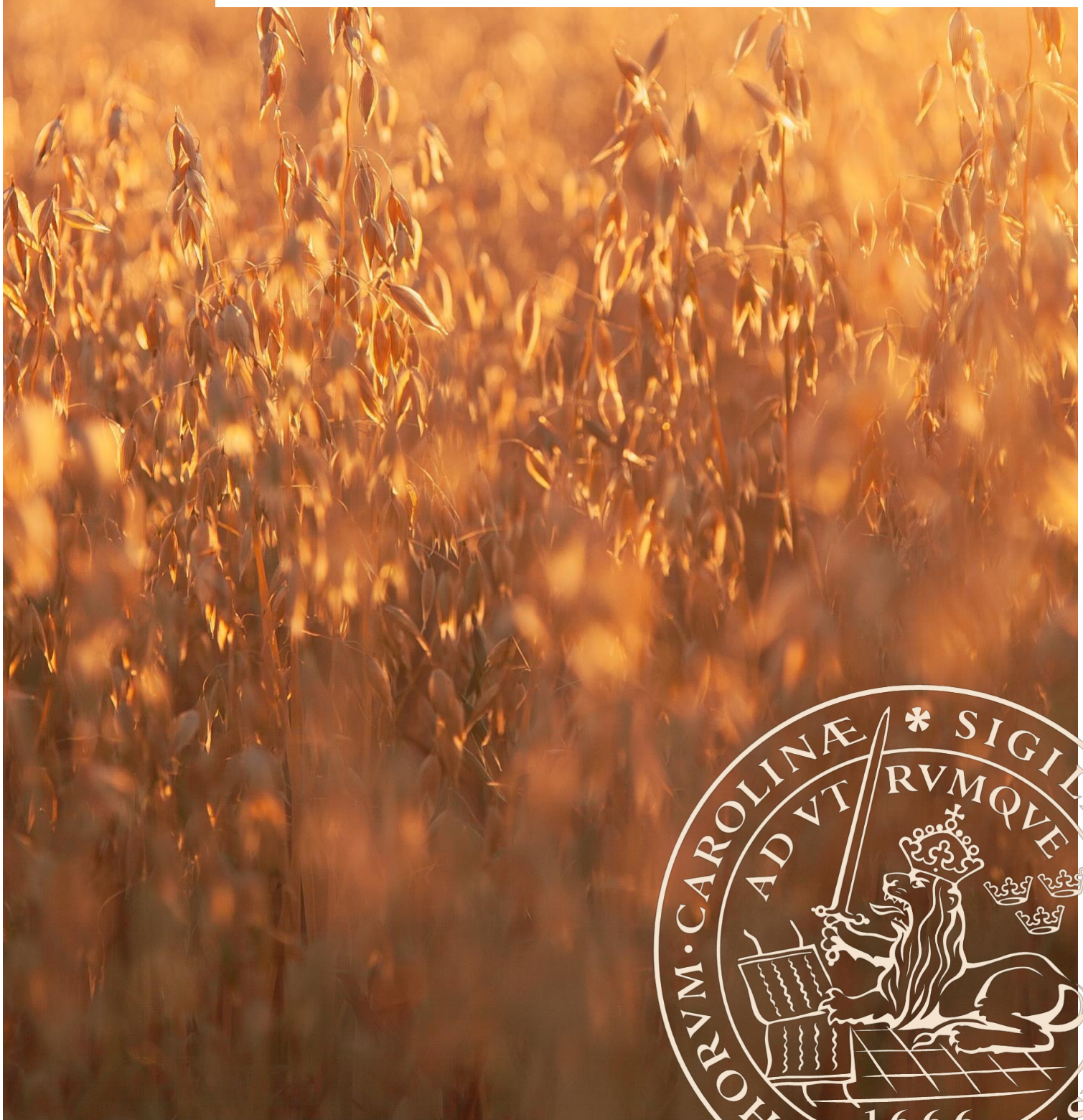
Alkaline Protein Extraction of Oat Bran and Oat Endosperm Flour

The Effect of pH during Extraction and Precipitation

DEPARTMENT OF FOOD TECHNOLOGY, ENGINEERING AND NUTRITION | LUND UNIVERSITY
ALVA RODHE & BELLA NIELSEN | MASTER THESIS IN FOOD ENGINEERING 2021



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Master Thesis in Food Engineering

Alkaline Protein Extraction of Oat Bran and Oat Endosperm Flour – the Effect of pH during Extraction and Precipitation

by

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January – June 2021

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Lund 2021

Abstract

An increased understanding of the negative impacts of animal-based proteins, on both environment and health, has led to a rise in the demand for plant-based proteins. To meet the demands, conventional crops must be efficiently utilized. One possible crop is oat which is a good source of protein, fibre, and fat, but is currently mainly used for animal feed. To better utilize the proteins in oat, they can be extracted to produce oat powders with high protein concentration. The aim of this project was to evaluate alkaline extraction for its efficacy in protein extraction from both milled oat bran and oat endosperm flour. This work also aims to provide a literature survey on oat, oat protein and its functional properties as well as protein extraction methods.

The experimental set-up consisted of alkaline extraction followed by isoelectric precipitation, with focus on the effect of pH on yield, protein content and dry matter content. Two extraction pH, two precipitation pH and constant versus non-constant pH during the alkaline treatment was investigated. In addition, a differential scanning calorimetry (DSC) test was performed to analyse the proteins.

Alkaline extraction resulted in high protein yields of 80-83% for oat endosperm flour and 66-70% for milled oat bran, where the range includes the results from all pH-levels tested. The protein content in the sediment extracted from oat endosperm flour and milled oat bran was 73-75% and 75-77% respectively. Precipitation pH 4.5 resulted in higher dry matter content for both raw materials, compared to pH 5.5. Constant pH during alkaline treatment did not improve protein yields, which was unexpected. However, the protein content was improved wen using constant extraction. The thermal properties analysis (DSC) of the proteins revealed higher peak denaturation temperatures for proteins precipitated at pH 5.5. This applied for both milled oat bran and oat endosperm flour.

The results from this project indicate that protein extraction from oat is comparably easy, relative to other plant sources. No optimal extraction can be developed as it depends on the intended use of the proteins and would require analysis of functional properties before any definitive conclusions can be drawn. Rather, the results of this study can be used in further development of alkaline extraction of oat. The conclusions are then that the yield is mostly unaffected by the choice of pH, within the interval, and does not require constant pH to be high. Although constant pH is preferable for protein content, it is more affected by the choice of pre-treatment than it is by extraction pH. For an energy efficient process precipitating pH at 4.5 should be used to shorten the following drying process, and precipitation pH 5.5 is preferred if the proteins need to be more heat tolerant.

Sammanfattning

En ökad förståelse för den negativa påverkan som animalie-baserade proteiner har, på både vår hälsa och miljö, leder till en ökad efterfrågan av växtbaserade proteiner. För att möta efterfrågan måste konventionella grödor användas på ett effektivt sätt. En möjlig gröda är havre, som är en bra källa till protein, fiber och fett, men som idag används främst för djurfoder. Proteinerna i havre kan bättre utnyttjas genom att extrahera dem för att producera ett pulver med hög proteinhalt. Målet med detta projekt var att utvärdera alkaliska metoder för proteinextraktion från två typer av havrematerial, malet havrekli samt endospermmjöl från havre. Projektet syftar även till att bidra med en litteraturstudie av havre, havreproteiner och dess funktionella egenskaper samt metoder för proteinextraktion.

Den experimentella metoden bestod av alkalisk extraktion följt av iso-elektrisk utfällning, med fokus på effekten som pH har på utbyte, proteinhalt och torrsubstans. Två extraktions-pH, två utfällnings-pH och konstant jämfört med icke-konstant pH under den alkaliska behandlingen undersöktes. Utöver det gjordes även en differentiell svepkalorimetri (DSC) för att analysera proteinernas värmetålighet.

Alkalisk extraktion resulterade i utbyten på 80–83% för havre-endospermmjölet och 66–70% för det malda havrekliet. För proteinhalten (torr basis) så uppnåddes nivåer av 73–75% för havre-endospermmjölet och 75–77% för det malda havrekliet. Utfällnings-pH 4.5 gav betydligt högre torrsubstans i sedimenten för båda råmaterialen. Konstant pH under den alkaliska behandlingen bidrog inte till högre utbyte, vilket var oväntat, däremot så var proteinhalten högre vid konstant extraktion. Den termiska analysen (DSC) av proteinerna visade att de protein som fällts ut vid pH 5.5 har högre denatureringstemperatur. Detta gällde för både det malda havrekliet och havre-endospermmjölet.

Resultaten från denna underökning visar att havre är enkelt att extrahera proteiner från om man jämför med andra material. En enda optimal extraktionsmetod kan inte tas fram då det är beroende av hur proteinet ska användas, vilket kräver att funktionalitet hos proteinerna undersöks. Snarare kan resultaten användas för att vidareutveckla alkalisk extraktion av havre. Slutsatserna är då att utbyte inte påverkas särskilt mycket av pH inom de intervall som användes här, och att konstant pH under extraktion inte behövs för att få ett högt utbyte. Även om konstant pH kan bidra till högre proteinhalt så ses en större effekt snarare av vilket förbehandling som tillämpas. För en energisnål process så bör utfällnings pH 4.5 användas då det förkortar den följande torkningsprocessen, och utfällnings pH 5.5 är lämpligt om proteinerna ska ha hög värmetolerans.

Popular Abstract – Extraction of protein from oat

A growing world population combined with an increased understanding of the negative effects of animal-based proteins on the environment as well as on health, means that the demand for new and sustainable plant-based proteins is rising. To meet the interest, food companies in Sweden turn towards readily available plant-sources, such as oat, which can be locally grown in our cooler climates. But how can protein be efficiently extracted from oat, and what decides how it can be used in food products?

Oat has become more popular lately due to its nutritional benefits. Amongst other, it contains high amounts of protein compared to other cereals, but still, its main application is as feed for animals and not for human consumption. If we want to utilize oat for its full potential, one way is to separate the proteins and use them to create high-protein food products.

In this study we investigated if protein could be extracted from different oat materials, from different parts of the oat grain, with varying amounts of protein. So far, oat protein has only been produced from the high-protein oat bran, whilst the largest part of the oat grain, the endosperm, has not been used. Our results show that protein can be more easily extracted from oat than other materials such as hemp, and a protein powder with a protein content of over 70% was obtained from both the milled oat bran and the oat endosperm flour. In addition, we found that protein was easier to remove from the oat endosperm flour than from the milled oat bran. Over 80% of the protein was able to be collected from oat endosperm flour whilst the milled oat bran yielded approximately 70%.

To extract the proteins, a so-called alkaline methodology was used, in which high pH separates the proteins from the oat flours. The impact of some key parameters on the extraction process were evaluated. In particular, we found that pH did not affect the protein yields as much as previously known, and that a gentler process can be used as the material is so easy to extract from. However, pH during the purification step of the process did influence how much water the protein retains, a factor which had not been previously observed.

Finally, a review of the properties of oat proteins based on results from previous studies was summarized. These properties include amongst other how easily the protein powder is dissolved in water, how high temperature the proteins can withstand, and whether the proteins can be used to create emulsions, foams, or gels. How well a protein can do any of these things dictates the potential applications in food products. Here it was found that oat proteins unfortunately perform comparatively poor, except for having a high temperature resistance. However, the properties can be improved by using different modification methods.

In conclusion, our results indicate that oat has great potential as a plant-based protein source, which may include additional uses for this Swedish-grown grain. While more work is required to improve the properties of the resulting protein, the final product may be successfully applied in a wide range of food products.

Preface

This master thesis was performed at the Department of Food Technology, Engineering and Nutrition at the Faculty of Engineering (LTH) of Lund university. The project was a collaboration together with Lantmännen. The thesis covers 30 credits and lasted from January to June of 2021, practical work was performed at the department.

A special thanks to our LTH supervisor, Jeanette Purhagen, for your involvement, valuable comments and discussions which guided and supported us through the project. Thanks to our supervisors at Lantmännen: Christian Malmberg, Karin Arkbåge & Mats Larsson, for giving us this project, and your enthusiasm, questions, and comments which has improved our work. Thanks to Amanda Helstad for your kind help during our practical work. Also, thanks to the Department of Food Technology, Engineering and Nutrition for allowing us to perform our practical work during these circumstances and thanks to Hans Bolinsson and Olexandr Fedkiv for your help.

We would also like to thank our Examiner, Marilyn Rayner, for your helpful comments and feedback. Thanks to our opponents, Nicki Holmgran & Jens Svendsen, for your interesting questions and useful comments during our presentation.

Finally, we would like to thank each other for excellent teamwork!

Thank you!

Alva & Bella

Abbreviations

ANOVA	Analysis of Variance
DF	Defatted
DM	Dry matter content
DSC	Differential scanning calorimetry
EAI	Emulsion activity index
EC	Extraction coefficient
ESI	Emulsion stability index
FBC	Fat-binding capacity
IEP	Iso-electric precipitation
NDF	Non-defatted
OBM	Milled oat bran
OEF	Oat endosperm flour
OPC	Oat protein concentrate
OPI	Oat protein isolate
PC	Precipitation coefficient
PDCAAS	Protein digestibility corrected amino acid score
PER	Protein efficiency ratio
PLS	Partial least-squares regression
PPS	Percent protein solubility
R^2	Coefficient of determination / goodness-of-fit
T_d	Peak denaturation temperature
UF	Ultrafiltration
US	Ultrasound
WHC	Water-holding capacity

Table of Content

1. Introduction

Due to a growing world population and increased comprehension of the negative effects of animal-based proteins on environment and health, the demand for sustainable and innovative plant-based proteins is rising. In order to succeed in this protein shift, more knowledge on how to efficiently utilize and extract protein from readily available plant-sources is needed.

Lantmännen is an agricultural cooperative in northern Europe active in the fields of agriculture, machinery, bioenergy, and food. The food sector at Lantmännen performs research and development in a number of areas, including the fractioning of grains to produce new food components and products. An example of innovations from such research is the product PrOatein™ which is an oat-based protein powder. The protein powder is derived through an enzymatic protein extraction of the oat bran. Lantmännen now seek to explore alkaline protein extraction as an alternative method. Additionally, performing extraction on both the milled oat bran as well as the oat endosperm flour in order to find an efficient process which extracts oat protein with high yield, high concentration and maintained functionality.

1.1 Scope

There is a wide range of factors influencing both protein extraction, the extraction efficiency as well as the functionality of the resulting protein. Due to limitations caused by the pandemic, the laboratory work for this project focused on investigating only few, select processing parameters and their effect on extraction efficiency. These parameters were the pH during extraction and precipitation, as well as the effect of keeping pH constant during the extraction process. Unfortunately, no experimental investigation into the effect on protein functionality could be carried out, except for denaturation temperature through DSC. Instead, through a thorough literature review on oat proteins, protein extraction methods and protein functionality the project provides a survey of existing results. The experimental and theoretical investigation into alkaline protein extraction of oat explores some parameters of interest and creates a foundation for future work and development within the subject.

1.2 Aim

The overall aim of the project was to evaluate alkaline methodology for its efficacy in protein extraction from oat materials. In addition, the work aims to present a literature review of oat, with a focus on oat protein, functional properties of proteins, protein extraction methods, and finally ways of improving functionality as well as extraction efficiency.

The aim of the practical experiments is to test and evaluate the following hypothesis:

- Alkaline protein extraction would successfully extract proteins from milled oat bran and oat endosperm flour.
- The use of different extraction and precipitation pH would result in a protein yield difference.
- Keeping the pH constant during the alkaline treatment would result in higher protein yields compared to a non-constant method.

2. Background

Oat, *Avena Sativa*, is grown and used for both food products and livestock feed, where roughly 75% is used for feed today. It can be grown in cool, moist climates such as Sweden and is well adapted for varying soil qualities and crop rotations. Oats have a high protein and fat content when compared to other cereals and are a good source of fibres, minerals and vitamins [1].

The use of oats in food production has grown over the last decades due to an increased understanding of nutritional benefits associated with oats. The increased interest from consumers creates incentive for development of new oat products and production methods. Oat fractioning is of special interest as it allows for separation and extraction of the different components in the oat, such as oat protein and β-glucan, which can then be used for their functional properties in food products. Raised demand of oat for food production also has the potential of increasing the profitability for farmers [1].

2.1 Oat Grain Composition

In Figure 1 a cross section of the oat grain can be seen. The outer layer, or the hull, acts as a protective barrier for the groat inside. The groat is divided into three major components, the bran, endosperm and the germ [2]. The bran contains most of the minerals and vitamins found in the oat kernel [2], and it also has a higher protein content of around 18-26% compared to the endosperm which contains mostly starch and around 12% protein [3]. The endosperm also contains lipids distributed throughout it unlike other cereals where most fat is found in the germ [4]. The highest protein content is found in the germ, with around 26-44% protein, however the germ only accounts for around 1% of the total weight of the groat so the total amount of protein in the germ is low compared to the bran and endosperm [5].

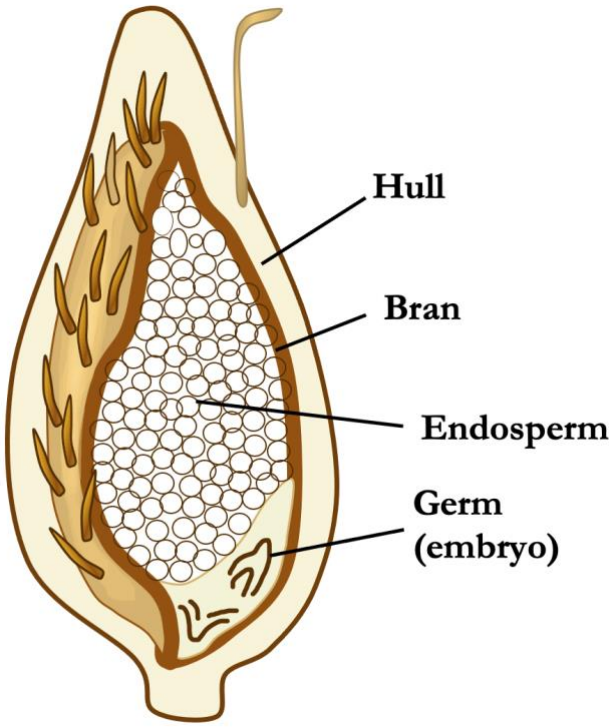

Figure 1: Cross section of oat grain showing the main components. Modified figure from [6].

Oat differs from other cereals in their protein composition as the major storage proteins are globulins, at 50-80% compared to prolamins in wheat, barley and rye. Prolamins are also found in oat, in the form of avenins, but in smaller amounts of around 4-15% of the total protein content. The second biggest fraction is albumins which are present in levels of 1-20%, and the remaining part is glutelins [6],[10],[8]. The proteins and amino acid composition differ between the endosperm and the bran, which is discussed in section 2.2.2.

The prolamins, called avenins, found in oats are low in proline and glutamine compared to the prolamins in wheat, gliadin and glutenin [6],[12]. This is believed to be a reason why oat can be tolerated by people with coeliac disease [3], who otherwise suffer severe immune reactions to gluten proteins [4]. However, in many cases oat is contaminated with gluten-containing cereals and therefore not suitable depending on the severity of a patient's coeliac disease [3].

2.1.1 Oat Products

Oat can be used for several types of food products such as oat drinks and different types of flours. However, both the protein and the β-glucan can be extracted from oat and thereby has a great potential to be used in new plant-based food products. In Figure 2, a schematic overview of the production process, from dehulling and milling into the β-glucan and the protein powders are presented [10]. The dry processes, consisting of dehulling and milling, separates the three major components of the oat grain, described previously in section 2.1. Dehulling separates the hull from the groat, creating the husk fraction, the groat then goes into milling which produces an oat flour of the starchy endosperm and an oat bran fraction. The germ is most likely included in the oat bran fraction, however part of it may end up in the oat flour [10].

Today the oat flour is sold and used in production of feed and alcohol, whilst oat bran is further processed in a wet process that uses enzymatic treatment to extract oat protein which is sold as the product PrOatein™[1] and oat β-glucan which is sold as the product PromOat®[2] [10]. These two powders have previously been studied by Vikenborg & Stensson [11] in a master thesis where the functionality and possible food uses for the products were analysed. In their thesis they concluded that PrOatein had low solubility of around 5% which was the main limiting factor for its use in food products, and it was believed that the low solubility was partly due to the heat treatment that the protein goes through during the wet process which causes denaturation [11]. PrOatein has a protein content of 50-60%, 16-19% naturally occurring lipids, 20-24% oat maltodextrins and 2-3% oat β-glucan [12].

[1] PrOatein™ is a trademark by Lantmännen and applies to all times where "PrOatein" is mentioned in this report.
[2] PromOat® is a trademark by Lantmännen and applies to all times where "PromOat" is mentioned in this report.

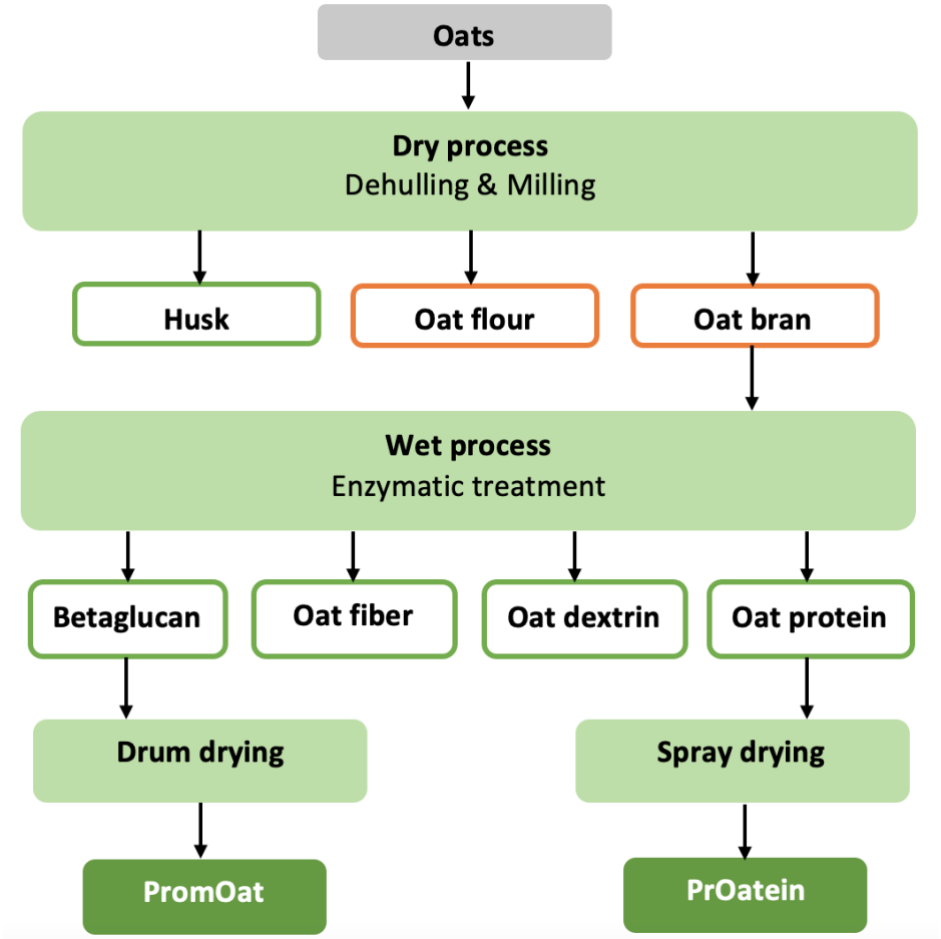

Figure 2: Processing scheme for oat fractioning [10].

2.2 Oat Proteins

This section contains a background on protein structure, quality, and protein fractions in oat.

2.2.1 Protein Structure and Shape

Protein structure is commonly divided into four levels. The primary structure is referring to the sequence of amino acids in the polypeptide. It starts with the N-terminal and ends with the C-terminal. The polypeptide can then fold into specific repetitive patterns, which is called the secondary structure. The main folding features are α-helics, β-sheets and hairpin turns (containing β-turns). α-helices and β-sheets are displayed in Figure 3. These structures are determined by dihedral angles in the bonding points of the peptide and stabilized by hydrogen bonds. The tertiary structure is a description of how the peptide folds in space. Different types of bonds and interactions will affect this global conformation of a protein. Some types of bonds are e.g., disulphide, salt bridges, hydrogen bonds, and hydrophobic interactions. If a protein consists of several polypeptide chains, their conformation in subunits is described by the quaternary structure [13].

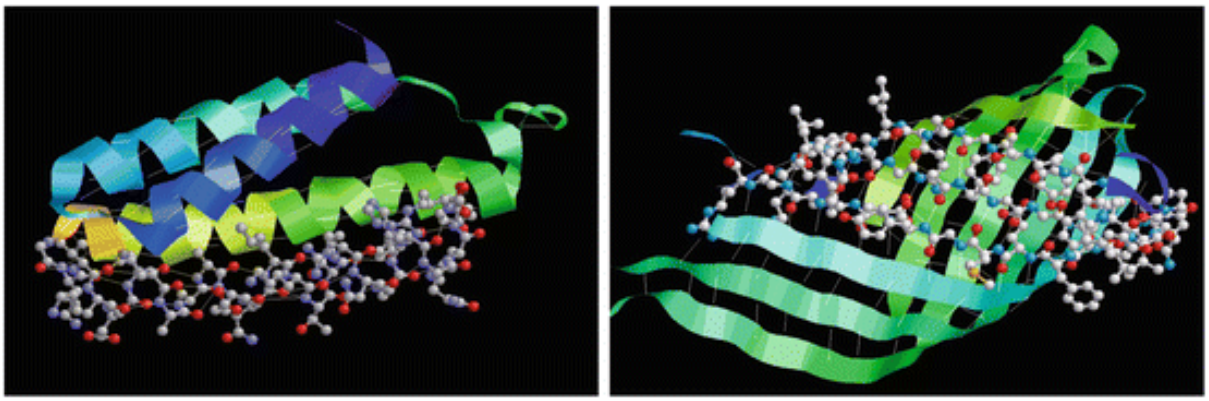

Figure 3: Secondary structures of proteins, α-helices (left), and β-sheets(right) [13].

The secondary structure of oat proteins has not yet been the subject of many research studies, but Liu *et al.* [14] investigated the content of different structure-components in oat protein isolates (OPI). The study revealed through Fourier transform infrared spectroscopy that β-sheets are the most abundant structural feature in OPI at 74%, followed by α-helices and β-turns at 19% and 7% respectively.

The shape of OPI has been determined to mainly consist of the disk-like shape and the ellipsoidal shape. When low protein concentrations occur, the OPI exists in isolated form and has a size of roughly 12-15 nm. If the OPI concentration increases towards 1.0 mg/mL, the proteins start to self-assemble into clusters. This aggregation is the reason why the average protein size is found to increase with increasing OPI concentration [14].

2.2.2 Oat Protein Quality

Glutamine-glutamic acid is the most common amino acid in oats and composes almost 25% of the total amino acids. Aspartic acid and leucine are also quite abundant at 8.9% and 7.4% respectively [3]. When compared to other cereals such as wheat and rye, oats contain the essential amino acids in larger amounts, except for tryptophan. The amino acid that is present in lowest amount in relation to the need for most cereals, thus the limiting amino acid, is lysine [15]. Oat contains substantially more lysine compared to both wheat and rye, but the amount is still not enough to reach the dietary reference standard, lacking 1.0g of lysine per 100g protein [6],[7]. All indispensable amino acids in oat, compared with the dietary reference standard for adults, are presented in Table 1 below. Cysteine and Tyrosine are not truly essential amino acids as they can be synthesized in the body, but for this, methionine and phenylalanine are required as precursors. Therefore, they are simultaneously taken into account when considering the dietary requirements [4].

Table 1: The amount of essential amino acids (grams/100 grams of protein) in oat compared to the dietary reference standard.

Essential Amino Acid	**Oat** [6]	**Dietary Reference standard** [16]
Histidine	2.1	1.7
Isoleucine	3.8	2.3
Leucine	7.2	5.2
Lysine	3.7	4.7
Methionine + Cysteine	4.5	2.3
Phenylalanine + Tyrosine	8.4	4.1
Threonine	3.4	2.4
Tryptophan	1.3	0.6
Valine	5.1	2.9

The different tissues in the oat groat can differ in their amino acid composition. An overview of the differences between the relative amounts of amino acids in the oat bran and endosperm is displayed in Table 2. It can be observed that they are quite similar, but the oat bran proteins contain more of proline, glycine and alanine while the oat endosperm proteins consist more of glutamic acid, isoleucine and phenylalanine [17].

