Fractionation of Wheat Bran for Utilisation in Biorefinery

by

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Abstract

Wheat bran is a by-product from the production of wheat flour. Nowadays wheat bran is mostly used as feed for animals, in the food and baking industry or it is simply burned in open air. However, wheat bran could be used in biorefineries for production of value-added products such as bioplastics, biofuels and chemicals as a substitute for fossil feedstocks as petroleum. Since wheat bran is an agricultural by-product it will not compete for field area with other crops. Furthermore, using biomass for the production of energy will not result in an increase of greenhouse gases since the carbon dioxide released in the production of fuels is re-used by the new crops in their photosynthesis.

The composition of wheat bran can vary but it consists among other things of starch, protein, lipids, cellulose, hemicellulose and lignin. To enable valorization of wheat bran it needs to be separated into its different components. The components can then be used for different purposes. Starch and cellulose can for example be used in the production of biodegradable packaging materials or for bioethanol production.

The aim of this thesis is to develop and compare different methods for extraction and separation of starch, protein, lipid and lignin with the goal that the different components end up in separate fractions with low amounts of impurities.

From this thesis work it can be concluded that starch, lipids, protein and lignin can be extracted from wheat bran. During this thesis, starch was extracted through leaching with warm water during different incubation times. The longer incubation time used, the more starch could be extracted. Different solvents, ethanol and hexane, were used in Soxhlet extraction of lipids. It was discovered that starch might be co-extracted with the lipids when using ethanol. This made hexane more suitable to use. Protein was extracted with NaOH and de-ionized water during a three step procedure, where most of the protein was extracted during the first step. Overall this method resulted in a 95 % protein yield. A reduced factorial design with different combinations of temperature, residence time, alkali concentration and solids loading was used for lignin extraction with microwave treatment. It was possible to extract lignin with this microwave treatment; however, there were major difficulties with the separation of the lignin from the rest of the slurry. Several separation techniques were tested to solve this issue. It was discovered that samples treated at 170 °C gave the highest yields. These samples were also the easiest to filter. Sugar analysis on the filtrate showed a very low presence of soluble sugars, which indicates a good separation between lignin and cellulose plus hemicellulose.

Sammanfattning

Vetekli är en biprodukt från produktionen av vetemjöl. Nuförtiden används vetekli mestadels som djurfoder, inom livsmedelsindustrin eller så eldas den helt enkelt upp. Dock skulle vetekli kunna användas i bioraffinaderier för produktion av värdefulla produkter så som bioplast, biobränsle och kemikalier som ett substitut till fossila råvaror som petroleum. Eftersom vetekli är en jordbruksrest behövs ingen ny åkerareal användas för att producera det. Dessutom innebär användandet av biomassa för energiproduktion inte en ökning av växthusgaser eftersom den mängd koldioxid som släpps ut från produktion av bränsle tas upp av nya växter genom fotosyntes.

Sammansättningen av vetekli kan variera men det består bland annat av stärkelse, protein, lipider, cellulosa, hemicellulosa och lignin. För att möjliggöra förädling av vetekli måste det separeras i sina olika beståndsdelar. Dessa beståndsdelar kan sedan användas för olika ändamål. Stärkelse och cellulosa kan till exempel användas i produktion av nedbrytbara förpackningsmaterial eller i produktion av bioetanol.

Syftet med detta examensarbete är att utveckla och jämföra olika metoder för extraktion och separation av stärkelse, lipider, protein och lignin med målet att få alla beståndsdelar i separata fraktioner med så lite orenheter som möjligt.

En slutsats från det här examensarbetet är att stärkelse, protein, lipider och lignin kan extraheras från vetekli. Under examensarbetet extraherades stärkelse genom lakning med varmt vatten under olika inkubationstider. Ju längre inkubationstid, desto mer stärkelse kunde extraheras. Olika lösningsmedel, etanol och hexan, användes i Soxhlet-extraktion av lipider. Det visade sig att stärkelse eventuellt extraheras tillsammans med lipiderna när etanol används. Detta gjorde att hexan ansågs mer lämplig att använda. Protein extraherades med NaOH och avjoniserat vatten i en tre stegs-process, där majoriteten av proteinet extraherades i första steget. Denna metod gav ett utbyte på 95 %. Ett reducerat faktorförsök med olika kombinationer av temperatur, uppehållstid, alkalikoncentration och förhållande mellan fast material och vätska användes för ligninextraktion med mikrovågsbehandling. Det var möjligt att extrahera lignin med den här metoden. Dock innebar det även stora svårigheter med separationen av lignin från resten av materialet. Flera olika separationstekniker testades för att lösa problemet. Det visade sig att prover som behandlats vid 170 °C gav de största utbytena. Dessa prover var även de lättaste att filtrera. Sockeranalys av filtratet visade att det knappt fanns något lösligt socker i filtratet, vilket indikerade att separationen av lignin från cellulosa och hemicellulosa var väldigt bra.

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1. Introduction

1.1 Project description

This master thesis is a part of the Farm2Furan project at Lund University. Farm2Furan focuses on developing value chains for agricultural by-products and surplus biomass. One of the target products is 5-hydroxymethylfurfural (HMF) which is a valuable platform chemical. The fact that HMF is a platform chemical means that it can be used for many different purposes. Some of the areas where HMF can be used are in the fuel industries and as a building block in the plastics production. To be able to fully utilise biomass to obtain the desired value-added products, smart methods for processing, extraction and separation are required.

This master thesis project focuses on developing and comparing different extraction methods for separation of the different components in wheat bran. This is to enable valorization of the wheat bran.

1.2 Aim

This master thesis reports possibilities of fractionating wheat bran into its different components such as starch, lipids, protein and lignin.

The aim of this study was to gather valuable knowledge about the composition of wheat bran and what methods that can be used to refine it. This was done with the goal that the wheat bran components will end up in separate fractions, having low amounts of impurities. The research questions (RQ) for the master thesis were:

RQ1: What is possible to extract from wheat bran?

RQ2: How well can a fractionation of wheat bran be made?

1.3 Scope

The scope of the thesis work included performing starch extraction and compare how different incubation times affected the possibility to extract starch. Different solvents for extraction of lipids in a Soxhlet were also investigated. A three-step protein extraction was also made. Furthermore, a reduced factorial design was made to study which of the parameters such as temperature, incubation time, solids loading and NaOH concentration would have the largest impact on lignin extraction. Finally, it was shown that filtration of microwave treated wheat bran was rather difficult. Therefore, investigating this was also added to the scope.

Some delimitations were made due to time limitation. Therefore, the following tasks were not included: finding a suitable and reliable method for analysing lipids, and separation of cellulose from hemicellulose and lignin was not included either.

1.4 Disposition

The report has six chapters. The first part gives an introduction to the problem and describes the aim of this thesis work. The background then explains how the environment and economy are affected by petroleum refineries and how wheat bran can be part of a solution to the problem. A description of the composition of wheat bran is also given. The third part covers the extraction and analysis methods. Furthermore, the results are presented and discussed. In part five, the conclusions are summarized and finally, an appendix with additional equations and a pre-study for the lignin extraction is presented.

2. Background

2.1 The environment and fossil fuels

A major part of the industrial products and energy carriers produced worldwide are dependent on petroleum refineries. During 2018 coal, oil and natural gas together accounted for more than 80 percent of the total energy supply (IEA, 2020). However, the availability of fossil resources such as oil and gas is declining (Apprich et al., 2014).

Due to depletion of gas and oil reserves, the price of such fossil fuel resources will in a near future be affected negatively. Industry is therefore being encouraged to see bio-based systems as valuable alternatives or even substitutes for fossil resources (ElMekawy et al., 2013).

Another reason why it is interesting to investigate the possibilities of using biomass in refineries is related to carbon emissions of greenhouse gases. During combustion, the carbon present in the fuels is released as carbon dioxide to the atmosphere. When burning fossil fuels the carbon that has been stored underground for centuries is liberated, adding carbon into the atmosphere and increasing the amount of greenhouse gases. When biomass is burnt, it releases no new carbon to the atmosphere. Instead, it releases what would be liberated naturally, if the biomass was decomposed. It also forms a closed loop since the carbon dioxide released during combustion is used by other plants in their photosynthesis to grow. The net carbon dioxide emission is zero which makes biomass a carbon-neutral energy source (Linde, 2007, Palmarola Adrados, 2004).

2.2 Biorefinery

Biorefinery is a facility that produces bioenergy, biofuels and valuable chemicals from biomass. A biorefinery works in the same way as a petroleum refinery but uses renewable biomass instead of oil as feedstock. Biorefineries aim to substitute petroleum refineries. Profitable products from a biorefinery could for example be bioplastics, chemicals, biofuels and textiles (ElMekawy et al., 2013).

A drawback with biorefineries is that large field areas are required for the cultivation of crops for biofuel production. Furthermore irrigation and artificial fertilizers might be needed. This problem can be solved by using by-products from these crops in the biorefinery, for example wheat bran (ElMekawy et al., 2013).

The transformation of by-products to useful chemicals, fuels and energy with a specific regard to the environment is called valorization. In other words, valorization is when low-value biomass is used in the production of value-added products. Some examples of low-value biomass and value-added products can be seen in *figure 1* (ElMekawy et al., 2013).

