Optimization of Protein Recovery Process from Rapeseed Cake

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6th June, 2020

LUND
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Abstract

The need for sustainable sources of food is brought about by the never-ending exploitation of natural resource that our planet has provided. The heavy dependence on animal-based foods for protein has led to major depletion of the earth's resources. The search for a sustainable protein source has led to the increase in utilization of the rapeseed cake which is a byproduct of rapeseed oil production. What would be used as animal feed is now considered as a sustainable product for the future due to its high protein and fiber content. Rapeseed cake is rich in cysteine and methionine which are essential amino acids usually lacking in most plant foods. The proteins in the cake however are not utilized to their full potential due to the presence of antinutrients in the form of phytic acid, phenolics etc. The main goal of this project therefore was to reduce the level of phenolics and attempt to reduce the total time for the process by testing different alkaline leaching times. After all, a protein concentrate for the customers must be rich in the essential amino acids and well balanced nutritionally, has a light color and does not have a bitter taste.

Previous work done on this field, shows potential in obtaining high yields of protein via alkaline assisted leaching followed by isoelectric precipitation to obtain a protein concentrate. By building on this platform, the protein leaching process which originally was set to be as long as 4hours, was cut down to one hour, as the extraction yield stayed constant at around 61-65%, and the protein yield in the final concentrate at around 41-43%.

A washing step was introduced where the rapeseed cake was allowed to soak in ethanol for one hour in order to leach out some of the phenolic. The results showed that soaking in ethanol in concentrations anywhere between 30% and 60% resulted in sufficient extraction of phenolics without too much of a drop in the protein yield. Additionally, the color of the protein concentrates was also of concern, and it showed that at washing at 30% resulted in the least loss in protein precipitate yield and extract as well as a comparably high loss of phenolic compounds.

Due to the success of extracting phenols using ethanol, a combination of a lower pH and ethanol was attempted, but there was no improvement in the yield at a lower pH, although it did show a higher purity of around 80-82% than the purity at pH10.5 which was around 60-70%. The color of the protein concentrates also were lighter in color at a lower pH because it is likely that the phenolic oxidation occurs more at more alkaline pH.

The use of rapeseed cake as an alternative to animal-based products for the requirement of protein seems like it still has a long way to go, but the following results show that there is a lot of potential in the quality and yield of protein obtained.

Contents

1. Introduction

Sustainability is the key to successive generations enjoying the same resources that was once enjoyed by the current generations. Proteins are the building blocks of cells in our body are responsible for the growth and maintenance of body tissue, indispensable in the modern world. Considering that the global food demand for meat and dairy will increase tremendously by 2050 according to the Food and Agricultural Organization (Campbell et al.,2016) there is a need for sustainable sources of protein. Soybean is one of the most widely used non-animal-based protein sources especially since the 20th century onwards (Thrane et al., 2017). But considering that soy is heavily responsible for deforestation in some parts of South America (Reuters, 2018), and the added cost of transporting soybean all the way to Sweden, the need for an alternative source is deemed necessary.

Rapeseed is one of the major oilseeds of the world, and its meal, generated after the oil extraction, is renowned for its high protein (40%) and fiber content (Mansour et al 1992). It is said to have a potential of 1.12 million tons of crude protein extracted from rapeseed press cake (RSPC) per year (Hald. C et al., 2019). Its high quality of protein, rich in limiting amino acids like cysteine and methionine, makes it the ideal choice for a sustainable protein source for the future generations (Rutkowski, 1971). The main obstacle however from creating products for the masses is the problem with antinutrients, which consist of phenolics, glucosinolates and phytic acids, which collectively reduce the nutritive value of the rapeseed-based foods and result in poor organoleptic properties. This proves to be unfavorable for the consumer and therefore there is a need to improve protein purity and reduce antinutrients to create an product with an enhanced product quality.

The research focus in the past has been for improving oil quality and yield improvement but the goal of this project is to optimize the yield and the purity of the concentrate.

1.1 Rapeseed

Rapeseed (*Brassica napus*), is one of the top oil crops in the world, with annual production levels of around 71.3 million metric tons worldwide. It remains to be one of the few oilseed crops that can grow in Scandinavia, predominantly in Denmark and southern parts of Sweden and Finland despite its harsh weather conditions (Daun et al, 2011). Out of all the major oil crops in the world, rapeseed is renowned for its high oil quality considering its high content in mono and poly unsaturated fatty acids and tocopherols (Laguna et al., 2018). The composition of RSPC is mentioned in Table 1. There are three oil extraction methods from rapeseed, two mechanical processes which are cold pressed extraction and warm pressed extraction, and solvent extraction. Solvent extraction is a method used to extract oil from seeds, using non polar solvents like n-hexane, due to its low latent heat of vaporization and high selectivity. (Kumar et al., 2017). Kumar et al, also state that the use of green solvents is highly recommended as a future solution to combat the problems that arise with the use of organic solvents such as pollution and toxicity. The byproduct, as a result of the oil extraction, is RSPC, and it yields an annual produce of 39.8 million metric tons in the world (Fetzer et al., 2019). Currently, the RPSC is being used as animal feed due to its high protein and calorific value. It is however high in indigestible fiber which affects non ruminants like

chickens. The antinutritional content like glucosinolates also has an effect on the growth of animals as it affects their protein intake (Rutkowski et al., 1971). Furthermore, RSPC is also currently being used as an organic fertilizer, providing nutrients to the soil and thereby improving crop production (Park.W. et al., 2017). One advantage rapeseed will have over other oilseeds, is the ability to be cultivated as an alternating crop due to its low cost (Kozlowska et al., 1990).

According to the Protein Digestibility Corrected Amino Acid Score which is the benchmark for measuring protein quality, RSPC is one of the highest non animal based sources of protein with soy and peanut the only non-animal-based protein sources above it (FAO, 2017). Besides this, RSPC is rich in fibers, minerals and vitamins like tocopherols, vitamin B and choline (Vuorela.S, 2005).