Table 2: Amino acid composition in oat bran and oat endosperm, as well as the total protein content [14].

Amino acid composition (% of total amino acids)		
Amino acid	**Oat bran**	**Oat endosperm**
Lysine	4.1	3.7
Histidine	2.2	2.2
Ammonia	2.5	2.9
Arginine	6.8	6.6
Aspartic acid	8.6	8.5
Threonine	3.4	3.3
Serine	4.8	4.6
Glutamic acid	21.1	23.6
Proline	6.2	4.6
Half-cystine	2.4	2.2
Glycine	5.4	4.7
Alanine	5.1	4.5
Valine	5.5	5.5
Methionine	2.1	2.4
Isoleucine	3.8	4.2
Leucine	7.4	7.8
Tyrosine	3.5	3.3
Phenylalanine	5.1	5.6
Total protein content	18.8	9.6

An important protein quality parameter to consider is that not all proteins can be utilized efficiently in our bodies. How well a protein can be digested in the gastrointestinal tract is described by its digestibility percent. Oat proteins have a high digestibility score of 90%, which is in level with those for rice and corn [4].

Different food items are considered to have different protein quality depending on their amino acid composition. One commonly used method to evaluate this is the protein digestibility corrected amino acid score (PDCAAS). It uses the concentration of the limiting amino acid, lysine in the case of oats, which is compared to a reference pattern and corrected for the protein digestibility. The PDCAAS for oat was calculated to 45-51%, which is high compared to other cereals such as wheat (PDCAAS 42%), but low in relation to soy protein (PDCAAS 91-93%) and proteins of animal origin [3]. To obtain a diet with high quality protein, oat could be complemented with legumes that are rich in lysine but low in sulphur amino acids [18].

Another food quality evaluation method is the protein efficiency ratio (PER). It is calculated as the weight gained of the subject per gram protein that is consumed [19]. The PER value from oat proteins is 2.3 g/g, which can be compared to milk proteins that are considered to be of high quality and have a PER of 3.1 g/g. Lower scores are obtained from other plant based food such as wheat proteins (1.5 g/g) and soy proteins (0.46 g/g) [3].

2.2.3 Oat Protein Fractions

Osborne fractionation is a frequently used technique to classify oat proteins. They are divided based on different solubilities into albumins, globulins, prolamins and glutelins [17]. How the oat proteins are divided into the different fractions varies some between different studies and different extraction methods, but generally the globulins comprise approximately 50-80%, prolamins 4-15%, albumins 1-20% and glutelins <10% of total proteins [10],[11]. The amino acid composition of the protein fractions can be found in the Appendix section A.1.

These fractions also differ quite a lot in the isoelectric point (pI) of the proteins.

Table 3 displays these variations where albumins have the lowest pI and prolamins the highest [3]. In the following sections these oat protein types are discussed more in depth.

Table 3: Isoelectric points for the different oat protein fractions [3].

Oat protein fraction	pI
Albumins	4.0-7.5
Globulins	~5.0
Prolamins	5.0-9.0
Glutelins	-

2.2.3.1 Globulins

The major part of the oat storage proteins consist of globulins, which are salt-soluble. Globulins of 3, 7 and 12 S have been identified in oats, with 12 S globulin being the most abundant one. 3S and 7S globulins are most abundant around the embryo and 12 S globulins in the endosperm. 12S globulins possess two dominant polypeptides, the acidic α-polypeptide and the basic β-polypeptide, with a combined molecular weight of 54 000 Da of one subunit. The protein is

composed of six subunits, making it a hexamer with a total molecular weight of 322 000 [8]. Each subunit is held together by disulphide bonds, and the subunits in globulin use non-covalent interactions to stay in place [3]. The quaternary structure of 12 S globulins is comparable to that of 11 S globulins in soy and leguminous seeds [8][3].

In terms of amino acids, the polypeptides in oat globulins contain high amounts of amidated amino acids and are low in amino acids that contain sulphur [17]. The peptides are internally held together mainly by β-sheets (74%), but also a few α-helices (19%) and β-turns (7%) as well. It has been observed that this secondary structure can be altered through changes in pH, with β-sheets as an example that will transition to random coil when the environment changes from neutral pH to very acidic or basic. Also, the tertiary structure undergoes structural changes due to alterations in pH, but it is mainly sensitive to low pH. Globulin's stability at alkaline pH is thought to possibly be a result of fewer acidic amino acids in between the subunits, which would then lead to less ionization at high pH and less electrostatic repulsion [3].

2.2.3.2 Prolamins

The alcohol-soluble protein fraction are the prolamins, which are called avenins in oat. Their molecular weight differs from 17 - 34 000 Da and contain multiple polymorphic components [17]. Oat prolamins are sulphur-rich proteins and in respect to their amino acid composition they contain proline and glutamic acid in higher amounts, and low amounts of basic amino acids. The leucine and valine content is also quite high, resembling that of rice [8]. Eight residues of cysteine were discovered in each avenin part, and they are responsible for forming disulphide bonds within the chain [3], [17]. Furthermore avenins have a low net charge at almost any pH, a feature resulting from the low content of charged amino acids [3]. Overall, the amino acid composition in the avenin polypeptides were found to be homologous [8].

The primary structure of avenins is characterized by two repetitive regions, which are rich in proline [3]. This can be compared to other members of the subfamily *Festucoideae*, to which oat belongs, that only have one proline-rich region [8]. Concerning the prolamin secondary structure, there is a high presence of α-helices in the non-repetitive sequences and the proline rich parts creates β-reverse turns [3].

2.2.3.3 Other Proteins in Oat

Albumin comprises the water-soluble protein fraction in oat and contains many of the oat proteins that are metabolically active. Most of them are enzymes, such as proteases, lipases, maltases, phosphatases and α-amylases [8]. The lipase activity is high in oat and it usually needs to go through a treatment, for example heat treatment, to inactivate the enzymes to prevent fat degradation and rancidification of the food product [20]. The lipases are not evenly distributed in the oat kernel, with around 90% in the oat bran fraction and only 10% in the endosperm. Among the albumins there are also proteinase inhibitors. Their function is to control protein hydrolysis and are considered antinutritional. The albumins have a more balanced amino acid composition compared to the other protein fractions, due to that the levels of lysine and alanine among others are high [8].

Glutelins are the last protein fraction, and these proteins are soluble in alkaline buffer solutions, possibly also containing reducing agents and detergents. These proteins have a low molecular weight of approximately 9 000 Da [8].

2.3 Protein Functionality

Proteins have different physicochemical functions that will contribute to how they behave in food systems, and this is referred to as protein functional properties. The functional properties will also affect the quality of food and the sensory attributes [21]. These functional features are decided by several characteristics of proteins, such as molecular size, charge distribution, structure and interaction with other food components [3]. The environment that the proteins are located in, as well as changes to that environment, can further affect the functional properties [22].

The most important functional properties of proteins are solubility, water-holding capacity, fat-binding capacity, gel forming, emulsification, rheological behaviours and foaming ability [22]. These properties and what affects them will be discussed further in this section.

2.3.1 Solubility

The amount of protein that can be dissolved into a solution is considered the protein solubility [21]. This is an important functional property since it often is a prerequisite for other functional attributes, and it is also critical if the protein is to be included in liquid food or beverages [22], [23]. For proteins, which almost always includes some hydrophobic parts, there will be hydrophobic interactions favouring protein aggregation. When the electrostatic repulsion between units is stronger than the hydrophobic interactions, the protein is soluble in the solvent. Furthermore, the amino acid composition in the protein can influence solubility, where charged amino acids contribute to electrostatic repulsion, thus increasing solubility. In aqueous solutions, charged amino acids contribute to increased interactions with water molecules [23]. In addition, the structure of the protein and how it folds and assembles is decisive for the solubility since it can determine what amino acids will be located on the surface, and hence in contact with the solvent [24].

The pH of the medium is an additional crucial factor for protein solubility. At the isoelectric point where proteins have a zero-net charge, they are the least soluble [22]. This is due to less charge that can result in electrostatic repulsion, meaning protein attraction will dominate [23]. pH is therefore a useful tool to control and adjust solubility. Protein solubility is also susceptible to temperature and ionic strength.

2.3.2 Water Holding

Water-holding capacity, WHC, is a functional characteristic that measures the amount of water one gram of protein can absorb. The main mechanism behind it is direct protein – water interactions, such as hydrogen bonding. Reducing these interactions will make proteins aggregate and lower the WHC [25]. Water holding capacity is an important factor in food formulation since it can greatly affect the texture of food. Low WHC can make food products susceptible to humidity, and high WHC can dehydrate other components in the food product [26].

2.3.3 Emulsification

An emulsion is when one liquid is dispersed in another, in the form of droplets. They can be either water-in-oil or oil-in-water emulsions [27]. Emulsions are thermodynamically unstable, but proteins with emulsification capacity can be used to increase the stability [25][27]. Proteins adsorb at the interface between the two phases and lowers the surface tension. This inhibits coalescence of droplets, and the emulsion stays intact. For a protein to work as an emulsifier, it should be amphipathic and have good surface activity. The protein structure at the interface is also an

important feature. Furthermore, the properties of the surrounding environment, such as pH and ionic strength, additionally influences the emulsifying capacity of the protein [25]. Food products such as processed meat, salad dressings and mayonnaises require emulsifiers to prevent phase separation [27]. Emulsification can be measured through emulsion activity index (EAI) and emulsion stability index (ESI). EAI can be defined as how much oil a certain amount of protein can emulsify and ESI as the speed of the phase separation that occurs when the emulsion is stored [28].

2.3.4 Foaming

A foam is created when air bubbles are incorporated and dispersed in a liquid. For this to be feasible, protein surfactants can be added to adsorb at the air-water interface and reduce the surface tension. Without surface-active molecules at the interfacial layer, air bubbles will collapse or coalesce due to the liquid film layer between them not having the required strength. Therefore it is reasonable that the ability to form this film is an important characteristic for proteins' foaming capability [27]. Proteins with small and flexible structures have been observed to be useful as emulsifiers in foams, and protein-protein interactions between them is crucial for the stability [25]. Foaming ability is an important functional property in the food industry, since it enables the creation of ice creams, whipped toppings and cakes to mention a few [27].

2.3.5 Gelation

Gel formation is a functional property that is used in development of e.g., baked products, sausages and puddings [25]. It contributes to elasticity and texture of food products, and can be described as a mass that is well-hydrated [25], [27]. A gel is a three-dimensional network created by proteins and sometimes also polysaccharides. As the proteins unfold, the hydrophobic regions come into contact with the water. The proteins then tend to aggregate, which is facilitated through different interactions, for example hydrogen bonding, disulphide bridges and hydrophobic interactions. Gel formation can be induced by enzymes, chemicals or heat treatments [25].

2.3.6 Fat Binding

How much oil that can be absorbed per grams of protein is referred to as the fat-absorption capacity, or sometimes also the fat-binding capacity (FBC). A protein with high amounts of hydrophobic amino acids often has a high fat-absorption capacity since they can bind more hydrocarbon chains. The size of the protein is also of great matter, where small proteins with low density are favourable. Fat-holding is an important property to achieve a desirable texture of a food product, and retain fat during cooking [25].

2.3.7 Thermal Properties

Thermal properties of proteins can be determined through differential scanning calorimetry (DSC). It is based on that a material either can adsorb or radiate energy when going through a transition. DSC is an instrument where a sample and a reference is heated up in a linear heating rate, and the difference in temperature of the two materials is measured. This method provides information regarding how the physical properties of the protein's changes with temperature and time, and is commonly used to observe peak denaturation temperatures, phase transitions and conformations in biomolecules [29]. DSC can also give information about purity and amounts of the components in a material.

2.3.8 Functionality of Oat Proteins

The functionality of oat proteins has previously been analysed in several scientific articles. Even though similar methods are used in the analysis, it can be difficult to compare the result since the extractions have been executed in varying ways, with varying aims. Also, different parts of the oat grain, and different species of oat have been used. Therefore, the results in this section should be interpreted with care.

Oat proteins are considered to have a lower solubility compared to other plant proteins, and at the pH in most food (neutral or slightly acidic) it is quite limited. One suggested explanation to this is that they have less charged amino acids, and that the C-terminus of the α-subunit contains a lot of glutamine [3], [22]. With this glutamine-rich sequence at the surface of the protein, it becomes less hydrophilic which decreases the solubility [3]. Chen *et.al.* suggested that the percent protein solubility (PPS) can range between approximately 10-20%, depending on the oat species, in oat protein concentrates (OPC), which is extracted protein with a protein content of 65-90%. Defatted OPIs, which have a protein content of over 90%, exhibit a higher PPS, due to both less lipids in the material and also that the subunit composition differs some between protein concentrations [30].

Oats generally have lower or similar functionality compared to other cereals such as wheat and barley. One exception is the foam stability, where oat proteins created a more stable foam. This is thought to be due to oats having higher levels of albumin and globulin proteins compared to the other two cereal types. These proteins can then migrate and stabilize the air-water interfaces in the foam quicker than prolamins, thus decreasing the degree of foam collapse [31].

In Table 4, different functional properties of oat protein isolates are presented. The result is from four different studies that all used alkaline extraction to obtain the protein isolates. All oats were defatted prior to extraction to facilitate extraction.

Table 4: Oat protein isolates functional properties from four different scientific articles. EAI stands for emulsion activity index, ESI for emulsion stability index, WHC for water holding capacity and FBC for fat binding capacity.

Oat protein isolate functionality						
Source	**EAI [m²/g]**	**ESI [min]**	**WHC [g/g]**	**FBC [mL/g]**	**Foam capacity [ml]**	**Foam stability [% after 30 min]**
Biresaw et al [32]	60.8 ± 3.9	29.0 ± 1.2	0.092 ± 0.0	-	106 ±7	-
Ma [33]	23.2	-	0.8	1.4	-	50
Kadivar et. al. [34]	49.0 ± 4.76	62.6 ± 0.3	1.27 ± 0.06	-	280 ±9.48	Approx. 85
Ma [35]	40.4	6.2	2.00	2.10	-	30

The peak denaturation temperature (T_d) can be used to evaluate thermal properties of oat proteins. A study performed by Chen *et al.* [30] revealed a T_d of approximately 109-110°C for different varieties of native oat proteins. Compared with other plant proteins, such as peas (T_d of 85°C) and hemp (T_d of 89°C), oat has a high temperature, and it was observed that it corresponds with that

of globulins in oat. A high peak denaturation temperature could indicate that the oat proteins could be useful in thermal processed food systems. Oat proteins with a high amount of lipids tend to exhibit a lower peak denaturation temperature compared to more concentrated oat proteins. This could be due to hydrophobic protein-lipid interactions [30].

2.3.9 Functionality of Protein Classes

The functional properties of different oat protein fractions have not been investigated thoroughly, but Ma & Harwalkar [36] performed a study examining this topic in 1984. Regarding the solubility, albumins were found to have the highest solubility since they were soluble over a large range of pH-values, as can be observed in Figure 4. Both globulins and prolamins revealed bell-shaped curves for the solubility, where globulins have their solubility minimum at pH 6-7 and prolamins at 5-6. Glutelins have the lowest solubility with poor solubility in pH ranging from 3 to 8. Globulins, prolamins and glutelins all exhibited higher solubility at alkaline pH compared to acidic [36].

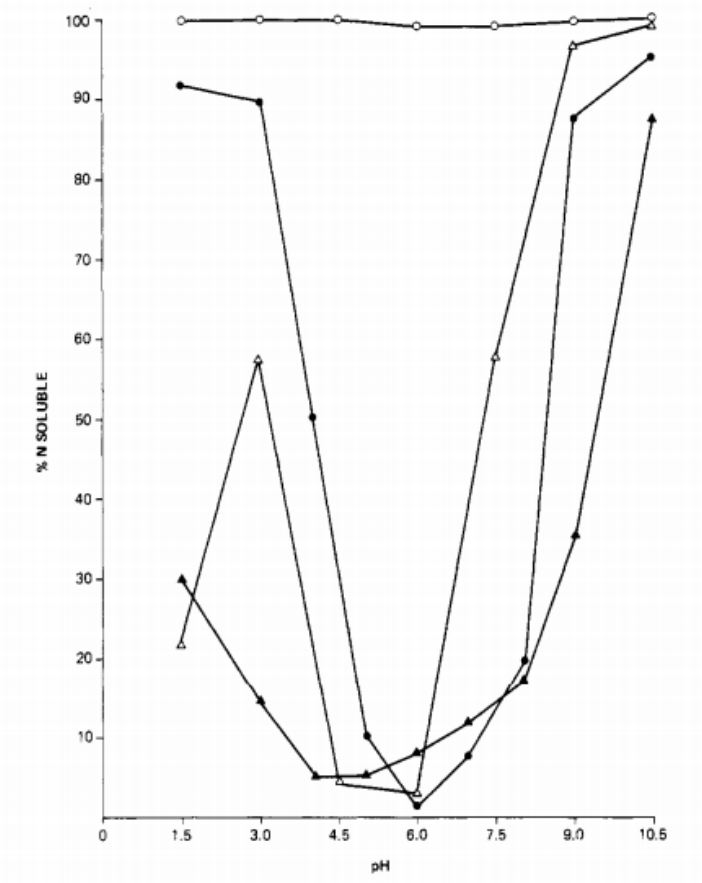

Figure 4: pH-solubility for the different oat protein fractions; albumins (circle), globulins (filled circle), prolamins (triangle) and glutelins (filled triangle) [36].

Furthermore, emulsifying properties, fat-binding capacity, water hydration capacity and foaming properties were also investigated in the study [36], and the results obtained are presented in Table 5. Albumins are clearly ahead in foam-ability, WHC and FBC, while glutelins have the best EAI and prolamin are superior in ESI and long-time foam stability. Globulins only exceed the other fractions in short-time foam stability [36].

Table 5: Functional properties of oat protein fractions [36]. EAI stands for emulsion activity index, ESI for emulsion stability index, WHC for water holding capacity and FBC for fat binding capacity.

Protein fraction	EAI [m^2/g]	ESI [min]	FBC [mL/g]	WHC [mL/g]	Foam-ability [%]	Foam stability [%]	
						30 min	60 min
Albumins	31.2	3.2	2.8	2.4	240	70	47
Globulins	27.6	3.0	1.6	0.8	100	73	60
Prolamins	36.8	9.5	1.7	0.9	50	67	63
Glutelins	45.0	2.0	2.1	1.9	45	37	27

Ma & Harwalkar [36] also investigated the protein fractions using DSC. Both albumins and globulins presented endothermic peaks, where albumins had a T_d at approximately 87°C and globulins at approximately 110°C. The shapes of the peaks were quite different since the albumin fraction displayed a very broad peak while the globulins revealed a narrower and sharper peak. This indicates that the globulin fraction is more homogenous compared to the albumins. Prolamins and glutelins did not present any thermal reaction that could be detected in the analysis. A possible reason for this in the case of glutelins is that they could have been thoroughly denatured in the extraction process and hence loses its ability to undergo thermal reactions. For prolamins it could be that many hydrophobic bonds were disrupted which is an exothermic reaction, and therefore those reactions could have cancelled out the endothermic reactions [36].

2.3.10 Protein Modifications for Improved Functionality

Modifications of proteins is a tool that can be used to improve the applicability of oat proteins in food, and can be done through increasing the functional properties such as the solubility [32].

Acylation is a chemical modification method where an acyl group is added to the protein [25]. This can be performed in two ways depending on the acylating agent used, acetic anhydrides in acetylation or succinic anhydrides in succinylation. In acetylation, a basic group on the amino acid is altered into a neutral one, which results in a change towards a more negative net charge of the protein. Succinylation also shifts the net charges of the protein to a more negative one, but by transforming positive charges to negative charges. This can lead to subunit dissociation in oligomeric proteins such as globulins. All amino acids can be acylated, but lysine has been observed as more accessible to the treatment [3]. Both acylation and succinylation improved the solubility of oat proteins at pH 5 and pH 7, although the effect of succinylation was more pronounced. At pH 3 the solubility was increased at low levels of acetylation but decreased at high levels and with succinylation [37]. In addition, acetylation was found to improve other functional properties such as EAI, ESI, FBC and foam ability, while foam stability was decreased [35]. The effect of acetylation on oat proteins differs between studies, where both positive and negative results have been achieved [35], [37]. Succinylation was observed to increase EAI, WHC and FBC for defatted

oat protein, while the ESI decreased significantly and the foaming capacity remained unchanged, according to Mirmoghtadaie *et al.* [34]. Another study found that also the ESI was improved by the succinic anhydride treatment [35]. A downside of acylation is that the bioavailability of lysine can be decreased, which would lower the protein quality [3].

Another common modification method is deamidation. During deamidation, an amide group is removed from amino acids which leads to an upturn in carboxyl groups. The free carboxyl groups are then transformed into carboxylate ions and the net charge is increased [38]. Deamidation can be obtained both from enzymatic, physical, and chemical treatments. Examples of enzymes that can deamidate food proteins under certain conditions are transglutaminase and proteases [3]. Chemical deamidation methods with hydrochloric acid (HCl) have been shown to increase the solubility of oat proteins in several studies [34], [38]. This is thought to be due to decreased molecular size and increased protein-water interaction as a result of the increased net charge. Deamidation also improved EAI, WHC, FBC and foaming capacity [34].

Enzymes can also be used for hydrolysis treatment of oat proteins. Alcalase, pepsin and trypsin are examples of enzymes that are commonly utilized industrially for this purpose [39]. Enzymatic hydrolysis is a process with mild conditions, and it is effective since it provides low formations of by-products. The degree of hydrolysis (DH) is of importance when considering the method since different DH can give different effects on the functional properties. Too high a degree of hydrolysis can lead to formation of undesirable flavours. Hydrolysis causes the surface net charge to increase, which in turn leads to improved emulsion capacity and emulsion stability [40]. It has also been observed that hydrolysis by trypsin and alcalase can significantly improve the solubility of OPIs at pH 4, but at neutral pH no difference was obtained. In addition, trypsin hydrolysis positively affected the foaming properties of the proteins at pH 4, which normally is quite limited. This broadens the possible applications of OPIs in food products [41]. When it comes to gelation properties, Chen *et al.* [39] found that partially hydrolysed oat proteins are less likely to form gels compared to untreated OPI, but the gels that form can have high mechanical strength in levels with egg white proteins [39]. Other positive outcomes of hydrolysis is that the antioxidant activity can be increased and it can contribute to decreased deterioration of food that contains a lot of oil [40].

2.4 Applications of Oat Protein

A combined demand for high-protein products and plant-based diets increases the need for sustainable protein sources such as oat protein [42],[43]. Thus the global oat protein market is expected to grow at a compound annual growth rate of 4.0% between 2019-2027 [44]. However, finding the right application where oat protein fulfils the nutritional, technical and sensory requirements can be a challenge [45]. The most important role of protein in food is to provide nutrition, however they are also implemented for their physicochemical properties such as forming and stabilizing foams, emulsions, suspensions and gels [24]. The main limitation of oat proteins is their low solubility within the pH-range of most food, see section 2.3.8, making it more difficult to use in products with a high water content such as beverages. Despite this, the beverage market for oat proteins is seen as a major market segment in future years, as the beverage market has the fastest growth rate [43]. However, this may require protein modifications for improving solubility in order to secure the market stance for oat proteins in beverage application.

Other possible applications of oat proteins are in baked products where they could be used to increase protein content, influence loaf volume and has been seen to decrease starch retrogradation [3]. The high peak denaturation temperature of oat globulin means they can be used in food products undergoing thermal processes [30], such as meat analogues. Oat proteins have also been tried in fermented yoghurt products which saw positive effects on the nutritional and sensorial qualities of the yoghurt [46].

Even though oat proteins have had some successful applications, the use of oat protein is still limited due to their poor functionality. Therefore, efforts need to be made moving forward to improve the functionality of oat proteins to meet the standards of the food industry [46].

2.5 Protein Extraction

Protein products can be divided into three categories depending on their protein concentration, flours (<65%), concentrates (65-90%) and isolates (>90%) [42]. For simplification the term 'protein powder' is used in this report as a joint label for the three types of protein products, independently of their protein concentration.

Proteins in plants are most often found in protein bodies within cell walls, which means cell disruption is needed for proteins to be released, enabling solubilization and extraction [47]. Methods to produce plant protein powders can be separated into wet and dry fractionation methods, where wet methods often produce powders of higher purity, although requiring large quantities of water, solvent and energy for drying compared to dry methods [3]. Wet fractionation methods traditionally uses a liquid of either alkali, acid, saline or water to extract the protein which is then precipitated at its isoelectric point or separated using membrane filtration before it is dried [3]. Dry methods often utilize milling and separation techniques based on density such as air classification, or newer technologies e.g., electrostatic separation [42].

Choosing the type of extraction process depends on the required yield, protein purity, intended use and resources that are available. Other than this, the environmental impact is important to consider.

Protein extraction from oat has been evaluated multiple times, as can be seen in Table A 2 in the appendix. In studies on wet protein extraction it is most common to use an alkaline solution to solubilize the protein as it has shown to give a higher protein yield compared to salt, acid and water [33], [48], [49]. Although salt provides a milder extraction with less risk of denaturing proteins, the yield is very low, and according to Ma [33] the amino acid profile and functionality was better with the alkaline extraction compared to salt. Also, the high fibre content in oat bran requires alkaline treatment in order to achieve a good protein yield [50]. However, alkaline extraction requires large amounts of alkali solvents, energy and produces sodium salts during isoelectric precipitation [42]. The harsh environment that alkaline extraction subjects the proteins to can lead to changes in functionality due to either denaturation or other alterations in the protein structure [3]. Alkaline extraction is the focus of this thesis and is discussed further in section 2.5.1.

It has also been investigated whether a combination of acid and alkali treatment can be more effective than a single-step alkali extraction. Kadam *et al.* [51] performed protein extraction on Irish Brown Seaweed with different combinations of acid and alkali. The study found that a treatment of acid first followed by alkali was most effective and was believed to be due to that the

acid helped release complex polysaccharides that then allowed for easier solubilization of the protein with alkaline treatment. Single step alkali extraction also showed high protein yield, while the extraction was lowered by using an alkaline treatment followed by acid. Lowest yield overall was found with single step acid extraction. The acid treatment can be compared with pre-treatment methods such as ultrasound, enzymes and more which are discussed further in section 2.5.2.

A newer type of wet extraction method is to use enzymes to degrade plant cell walls and solubilize protein. Enzymatic protein extraction is often considered a milder alternative to alkali, and also has a lower environmental impact [52]. However, it associated with high processing costs, mainly due the high price of enzymes, as well as close monitoring of extraction conditions [42]. The protein yield in enzymatic extraction is also generally lower than with alkaline methods[53], although if enzymes are used in combination with alkali, e.g., pre-treatment, the yield is often higher as discussed further in section 2.5.2.2. Alternatively, the raw material could be processed with mechanical methods such as sonication, which could increase the yield during enzymatic extraction [53]. Enzymatic extraction is what Lantmännen uses today in their production of PrOatein, however their process includes a heat treatment which is thought to cause some denaturation of the protein leading to reduced functionality.

Alternative to the wet extractions methods is dry fractionation as mentioned earlier. Most common of those is air classification which has been performed on oat [54]. Although dry fractionation often produces protein powders of lower protein content it is argued that there are few food product applications which need such high protein concentrations. A slightly lower protein purity may therefore still be useful for many food applications, and air classification has the benefit of lowering both energy and water consumption [55]. Sibakov *et al.* [54] showed in their study the possibility of using air classification to obtain a protein concentrate of 73% purity from the otherwise low-value endosperm flour fraction. The mass yield was 5% which accounts for a protein yield of around 20%. However, the material must be defatted prior to pin milling and air classification to be able to obtain a protein concentrate.

New and innovative extraction techniques have been widely studied in the last decades. They have the objective of being more environmentally friendly in comparison to traditional methods such as alkaline extraction. However, they are still mostly studied and performed in lab scale and more studies on pilot and industrial scale needs to be done to know their economic viability [42].

2.5.1 Alkaline Extraction

Alkaline extraction works by degrading cell wall components which helps release the proteins into the solution [56]. However, alkali is only able to degrade hemicellulose and lignin in the cell wall whilst cellulose is relatively unaffected [57]. According to a study by Sari *et al.* [56] where alkaline protein extraction was performed on sixteen types of biomass, oat not included, cellulose showed a strong negative correlation with protein extractability. To increase extraction yield in plant material with high levels of cellulose, a pre-treatment with cellulose-degrading enzymes can be performed as discussed previously in section 2.5.2.2. The study also showed that oil content was negatively correlated with protein yield, which was believed to result from oil acting as a barrier for protein in the cell [56]. Another suggestion for the negative influence of oil on protein extraction is that it forms lipid-protein complexes [58]. Due to this, defatting can be used as a pre-treatment method, discussed in section 2.5.2.1.