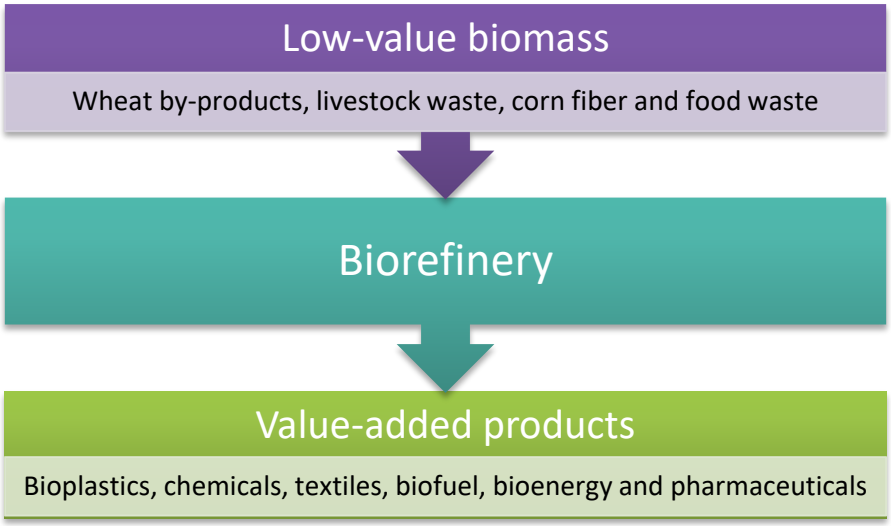

Figure 1. Low-value biomass that can be used in biorefineries and some potential value-added products.

A value-added product that can be derived from biomass, by dehydration of sugars such as glucose and fructose, is 5-hydroxymethylfurfural (HMF). Its many intermediates and end-products can be used as substitutes for petroleum-based building blocks for production of polymers, fine chemicals and fuels (Apprich et al., 2014).

2.3 Wheat bran production and composition

Wheat bran, which is the hard outer layer of the wheat kernel, is a by-product from the wheat milling process in the production of wheat flour (Apprich et al., 2014, Ardell, 2017). Sweden's annual production of wheat is 3 million tonnes/ year. Of this 10 % is wheat bran (Ardell, 2017). A portion of the wheat bran is used in the food and baking industry or for cooking at home (Onipe et al., 2015). Another fraction, 128 000 tonnes, of wheat bran is used as feed in Sweden every year (Jordbruksverket, 2020). In many countries a large amount of agricultural residues are burnt in open air resulting in the release of harmful gases (ElMekawy et al., 2013, Levine, 2014).

Agricultural biomass mainly consists of starch, cellulose, hemicellulose, protein and lignin. The amount of each component differs between different biomass types. Both starch and cellulose are polymers of glucose units, but the bonds between the glucose molecules differ between them. The sugars that hemicellulose consist of are mainly arabinose, xylose, galactose, glucose and mannose. The dominating polysaccharide in agricultural hemicellulose is arabinoxylan, which is made up of units of arabinose and xylose. It is the lignin, together with the cellulose, that gives plants and trees their rigidity. The composition of lignin is much more complex and still not fully known (Linde, 2007).

The composition of wheat bran can vary a lot. That could be due to different cultivation and harvesting conditions. For example, if the wheat is left on the field after harvesting, the cellulose might be degraded by microbes. Rainfall can cause leaching of minerals. In addition, different analytical methods can be used to determine the composition of wheat bran, resulting in different information about the composition given in literature (Linde, 2007).

Wheat bran approximately consists of 14-25 % starch, 13-18 % protein, 3-4 % lipids, 3-8 % minerals, 6 % lignin and 55-70 % non-starch carbohydrates. Out of these non-starch carbohydrates, 52-70 % is arabinoxylan, 20-24 % is cellulose and 6 % is β-glucan. An overview of the composition can be found in *figure 2* (Hell et al., 2014, Merali et al., 2015).

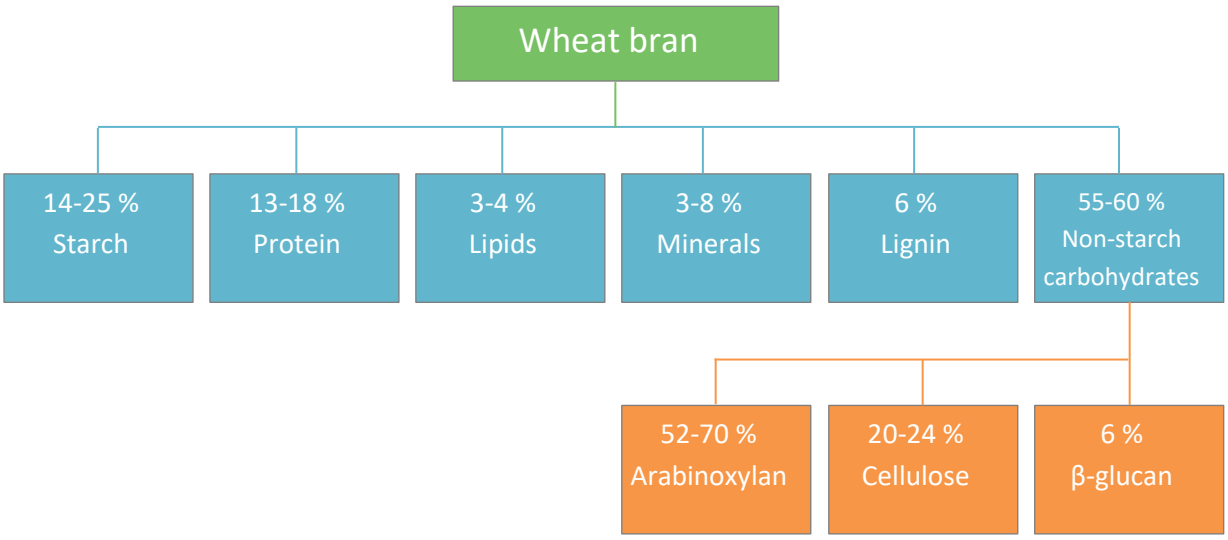

Figure 2. The composition of wheat bran.

As mentioned before, wheat bran was originally used for animal feed and human food, but considering its availability and composition it could definitely also be used as a feedstock in the production of value-added products (Apprich et al., 2014).

The relatively high protein content and the easily accessible starch add extra value to the wheat bran as a feedstock compared to other lignocellulosic resources like straw or wood (Apprich et al., 2014). Wheat bran-derived starch and cellulose can for example, in combination with other materials, make constituents for biodegradable packaging materials (Xie et al., 2008). Furthermore, sugars from starch and cellulose can be used in the production of bioethanol (Erdei, 2013). Hemicellulose is another valuable component of wheat bran, which can be used in many different products such as in oxygen barrier films in food packaging, foams and emulsifiers (Thuvander, 2018).

2.4 Extraction methods

To be able to fully utilise biomass to obtain the desired value-added products, extraction and separation of wheat bran's different components are required. There are many different extraction methods to choose between.

2.4.1 Starch

Starch could be extracted through leaching by mixing warm water with the wheat bran and then incubating the solution. The water is then removed by vacuum filtration and the remaining wheat bran residues are washed and dried. In a study by Sardari et al. (2019), this method was tested at different incubation temperatures and times. First, an incubation at 30 °C for 30 minutes was tested and later an incubation at 55 °C for 4 hours. The amount of starch that could be extracted using these methods was shown to depend on the incubation temperature used. The starch yields were 68 % and 82 % starch, for 30 °C and 55 °C, respectively. The results, however, show that extraction at 55 °C was less favourable from a separation perspective since it was more difficult to remove the water and separate the starch from the protein than during extraction at 30 °C. At 30 °C, the major part of the extracted starch in the water phase was dispersed in insoluble form, which allowed separation from the remaining wheat bran by filtration. The filtered starch was then separated from the water by sedimentation or centrifugation. Extraction at 55 °C resulted in much more water-soluble starch compared to extraction at 30 °C. When the starch is soluble in the water, it is much more complicated to separate (Sardari et al., 2019).

2.4.2 Starch and protein

A starch and protein removal process that has been used on barley bran by Karimi et al. (2018) is enzymatic hydrolysis. Different combinations of the enzymes α-Amylase, Protease and Amyloglucosidase have been tested. Maltose released by α-Amylase and tyrosine released by protease were indicators of the starch and protein removal, respectively. The glucose released by the Amyloglucosidase is an indicator of remnant starch removal. They have been used in different concentrations and were used in the treatment for different lengths of time. The experiments resulted in a starch removal between 64 % and 87 %. The experiment that resulted in the largest starch removal of 87 % starch was with sequential addition of protease, α-Amylase and Amyloglucosidase during a total time of 315 minutes. Before performing the enzymatic hydrolysis a pretreatment needs to be carried out. For example, the pH needs to be modified to suit the enzymes better (Karimi et al., 2018).

2.4.3 Protein

In a study by Sari et al. (2015), protein extraction with NaOH was tested on 16 different types of biomass, of which barley mill run and wheat middling were the two types that had the most similar composition to wheat bran. The protein extraction was conducted at three increasing incubation temperatures, starting at 25 °C for one day, followed by 60 °C for one hour, and ending with 120 °C for another hour. NaOH was added in the first and second step whilst de-ionized water was added during the final step. After each incubation step, the solution was centrifuged and the supernatant was collected for analysis. The solid phase was transferred to the next incubation step. This method resulted in three supernatants and a solid part, all containing proteins. Analysis of the supernatants resulted in a protein extraction of 69 % and 80 % from barley mill run and wheat middling, respectively. The majority of the proteins from wheat middling were extracted during the first step at 25 °C. For barley mill run, one third of the extracted protein was extracted at each step (Sari et al., 2015).

