube. All experiment was performed in triplicate.

4.3 Establishing a baseline

The initial protein extraction from field cress press cake was conducted using the pH-shift method, following a protocol based on (Helstad et al., 2022). In this procedure, fifty grams of defatted FCPC was milled into fine powder using a knife mill (Grindomix GM 200, Retsch, Germany) at twenty seconds total, in 5-second intervals with 10 rpm, and then mixed with 450 grams of tap water. The pH was thereafter adjusted to 10.5 using 2M NaOH while stirring with a magnetic stirrer at 750 rpm to ensure uniform mixing. The mixture was then centrifuged (Beckman Coulter, Avanti®J-15R Centrifuge, Brea, CA, USA) at 4700 rpm for 10 minutes at 20 °C to separate the soluble protein fraction from the insoluble residue. Subsequently, the supernatant was acidified to pH 4.5 with citric acid, indicating protein precipitation at their isoelectric point. The precipitated proteins were then collected by a second centrifuge for further analysis. The extraction was performed in triplicate.

4.4 Screening of the enzyme

Five commercial enzymes were selected based on their catalytic activities and were tested under controlled conditions (Table 4.1). The screening of the enzyme was done on non-defatted FCPC, due to the limited amount of defatted FCPC samples. Later, it turned out that there was

no starch in FCPC. This was not known by the time of the enzyme-assisted extraction, since the samples that were sent to the external laboratory had been stuck in the customs office for administrative checks for multiple weeks. After evaluating the amylases, pectinase, and cellulase were used to evaluate the viscosity of the slurry.

*Table 4.1. List of enzymes used for screening to optimize protein extraction from field cress press cake, including enzyme type, amount added, optimum pH, incubation temperature, and deactivation temperature. *The amount of enzymes added to batches of 50 grams of FCPC.*

Enzyme Name	Enzyme Type	Amount* (ml)	Optimum pH	Incubation Temp °C	Deactivation Temp °C
AMG® 300L	Glucoamylase	0.25	4.5	60	80
Fungamyl® 800L	α -amylase	0.63	5.5	55	80
BAN® 480L	α -amylase	0.63	5.89	70	80
Cellic® CTec 3 HS	Cellulase	4	4.5-5.0	50-55	65
Pectinex® Ultra SPL	Pectinase	4	4.5-5.0	50-55	65

The batches of non-defatted FCPC (25 g) were mixed with 225 g of tap water to prepare a slurry. The slurry was thereafter adjusted to each enzyme's optimum pH. Afterwards, each slurry was heated to the enzyme-specific temperatures while stirring with a magnetic stirrer at 500-750 rpm to ensure uniform heating. Appropriate enzyme volumes were added and incubated at the targeted temperature for 30 minutes to allow enzymatic activity. After incubation, enzymes were inactivated by heating the mixtures to the enzyme's specific temperature for about 30-60 seconds. The samples were cooled rapidly in an ice bath and stored for further analysis.

Subsequent viscosity measurement was performed with a rheometer (Kinexus Pro, Malvern, Worcestershire, UK) equipped with a 25 mm parallel plate geometry (Upper: 4V14 SS0089; PC25 C0052 AL; lower: PC25G A0008 AL). Approximately 34 mL of each sample was loaded onto the lower plate, equilibrated at 25°C for 5 minutes, and subjected to a flow ramp test. A flow curve was recorded by applying a shear stress ranging from 0.001 to 1 Pa to determine the viscosity profile. The shear viscosity was calculated at each shear stress value, using measurement number 07 (Shear stress ramp rseq) in the rFinder software.

4.5 Protein extraction and recovery

The protein extraction was conducted based on Helstad et al. (2022), with minor modifications. Fifty grams of non-defatted FCPC was milled into fine powder using a knife mill (Grindomix GM 200, Retsch, Germany) at 20 seconds total, in 5-second intervals with 10 rpm. Table 4.2 shows the flow and variation of the treatments for protein extraction of FCPC.

Table 4.2. The variation of treatment and the values used in the extraction of the field cress press cake

	Varying precipitation pH	Varying alkali extraction pH	Varying amount of enzyme added
No. of replicates	3	3	3
Amount of FCPC	50 g	50 g	50 g
Amount of water	450 g	450 g	450 g
Amount of cellulase	2 mL	4 mL	1.2, 2.0, 2.4 mL
Impeller	Rushton 30 mm	Rushton 30 mm	Rushton 30 mm
Stirring speed	750 rpm	750 rpm	750 rpm
Alkali extraction time	1 h	1 h	1 h
Alkali extraction pH	10.5	10, 11, 12	10.5
Precipitation pH	3.5, 4.0, 4.5, 5.0, 5.5	4.0	4.0

Protein extraction was performed using enzymatic treatment. Initially, various enzymes, including AMG 300L, Fungamyl 800L, BAN 480L, and Pectinex[®] Ultra SPL, were tested but showed no significant improvement in protein yield. Ultimately, Cellic[®] CTec 3 HS (cellulase) was selected as the most effective enzyme for the process.

4.5.1 Varying precipitation pH

To determine the isoelectric point of protein from FCPC, a screening study was carried out by varying pH between 3.5 and 5.5 in increments of 0.5 units during the precipitation step. Initially, fifty grams of the milled non-defatted press cake was mixed with 450 mL of tap water (1:10 w/v). The pH of the slurry was adjusted to the range of the enzyme's optimal condition to pH 4.5-5.0 using citric acid, and the mixture was stirred at 750 rpm while heating to 50 °C on a magnetic stirrer with a hot plate (RCT ST S000, IKA[®] LLG Labware, Meckenheim, Germany). At this temperature, 2 mL of cellulase was added and then incubated for 30 minutes. Enzyme deactivation was achieved by increasing the temperature to 65 °C for approximately 30-60 seconds, followed by immediate cooling to 25 °C using an ice bath. The pH of the slurry was thereafter increased to an alkaline level of 10.5 using 2M NaOH, then stirred for 60 minutes at room temperature using a Rushton impeller (MICRO-ST 7.5C S001, IKA[®], Staufen, Germany). The mixture was then centrifuged (Beckman Coulter, Avanti[®]J-15R Centrifuge, Brea, CA, USA) at 4700 rpm for 10 minutes at 20 °C. The supernatant (light liquid phase, LLP) was collected for further processing, while the residue (spent solid fraction, SSF) was retained for protein analysis. Protein in the LLP was then precipitated by adjusting the pH to different pH levels from 3.5 to 5.5 using citric acid. The solution was then centrifuged under the same conditions (4700 rpm, 10 minutes, 20 °C). The resulting sediment/precipitate was collected and analyzed for protein content, and the supernatant was discarded.