Alkali not only degrades cell walls, but it also acts as a solubilizer of protein as the proteins have a higher solubility at alkaline pH [59]. Proteins' pH dependency is often utilized in the extraction process to separate the protein from the solution through isoelectric precipitation (IEP), where pH is lowered to around the isoelectric point of proteins where their net charge is zero. This causes the proteins to aggregate and precipitate in the solution, and these can then be collected through e.g., centrifugation and dried creating a protein powder [3], [42].

An alternative to IEP as separation technique is ultrafiltration (UF), which uses a membrane that utilizes the hydrodynamic properties of proteins to separate them from the extraction solution [60]. A study performed by [61] on rice endosperm showed that UF provided milder separation conditions that resulted in improved solubility and emulsification properties compared to IEP. Filtration also avoids the generation of salts [59]. However, the yield and protein content were lower for UF than IEP. Another study focusing on legume proteins showed similar result, ultrafiltration did not improve protein yield or content but generally enhanced functionality [60]. Although a problem that can occur with alkaline extraction of oats is that it can often become highly viscous, making filtration difficult [62].

2.5.1.1 Effect of Processing Conditions on Yield

How much protein is extracted depends on the processing conditions, and some parameters will also affect the functionality and protein composition of the resulting protein powder. The main processing conditions for alkaline extraction are pH, time, temperature and ratio of solid-to-solvent [59].

The effect of temperature during extraction on protein yield is mainly connected to time, where an increase in either time or temperature results in higher yield [59]. This is due to that reaction speed increases with temperature as the collision rate of reacting molecules increases [63]. Although, a high temperature may also lead to denaturing of proteins resulting in impaired functionality. Studies performed on oats have varying extraction temperatures of around 20-50°C, Table A 2 in appendix, but none of the studies specifically evaluated temperature effect. Sari *et al.* [56] found that most protein is extracted already at 25°C for many types of biomasses. However, those extractions were performed with an incubation time of several hours. Most of the studies performed on oats, Table A 2 in appendix, has been done during a timeframe of under one hour, and Cluskey *et al.* [49] concluded in their study that no significant difference in yield was obtained between incubation time of 25 and 60 min. However, alkaline extraction performed on other biomass material such as tea [64] has shown that increasing extraction time from one to six hours significantly increased the protein yield. This is believed to be due to that the longer time allows for more protein to be released into the solvent [59]. Nonetheless, a study on alkaline protein extraction of hempseed press cake did not show any significant differences in yield between one and four hours of incubation time [65].

The ratio of solvent to solid effects the protein yield as the protein concentration in the biomass shall be higher than in the solvent to maintain the driving force for protein to be solubilized [59]. Both Cluskey *et al.* [49] and Paraman *et al.* [61] found that increasing the ratio of solvent increased the protein yield during alkaline extraction. However, both studies conclude that the optimum ratio is not only determined by the yield but is a balance between yield and cost of solvent and waste disposal which makes industrial implementation more difficult. Higher amounts of solvent

also increases cost of downstream processing of e.g., drying to obtain a protein powder, and increases the environmental impact of the process due to generation of large amounts of sodium salts [59].

pH largely effect the protein yield during extraction and earlier studies [48], [49] on oat protein have found that an optimum pH lies around 9-9.5 which has then been utilized in later studies [33], [50], [66]. As discussed in section 2.3.1 the solubility of proteins is better further from their isoelectric point, which is at more acidic pH. Therefore, alkaline pH is effective for protein extraction. However, if the pH is too high it risks denaturing the protein which will impact the functionality [59]. The impact of processing parameters on protein functionality is discussed in section 2.6.

In a master thesis by Forsén [65], alkaline protein extraction from hempseed press cake was optimized. The study evaluated, amongst other, the effect of keeping pH constant during the alkaline treatment as opposed to adjusting it in the beginning and then letting it fluctuate during the incubation which is the traditional method. The result showed that keeping a constant pH resulted in significantly higher yield, especially at lower pH levels. Constant pH allowed for using a lower alkaline pH during the extraction whilst still obtaining a high yield, thus creating a milder process.

When it comes to pH for isoelectric precipitation it varies between studies on oat, Table A 2 in appendix. The studies all lie within the range of pH 4.0-6.0, with many of them around pH 5.5. Although, the choice of pH is seldom discussed or motivated. In an earlier study by Wu *et al.* [48] a maximum amount of protein was found to be precipitated at either pH 5.0 or 5.7 depending on the type of oat used. However, later studies have obtained similar yields using other precipitation pH [30], [33], [62], [66]. The isoelectric point differs between oat protein fractions as can be seen in Table 3. Globulins have their isoelectric point around pH 5.0, and this could explain why many of the studies have performed their precipitation around this point as globulins are the largest protein fraction.

Lastly agitation rate has been examined for its effects on protein yields during extraction of soy protein [67]. Rates between 200-9000 rpm were examined, however no differences in protein yield were seen. In addition, the power consumption increases significantly with increased agitation rate thus affecting the cost of production.

2.5.2 Pre-treatment Methods

This section reviews some pre-treatment methods that can be used in combination with alkaline extraction.

2.5.2.1 Defatting

Defatting is the method of removing lipids from the plant material before extraction to increase the protein yield, concentration [56] and functional attributes [68]. Most studies performed on oat use some sort of defatting technique before extraction, but only two of the studies have examined the effect of defatting with hexane compared to non-defatted groat. Liu [62] found that the protein content in the resulting powder was higher when defatting but the yield was not improved. Yue *et al.* [30] had similar result, the extracted protein was of higher purity when defatting was applied, however the yield was lower. The lower yield was believed to be caused by either protein

denaturation during defatting, or that phospholipids in non-defatted oat aided in the protein extraction. Yue *et al.* also found that the functionality of the protein powder was improved when defatting. Lipids in oat protein powders are found to impact the foaming capacity, where nonpolar lipids have an especially detrimental effect [3].

There are various methods for defatting, but most commonly hexane is used, due to that it is both readily available and highly efficient at extracting oil [69]. Hexane has also shown to give improved functionality compared to other defatting solvents such as ethanol, water and methanol. Especially emulsification, foam stability and amino acid composition was better in the resulting protein powder [68]. Although, when Wu *et al.* [48] compared hexane and butanol as defatting solvents for oats, prior to protein extraction, butanol was considered better due to that it is able to remove both bound and free lipids whilst hexane is only removes free lipids. This resulted in both higher yield, protein content, emulsifying properties and amino acid profile. However, later studies on oat all use hexane for defatting, Table A 2 in appendix.

Although hexane is commonly used, there are many disadvantages with it. One is that residuals of hexane left after defatting need to be removed in order to be safe for use in food as it is connected to several severe health effects. Also, the removal of hexane requires a lot of energy, combined with that hexane comes from fossil sources and is considered a pollutant means that it is not a sustainable method for defatting [69].

An alternative to hexane is supercritical carbon dioxide (SC-CO_2) which has the advantages of being nontoxic, both nutritionally and environmentally, can be removed from food easily and is relatively inexpensive. However, the oil-extraction efficiency has varied between studies and it is questioned how well it will work in industrial scale [69].

2.5.2.2 Enzymes

Enzymes can be used as pre-treatment of plant tissue in preparation of protein extraction by degrading cell walls and cellular structures that are otherwise hard to break down. This subsequently increases the protein yield by facilitating the release of protein into the extraction solution [70]. Cell wall degrading enzymes that can be implemented include cellulase, hemicellulase and pectinase. Often these enzymes need to be used in combination to achieve optimal effect and therefor pre-made enzyme blends are produced such as Viscozyme® L which has previously been studied for its potential effect on plant-protein extraction [66], [70]. Viscozyme L contains a variety of carbohydrases which degrades cell wall components [71] without causing hydrolysis of protein [70]. Guan and Yao [50] found that using enzymatic pre-treatment of oat bran resulted in a significantly increased protein yield (~56%) in subsequent alkaline extraction, compared with untreated oat bran (~15%). In another study by Jodayree *et al.* [66] defatted oat bran was treated with four different carbohydrase-containing enzymes (Viscozyme L, alfa-amylase, amyloglucosidase and Celluclast®) before an alkaline extraction was performed to obtain protein powders. The study showed that amyloglucosidase, alfa-amylase and Viscozyme L all increased the protein content compared with untreated oat bran. Celluclast however, did not have any effect on protein content.

Apart from cell degrading enzymes, proteolytic enzymes, protease, can also be used to increase protein yield. They work by reducing protein size, enabling extraction. However, the high cost of these enzymes reduces the potential for industrial applications [59]. Also, the hydrolysis that the

protease causes can impact the functionality of the proteins. In some cases, the effect is positive, however it depends on the degree of hydrolysis as discussed in section 2.3.10. Enzymatic treatment using carbohydrases seems to be most beneficial in order to retain functionality, however the yields are lower than when using proteases [53].

2.5.2.3 Ultrasound

Ultrasound (US) can be considered a novel pre-treatment method that is economic, process and energy efficient [42]. Ultrasound works as a cell disruption technique, enabling the release of protein into the solvent during subsequent extraction. Several studies [42], [51], [72]–[74] of different types of biomass, have evaluated the efficacy of using ultrasound-assisted protein extraction both in terms of yield as well as functionality of the resulting protein powder. Due to its ability to create pores in the cell membrane the protein yield during extraction is higher compared with single-step alkaline extraction [51], [72], [74]. However, ultrasound treatment may alter protein structure, resulting in changes in functionality. A study on peanut protein [72] showed significantly lower solubility for US treated powders, as well as lower nitrogen solubility believed to be linked to denaturation of the proteins. Although, the foam activity was higher following US treatment. Another study on soy protein instead showed increased solubility, but lower emulsification and foam capacities [74]. Changes in protein functionality is believed to be caused by conformational changes, such as exposing hydrophobic groups [42].

2.5.2.4 Particle Size Reduction

The effect of particle size on protein extraction has been studied using soybean [67], [75]. Both studies found that a reduced particle size, achieved through e.g., milling, increased the protein yield. Russin *et al.* [75] found that a reduction in particle size from 223 to 89.5 μm increased the protein yield by 30% without affecting the purity of the protein powder. Rosenthal *et al.* [67] saw a difference in extraction yield from ~25% to ~75% when reducing the particle size from 1200 to 150 μm. They suggested that the effect particle size has on extraction yield is mainly due to the disruption of cells allowing for penetration of the extraction liquid.

2.5.3 Alternative Alkaline Solvents

Sodium hydroxide (NaOH) is the most common solvent used in alkaline extraction, however it produces large amounts of sodium salts which makes it a less sustainable option as it requires downstream processing to remove the salt [42]. Zhang *et al.* [76] investigated the possibility of using alternative alkaline solvents for protein extraction from green tea residue. The solvents considered were KOH and $Ca(OH)_2$, and it was also evaluated how a pre-treatment with either weak alkali or Viscozyme L would affect the environmental impact and costs related to the extraction. The results showed that a classic extraction using NaOH had the highest environmental impact compared to profit gained, then all other options analysed. Although, the effect is significantly lower when using a pre-treatment method, mainly due to the increased yield during extraction. KOH is a more expensive solvent compared to NaOH, but the potassium salts generated can either be recycled or used directly as a crop fertilizer, which will limit the costs related to waste management or fertilization. KOH also has a lower environmental impact compared to NaOH. $Ca(OH)_2$ differs from the other two in its extraction mechanics, and is less efficient resulting in a low protein yield. However, the costs is very low and the generated calcium salts can be recycled by bubbling CO_2 through, creating $CaCO_3$, which has the possibility of lowering the need for acids during isoelectric precipitation of the protein [76].

2.6 Relationship between Processing and Protein Functionality

Oftentimes protein yield is invertedly correlated to protein functionality, meaning that extraction parameters that give high protein yields result in lowered functionality of the proteins. For instance, high temperature and pH risks denaturing or altering proteins, leading to impaired functionality, whilst it makes extraction more efficient. High temperature and alkali can also lead to racemization which can reduce the nutritional value of the proteins; thus a milder protein extraction is preferred if the protein is to be used in food applications [59].

In order to produce a commercial protein powder, when using wet methods, the protein-rich material must be dried. A few different methods can be used when drying, the three most common types of drying is freeze, spray and vacuum, where spray drying is the most popular method used in the food industry as it is efficient. However, it can cause some protein deterioration whilst freeze drying preserves quality better but is more time-consuming and expensive. Vacuum drying can cause some degradation from heat and is not as suitable for large scale production but is nevertheless popular [77]. In a study on quinoa protein isolate by Shen *et al.* [77] the previously mentioned drying methods were evaluated for their effect on functionality. It was concluded that freeze drying was optimal from a functionality perspective and caused the least denaturation. Another method for drying is oven drying, however the high temperatures needed to dry make it an unsuitable alternative for protein powders. Although, it is the method used in this report as the dried protein is only used for protein content analysis and dry matter calculations, and not analysed for functionality.

2.7 Learning Outcomes from Literature Study for the Experimental Plan

Important factors when evaluating a protein extraction method is the protein yield and content of the resulting powder to determine the process efficiency and economic viability. Additionally, functionality of the protein is of significance to ensure successful application in food products. The literature review has shown that functionality is dependent on the extraction method, and it often becomes a balance between obtaining a high enough yield and still maintain protein functionality. As an original objective with the study was to find a milder extraction method leading to improved functionality compared with the method used by Lantmännen today, the relation to functionality must be taken into consideration when choosing parameters for the extraction.

Temperature affects protein extraction as increased temperature leads to faster reactions, and it therefore allows for higher yields with a shorter incubation time. However, a high temperature also risks denaturing proteins thus decreasing functionality. Previous studies on oat protein extraction have shown that yields over 50% can be obtained even at room temperature with an incubation time of one hour. Therefore, room temperature seems to be a preferable option.

Yield during extraction is highly dependent on pH as it determines the amount of protein that is solubilized. Previous studies on oat have found a pH optimum around 9-9.5, however none of them have performed extraction using constant pH during the entire incubation time. A study on hempseed press cake showed that a constant pH can allow for lower pH whilst maintaining high yield. A lower pH is desirable as it is milder on both the protein and equipment.

Isoelectric precipitation is a common purification method to use in combination with alkaline extraction as it utilizes the proteins low solubility at its isoelectric point to separate the protein from the extraction solution. Studies performed on oat differ in the pH used for IEP and no real

consensus can be found what is optimal. Most studies lie within the range of pH 4-6, however no information could be found on whether variations within the range lead to differences in yield or precipitation efficiency.

When aiming to maintain protein functionality whilst still obtaining high yields through alkaline extraction, focus should lie on optimizing the extraction at low temperature and pH to provide a mild treatment. Apart from this there are methods for improving functionality that can either be used prior to extraction, defined as pre-treatments, or after extraction through different protein modifications. However, neither pre-treatments nor modifications will be applied in the experiments for this report due to the limitations of the project.

3. Materials and Method

This section describes the methods for the experimental work, along with the methods used for calculations, data interpretation and statistical analyses.

3.1 Materials

Oat endosperm flour (OEF) and oat bran were produced as described in section 2.1.1 and collected from the same batch at Lantmännen, Kimstad. The oat batch was produced in February 2021 and stored at room temperature. The chemicals used were provided by the faculty and presented in Table 6.

Table 6: Presents all chemicals used during the experimental part.

Chemicals
Technical buffer pH 4.01 – Mettler Toledo GmbH, Switzerland
Technical buffer pH 7.00 – Mettler Toledo GmbH, Switzerland
Technical buffer pH 11.00 – Mettler Toledo GmbH, Switzerland
2 M Sodium hydroxide (NaOH) – Merck, Germany, CAS-nr: 1310-73-2
Citric acid (powder form) – Merck, Germany, CAS-nr: 77-92-9
Aspartic acid (granular form) – Thermo Electron, Italy, CAS-nr: 56-84-8

3.2 Experimental set-up

Based on the literature review the parameters of highest interest were extraction and precipitation pH, using constant pH in the preliminary extractions. An experimental set-up was developed based on this, as can be seen in Table 7. To be able to compare the results with studies reviewed in section 2.5.1, one more extraction was performed on each material using non-constant pH instead. The set parameters used in the second extractions were chosen based on the results from the first round.

Table 7: Experimental set-up for the laboratory work.

Test nr.	Raw material	Extraction pH	Precipitation pH	Method
1	Oat endosperm flour	9.5	4.5	Constant
2	Oat endosperm flour	9.5	5.5	Constant
3	Oat endosperm flour	9.0	4.5	Constant
4	Oat endosperm flour	9.0	5.5	Constant
5	Milled oat bran	9.5	4.5	Constant
6	Milled oat bran	9.5	5.5	Constant
7	Milled oat bran	9.0	4.5	Constant
8	Milled oat bran	9.0	5.5	Constant
9	Oat endosperm flour	9.0	4.5	Non-constant
10	Milled oat bran	9.0	4.5	Non-constant

3.3 Methods

This section details the experimental work in chronological order as they are performed.

3.3.1 Preparation of Sodium Hydroxide Solution

A 1 L measuring flask was filled with deionized water to a volume of slightly less than 1 L to avoid spilling while stirring. 80g NaOH, which is two times the molar weight for NaOH, was weighed and added to the water to obtain a 2M NaOH solution. The mix was stirred until all NaOH was dissolved, and deionized water was then added to reach a total of 1 L in the flask.

3.3.2 Milling of Raw Materials

The oat bran was milled using a Laboratory Mill 120 (Perten Instruments, Sweden) prior to extraction in order to decrease particle size, facilitating easier extraction and making it more similar in size to the oat endosperm flour. The milled oat bran (OBM) was then stored in a plastic bag and used in subsequent protein extractions.

3.3.3 Flow Chart

In Figure 5 below a flow chart for the extraction process performed in lab scale can be seen. The same process was used for both oat endosperm flour and milled oat bran. Proteins were extracted from the raw material through an alkaline treatment with sodium hydroxide (NaOH), these are then separated from the non-soluble components through centrifugation and further purified in a precipitation step utilizing the proteins isoelectric point rendering them insoluble. Through a second centrifugation a protein-rich sediment was obtained.

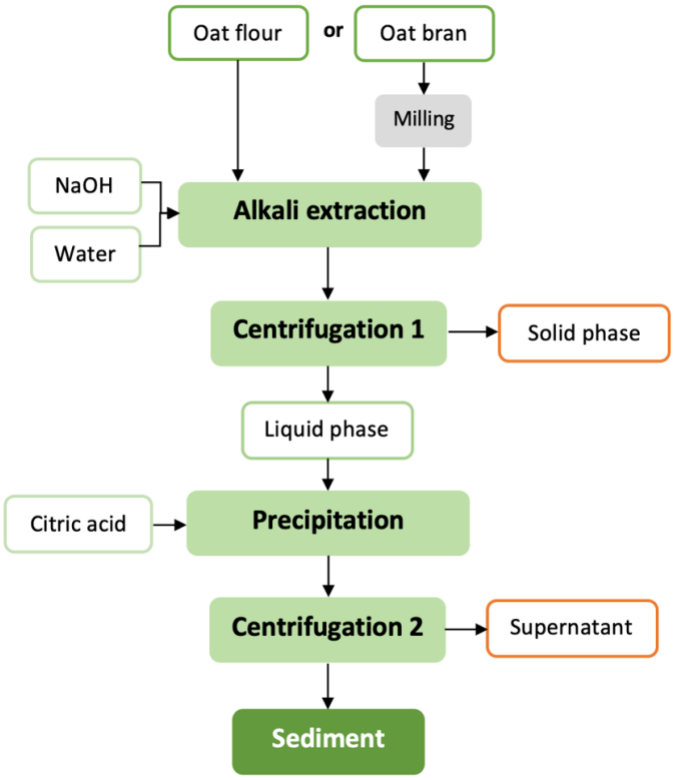

Figure 5: Flow chart of the protein extraction method for both milled oat bran (OBM) and oat endosperm flour (OEF). The sediment is the protein-rich fraction that can be dried to obtain a powder.

3.3.4 Main Extraction Method

The laboratory experiments were performed in the Food & Bioprocesses laboratory at the Kemicentrum at Lund University.

The extraction method used for the experiment is based on the method used by Forsén [65], and parameters decided from the literature review. This general method was used for all experiments, only the targeted pH value was altered according to the experimental set-up in Table 7. All extractions were performed at room temperature and done in triplicates.

50g of raw material was weighed (Sartorius Entris Laboratory balance) and mixed with 450g of water in a 1000mL plastic beaker, giving a solid-to-solvent ratio of 1:10. A Rushton impeller (IKA Microstar 7.5 control), 50 mm diameter, was used to stir the slurry at 750 rpm. The pH was adjusted to the target value, 9.0 or 9.5, by adding 2 M NaOH and was then kept constant by a bioreactor (Belach Bioteknik AB, Model CP10/ SARA), supplying 2 M NaOH as needed during the entire extraction time of 1 hour. The extraction was performed at room temperature.

After the alkaline treatment was completed, the mixture was poured into centrifuge tubes (750ml) and centrifuged (Beckman Coulter Avanti J-15R Centrifuge) for 20 minutes at 4700 rcf (relative centrifugal force) and 20°C. The centrifugation yielded a liquid phase which was transferred into a new container whilst samples were taken of the solid phase for later analysis.

The liquid phase was further separated through isoelectric precipitation by adding citric acid to lower the pH to the target precipitation value, either 4.5 or 5.5. The solution was manually stirred during the precipitation. Centrifugation (Beckman Coulter Avanti J-15R Centrifuge) at 4700 rcf for 20 minutes, at 20°C, was then performed to separate the supernatant from the sediment, and samples from both phases were collected. All samples gathered from the experiment were transferred into plastic containers and stored in a freezer at -18°C.

3.3.5 Extraction Method for Non-constant pH

The method followed the main extraction protocol, as described over, with the only difference being that no bioreactor was used to keep the pH constant during the extraction step. Instead, the pH was manually set to the desired pH using NaOH and after 10 min it was re-adjusted manually to the initial pH. After the adjustment, the slurry was left during the rest of the extraction time and changes in pH were noted every 10-15 min. Otherwise, the experiment was performed in the same procedure as stated above in section 3.3.4.

The pH for the extraction and precipitation in the non-constant pH method were chosen based on results from the previous extractions, see section 0.

3.3.6 Calculation of Dry Matter

The raw material, solid phase and sediment were oven dried in order to determine the dry matter content, triplicates were taken from each replicate of the sediment and duplicates from solid phase. For raw material triplicate samples were taken from each of the materials. The samples were thawed and approximately 3-5 grams per sample was transferred into numbered metal containers and weighed (Sartorius Entris Laboratory balance). The containers were then placed in a convection oven (Termarks) and dried at 103 °C for at least 16 hours. After drying, the samples were transferred to a desiccator to cool off and to ensure no moisture was taken up by the sample

before they were re-weighed. All samples were stored in the desiccator at room temperature until further analysis.

3.3.7 Protein Content Analysis

Protein content analysis was performed on the oat flour, oat bran, solid phase as well as the sediment. The raw materials were analysed in triplicates, whilst all extraction samples of solid phase and sediment were done in duplicates and triplicates respectively. The dried material from sediment and solid phase were used. The samples were ground manually, using a mortar and pestle, and between 25 and 50 mg was weighed (Entris Mettler AE 163) and placed in a tin capsule before the nitrogen content was measured (FlashEA® 1112 N/Protein Nitrogen and Protein Analyzer) using the DUMAS method. Here, the dynamic flash combustion technique is used where the sample is combusted at high temperature [78]. During this process the nitrogen in the sample is converted into N_2 and separated from other gases produced [79]. The amount of nitrogen is then measured and converted into an amount of protein using a conversion factor, in this case 6.25. Aspartic acid was used as the reference material.

3.3.8 Differential Scanning Calorimetry (DSC) Analysis

For the differential scanning calorimetry measurements, a Seiko 6200 differential scanning calorimeter (Seiko Instruments Inc. Shizuoka, Japan) with the EXSTAR600 Thermal Analysis System was utilized. The instrument was calibrated with indium (M_p = 156.6 °C) and a scanning rate of 10°C/min in an interval of 20-200°C was applied. The DSC was performed on sediments from all extraction methods (1-2 samples each), but not all replicates. This was due to limited access because of Covid-19, as well as that this number of measurements would still enable the observations required to get information about if undenatured, active, proteins exist in the sample and the denaturation temperatures these proteins had. The samples were frozen, thawed, and placed in coated aluminium pans (TA Instruments, New Castle, Delaware, USA), and an empty pan was used as a reference.

The peak denaturation temperatures (T_d) for the samples were determined and compared between the different materials and methods used in the experiment, and with results from other studies. Another parameter that was used in the analysis of the data is how wide the peaks are, which is denoted as ΔT. A wide peak indicates that the sample is more heterogeneous compared to a narrower peak and a larger peak indicates that more protein of that kind is present. Enthalpies from the phase transitions were also provided from the measurements, but due to insufficient information and restricted laboratory access those data could not be used for analysis [80].

3.4 Calculations

To obtain more information from the experiments, some calculations were performed on the raw data.

Equation 1 below was used to calculate the dry matter (DM).

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

The protein analysis provided the protein concentration in the dried material, $P_{dry\ matter}$. This was then used together with the weights of the sediment before and after drying in Equation 2, to calculate the protein concentration in the sediment, $P_{sediment}$. The same equation was used to calculate protein content in the solid phase, $P_{solid\ phase}$, but with the dry matter protein content and weights for the solid phase.

$$P_{sediment}(\%) = \left(\frac{P_{dry\ matter} \times weight_{after\ drying}}{weight_{before\ drying}}\right) \times 100 \quad (2)$$

To calculate the protein yield of the extractions, Equation 3 was used. The yield compares the amount of protein in the sediment to the amount in the raw material and gives information on how much of the protein in the raw material that was successfully transferred into the sediment. The protein concentration in the raw material is referred to as $P_{raw\ material}$.

$$Yield\ (\%) = \left(\frac{P_{sediment} \times weight_{sediment}}{P_{raw\ material} \times weight_{raw\ material}}\right) \times 100 \quad (3)$$

Since not all proteins end up in the liquid phase, the extraction coefficients and precipitation coefficient can be used to determine where the proteins are located. For the extraction coefficient (EC) it is a comparison between the liquid phase and the solid phase, which is calculated using Equation 4. The precipitation coefficient compares how much of the protein in the liquid phase that is precipitated into the sediment, Equation 5 was utilized to obtain the precipitation coefficients (PC).

$$EC\ (\%) = \left(\frac{P_{raw\ material} \times weight_{raw\ material} - P_{solid\ phase} \times weight_{solid\ phase}}{P_{raw\ material} \times weight_{raw\ material}}\right) \times 100 \quad (4)$$

$$PC(\%) = \left(\frac{P_{sediment} \times weight_{sediment}}{P_{raw\ material} \times weight_{raw\ material} - P_{solid\ phase} \times weight_{solid\ phase}}\right) \times 100 \quad (5)$$

3.5 Statistical Analysis

Triplicate, or duplicate, samples were taken from sediment and solid phase of each replicate extraction to enable determination of mean and standard errors of each extraction method. Analysis of Variance through one-way ANOVA was performed on dry matter content, yield, protein content, EC and PC separately to determine significant difference between the four different extraction methods. Calculations were done on the two types of raw materials separately, but comparisons were also made between the two. A post-hoc analysis using Tukey-Kramer HSD was performed in cases where significance occurred.

The data from the non-constant extractions were compared to the constant extractions at the same extraction and precipitation pH using one-way ANOVA. The results were not compared with the other constant extractions in order to see only the effects of constant versus non-constant.

A multivariate analysis using multi-way ANOVA and post hoc analysis with multiple comparison was performed in Matlab to further analyse the effect of different parameters on the responses yield, protein content and dry matter.

For better visualization of the relationship between variables and responses, partial least squares regression (PLS) was used to plot the variables and responses together.

3.5.1 Outlier Investigation

Grubb's outlier test was performed using GraphPad Outlier Calculator [81]. The outlier investigation was performed on each data set from the different extractions, a dataset comes from a specific test number as described in the experimental set-up in Table 7 and could be either of the protein content, dry matter content or yield from a specific phase. The variation within one dataset was also compared to the variation in other datasets when deciding on whether to keep a datapoint that the Grubb's test declared as an outlier.

One outlier was found in the protein content dataset for oat bran sediment 9.5/5.5 constant extraction. However, it was decided that the datapoint should be kept as the variation in the dataset, if the outlier was removed, was lower compared to the variations within the other datasets. A very low variation in a dataset increases the risk for a specific data point to be classified as a false outlier.