2.4.4 Lipid

Supercritical fluid extraction (SFE) with supercritical carbon dioxide (SC-CO_2) can be used for lipid extraction. In a study by Sardari et al. (2019), the extraction was performed at 50 °C at a pressure of 150 bar and 350 bar. The yield was not shown to increase with increasing pressure. Therefore, a pressure of 150 bar was considered to be the most favourable. This is why 150 bar is the pressure chosen for the experiments on the de-starched wheat bran. The results of the experiments on the de-starched wheat bran showed that some of the initial lipids were lost. In fine wheat bran, 22 % of the lipids were lost. In coarse wheat bran, it was 31 % lipid loss. The extracted lipids yield was 55 % and 46 % for untreated respective de-starched fine wheat bran. The lower yield shows that some of the lipids already have been co-extracted with the starch. The extracted lipids yield for coarse wheat bran was 53 % when it was untreated and 63 % when being de-starched (Sardari et al., 2019).

Furthermore, Soxhlet extraction with different kinds of solvents can be performed. In Soxhlet extraction, the solid material is put in porous thimbles. The solvent is heated up so that it boils and is then flowing over the thimbles. The lipids will eventually be dissolved into the solvent. This is performed utilising a reflux of the solvent. In a study by Sardari et al. (2019), Soxhlet extraction was performed with hexane. Compared to SFE, this method was much more effective resulting in almost 100 % lipid yield. However, co-extraction of starch was shown, which is why it was recommended having extraction of starch in a previous step. A drawback using hexane as solvent is that it is not healthy for humans and the environment. A suggestion would be to use ethanol instead (Sardari et al., 2019).

2.4.5 Lignin

Two of the most common methods for lignin extraction are acidic and alkaline hydrolysis. For acidic hydrolysis, sulfuric acid can be used. In a study by de Carvalho and Colodette (2017), acid hydrolysis was performed with sulfuric acid in two steps. First sulfuric acid with a concentration of 12 mole/L, or ~72 % (w/w), was used. In the second step 0.41 mole/L, or ~3 % (w/w), sulfuric acid was used. During the first step, which was performed at 30 °C for one hour, the crystalline cellulose structure was broken. In the second step performed at 121 °C for one hour, the lignin and polysaccharides were hydrolysed. In the study, hydrolysis was performed on sugarcane bagasse, sugarcane straw and eucalyptus. The amount of lignin extracted increased with increasing reaction time and greater acid concentration for all types of biomasses (de Carvalho and Colodette, 2017).

Biomass containing lignin can also be treated with an alkali such as calcium, potassium, sodium and ammonium hydroxides. Among the different alkalis, sodium hydroxide is the most common. In a study by Vincent et al. (2020), the following conditions were recommended: 60-180 °C, 5-60 minutes, 10-30 % (w/v) solid:liquid ratio and 0.5-10 % (w/v) sodium hydroxide concentration. These conditions resulted in an extraction of 50-80 % lignin and hemicellulose (Vincent et al., 2020).

3. Methods

3.1 Soxhlet extraction of lipids with ethanol and hexane

A method that can be used for extraction of lipids for analytical purposes is Soxhlet extraction. The basics of this method are that a solid sample is added to a porous thimble and a solvent is added to the solvent beaker. The solvent boils and condenses down on the solid sample. The solvent drips through the thimble and re-enters the solvent beaker. This process is repeated many times during the procedure which usually is several hours (Sardari et al., 2019). This procedure lasted 7 hours.

Figure 3. Soxhlet extraction apparatus set up.

For this experiment, four samples of known amounts of approx. 5 g wheat bran were added to thimbles, which then were placed in the extraction chamber in the Soxhlet extractor (Extraction system B-811, Büchi, Switzerland). A picture of the Soxhlet extractor is shown in *figure 3*. Two different solvents, ethanol and hexane, were used to see how the choice of solvent would affect the extraction. The same amount of solvent, 150 ml, was poured into all solvent beakers. The amounts were chosen according to previously performed experiments (Sardari et al., 2019).

The boiling point of the solvent affects the amount of heating needed in the process. Since the two different solvents have different boiling temperatures the heating was set so that both of the solvents were boiling. The heating was first set to 12 but later decreased to 10 since the solvents were boiling too vigorously at 12. The reflux time was set to 7 hours.

To be able to find out the mass of extracted lipids, the liquids containing solvent and dissolved lipids were evaporated. The evaporation was performed using a Multivapor (Multivapor P-6, Büchi, Switzerland). The empty Multivapor bottles were weighed before the liquids were poured inside.

The pressure in the Multivapor was first set to 350 mbar. When all hexane had evaporated the pressure was lowered to 170 mbar so that the ethanol would evaporate faster. When nearly all solvent was evaporated, the Multivapor bottles were put in a 45 °C oven overnight followed by one day in the desiccator. The lipids had then become completely dried. The Multivapor bottles containing extracted lipids were weighed once again and the mass of the extracted lipids were calculated.

3.2 Starch leaching with water

Starch leaching was carried out with warm water. The experiment was performed by filling six bottles with known amounts of approx. 10 g wheat bran and 100 ml water, respectively. The bottles were put in a 30 °C water bath for different amounts of time. Two bottles were in the water bath for 30 minutes, two bottles were in for one hour and two bottles were in for three hours. The samples were constantly stirred. When the leaching process was done the solutions were vacuum filtered (Sardari et al., 2019).

The liquids were poured in plastic bottles and the glucose concentration of the liquids were later analysed in a High Performance Liquid Chromatography (HPLC) according to the method described in chapter 3.3.

3.3 Starch analysis of samples from starch leaching and the raw feedstock

Starch analysis was performed on the raw wheat bran as well as on the filtrate from the starch leaching with warm water.

The raw wheat bran was analysed for starch content. The wheat bran was first milled with a knife mill to pass a 0.5 mm screen. A known amount of approx. 100 mg wheat bran was put into two sample tubes. An amount of 10 ml buffer, containing sodium acetate buffer (100 mM, pH 5.0) and calcium chloride (5 mM), were added to the tubes and the tubes were vortexed for a few seconds. After that, 0.1 ml α-Amylase (Sigma-Aldrich, USA) was added to the tubes and the tubes were vortexed once again. The tubes were loosely capped and put into a 100 °C water bath. After 2 minutes the tubes were vortexed again and the caps were tightened. The samples were put back into the water bath. The tubes were vortexed again after 5 and 10 minutes. After a total time of 17 minutes in the 100 °C water bath, the sample tubes were moved to a 50 °C water bath. The temperature was allowed to equilibrate for 5 minutes before 0.1 ml Amyloglucosidase (Sigma-Aldrich, USA) was added to the tubes. The tubes were vortexed and put back into the water bath. After 30 minutes the tubes were taken out from the water bath to cool down. The samples were filtered through a 0.2 µm syringe filter and 2 ml of the liquids were put into microcentrifuge tubes in the freezer until they were analysed. The samples were analysed in an HPLC (Shimadzu, Japan) instead of in an UV-VIS spectrophotometer as was done in the Megazyme pamphlet. During the HPLC

analysis, a lead column and water as eluent were used. A volume of 1 ml of the liquid samples was put into vials and inserted into the HPLC (Megazyme, 2020).

The six filtrate liquids from the starch leaching were also analysed to find out how much starch that had been extracted. From the filtrate, 5 ml were taken from five out of the six bottles and put into sample tubes. Since one of the bottles did not contain as much filtrate as the rest, 2.5 ml of the liquid was transferred into a sample tube as well as 2.5 ml de-ionized water (DIW) to get the same volume in all sample tubes. To each sample tube, 5 ml buffer, containing sodium acetate buffer (200 mM, pH 4.5) and calcium chloride (5 mM), was added. The tubes were vortexed for a few seconds. After that, 0.1 ml α-Amylase was added to the tubes and the tubes were vortexed again. The tubes were loosely capped and put into a 100 °C water bath. The rest of the procedure was the same as described in the previous paragraph. These samples were also analysed in the HPLC (Megazyme, 2020).

3.4 Sequential extraction of starch and lipids

An investigation of whether the order of the starch and lipids extraction affects the amounts of starch and lipids that can be extracted was made. Starch leaching was made with an incubation time of 3 hours and lipids extraction was made with hexane as solvent. The process where starch extraction is followed by lipids extraction will later be denoted “S-L”. The reversed process where lipids extraction is followed by starch extraction will later be denoted “L-S”.

3.4.1 Starch leaching followed by lipid extraction (S-L)

In the first experiment, starch leaching was followed by lipid extraction. Two bottles were filled with 10 g of raw wheat bran and 100 ml tap water, respectively. The bottles were put into a 30 °C water bath for 3 hours. The samples were then vacuum filtered. The bottles were rinsed with the filtrate to flush out all wheat bran. When all the liquid had passed through the filter the filtrate was saved for later analysis. The filtrate was analysed for starch according to the same procedure as the method described in chapter 3.3.

