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Protein Recovery from Alkaline Extracts: A Comparative Analysis of Isoelectric Precipitation and Ultrafiltration

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Popular Science Summary

The Nordic Council of Ministers for Nutrition states that an adult requires ca. 0.8 - 1.5 g protein for each kg of their body weight daily, and the World Health Organization and the Food and Agriculture Organization define the daily intake requirements for the essential amino acids. As concerns about health and the environment have been on the rise in recent years, the current food system has started to show vulnerabilities. With increasing population size, production levels need to catch up. To develop a new source of healthy food that can cover all these aspects, valorising the waste/by-products of food production, often rich in proteins and other beneficial biomolecules, has been in focus. The waste from rapeseed oil production (rapeseed press cake) and the leftover grains (Brewers' spent grains) from beer production are two materials with a balanced amino acid profile to cover the daily intake requirements.

This work focuses on evaluating and comparing isoelectric precipitation and ultrafiltration combined with diafiltration as two protein recovery methods. The comparison was made based on the dry matter and protein content and the recovery yield of the final product from both methods. The proteins were extracted from the raw materials as a solution, using alkaline conditions, and then subjected to the two recovery methods. Isoelectric precipitation uses acidic pH to decrease the solubility of proteins, allowing their separation using centrifugation. On the other hand, ultrafiltration removes the solvent and concentrates the protein solution, followed by diafiltration for removing undesired impurities such as salts, minerals, etc. and improving the product purity.

The isoelectric precipitation method is ideal for recovering a high amount of protein from rapeseed cake protein extract. Still, the purity of the final product is not as good as the one made from ultrafiltration and diafiltration. When the rapeseed cake is treated with ethanol before protein extraction, the purity and content of protein in the final product are poor, irrespective of the type of recovery method used. However, if the yield is to be considered, isoelectric precipitation has the edge over ultrafiltration and diafiltration.

Due to an error in the experimental design, the spent grains could only be analysed qualitatively. The isoelectric precipitation can be considered to be ideal for the spent grains since the protein content recovered was higher. However, a study with the correct methodology is needed to have conclusive results.

The results of this work show that both methods considered here can provide good results. Still, the intended end use, the type of starting material, how it is processed, and operating costs should also be factored in when choosing the recovery method.

Abstract

Proteins are one of three macro-components of a diet. An increasing demand for products that can be a complete protein source exists. The vulnerability of the current food system towards environmental impacts and the inability to keep up with the rising population has shifted the focus on valorising underutilised by-products of the food industry. The rapeseed cake (a leftover from rapeseed oil production by mechanical pressing) and spent grains (a by-product of the brewing process) are rich in proteins and a balanced amino acid profile for a complete protein product. This work used protein extraction under alkaline conditions optimal from rapeseed cake and brewers' spent grains, respectively, and investigated the difference between the protein quantity and quality recovered using the isoelectric precipitation method and ultrafiltration combined with diafiltration.

The isoelectric precipitation method gives a good yield for rapeseed cake that has been treated with ethanol, but the purity of the proteins could be better. Ultrafiltration and diafiltration also have poor results with the same raw material. However, on the other hand, if rapeseed cake is used as is for extraction and recovery, either of the methods is good, with ultrafiltration and diafiltration providing more yield. Qualitative analysis of the results for the spent grains shows isoelectric precipitation to be ideal. However, a study with the correct methodology is needed to have conclusive results.

The choice of protein recovery method depends on the starting material used for protein extraction. Additional factors such as the intended use, further downstream processing requirements, and costs must also be considered when deciding the type of recovery process.

List of Abbreviations

AA	Amino Acid
BSG	Brewers' Spent Grains
CF	Concentration Factor
CV	Coefficient Of Variance
db	Dry Basis
DF	Diafiltration
DV	Diavolume
ERPC	Ethanol-soaked Rapeseed Press Cake
IPI	Isoelectric Protein Isolate
MW	Molecular Weight
N	Nitrogen
NAC	N-acetyl Cysteine
NaOH	Sodium Hydroxide
pI	Isoelectric Point
RPC	Rapeseed Press Cake
SSF	Spent Solid-fraction
UF	Ultrafiltration
URPC	Untreated Rapeseed Press Cake

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I want to thank Olexandr Fedkiv and Hans Bolinsson for guiding the lab and helping me set up the dry-freezing equipment each time it was needed.

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1. Introduction

With increasing concerns over the vulnerability of the food system and visible environmental impacts alongside the system's inability to meet the rising dietary demands of the global population (29), the focus has shifted towards valorising underutilised by-products of the food industry. Proteins, one of the three macro-components (the other two being carbohydrates and lipids) of any living organism or food item, can be hydrolysed into monomeric units - amino acids (AA) by hydrolytic enzymes in the human digestive tract. These AAs can be divided into two categories: essential AAs (those that must be supplied externally) and non-essential AAs (those that can be synthesised in the body with an adequate supply of carbon and nitrogen from the diet) and are required by our body for the production of the requisite proteins for normal cellular functions such as material transport (inter- & intra- cellular), enzymatic activities (6).

A meal providing all the essential AAs is considered a ‘complete protein’ source (19). As per the recommendations set by the Nordic Council of Ministers for nutrition, an adult requires ca. 0.8 - 1.5 g protein for each kg of their body weight daily (20). A vegetarian/vegan diet may be more sustainable for the environment than an animal-based one; however, ensuring adequate intake of the necessary quantity of protein is challenging due to the low amount of protein available in such a diet's different components and the reduced bioavailability due to anti-nutrients like phytate. A variety of protein sources or a high intake of low-protein sources are the best options in a plant-based diet to overcome insufficiency from a single source (17). A source that covers the complete protein profile by providing all nine essential amino acids in adequate amounts can significantly contribute to daily protein intake requirements. Various studies into rapeseed cake (a leftover from rapeseed oil production by mechanical pressing) and spent grains (a by-product of the brewing process) have indicated high levels of leftover proteins and a balanced AA profile for meeting the daily protein (and AA) requirements as indicated by the World Health Organization and the Food and Agriculture Organization (23).

Proteins are susceptible towards pH changes in their environment. One conventional technique for extracting proteins from any material is alkaline extraction, which precedes isoelectric precipitation. The method involves increasing the pH value high enough (usually 8- 11) to impart a negative charge to the proteins, thereby increasing their solubility in the solvent used for extraction. Non-protein components are separated from the proteins using simple filtration or centrifugation. Afterwards, changing the pH of the protein-rich liquid to the pI of the proteins effectively removes any charge on them. It leads to protein precipitation, creating an ‘Isoelectric protein precipitate’ and a ‘soluble protein’ fraction.

Alternatively, the other option is the concentration and purification of the alkaline extract through methods like ultrafiltration (UF) and diafiltration (DF). UF helps to remove excess solvent and any small non-protein impurities, while DF further purifies the extract (11).

2. Objectives

This work aims to compare two protein recovery methods for alkaline extracts made from Rapeseed Press Cake (RPC) and Brewers' Spent Grains (BSG) by evaluating and comparing the protein quantification measurements of the recovered proteins.

The specific objectives are:

- To create an alkaline extract for both raw materials and compare its protein quantification values with the base work used for defining the extraction method.
- To use the two methods for protein recovery and measure the protein content for recovered protein fraction(s).
- To compare the recovery efficacy of the methods in question.

3. Background

3.1. Raw Materials

3.1.1. Rapeseed Press Cake

Rapeseed (*Brassica napus*) is a rapeseed/ canola oil source, and it is one of the top three most cultivated crops for oil production. The oil is primarily used in cooking, while the leftover seed material is mostly fodder or fertiliser. Due to the nature of the oil extraction process, the leftover material, commonly called "Rapeseed Press Cake" or RPC, is a potentially good source of proteins and is often underutilised (27).