Three outliers were discovered when analysing the datasets for protein yield, for constant extractions. One for oat endosperm flour 9.0/5.5, one for milled oat bran 9.5/4.5 and the last one from oat bran 9.0/5.5. As with the outlier discovered in protein content it was decided that these datapoints should be kept, as the variation within the rest of the dataset was very low compared to the variation in other datasets.

No outliers were found for the non-constant extractions.

3.5.2 One-way ANOVA

One-way ANOVA is used to determine significant differences between the means of independent groups, in our case the four extraction methods for each raw material, as well as the constant versus non-constant extractions. The null hypothesis in ANOVA is that the groups do not differ in their means. Using a significance level of α=0.05, a p-value below α discards the null hypothesis with 95% certainty, resulting in significant difference between the methods.

Some assumptions are made when applying ANOVA, the first one being that the samples in each group come from a population which is normally distributed. As each of the groups only contain nine samples, it is difficult to check for normality. However, some histograms were made to check, and the data was assumed to be normally distributed if the histogram had a somewhat resemblance of normality. Other assumptions are that samples are independent and that the variance is equal for the different groups. [82]

One-way ANOVA was performed in Microsoft Excel (Version 16.48).

3.5.3 Tukey-Kramer HSD post-hoc Analysis

ANOVA analyses if a significant difference between a set of groups exist, however it does not describe where this difference is. In order to understand which groups that differ significantly, a post-hoc analysis is needed. Tukey-Kramer HSD tests were performed in those cases where ANOVA resulted in $p < 0.05$. The Tukey's test performs pairwise comparisons of the means of all groups, calculating a Student's q-value for each pair that is compared to a critical value (q_{crit}) which depends on the number of groups and the significance level (α=0.05). Significant difference occurs for $q > q_{crit}$ [83]. The post hoc analysis was performed using an online software [84].

3.5.4 Multi-way ANOVA

The data from both the constant and non-constant pH experiments were further analysed through multivariate analysis using multi-way ANOVA in Matlab (anovan). This was done in order to understand what factors have significant effects on different responses. The multi-way ANOVA in Matlab returns an ANOVA-table with information on the p-value for all main factors as well as interaction pairs.

For the constant pH experiments the data was analysed separately for milled oat bran (OBM) and oat endosperm flour (OEF). The factors tested for were all categorical and were as follows: extraction pH (9.5 or 9.0), precipitation pH (4.5 or 5.5) and replicate (1, 2 or 3). The reason that replicate is included is that it gives information on which bioreactor that was used, amongst others, and can indicate if there are big differences in the sediments obtained from the different bioreactors. Large differences between replicates could be an issue if they dominate the data and hide differences between extraction methods.

Interaction factors for all pairwise interactions as well as the three-term interaction were also included. This analysis was done in order to see which of these factors have a significant effect on the responses: yield, dry matter content and protein content. A three-way ANOVA was performed for each of the responses, for both OBM and OEF.

For the data from non-constant pH extractions, it was tested against the constant data at the same pH for both extraction and precipitation in order to see if the categorical factors: method (constant or non-constant) or replicate (1, 2 or 3) had a significant effect on the responses: yield, dry matter and protein content. In this case a two-way ANOVA was performed in Matlab for each of the responses and materials.

3.5.5 Multiple Comparison Test and Interaction Plots

For all factors where a significant effect was seen ($p<0.05$), a multiple comparison test was performed in Matlab (multcompare) as a post-hoc analysis, in order to investigate which conditions give rise to a higher or lower response. The test returns a graph with the means of each group and its comparison interval, two groups are significantly different if their intervals do not overlap.

In some cases where a significant effect was observed in interaction pairs, interaction plots using the data means were performed in Microsoft Excel and analysed to further understand the interaction effect. An interaction effect occurs when the combination of two, or more, main effects change the result in a specific response. This means that the main effects cannot be analysed on their own and therefor the interaction effect must be examined instead.

3.5.6 Visualization with Partial Least Squares Regression (PLS)

For further analysis, and better visualization of the relationships between the different variables and responses (yield, protein content, dry matter), partial least squares regression (PLS) was performed in Matlab using a function provided in the Chemometrics course KLGN10, Lund University. PLS is useful as it finds relations between two matrices, one containing the variables and the other containing the responses. The modelling tool uses latent structures, termed principal components, upon which the data is projected using linear regression and partial least squares methodology. The data can then be plotted using the main principal components in order to visualize relationships [85]. A small angle between two points, and a long distance to the origin indicate a positive correlation between two points. Whilst a 90° angle between two points indicate no statistical correlation, and an angle over 90° indicate negative correlation [86]. If a variable is placed closely to the origin, no conclusions can be drawn about the variable and its relationship to others [87]. Important to mention is that the results from a PLS only gives information about correlations between responses and variables, no conclusions about causation can be made.

The PLS was performed using the data from both materials together. The variable matrix contained categorical factors, "dummy variables" for extraction pH, precipitation pH, replicate, constant or non-constant extraction and raw material. The response matrix contained three columns (yield, protein content and dry matter). Before performing the PLS the data was pre-processed by mean centering and standardizing both matrices using Matlab (zscore).

Since there are multiple responses, PLS2 was used to be able to model upon all responses simultaneously, as opposed to PLS1 which is performed with one response column at a time [88]. The aim when applying PLS in this study is to provide visualization which is most easily done by using the first two principal components to plot the variables and responses together in a 2D-biplot termed W*Q-plot. However, before doing so it is important to establish how well the PLS model explains the variation in the data when using only the first two principal components. Therefore, the coefficient of determination (R^2) is calculated for different numbers of components which gives an indication of how well the model fits the data.

4. Results and Discussion

The results obtained in the experimental trials are presented and discussed in this section.

4.1 Raw Material Characterization

The two types of raw materials, oat endosperm flour and milled oat bran, were analysed for dry matter content and protein content in the original material. The result, Figure 6, shows that the milled oat bran has a higher protein content of 16.3% whilst the oat endosperm flour has a protein content of 9.1%. These values are both on the original materials that are not dried. The protein content for both OBM and OEF is slightly lower than the theoretical values which were 18-26% and 12% respectively [3]. However, the protein content of OEF is similar to the results of an analysis performed on oat flour from Lantmännen in 2019, showing a protein content of 9.5% [89]. Also, it is not known if the theoretical protein content values are in dry basis or from non-dried material. The dry basis protein content for OEF is 10.1% and 18.5% for OBM, which places milled oat bran in the range of the theoretical values, but oat endosperm flour is still slightly below.

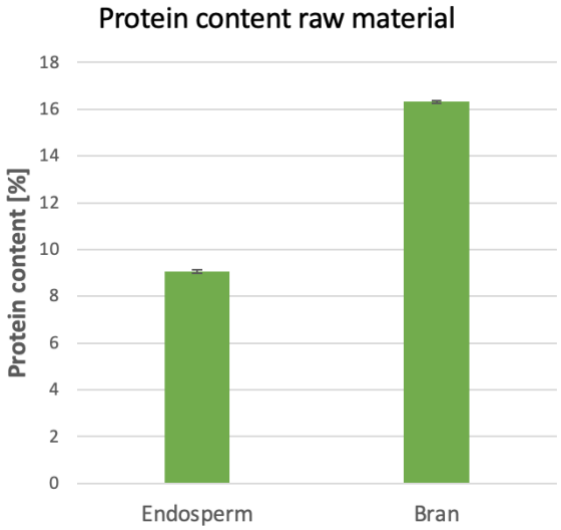

Figure 6: Protein content in the raw materials, oat endosperm flour (OEF) and milled oat bran (OBM).

4.2 Different Extractions Methods – Constant pH

Different pH during extraction and precipitation were analysed in order to find a method that produces a protein sediment of high purity and yield which allows for an efficient and mild process to maintain protein functionality.

4.2.1 Dry Matter Content (DM) – Constant pH

Dry matter content (DM) of the sediments, Figure 7, show the same trend for both raw materials, where precipitation at pH 5.5 yielded significantly lower DM than precipitation at pH 4.5. For oat endosperm flour the difference was a DM of around 32% for pH 4.5 and 26% for pH 5.5, and for milled oat bran 28% versus 22%. The DM did not vary significantly with different extraction pH. The reason for the lower dry matter content when using precipitation pH 5.5 is unknown. However, as the yield did not differ significantly between the different precipitation pH, as seen in section 4.2.3, it seems that the sediment obtained with pH 5.5 contained more water.

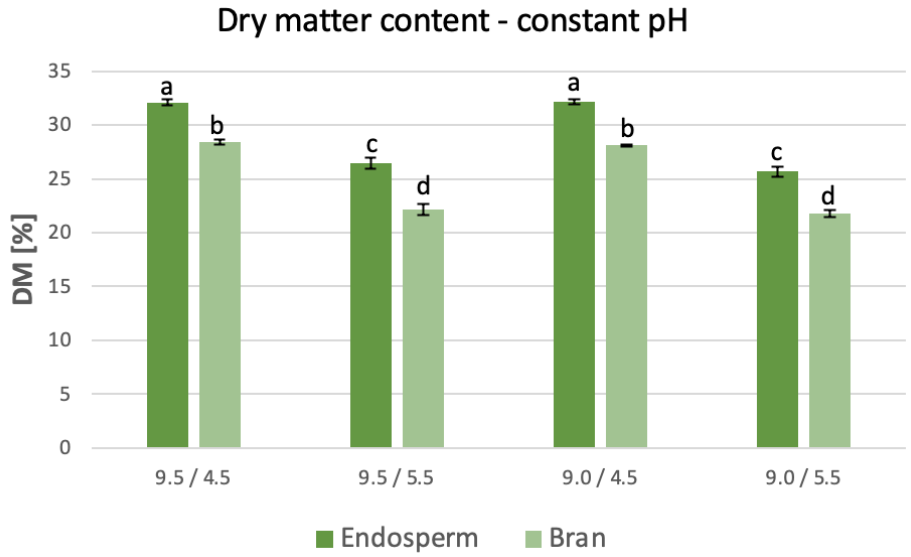

Figure 7: Dry matter content (%) in sediments from oat endosperm flour (OEF) and milled oat bran (OBM) obtained with different extraction and precipitation methods. Different letters (a-d) represent significant differences ($p<0.05$).

4.2.2 Protein Content – Constant pH

The protein content in the dried sediments obtained from oat flour, seen in Figure 8, show significant difference between 9.0/4.5 and the two methods using extraction pH 9.5. Although the difference is significant, it is still only a small difference with 9.5/4.5 showing the highest protein content of 74.8% and 9.0/4.5 the lowest content of 72.9%. For the sediments obtained from oat bran, also Figure 8, the second method (9.5/5.5) showed significantly higher protein content of 77.2%, than the other three methods which were around 75%. Also in this case, the difference is significant but still small.

A one-way ANOVA was performed on all extractions as well, to compare differences between the two raw materials for each extraction method. The analysis showed that sediments from oat bran had significantly higher protein content than their respective sediments from oat flour, except for the first method (9.5/4.5) where there was no significant difference.

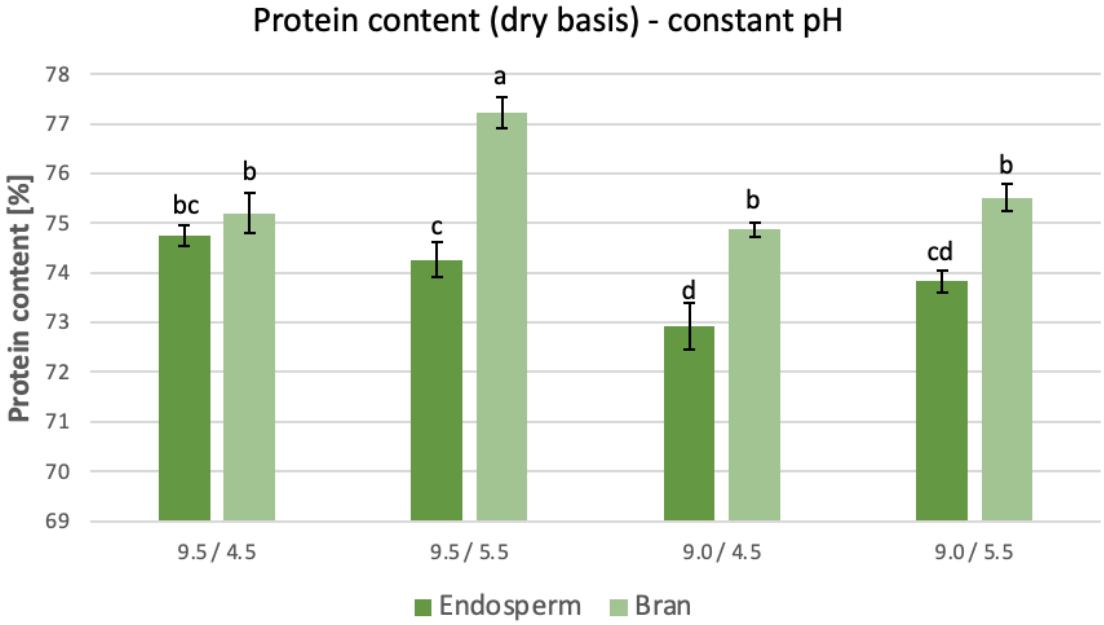

Figure 8: Protein content in the dried sediments from oat endosperm flour (OEF) and milled oat bran (OBM) using different extraction methods. Different letters (a-d) indicate a significant difference ($p<0.05$).

4.2.3 Protein Yield – Constant pH

Figure 9 show the protein yield for oat endosperm flour and oat bran using different extraction methods. The results show no significant difference in yield for OEF when comparing the methods. For milled oat bran there is a significant difference between method two (9.5/5.5) and method four (9.0/5.5), however no other significance is found. When comparing the two raw materials, the protein yield is significantly higher for OEF than OBM, independently of extraction method. The yield is over 80% for OEF, whilst OBM is in the range of 66-70%.

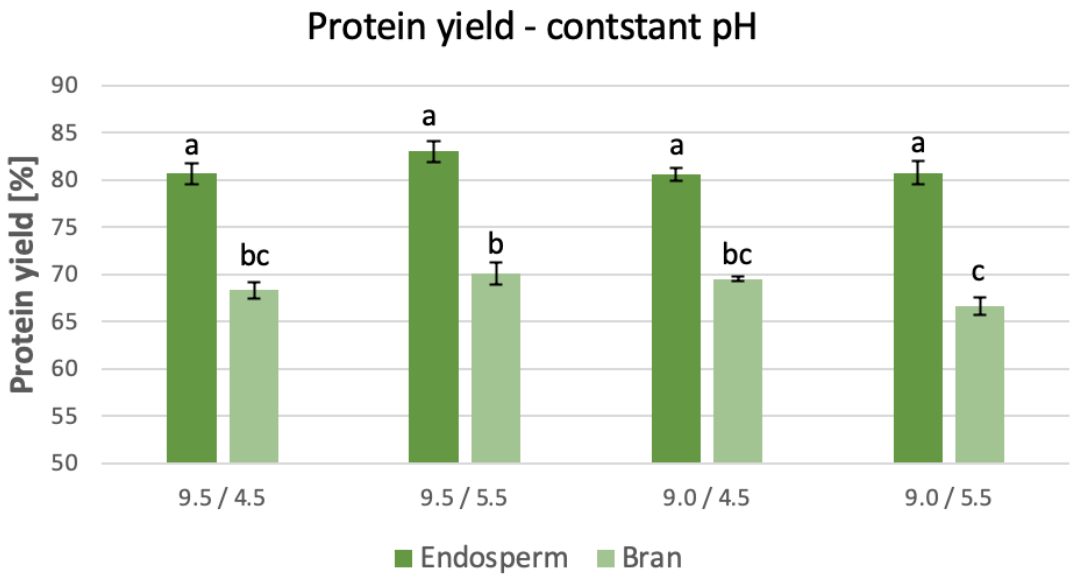

Figure 9: Protein yield using different extraction methods on oat endosperm flour (OEF) and milled oat bran (OBM). Different lettering (a-c) indicates significant difference between methods ($p<0.05$).

4.2.4 Extraction Coefficient (EC) – Constant pH

The extraction coefficient (EC) is a measurement of the amount of protein that is extracted to the liquid phase and gives an indication of the extraction efficacy. As seen in Figure 10 the EC is higher for oat endosperm than for milled oat bran, which is consistent with the yield which was higher for OEF. When comparing the different extraction methods, OEF significantly differed in EC at 85.7% for method 9.0/4.5, which was lower than for the other methods which were around 88%. For milled oat bran, the EC did not differ significantly between methods and were in the range of 79.9-81.3%. For both methods the extraction coefficient is high when compared with other material such as hemp seed where a previous study [65] found that the highest extraction coefficient was around 70%.

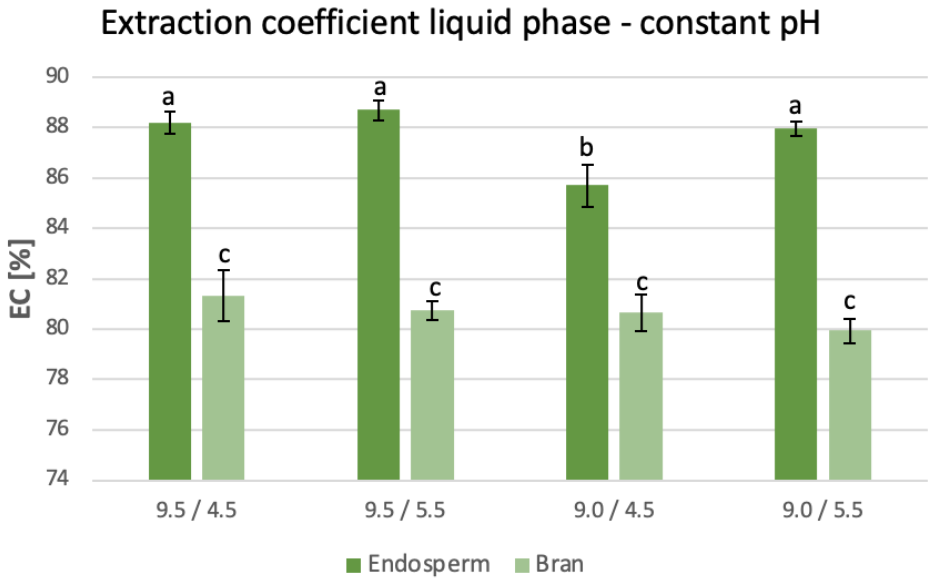

Figure 10: Extraction coefficient for different methods of protein extraction from oat endosperm flour (OEF) and milled oat bran (OBM). Different letter (a-b) indicates significant difference ($p<0.05$).

4.2.5 Precipitation Coefficient (PC) – Constant pH

The precipitation coefficient is a measurement of the precipitation efficacy, thus how much of the protein which was extracted to the liquid phase that is precipitated. As can be seen in Figure 11, the PC for oat endosperm flour does not differ significantly between precipitation methods. Also, the precipitation efficacy is generally high and in the range of 91-94%. The PC is lower for milled oat bran, Figure 11, in the range of 83-86%. Again, the methods do not differ significantly in precipitation efficacy. This shows that both pH 4.5 and 5.5 can precipitate much of the available protein. Although, as seen in the dry matter calculations the sediment in pH 5.5 has a much lower DM which must be taken into consideration.

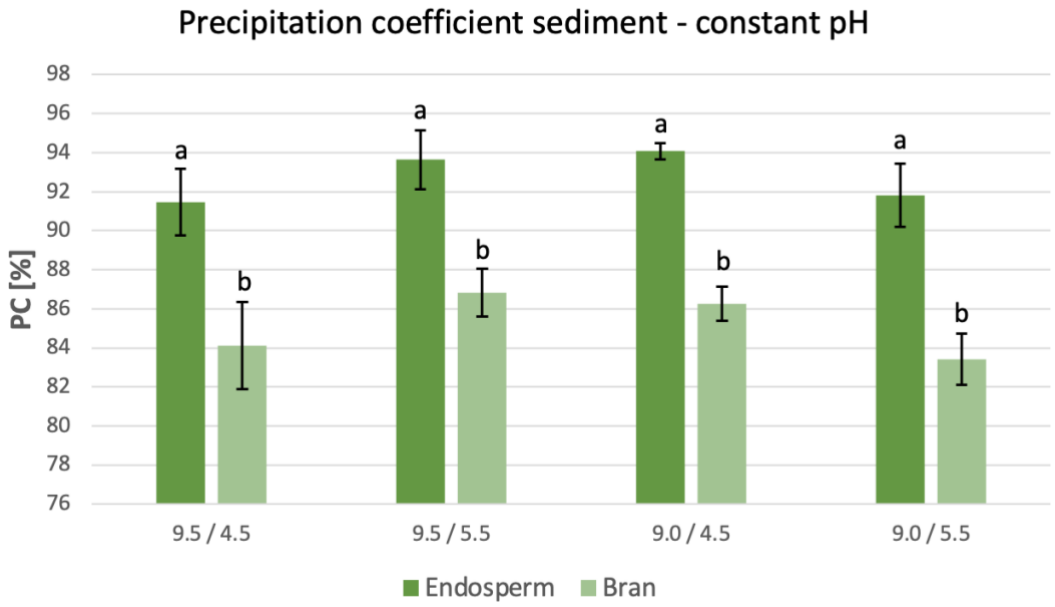

Figure 11: Precipitation coefficient for different methods of protein precipitation from oat endosperm flour (OEF) and milled oat bran (OBM). Same letter (a/b) indicate no significant difference ($p>0.05$).

4.2.6 Differences Between the two Materials

The oat endosperm flour obtained a higher yield compared to milled oat bran. The difference in extractability is also revealed in the EC result, where oat endosperm flour had more of the protein extracted into the liquid phase. This outcome is consistent with the literature since milled oat bran contains more fibre, and fibre has been found to be a component that complicates the protein extraction due to the fact that they can bind to protein bodies [90].

The higher yield from OEF might seem contradictory since a higher protein content was obtained from OBM, but this can be attributed to the amount of protein in the raw material. The raw material analysis showed that milled oat bran contains nearly twice as much protein compared to the oat endosperm flour. Thus, less impurities must be removed from OBM in order to achieve the same protein content as in OEF. However, the protein in OBM is also harder to extract, meaning that more protein will be left in the first solid phase which is discarded, thereby lowering the protein yield.

When comparing the dry matter content for the two materials, the oat endosperm flour has a higher percentage dry matter in the sediment. A possible explanation for this dissimilarity could

be that milled oat bran proteins could hold more water, or that the impurities left in the protein concentrate differs between the two materials, where the OBM concentrate contains compounds with higher water holding properties. For example, β-glucan is a soluble fibre found in oat bran that can increase the water holding capacity of a product [91], so perhaps some of it could have been co-extracted with the proteins.

The precipitation coefficient analysis for the sediment revealed that OBM had lower proportion of proteins precipitated from the liquid phase to the sediment. A possible reason for this could be that the two materials do not contain the same types of proteins. As highlighted in the theoretical background, the amino acid composition is a bit different which could indicate that different amounts of the protein classes are incorporated in the raw materials. If that is the case, the protein fractions could be affected to varying extent by the precipitation pH and therefore influence the results.

4.2.7 Optimal Extraction Method

Based on the results from the different extractions methods at constant pH, the optimal extraction method was to be identified and later used for the analysis with non-constant pH.

Both for the oat endosperm flour and the milled oat bran, the precipitation pH had a significant effect on the dry matter content, where pH 4.5 gave a higher DM than pH 5.5. Since there are no obvious differences regarding the protein content, this indicates that the sediment after precipitation at pH 5.5 contains more water. This could argue that this protein sediment can retain water more efficiently, which could be a useful functional property. However, it also means that more water needs to be evaporated during an eventual drying process in the production of oat protein concentrates, which makes the energy consumption much greater. Since the yield results presented no clear significant differences, it was decided that precipitation pH 4.5 was the optimal pH based on maintaining a more efficient production process.

The yield did not show any significant trends regarding which extraction pH was more effective. In addition, the protein content and dry matter content also complied with this theory. Therefore, the choice of optimal extraction pH was instead based on what would be more favourable for protein functionality. To maintain as much functionality as possible, proteins should be treated mildly to avoid denaturation. The extraction pH 9.0 was then viewed upon as the most suitable option since less NaOH would be used and the pH would not be as high. This option is also best suited for safety reasons, due to minimization of chemical-use, and most likely less salt formation.

4.3 Non-constant pH Extractions

The results from the extractions using non-constant pH are presented below and compared with the constant extractions at pH 9.0 and precipitation pH 4.5.

4.3.1 pH Variation during Extraction

The pH was tracked during the entire extraction time, and recorded at intervals of around ten minutes, in order to see how it would change when not kept constant by the bioreactor. The pH for all three replicates of each extraction can be seen in the graphs below in Figure 12 and Figure 13. The pH was adjusted up to 9.0 after 10 minutes by adding more NaOH, and then it was left for the rest of the extraction time. As can be seen in the figures below, the pH dropped quickly during the first ten minutes, but after being adjusted it sank more slowly. The pH went down more for milled oat bran than for oat endosperm flour, with the lowest pH for OBM being 8.6 and 8.8 for OEF. Interestingly, OBM had a higher pH than OEF, 6.27 compared to 5.68, before NaOH was added to the slurry. However, the reason for the difference in pH drop is not known.

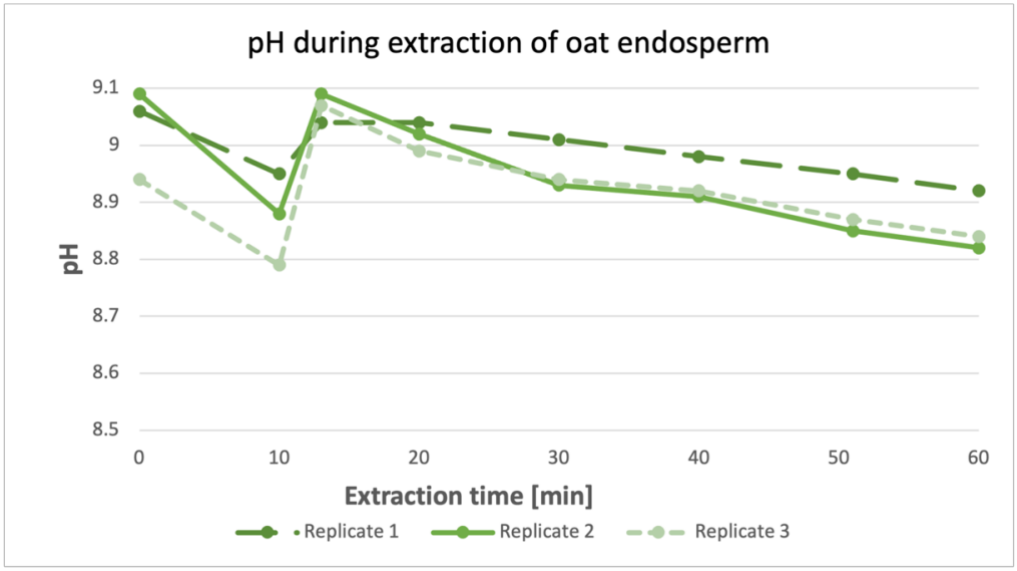

Figure 12: pH during extraction of oat endosperm flour (OEF) using original pH of 9.

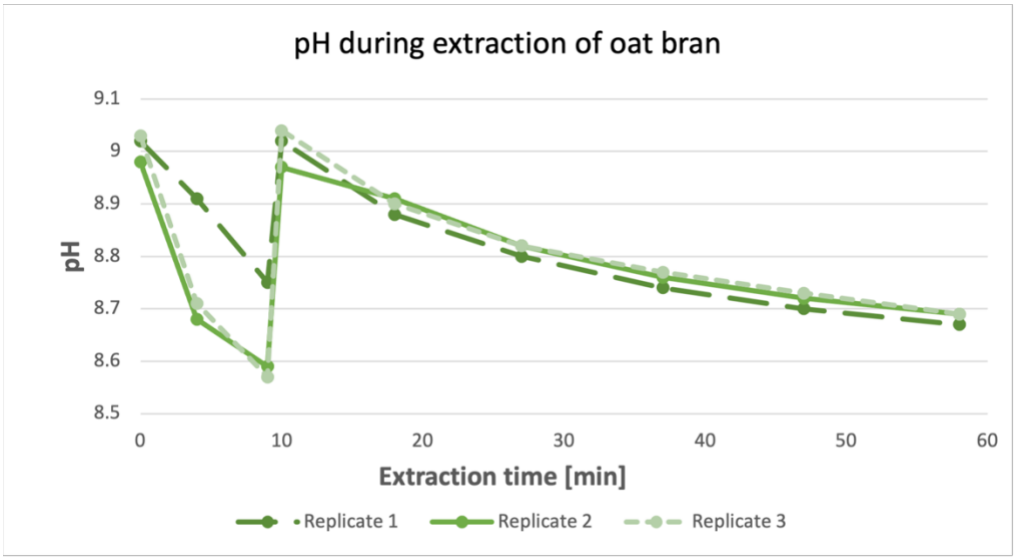

Figure 13: pH during the extraction of milled oat bran (OBM), using an original pH of 9

4.3.2 Dry Matter Content – Non-constant pH

In Figure 14, the dry matter content of the non-constant method is compared to the constant method with the same pH for extraction and precipitation. The DM was higher for both the oat endosperm flour and the milled oat bran with the non-constant method. The difference is significant for OEF where the DM was 32% for the constant method and 34% for the non-constant method. For OBM the values are slightly lower at around 28% with no significant difference between the two methods.