The solid samples were washed with 55 °C water two times to get a solid residue free from as much starch as possible. The solid samples were put in a 45 °C oven over night.

Lipid extraction was made with the same method described in chapter 3.1, with a Soxhlet extractor. The dry content of the solid samples from the 45 °C oven were measured in a moisture analyser (LP16, Mettler, USA) and known amounts of approx. 10 g of the wheat bran samples were put into thimbles. The same amount of hexane, 150 ml, as used in chapter 3.1 was poured into the solvent beakers and just as before a residence time of 7 hours was used.

The liquids in the solvent beakers were poured into previously dried and weighed Multivapor bottles to evaporate the liquid so only the lipids remained. The pressure was set to 382 mbar. According the method described in chapter 3.1, the Multivapor bottles were then dried in a 45 °C oven and put in a desiccator before being weighed once again.

3.4.2 Lipid extraction followed by starch leaching (L-S)

The solid samples from the lipids extraction described in chapter 3.1 were used during this procedure. However, only the samples extracted with hexane were used in the starch extraction. The solids from the lipids extraction were dried in room temperature for over two weeks. The dry contents were measured in a moisture analyser (LP16, Mettler, USA).

For the starch leaching, known amounts of approx. 3.3 g of wheat bran sample and 33 ml tap water were put into two 50 ml serum bottles, respectively. The reason for these amounts was that there were not enough samples for using 5 g as used before. Furthermore, the ratio between the mass of wheat bran and the volume liquid was desired to be the same as previously. The samples were incubated in a shake incubator (Lab-shaker, Lab-therm, UK) for 3 hours before being vacuum filtered. The filtrates were analysed according to the same procedure as described in chapter 3.3

3.5 Protein extraction with NaOH

The protein extraction was made in three steps where NaOH (55 mM) was used in the first and second step and DIW was used in the last step. Known amounts of approx. 10 g raw wheat bran were added to a 500 ml blue cap bottle together with 90 ml NaOH (55 mM). A duplicate of the sample was used. Magnetic bars were put into the bottles and the bottles were put on a magnetic stirrer for one day. The samples where centrifuged in a centrifuge at 4000 rpm for 45 minutes. The supernatants were removed with a pipette from the samples and saved in falcon tubes. The solid residues were put in new 500 ml blue cap bottles and another 90 ml NaOH (55 mM) were added to them. The bottles were put in a 60 °C water bath with constant stirring for one hour. The samples where once again centrifuged in a centrifuge at 4000 rpm. The samples where first centrifuged for 15 minutes and the supernatants were removed and then centrifuged for another 15 minutes and the rest of the supernatants were removed. The solid residue was again put into new 500 ml blue cap bottles. The third time, 90 ml DIW was added to the bottles instead. The bottles were put in an autoclave (DX-150, Systec, Germany) at 121 °C and one hour. The bottles were cooled to room temperature before the samples were centrifuged at 4000 rpm again. The samples were centrifuged for 15 minutes twice. The supernatants were removed from the samples both times. Finally, the samples were vacuum filtered and the liquids were collected (Sari et al., 2015). The liquids were later analysed according to the Bradford method described in chapter 3.7.

3.6 Dumas method for analysing proteins in solids

The method used for analysing protein content in solid samples is called the Dumas method. In this method, the solid sample is combusted at a high temperature in presence of oxygen. The combustion results in a release of carbon dioxide, water and nitrogen. The gases are then passed through one column that absorbs the carbon dioxide and another that absorbs water. The nitrogen content in the remaining gas is measured (Saint-Denis and Goupy, 2004). Finally the protein content can be calculated with a conversion factor of 6.25 (Sardari et al., 2019).

A protein analyser (FlashEA 1112 N/Protein Analyzer, Thermo Fisher Scientific, USA) was employed for the analysis. Known amounts of approx. 25 mg and 50 mg of aspartic acid were used for the standard curve. These were the first two samples inserted in the sample holder of the machine. The

third sample of aspartic acid measured had a weight between 25 and 50 mg. After these three samples of aspartic acid, the wheat bran samples were analysed. Known weights of approx. 30 mg of the unknown wheat bran samples were analysed. The samples analysed with this method were raw wheat bran, the solid residue from the protein extraction and the solid residues from the sequential extractions. The temperatures of the two ovens were set to 950 °C and 850 °C, respectively (H. Bolinsson 2020, pers. comm., 3 Nov).

3.7 Bradford method for analysing proteins in liquids

To analyse the protein content in the three liquids from the protein extraction with NaOH, the Bradford method was used. With the Bradford method, the protein concentration is determined by measuring the absorbance of the unknown samples as well as of protein standards. The concentration is given by plotting the absorbance of the standards versus their concentration (Bio-Rad Laboratories).

The ideal protein to use as a standard is a purified sample of the protein being analysed. But if only relative protein values are desired any purified protein can be used as a standard. Therefore, Bovine Serum Albumin (BSA), which is one of the most common protein standards, was used. BSA was diluted into different concentrations with DIW. In *table A1* in appendix A it can be seen that standards with seven different concentrations was first used (Bio-Rad Laboratories). However, only the five lowest concentrations were included in this experiment. The concentrations of the standards used can be seen in *table 1*.

Table 1. Protein concentration of BSA standard 3, 4, 5, 6 and 7.

Standard	Protein concentration (µg/ml)
3	1000
4	750
5	500
6	250
7	125

Some of the samples were first diluted 1:1, 1:5, 1:10 and 1:20 with DIW. This was to find out what dilution factor was necessary to obtain an absorbance below 1. It was found to be necessary to dilute the samples 1:10. Therefore, all the samples were then diluted 1:10.

The samples and standards were mixed with the dye Coomassie Brilliant Blue G-250 (Bradford Reagent, Sigma-Aldrich, USA) consisting of methanol and phosphoric acid. This reagent binds to the proteins and forms a complex that can be detected at 595 nm. The reagent was allowed to reach room temperature before being used. The reagent bottle was also inverted a few times to ensure even mixing (Bio-Rad Laboratories).

The dye has some requirements regarding the concentration of other compounds present. The samples must have a sodium hydroxide concentration less than 0.1 M (Bio-Rad Laboratories). This requirement is fulfilled since a sodium hydroxide concentration of 55 mM has been used. There is

another requirement on the sucrose content. It needs to be less than 10 % (Bio-Rad Laboratories). Since wheat bran consists of approx. 30 % starch, which can be seen in *table 5*, this means that in the 10 g wheat bran sample there could be 3 g of glucose. If all glucose was extracted in the first step when adding 90 ml of NaOH there would be a glucose concentration of 33 g/l or 3.3%. This sample is as mentioned before also diluted to 1:10 which would mean a concentration of 0.33 % which is well below 10 %.

According to the recommendations, 100 µl of the standards and the samples were mixed with 5 ml reagent, respectively. The sample-dye mixtures were allowed to incubate in room temperature, for at least 5 minutes but less than 1 hour, before being analysed. The samples were poured into cuvettes and analysed in a UV-VIS spectrophotometer (UV-160, Shimadzu, Japan) at 595 nm (Bio-Rad Laboratories).

The protein concentration in the samples was calculated with the equation given from the standard curve.

3.8 Structural carbohydrates and lignin

The amount of structural carbohydrates, such as cellulose and hemicellulose, and lignin were determined according to a procedure from National Renewable Energy Laboratory (NREL) called "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al., 2012). The samples analysed were the solid samples from the sequential extractions. The samples from the S-L extraction and from the L-S extraction were both analysed according to the same procedure. The samples were analysed in triplicates.

3.8.1 Preparation and acid hydrolysis

Six filtering crucibles were prepared by de-ashing them at 575 °C. A muffle furnace oven program with temperature ramping was used. The first step of the muffle furnace oven program was the heating of the muffle furnace oven from room temperature to 105 °C followed by the temperature being held constant at 105 °C for 12 minutes. The temperature was increased 10 °C/minute until 250 °C was reached and the temperature was then held constant at 250 °C for 30 minutes. In the last heating step the temperature was increased 20 °C/minute until 575 °C was reached and the temperature was held constant at 575 °C for 180 minutes. Finally, the temperature was allowed to drop to 105 °C and then held at 105 °C until the samples were removed.

The empty and de-ashed crucibles were put in a desiccator for one hour to allow the temperature to stabilize before weighing them. A known amount of approx. 300 mg of each solid sample was put into large glass tubes and 3 ml of 72 % sulfuric acid was added into each tube. The samples were stirred with Teflon stir rods until the samples were thoroughly mixed. The tubes were heated in a 30 °C water bath for one hour. The Teflon stir rods were used to mix the samples every 10 minutes. After 60 minutes, the tubes were removed from the water bath and 84 ml of DIW were added with an automatic burette to each tube to dilute the sulfuric acid. The tubes were inverted several times (Sluiter et al., 2012).

Since there might be some losses due to destruction of sugar components during dilute hydrolysis, some sugar recovery standards (SRS) were prepared. The SRSs were later used to calculate how much of each sugar component that was recovered after the dilute acid hydrolysis (Sluiter et al., 2012).

The sugar recovery standards contain the sugars glucose, arabinose, xylose, mannose and galactose. The standards were thawed and vortexed before being prepared according to *table 2*.

Table 2. Preparation of sugar recovery standards SRS0, SRS1 and SRS2.