If cold-pressed (at a temperature less than 60℃) (10), RPC can contain 38–45% protein; if heat is applied, it may contain 28–31% protein. RPCs proteins' AA composition (see Table 1.) is rich with high sulphur-containing AA levels (24). The extractable proteins from the seeds are mainly found in the form of "storage proteins", followed by structural ones.

The predominant storage proteins found in the plant, 300-350 kDa cruciferin (11S or 12S globulin) and 12-16 kDa napin (1.7S or 2S albumin), are present at a ratio of 6:2. While the remaining ones, a class of "Oil Body Proteins", viz. Oleosins, steroleosins and caleosins stabilise the oil storage organelles and comprise the remaining ca. 20% of the total proteins (1, 28, 30). The salt-soluble cruciferin has a pI of 7.2, while the water-soluble napin has a pI of pH 11. However, the most commonly accepted pI range for RPC proteins is 3.5-6 (1).

Rapeseed isolate proteins are ideal for food product applications due to their emulsifying and oxidative properties (24). Since these proteins start to denature above 75℃, cold-pressed RPCs are a better

option than heat-pressed ones, which usually require temperatures well above the denaturation temperature of proteins. Apart from functional favorability, the cold-pressed method has a lower environmental impact and overall costs (25).

Table 1. *Essential AA composition of RPC and its comparison to the recommended requirements based on daily protein intake recommendations by WHO (1, 23).*

S.No.	**Essential AA**	**Essential AA requirement**		**RPC**	**Covered**
Unit		**mg/ g protein**	**g per 100 g protein**	**g per 100 g**	**%**
1	Histidine (H, His)	15	1.50	2.9	193%
2	Isoleucine (I, Ile)	30	3.00	4	133%
3	Leucine (L, Leu)	59	5.90	7.4	125%
4	Lysine (K, Lys)	45	4.50	6.4	142%
5	Methionine (M, Met) + Cysteine (C, Cys)	22	2.20	4.6	209%
	Methionine (M, Met)	16	1.60	2.1	131%
	Cysteine (C, Cys)	6	0.60	2.5	417%
6	Phenylalanine (F, Phe) + Tyrosine (Y, Tyr)	38	3.80	7.4	195%
	Phenylalanine (F, Phe)	-	-	4.2	-
	Tyrosine (Y, Tyr)	-	-	3.2	-
7	Threonine (T, Thr)	23	2.30	5	217%
8	Tryptophan (W, Trp)	6	0.60	1.6	267%
9	Valine (V, Val)	39	3.90	5.4	138%
TOTAL		277	27.7	44.7	**161%**

3.1.2. Brewers' Spent Grains

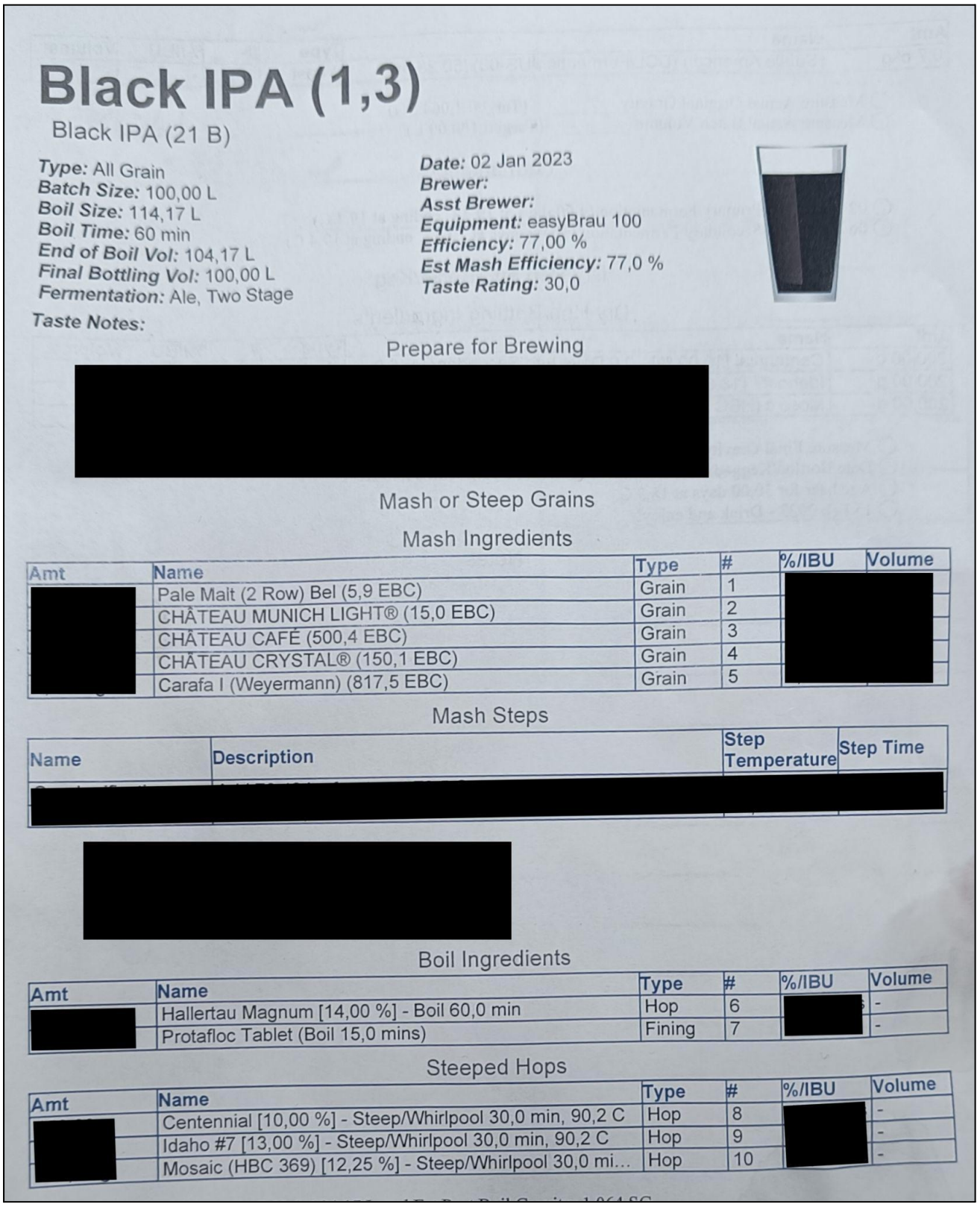
Brewing is a process that involves using the sugar-rich wort made from cereal(s) to produce alcohol via fermentation. The source of sugars determines the end product. Grapes and rice are used for wine production, while barley is the standard source for producing beer. Depending on the intended final product, various materials (adjuncts) can be a part of the cereal mix. The BSG can be of two types, based on the temperature used for kilning, 'black BSG' and 'pale BSG', respectively. The BSG used in this work is the Black Indian Pale Ale (IPA) production line waste, which has many barley malts and hops in the wort cereal mix (see Fig. 1). Since the only components needed for the process are the sugars and starch, each brewing cycle leaves a large quantity of biomass. Beer brewing leaves behind the bio-mass originating from the steeped malt, known as Brewers' Spent Grains. BSG to beer ratio for each brew is 20 kg to 100 L, which, as of 2016, resulted in ca. 39 Mt of BSG as a waste product (26). Although BSG has found its use as a fertiliser and animal feed, it remains underutilised. It is a rich source of fibres, micronutrients and, most importantly, proteins (15).

Fibres are a significant part of BSG, followed by proteins, which account for ca. 65% of malt proteins. These proteins are rich in essential AAs (see Table 2). Of the total protein, 43% is prolamins (hordeins) with high proline and glutamine content; the remaining is glutelin (9). The barley storage proteins, hordeins, can be classified into five categories (A, B, C, D and γ) based on their amino acid composition and electrophoretic mobilities (5). They have a MW range of <15 kDa, 30–50 kDa, 55 - 80 kDa, 85 - 90 kDa and 35- 45 kDa, respectively (4, 7). B-hordeins comprise most of the hordein fraction and are much richer in sulfur than the smaller C-hordeins fraction. D -hordeins have disulfide bonds. The rest of the sub-types only account for a negligible fraction of the total hordein pool (31).