The collected precipitate and SSF in each experiment were analyzed for their protein content in triplicate. The Flash EA 1112 Series N/protein analyzer (Thermo Electron Corporation, Waltham, MA, USA) measured the protein content using the Dumas method (AOAC 968.06) with aspartic acid as the standard. Samples were ground manually with a mortar and pestle, and approximately 25 mg of each sample was weighed and placed in a 30 mm diameter tin capsule for analysis. Aspartic acid served as the reference standard, and a nitrogen-to-protein conversion factor of 6.25 was applied.

4.5.2 Varying alkali pH

The extraction pH optimization was conducted afterwards to find the highest protein recovery yield and combination. The alkali pH varied from 10 to 12 with a one-unit increment. In this experiment, 4 mL of enzyme was used. The amount of enzyme applied in this stage differed from the precipitation pH, since the exact results of cellulose in FCPC were not received.

The initial steps were similar to what had been done in the previous stage. However, instead of 2 mL of cellulase added, at this stage, 4 mL of cellulase was added and then incubated for 30 minutes. Enzyme deactivation was achieved by increasing the temperature to 65 °C, followed by immediate cooling to 25 °C using an ice bath. The pH of the slurry was thereafter increased gradually to alkaline levels 10.0, 10.5, 11.0, and 12.0 (see Table 4.2) using 2M NaOH, then stirred for 60 minutes at room temperature using a Rushton impeller (MICRO-ST 7.5C S001, IKA®, Staufen, Germany). Then, a similar treatment from the previous stage was applied until the sample was collected and analyzed. However, the precipitation pH was chosen at 4.0 instead of varying the pH.

4.5.3 Varying amount of enzyme added

After the results from dietary fibre analyses were received, revised enzyme dosages were applied in this study to achieve the optimal ratio of enzyme-to-substrates (cellulase-to-cellulose). The cellulase was calculated at 0.15 grams of enzyme per gram of cellulose. Then, the 2.4 mL, 2 mL, and 1.2 mL were selected during the initial step of FCPC extraction. All subsequent extraction procedures were carried out under the same procedure from varying precipitation and alkali pH, but the extraction pH was set at 10.5 and the precipitation pH at 4.0.

4.6 Calculations

The calculation for protein recovery yield was previously described by Östbring et al. (2020). First, the amount of protein in LLP was calculated from the amount of protein in FCPC and the SSF, since the LLP was not directly measured with the protein analyser (Equation 1). Afterwards, the light liquid phase (LLP) extraction coefficient (EC) quantifies the proportion of protein extracted from the FCPC into the LLP (Equation 2).

$$\text{Amount of Protein in LLP} = \text{amount protein in FCPC} - \text{amount protein in SSF} \quad (1)$$

$$EC (\%) = \frac{\text{amount of protein in LLP}}{\text{amount of protein in FCPC}} \quad (2)$$

Then, the precipitation coefficient (PC) measures the protein precipitated from LLP after the second centrifugation (Equation 3).

$$PC (\%) = \frac{\text{amount of protein in the precipitate}}{\text{amount of protein in LLP}} \quad (3)$$

The protein recovery yield was then calculated based on the proportion of protein initially present in the FCPC that was recovered from the precipitate fraction (Equation 4).

$$\text{Protein Recovery Yield (\%)} = \frac{\text{amount of protein in the precipitate}}{\text{amount of protein in FCPC}} \quad (4)$$

4.7 Statistical analysis

The data were presented as mean $\pm$ standard deviation of each analysis. All statistical analyses were performed using R software and RStudio version 2024.12.1+563 (PBC, Boston, MA, USA). The t-test and one-way ANOVA with Tukey's post hoc were performed to determine the samples' differences.

5 Results and Discussions

5.1 Proximate analysis

The proximate analysis of the non-defatted and defatted FCPC can be seen in Table 5.1.

Table 5.1. Proximate composition of non-defatted and defatted FCPC. The distinct superscript letters within a row indicate statistically significant differences ($p < 0.05$), and the data are presented as mean $\pm$ standard deviation of triplicate

Parameter (%)	Non-defatted FCPC	Defatted FCPC
Moisture	10.24 $\pm$ 0.04 ^a	8.51 $\pm$ 0.03 ^b
Crude protein	17.53 $\pm$ 0.45 ^b	22.92 $\pm$ 0.78 ^a
Fat	12.42 $\pm$ 0.04 ^a	2.33 $\pm$ 0.10 ^b
Ash	7.38 $\pm$ 0.58 ^a	8.05 $\pm$ 0.05 ^a
Carbohydrate by different	62.67	66.70

Field cress press cake contained 8-10% moisture, 17-23% proteins, 12% fat, 7-8% ash, and around 63-67% carbohydrates. However, the fat content in the defatted field cress press case was only 2.3%. These results align with the previous studies in the field of cress seeds by Ivarson (2016), which has almost the same proximate composition. In this study, the non-defatted samples exhibited higher moisture and fat content but lower protein and carbohydrate concentrations than defatted samples, while ash remained the same between the two samples.