The downside however for Brassica seeds, the family of the seed in consideration, is that they contain the highest amount of phenolic compounds of all the oilseeds with 0.6-2.0% by weight of the rapeseed meal (Vuorela.S, 2005). Phenolic compounds are those compounds that lead to astringent flavor and an undesirable color, more of which will be mentioned in section 1.1.2.

Current processes for obtaining the protein from RSPC in the form of Rapeseed Protein Concentrate (RPI) or Rapeseed Protein Extract (RPE) are using the relationship between protein solubility, pH and ionic strength (Yonsel et al., 2018).

Table 1: Composition of cold pressed rapeseed cake from Gunnarshögs Jordbruk AB (Dahlberg, 2018)

Component	g/100g press cake
Water	10.3±10%
Ash	6.04±10%
Protein	27.3±10%
Fat	15.5±10%
Carbohydrates	10.8
Fibers	30.1±15%

1.1.1 Proteins

The protein content in rapeseed oilseeds ranges from 37-41% in the Canadian variety (Wanasundara et al., 2016) composed mainly of two components, namely, the cruciferin (12S) and napin (2S) proteins, which are the storage proteins. Cruciferin is the more dominant protein, with 60% of the total protein while napin stands for only 25%. The rest of the protein present includes, oleosins and caleosins which are also called oil body (OB) proteins which assists in preventing the coalescence of oil droplets. (Dahlberg, 2018). Majority of the protein is secreted in the cotyledon section of the rapeseed of around 25% (Yonsel et al.,2018). Cruciferin has a molecular weight of 300-370kDa and an isoelectric point of pH7.25 while napin has a molecular weight of 8-15kDa and an isoelectric point higher than pH10 (van der Haar et al., 2014)

The warm pressing process occurs at 80-105degC, and the denaturation temperature of these proteins are at around 75-86degC, so for this reason, cold pressed rapeseed cake is preferred over warm-pressed (Östbring et al., 2019) as a raw material for protein isolation. Moreover, cold pressed rapeseed cake has a more delicate impact on the environment in terms of energy, lesser manpower, no organic harmful solvents as compared to solvent extraction, as well as low overall cost (Çakaloğlu et al., 2018). For feed purposes however, the denaturation of the proteins is not a concern, so any form of RSPC can be used. On the other hand, for the product development purposes and for improved protein solubility, native proteins are preferred.

Rapeseed protein is known to have a more multifaceted protein composition as compared to its other oilseed counterparts, with different isoelectric points and molecular weights (Diosady et al., 1989). Additionally, rapeseed protein is rich in most essential amino acids (except tryptophan), like methionine and cysteine for example, which is lacking in other plant-based products such as pulses and cereals, and even providing competition to animal-based protein sources like meat and eggs in this regard. (Wanasundra et al., 2016).

1.1.2 Antinutrients

Antinutrients are those compounds present in foods that come in the way of absorption of nutrients by the body. In this case, oilseeds, like legumes, are home to a wide variety of antinutrients. Rapeseed is known to contain antinutrients such as phenolic acid esters and glucosinolates (Fenwick et al., 1986). Phenolics are secondary metabolites produced by plants in response to harsh treatment, whether it be pH, temperature or UV radiation, and they include tannins, lignins and phenols and phenolic acid (Naczk et al., 2004). Currently, glucosinolates levels are being continuously reduced by plant breeding techniques and have been reduced tenfold since the 1970s (Östbring et al., 2019).

Studies have shown that rapeseed is known to have the highest amount of phenolics of all oilseeds, around 30 times the amount found in soybean flour as shown in Table 2 (Kozlowska et al., 1990) The dark color and bitter flavor that arises in rapeseed meal, arises due to the presence of phenolics. Besides being considered unattractive and undesirable, phenolics are known to have positive effects as well, such as their antioxidant capacity (Li et al., 2016).

Table 2. Total content of Phenolic Acids in Oilseed Meals (Adapted from Canola and Rapeseed, Chapter 11, Fereidoon Shahidi)

Meal	Total Phenolics (mg/100g meal, on dry basis)
Soybean	23.4
Cotton	56.7
Peanut	63.6
Rapeseed	639.9

The phenolic composition of RSPC is around 0.6-2% dry matter (Vuorela.S, 2005). The phenolics are composed of a variety of compounds, with Sinapine, being the dominant one,

with 80% of the dry matter of the phenolic compounds. Besides, sinapine, the rest of the phenolics are esterified sinapic acid with sugar and kaempferol (Laguna et al., 2018). Sinapine is one of the main phenolic compounds that leads to the bitter taste and astringency, affecting palatability of the protein (Niu. Y et al., 2015). Kaempferol 3-O-(2‴-O-sinapoyl-β-sophoroside) has been identified as one of the key compounds that contributes the bitter flavor. (Hald. C et al., 2019)

1.2 Background of the Process

The core process for most of the experiments conducted in this thesis is based on the work of previous studies carried out on RSPC in the past involving extraction and isolation of rapeseed protein (Zhang. Z et al., 2020).

Previous studies dictate the importance of the use of cold pressed rapeseed cake over warm pressed rapeseed cake due to the impact that heat has on the protein quality and yield (Östbring et al., 2019). This is due to the partial denaturation of protein in the warm pressed cake.

The RSPC is soaked in an alkaline solution, and centrifuged. The process relies on solubilizing the proteins in solution, so that insoluble non-protein material can be separated by centrifugation. What happens in this process is that the sulfhydryl and hydrophobic groups are exposed and the amino acids start to associate with water, which results in them dissolving in solution, while the high pH increases the water solubility of the proteins, giving the protein surface a net negative charge. Centrifugation, as a result separates the heavy phase consisting of husk, intact cells and uncharged protein, and the light phase which predominantly consists of solubilized protein and some fiber.

Despite the highest yield coming at pH 12 (Ahlstrom, 2019), previous work conducted had taken 4 hours at an initial adjustment to a lower pH of 10.5 so that the protein is not as affected by the high pH. The isoelectric precipitation step was done at a pH of 3.5, and this is dependent on the type of side chains present in the protein. Proteins tend to precipitate at the isoelectric point as the net charge on the protein is neutral.