Since many components like lignin and polysaccharides are present in BSG, proteins get trapped in the network of fibres, and the extraction efficiency is generally low. As a result, pre-treatment steps, such as hydrothermal treatment, are required, as suggested by Qin et al. (26).

Table 2. *Essential AA composition of BSG and its comparison to the recommended requirements based on daily protein intake recommendations by WHO (3, 23).*

S.No.	Essential AA	Essential AA requirement		BSG	Covered
Unit		mg/ g protein	g per 100 g protein	g per 100 g	%
1	Histidine (H, His)	15	1.50	2.1	140%
2	Isoleucine (I, Ile)	30	3.00	3.6	120%
3	Leucine (L, Leu)	59	5.90	6.9	117%
4	Lysine (K, Lys)	45	4.50	3.4	76%
5	Methionine (M, Met) + Cysteine (C, Cys)	22	2.20	3.1	141%
	Methionine (M, Met)	16	1.60	1.5	94%
	Cysteine (C, Cys)	6	0.60	1.6	267%
6	Phenylalanine (F, Phe) + Tyrosine (Y, Tyr)	38	3.80	7.3	192%
	Phenylalanine (F, Phe)	-	-	4.4	-
	Tyrosine (Y, Tyr)	-	-	2.9	-
7	Threonine (T, Thr)	23	2.30	3.4	148%
8	Tryptophan (W, Trp)	6	0.60	1.1	183%
9	Valine (V, Val)	39	3.90	4.8	123%
TOTAL		277	27.7	35.7	**129%**



Black IPA (1,3)

Black IPA (21 B)

Type: All Grain
Batch Size: 100,00 L
Boil Size: 114,17 L
Boil Time: 60 min
End of Boil Vol: 104,17 L
Final Bottling Vol: 100,00 L
Fermentation: Ale, Two Stage

Date: 02 Jan 2023
Brewer:
Asst Brewer:
Equipment: easyBrau 100
Efficiency: 77,00 %
Est Mash Efficiency: 77,0 %
Taste Rating: 30,0

Taste Notes:

Prepare for Brewing

[redacted]

Mash or Steep Grains

Mash Ingredients

Amt	Name	Type	#	%/IBU	Volume
[redacted]	Pale Malt (2 Row) Bel (5,9 EBC)	Grain	1	[redacted]	
	CHÂTEAU MUNICH LIGHT® (15,0 EBC)	Grain	2		
	CHÂTEAU CAFÉ (500,4 EBC)	Grain	3		
	CHÂTEAU CRYSTAL® (150,1 EBC)	Grain	4		
	Carafa I (Weyermann) (817,5 EBC)	Grain	5		

Mash Steps

Name	Description	Step Temperature	Step Time
[redacted]			

[redacted]

Boil Ingredients

Amt	Name	Type	#	%/IBU	Volume
[redacted]	Hallertau Magnum [14,00 %] - Boil 60,0 min	Hop	6	[redacted]	-
	Protafloc Tablet (Boil 15,0 mins)	Fining	7		-

Steeped Hops

Amt	Name	Type	#	%/IBU	Volume
[redacted]	Centennial [10,00 %] - Steep/Whirlpool 30,0 min, 90,2 C	Hop	8	[redacted]	-
	Idaho #7 [13,00 %] - Steep/Whirlpool 30,0 min, 90,2 C	Hop	9		-
	Mosaic (HBC 369) [12,25 %] - Steep/Whirlpool 30,0 mi...	Hop	10		-

Fig. 1. *Ingredients added to BSG sourced from Brygghuset Finn. (Image, courtesy of Joacim Larsen from Brygghuset Finn)*

3.2. Protein Extraction

Plant material is a complex matrix of many components, such as water, fibre and lipids, which can trap the target/ molecule(s) of interest while extracting them from the material. As a result, it is essential to plan appropriate 'pre-treatment' step(s), which can help to increase extraction efficiency. Some potential pre-treatment methods can be heat/ freeze drying, shearing, hydrothermal, and enzymatic, but the choice depends on the type of material(s) and their target components (14). The materials used in this work have been convection-dried and ground to provide homogenised samples for processing. While drying prevents enzymatic activity, grinding helps to break the cell structure and any complex matrix like fibre, making proteins more accessible during the extraction phase. BSG, in particular, is rich in lignocellulose and hydrothermal pre-treatment step, which causes swelling and further disruption of the lignocellulosic matrix and aids protein extraction. Various studies have optimised the pre-treatment (s), temperature, feed/solvent ratio, reagent type and concentration requirements for an increased protein extraction efficiency of ca. 60%. According to Qin et al. (26), a temperature of 60°C for 1 h to 24 h is very suitable for hydrothermal pre-treatment, irrespective of the feed/solvent ratio used, and will not result in protein degradation. However, further research is still needed to determine how short the duration can be. Shearing further helps the process as the mechanical stress ruptures the lignocellulosic matrix in BSG, and 11 000 rpm for 60 s can increase the yield by ca. 1.5 times for 'black BSG' and threefold for 'pale BSG'.

The salt and pH-shift methods are two common ways to extract proteins. Salt extraction uses the principle of “increasing the ionic strength to break salt bridges and shielding of the surface charge of the protein”. The shielding effect reduces protein-protein interactions while enhancing protein-media interactions and increasing protein solubility as a result. The extract can be UF and DF, and the concentrated and purified proteins will precipitate once de-ionised water is added to decrease the ionic strength (1).

In the pH-shift method, a potent alkaline agent curbs the positive charge on the protein surface and promotes AAs with either hydrophobic or sulfhydryl groups to interact more with the solvent, destabilising the protein structures and promoting water-protein interactions. A net negative charge promotes electrostatic interactions between the solvent and proteins, increasing solubility. Once solubilised, centrifugation can easily separate the non-protein fraction (husk, fibre, etc.) from the solubilised protein fraction. A suitable acid like citric acid to lower the protein extract pH to the pI value effectively neutralises the net charge on the protein molecules, causing their precipitation, resulting in an isoelectric precipitate fraction and soluble protein fraction (1).

Another final protein recovery approach can be using UF and DF on the alkaline extract to purify and concentrate the proteins and then adjusting pH to a desired value for further processing. Membrane-based processing technologies, such as UF and DF, are industrially favoured for separating, concentrating and purifying liquid products. UF allow molecular size selectivity for separating the feed's desired component(s) and concentrating them. DF uses a continuous supply of fresh solvent to purify the retentate. The added

solvent is simultaneously removed to carry out any undesired components, like salts, with each cycle. UF and DF use the application of positive pressure (force), either using a pump or compressed neutral gas (like nitrogen), allowing particles below the molecular size cut-off of the membrane to pass the membrane while retaining the larger molecules. This gives them an advantage over the isoelectric method since no additives (pH regulators/ modifiers) are needed for the process, and the native environment of the proteins can be maintained, resulting in more purified native protein recovery. While BSG extracts have been subjected to UF and DF for protein recovery, most of the reported work uses novel techniques like ultrasonication for feed (protein extract) preparation (8, 12).

The pH-shift method is well-studied for BSG protein recovery and is very efficient in extracting hordeins and glutelin fractions. In their work on BSG, Connolly et al. (5) discovered that using N-acetyl Cysteine (NAC) as a reducing agent combined with applying heat during extraction can enhance the efficiency of the process. Applying heat with NAC is crucial as only using the reducing agent can reduce the extraction yield of black BSG.