These compositional shifts highlight the impact of defatting treatment, which significantly reduces fat content and increases the protein content due to the percentage of total mass of protein becoming higher (Senarathna and Malalgoda, 2024; Yue et al., 2021). Similar trends have been observed in oat protein, where defatting not only raises protein content but also enhances purity and functional properties (Senarathna and Malalgoda, 2024; Yue et al., 2021).

Table 5.2. Polysaccharides fraction analysis of field cress press cake

Parameter	Non-defatted FCPC
Dietary fibre (g/100 g)	50.15 $\pm$ 1.06
Soluble fibre (g/100 g)	<1
Insoluble fibre (g/100 g)	50.4 $\pm$ 1.31
Starch includes free glucose (%)	1
Acid detergent fibre (ADF) (%)	36.05 $\pm$ 0.35
Cellulose (%)	17.8 $\pm$ 0.25
Lignin (%)	18.25 $\pm$ 0.07

As shown in Table 5.1, carbohydrates constitute 63-67% of FCPC, with dietary fibre accounting for about 50% of the total mass (Table 5.2). This high fibre content is primarily correlated with the thick seed coat of field cress, which can carry 400 g of dietary fibre per kg (Andersson et al., 1999; Merker et al., 2010). During the oil extraction, this thick coat was not removed with a specific treatment, so it remained in the press cake.

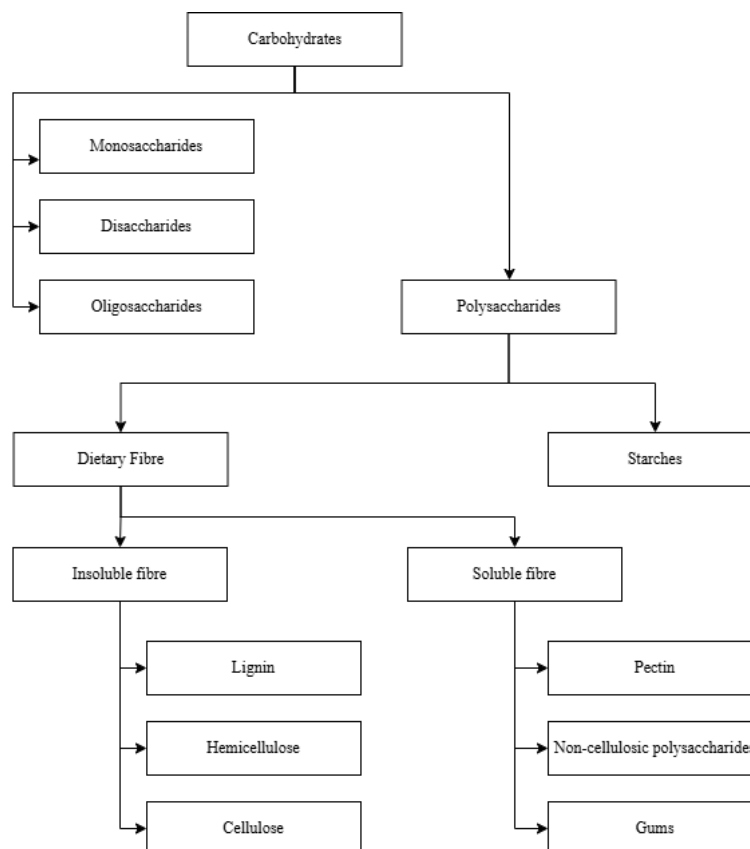


Figure 5.1. Classification of carbohydrates based on molecular complexity and dietary functionality. Illustration made based on the carbohydrate classification described in the book and the article related to carbohydrates (George et al., 2020; Zeece, 2020).

To deepen the understanding of the carbohydrate fractions, Figure 5.1 illustrates the classification of the dietary fibre in plant cells. The fibre fraction is predominantly insoluble, comprising lignin, cellulose, and hemicellulose. In FCPC, the insoluble fibre dominated the total fibre of 50.4%. Moreover, the cellulose and lignin content both covered up to approximately 18% of the total dietary fibre. In another study, this high content of dietary fibre, especially the pectic polysaccharides, suggested an ability of field cress seeds to form a gel, a viscous gel (Manneteg, 2017). However, that previous study did not classify the dietary fibre fractions on FCPC, and this study confirmed that the soluble fibre (including pectin fraction) is lower than the insoluble fibre. On the other hand, starch content was very low, which was caused by the plant field cress's adaptation to prioritise storing oil and protein over starch storage (Andersson et al., 1999). Compared to sesame press cake, which contains approximately 22% insoluble fibre, 3% soluble fibre, and 3% remaining carbohydrates (Melo et al., 2021), FCPC has higher carbohydrate fractions.

5.2 DSC thermogram

Differential Scanning Calorimetry (DSC) was used to assess thermal transitions across a temperature range of 30 °C to 120 °C, allowing the identification of endothermic peaks related to protein denaturation. The DSC thermogram displays the heat flow (mW) as a function of sample temperature. After knowing the range of denaturation protein, it can help to choose a proper temperature of enzyme deactivation without denaturing the protein of FCPC.