Industrially, the use of ethanol-water extraction consumes a lot of organic solvent and alternatives in the form of Reverse Micelle treatment or a combination of membrane separation and alkaline extraction are expensive and complex. Therefore, the traditional method of extraction using alkali before precipitating with acid has been preferred. (Zhang. Z et al., 2020).

This process was considered sub-optimal, and there seemed to be room for improvement. The time taken for the leaching process was 4 hours coupled with around 1-2 hours for the rest of the process, accounting for the time taken for the milling, separation and precipitation. Moreover, during the leaching step, the high pH might also have an impact on the yield obtained at the end of the leaching process. Research indicates that the poor yield is due to protein denaturing as well as lysine alanine formation which is toxic and leads to poor nutritional value of the protein. Studies conducted (Wanasundara et al., 2011) show

that there is promise in getting an improved nitrogen solubility and thereby a greater yield at pH7, if the ionic strength is increased using a salt like sodium chloride.

Another observation in the rapeseed studies is the presence of antinutrients in the form of phenols. The phenols can be oxidized in the leaching process, and can then react with the protein, which leads to undesirable off colors and flavors. An observation made by Cilliers et al, 1989, was that the oxidation of phenolic acids is increasing with increasing pH. During leaching, due to enzymatic and non-enzymatic oxidation, the phenols form quinones, which, when interacting with proteins lead to the formation of dark green or brown undesirable colors. (Xu et al., 2000). Polar solvents such as ethanol in aqueous media have a tendency to extract polyphenols out of solution. Ethanol is also safe for human consumption and would not be a problem when formulating the end-product (Diem Do et al., 2014)

1.3 Aim

This project will be conducted in 3 major steps, which are,

1. Cutting down the time taken for the total process by reducing the 4-hour duration of the leaching step.
2. Reducing the amount of antinutrients, in this case, phenols, by adding a pre-treatment soaking step with ethanol, by trying different concentrations of ethanol.
3. Attempting to lower the pH during the protein leaching step, in order to reduce the impact of high pH on the protein as well as the economic impact by using less NaOH.

1.4 Hypothesis

The hypothesis is that the polar solvent in the form of ethanol will have an effect on the phenolic concentration in the concentrate and as a result influence the color of the same. Additionally, lower pH would result in less damage to the protein and hopefully greater yield.

2. Materials and Methods

2.1 Chemicals and Materials used

Cold pressed RSPC, a gift from Gunnarshögs Jordbruk AB was used as the starting material for all the aforementioned experiments. For the general leaching process, the cake was milled using a knife mill (Retsch GRINDOMIX GM200), for size reduction and the chemicals used were 2M Sodium Hydroxide for alkaline leaching and Citric acid (Merck, Darmstadt, Germany) for acid precipitation of the protein. A bioreactor control (SARA small bioreactor control series 2000 (Belach Bioteknik, Sweden)) was used to regulate the pH during the leaching step. The samples are centrifuged using a Beckman Coulter Avanti 3-15R Bench top centrifuge. The protein concentration in each of the samples were identified using a protein analyzer (Thermo Electron Corp., Flash EA, 1112 series), while aspartic acid (Thermo Electron, Milan, Italy) was used as a standard. 95% Ethanol (Solveco, Sweden) was used to make the different concentrations of ethanol. Folin's reagent (Sigma, St.Louis, USA), diluted 1:9 with water, and anhydrous sodium carbonate (VWR, Sweden).

2.2 Protein Leaching from Rapeseed Cake

For the first part of the project, the RSPC was first collected after being stored at -18°C. The RSPC was finely ground using a knife mill, for 5 pulses of 4 second intervals at a speed of 2500 rpm, in 50g batches. Then, the powdered cake was dissolved in tap water, in a ratio of 1:10(w/w), while the pH was adjusted to 10.5. The pH is kept constant using a small bioreactor control with 2M NaOH used as the alkali for pH maintenance. The solution was kept constantly stirred using an IKA Microstar 7.5 control stirrer. The leaching step was allowed to continue for 5 hours, using a big batch of 300g RSPC in a 1:9 ratio, with water, while samples were taken after each hour. Each sample was separated to test the difference in protein yield at different times. The light phase is then precipitated with a low pH, followed by another centrifugation step in order to separate the precipitated proteins. (Tzeng et al., 2006). The experiments were done in triplicates.

The optimal time was then utilized for the following experiments.

2.3 Protein Isolation from Rapeseed Cake

Protein isolation is achieved by taking the supernatant from the leaching step, and adding citric acid, until the pH is adjusted to 3.5. The precipitated proteins are then separated by centrifugation and obtained as a protein concentrate. Figure 1 shows the process in the form of a flow chart.