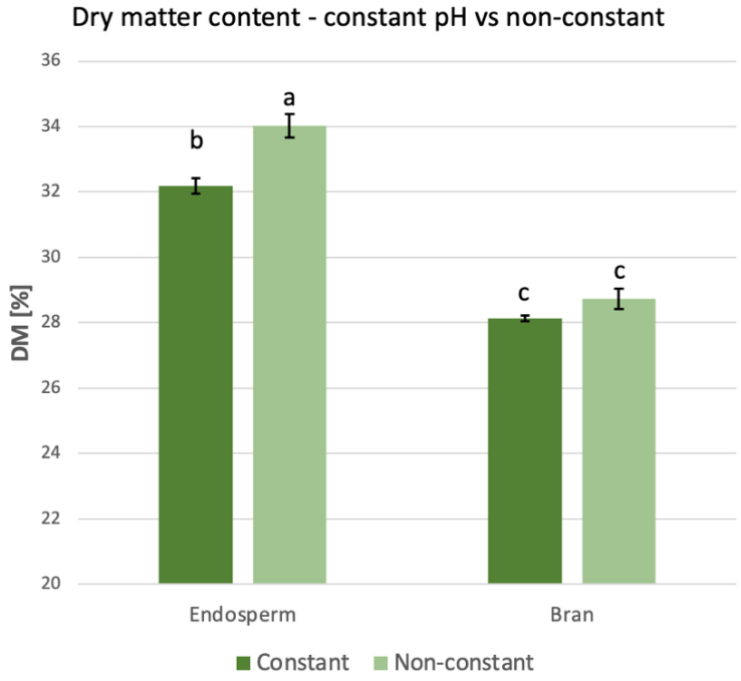

Figure 14: Dry matter content for protein concentrates obtained through extraction with constant pH 9.0 and non-constant pH 9.0 with precipitation (IEP) at pH 4.5, for both milled oat bran (OBM) and oat endosperm flour (OEF). Different letters (a-c) indicate significant difference ($p<0.05$).

4.3.3 Protein Content – Non-constant pH

For the protein content, Figure 15 shows that it is significantly higher for the method with constant pH compared to the one with non-constant. This applies to both the oat endosperm flour and the milled oat bran. Although the non-constant protein contents are lower, they are still quite high at 71% for OEF and 72% for OBM compared to constant extractions which gave 73% and 75% respectively.

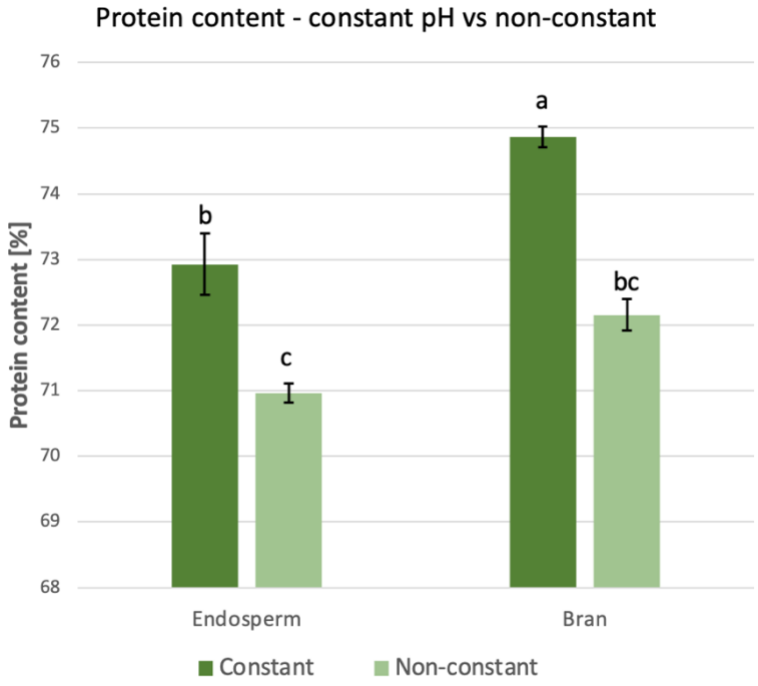

Figure 15: Protein content for protein concentrates obtained through extraction with constant pH 9.0 and non-constant pH 9.0 with precipitation (IEP) at pH 4.5, for both milled oat bran (OBM) and oat endosperm flour (OEF). Different letters (a-c) indicate significant difference ($p<0.05$).

4.3.4 Protein Yield – Non-constant pH

The protein yield displays a similar pattern as the dry matter content, as is presented in Figure 16. The method with non-constant pH gave a higher yield for both raw materials, however, the difference is not significant in the case of milled oat bran. The yield was around 70% for OBM and 80% versus 83% for OEF.

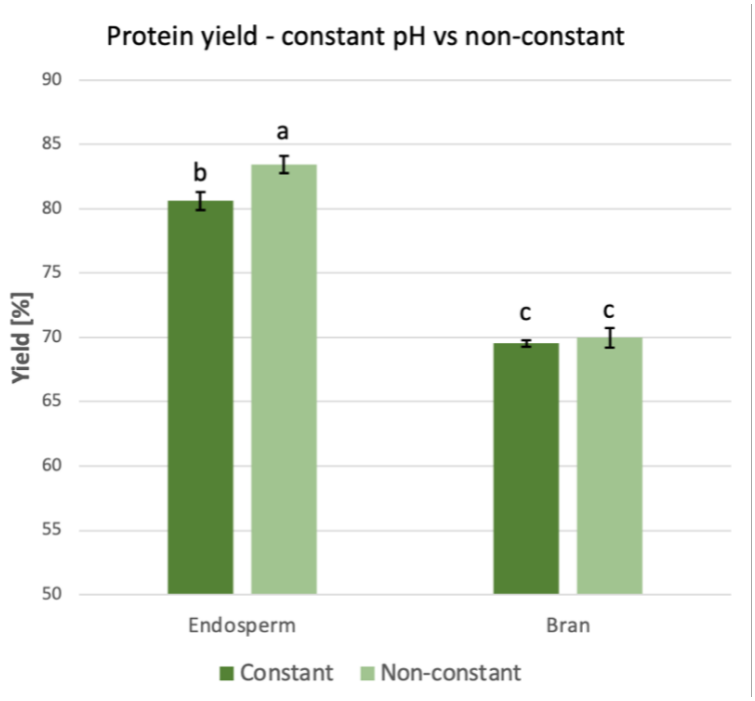

Figure 16: Protein yield for protein concentrates obtained through extraction with constant pH 9.0 and non-constant pH 9.0 with precipitation (IEP) at pH 4.5, for both milled oat bran (OBM) and oat endosperm flour (OEF). Different letters (a-c) indicate significant difference ($p<0.05$).

4.3.5 Extraction Coefficient – Non-constant pH

As mentioned earlier, extraction coefficient (EC), gives an indication of how efficient the extraction is by comparing the amount of available protein that is extracted to the liquid phase. As can be seen in Figure 17 below, the EC is higher for non-constant extraction for both raw materials. The difference is significant in both cases, for oat endosperm flour the EC went up from 86% for constant pH to 89% with non-constant. Whilst for milled oat bran the previous EC was 81% and with non-constant pH it reached 84%.

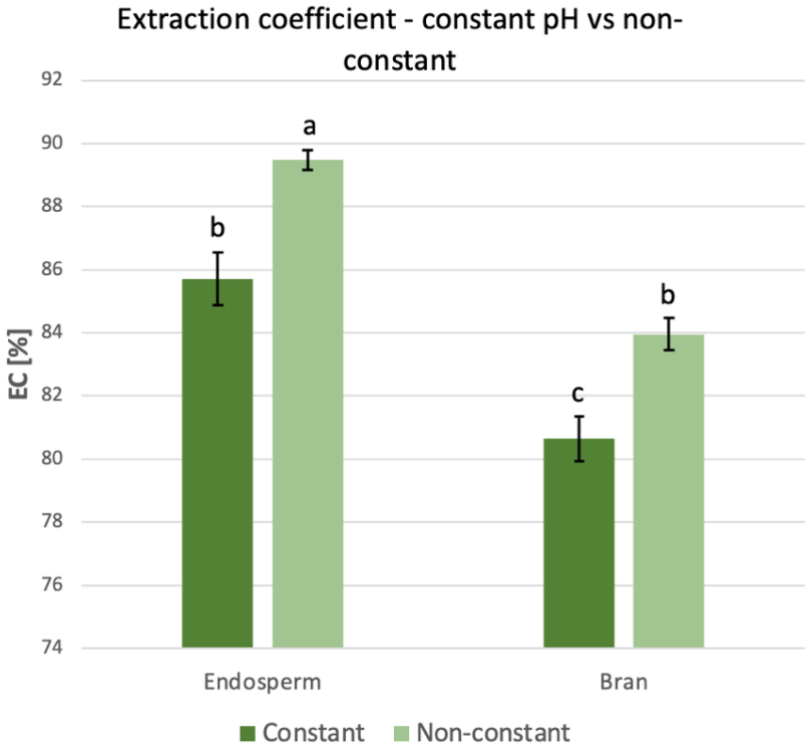

Figure 17: Extraction coefficient for protein concentrates obtained through extraction with constant pH 9.0 and non-constant pH 9.0 with precipitation (IEP) at pH 4.5, for both milled oat bran (OBM) and oat endosperm flour (OEF). Difference letters (a-c) indicate significant difference ($p<0.05$).

4.3.6 Precipitation Coefficient – Non-constant pH

Precipitation coefficient (PC) is as mentioned a measurement of how much of the available protein is precipitated, and therefore ends up in the sediment. No changes were expected between the constant and non-constant measurements as the precipitation method is the same for both. However, as seen in Figure 18 below, the PC is slightly lower for the non-constant. The difference is only significant for milled oat bran, where the constant pH material has a PC of 86% and the non-constant 83%. For oat endosperm flour the PC is around 93-94%.

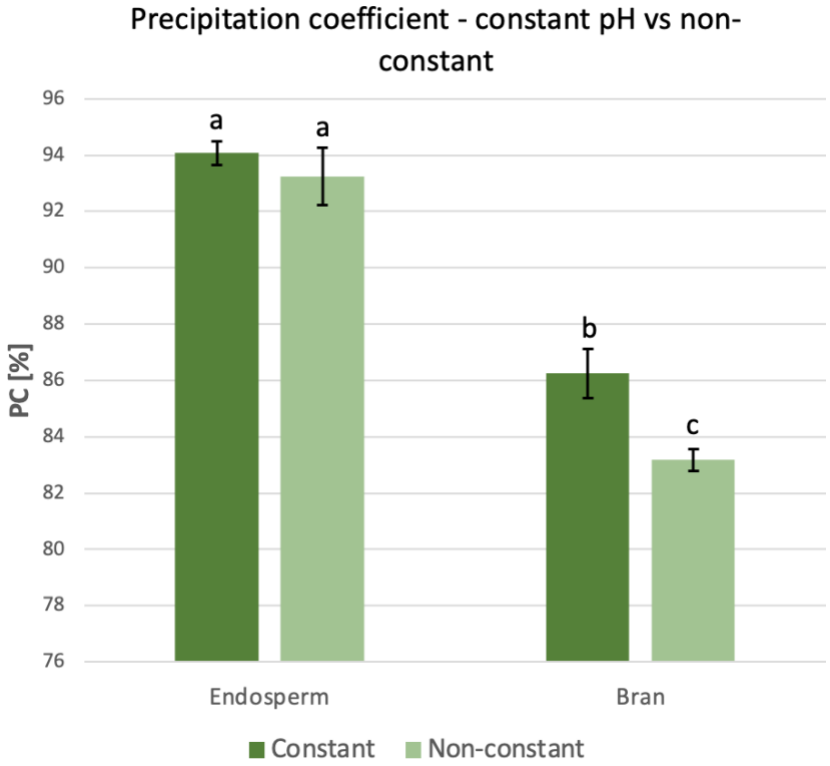

Figure 18: Precipitation coefficient for protein concentrates obtained through extraction with constant pH 9.0 and non-constant pH 9.0 with precipitation (IEP) at pH 4.5, for both milled oat bran (OBM) and oat endosperm flour (OEF). Different letters (a-c) indicate significant difference ($p<0.05$).

4.3.7 Differences between Constant and Non-constant pH

The results used when comparing the effect of constant pH and non-constant pH during the extraction was obtained from trials with the same pH, 9.0 in the alkaline treatment and 4.5 in the precipitation step as motivated in section 0.

For both materials, the protein content was higher when using constant pH. On the other hand, when looking at the dry matter content it can be noticed that the oat endosperm flour revealed a higher DM from the non-constant trial, whereas the milled oat bran showed no significant difference between the two methods. This could indicate that the samples obtained from the non-constant method were less pure and more heterogeneous compared to the ones from the constant method. More unwanted materials were then extracted in the process.

For OEF, the yield was significantly lower for the constant method compared to the non-constant method. For OBM, no significant difference was observed between the two approaches. This result could argue that keeping the pH constant over a longer time is unnecessary for oat protein extraction, and that there is a possibility to shorten the extraction time which would be favourable in a process viewpoint. The pH curves in Figure 12 and Figure 13 also contribute to this conclusion, since the pH drop is higher in the first ten minutes compared to the last 50.

The extraction coefficient was lower at constant pH for both materials. Why more proteins were transferred into the liquid phase with the non-constant method could be that the constant method creates a harsher environment for the proteins due to higher pH. As a result of this, it is possible that more proteins have been denatured and hence will not be soluble in the liquid phase.

The result obtained from the precipitation coefficient calculations revealed higher values for the constant method, although the difference was only significant for the milled oat bran. This change was not expected as the precipitation step was identical for the two methods, but it could be attributed to a possible difference in what protein types that were extracted. The same precipitation pH could then have different effects on the proteins.

4.4 Multivariate Analysis – Multi-way ANOVA

This section contains the results from multivariate analyses using multi-way ANOVA, the section is divided into comparison between constant pH extractions, and non-constant versus constant.

4.4.1 Constant Extraction pH

As mentioned in section 0, three-way ANOVAs were performed on both materials for each of the responses: yield, dry matter, and protein content. The results of all ANOVAs and relevant multiple comparison plots can be seen in appendix A.2. The factors analysed were extraction pH, precipitation pH and replicate.

4.4.1.1 Effects on Yield

For oat endosperm flour the ANOVA-table, Table A 3 in appendix, shows no significant effect for any of the factors on the protein yield. Meaning that the yield was not significantly affected by which extraction or precipitation pH was chosen. For milled oat bran, Table A 4 in appendix, no significant effect was seen from any of the main factors. However, a significant effect was seen for the interaction between extraction and precipitation pH. Therefore, an interaction plot was performed, which can be seen below in Figure 19. The plot shows that the effect of precipitation pH on the yield depends on which extraction pH that has been used and changes direction. A positive relationship between precipitation pH and yield is seen for extraction pH 9.5, whilst for 9.0 the relationship is negative. Reasons that the relationship differs is not known. One explanation could be that differences in protein fractions exist after solubilization at 9.5 and 9.0, which could in turn be affected differently by the precipitation pH.

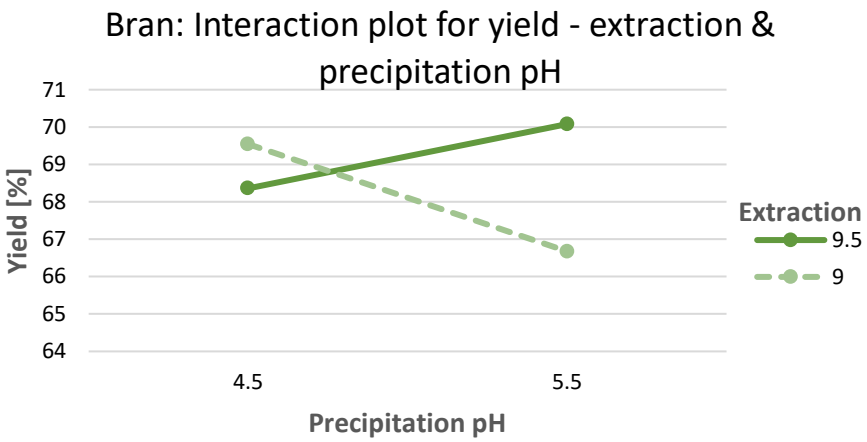

Figure 19: Interaction plot for the effect on yield from the interaction of extraction and precipitation pH on the protein extraction of milled oat bran (OBM).

4.4.1.2 Effects on Dry Matter Content

For oat endosperm flour the dry matter was significantly affected by two main effects: precipitation pH and replicate, as seen in Table A 5 in appendix. Milled oat bran showed similar results in the ANOVA, Table A 6 in appendix, but a significant interaction effect between extraction pH and replicate was also seen.

The significant effect from precipitation on dry matter content was analysed further through multiple comparisons, Figure A 1 and Figure A 2 in appendix. The result shows higher DM for pH 4.5, which is consistent with the earlier data analyses in section 4.2.1, and further strengthens the conclusion that the sediment created with higher precipitation pH of 5.5 has a higher water binding capacity.

The significant effect of replicate on dry matter for both materials was analysed further through multiple comparisons, Figure A 3 and Figure A 4 in appendix, showing that replicate 1 has a higher DM than 2, and replicate 2 is slightly higher than 3. This is not an effect that was expected, and the reason for this is unknown. What differentiates the replicates is which bioreactor that has been used during the extraction, as well as in what order the precipitation was performed leading to some differences in precipitation time. However, it is most likely that the variation between replicates is a result of error. In that case the replicate shall not be seen as having a significant effect on DM.

The interaction effect for milled oat bran between extraction pH and replicate was not further analysed through interaction plot, as the main effect of extraction was not significant. And the effect from replicate is likely due to error. However, the interaction means that if the effect of replicate were to be analysed further, the relationship to extraction pH needs to be taken into consideration.

4.4.1.3 Effects on Protein Content

The result from the ANOVA on oat endosperm flour can be seen in Table A 7 in the appendix, it shows a significant effect from both extraction pH and replicate, as well as all three two-way interaction effects. The interaction effects mean that it is difficult to analyse the main effects in themselves as the response is dependent upon the interaction of the two.

For the interaction of extraction and precipitation pH it was further analysed with an interaction plot, Figure 20. The plot shows a positive relationship between precipitation pH and protein content for extraction pH 9.0, whilst a negative relationship for extraction pH 9.5.

The two interaction effects connected to replicate were not further analysed as the effect from replicate is likely due to error. However, it is important to take into consideration that replicate has a significant effect both as a main effect and in interactions. Therefore, the results on protein content from OEF is more difficult to analyse for the differences between the different methods.

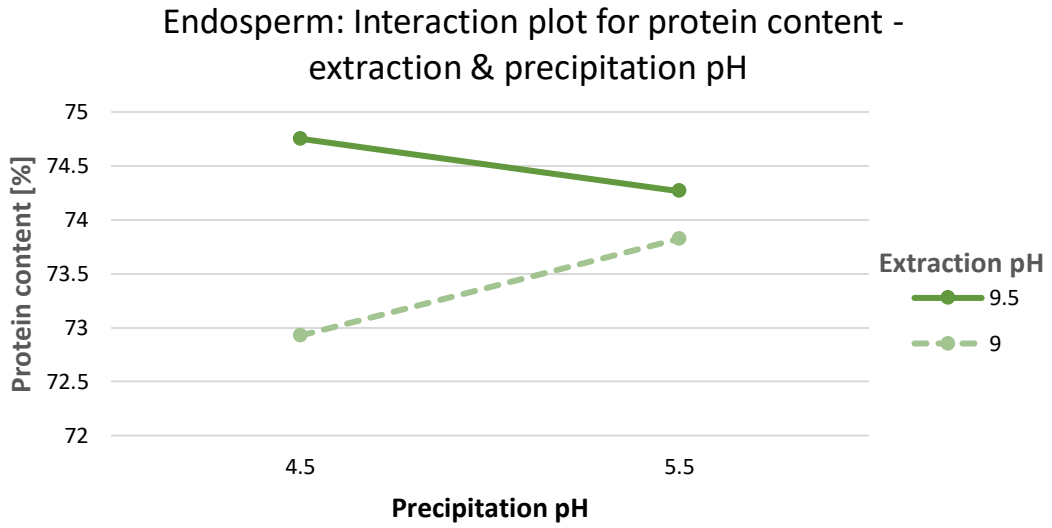

Figure 20: Interaction plot for the effect on protein content from the interaction of extraction and precipitation pH on the protein extraction of oat endosperm flour (OEF).

For milled oat bran, the ANOVA, Table A 8 in appendix, showed significant effect from extraction pH, precipitation pH as well as two interactions. One interaction effect between extraction and precipitation pH, and one between precipitation pH and replicate.

The interaction effect between extraction and precipitation pH means that the two main effects should not be analysed on their own. Their interaction was therefore further analysed through an interaction plot, Figure 21, showing that for both extraction pH the relationship between precipitation and protein content was positive. However, the effect from precipitation is stronger for pH 9.5 than for 9.0, meaning that the proteins extracted with 9.5 are more affected by what precipitation pH that is used.

The interaction between precipitation pH and replicate was not further analysed.

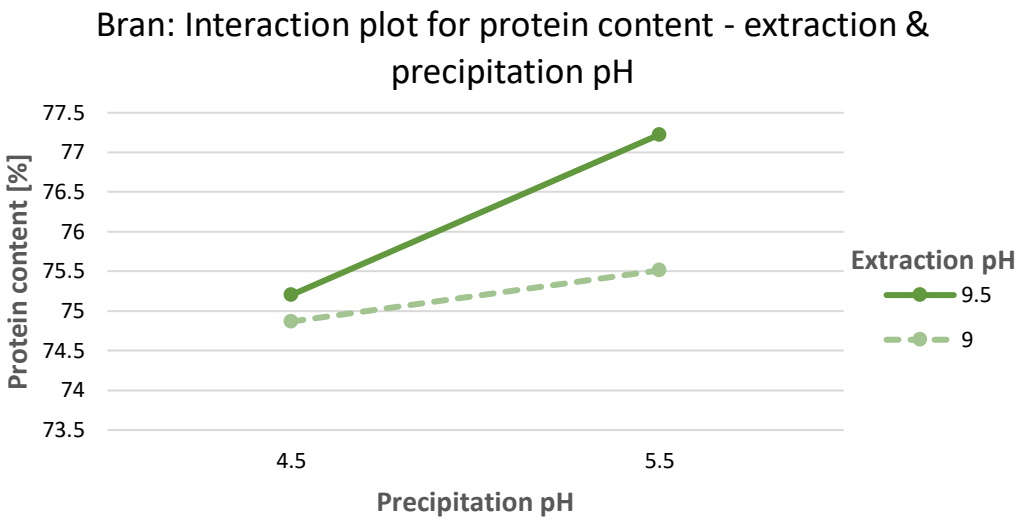

Figure 21: Interaction plot for the effect on protein content from the interaction of extraction and precipitation pH on the protein extraction of milled oat bran (OBM).

4.4.2 Non-constant versus Constant

For the oat endosperm flour, the two-way ANOVA showed significant effect of the type of method on dry matter, protein content and yield. This is presented in section A.2.2.1 in the appendix, along with the multiple comparisons' tests. The multiple comparison test revealed that the condition non-constant method gave a higher response value for dry matter and yield, whereas the condition constant method gave a higher response value for protein content. The multivariate analysis results also clarified that the replicates did not influence the responses.

On the other hand, two-way ANOVA on the milled oat bran only revealed a significant effect between the types of method for the protein content, section A.2.2.2 in appendix, no significant effects were observed for yield or dry matter. In further analysis through multiple comparisons, figures found in section A.2.2.2, it was seen that the method with constant pH provided a higher response value for the protein content.

No interaction effects between factors were found in the investigation and the multivariate analysis thereby confirmed the results from the previous statistical analysis.

4.5 Multivariate Analysis – Partial Least Squares Regression (PLS)

As stated in section 3.5.6, PLS modelling was performed in order to provide visualization of the relationships between variables and responses that have been analysed in the multi-way ANOVA. In order to control that the first two principal components of the model explain enough of the variance in data to allow for a meaningful biplot of the responses and variables, the coefficient of determination (R^2) is calculated for different number of components. This can be seen in Figure 22 that shows that the first two principal components have an R^2 >99.9%, which tells us that the model describes the variance in the data very well as it is able to replicate it in 99.9% of cases. This high R^2-value could indicate that the model is even overfitted to the data, although in this case this is not an issue as the model is used for visualization and will not be used for future predictions where overfitting could become an issue.

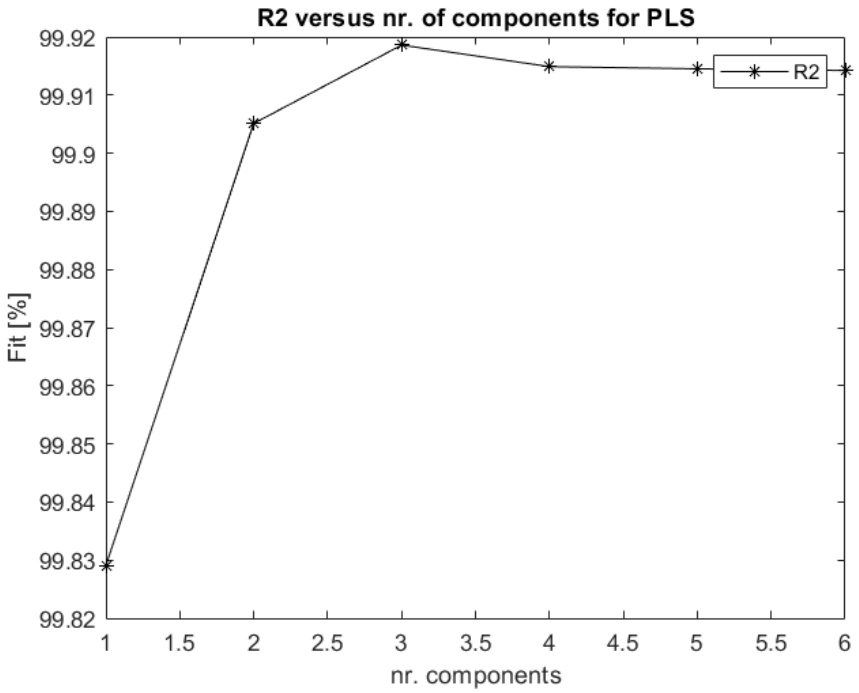

Figure 22: The coefficient of determination (R^2) as a function of the number of principal components.

The W*Q-plot, Figure 23, describes the relationship between variables and responses through a biplot. When looking along the horizontal axis (Principal component 1) a clear interpretation of the relationship to the responses "protein content" and "Dry matter" can be made. This means that for the variables, a placement to the left of the horizontal axis indicates a positive correlation with protein content, and a negative correlation with dry matter. A placement to the right indicates the opposite. For yield the interpretation, and its correlation with variables is more complex, however a higher placement on the vertical axis (Principal component 2) together with a high placement on the horizontal axis implies positive correlation.

When comparing the two raw materials, the figure shows that oat endosperm flour has a strong positive relationship to yield, whilst milled oat bran has a negative relationship. This is consistent with the earlier analyses which show that OEF has a higher protein yield than bran independently of extraction method, section 4.2.3. OBM also shows a positive correlation with protein content, whilst the correlation is negative with OEF. This is consistent with earlier results, section 4.2.2, showing that OBM had a significantly higher protein content in almost all extractions.

None of the variables, except for raw material, show a strong correlation to yield as the smallest angle is close to 90°. This is consistent with earlier results as yield did not differ significantly between different extraction methods, in most cases.

Variables that correlate positively to protein content are extraction pH 9.5, precipitation pH 5.5 and constant pH during extraction. Whilst the lower extraction and precipitation pH, and non-constant pH, show a positive correlation with dry matter. The correlation between precipitation pH and dry matter is consistent with earlier results. For protein content it is important to remember the interaction effect that was seen between precipitation and extraction pH in section 4.4.1.3, making the interpretation more difficult.