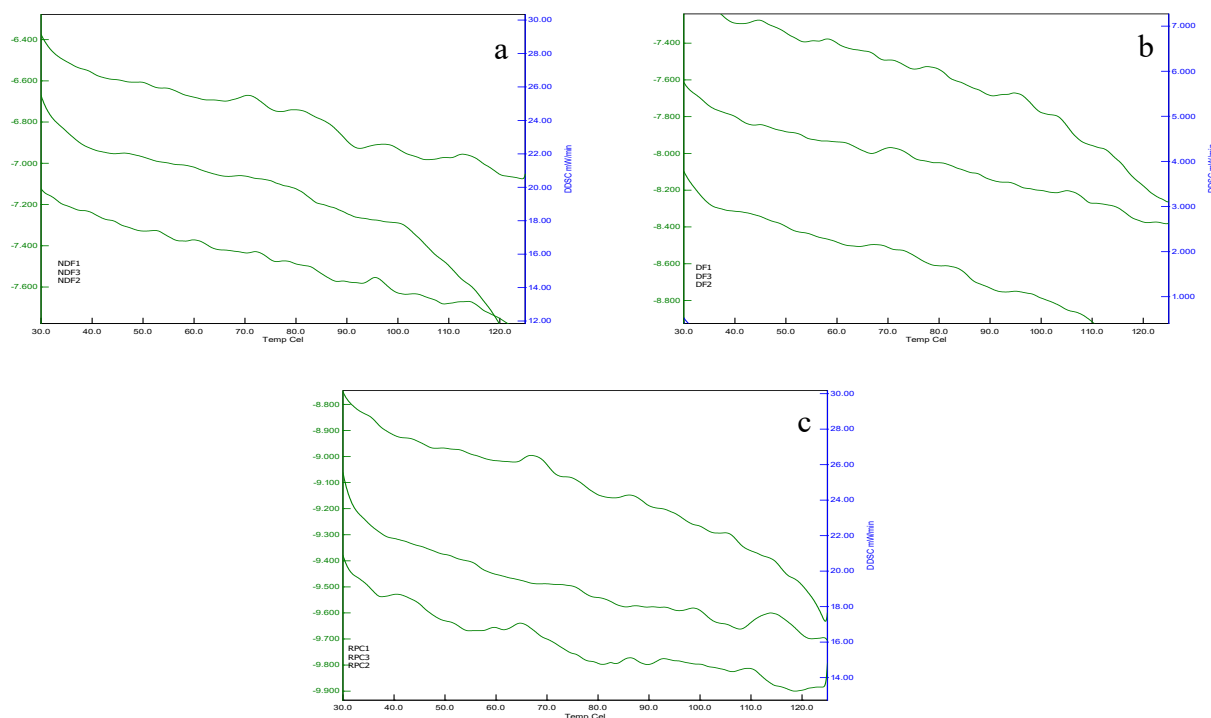


Figure 5.2. Differential Scanning Calorimetry (DSC) thermogram of three replicate samples showing endothermic transitions associated with protein denaturation. (a) Non-defatted field cress press cake; (b) Defatted field cress press cake; (c) Rapeseed press cake.

In the non-defatted FCPC samples (Figure 5.2a), endothermic transitions were observed, with denaturation occurring between 84°C and 96°C, and the denaturation peaks (T_d) occurred at 92°C. The defatted FCPC samples (Figure 5.2b), exhibited a similar range from 80°C to 98°C. The peak of protein denaturation in this sample occurred at 95°C. These results suggest that the deactivation of the enzyme used should be below these T_d . Conversely, rapeseed press cake exhibited endothermic peaks at lower temperatures than other samples. It can be seen on the third graph (c) in Figure 5.2 that the onset temperature (T_o) for rapeseed press cake protein denaturation was 71°C, and its peak was at 83°C.

Among the three samples, defatted FCPC exhibited the highest peak denaturation temperature, followed narrowly by the non-defatted samples, while rapeseed press cake showed the lowest. These findings, especially between the non-defatted and defatted FCPC, suggest that the defatting process did not significantly affect the thermal properties of the press cake. In addition, the different prior heat exposure during oil extraction in FCPC and rapeseed, and the different protein composition, make the rapeseed have lower denaturation temperatures. The composition of protein in the FCPC has not been evaluated yet, so it raises questions about what kind of protein constitutes the FCPC. The rapeseed protein consists primarily of cruciferin and napin, and both proteins display high thermal stability with a denaturation temperature (T_d) of

approximately 91°C and 110°C (Jia et al., 2021). However, all the results showed that the peaks varied among their replicates, and some of them had broader peaks while others had narrower valleys. The heterogeneous press cake, a tiny amount of which was put onto the crushable plate, might be the reason for these results. A clear comparison of the samples can be seen in Table 5.3.

Table 5.3. Thermal transition parameters of non-defatted FCPC, defatted FCPC, and rapeseed press cake as determined by DSC. Values in the same row bearing different letters differ significantly ($p < 0.05$), $n=3$.

Paramemeter	Non-defatted FCPC	Defatted FCPC	Rapeseed Press Cake
Onset denaturation temperature, T_o (°C)	83.77 ± 2.55^a	80.49 ± 5.93^{ab}	71.04 ± 5.23^b
End denaturation temperature (°C)	96.17 ± 1.02^a	98.42 ± 5.52^a	90.66 ± 7.34^a
Peak denaturation temperature, T_d (°C)	92.04 ± 0.91^{ab}	95.36 ± 6.71^a	83.17 ± 2.25^b
Enthalpy change ΔH (mJ/mg)	1.2 ± 0.06^a	1.9 ± 0.89^a	1.71 ± 0.49^a

A DSC thermogram can also be used to calculate the enthalpy change (ΔH) of the area under the peak. This reflects the energy required to unfold the protein, and a higher ΔH indicates stronger intramolecular bonds and stability of the protein (Durowoju et al., 2017). Defatted FCPC has the highest ΔH (1.9 mJ/mg), although not statistically significant compared to the others. This suggests a higher energy requirement to denature the protein, due to structural changes in the protein after defatting and oil extraction temperature.

The exit temperature of oil extraction on FCPC was 70-80°C, and it only stayed at that temperature for the first minute, then it went down to 60°C. Therefore, the defatting process has been done by boiling the FCPC in hexane at 69°C and then filtering and washing with fresh hexane at room temperature. It is not exposed to higher temperatures than that. In contrast, rapeseed seeds start to press at 35°C, and the temperature of the oil goes up to 45°C. After the extraction, the rapeseed press cake is 65-75°C at the exit temperature. Furthermore, the different processing temperatures of each sample increase the likelihood of altering the protein denaturation temperature.

5.3 Extraction process at baseline

The baseline of FCPC protein extraction using traditional pH-shifting yielded only approximately 3% recovery, primarily due to inefficient protein release from the FCPC matrix and challenges in phase separation. This step aimed at understanding the characteristics of the field cress press cake during the extraction process.