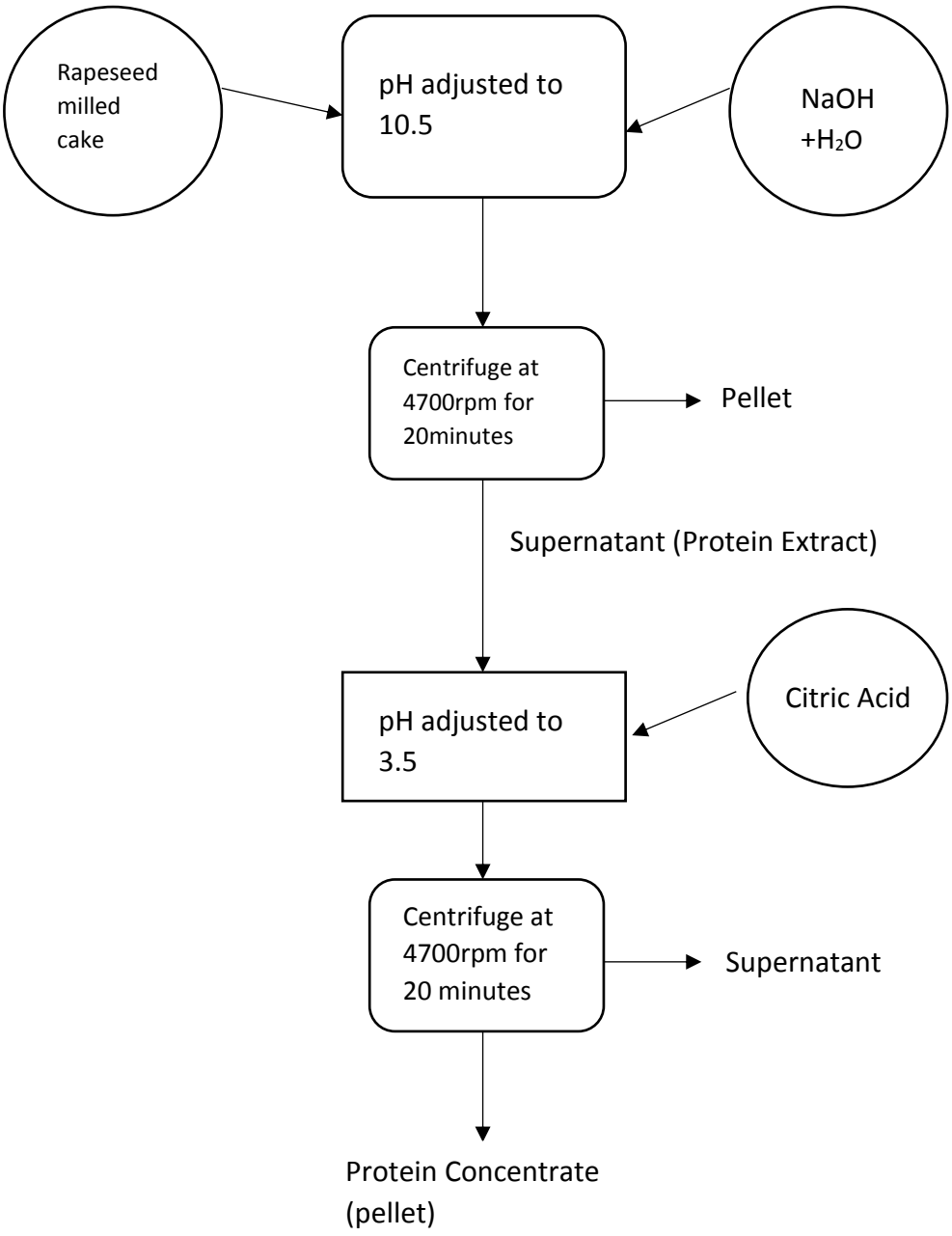

Figure 1: Flow Chart of Rapeseed Protein Recovery Process

2.4 Ethanol Extraction of Phenols

For improved color and flavor of the rapeseed protein concentrate, the RSPC is first soaked in different concentrations of ethanol to find out which concentration extracts the highest polyphenols. The RSPC to water ratio was set to 1:9.

The RSPC is soaked in 0%, 30%, 60% and 90% ethanol (v/v). The rest of the process is completed in its entirety, to obtain the protein concentrate.

The experiment also involved testing out different combinations of solutions at different pH and ethanol concentration to see which arrangement has the best output yield and purity.

3. Analysis

3.1 Dry Matter Content

The dry matter content of the RSPC, protein extract, concentrate as well as the pellet from the leaching and the supernatant from the isolation step, was calculated in order to estimate the total yield. The drying was performed by weighing 3-4g of solid aliquots and 5-6g of liquid aliquots in metal cups, and stored in an oven at 103°C overnight. The experiment was performed in triplicates.

The dry matter content is calculated by using the formula in equation 1.

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

3.2 Protein Estimation

The protein content is measured by using a protein analyzer which combusts the sample and calculates the amount of nitrogen which can be multiplied by a protein factor which in this case is 6.25. Aspartic acid is the standard used and the blank sample is air. 25-30mg of sample is used in 33mm tin discs for the analysis. The analyses were done in triplicates.

The amount of protein in each sample is quantified by first identifying how much protein is present in the RSPC used (Equation 2).

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

The amount of protein solubilized in the leaching step or concentrated in the precipitation step with acid(ppt) can be calculated by using the following equation (Equation 3).

$$Protein\ extracted/concentrated=\frac{Dry\ Solids\ of\ sample\times weight\ of\ original\ extract/ppt\times sample\ protein\ concentration}{100} \quad (3)$$

After calculating the protein extracted or concentrated, the yield can be estimated (Equation 4).

$$Protein\ yield(\%) = \frac{Protein\ Concentrate/Extracted}{Ingoing\ Protein} \times 100 \quad (4)$$

3.3 Colorimetric Analysis

The color of the protein extract and concentrate is very important for customer appeal, and hence, the color of the samples is tested using a color measuring instrument (Konica Minolta SpectroPhotometer CM700D) for every sample and to test if the ethanol washing shows any improvement on the basis of the color. Before measurement, the camera was calibrated using ambient light and white measurements. The results are declared in L*(brightness), a*(green to red), and b*(blue to yellow) units. After measuring the color values, using the spectrophotometer camera, the values were obtained in L*, a*, b*. The values were converted in terms of the ΔE values calculated by,

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2}$$

Fig2. CIE76 formula for calculating the color difference between 2 coordinates (Sharma.G, 2003)

Using this formula, the distance from the white point (100,0,0) was calculated, and the further the distance, the darker or more unpleasant the sample was.

3.4 Phenolic Estimation

The polyphenolic content is then analyzed using a UV/Vis Spectrophotometer (U-1500, Hitachi, Japan) at 765nm. The standards used for the process are gallic acid (Sigma, St. Louis, USA) dissolved in methanol. After measuring, a standard curve as shown in Figure 3. The method (Folin–Ciocalteu (F–C) assay) is a colorimetric method based on oxidation of the phenolic compounds by Folins' reagent (Rangel et al., 2013). This in turn gives rise to a color for which the absorbance is checked. In order to create the absorbance curve, the standards were created as different concentrations of gallic acid- methanol solutions.

First, 1.4ml of water was added to 0.6ml of standard to make the volume up to 2.0ml. In case of the sample, the same steps are followed except, the amount of water added to make it to 2.0ml must be adjusted accordingly. To that, 2ml of Folin's reagent (prediluted 1:9 with water) was added. After vortexing and an incubation period of 5 minutes, 2ml of 7% sodium carbonate and 0.8ml of water was added. The sample is then vortexed and incubated for 30minutes. Finally, the samples/standards are measured at 765nm.