Both salt and pH-shift methods have been previously used for “non-defatted” RPC and “defatted” RPC and are pretty efficient; however, it has been observed that using cold-pressed RPC results in higher protein yield levels (24). Based on the findings of Ahlström et al. (1) and Pillai (25), a modified pH-shift process, with only 1h of incubation time with the alkaline solution, was used. RPC was soaked twice in an alkaline solution, and centrifugation collected the liquid phase from both runs. The extraction pH was also reduced to 9, even though a pH of 12 offered the highest extraction efficiency. These two conditions helped to minimise the problem of protein denaturation as the process created less harsh conditions. A close-to-neutral pH could also be used, but it is not ideal since it results in poor protein recovery.

4. Materials and Methods

4.1. Raw Materials

Cold-pressed Rapeseed Press Cake (RPC), available at the Department of Food Technology, Engineering and Nutrition, and brewers' spent grains from beer production (BSG), was provided by Brygghuset Finn, Landskrona, Sweden, were used as the starting raw materials for all experimental work reported henceforth. RPC was held in cold storage at -20 ℃. Freshly acquired BSG was frozen using a blast freezer (Normann Nortech^{+} Plus 5, La Croce, Italy) till -20 ℃ and placed in -20 ℃ storage.

4.2. Sample Preparation

Both materials were oven-dried in a convection oven (Termaks, Bergen, Norway) (see Section 4.3). They were milled using a hammer-type mill (Laboratory Mill 120, Perten Instruments AB, Stockholm, Sweden) as mechanical pre-treatment to increase the surface area during the protein recovery. The milled materials were stored in -20 ℃ storage before carrying out the experiments.

4.3. Dry Matter Analysis

The dry matter content of raw materials and all final isolate/soluble protein fractions can be calculated with equation 1. The measurements were done in triplicates.

$$Dry\ Matter\ (\%) = \left(1 - \left(\frac{Sample\ before\ drying(g) - Sample\ after\ drying(g)}{(Sample\ before\ drying(g))}\right)\right) \times 100\ \% \quad (1)$$

The analysis was done based on the AOAC standards 945.32C(b) and 935.29(C) (20, 19). 3g of RPC, in triplicates, was allowed to reach room temperature and then placed in the convection oven (Termaks, Bergen, Norway) at 105 ℃ for 6 h. Afterwards, they were allowed to cool down inside a desiccator. The weight of samples was recorded before and after drying to calculate the dry matter content (16).

15g of BSG, in triplicates, was kept at room temperature to de-freeze, then placed in the drying oven at 60 ℃ for ca. 1.5 h. Afterwards, they were further dried at 105 ℃ for 3 h. Samples were allowed to cool down inside a desiccator. The weight of the samples was recorded before and after drying to calculate the dry matter content (18, 21, 22).

All extracts and final isolate/soluble protein fractions were freeze-dried to measure the dry matter content. A 1 cm thick layer of the samples was weighed in duplicates and then blast-freezed in the blast chiller (Normann Nortech^{+} Plus 5, La Croce, Italy) until solid. The frozen samples were placed in the freeze dryer (Labconco Lyph Lock 18, Kansas City, MO, USA) for 4 days. After the cycle, the dried samples were kept in a desiccator for 24 h before analysis.

4.4. Protein Extraction

The pH was controlled manually during the extraction steps for both materials. A benchtop centrifuge (Beckman Coulter Allegra X-15R, California, USA) for sample centrifugation was used. A magnetic stirrer was used during incubation periods to maintain homogeneity. The measurements were taken in duplicates.

4.4.1. Rapeseed Press Cake

Using a modified method based on the work described by Ahlström et al. (1, 2) and Pillai (25) for protein extraction, the milled RPC sample (see section 4.2) was dispersed in 1:10 (w/v) tap water. A solution of 1 M NaOH was used to adjust the pH of the dispersion to 9 and maintained while continuously stirring with a magnetic stirrer for 1 hr. Afterwards, the dispersion was centrifuged to separate the heavier SSF and the lighter protein fraction. The SSF was re-dispersed in the same ratio as initially with the tap water, and the exact steps were followed one more time (see Fig. 2). The lighter fractions from both were combined for further processing and analysis work.

Before NaOH treatment for protein extraction, a pre-treatment step was also tested (see red box in Fig. 2). The milled RPC was dispersed in a 1:9 (w/v) 30% ethanol and incubated for one hour with continuous stirring. Afterwards, the dispersion was centrifuged at 5141 G for 20 min at 4 ℃, and the solid phase was air-dried overnight before conducting the NaOH treatment as described before.

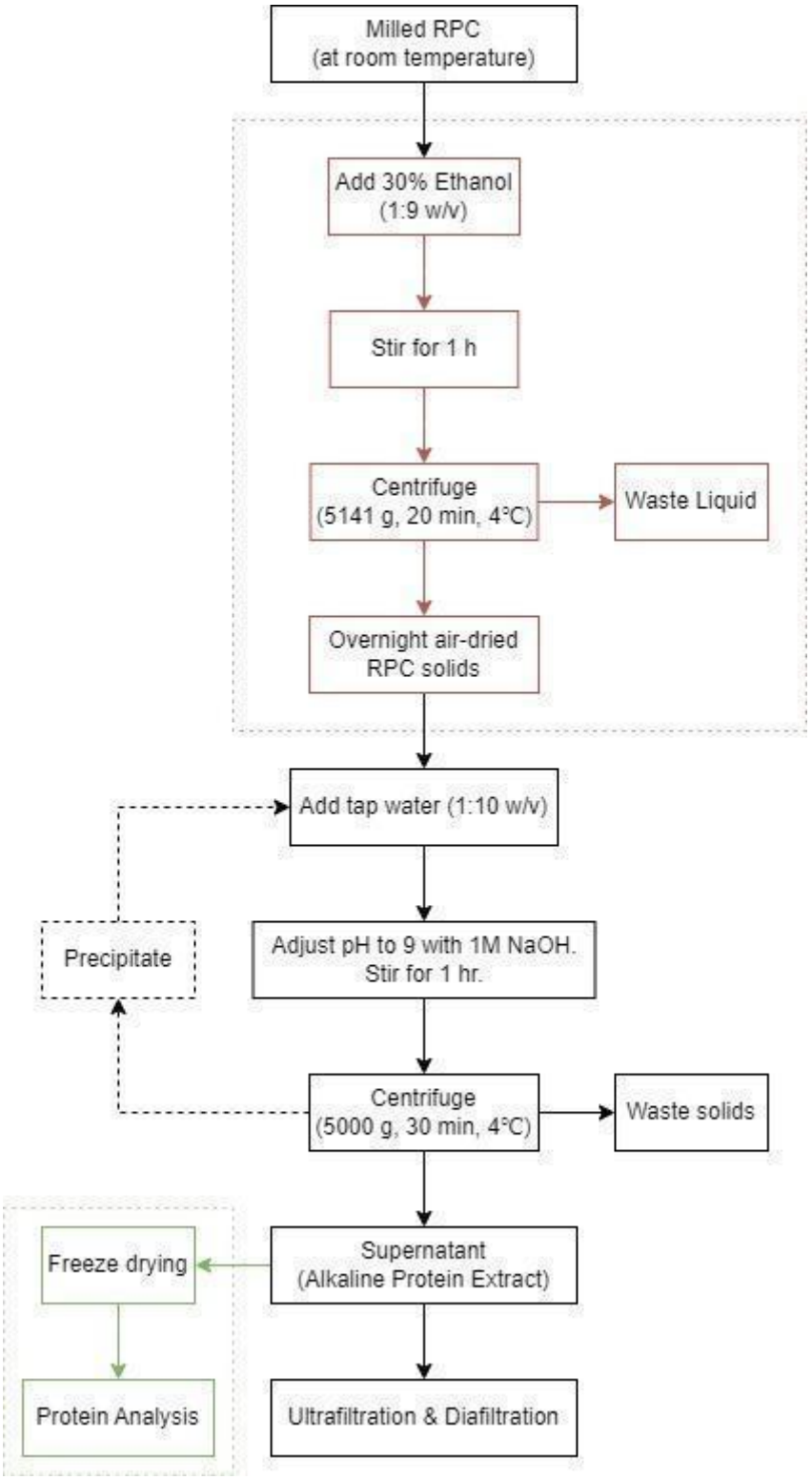


Fig. 2. *A flowchart describing alkaline protein extraction from RPC. The dotted lines show the re-circulation of the solid phase. The red boxes show ethanol pre-treatment steps, while the green boxes indicate the sample taken for protein analysis.*