Sample	SRS standard (ml)	DIW (ml)	72 % sulfuric acid (ml)
SRS0	1	86	0
SRS1	1	83	3
SRS2	1	83	3

The tubes with the six wheat bran samples and the two SRS samples containing sulfuric acid, SRS1 and SRS2, were sealed and incubated in an autoclave for one hour at 121 °C. The samples were later allowed to cool to room temperature. The sugar concentration of SRS0, that had not been hydrolysed, was later compared to the sugar concentrations of SRS1 and SRS2 to calculate sugar recovery (Sluiter et al., 2012).

3.8.2 Analysis of acid insoluble lignin

The wheat bran samples were vacuum filtered through the previously weighed filtering crucibles. The filtrates were collected into 100 ml blue cap bottles. The filtrates were later used for acid soluble lignin determination. To rinse out the remaining solids from the tubes, 55 °C DIW was used. In total, the crucibles were washed three times with 100 ml DIW. The crucibles with the acid insoluble residue were put in an oven at 105 °C over night to dry the residue. Then, the crucibles were put in a desiccator for one hour and the weight was recorded. Finally, the crucibles were incinerated in a muffle furnace, with the same oven program with temperature ramping as mentioned in chapter 3.8.1, from room temperature to 575 °C. The crucibles were allowed to cool to 105 °C before they were put in a desiccator once again. The final crucible weight was recorded (Sluiter et al., 2012).

3.8.3 Analysis of acid soluble lignin

The amount of soluble lignin was determined utilising a UV-VIS spectrophotometer at 320 nm. The blank used consisted of DIW (Sluiter et al., 2012). All of the filtrates were filtered through 0.2 µm syringe filters. The wheat bran samples were then analysed in two different UV-VIS spectrophotometers. The samples that were S-L extracted were analysed in an UV-VIS spectrophotometer (UV-160, Shimadzu, Japan) and were not diluted. The samples that were L-S extracted where analysed in an UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan). These samples were diluted so that three parts of the sample were mixed with one part DIW.

Since the samples were measured at 320 nm, which is in the UV light range, UV cuvettes were used. The cuvettes were washed with the samples twice to make sure that there were not any residues left from the sample analysed before. All samples were analysed in duplicates.

3.8.4 Analysis of structural carbohydrates

Analysis of sugars originating from structural carbohydrates such as cellulose and hemicellulose was made in an Ion Chromatography (ICS-3000, Dionex, USA) according to the following procedure. A small amount of the filtrates from the 100 ml blue cap bottles were filtered through 0.2 µm syringe filters. The liquids were diluted 20 times by mixing 50 µl of the sample with 950 µl DIW in microcentrifuge tubes. The tubes were vortexed and transferred to vials. The standards used contained glucose, xylose, galactose, arabinose and mannose.

3.9 Lignin extraction utilizing microwave heat treatment with NaOH as catalyst

Alkaline fractionation is one of the most common methods for extracting lignin. The biomass can be treated with alkalis such as sodium, calcium, potassium and ammonium hydroxides. Sodium hydroxide is typically preferred since it gives higher extraction yields for lignin and hemicelluloses. According to literature, the parameters that affect extraction the most are temperature, duration of treatment, base concentration and solid:liquid ratio (Vincent et al., 2020).

Lignin extraction was therefore performed by mixing wheat bran with NaOH and heating the solution in a microwave oven (Ethos Plus microwave labstation, Milestone, Italy) for a specified time. To investigate how the parameters temperature, residence time, solid: liquid ratio and NaOH concentration affected the amount of extracted lignin, a reduced factorial design with 17 different experiments was made.

Several different values of the parameters, such as incubation time, temperature, solids loading and NaOH concentration, were tested before determining the final values of parameters in the experiments. Different methods for filtering the mixtures after the treatment in the microwave oven were also tested. These can be found in appendix C. The method and procedures finally used are described here.

The ranges that were investigated were 54-276 min, 121-179 °C, 8-19 wt % solids loading and NaOH concentrations of 2-13 wt %. In every experiment, 150 ml of NaOH solution was used. Instead of changing the volume of NaOH solution the mass of wheat bran was varied to get different solids loadings. The complete reduced factorial design can be seen in *table 3*.

For experiment E8 and E9, the exact same values were selected to be able to estimate the errors of the model.

Table 3. Reduced factorial design for lignin extraction utilizing microwave heat treatment with NaOH as catalyst. Solids loading and NaOH concentration are expressed as weight percentage per volume.

Experiment No.	Residence time (min)	Temperature (°C)	Solids loading (% wt/V)	NaOH concentration (% wt/V)
E1	54	150	14	2
E2	90	130	10	8
E3	90	130	17	8
E4	90	170	10	8
E5	90	170	17	8
E6	168	121	14	2
E7	168	150	8	2
E8	168	150	14	6
E9	168	150	14	6
E10	168	150	14	13
E11	168	150	19	2
E12	168	179	14	2
E13	240	130	10	8
E14	240	130	17	8
E15	240	170	10	8
E16	240	170	17	8
E17	276	150	14	2

After treating the samples in the microwave oven, the samples were vacuum filtered through a filter paper (Ahlström-Munksjö, Finland) with grade 5. After all the liquid had been filtered through the filter paper, the residue was washed two times with 100 ml 55 °C NaOH solution with the same concentration used during the incubation. This was to dissolve as much lignin as possible.

3.10 Analysis of soluble sugar in filtrate from lignin extraction

The samples that were chosen for further analyses were sample E4, E5, E7, E15 and E16. The reason for only analysing 5 of the 17 samples is because of filtration problems, which are discussed more thoroughly in chapter 4.7 and in appendix C. In these chapters, it is mentioned that several separation techniques were tested, including centrifugation, vacuum filtration and use of filter aid.

The filtrates from the lignin extraction with NaOH, sample E4, E5, E7, E15 and E16, were put into microcentrifuge tubes and centrifuged for 5 minutes at 13000 rpm. The supernatants were collected and filtered through syringe filters. Sample E4, E5, E15 and E16 could be filtered through a syringe

filter with a size of 0.20 µm. However, sample E7 was difficult to filter and had to be pre-filtered through at syringe filter with a size of 0.45 µm.

The filtered liquids were diluted 20 times with DIW and analysed in an Ion Chromatograph (ICS-3000, Dionex, USA), as described in chapter 3.8.4, with glucose, arabinose, xylose, mannose and galactose as standards.

3.11 Analysis of lignin in filtrate from lignin extraction

To compare how the different parameters affected the amount of lignin that could be extracted, the following analysis method was performed. Due to the same reason as previously mentioned in chapter 3.10, only sample E4, E5, E7, E15 and E16 were analysed. Five empty porcelain crucibles with lids, one for every sample of E4, E5, E7, E15 and E16, were put in a 575 °C muffle furnace oven with temperature ramping for approx. four hours. The temperature ramping mentioned before was used here as well. This was made to make sure that there were not any residues left in the crucibles. The porcelain crucibles were put in a desiccator and then weighed. A volume of 15 ml of each filtrate was transferred into the porcelain crucibles. The crucibles with the samples were weighed. The weight given at this point was the weight of the liquid samples, i.e. water, lignin and NaOH. The samples were put in a 105 °C oven during seven days to allow all the liquid to evaporate. The crucibles were then put in a desiccator and weighed again. This corresponded to the weight of a dry sample, i.e. lignin and NaOH. Finally, the porcelain crucibles were put back into the 575 °C muffle furnace with the same temperature ramping as before. After the four hours, the crucibles were once again put in a desiccator and then weighed. The final weight given is the weight of NaOH, allowing the amount of lignin to be calculated.

4. Results and discussion

4.1 Soxhlet extraction of lipids with ethanol and hexane

The weights of extracted lipids from Soxhlet extraction are presented in *table 4*. The dissolved material from the Soxhlet extraction was assumed to consist of only lipids. Therefore it was expected that the weight of the dissolved material would be around 3-4 g/ 100 g (Hell et al., 2014). However, the weight given with ethanol as solvent was 8.9 g/ 100 g wheat bran, which is unreasonably high. An explanation for this could be that other material than lipids, for example starch, also have been extracted with the ethanol.

The amount resulting from extraction with hexane, 3.1 g/ 100 g wheat bran is on the other hand reasonable. Since wheat bran normally contains around 3-4 g lipids/ 100 g wheat bran the given extraction of 3.1 g lipids/ 100 g wheat bran results in a lipids yield of 77.5-100 % (Hell et al., 2014). Therefore, extraction with hexane was chosen for the sequential extractions. However, since time did not allow finding a reliable lipids analysis method, it is not possible to be completely sure that this method only extracts lipids. Further analysis is therefore required.

Lipid analysis could for example be done with gas chromatography. The basics of this procedure are that the fatty acids are converted to more volatile compounds, fatty acid methyl esters (FAME), by esterification. These are then inserted into a gas chromatograph were their retention times are compared with the retention times of known pure standards. The amounts of FAMEs are usually determined by measuring the peak areas (Eder, 1995). However, the only information necessary from the analysis is the quantity of the lipids, not what kinds of lipids that are present.

Table 4. Mass of lipids extracted per 100 g wheat bran.