The concentrations and the absorbance values for the standards are found in the Appendix A.1.

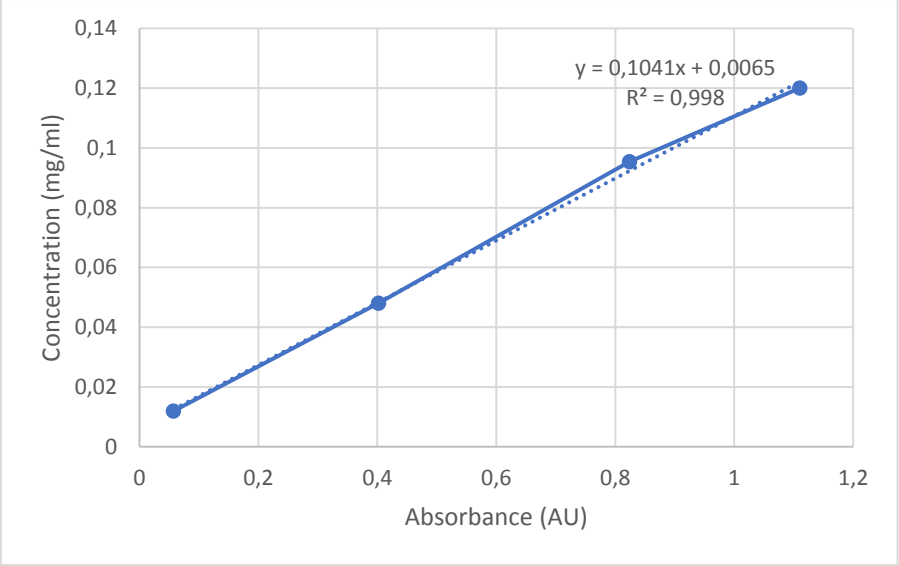

Figure 3: Standard Curve of Gallic Acid dissolved in Methanol at 765nm using Folin's assay

4. Results and Discussion

4.1 Raw Material Composition

The RSPC after being milled was dried in the oven, before being added to the protein analyzer so as to estimate the ingoing protein during the leaching step. The dry solids and the protein content of the cold pressed RSPC used are 0.916±0.002 g dry matter/g RSPC and 33.06±0.27% respectively.

4.2 Leaching Time

Originally, the time used for leaching and isolation of protein would be around 4 hours. By conducting experiments, where the protein yield at different times could be determined, the optimal time for leaching was obtained.

The leaching step was considered key to reducing the total process time, as it alone took 4 hours to complete. Figure 4 show the results plotted in a graphical format, showing the minor difference in yield as compared to the different leaching times. The extraction yield is constant between 61-65%, while the concentrate yield is constant at 40-43%, at all times.

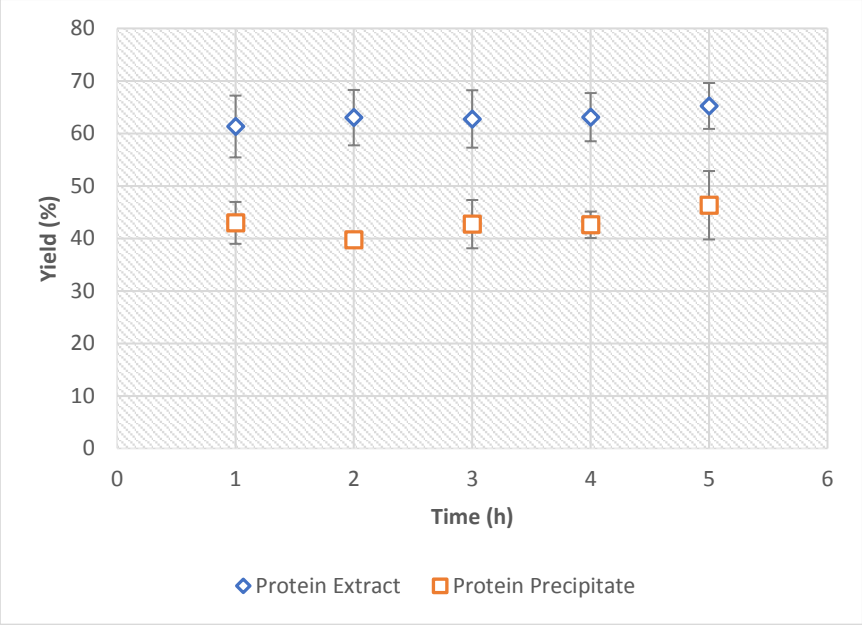

Figure 4: Protein Extract and Concentrate Yield as a function of Time

The purity of each of the concentrates precipitated from each experiment were analyzed as well, using the Protein Analyzer. The following results were obtained, since the experiments were done in triplicates,

Table 3: Purity of Concentrate produced after different times of leaching

Time(h)	Protein Purity (%)
1	61.5 ± 3.6
2	60.3 ± 0.9
3	59.6 ± 0.9
4	56.7 ± 2.9
5	56.4 ± 1.4

The purity at all 5 time points were not very different from each other, except for a slight reduction of around 5% units between 1 hour and 5 hours. This provides further proof that the time is not of prime importance and can be shortened to 1 hour.

The color of the concentrates at each hour were tested using the spectrophotometer, and the following results were obtained.