The baseline result showed that the alkaline pH-shift method failed to sufficiently solubilize proteins, leaving a dominant spent solids fraction (SSF) after the first centrifugation. The SSF mostly contains husk, unbroken cells, and protein that were not dissolved (Ahlström et al., 2022b). Only a small amount of protein in the light liquid phase (LLP), limiting the protein perceptibility potential.

The intact matrix of FCPC also likely inhibits protein extraction. The mechanical treatment already used by grinding the FCPC using a knife mill can increase the surface area. However, this treatment was not enough to get more protein from FCPC and degrade the cell wall. Thus, enzyme-assisted extraction of protein was chosen to optimize the protein recovery yield from FCPC. The behavior of FCPC was then evaluated by analyzing its least gelling concentrations and viscosity. Therefore, the enzyme screening was also carried out to find the right enzyme.

5.4 Least gelling concentration (LGC)

Another characteristic analyzed was the least gelling concentration (LGC). The results are presented in Table 5.3, which shows a comparison between field cress press cake, flax seeds, and rapeseed press cake. Both non-defatted and defatted samples exhibited an LGC of 6% (w/v), while flax seeds witnessed the gel formation at a lower concentration of 4% (w/v). On the other hand, rapeseed press cake exhibited a distinct phase separation at a concentration of 5%-7% (w/v), the samples formed a heterogeneous layer with a dark layer on the top, clear and yellowish liquid in the middle, while the lighter phase was on the bottom of the layer. At the higher concentration, the rapeseed press cake can form a homogenous mixture that cannot retain its gelling structure at 8% and 9% (w/v), and at 10% (w/v), a homogenous and firm gel was formed.

The LGC measurement gives the baseline for the gel-forming ability of the native protein matrix in the samples. This is valuable for comparing how different food matrix results in different protein functionality, especially gelation, which is one of the key properties for food applications. In addition, the LGC evaluation helps optimize the extraction methods that can be used to develop food ingredients. In this study, the LGC of FCPC was low, which helped to decide to use an extra step on the pH-shifting method, which was enzyme-assisted protein extraction.

In a previous study, rapeseed press cake was extracted using the pH-shifting method without the use of enzymes (Ahlström et al., 2022a). The results showed that rapeseed press cake did not form a gel at a ratio of 1:9 with water (w/w), which facilitated more efficient extraction compared to FCPC (Ahlström et al., 2022a). In the present study, the ratio of FCPC to water was maintained at 1:9 (w/w) to minimize environmental impact and enhance economic feasibility. However, at this concentration, the gel was formed, resulting in a thicker slurry that hindered extraction. Thus, the LGC evaluation provided the foundation to use the enzyme-assisted protein extraction method to break down the gel and reduce the viscosity, aiming for more feasible protein extraction.

Table 5.4. The least gelling concentration of non-defatted FCPC, defatted FCPC, flax seeds, and rapeseed press cake.

Concentration (w/v)	Non-defatted FCPC	Defatted FCPC	Flax seeds	Rapeseed Press Cake
3%	Slipped down	Slipped down	Slipped down	-
4%	Slipped down	Slipped down	Formed Gel	-
5%	Slipped down	Slipped down	Formed Gel	Partially gelling
6%	Formed Gel	Formed Gel	Formed Gel	Partially gelling
7%	Formed Gel	Formed Gel	Formed Gel	Partially gelling
8%	Formed Gel	Formed Gel	Formed Gel	Slipped down
9%	Formed Gel	Formed Gel	Formed Gel	Slipped down
10%	-	-	-	Formed gel

5.5 Viscosity of enzyme-treated field cress press cake

Enzyme screening was a preliminary step in the protein extraction process to identify the most effective enzymes for enhancing protein recovery from field cress press cake. The screening aimed to determine which enzyme could effectively break down plant cell wall components and improve protein release. Plant cell walls are complex structures made of cellulose, hemicellulose, pectin, lignin, etc. These structures create a physical barrier that makes it difficult for the protein to be extracted. The right combination of enzymes can effectively degrade these barriers, making the protein more accessible for extraction (De Vries and Visser, 2001). Figure 5.5 shows the viscosity of extracted protein-rich sediment derived from corresponding enzymatic treatments. The viscosity measurements were performed at a shear stress of 1 Pa using a rheometer at 25 °C. Background colour bands on the graph indicate viscosity ranges, blue for low viscosity, pink for moderate viscosity, and red for high viscosity.

One expected outcome of enzyme activity was a reduction in the slurry's viscosity, indicating effective breakdown of the press cake's cell wall components. Reducing the viscosity indicates that enzymes disrupt the cell wall and release the protein (Sheng et al., 2023). Among the enzymes tested, glucoamylase (AMG[®] 300L), α -amylases (Fungamyl[®] 800L, BAN[®] 480L), cellulase (Cellic[®] Ctec 3 HS), and pectinase (Pectinex[®] Ultra SPL), only cellulase significantly reduces the viscosity of FCPC slurry. Each enzyme targets a particular component of the plant cell wall. Glucoamylase (AMG[®] 300L) and α -amylase (Fungamyl[®] 800L and BAN[®] 480L) target starch. Glucoamylase hydrolyses both α -1,4 and α -1,6 glycosidic bonds, while α -amylase primarily cleaves the internal α -1,4. These enzymes help degrade residual starch, improving the accessibility of protein within the cell wall (Pandey, 2006). The lack of viscosity reduction with α -amylase and glucoamylase indicates the limited starch content in FCPC (Andersson et al., 1999). This was confirmed by the analysis of the carbohydrate fraction in Table 5.2. For this reason, these three enzymes were excluded from further use in the study.