Solvent	g lipids/ 100 g wheat bran
Ethanol	8.9
Hexane	3.1

4.2 Starch leaching with water

As can be seen in *figure 4,* the amount of extracted glucose increases with incubation time. The longer time the wheat bran is in contact with the 30 °C water, the more starch will be released in the water. The starch analysis on the raw wheat bran showed that it contains 30.2 % starch which is seen in *table 5*. The largest amount of extracted glucose was given by the 3 hour process. The amount of glucose extracted from that process was of 8.7 g/ 100 g wheat bran, which is less than 30 % of the starch content of the wheat bran.

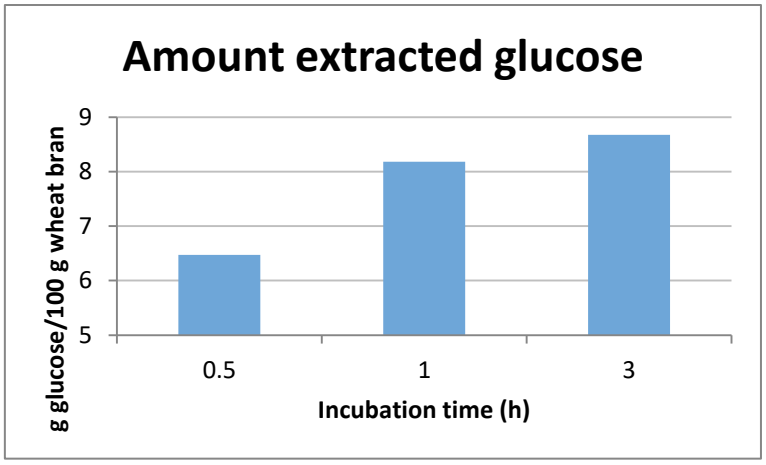

Figure 4. Amount extracted glucose after leaching with 30 °C water for 0.5 hour, 1 hour and 3 hours.

Table 5. Mass starch in 100 g raw wheat bran.

Sample	g starch/ 100g wheat bran
Raw wheat bran	30.2

To increase the yield, a longer incubation time or higher temperature is probably required. Although increasing the temperature also means increasing the risk of gelatinization. This would then lead to problems with vacuum filtration (Sardari et al., 2019). Another way of improving the extraction could be to add an enzymatic hydrolysis step.

4.3 Sequential extraction of starch and lipids

In *figure 5,* the results of the sequential extractions are shown. The procedure where starch extraction is followed by lipids extraction is denoted “S-L” and the reverse procedure, were lipids extraction is followed by starch extraction, is denoted “L-S”.

During L-S extraction, 4 g starch/ 100 g wheat bran could be extracted. In the reversed process, S-L, 6.7 g starch/ 100 g wheat bran could be extracted. This means that some of the starch was lost during the lipid extraction. The reason for this could be that some of the starch is lost in the Soxhlet solvent. After all, the temperature is much higher and the extraction time is much longer in the Soxhlet extraction than in the starch extraction. The temperature is at least 69 °C, (the boiling point of hexane at atmospheric pressure), and the extraction time is 7 hours. This means that there is a risk that the hexane has dissolved both lipids and starch. A starch analysis on the hexane solution according to the procedure in chapter 3.3 is required to find out if the solvent contains any starch or not.

There is a similar problem when it comes to loss of lipids. The S-L extraction resulted in that 1.5 g lipids/ 100 g wheat bran could be extracted. This is less compared to the L-S extraction where 3.1 g lipids/ 100 g wheat bran could be extracted. The lipids are less prone to be affected by adding hot water first to extract starch, since lipids are not water-soluble. It is more likely that the lipids stay in

the solid fraction. One explanation for that the weight of the dissolved lipids is lower when starch has been extracted first (S-L extraction) is that the "lipids weight" actually might be the weight of dissolved lipids and some starch. The more starch that is extracted before the lipids, the lower the "lipids weight" then would become.

The best sequence of starch and lipids extraction to choose depends on what is prioritized. But as mentioned above, it is required to begin with analysing the Soxhlet solvent to see if it contains any starch or not.

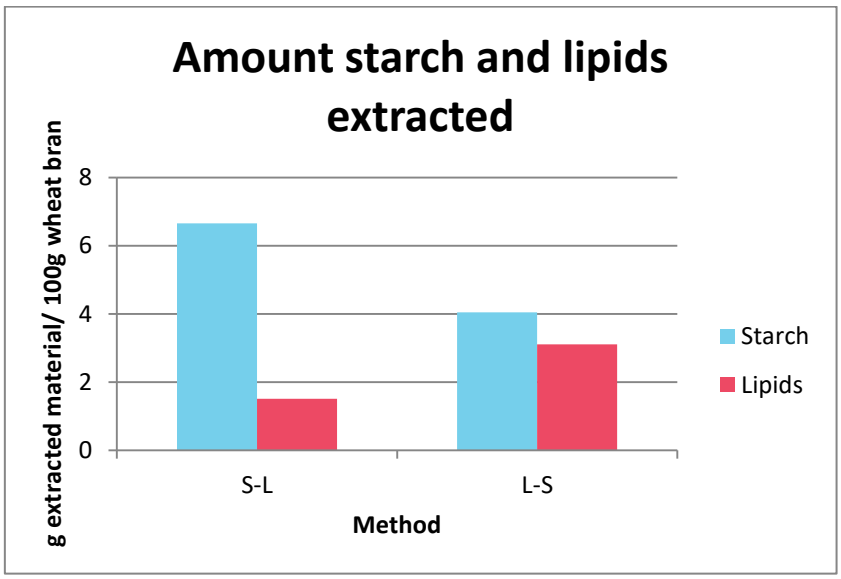

Figure 5. Amount of extracted starch and lipids from the sequential extractions.

4.4 Dumas method for analysing proteins in the feedstock and the solid residues from the sequential extractions

The amounts of protein in the samples from the protein analysis are presented in *table 6*.

Table 6. Protein content in raw wheat bran and solid residues from S-L, L-S and protein extraction.

Sample	g protein/ 100 g solid sample
Raw wheat bran	16.0
Solid residue from S-L extraction	12.6
Solid residue from L-S extraction	13.5
Solid residue from protein extraction	3.2

To be able to actually compare the numbers with each other, it is necessary to calculate backwards to the composition of the wheat bran sample before compounds were extracted from it. The true compositions can be seen in *table 7*.

Table 7. True protein content in raw wheat bran and solid residues from S-L, L-S and protein extraction.

Sample	g protein/ 100 g wheat bran
Raw wheat bran	16.0
Solid residue from S-L extraction	11.6
Solid residue from L-S extraction	12.5
Solid residue from protein extraction	2.8

As can be seen in *table 7*, there is 16 g protein/100 g wheat bran. When extracting starch and lipids with the methods described, some of the protein is lost from the solid fractions during the treatments. The solid sample from the S-L extraction contains less protein than the solid sample from the L-S extraction. This means that there is a slightly larger protein loss from the solid fraction during the S-L extraction. But since the difference is so small the extraction sequence does not seem to affect the protein content that much.

It can also be seen that the solid residue from the protein extraction consists of 2.8 g protein/ 100 g wheat bran instead of 16 g protein/ 100 g wheat bran as before. This implies that a large quantity of protein, 13.2 g protein/ 100 g wheat bran, actually was extracted with the protein extraction method described in chapter 4.5.

4.5 Extracted protein from NaOH extraction

As mentioned in chapter 3.7, the liquid samples from the protein extraction were diluted before being analysed in the UV-VIS spectrophotometer, to be in the same range as the standards. The absorbance and protein concentration of the samples are presented in *figure 6*.

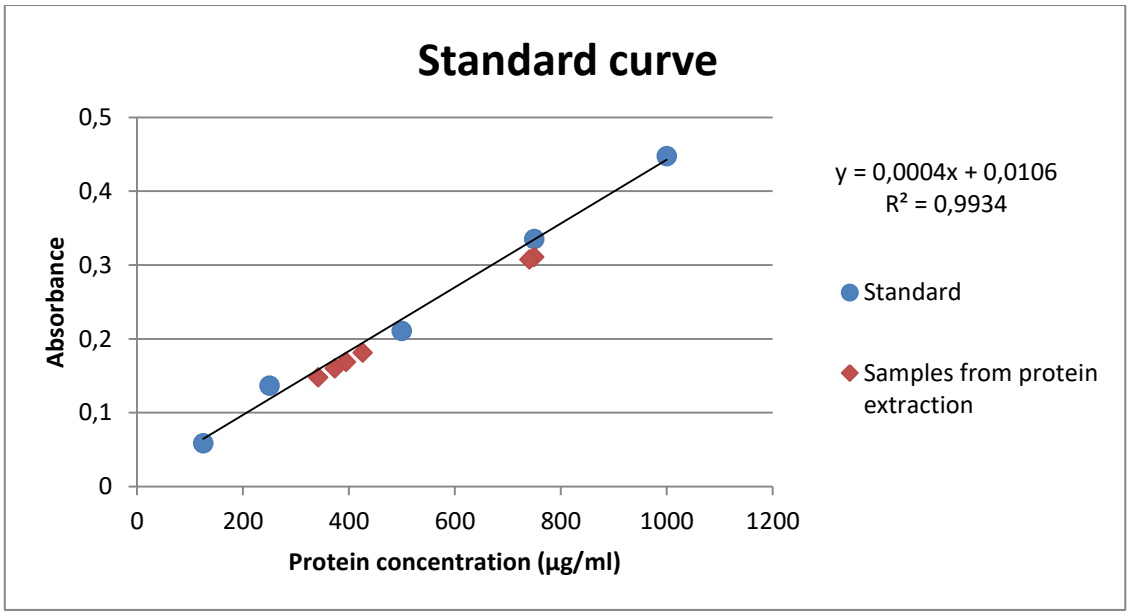

Figure 6. Standard curve with BSA standards and unknown protein samples.