As for the effect and correlation to replicate no conclusions can be drawn as all three replicate points are clustered around the origin. This indicates that the earlier significant effects seen in some cases of the multi-way ANOVAs perhaps aren't as significant as what was first believed, and further strengthens the idea that the variation between replicates is the result of error.

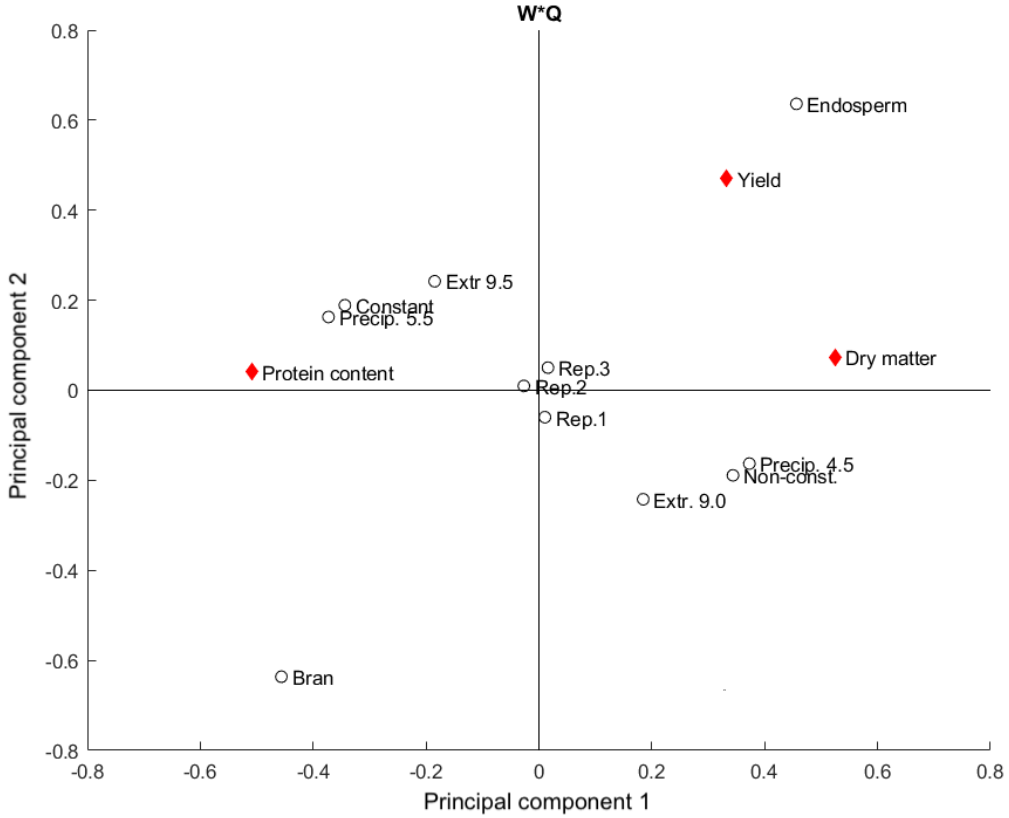

Figure 23: W*Q-plot describing the relationship between variables (black circles) and responses (red filled diamonds).

4.6 Differential Scanning Calorimetry

All graphs obtained from the differential scanning calorimetry trials are found in section A.3 in the appendix.

4.6.1 Constant pH Method

All graphs obtained in the DSC analysis show peaks that indicate presence of active, undenatured proteins. This means the extraction process is not too damaging for the proteins, and a good sign in terms of functionality. The peak that is found between 100 – 108 °C is the largest peak and is therefore believed to belong to the oat globulins. This is supported by the literature since it has been found that oat globulins have a high T_d of around 110 °C, which is close to the measured value. The most distinct trend observed from the DSC graphs for both milled oat bran and oat endosperm flour is that the globulin peak had a lower T_d for the methods with precipitation pH 4.5 compared to the methods with pH 5.5. The difference in T_d for oat globulins was more enhanced for OBM compared to OEF. Since the different samples exhibit different peaks, it can be difficult to compare them to each other, but overall, the trend of higher denaturation peak temperatures with higher precipitation pH seems to also apply for the rest of the peaks.

This could argue that the precipitation pH would affect the proteins that are transferred into the sediment. Since stronger bonds require higher temperatures to break them [92], this could be an

indication that more bonds are intact in the globulin fraction after a precipitation step at a higher pH. Possibly the more acid environment at pH 4.5 harms the proteins to a greater extent.

Another conceivable explanation for the difference in denaturation temperatures is that the purity of the proteins could differ. Impurities in the samples lowers the melting points of the proteins, which could then lead to this alteration in temperature. The width of the peak is an indication of a less homogenous sample [93], and after measurements in the oat endosperm flour graphs it was observed that the globulin peaks where 4.5 were used as precipitation pH were wider compared to those with pH 5.5. Based on this, an assumption that the higher precipitation pH provided higher purity in these samples.
The width of the main peaks for OEF also revealed a difference between the two extraction pH-values at the same precipitation pH. The samples where extraction pH 9.0 were used showed narrower peaks, which could contribute to the conclusion that lower extraction pH gave a more homogeneous sample for endosperm flour.

The other material, the milled oat bran, exhibited the reverse result regarding the width of the peaks compared to the oat endosperm flour. Here the peaks were narrower at precipitation pH 4.5 and at extraction pH 9.5, showing that those parameters provided more homogenous protein samples.

All samples for both materials exhibited a high denaturation peak temperature at the end of the temperature range, which varied between 163 – 170 °C. This is most likely due to formation of a new complex after the main phase transition of the proteins. The complex then undergoes another phase transition that give rise to this high temperature peak [92].

4.6.2 Non-constant pH Method

For the oat endosperm flour, the DSC-analysis revealed a very small difference in the denaturation temperatures between the constant pH method and the non-constant pH method, where the non-constant method had the lower temperature. The milled oat bran revealed the opposite result where the non-constant method yielded a slightly higher denaturation temperature. But since only one replicate was tested it is not possible to know if these temperature differences are significant. In terms of peak width, both raw materials had wider peaks for the non-constant method. This suggests that keeping the pH constant during the extraction would provide a more homogeneous protein sample.

4.7 Comparison to literature review

Earlier studies on alkaline protein extraction from oat have been performed on oat bran and oat groats, but not endosperm on its own, and often use a defatting treatment prior to extraction. However, some studies have been performed on both defatted (DF) and non-defatted (NDF) oat [30], [62] and can therefore be compared more easily to the results of this study.

Liu [62] performed extraction on groats which resulted in oat protein with a protein content of ~72% for NDF and ~93% for DF. Yue *et al.* [30] saw a similar difference between NDF and DF oats, but with slightly lower protein contents of ~66% and ~88% respectively. Assuming both studies used non-constant pH during extraction, the result from this study compares well with protein contents from non-constant at ~71% for oat endosperm flour and ~72% for milled oat bran. And constant pH significantly increased protein content with levels of ~74% for OEF and ~75% for OBM.

Studies that have performed extraction on only defatted oat (groats or bran) differ in protein content but are generally at the same level or higher, see Table A 2 in appendix, than the oat protein from this study as well as the other NDF results. This shows that protein contents of 80-90% is only achieved through a defatting pre-treatment.

The effect of extraction pH on protein content in previous studies varies slightly. Cluskey *et al.* [49] performed extraction at both pH 8.9 and 9.5, where the higher pH gave lower protein content at 64.5% compared to 72.1%. Wu *et al.* [48] saw a similar trend when carrying out extractions at pH 9.1 and 9.8 with protein contents of 90% and 88% respectively. Both studies used defatted groats as raw material. These results differ from this study where a higher pH of 9.5 tended to give slightly higher protein content than 9.0, although differences were not significant in all cases, Figure 8. An even bigger difference was seen between constant and non-constant pH, with constant pH contributing to higher protein content.

The protein yield from this study, for both oat endosperm flour (>80%) and milled oat bran (66-70%), is consistent with earlier studies on oat. Yue *et al.* [30], who used an extraction method similar to this study, got yields of around 64% for NDF groats, whilst DF got ~48%. Their conclusion was the same as Liu [62], which stated that defatting did not improve yield. However, some studies using DF oats show higher yields than those obtained by Yue *et al.* [30], and are closer to what was achieved in this study.

Interestingly, a study by Guan & Yao [50] using enzymatic pre-treatment prior to alkaline extraction showed similar or lower yields than this study, even though enzymatic treatments aim to increase protein yields, section 2.5.2.2.

The only study found which utilizes oat endosperm as raw material, uses dry fractionation techniques instead. They achieve a protein content similar to this study, ~73%, although using defatting treatment, and also a much lower yield of around 20%. This shows that alkaline extraction is much more efficient for protein extraction, as yield of over 80% can be achieved for endosperm [54].

Compared to other raw materials, oats appear to be easy to extract protein from as it can achieve relatively high protein contents and yield from alkaline extractions without using pre-treatments other than particle size reduction. Compared to rice endosperm flour, [61], oat endosperm flour

requires lower pH and gives higher yield, >80% compared to 43%. The effect of constant pH during extraction on protein yield is also much less prominent, and for oat endosperm flour reversed, compared to its effect on hempseed. Protein yield from hempseed press cake, [65] was increased to almost the double for pH 10 and 10.5 when using constant pH. Also, hempseed still only reached a maximum yield of ~60%, whilst oat endosperm flour and milled oat bran reached yields of 83% and 70% whilst using non-constant pH.

4.8 Discussion

When analysing the effect of different processing factors on yield, protein content, dry matter and protein denaturation it becomes evident that one optimized extraction process does not exist. Instead, the extraction process can be optimized differently depending on the desired result. How different parameters can be optimized according to the results of this study is discussed below.

Protein yield is an important factor as it essentially determines the amount of product that can be obtained from a set amount of raw material; thus, it is a crucial factor for process and cost efficiency. The results from the experimental study on oat are rather vague when it comes to yield, and differences between alkaline extraction methods are insignificant in many cases. However, what can be said is that protein yield from oat is generally high when compared to studies done on other materials, and the yield is higher for oat endosperm flour than for milled oat bran. When it comes to processing parameters, non-constant pH seems to be more beneficial possibly due to a milder environment with less protein denaturation during the solubilization. Otherwise, the choice of extraction and precipitation pH does not have a significant effect on oat endosperm flour, and for milled oat bran an interaction effect was seen making the effect of the two variables dependent on each other.

Protein content differed between extraction methods, where higher purity was achieved at higher extraction pH and using constant extraction. The negative impact of non-constant extraction could be caused by the dip in pH that occurs during the extraction time. Precipitation at pH 5.5 resulted in a higher protein content in most cases, however the effect is dependent on extraction pH. Raw material also affects the protein content, with OBM generally having a slightly higher purity of 72-77% compared to OEF 71-75%. This probably comes from OBM having a higher protein content in its raw form, meaning that there are less impurities to remove. Ultimately the effect that the processing conditions in this study has on the protein content is small when looking at it from a food application perspective. In order to achieve protein contents of over 80 or 90%, pre-treatment such as defatting is required. However, it can be argued that such high protein purity is not needed for most food applications, and that the contents achieved in this study is sufficient. Also, the processing costs increase when using pre-processing such as defatting due to the requirements of downstream processing to remove solvents, not to mention the environmental impact of using solvents such as hexane.

When comparing the protein content from these extractions to that of Lantmännen's existing oat protein product PrOatein, the protein content is higher using the alkaline extractions. PrOatein contains 50-60% protein and is produced from oat bran which is comparably lower than the contents ~75% which were achieved in this study.

Process efficiency is important in order to lower economic and environmental costs of producing oat protein. Process optimization in terms of material and energy usage, and time efficiency was

not included in the scope of this project and would require upscaling of the process in order to be evaluated. However, some observations were made in the lab-scale process that are of interest when discussing process efficiency. The first one is dry matter which was significantly affected by precipitation pH. A high dry matter is preferable from a process perspective as it requires less energy to be dried simply because the material contains less water. From this perspective, pH 4.5 is thus more process efficient. Another factor that affects efficiency is extraction time, which was a fixed parameter in this project. However, when performing non-constant extractions, it was seen that the yield surprisingly was higher. This indicates a possibility of reducing the extraction time, seeing as a high pH held over a long time does not improve the yield. Earlier studies have also found that yield is not affected when lowering extraction time from 60 to 20 minutes, as discussed in section 2.5.1.1. A third factor which affects production costs is the amount of chemicals, such as NaOH and citric acid, required. There, a low extraction pH and higher precipitation is theoretically beneficial as they should require less use of NaOH and citric acid respectively. However, the amounts used during the extractions were not monitored closely and therefore the difference in chemical usage is not analysed.

In general, oat protein possesses quite limited functional properties. Especially protein solubility, which is a very important property to function in all liquid food or beverages, is restricted. Due to time limitation in the lab, not many laboratory exercises were performed testing the functionality of the proteins after the alkali extraction, but one aspect that was observed is that the oat sediment obtained after precipitation at pH 5.5 contained more water compared to that of pH 4.5. This could indicate that the protein precipitated at the higher pH has a higher water holding capacity, which could be an important feature depending on the application of the oat protein. Furthermore, it is also thought that the use of chemicals could damage the proteins during the extraction process, and hence lower many of the functional properties of the proteins. Based on this theory, the method with pH 9.0 and 5.5 would provide a less harsh environment and would be superior from the functionality point of view. If the functionality does not reach the required level, there are several modifications that can be done to increase it through enzymatical, physical or chemical treatments, which is discussed more in section 2.3.10.

As mentioned earlier in section 2.4, plant proteins are currently of high interest and a new trending application is its use in meat analogues. For a protein to function well in this purpose, it is advantageous if it has good thermal stability. If the aim of an oat protein extraction is to create a heat tolerant food product, it could be wise to take the result from the differential scanning calorimetry into consideration. The results clearly showed that the precipitation pH had a large impact on the peak denaturation temperature and that pH 5.5 increased the temperature where the proteins went through a phase transition. Therefore, the precipitation pH is a good variable to use when desiring to alter the extraction method to suit the properties of the oat proteins.

5. Conclusion

This project evaluated alkaline methodology for its efficiency of extracting protein from milled oat bran as well as the lower value oat endosperm flour. Focus lay on analysing the effect of pH during extraction and precipitation on extraction efficiency in terms of protein yield, concentration and dry matter, along with thermal stability through DSC. The result suggests that protein is comparatively easy to extract from oat, in particular from oat endosperm flour, relative to other plant materials. Thus, the hypothesis is confirmed that alkaline methodology would successfully extract proteins from both milled oat bran and oat endosperm flour.

However, the two other hypothesizes are discarded. Previous studies conclude that pH is a key factor, but neither extraction nor precipitation pH significantly affected the extraction yields within the ranges in this study. Thus, discarding the hypothesis that the different pH would result in different protein yields. The same goes for the effect of constant versus non-constant pH during extraction. Constant pH, which has proved effective for other plant materials, did not improve protein yields for oat. Using a milder pH of 9.0 and non-constant extraction at room temperature provided yields of over 80% for oat endosperm flour and around 70% for milled oat bran. These yields are not improved with a harsher extraction at constant pH 9.5.

Extraction pH did however affect the protein purity, where higher purities were achieved using higher, and constant, pH. Although, differences are only in the range of 2-5 percentage points, and earlier studies have shown that higher protein contents would require pre-treatments such as defatting. Process efficiency was mainly affected by precipitation pH, as pH 5.5 provided a significantly lower dry matter content, thus requiring more energy during drying. This effect has not been discussed in previous studies on oat protein. Also, precipitation pH affected both denaturation temperature and possibly water holding capacity, indicating that precipitation can influence functionality.

Moreover, it is concluded from the literature review that oat proteins' functionality is poorer than what would be preferable for utilization in food products. Particularly problematic is the protein solubility which is crucial for many food applications as well as a prerequisite for several other functional properties. To improve the solubility and other properties, protein modifications were found to be a possible solution to enhance the applicability of oat proteins.

The literature review, show that the relationship between the extraction process and protein functionality is mainly affected by two parameters – temperature and pH. Conditions where those variables are high increases the risk for protein denaturation or alteration, thus contributing to reduced functionality. Therefore, a mild protein extraction process is preferred from a protein functionality point of view. While the ongoing pandemic limited the experimental investigation of functional attributes, a potential correlation between water holding capacity and precipitation pH was found. This motivates further work regarding the effects on oat protein functionality.

In conclusion there is not one singular optimized extraction process for oat protein, but rather this study finds that the process can be customized depending on the desired properties and economic requirements. Furthermore, alkaline methods prove successful in extracting protein from oat, providing a valuable use for the today low-value oat endosperm flour. However, more knowledge on the functionality of the resulting oat proteins is needed in order to establish successful, final applications on the market.

6. Future Outlook

Firstly, the impacts of the Covid-19 restrictions on the laboratory work of this study naturally opens for future extensions. While the functional attributes of oat proteins are essential to determine when developing a successful extraction method, we were unfortunately unable to investigate functionality in this project. This would therefore need to be the focus if moving forward with the subject.

In particular, we suggested that the functionality of proteins extracted using the methods from this study should be investigated and compared to previous analyses on oat protein, as well as other raw materials. Although, given the findings of our theoretical investigation it seems as if functionality of oat proteins is generally poor compared to other plant proteins. Therefore, it is likely that pre-processing or protein modifications are required if improved functionality is needed for food applications.

Regarding possible pre-processing methods, the most prevalent is defatting, wherein lipids are removed from the raw material. Defatting has been found to increase protein content as well as improve functional attributes such as foaming capacity. The method however has drawbacks, including decreasing yields slightly. The most common defatting method is using hexane which is not environmentally sustainable. Therefore, perhaps more novel methods such as using supercritical CO_2 could be evaluated if pre-processing using defatting is explored.

Other pre-treatment methods such as ultrasound or enzymes are not as applicable since their main function is to increase protein yields, which is not a main priority in this case. Ultrasound may also alter protein structures, resulting in conflicting results of both impaired and improved functionality across studies. Enzymatic treatment can aid in maintaining functionality only if the alkaline process can be made milder, an effect which has not yet been studied fully.

If the functionality of the proteins is of greatest importance, then protein modifications appear to be a more successful solution relative to extraction method optimization. Based on the literature review, we conclude that protein modifications have a potentially large impact on functionality and therefore constitutes an interesting parameter for further research.

Other areas to analyse, beyond the scope of this study, include investigations into differences between the two raw materials (oat endosperm flour and milled oat bran) in terms of protein fractions and functionality, both prior to and after extraction. Similarly, exploring optimization of extraction time is of interest as the high yields identified during non-constant extractions suggests a possibility of lowering treatment time significantly.

Finally, the environmental impact of the extraction method should not be overlooked. This includes everything from the water and energy consumption to the choice of alkaline solvent. A sustainable protein powder is only ever as sustainable as its method of production.

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A. Appendix

A.1 Background

Table A 1: Amino acid composition in oat protein fractions, presented in % of recovered protein.

Amino acid	Albumin	Globulin	Prolamin	Glutelin
Lysine	7.5	5.6	3.0	4.9
Histidine	2.6	2.9	1.6	3.0
Arginine	4.8	9.8	4.4	9.2
Aspartic acid	11.2	8.9	3.1	9.7
Threonine	5.1	3.6	2.1	4.3
Serine	6.0	4.9	2.7	4.9
Glutamic acid	12.5	20.3	34.7	17.0
Proline	5.6	5.5	8.4	5.3
Cystine	1.3	1.3	3.8	0.8
Glycine	6.1	5.4	2.2	4.9
Alanine	7.3	5.7	4.1	4.9
Valine	6.0	4.9	5.4	5.3
Methionine	2.2	1.8	3.4	1.7
Isoleucine	4.4	4.3	3.4	4.8
Leucine	7.8	6.9	9.8	7.8
Tyrosine	2.8	2.4	1.6	4.8
Phenylalanine	6.6	5.9	6.6	6.6

The different amino acids are grouped based on four attributes and marked with different colours accordingly. Yellow represents positively charges amino acids whilst the negatively charged amino acids are coloured in orange. The blue colour presents amino acids with a polar group and green those that are hydrophobic. The amino acids marked with grey are special cases and cannot be categorized into any of the other categories [94].

A.1.1 Summary of studies

Table A 2: Summary of previous studies, discussed in this report, which have performed extractions on oat, or other raw materials.

Authors	Title	Year	Extraction method	Protein source	Parameters	Comments	Summary of results	Conclusions
Ma [33]	Preparation, Composition and Functional Properties of Oat Protein Isolates	1983	1: Isolectric precipitation of alkaline extract 2: Dilution of a salt extract	Oats: dehulled groats (Hinoat and Sentinel), defatted (hexane)	1: pH 9.5 (NaOH, 0.015 N), solid-to-solvent (1:8), 60 min, room temp, IEP with HCl pH5.5 2: 0.5 M CaCl2, solid-to-solvent 1:10, 60 min, room temp,	Functionalities of isolates compared to wheat gluten and soy protein. Salt only extracts glubulins. Kjeldahl nitrogen method (5.8 factor)	Both isolates > 90% protein Yield higher in alkaline >60%, salt 25% Similar amino acid comp. Alkaline higher lysine and total e.a.a Both isolates high fat binding and foaming prop.	Possibly some denaturation from alkali, however the yield is significantly higher which makes it economically superior
Guan & Yao [50]	Optimization of Viscozyme L-assisted extraction of oat bran protein using response surface methodology	2008	Enzymatic pretreatment using Viscozyme L Alkali extraction of pretreated and untreated oat bran	Oat bran: defatted (hexane)	Viscozyme L conc: 6-30 FBG pH 3-5 Time: 0.5-2.5h Temp 35-55°C Alkali extraction: pH 9.5 (NaOH 2M), 30min, 50°C	Response surface methodology used to determine optimum pretreatement Kjeldahl method for protein content determination (factor of 6.25) Protein content was measured in supernatant, not with IEP	Extraction mainly effected by pH and temp. Optimum conditions: Viscozyme L 30FBG/10g of oat bran, pH4.6, 2.8h at 44°C Yield: 56.2% (predicted 55.7%) , significantly higher than only alkaline extraction (14.7%)	Enzymatic pretreatment can significantly enhance the protein extraction, consistent with previous studies. Degradation of β-glucan in oat bran may be reason for increased extraction
Wu *et al.* [48]	Protein isolate from high-protein oats: preparation, composition and properties	1977	Alkaline extraction	Oat: defatted high-protein groats	NaOH pH 7.4-11.1 solid-to-solvent 1:6 isoelctric precipitation: pH 5-7	Different solutions used for extraction (HCl, water, NaOH) however alkaline was significantly better. Tested butanol and hexane for defatting.	HCL: yield 39%, protein content 65% Water: yield 9%, p.c 41% NaOH: higest yield (75%) at pH 10.8 (but lower content 78%), highest content 90% (yield 67%). Defatting: butanol highest yield (12%) and protein content (103%), hexane (yield 10%, content 101%)	Optimum pH 9.2, IEP at 5.7, protein content 94-103%, yield 53-67% butanol removes bound and free lipids compared to hexane which only removes free lipids. Removing bound lipids improves protein yield and content, also better emlusifying prop.
Cluskey *et al.* [49]	Oat protein concentrates from a wet-milling process: Preparation	1973	Alkaline extraction	Oat: Groats, defatted (butanol)	NaOH: pH 6.25-11.71 Time: 4-60 min solid-to-solvent: 1:2 - 1:20 IEP at pH 6.0	Different solutions used (HCl, acetic acid, water, NaCl, NaOH) but alkaline most efficent Protein conversion factor 6.25	Solvent: pH / yield / protein content HCL: 2.3 / 13% / 36% Acetic acid: 2.7 / 22 % / 36% Water: 6 / 17% / 31% NaCl: 5.5 / 38% / 8.5% NaOH: higest yield 88% and content 72% at pH 8.9	Optimum yield of protein at pH 9 (88%) Time had effect on yield in the lower times, but no significant effect between 25-60 min. Higher solvent ratio gives higher yield, however more costly. Optimum chosen 6:1
Jodayree *et al*. [66]	Use of carbohydrase to enhance protein extraction efficiency and antioxidative properties of oat bran protein hydrolysates	2012	Enzymatic pretreatment & alkaline protein extraction	Oat bran, defatted (hexane)	2 M NaOH: pH 9.5 IEP at pH 4.0	Four different enzymes tested: Viscozyme L, alfa-amylase, amyloglucosidase and celluclast®	Highest protein content from each enzyme No enzyme: 54% Viscozyme L: 70% Alfa-amylase: 72% Celluclast: 53% Amyloglucosidase: 82%	Viscozyme L, alfa-amylase and amyloglucosidase gave higher protein content in isolates Cellucast had no effect on protein content

Authors	Title	Year	Extraction method	Protein source	Parameters	Comments	Summary of results	Conclusions
Liu [62]	Fractionation of oats into products enriched with protein, beta-glucan, starch, or other carbohydrates	2014	Alkaline protein extraction	Oat: groats some defatted (hexane)	0.075 M NaOH (pH not specified) IEP precipitation with HCL pH 5.2	Fractionation of oats Reduce viscosity Analyze effect of defatting (DF) and centrifugal force (CF)	Protein content depending on processing: Groats low CF: 72.4% Groats high CF: 72.6% DF groats low CF: 92.5% DF froats high CF: 92.6%	Defatting significantly improved protein content but not yield Centrifugation before filtration (fiber recovery) reduced viscosity Centrifugal force did not effect protein recovery or content
Yue *et al.* [30]	Impact of defatting treatment and oat varieties on structural, functional properties, and aromatic profile of oat protein	2021	Alkaline extraction & isoelectric precipitation	Oats: naked or hulled, non-defatted (NDF) and defatted (DF) (hexane)	1 M NaOH, pH 9.5, solid-to-solvent 1:15, 1 h, room temp (23°C) IEP at pH 4.5 (HCl) Freeze dried	Comparison between defatted and non-defatted oat in protein yield, composition and functionality	DF gave a higher protein content (~88%) than NDF (~66%). Protein yield was higher in NDF (~64%) than in DF (~48%) Peak denaturation temp: 109-111°C Higher solubility in DF than NDF, but still low below 25% Some conformational changes in DF Better emulsion and foam capacity, and emulsion stability in DF. NDF had better foam stability.	Defatting improved protein content but lowered yield. Generally better functionality from defatted oats, as well as improved aromatic profile.
Sibakov *et al.* [54]	Lipid removal enhances separation of oat grain cell wall material from starch and protein	2011	Dry fractionation: air classsification	Oats: protein concentrate from endosperm Some defatted with SC-CO_2	Pin disc milling and air classification gave fine and corse fractions. The fine fraction (endosperm) was air classified again to create a protein and a starch fraction.	Experiment performed on non-defatted and defatted oat. The defatting process was done using only SC-CO_2 or with ethanol in compination. In pilot scale lipid extraction was done with temperature of 70°C, and industrial with 40°C.	~65% of lipids were extracted with SC-CO_2 in pilot scale, ethanol increased the extractability of lipids. 80% were extracted in industrial scale. Non-defatted oats were difficult to pin mill For industrial scale only the defatted endosperm flour could be reclassified into a protein concentrate. Protein concentrate of 73% protein content, 5% mass yield (accounts for ~20% protein yield)	Protein rich concentrate could be obtained from the low value endosperm fraction. However, the material needs to be defatted.
Kadam *et al.* [51]	Extraction and characterization of protein from Irish brown seaweed Ascophyllum nodosum	2017	Acid, alkali and combined acid-alkali extractions With and without ultrasound (US) pretreatment	Irish brown seaweed (*A. nodosum*)	Acid: HCL (0.1-0.4 M) Alkali: NaOH (0.1-0.4 M) Combined acid + alkali: 0.4 M for both	Molecular weight of proteins and amino acid profile was analyzed	Highest protein content for each extraction method Acid: 17% Alkali: 56% Acid-Alkali: 59% Alkali-Acid: 51% Acid + US: 43% Alkali + US: 57%	Combined acid-alkali most efficient, followed by alkali+ultrasound. Ultrasound increased yield somewhat for the single-step extractions Alkali gave optimum amino acid profile
Paraman *et al.* [61]	Preparation of Rice Endosperm Protein Isolate by Alkali Extraction	2008	Alkali extraction isoelectric precipitation or ultrafiltration	Rice endosperm (flour from long-grain rice)	pH 9-11, temp 30-50°C, solid-to-solvent 1:8-1:12	Alkali extraction method was optimized	Optimum extraction: pH 11.0, 40°C, 3h 1:8 solid-to-solvent Mamimum yield 43.1% UF: 71% protein, higher solubility and emulsifying prop. IEP: 86% protein	UF provides milder and improved functionality but lower protein conc. Yield decreased with decreased solid-to-solvent. However, increases waste and costs.