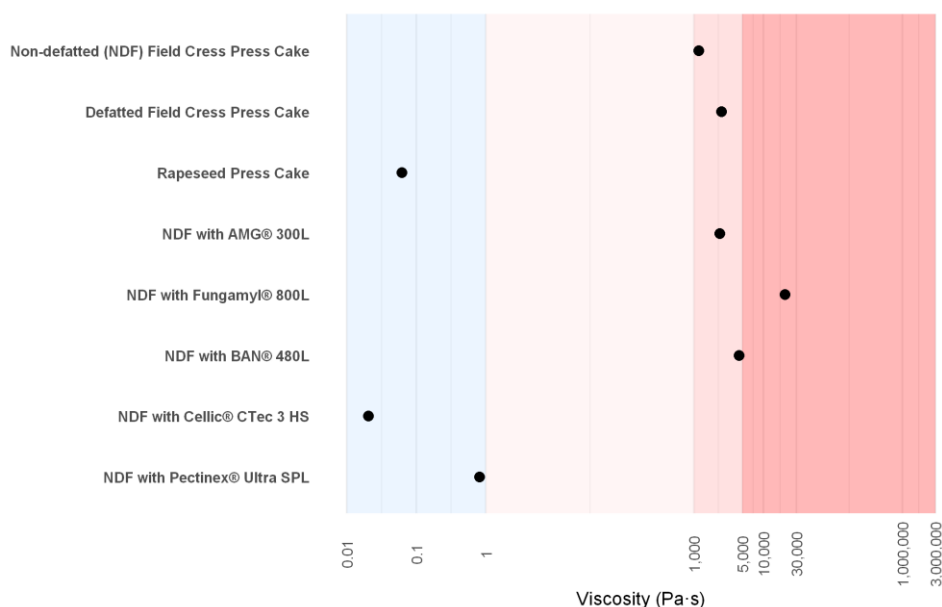


Figure 5.3. Apparent viscosity (Pa·s) of non-defatted, defatted FCPC, rapeseed press cake and NDF FCPC treated with different commercial enzymes. Enzymatic treatments included

glucoamylase (AMG[®] 300L), α -amylase (Fungamyl[®] 800L, BAN[®] 480L), cellulase (Cellic[®] Ctec 3 HS), and pectinase (Pectinex[®] Ultra SPL) applied to the non-defatted fraction (NDF) of FCPC.

Cellulase (Cellic[®] Ctec 3HS) was identified as the most effective enzyme due to its ability to degrade cellulose, thus significantly enhancing the protein extraction. Cellulase operated efficiently under favourable conditions (pH 4.5-5.0, incubation temperature 50 °C, and deactivation temperature 65°C) and was therefore selected for use in the main extraction process. Pectinase showed moderate effectiveness but did not surpass cellulase in reducing mixture viscosity. This is because most pectin is abundant in soft plant tissue like fruits and vegetables, but less prevalent in seed press cake (Mudgil and Barak, 2013; Tamaki et al., 2008). Pectinase targets pectin, which binds the plant cell wall together. It breaks down the pectic substances, making the cell wall lose its structure, making it easier to extract protein (Pandey, 2006).

According to some previous studies, cellulose, hemicellulose, and pectin are the most abundant non-starch polysaccharides in the plant cell wall (Andersson et al., 1999; Manneteg, 2017; Merker et al., 2010). However, at the mature stage of the plant, cellulose accounts for 70% of the cell wall and becomes the main component. The native cellulose is very insoluble; its tight hydrogen bonds prevent water from penetrating its structure. However, chemical modification can alter the cellulose structure and its functionality, such as microcrystalline cellulose, which can be used as a thickening agent (Zeece, 2020). Another study mentioned that *Lepidium campestre* produces mucilaginous seeds that are sticky and hydrophilic. This mucilage contains cellulose and pectin, which help them to germinate (Mohammed and Mummenhoff, 2024). Furthermore, these polysaccharides can remain in the press cake after the oil extraction process, which is one reason for the high viscosity. This was logical and justified by viscosity reduction, which highlighted the effectiveness of the cellulase enzyme in cell wall degradation and enhanced protein recovery.

5.6 Protein recovery yield

The extraction coefficient, which indicates the proportion of protein in the solid-state fraction (SSF) and light liquid phase (LLP), can be seen in Figure 5.6. The proportion of protein in LLP is relatively low across all pH values and enzyme concentrations tested. At acidic precipitation pH, the amount of protein in LLP was almost similar in all samples, since the extraction pH, which was used for extracting the protein, was the same, which was at pH 10.5. However, at pH 4.5, it gave a slightly higher extraction coefficient than at other pH.

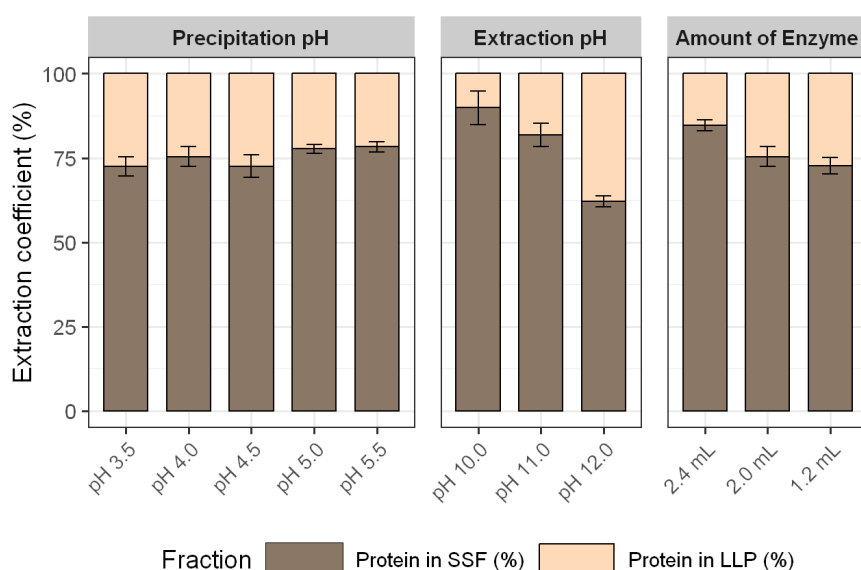


Figure 5.4. The extraction coefficient from solid-state fraction (SSF) and light liquid phase (LLP) of FCPC, which indicates the amount of protein in SSF and LLP in different treatments, $n=3$.