The concentration of the protein samples were calculated utilizing the standard curve. The total amount of extracted protein from the 3-stage protein extraction with NaOH and DIW, and the protein yield are presented in *table 8* below. The yield is calculated based on the protein content in raw wheat bran (cf. *table 6*).

Table 8. Total amount of extracted protein and protein yield from extraction with NaOH.

Sample	Total mass extracted protein (g protein/ 100 g wheat bran)	Protein yield (%)
1	15.2	95.4
2	15.2	95.3

That this method can extract 95 % of the protein present in wheat bran is a very good result. However, the results in *table 7* imply that 13.2 g protein/ 100 g wheat bran should have been extracted with this method. Therefore, it can be seen that there is an uncertainty with the analysis methods. The protein analysis of the filtrate, described in chapter 3.7, is performed on three different liquids from the three extraction steps. Furthermore, the liquids are diluted before being analysed. Since there are a lot more steps in the analysis of the filtrate than in the analysis on the solid residues, described in chapter 3.6, the results given from this method might be less reliable. That explains why these methods give slightly different results.

In *figure 7,* the contribution of each protein extraction step is presented. The three steps together correspond to 95.4 % and 95.3 % protein yield, which also is mentioned in *table 8*. It can be seen that the majority of the protein extracted, is extracted during the first step, at 25 °C. Around 47 % of the protein present in wheat bran is extracted during this step. The rest is equally distributed between step 2 and 3.

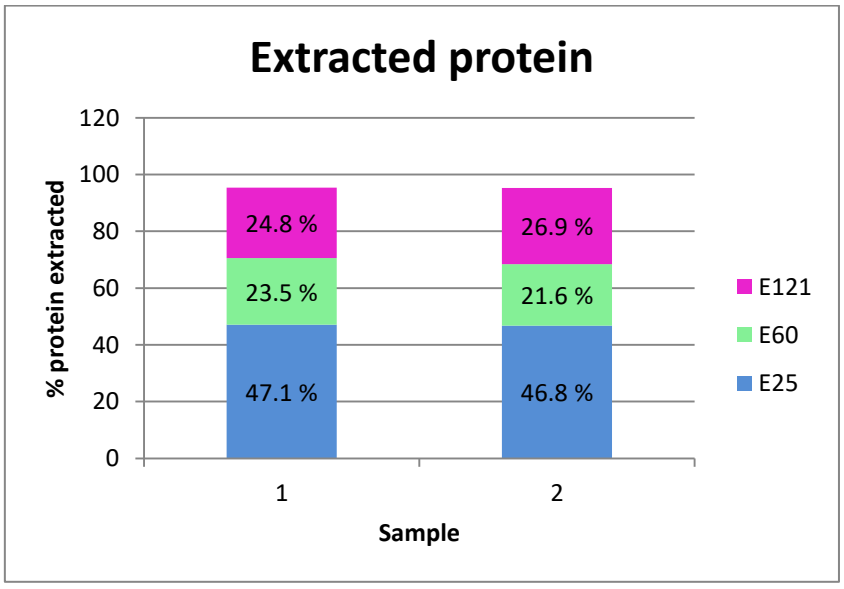

Figure 7. Percentage protein extracted in the three steps, E25 (25 °C), E60 (60 °C) and E121 (121 °C).

4.6 Structural carbohydrates and lignin

The amount of acid insoluble lignin (AIL), acid soluble lignin (ASL), cellulose and hemicellulose were calculated employing the *equations B1-B10* in appendix B. The amounts in the different solid residues can be seen in *table 9*. The range of values for AIL depends on where the protein content of the sample ends up, i.e. in the filtrate or in the solid residue. In *equation B1* in appendix B, there is a term for protein mass. If it is assumed that 100 % of the protein in wheat bran exists in the solid residue after this process, subtraction of the protein mass from the solid residue mass is required, when calculating the amount of AIL. This is the lower limit of the AIL content (0 %). If it is assumed that no protein is in the solid residue, but is in the filtrate instead, this term can be ignored. This is the upper limit of AIL contents, 8.3 and 10.6 %.

The liquids analysed for ASL in chapter 3.8.3 and the solids that were dried and ashed in chapter 3.8.2 need to be further analysed to clarify where the protein is found. This could be done with the Dumas method described in chapter 3.6 and the Bradford method described in 3.7.

Table 9. Amount of AIL, ASL, cellulose and hemicellulose from S-L and L-S extraction.

Extraction method	AIL (%)	ASL (%)	Cellulose (%)	Hemicellulose (%)
S-L	0 - 8.3	0	23.1	35.0
L-S	0 - 10.6	0	24.8	40.9

A small difference in lignin, cellulose and hemicellulose content between the two extraction methods can be seen in *table 9*. It could be seen in *figure 5*, that the S-L and L-S extraction methods resulted in different amounts of extracted starch and lipids. This will later affect the final weight of the sample and therefore affect the amounts of lignin, cellulose and hemicellulose that are present. The true lignin, cellulose and hemicellulose content of wheat bran are probably between these two.

4.7 Lignin extraction after microwave treatment with NaOH as catalyst

After treating the wheat bran in NaOH with different concentrations, temperatures and residence times, it was discovered that many of the samples were very hard to vacuum filter or practically not possible to filter. Several different separation techniques were tried including centrifugation, vacuum filtration and the use of filter aid. The separation problem is described more thoroughly in appendix C.

Therefore, it was decided to focus on the samples that were the easiest to filter. These samples were sample E4, E5, E7, E15 and E16. The parameters of these samples can be seen in *table 10*.

Table 10. Samples from the lignin extraction utilizing NaOH as catalyst that could be further analysed. Solids loading and NaOH concentration are expressed as weight percentage per volume.

Experiment No.	Residence time (min)	Temperature (°C)	Solids loading (% wt/V)	NaOH concentration (% wt/V)
E4	90	170	10	8
E5	90	170	17	8
E7	168	150	8	2
E15	240	170	10	8
E16	240	170	17	8

In *figure 8* sample E6 is shown as an example of a sample that was very thick and hard to filter. Sample E6 was microwave treated at 121 °C for 168 minutes with 14 % solids loading and 2 % NaOH solution. Sample E16 on the other hand was much easier to filter. Sample E16 was microwave treated at 170 °C for 240 minutes with 17 % solids loading and 8 % NaOH solution.

Figure 8. Samples microwave treated. Left: Sample E6. Right: Sample E16.

4.7.1 Analysis of soluble sugar in filtrate after microwave treatment

It can be seen in *figure 9* that most of the liquid samples did not contain much sugar. However, sample E7 contained far more arabinose that the other samples. A reason for this may be that arabinose is broken down at 170 °C and therefore is not present in the other four samples. This might also be the explanation to why sample E7 was much harder to filter. The fact that sample E7 contained more arabinose which originates from hemicellulose might affect the filtration resistance. A reason for why these five samples were the easiest to filter might be that the soluble sugars start to break down at 150-170 °C.

Even though sample E7 contained more arabinose than the other samples, the samples still did not contain very much soluble sugars. This means that the lignin is partially solubilized, while hemicellulose and cellulose are still in the solid fraction. Therefore, it may be possible to fractionate the compounds, if the filtering challenge can be solved.

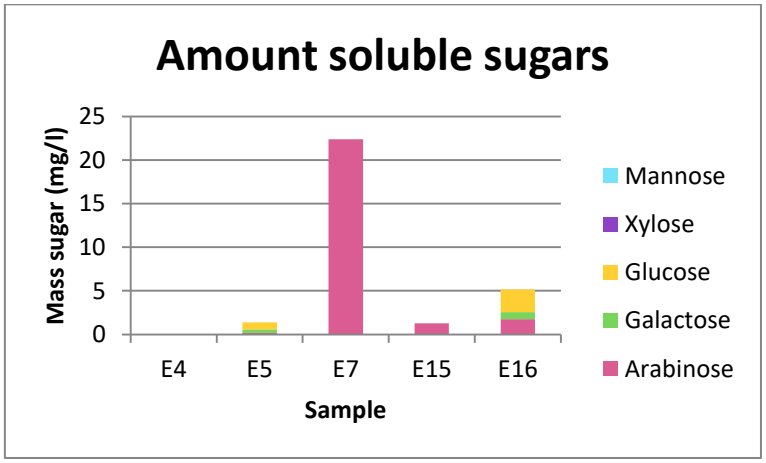

Figure 9. Soluble sugars present in the filtrate of sample E4, E5, E7, E15 and E16 after heat treatment.

4.7.2 Analysis of lignin in filtrate from lignin extraction after microwave treatment

The amount of lignin extracted from the wheat bran samples E4, E5, E7, E15 and E16 after microwave treatment can be seen in *figure 10*.