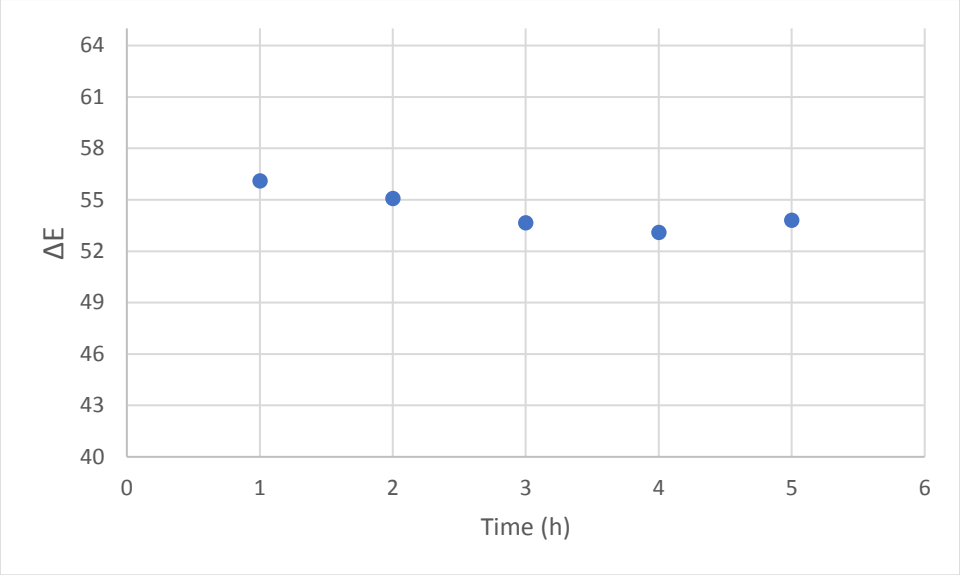

Figure 5: Color of concentrates at different times during leaching

The color of the concentrates was not noticeably different at different times. Although there does seem to be some evidence showing a brighter color at 3 and 4 hours as compared to the samples at 1 and 2 hours. There wasn't enough improvement in color at 3 and 4 hours, as compared to the obvious benefits of shortening the time.

4.3 Ethanolic Extraction of Phenolics

For the successful extraction of phenolics, ethanol at different concentrations were chosen as the solvent for extraction. The rapeseed cake was soaked at 0%, 30%, 60% and 90% ethanol for one hour before being centrifuged to separate the ethanol that potentially would be carrying the phenolics that have been extracted. From then on, the leaching process is carried forward.

The concentrations between 0 and 60% show a high concentration of phenolics as compared to 90%, while 90% shows low concentrations of phenolics.

The yield of protein was another characteristic that was looked into, and to see how much of an impact the ethanol washing step had on it. The samples were again run through the protein analyzer, to find out the concentration of protein and therefore the yield.

The extraction yield as well as the concentrate yield were affected by the increase in concentration of ethanol, as shown in figure 6. The extraction and isolation yield are highest at 30%, with 51% extraction yield, and 41% isolation yield. At 60%, despite having a higher concentration of phenolics in the washing supernatant, the loss in extract yield is considerable and would not be ideal.

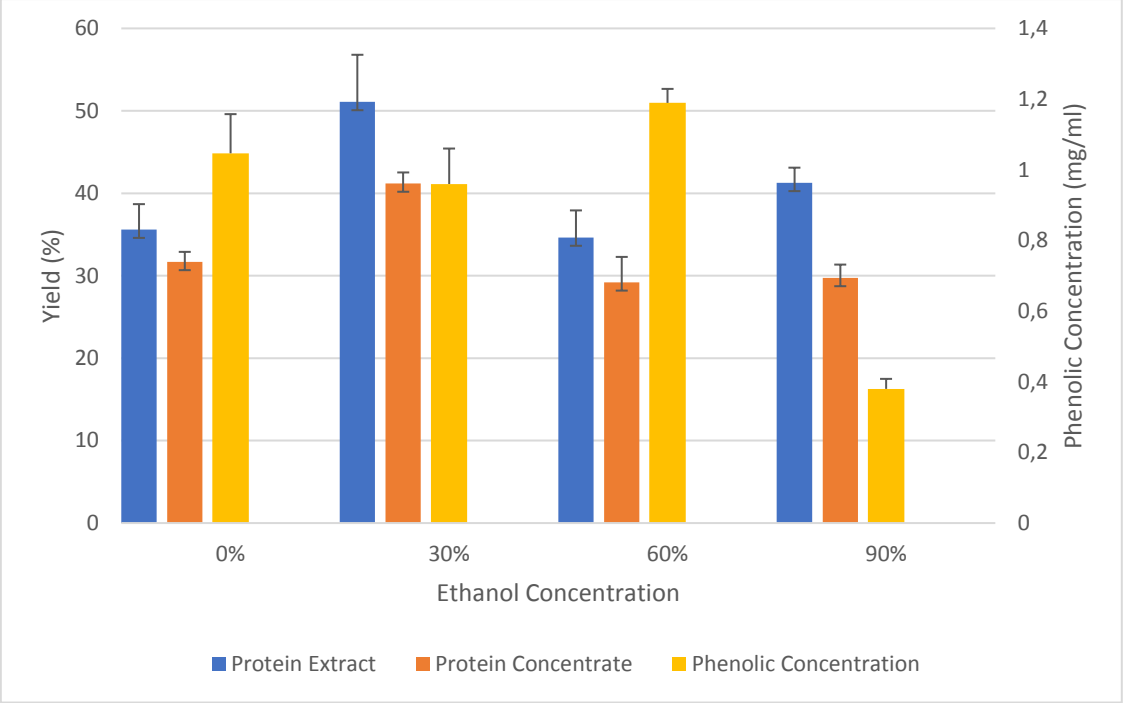

Figure 6: Comparison between the phenolic concentration and the protein yields

For the ethanolic extraction, the protein purity was also measured to find out whether the ethanol had an effect on the protein quality. Table 4 shows the results from the measurements.

Table 4: Purity at different Concentrations of Ethanol

Concentration	Protein Purity (%)
0%	52.5 ± 3.4
30%	60.2 ± 0.8
60%	69.0 ± 2.6
90%	72.4 ± 2.1

There is a linear increase in the purity of the protein concentrate obtained, despite having a higher phenolic removal at lower concentrations. This is something that needs to be further investigated, but one of the reasons could be that ethanol improved the solubility of the protein as well, and contributed to a higher protein content of 72%.

Finally, the color of each of the concentrates at each concentration was measured and the following results were obtained.