In the extraction pH range (pH 10.0-12.0), the graph demonstrates an upward trend in the amount of protein released into LLP. Specifically, higher pH levels enhance the protein that could be extracted into LLP, reaching its highest percentage of approximately 40% at pH 12. These results align with another study on hempseed press cake protein extraction, which suggests that increasing the base extraction pH enhances the protein content that possibly can be precipitated in the next step (Helstad et al., 2022). The enhanced protein solubility observed at elevated pH conditions can create electrostatic repulsion, which increases the solubility of the protein (Potin et al., 2019). Similarly, a study conducted by Östbring et al. (2020) on rapeseed press cake study mentioned that increasing the extraction pH significantly enhanced the solubilization of solids and proteins, leading to higher extraction coefficients and higher protein recovery in the LLP.

In contrast, reducing the cellulase concentrations from 2.4 to 1.2 mL of cellulase tends to provide a greater extraction coefficient. Moreover, when comparing the amount of enzyme used in this study, a high concentration of enzyme reduces the protein release from the press cake, as seen in Figure 5.6, where different alkali treatments tend to have lower protein in LLP compared to different enzyme treatments with alkali extraction at pH 10.5. However, this result is not aligned with a previous study on enzyme-assisted protein extraction of residual products, in which Flaibam & Goldbeck (2024). It was discovered that increasing the enzyme-substrate ratio could quadratically increase the degree of protein hydrolysis. Yet, that study also reported that determining the enzyme-substrate ratio must be precisely evaluated for optimal results. So, further, and precise studies need to be performed to deepen our understanding of this enzyme concentration related to protein extraction in FCPC.

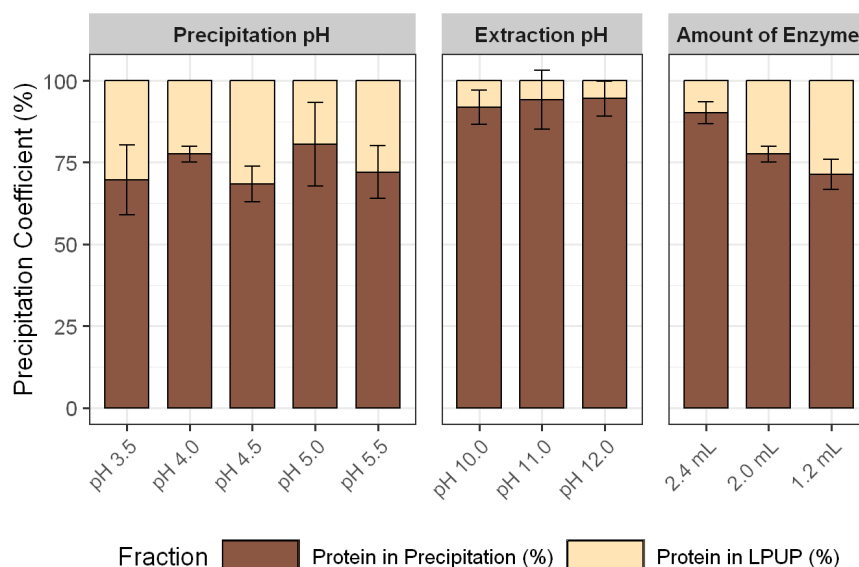


Figure 5.5. The precipitation coefficient of LLP, which indicates the amount of protein in the precipitation and liquid phase containing unprecipitated protein (LPUP) in different treatments, $n=3$.

The precipitation coefficient highlighted the proportion of protein that can be precipitated from LLP. The precipitation coefficient serves as a crucial indicator of the efficiency of protein recovery from LLP during fractionation. The results (Figure 5.7) demonstrate that both pH adjustment and enzymatic treatment significantly affect the precipitation of proteins. The variation resulted in different precipitation pH levels, with pH 5.0 being higher than the other acid pH treatments. Furthermore, the various alkali pH levels show that higher pH levels at the extraction stage can improve protein precipitation. The same trends also happened at a higher amount of enzyme used in the pre-treatment step. Adding 2.4 mL of cellulase resulted in a high precipitation coefficient compared to other enzyme treatments.

Among the acidic treatments, pH 5.0 led to a slightly higher precipitation coefficient than other conditions. This can be attributed to the proximity of pH 5.0 to the isoelectric point (pI) of many plant-based proteins, where proteins exhibit minimal solubility and are hence more likely to precipitate. This finding aligns with the observations of Zhang et al. (2020), who discovered enhanced protein precipitation around the pI in rapeseed press cakes.

Regarding the alkaline extraction, higher pH levels increased the precipitation coefficient. This trend suggests that more alkaline conditions during extraction may solubilize more protein, precipitating more effectively upon subsequent acidification. This behavior is consistent with studies by Tang et al. (2006) and Zhang et al., (2020) and reported enhanced protein solubility and recovery at high extraction pH values in soy, hempseed meals, and rapeseed press cake.

Regarding enzyme concentration variations, it shows that a much higher concentration has been used to degrade the cell wall, less protein could be freed into LLP, but a much higher protein proportion is in the precipitate. The comparison, not only between the different amounts of enzymes but also the utilization of 4 mL of enzymes during different alkali extractions, led to a high precipitation coefficient.