4.4.2. Brewers' Spent Grains

The protein was extracted using a modified method based on the work done by Connolly et al. (5) and Qin et al. (26). The milled BSG (see section 4.2) was dispersed in 1:20 (w/v) tap water and sheared using a homogeniser (IKA®-Labrotechnik, ULTRA-TURRAX T25). The slurry was incubated in a shaker hot-water bath (LAUDA, A120S) for one hour and then centrifuged to retrieve the solid fraction. 110 mM NaOH in the same ratio as tap water and 5% NAC (w/v) was added to the solid fraction, and the dispersion was incubated in the shaker hot-water bath for 1 h. Afterwards, the dispersion was centrifuged again to separate the heavier SSF and the lighter protein fraction. The SSF was re-dispersed with the reagents in the same ratio, and the exact steps were repeated (see Fig. 3). The lighter fractions from both were combined for further processing and analysis work.

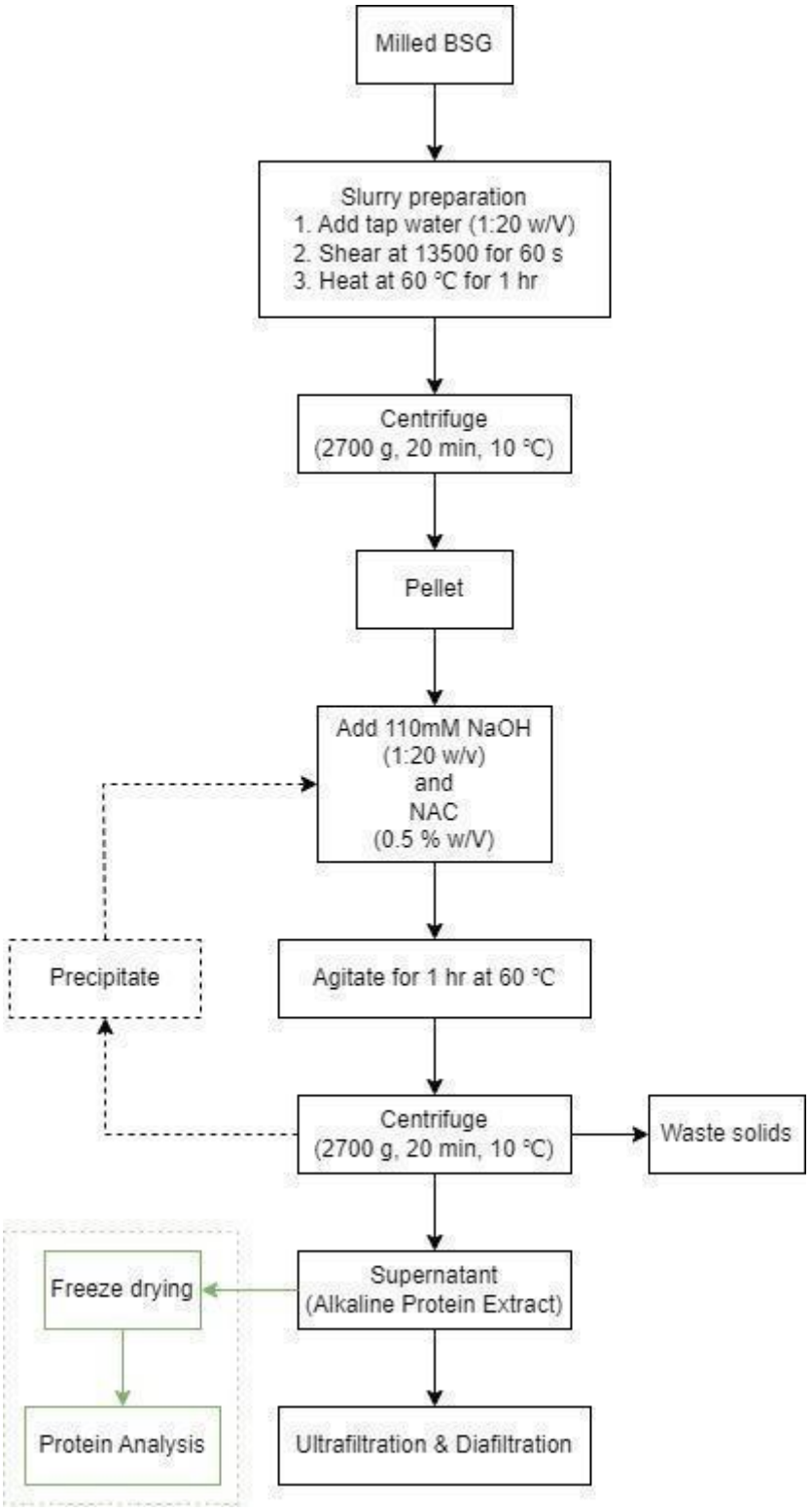


Fig. 3. *A flowchart describing alkaline protein extraction from BSG. The dotted lines show the re-circulation of the solid phase. The green boxes indicate the sample taken for protein analysis.*

4.5. Protein Recovery

The pH was controlled manually during the recovery steps for both materials. A benchtop centrifuge (Beckman Coulter Allegra X-15R, California, USA) for sample centrifugation was used. The measurements were taken in duplicates.

4.5.1. Isoelectric Precipitation

4.5.1.1. Rapeseed Press Cake

The pH of the alkaline extract was measured. The pH was adjusted to 3.5 with citric acid, the isoelectric point of the native proteins in RPC. The extract was centrifuged to separate the precipitated proteins (IPI) from the soluble proteins. Both protein fractions were freeze-dried (see section. 4.3) and stored before protein analysis (see section. 4.6). Due to its high protein concentration (data not showed), the soluble protein fraction was further subjected to UF and DF for concentration and purification, followed by protein analysis. Fig. 4 summarises the steps involved.