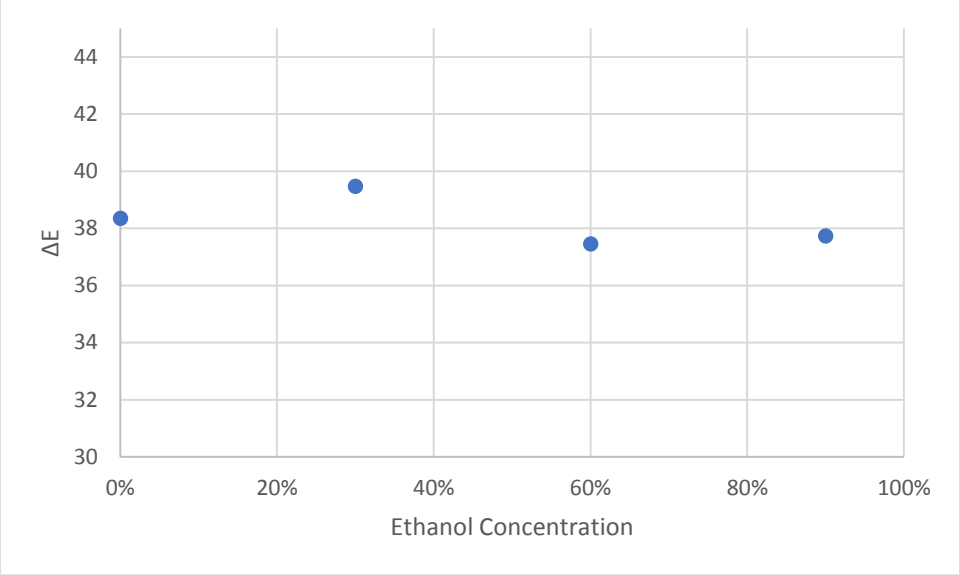

Figure 7: Color at different concentration of Ethanol

The color of the samples didn't vary too much at each of the different concentrations. But the color at 60% is closer to white, and the overall color in comparison to the unwashed step is closer to white. The unwashed samples had colors ranging from ΔE values of 53-56 to values of 37-40 for the washed samples. But one observation was that even washing with water (0% ethanol) had an impact on the color as compared to the unwashed samples, due to the water soluble phenols getting extracted. It also remains to be seen whether a major contributor to the color is just the polyphenols, or also the alkaline conditions that cause the phenols to be oxidized resulting in the off-color.

4.4 Combination of lower pH and washing with Ethanol

Experiments were run with a lower pH at pH7, so as to avoid exposing the protein to harsh conditions which could potentially denature the protein, as well as for economic reasons, to lower the amount of NaOH used to bring it to the higher pH 10.5.

From studies conducted (Wanasundara et al., 2011), we can clearly see an improved nitrogen solubility and thereby a greater yield at pH7 at certain ionic strengths.

Therefore, in order to test the following assumptions, the following experiments were conducted at pH7 with 60% and 30% concentration of ethanol and compared with the results at pH10.5.

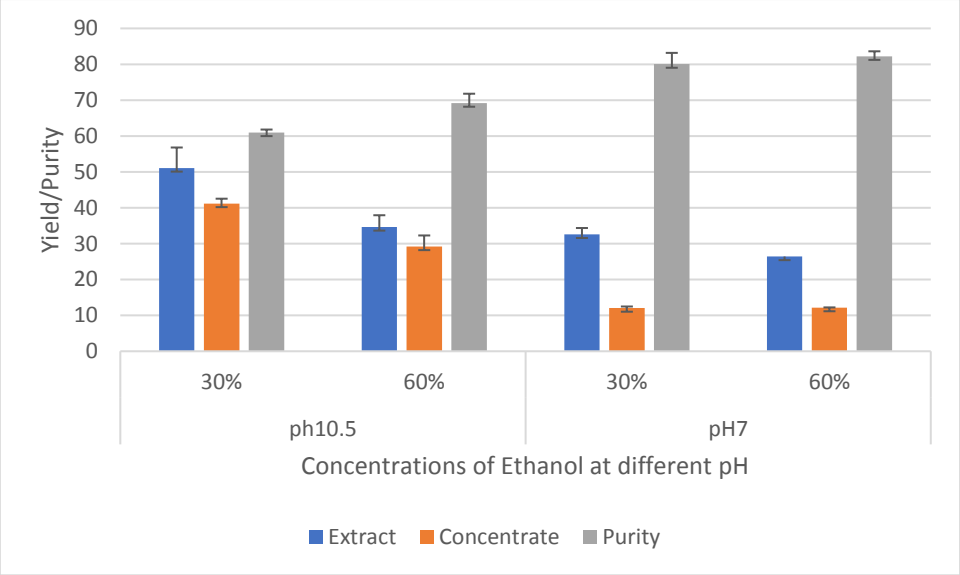

Figure 8: Comparison between Protein Yield and Phenolic Concentration at a combination of pH and Ethanol

The results obtained showed an obvious improvement in purity of the sample at pH7, but the yield of the extract decreased from around 34% at pH10.5 with 60% ethanol concentration to around 26% at pH7 with the same ethanol concentration. At 30% ethanol, likewise, the yield of the protein extract decreased from 50% at pH10.5 to 31% at pH7. Similarly, the yield of the concentrate reduces from 30-40% at pH10.5 to around 12% at pH7. This indicates the need for a higher pH maybe at 9 or altering the ionic strength of the solution to enhance the leaching process. Additionally, the yield is consistently higher at 30% so more effort can be made in the future to work with concentrations closer to 30%.

The colors of the samples were measured, and the following results were obtained.