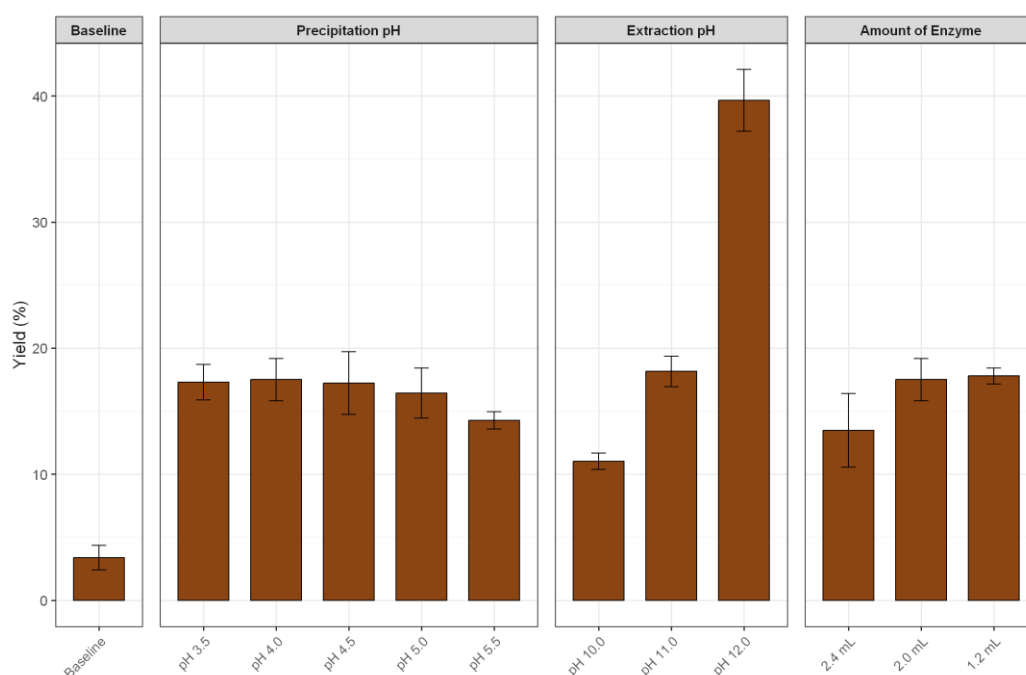


Figure 5.6. Protein recovery yield of FCPC in different treatments, varying the precipitation pH, extraction pH, and enzyme concentration used, $n=3$.

The protein recovery yield reflects the combined effects of extraction and precipitation conditions. Figure 5.8 presents the protein recovery yields. Overall, the protein recovery yields were higher than the baseline after using the enzyme-assisted protein extraction method. In comparison, the baseline was performed in the traditional pH shifting method. The baseline recovery yield was only around 3%, and the new applied method increased it multiple times. In the diverse precipitation pH treatments, the optimal pH for precipitating was pH 4.0. It was just above pH 3.5 and 4.5, and then the recovery yield was reduced as the pH increased. The pH 5.0 gave the highest precipitation performance, but pH 4.0 yielded more than other precipitation pHs. This indicates that the extraction coefficient and protein precipitation affect the protein recovery yields. However, at the extraction step, higher pH also resulted in higher yield; at pH 12, it is approximately 39%. This pH range, from 10-12, had a similar precipitation coefficient, but the highest extraction coefficient was achieved at pH 12. The overall yield was also strongly dependent on extraction pH, with pH 12.0 showing the highest recovery (~39%), followed by pH 10.0 and 11.0. This indicates that high extraction pH increases the quantity of recoverable protein. However, the higher extraction pH leads to the risk of protein denaturation, high chemical usage, and equipment damage, and environmental damage if the chemical is leaking (Helstad et al., 2022).

Interestingly, the protein recovery yield was higher at an enzyme concentration of 1.2 mL than at 2.0 and 2.4 mL. In contrast, the extraction coefficient at pH 2.4 mL was lowest compared to other enzyme treatments, which led to a lower amount of protein that could be precipitated. Nevertheless, in the 2.4 mL enzyme-treated sample, the precipitation coefficient was higher than the others. This phenomenon was remarkably interesting to investigate further. In another experiment, increasing enzyme concentration is generally expected to enhance cell degradation and facilitate protein release; excessively high levels can lead to over-hydrolysis of protein (Sari et al., 2013). However, too high levels of the enzyme can lead to saturation of the limited substrate and inhibit the release of protein from FCPC into LLP.

In contrast, at 1.2 mL, the enzyme concentration was sufficient to release protein from the cell wall matrix without extensively breaking it down, allowing more protein to aggregate and precipitate efficiently. Beforehand, adding 1.2 mL of enzyme was calculated based on the amount of cellulase and lignin present in the FCPC, preventing the substance's saturation and excessive use of enzyme. This observation aligns with a previous study where a moderate and sufficient amount of enzyme for targeted materials was shown to optimize protein extraction while preserving the integrity needed for effective precipitation (Sari et al., 2013).

6 Conclusion

This study discovered the optimization of the protein extraction process from field cress press cake, a byproduct of oil extraction from *Lepidium campestre*. The initial extraction process by the pH shifting method resulted in a protein recovery yield of approximately 3%. During the extraction processes, the viscosity of the FCPC was a big challenge and inhibited the protein release. This result led to a series of enzymatic screenings, and cellulase (Cellic® Ctec 3 HS) was identified as the most effective enzyme for reducing the viscosity and enabling the protein extraction. The optimal extraction condition was found at an alkali pH of 12.0, followed by acid precipitation at pH 4.0, resulting in the highest protein recovery yield of ~39%. However, the greater cost of using high alkali pH must be addressed by precise evaluation of alkali extraction pH. Notably, an enzyme amount of 1.2 mL provided a better yield than a higher dose, likely due to the optimum enzyme-substrate ratio. These findings highlight the potential of valorising FCPC into a valuable protein source, contributing to sustainable food system development.

7 Future Works

Future research should focus on assessing protein quality and functionality while optimising the extraction process. Protein quality assessment is needed to understand the amino acid profile of FCPC protein, and the functionality evaluation will deepen the understanding of the protein solubility, emulsification, foaming, and gelling. In addition, optimising the extraction process can be performed by exploring additional processes on raw materials or multi-stage extraction to further increase the protein yield and reduce waste.

8 References

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