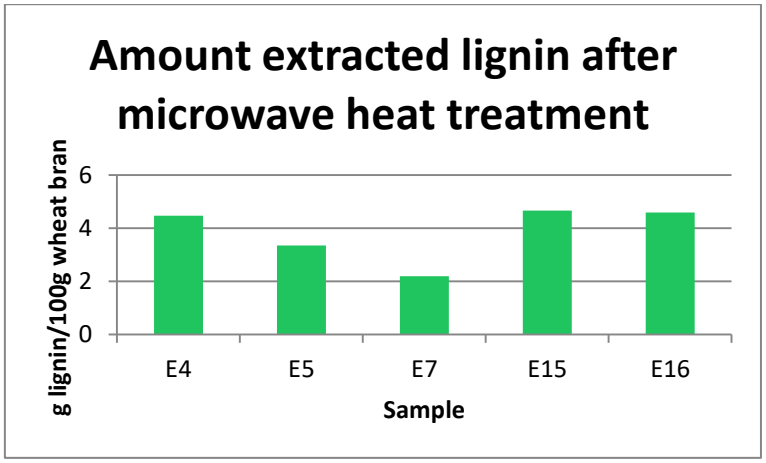

Figure 10. Amount extracted lignin in sample E4, E5, E7, E15 and E16 after microwave heat treatment.

The largest amount of lignin could be extracted from the two samples E15 and E16, both having the highest temperature and the longest incubation time, 170 °C and 240 minutes. From these samples

4.7 g lignin/ 100 g wheat bran and 4.6 g lignin/ 100 g wheat bran respectively could be extracted. Sample E15 therefore gave a lignin yield of around 44-100 % according to the interval of AIL in *table 9*.

The third and fourth highest yields resulted from samples E4 and E5. These samples were also incubated at 170 °C but for a shorter time, 90 minutes instead. These parameters resulted in extractions of 4.5 g lignin/ 100 g wheat bran and 3.3 g lignin/ 100 g wheat bran respectively.

The least amount of extracted lignin came from sample E7. From sample E7, 2.2 g lignin/ 100g wheat bran could be extracted. This could either be because of the lower temperature, 150 °C, or that the NaOH solution used had a lower concentration than the NaOH solution used for the other experiments.

In *figure 10* it can also be seen that the samples with 10 % solids loading, sample E4 and E15, gave higher lignin yield compared to the samples with 17 % solids loading, sample E5 and E16. This may be due to the fact that it is easier for the NaOH solution to come in contact with the wheat bran the smaller the solid to liquid ratio is. The more liquid that can come in contact with the wheat bran the easier it is for the temperature of the wheat bran to increase. Furthermore, diffusion is faster in a thinner solution.

5. Conclusion

To answer RQ1, from this thesis work it can be concluded that starch, lipids, protein and lignin could be extracted from wheat bran. Starch was extracted from wheat bran by leaching with warm water. The longer incubation time the larger amount of extracted starch was given. The largest starch yield given was 29 % starch.

Lipids could be extracted with a Soxhlet extraction, both with ethanol and hexane as solvents. It was discovered that starch might be extracted together with the lipids when using ethanol. This makes hexane a more suitable solvent to choose. The lipids yield given when using hexane was 77.5-100 %, depending on how much lipids that were present in raw wheat bran. However, further lipid analysis is required. A suitable method for lipid analysis could for example be to use gas chromatography.

Protein was extracted in a three step procedure with NaOH and DIW resulting in a 95 % yield. Analysis showed that the majority of the protein content was extracted during the first extraction step.

It was possible to extract lignin using microwave treatment and NaOH as catalyst. However, there were difficulties with separation of the lignin from the rest of the wheat bran due to the gel-like product formed. Several different separation techniques such as centrifugation, vacuum filtration and the use of filter aid were tested. It was shown that samples treated at 170 °C were easier to filter than samples treated at lower temperatures. This might be due to the fact that the hemicellulose had not been broken down at a lower temperature. Samples treated at 170 °C also gave the highest lignin yield. Solids loading was also shown to have an effect on the amount of lignin that could be extracted. From samples with 10 % solids loading, more lignin was extracted compared to samples with 17 % solids loading. The highest lignin yield given was 44-100 % depending on how much lignin the starting material contained. This yield was given when using the following parameters, 240 minutes, 170 °C, 10 % solids loading and a NaOH concentration of 8 wt %/ V.

Sugar analysis on the filtrate from the microwave treated wheat bran showed that almost no soluble sugars were present. This indicates a good separation of lignin in the filtrate and cellulose and hemicellulose in the solid residue.

To summarize the answers to RQ2, the following yields were obtained, 29 % starch, 77.5-100 % lipids, 95 % protein and 44-100 % lignin.

Further work regarding lignin extraction could include using higher temperatures in the microwave treatment as well as investigation of other separation techniques.

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Appendix A – Bradford analysis

In *table A1* protein concentration of BSA standard 1-7 can be seen.

Table A1. Protein concentration of BSA standard 1-7.

Standard	Protein concentration (µg/ml)
1	2000
2	1500
3	1000
4	750
5	500
6	250
7	125

Appendix B – Structural carbohydrates and lignin

Equations B1-B10 were used to calculate the amount of acid insoluble lignin (AIL), acid soluble lignin (ASL), cellulose and hemicellulose (Sluiter et al., 2012).

$$\%\ AIL = \frac{(Weight_{crucible\ plus\ AIR} - Weight_{crucible}) - (Weight_{crucible\ plus\ ash} - Weight_{crucible}) - Weight_{protein}}{ODW_{sample}} \times 100 \quad \text{(B1)}$$

$$\%\ ASL = \frac{UVabs \times Volume_{filtrate} \times Dilution}{\varepsilon \times ODW_{sample} \times Pathlength} \times 100 \quad \text{(B2)}$$

$$Dilution = \frac{Volume_{sample} + Volume_{diluting\ solvent}}{Volume_{sample}} \quad \text{(B3)}$$

$$\%\ Lignin_{ext\ free} = \%\ AIL + \%\ ASL \quad \text{(B4)}$$

$$\%\ Lignin_{as\ received} = (\%\ Lignin_{ext\ free}) \times \frac{(100 - \%\ Extractives)}{100} \quad \text{(B5)}$$

$$\%\ R_{sugar} = \frac{conc.detected\ by\ Dionex}{Known\ conc.\ of\ sugar\ before\ hydrolysis} \times 100 \quad \text{(B6)}$$

$$C_x = \frac{C_{Dionex} \times dilution\ factor}{\%\ R_{ave.\ sugar}/_{100}} \quad \text{(B7)}$$

$$C_{anhydro} = C_x \times Anhydro\ correction \quad \text{(B8)}$$

$$\%\ Sugar_{ext\ free} = \frac{C_{anhydro} \times V_{filtrate}}{ODW_{sample}} \times 100 \quad \text{(B9)}$$

$$\%\ Sugar_{as\ received} = (\%\ Sugar_{ext\ free}) \times \frac{(100 - \%\ Extractives)}{100} \quad \text{(B10)}$$

Appendix C – Pre-study for lignin extraction

The lignin extraction began with preparing six samples with different NaOH concentrations, 2, 5 and 10 wt% NaOH. All six samples had a solids loading of 17 %. The samples were incubated in a microwave oven (Ethos Plus microwave labstation, Milestone, Italy) at 120 °C for 180 minutes. One of the samples with 10 % NaOH solution was used for investigating the filtration possibilities.

To begin with the sample was centrifuged at 4000 rpm for 10 minutes. Since the mixture still was quite thick, 100 ml of 55 °C NaOH solution (5 % NaOH) was added and the sample was centrifuged once again. The supernatant was then collected. This was repeated once more. After the sample had been centrifuged three times, another 100 ml 55 °C NaOH solution (5 % NaOH) was added. The sample was vacuum filtered overnight. In the morning the day after only a small amount of liquid had passed through the filter. The filter cake was very thick and gel-like.

Two different types of filters were then tried, 100 µm nylon filter and a nylon cloth strainer, which typically is used for filtering juices. No further progress was made with these filters.

To improve the porosity and permeability of the filter cake, kieselguhr, which is a kind of filter aid, was added to the mixture (Purchas and Sutherland, 2002). Before vacuum filtering the mixture, 2 g of kieselguhr were added. At this point the filtration seemed to have improved.

A factorial design was then made. The lower and upper limits of the parameters used were 60-120 °C, 90-240 minutes, 10-17 % solids loading and 2-10% NaOH concentration. The values of the parameters were chosen according to recommendations in literature (Vincent et al., 2020). The first two samples that were incubated were samples with 10 % and 17 % solids loading. Both had a NaOH concentration of 8 % and they were incubated at 60 °C for 90 minutes.

After incubation, kieselguhr was added to the wheat bran mixture. The amount of kieselguhr added was the equivalent of approx. 2 % of the starting weight of wheat bran. The mixture was vacuum filtered with a filter paper with grade 5. To speed up the filtration, 100 ml of 55 °C NaOH solution (8 % NaOH) was added. The next morning only a little amount of the sample had dripped through the filter. One of the samples had even been solidified. The solidified sample can be seen in *figure C1*.

Figure C1. Wheat bran sample treated at 60 °C for 1.5 hour with 17 % solids loading and a NaOH concentration of 8 %. The sample was mixed with kieselguhr.

The factorial design was modified and the temperature interval was changed to 121-179 °C. This design became the final factorial design which can be found in *table 3*.