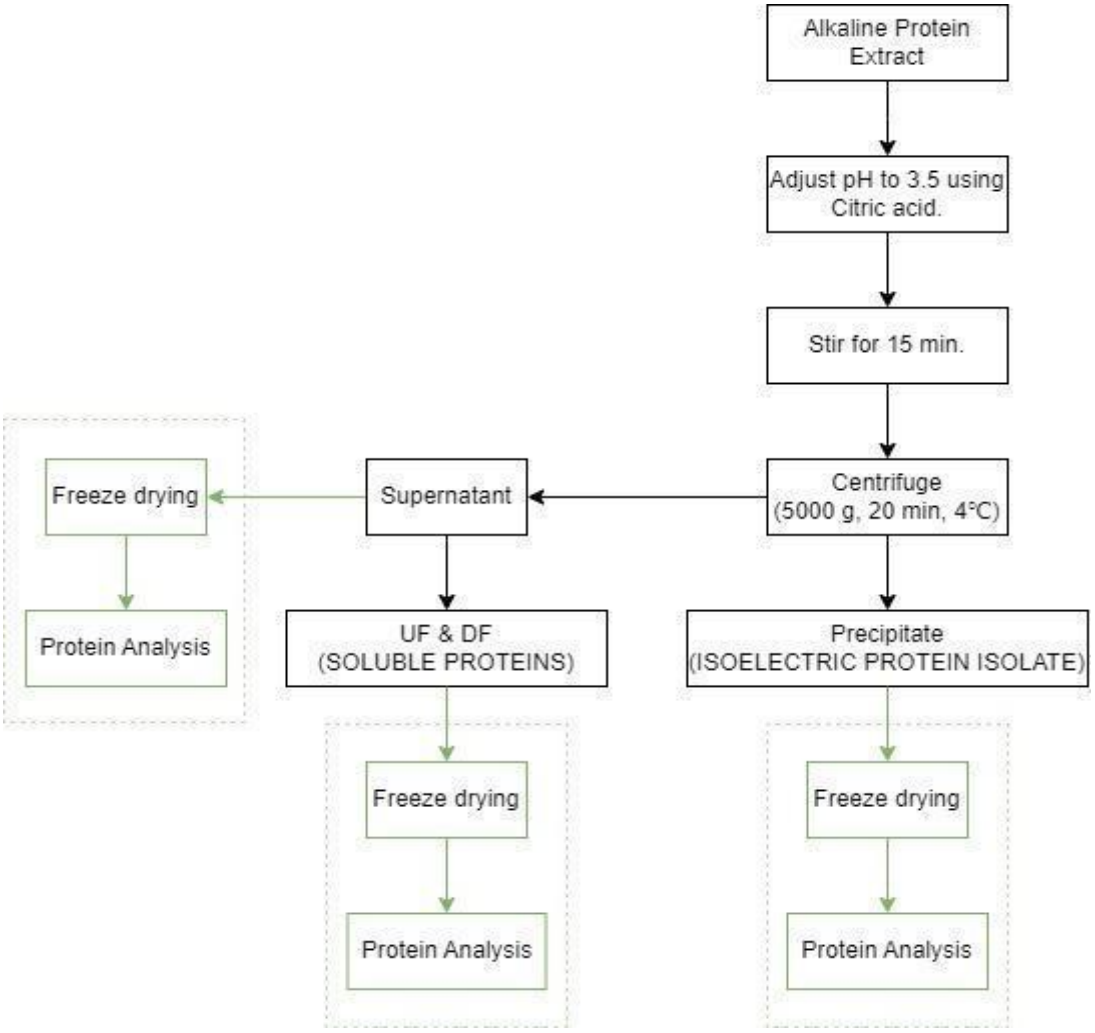


Fig. 4. *A flowchart describing the Isoelectric precipitation of proteins from the alkaline method-based protein extract of RPC. The green boxes indicate the sample(s) taken for protein analysis.*

4.5.1.2. Brewers' Spent Grains

The pH of the alkaline extract was measured and then adjusted to 3.8 with citric acid, the isoelectric point of the native proteins in BSG. The liquid was centrifuged to separate the precipitated proteins (IPI). The precipitated protein fraction and the liquid fraction (supernatant) were freeze-dried (see section. 4.3) and stored before protein analysis (see section. 4.6). Fig. 5 summarises the steps involved.

After protein analysis, it was observed that the liquid fraction (supernatant) had low protein content (data not shown). The fraction was not subjected to UF and DF. The reason for this decision was to maintain consistency in the experimental design across this work for comparison purposes. The CF value required for UF would have to be higher than that used on other samples (see section 4.5.2).

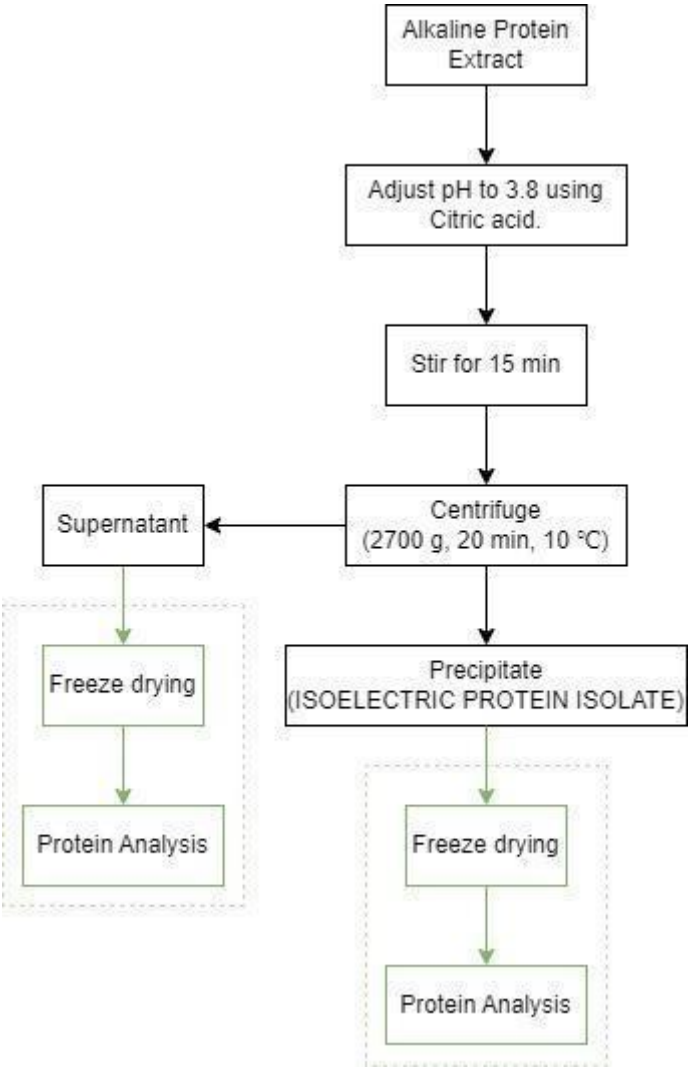


Fig. 5. *A flowchart describing the Isoelectric precipitation of proteins from the alkaline method-based protein extract of BSG. The green boxes indicate the sample(s) taken for protein analysis.*

4.5.2. Ultrafiltration and Diafiltration

The extracts (respectively from RPC and BSG) can contain some residual impurities (plant material), which must be filtered out separately before being used as a feed for UF. Pre-sieving through polyester sieves is a suitable method. Sequentially, each extract was passed through a 100 µm, 60 µm, and 10 µm sieve to remove any solid impurities.

The Albi module is a dead-end type which can do for both UF and DF. It has a stirrer that reduces concentration on top of the membrane and is pressurised with N_2 to provide the feed pressure. The Alfa Laval-GR90PP (Alfa Laval, Nakskov, Denmark) ultrafiltration membrane of polyethersulfone has a molecular cut-off of 5 kDa, which is ideal for retaining a majority of smaller proteins in the extract during the process.

The membrane is cut out to fit the Albi module and conditioned using 1% (v/v) Ultrasil 110 (Ecolab, Sweden) and de-ionised water for 1 hour. After washing with de-ionised water, the unit used a pressure of 5 bar during the UF process. The unit ran till a concentration factor (CF) of 3 was reached. The retentate from the UF step was the feed volume for DF.

De-ionised water with a similar pH to the retentate was used as the diavolume (DV) for the DF process at a value of 3. DF was done as a batch process. The final retentate was freeze-dried (see section. 4.3) and stored before protein analysis (see section. 4.6). Fig. 6 shows the process flow.