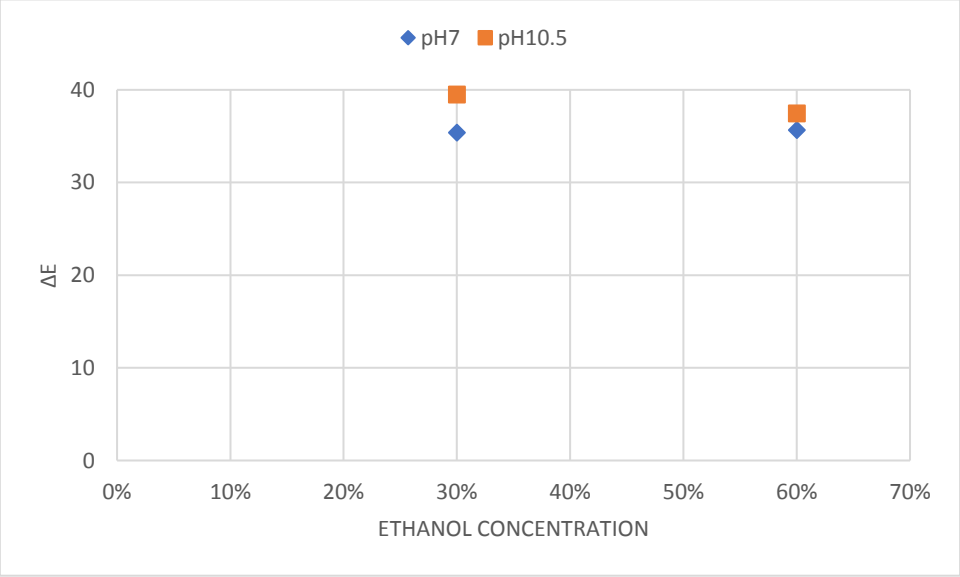

Figure 9: Comparison of color at combination of different pH and ethanolic concentration

The color of the sample at pH7 was brighter since there wasn't as much phenolic oxidation that occurs at a high pH. Since at pH7, we obtain high purity, as well as an improved color, further work can be done where the leaching process is done at pH7 at different ionic strengths.

5. Conclusion

Rapeseed continues to grow as a promising crop in terms of its use for oil as well as for its high protein value. Due to its high phenolic content however, its use as a protein source has been hindered lately. This study has shown that using rapeseed as a viable protein source has potential, but still has a long way to go in terms of being an actual product.

The leaching process initially, had taken 4 hours to complete. Now after conducting experiments, the leaching process has been shortened down to 1 hour, since the time didn't influence the yield as much.

Due to a high phenolic content, the products generated from rapeseed protein wouldn't have been appealing to the public. An ethanol extraction step proved to remove some amount of phenolics from the extract and concentrate and as a result, convert it to a more organoleptic product with a better color profile. The ethanol concentration of 30% (v/v) has shown the most promising results with a high reduction of phenolics, with not much of a compromise on the yield from the leaching or precipitation process. The major contributor in terms of color, is oxidation of phenols which still occurs under alkaline conditions during leaching.

Lastly, lowering the pH has previously shown to have some promising effects on the improvement of yield (Wanasundara et al., 2011). When attempted, the lower pH did show promising improvements on the purity of the concentrate, but the yield was a lot lower at pH7. This was because the protein was not solubilized in water as well as it was at alkaline conditions. Modulating the ionic strength however could be seen as a method to improve the solubility while keeping the pH low.

5.1 Future Development

The washing step did as much as extract phenols from the rapeseed cake, but to exactly know what was extracted into the ethanol solution, an HPLC process would be required. HPLC would separate the compounds based on differences in polarity. Further down the line, this process would assist in selectively extracting certain phenolics, especially those that cause the bitter flavor.

After conducting different extraction possibilities at different concentrations at ethanol, specifically, at 0%,30%, 60 % and 90%, it would be interesting now to test the extraction efficiency at concentrations between 30% and 60% to get the best possible outcome in terms of protein yield as well as phenolic extraction.

Studies have shown the potential of increased nitrogen solubility at certain ionic strengths between pH7 and pH10. The use of a salt to reach those ionic strengths, would result in a higher yield of protein as a result of increased solubility in solution.

Another interesting process that was not looked into was the ultrasonic assisted method of leaching of protein, and it's potential to give a greater yield as well as a more efficient process.

Moreover, enzyme extraction method would be something to look into as well, due to its high efficiency and the fact that it is a sustainable resource, the end result would greatly lower operation costs and make it a cheaper and more environmentally friendly product all in all.

Freeze drying the protein concentrate samples would be another idea, where the protein would not be as affected as compared to conventional drying techniques. But due to the problems that arise with freeze drying in terms of the complicated process and the cost, it wouldn't be ideal. The commercial product, however, would need some sort of alternative to oven drying, and spray drying or extrusion could also prove to be useful.

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Appendix

A1. Folin's Assay Standard

Concentration (mg/ml)	Absorbance (AU)
0.011898	0.057
0.048	0.402
0.095376	0.824
0.12	1.11

A2. Phenolic concentrations from Folin's Assay

Conc of ethanol.	Triplicate #1	Triplicate #2	Triplicate #3
0%	0.633	0.852	0.713
30%	0.722	0.562	0.747
60%	0.791	0.813	0.868
90%	0.282	0.298	0.337

A3. Spectrophotometer Camera Values

Table: Color of the ethanol supernatants from the washing step

Ethanol Concentration	L*	a*	b*
0%	66.89	-0.27	19.35
30%	65.33	0.26	18.86
60%	69.56	0.42	21.81
90%	73.42	-0.42	26.78

Table: Color of the samples taken at each hour from the leaching step

Time	L*	a*	b*
1h	60.02	2.26	27.25
2h	56.28	1.5	23.89
3h	57.86	2.01	20.89
4h	60.97	2.46	20.83
5h	56	3.09	20.55

Table: Samples at different concentrations of ethanol at a lower pH

Sample	L*	a*	b*
30%@pH7	71.013333	0.39	20.28
60%@pH7	73.983333	1.3466667	24.313333

A5. Protein Yield at different times

Table: Extract Yield and Concentrate Yield at different times

Time	Protein Extract Yield (%)	Protein Concentrate Yield (%)
1h	61.3 ± 5.8	42.9 ± 3.9
2h	63.0 ± 5.2	39.7 ± 1.1
3h	62.7 ± 5.4	42.7 ± 4.5
4h	63.1 ± 4.5	42.5 ± 2.5
5h	65.2 ± 4.3	46.3 ± 6.5

A6. Concentration of Phenolics at different concentrations of Ethanol

Table: Concentration of phenolics at different concentrations of ethanol

Ethanol Concentration	Concentration of Phenolics(mg/ml of sample)
0%	1.04 ± 0.11
30%	0.95 ± 0.10
60%	1.18 ± 0.03
90%	0.37 ± 0.02