A.2 Multivariate Analysis

A.2.1 Analysis of Constant Extractions

Blow the multi-way ANOVA and relevant multiple comparison tests can be found for the results from the constant pH extractions.

A.2.1.1 Yield

Table A 3: Multi-way ANOVA for the effect of variables on the response **'yield'** for oat **endosperm** flour.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Extraction pH	12.328	1	12.3279	1.46	0.2381
Precip. pH	14.219	1	14.2192	1.69	0.2062
Replicate	28.736	2	14.368	1.71	0.2029
Extraction pH*Precip. pH	11.157	1	11.1566	1.32	0.2611
Extraction pH*Replicate	11.486	2	5.7431	0.68	0.5152
Precip. pH*Replicate	24.922	2	12.4612	1.48	0.2478
Extraction pH*Precip. pH*Replicate	51.749	2	25.8744	3.07	0.0649
Error	202.151	24	8.4229		
Total	356.748	35			

Table A 4: Multi-way ANOVA for the effect of variables on the response **'yield'** for milled oat **bran**.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Extraction pH	11.065	1	11.0651	1.65	0.2114
Precip. pH	2.965	1	2.9647	0.44	0.5126
Replicate	17.756	2	8.8778	1.32	0.2851
Extraction pH*Precip. pH	47.341	1	47.3413	7.05	0.0138
Extraction pH*Replicate	0.577	2	0.2886	0.04	0.958
Precip. pH*Replicate	13.977	2	6.9886	1.04	0.3684
Extraction pH*Precip. pH*Replicate	26.551	2	13.2753	1.98	0.1603
Error	161.073	24	6.7114		
Total	281.305	35			

A.2.1.2 Dry Matter

Table A 5: Multi-way ANOVA for the effect of variables on the response **'dry matter'** for oat **endosperm** flour.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Extraction pH	0.994	1	0.994	1.22	0.2806
Precip. pH	331.261	1	331.261	406.06	0
Replicate	7.817	2	3.908	4.79	0.0178
Extraction pH*Precip. pH	1.687	1	1.687	2.07	0.1633
Extraction pH*Replicate	3.451	2	1.726	2.12	0.1425
Precip. pH*Replicate	3.092	2	1.546	1.9	0.1721
Extraction pH*Precip. pH*Replicate	3.94	2	1.97	2.41	0.1108
Error	19.579	24	0.816		
Total	371.821	35			

Table A 6: Multi-way ANOVA for the effect of variables on the response **'dry matter'** for milled oat **bran**.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Extraction pH	0.93	1	0.93	1.73	0.2005
Precip. pH	361.586	1	361.586	673.53	0
Replicate	6.008	2	3.004	5.6	0.0101
Extraction pH*Precip. pH	0	1	0	0	0.9797
Extraction pH*Replicate	5.985	2	2.992	5.57	0.0103
Precip. pH*Replicate	2.417	2	1.208	2.25	0.1271
Extraction pH*Precip. pH*Replicate	3.031	2	1.516	2.82	0.0792
Error	12.884	24	0.537		
Total	392.842	35			

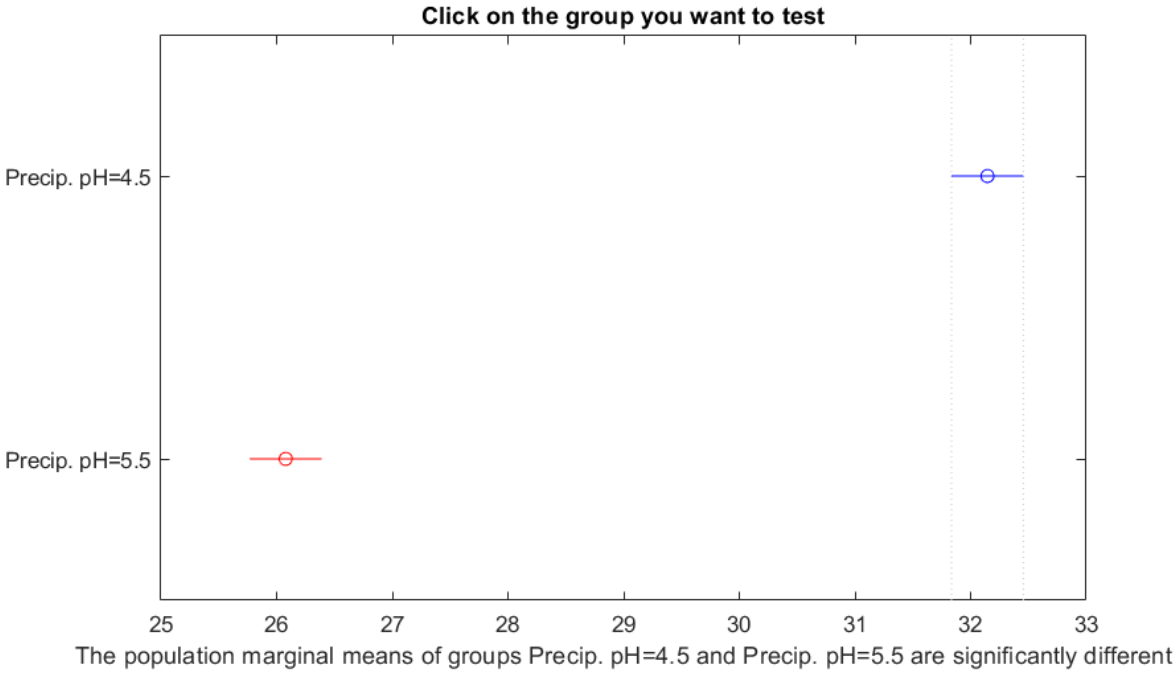

Figure A 1: Multiple comparisons test for the variable **'precipitation'** for its effect on **'dry matter'** for oat **endosperm** flour. Different colouring (blue/red) and separated intervals show significant difference.

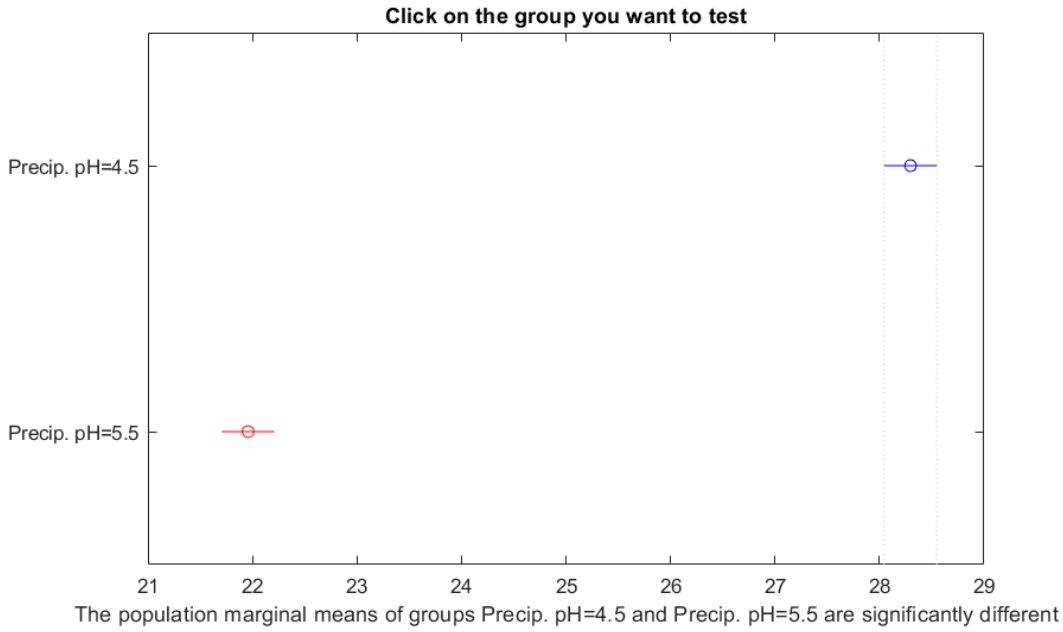

Figure A 2: Multiple comparisons test for the variable **'precipitation'** for its effect on **'dry matter'** for milled oat **bran**. Different colouring (blue/red) and separated intervals show significant difference.

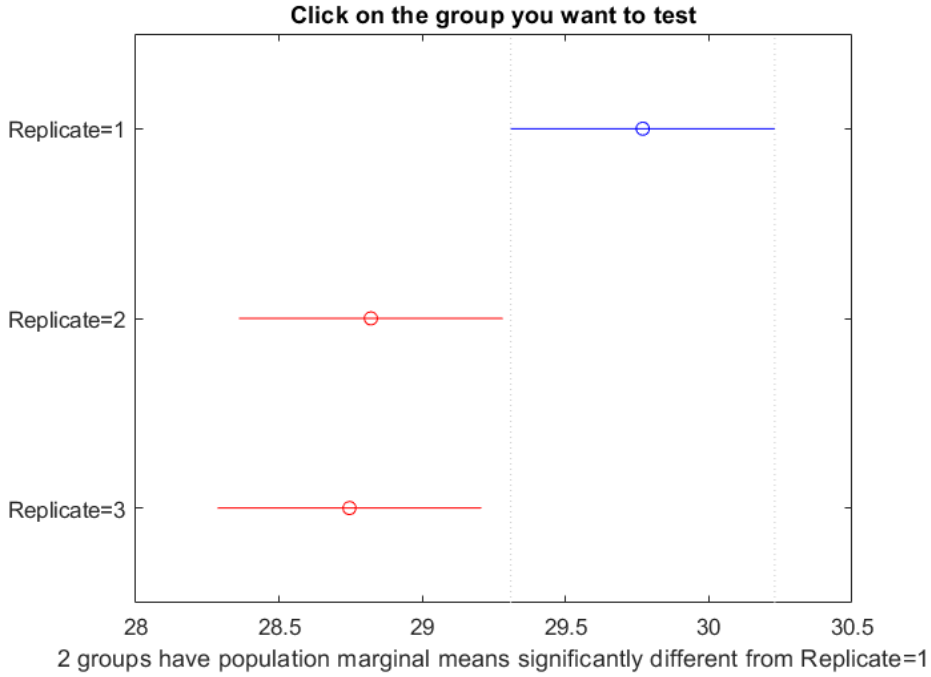

Figure A 3: Multiple comparisons test for the variable **'replicate'** for its effect on **'dry matter'** for oat **endosperm** flour. Different colouring (blue/red) and separated intervals show significant difference.

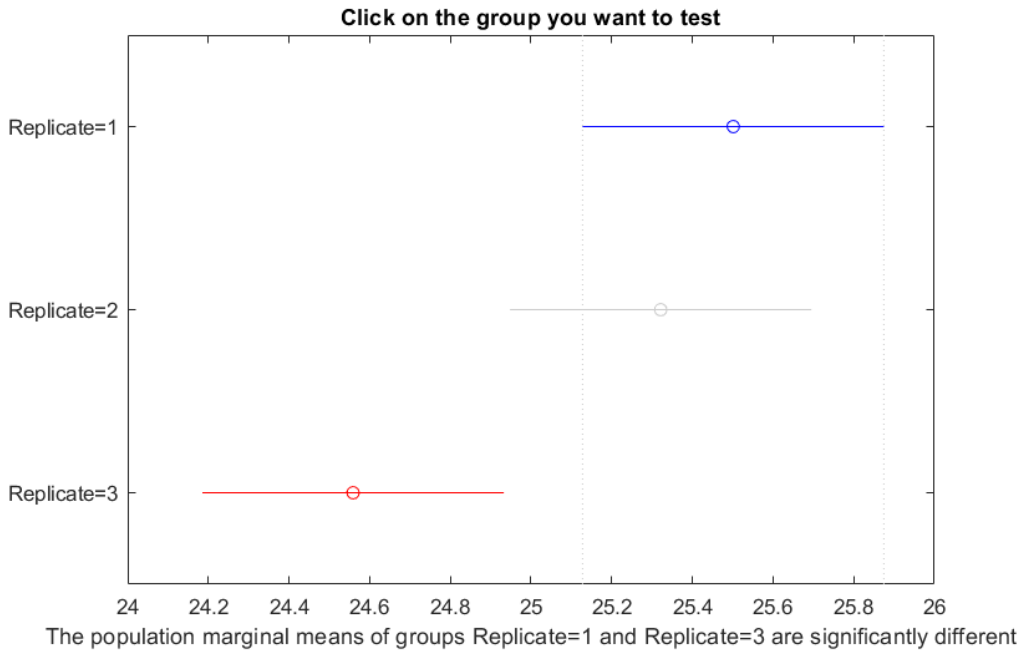

Figure A 4: Multiple comparisons test for the variable **'replicate'** for its effect on **'dry matter'** for milled oat **bran**. Different colouring (blue/red) and separated intervals show significant difference.

A.2.1.3 Protein Content

Table A 7: Multi-way ANOVA for the effect of variables on the response '**protein content'** for oat **endosperm** flour.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Extraction pH	11.5361	1	11.5361	31.15	0
Precip. pH	0.3797	1	0.3797	1.03	0.3214
Replicate	2.9077	2	1.4539	3.93	0.0335
Extraction pH*Precip. pH	4.3142	1	4.3142	11.65	0.0023
Extraction pH*Replicate	6.5179	2	3.2589	8.8	0.0014
Precip. pH*Replicate	12.1704	2	6.0852	16.43	0
Extraction pH*Precip. pH*Replicate	0.1521	2	0.0761	0.21	0.8157
Error	8.8882	24	0.3703		
Total	46.8663	35			

Table A 8: Multi-way ANOVA for the effect of variables on the response **'protein content'** for milled oat **bran**.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Extraction pH	9.4321	1	9.4321	14.72	0.0008
Precip. pH	16.012	1	16.012	24.98	0
Replicate	0.2087	2	0.1043	0.16	0.8507
Extraction pH*Precip. pH	4.262	1	4.262	6.65	0.0165
Extraction pH*Replicate	3.8905	2	1.9452	3.04	0.0668
Precip. pH*Replicate	5.3599	2	2.6799	4.18	0.0277
Extraction pH*Precip. pH*Replicate	0.5241	2	0.2621	0.41	0.6689
Error	15.3818	24	0.6409		
Total	55.071	35			

A.2.2 Analysis of Constant vs Non-constant

A.2.2.1 Oat Endosperm Flour

Table A 9: Multi-way ANOVA for the effect of **constant vs non-constant** extraction on the response **'yield'** for oat **endosperm** flour.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
method	33.849	1	33.8489	5.93	0.0314
Replicate	31.146	2	15.573	2.73	0.1054
method*Replicate	19.54	2	9.7699	1.71	0.2217
Error	68.467	12	5.7056		
Total	153.002	17			

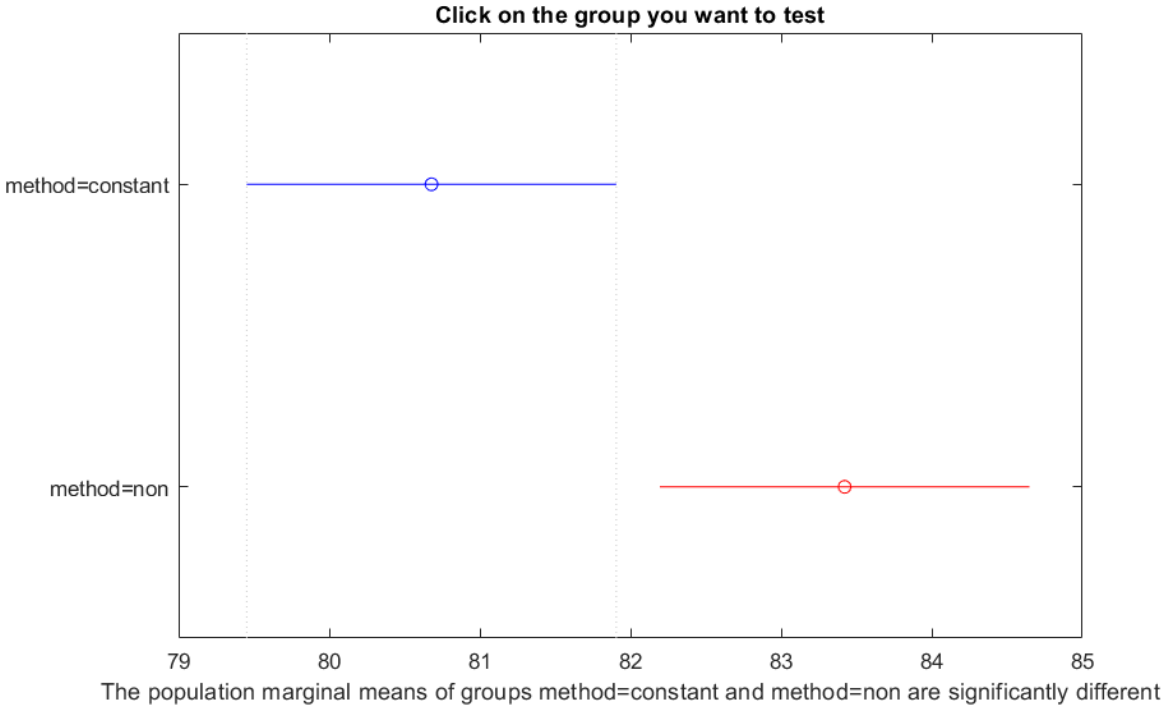

Figure A 5: Multiple comparisons test for the variable **'method'** for its effect on '**yield'** for oat **endosperm** flour. Different colouring (blue/red) and separated intervals show significant difference.

Table A 10: Multi-way ANOVA for the effect of **constant vs non-constant** extraction on the response '**dry matter**' for oat **endosperm** flour.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
method	16.7457	1	16.7457	20.27	0.0007
Replicate	2.3816	2	1.1908	1.44	0.2748
method*Replicate	3.333	2	1.6665	2.02	0.1757
Error	9.9141	12	0.8262		
Total	32.3744	17			

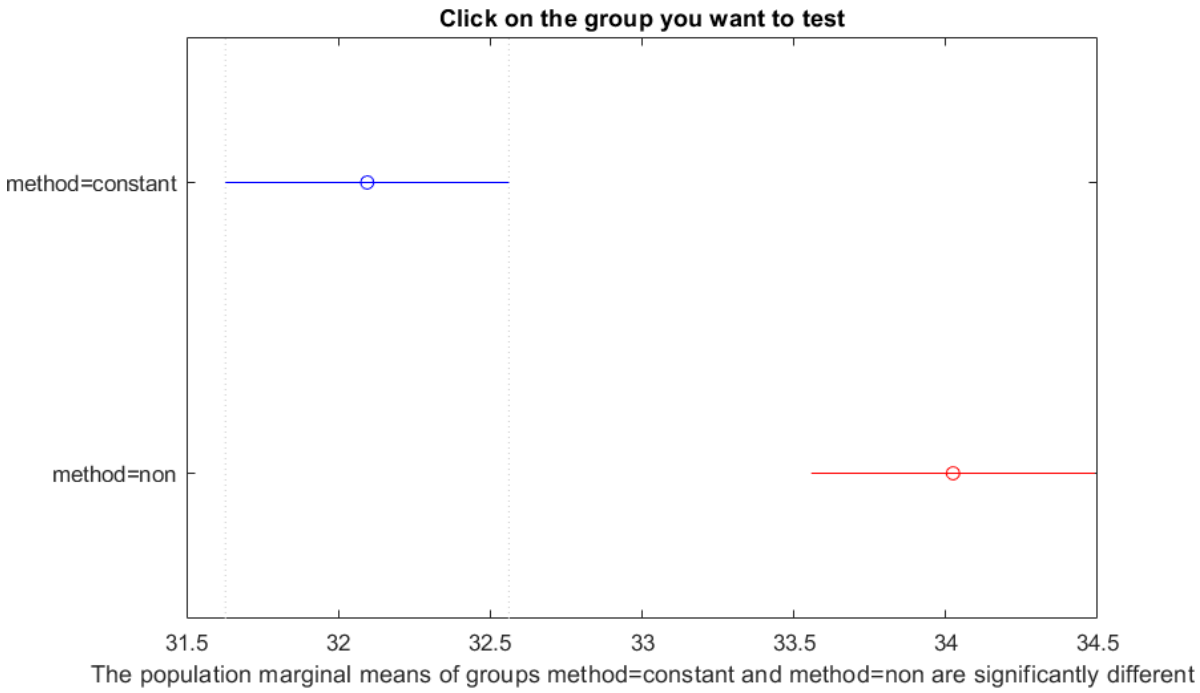

Figure A 6: Multiple comparisons test for the variable **'method'** for its effect on '**dry matter'** for oat **endosperm** flour. Different colouring (blue/red) and separated intervals show significant difference.

Table A 11: Multi-way ANOVA for the effect of **constant vs non-constant** extraction on the response '**protein content'** for oat **endosperm** flour.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
method	64.5381	1	64.5381	273.11	0
Replicate	1.1742	2	0.5871	2.48	0.1251
method*Replicate	0.5526	2	0.2763	1.17	0.3436
Error	2.8357	12	0.2363		
Total	69.1006	17			

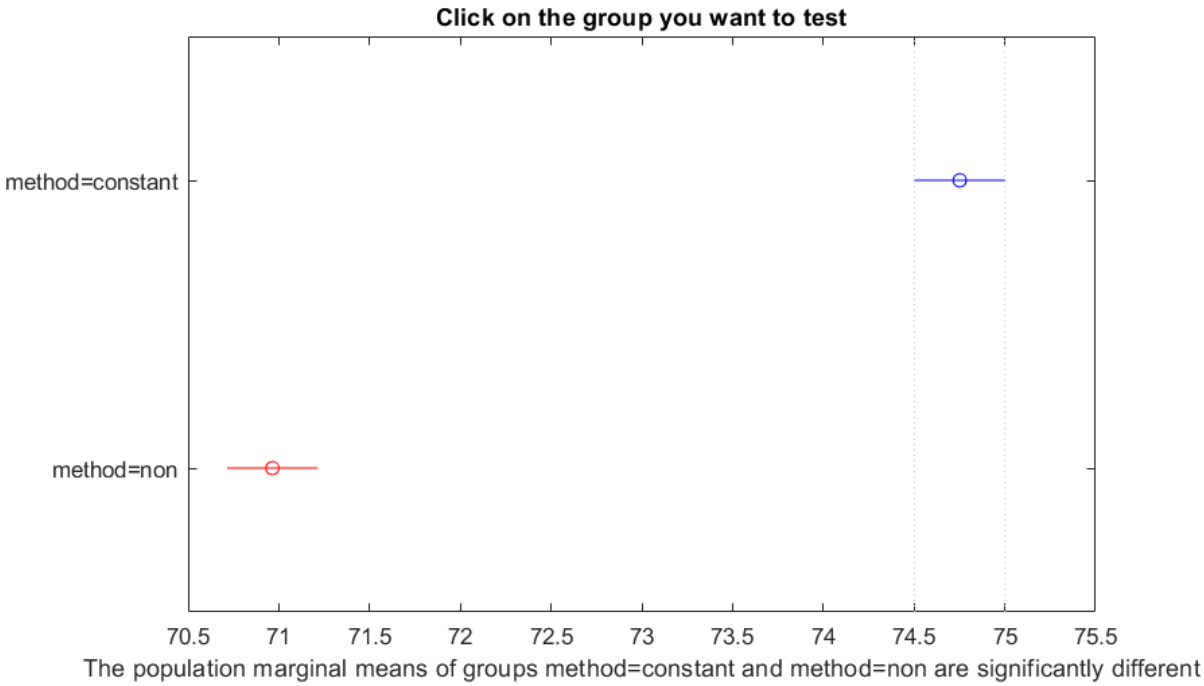

Figure A 7: Multiple comparisons test for the variable **'method'** for its effect on **'protein content'** for oat **endosperm** flour. Different colouring (blue/red) and separated intervals show significant difference.

A.2.2.2 Milled Oat Bran

Table A 12: Multi-way ANOVA for the effect of **constant vs non-constant** extraction on the response **'yield'** for milled oat **bran**.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
method	12.039	1	12.0386	2.01	0.182
Replicate	9.051	2	4.5257	0.75	0.4913
method*Replicate	14.511	2	7.2554	1.21	0.3323
Error	71.984	12	5.9987		
Total	107.585	17			

Table A 13: Multi-way ANOVA for the effect of **constant vs non-constant** extraction on the response **'dry matter'** for milled oat **bran**.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
method	0.3296	1	0.32961	0.59	0.4583
Replicate	0.6989	2	0.34943	0.62	0.553
method*Replicate	3.5875	2	1.79374	3.2	0.0771
Error	6.7341	12	0.56117		
Total	11.35	17			

Table A 14: Multi-way ANOVA for the effect of **constant vs non-constant** extraction on the response **'protein content'** for milled oat **bran**.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
method	41.7569	1	41.7569	44.94	0
Replicate	4.2113	2	2.1057	2.27	0.1463
method*Replicate	6.6731	2	3.3366	3.59	0.06
Error	11.1506	12	0.9292		
Total	63.792	17			

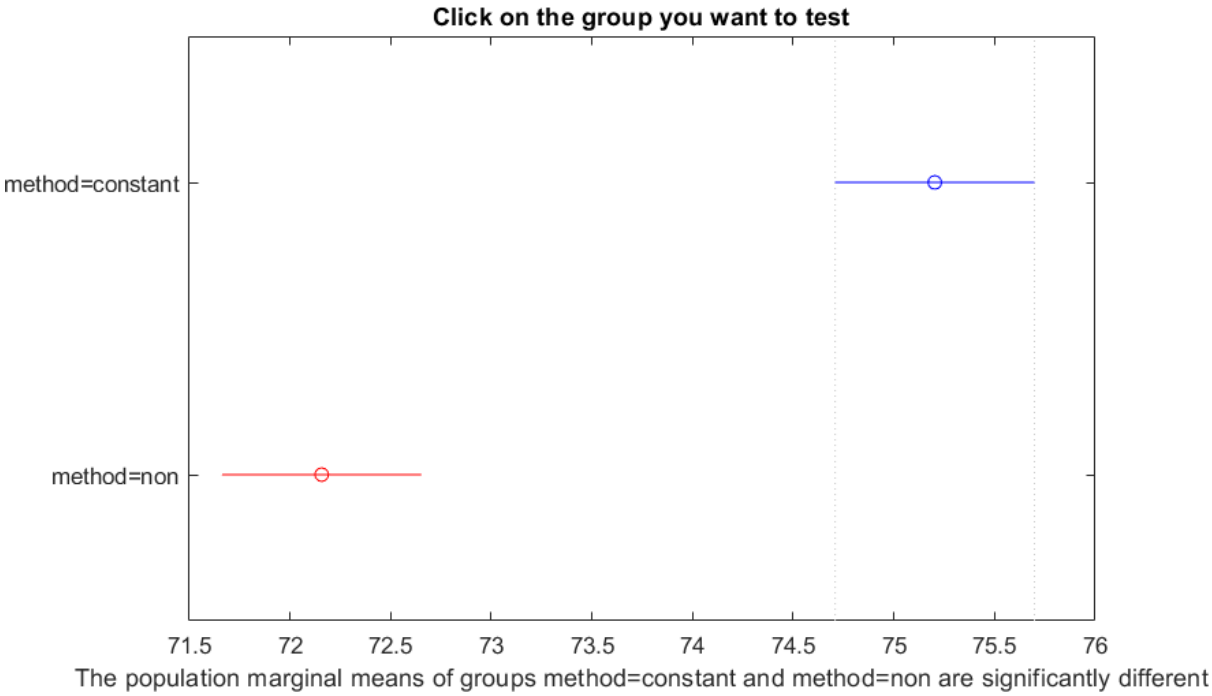

Figure A 8: Multiple comparisons test for the variable '**method**' for its effect on '**protein content**' for milled oat **bran**. Different colouring (blue/red) and separated intervals show significant difference.