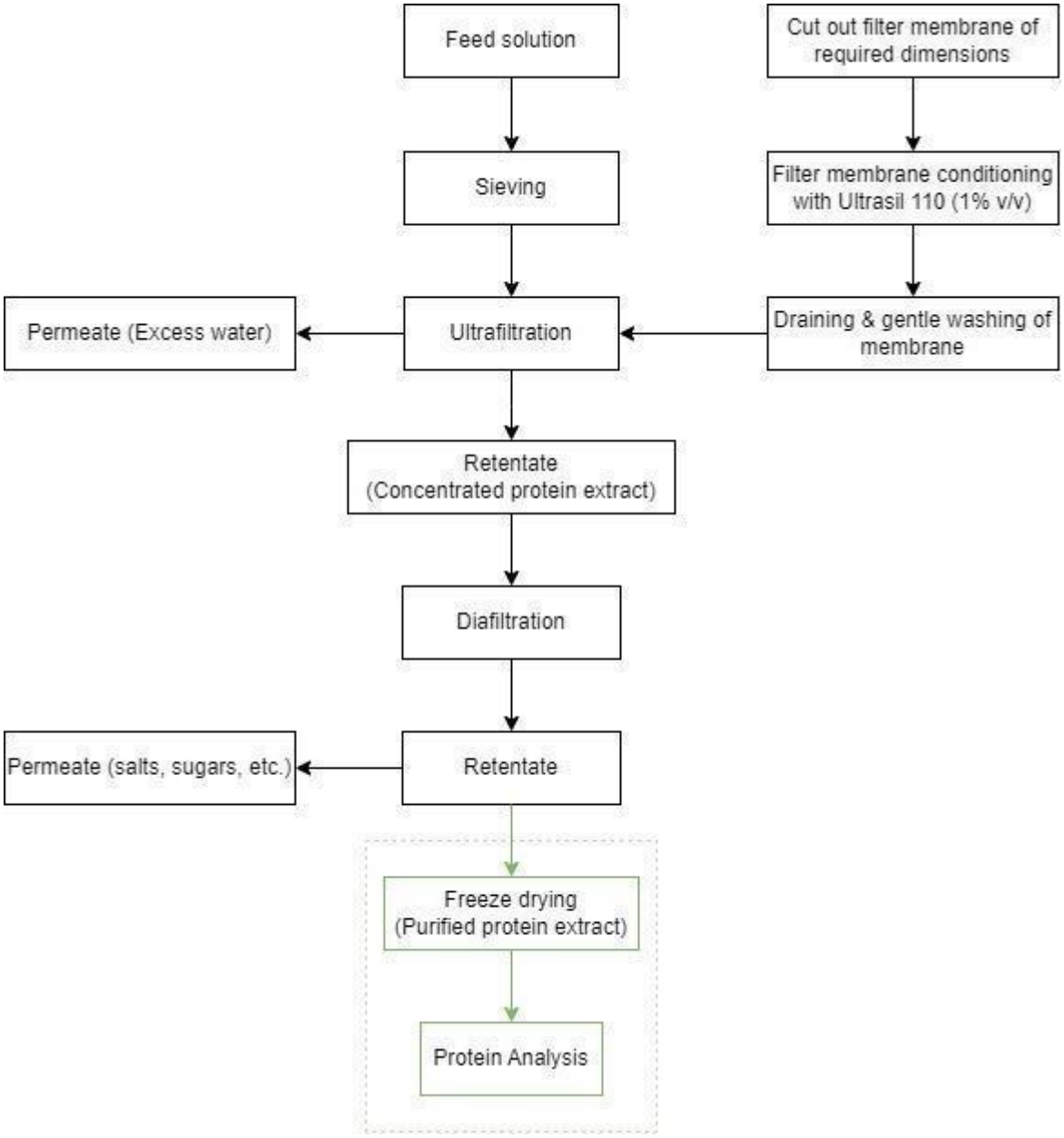


Fig. 6. *A flowchart describing the overall steps involved in the UF and DF process. Feed solution refers to the protein extracts from RPC and BSG (see section 4.4. Protein Extraction). The green boxes indicate the sample taken for protein analysis.*

4.6. Protein Quantification

The protein content (N*6.25) (% dw) was measured using a N/Protein Analyzer (Thermo Fisher Scientific, FlashEA 1112 series, Waltham, MA, USA), which combusts the sample in a pure O_2 environment. The software detects nitrous products from the reduction reaction of the fumes, which calculates the amount of nitrogen in the sample. The nitrogen content, when multiplied by Jones' factor of 6.25, provides an estimate for the protein content of the sample. The sample size used was ca. 25 mg. Aspartic acid was the standard for calibration, and air was a blank. The analysis was performed in duplicates.

Based on the amount of raw material used, the amount of protein present before extraction is quantified by Equation 2.

$$Ingoing\ protein\ content\ = \frac{Amount\ of\ raw\ material\ \ used \times dry\ matter\ content\ of\ raw\ material\ \times protein\ content\ of\ raw\ material}{100} \quad (2)$$

The amount of protein recovered/extracted from each step is calculated using Equation 3.

$$Protein\ recovered/extracted\ = \frac{Dry\ Solids\ of\ sample \times weight\ of\ original\ sample \times sample\ protein\ content}{100} \quad (3)$$

The protein extractability can be estimated using Equations 2 and 3 (Equation 4).

$$Protein\ extractability(\%)\ = \frac{Protein\ extracted}{Ingoing\ protein\ content} \times 100 \quad (4)$$

The final yield after protein recovery can be estimated using Equation 2 and 3 (Equation 5).

$$Protein\ yield(\%)\ = \frac{Protein\ recovered}{Ingoing\ protein\ content} \times 100 \quad (5)$$

4.7. Statistical Analysis

A Google Sheets extension, XLMiner Analysis ToolPak (Frontline Systems Inc., Incline Village, USA), was used to analyse the data using a T-test and ANOVA based on the data group size. Both tests were run with a 5% significance level ($p < 0.05$). The null hypothesis (H0) was: "There is no significant difference in the means of the groups".

5. Results and Discussion

5.1. Sample composition

Table 3 reports the dry matter content (see equation 1in section 4.3) and protein quantification values of unprocessed rapeseed press cake and brewers' spent grains used in this work. The protein content (N*6.25) (% dw) was obtained from the N/Protein analyser to estimate the 'ingoing protein content' (g dw), based on equation 2 (see section 4.6), for the extraction phase.

Table 3. *Dry matter and protein content of the raw materials.*

Sample	**Dry Matter Content**	**Protein Content (N*6.25)**
Unit	g dry matter/ 100 g sample	% dw
Raw RPC	90.0 ± 0.0*	30.5 ± 0.1
Raw BSG	26.0 ± 0.0*	18.5 ± 0.1

* - The reported value is a Standard error based on n=3.

5.2. Protein Extraction

Extraction was the initial step in isolating protein from plant materials. All cells are susceptible to environmental changes, such as pH level, and their plasma membrane usually breaks open when the change is too drastic. This work used a combination of mechanical stress, heat and pH change to extract the proteins from the complex plant matrix.

Table 4 reports the dry matter content (see equation 1 in section 4.3) and protein quantification values of the respective alkaline protein extract made from the raw materials. The protein content (N*6.25) (% dw) of the freeze-dried extracts was obtained from the N/Protein analyser to estimate the 'extracted protein content' (g dw), based on equation 3 (see section 4.6), after the extraction phase.

The protein extractability (see equation 4 in section 4.6) shows the efficiency of the extraction process based on the amount of protein extracted and the ingoing protein content of the starting material.

5.2.1. Rapeseed Press Cake

Ethanol is an excellent solvent for water-insoluble compounds such as lipids, carbohydrates, certain amino acids and minerals like zinc. These could have been eliminated during the ethanol treatment, resulting in a relatively lower dry matter content of ethanol-soaked RPC (ERPC) than the extracts from untreated RPC (URPC) samples.

While the difference between the protein content (N*6.25) (% dw) values for both cases was statistically insignificant ($P > 0.05$), the extraction yield (%) had a significant difference ($P < 0.05$).

Although the protein extraction yield for URPC was in line with the findings of Pillai (25), the ERPC's extraction yield, compared to the same ethanol concentration results, was lower. However, a lack of information about their experimental design makes it difficult to conclude the reasons for this observation. It was observed that protein extractability decreased by ca 40. % when ethanol pre-treatment was done. Although Pillai (25) stated that the extraction yield of proteins goes down due to the effect of ethanol pre-treatment, the scale was not quantified. Kalaydzhiev et al. (13) researched the effects of ethanol on protein extractability from RPC and found similar results where the overall value decreased. The primary cause is the disulfide bridge formations and ethanol-induced conformational changes. The resulting aggregates can be a reason behind poor extractability, as it reduces the solubility of proteins.

5.2.2. Brewers' Spent Grains

Due to an error in the experimental setup where proteins were lost due to solubilisation during the hydrothermal pre-treatment, compared to the work done by Connolly et al. (5), the protein content and the extractability were much lower. However, based on the work done by Qin et al. (26), the high solid-to-liquid ratio (5% in this work) and short hydrothermal treatment time seem to have decreased the protein loss to some extent; although further study is needed to obtain concrete results.

As a result of the experimental error, the data reported for the subsequent protein recovery steps of the BSG extract sample have a certain level of uncertainty. They cannot be taken at face value. Despite the known error, the data from the experiment offers insights into the potential protein composition and abundance under the given conditions.

Care needs to be considered for the hydrothermal treatment step for future work. One way is to combine the separated liquid fraction with the lighter protein fraction obtained from alkaline treatment steps. Another possible approach can be to combine hydrothermal treatment with an alkaline extraction step and prepare the slurry accordingly. However, selecting the better approach needs further research and analysis. Also, decreasing the solid-to-liquid ratio to 4% during alkaline treatment is suggested (26) to increase the extracted protein content.

Table 4. *Dry matter and protein quantification results from alkaline protein extraction from the raw materials.*

Sample		Dry Matter Content	Protein Content (N*6.25) (of the extract)	Protein Extractability
Unit		g dry matter/ 100 g sample	% dw	% dw
RPC	**Ethanol Pre-treated**	0.61 ± 0.0*	[a]35.1 ± 0.5** %	33.1 ± 0.0** %
	Ethanol Un-treated	1.4 ± 0.0*	[a]34.8 ± 0.0 %	77.6 ± 0.0 %
BSG		1.9 ± 0.0*	31.3 ± 0.0 %	40.3 ± 0.0 %

a - no statistical difference was observed. The P-value was >0.05 according to T-Test.

* - The reported value is a Standard error based on n=3.

** - The reported value is a Standard error based on n=4.

5.3. Protein Recovery

Once the proteins have been extracted successfully, they must be recovered and purified from the extraction mixture, which can contain carbohydrates, lipids, and minerals, to name a few. This work used two distinct methods. The isoelectric precipitation method (see Figures 4 and 5 in section 4.5) recovered the proteins as an isolate and a soluble fraction. In contrast, UF combined with DF concentrated and purified the alkaline extract without separating the proteins (See Figure 6 in section 4.5).

Table 5 reports the dry matter content (see equation 1 in section 4.3) and protein quantification values of the respective recovered protein fractions made from the extracts. The protein content (N*6.25) (% dw) of the freeze-dried recovered samples was obtained from the N/Protein analyser to estimate the recovered protein content (g dw) (see equation 3 in section 4.6) for the respective recovered protein fractions. The protein yield (see equation 4 in section 4.6) shows the efficiency of the recovery process based on the amount of protein recovered and the ingoing protein content of the starting material (calculated according to the results shown in section 5.1).

5.3.1. Isoelectric Precipitation

5.3.1.1. Rapeseed Press Cake

There was a statistically significant difference ($p < 0.05$) in the protein content (N*6.25) (% dw) and protein yield (% dw) of all the sample types for URPC and ERPC.

The yield of URPC was in the range with the findings of Pillai (25). However, the yield from ERPC was higher than expected. A lack of information about their experimental design makes it difficult to conclude the reasons for this observation. However, one speculation could be due to the SSF re-circulation done in this work. Further detailed analysis is needed to understand this difference resulting from ethanol treatment.

Another interesting observation from the results was the higher protein content (g dw) value in URPC compared to ERPC. In contrast, the results for the yield were opposite in comparison. Since protein extraction and recovery are related, as only the solubilised proteins during extraction can be recovered, this was expected. However, higher yield for ERPC suggests that ethanol positively affected the recovery process. Ethanol results in protein aggregate formation (13), which can promote a precipitation effect during the recovery phase. This reasoning is further backed by the low recovered protein content values of the soluble protein fractions obtained from ERPC rather than URPC.

5.3.1.2. Brewers' Spent Grains

Table 5 reports the dry matter content (see equation 1 in section 4.3) and protein quantification values of the protein isolate made from the alkaline BSG extract. The leftover solvent was also analysed for the soluble protein content (N*6.25) but had a minor quantity present and was not analysed further.

5.3.2. Ultrafiltration and Diafiltration

5.3.2.1. Rapeseed Press Cake

The results for both ERPC and URPC show an overall increase in all protein quantification results compared to their respective extracts. Only a slight increase in the ERPC's recovered protein content indicates that some protein might have aggregated and lost as fouling during the process. Another reason could be that the quantified protein content of the ERPC alkaline extract had a low quantity of protein. The non-protein species were probably lost as permeate due to their MW being lower than the molecular cut-off of the filter membrane (5 kDa).

The CF value for UF can be increased to concentrate the proteins in the recovered liquid further and polish the protein quality. Also, since the pH during extraction was 9 during this work, based on the findings of Zhang et al. (32), the low extraction pH could have negatively affected the yield. However, increasing the

extraction pH too much is not recommended as it could lead to unwanted changes in protein conformations due to the dissociation of peptide bonds.

5.3.2.2. Brewers' Spent Grains

Table 5 reports the dry matter content (see equation 1in section 4.3) and protein quantification values of the recovered protein liquid (retentate from UF and DF) made from the alkaline BSG extract. Due to the previously mentioned error in the experimental setup for protein extraction (see section 5.2.2), the results can not be discussed. However, they still provide insights into the recovery process's possible outcome. It appears that the CF value can be increased to concentrate the proteins more.

Table 5. *Dry matter and protein quantification results from protein recovery from the extracts.*

Sample			**Dry Matter Content**	**Protein Content (N*6.25) (of the recovered sample)**	**Protein Yield**
Unit			g dry matter/ 100 g sample	% dw	% dw
RPC	**Ethanol Pre-treated**	Isoelectric Protein Precipitate	25.6 ± 0.0*	44.8 ± 0.8 %	52.3 ± 0.0 %
		Soluble Proteins	1.5 ± 0.0*	41.9 ± 0.7 %	13.4 ± 0.0 %
		UF & DF	0.68 ± 0.0*	41.2 ± 0.5** %	8.0 ± 0.0** %
	Ethanol Un-treated	Isoelectric Protein Precipitate	25.2 ± 0.0*	52.7 ± 0.3 %	40.0 ± 0.1 %
		Soluble Proteins	5.8 ± 0.0*	36.3 ± 0.0 %	73.0 ± 0.0 %
		UF & DF	1.7 ± 0.0*	47.4 ± 0.3 %	19.6 ± 0.0 %
BSG		Isoelectric Protein Precipitate	10.0 ± 0.0*	55.7 ± 0.6 %	29.0 ± 0.1 %
		UF & DF	2.1 ± 0.0*	41.4 ± 0.0 %	32.0 ± 0.0 %

* - The reported value is a Standard error based on n=3.

** - The reported value is a Standard error based on n=4.

6. Conclusion

This work used protein extraction under alkaline conditions optimal from rapeseed cake and brewers' spent grains, respectively, and investigated the difference between the protein quantity and quality recovered using the isoelectric precipitation method and ultrafiltration combined with diafiltration.

The choice of protein recovery method depends on the starting material used during protein extraction. When the isoelectric precipitation method is considered, the protein content is more favourable for the protein isolate formed using untreated rapeseed press cake. Still, the yield is lower as most of the proteins remain soluble. A comprehensive study is required to determine how to increase the protein content since ethanol treatment increases protein yield. However, when ultrafiltration and diafiltration are used for protein recovery, ethanol treatment negatively impacts the protein content and the yield.

Although the quantification results for BSG are unreliable due to an experimental error, the qualitative insight shows that isoelectric precipitation can provide better results. However, a study with the correct methodology is needed to have conclusive results.

Additional factors such as the intended use, further downstream processing requirements, and costs must also be considered when deciding the type of recovery process.

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