A.3 DSC Graphs

A.3.1 Oat Endosperm Flour

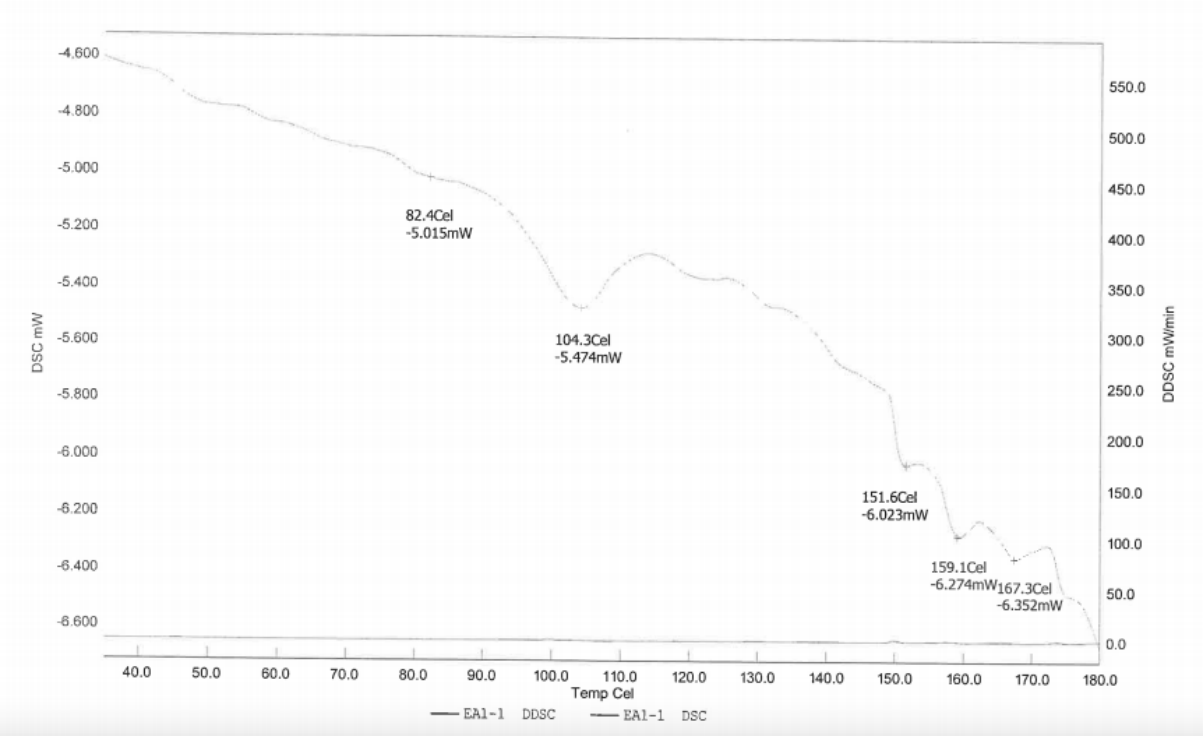

Figure A 9: DSC result from oat endosperm flour proteins, extracted using extraction pH 9.5 and precipitation pH 4.5 with the constant method.

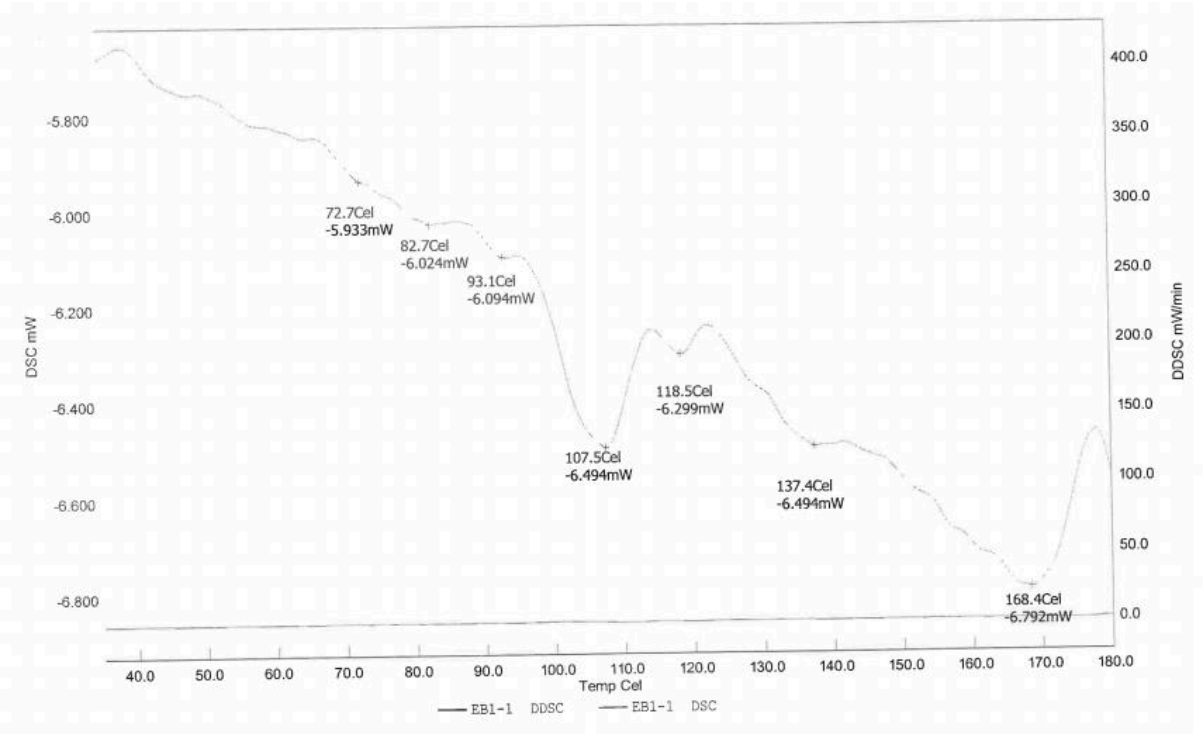

Figure A 10: DSC result from oat endosperm flour proteins, extracted using extraction pH 9.5 and precipitation pH 5.5 with the constant method.

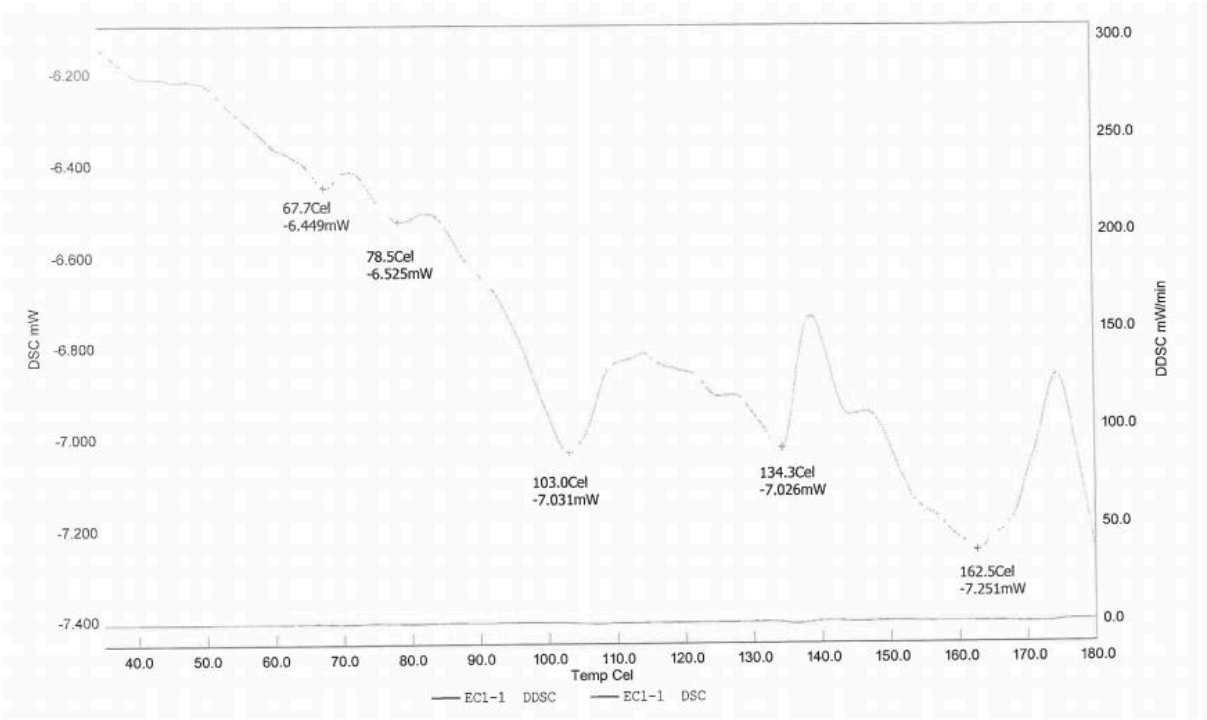

Figure A 11: DSC result from oat endosperm flour proteins, extracted using extraction pH 9.0 and precipitation pH 4.5 with the constant method.

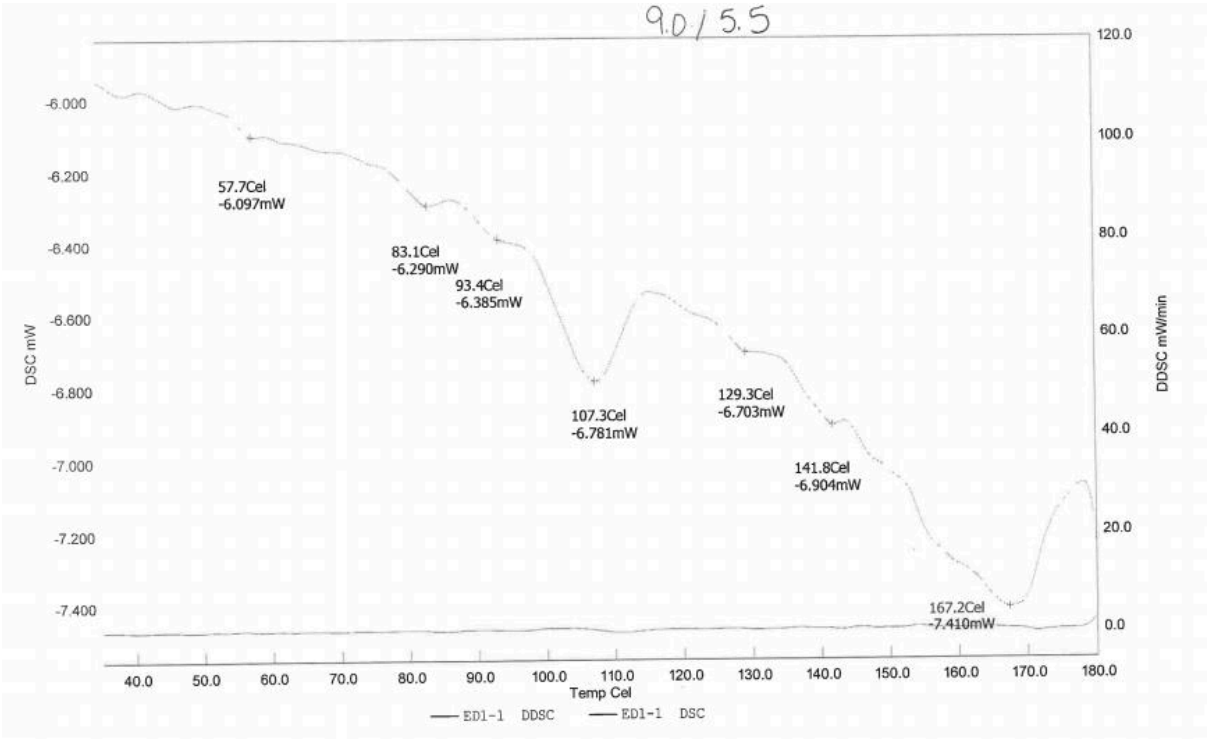

Figure A 12: DSC result from oat endosperm flour proteins, extracted using extraction pH 9.0 and precipitation pH 5.5 with the constant method.

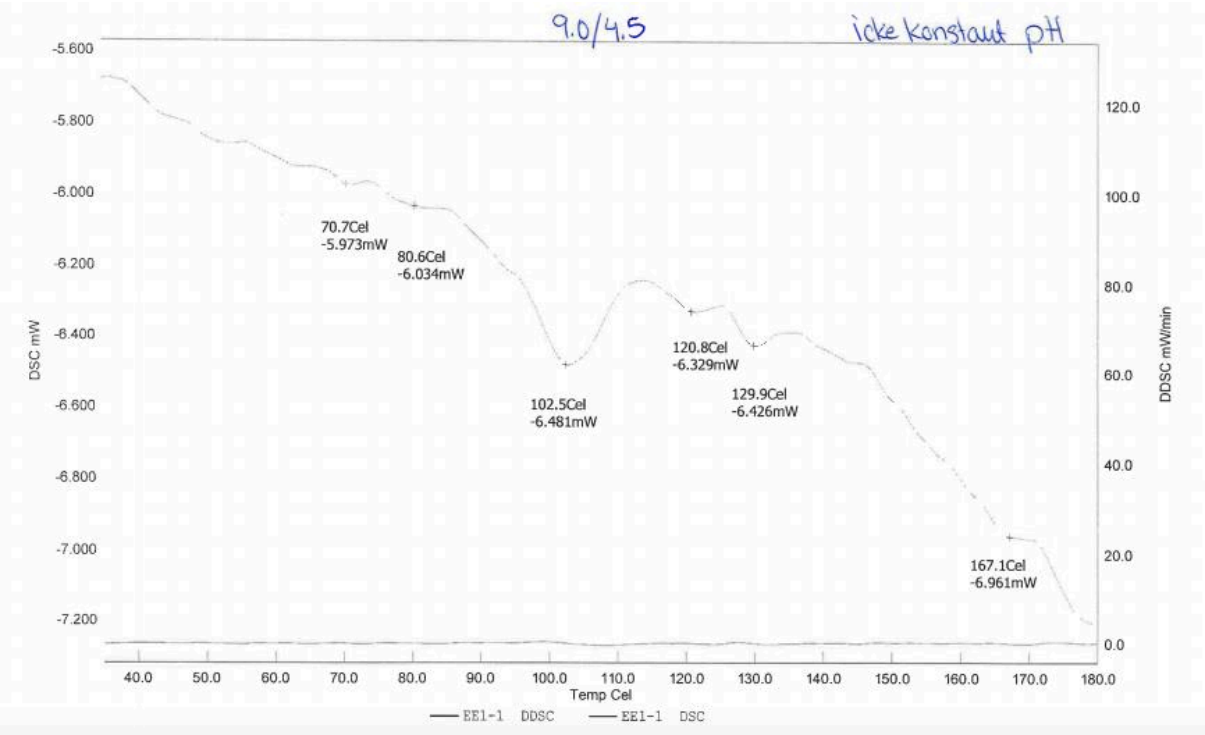

Figure A 13: DSC result from oat endosperm flour proteins, extracted using extraction pH 9.0 and precipitation pH 4.5 with the non-constant method.

A.3.2 Milled Oat Bran

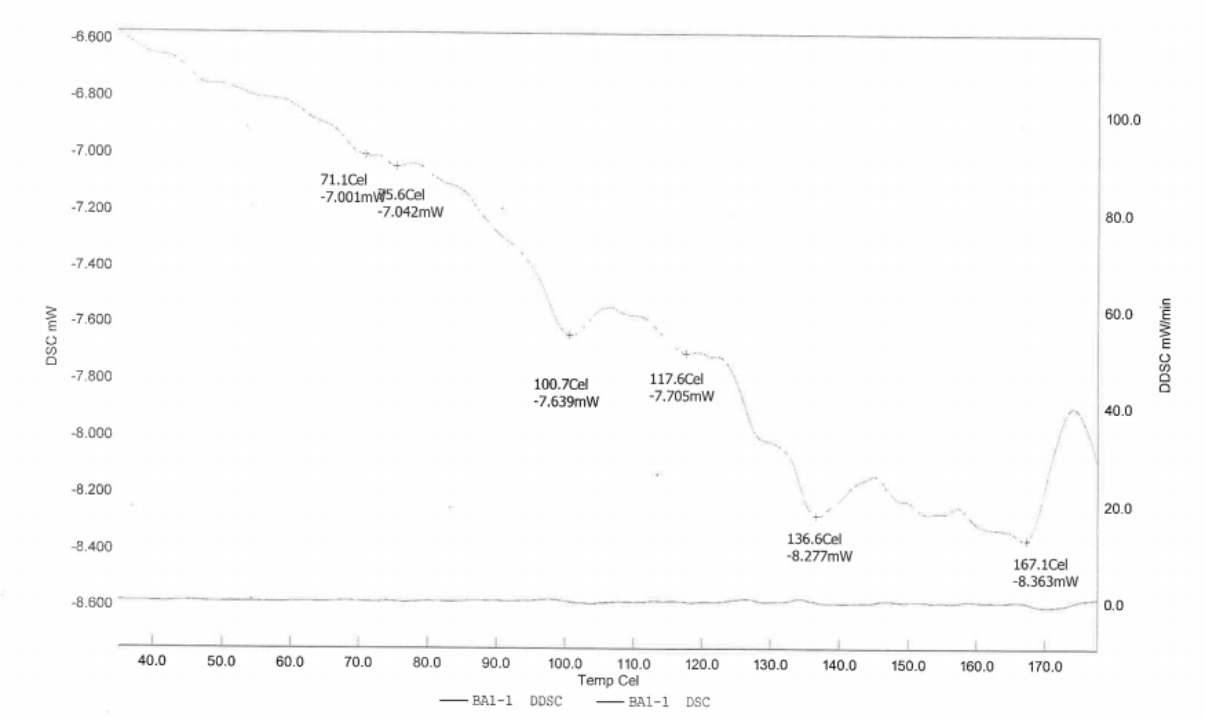

Figure A 14: DSC result from milled oat bran proteins, extracted using extraction pH 9.5 and precipitation pH 4.5 with the constant method.

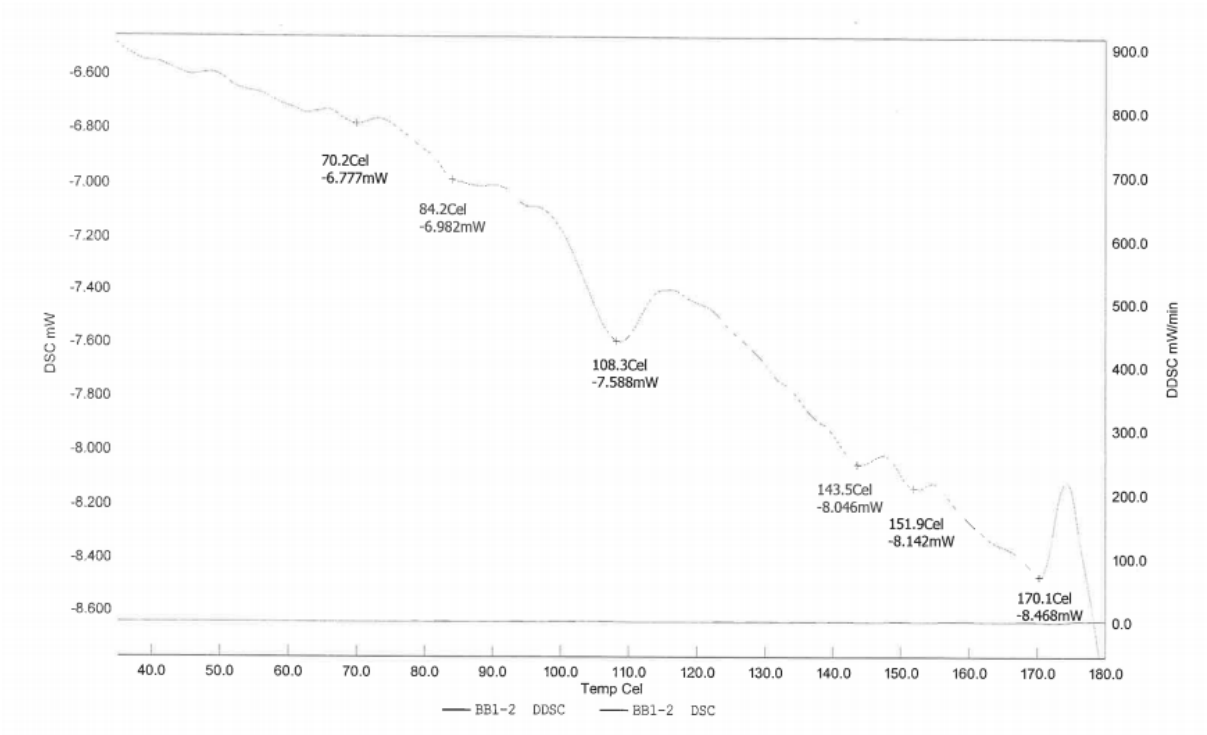

Figure A 15: DSC result from milled oat bran proteins, extracted using extraction pH 9.5 and precipitation pH 5.5 with the constant method.

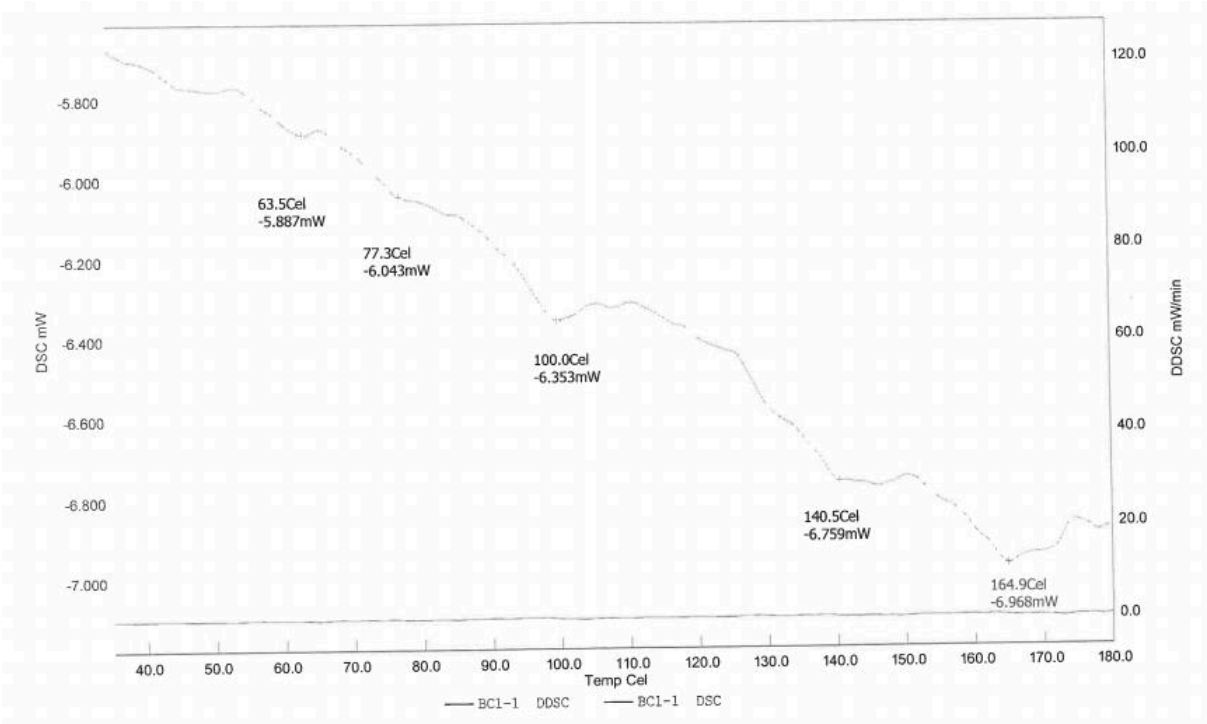

Figure A 16: DSC result from milled oat bran proteins, extracted using extraction pH 9.0 and precipitation pH 4.5 with the constant method.

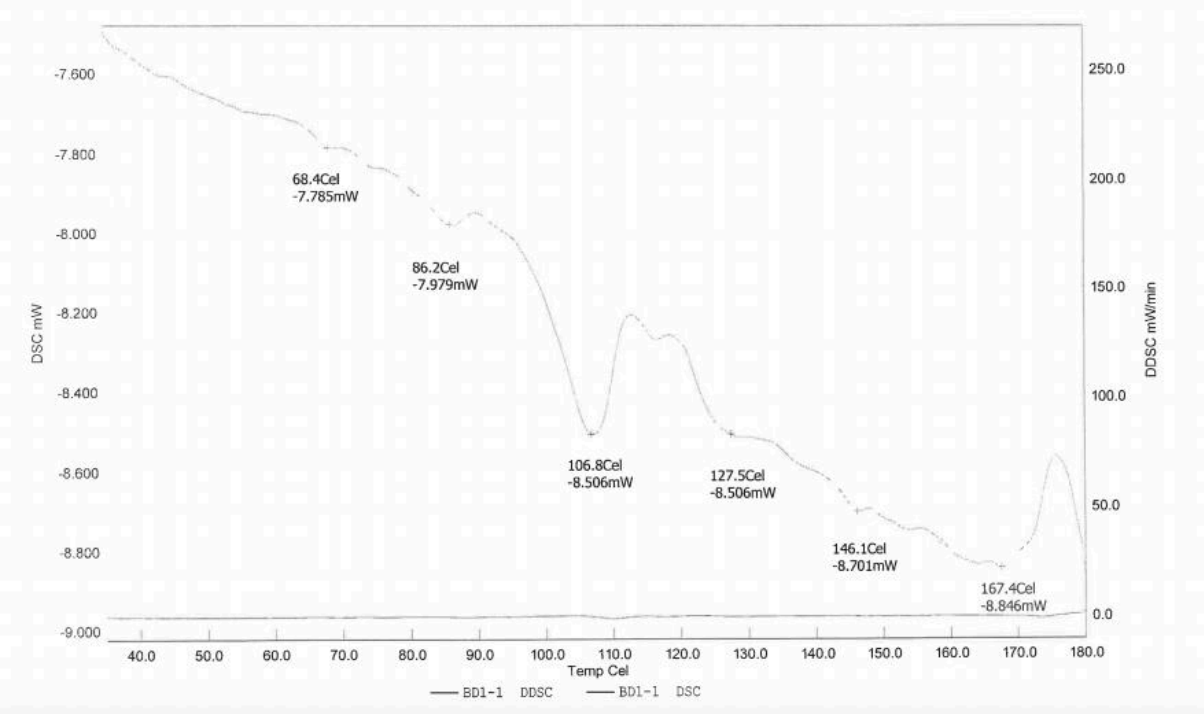

Figure A 17: DSC result from milled oat bran proteins, extracted using extraction pH 9.0 and precipitation pH 5.5 with the constant method.

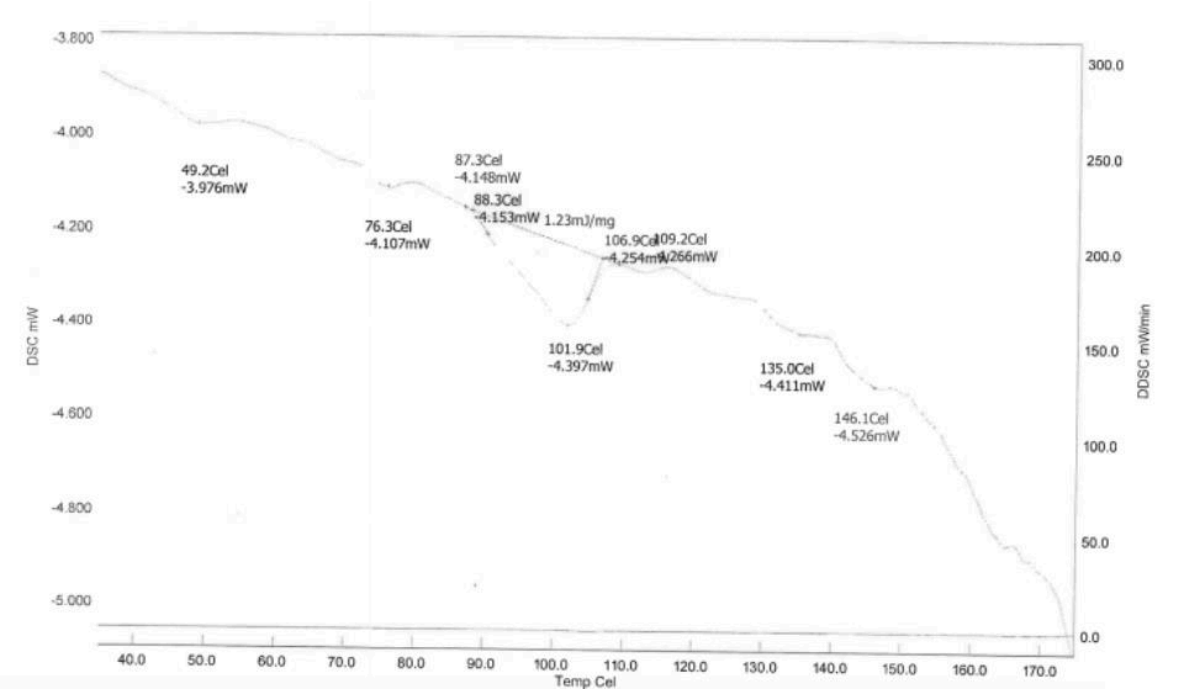

Figure A 18: DSC result from milled oat bran proteins, extracted using extraction pH 9.0 and precipitation pH 4.5 with the non-constant method.