Development of a Universal Method of Measuring Macromolecules in Oats.

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Abstract

The breeding of agricultural food crops has been of imperative importance for thousands of years and remains so today, with new methods being developed regularly. Some of these newer methods have some legal difficulties, forcing scientists to stay with the older, reliable yet slower methods. With one of these methods; mutation breeding, oat is being developed with new properties such as high content of proteins or healthy beta-glucan fibres for the market of the future. A challenge in this method is to quickly screen vast numbers of different oat lines to find these desired traits. In order to speed up this process I have strived to develop a method to store a preparation of oat flour for longer periods of time in a state that is compatible with the starch, beta-glucan and protein assays without degradation. Also, an alternate method for weighing oat flour on a scale has been attempted to be developed, since this too is a bottle-neck. The results show that by storing the oat flour in a fridge (+5°C), all three macromolecules remain stable for at least 5 years. Further, a heat sterilized or biostatic-containing slurry of oat flour in water shows promising potential of preventing degradation of all three macromolecules by being stored in a frozen state. In order to avoid the time consuming scale, a spectrophotometer's absorbance at 610 nm has been shown to correlate with the oat flour concentration with a sufficient accuracy for screening purposes.

Preface

This master thesis project has been performed at the department of applied biochemistry and the company CropTailor with the support of my mentors Prof. Olof Olsson and Andreas Hansson from september 3rd until January 9th. This project was initiated on the request of Prof. Olof Olsson as he and his research group would like to have a universal method for storing larger amounts of oat flour without degradation, ready for analysis of starch, mixed linkage ß-glucans and proteins since the grinding process is time consuming and a bottleneck for the analytical work.

Thanks

Hans olof for showing me around and introducing me to the ß-glucan method, for good company, discussions and advice.
Lars for showing me the protein assay and your jokes.
Andreas for your guidance.
Olle for your stable supervision and leadership.
Alfia, Svetlana and Sofia for your encouragement of my yoga practise.
My parents and friends for your never-ending support and being a continuous source of joy.

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1. Introduction

1.1. Oats

Oats (*avena sativa*) is a cereal crop grown in large quantities all over the world for its nutritious seed used for both human consumption and animal feed. Recently oats have been getting a lot of attention partly due to a number of studies being published that claim that the mixed linkage beta-glucan found in large quantities in oats lower low density lipoprotein (LDL), also known as 'bad' and total cholesterol, therefore possibly reducing the risk of heart disease.[2] Additionally many new products are being developed for vegans and dairy sensitive persons such as oat milk, cream, yogurt, and even ice cream; increasing the market cap of oats. The recent trend of eating gluten free is also a positive shift for oats since they contain no gliadin or glutenin which are the constituents of gluten. Because of this lack of gluten, oats have been proven safe to eat for sufferers of celiac disease with numerous experiments.[4] Furthermore the crop is frost resistant and rain tolerant, and thereby capable of cultivation in a colder and wetter climate, such as Sweden's, which is unfortunately tailored to suit oats and not humans. Cultivation of oats is also less pesticide and herbicide intensive than other cereals because of the few weeds and pests affecting this plant.[3] This opens up possibilities for organic production, becoming more and more requested by the environmentally-minded consumers of today. Yet, despite all these reasons the price of oats remains low. Also the high protein content of oats compared to other cereals 17% vs 13% for wheat makes it interesting as a possible plant based protein alternative to meat.[3,5] Besides the obvious ethical advantages of avoiding the slaughter of animals, it is also desirable because of the much lower cost and environmental footprint of plants compared to livestock.[17] Combining all these reasons makes it quite clear why there is so much interest in oats and why it is important to further develop this delicious and futuristic food.

1.2. GMOs and the Law

So now that we are convinced that oats are the way to go, how do we improve them further? How do we increase the amount of nutritious protein or beta-glucan fibres or the flavorful compounds giving oats the characteristic nutty taste? By breeding the plants of course! And now the legality starts to become an issue for health and ethical reasons, let's look a bit deeper into what constitutes a GMO (genetically modified organism) and what the law says about it. There is still a lot of controversy and ignorance regarding GMOs in the general public despite a strong unanimity among scientists.[10] This has often led to strange and inconclusive legal framework regarding what counts as a GMO and whether or not these organisms can be used for commercial agriculture. For this reason and many years of disinformation spread by several organisations has led only to further confusion among non-experts and a general consensus of the public of GMOs being 'unnatural' and 'bad'.[11] So what is a GMO according to the law? It's a tricky question to answer since we have been altering the genome of the plants and animals that constitute our food for thousands of years.[8] To aid us the European Commission have

created a list of conventional breeding techniques; that is ways of altering the genome that have been used for long enough to be considered traditional and their outcomes non-GMOs.[6]

On this list we will of course find techniques such as ‘simple selection’ where the best tasting, most beautiful or highest yielding crops’ seeds are selected every season and planted for the next. Another obvious candidate for this list is named ‘sexual crosses’ where the crops with desired traits are crossed with one another, mendelianly leading to even better tasting offspring. However this list also includes methods which are not so clearly belonging to traditional agriculture such as ‘somatic hybridization’. This technique aims to fuse the cells of two sexually incompatible plants by use of chemicals or electric shocks, thereby integrating both of the parents’ genetic material in one cell. Also included on this list is the method used by the research team I am a part of, namely ‘mutation breeding’. By use of a chemical (e.g. EMS) or physical (e.g. radioactive radiation) mutagen spontaneous random mutations are induced. Please note that in a natural system also mutations spontaneously arise over time, leading to evolution, yet while employing ‘mutation breeding’ this process is sped up.[6]

On to the next and final category of gene-altering methods: ‘techniques of genetic modification’, that is the techniques which legally create GMOs. Here we can find established tools for inserting genetic material such as the cool sounding ‘gene cannon’ which actually shoots metal particles coated with DNA into nuclei, in the hope of integration into the chromosomal DNA. Another established method of inserting genetic material into plants is by using ‘Agrobacterium tumefaciens’ which is a bacterium that naturally infects plants and redirects their metabolism by inserting DNA. This DNA is swapped in the bacteria for whatever gene you would like to insert and voila! You now have a GMO. Also new techniques are discussed here such as the very famous CRISPR system. This method was fairly recently developed from a bacterial immune system. It functions by cutting a specific DNA sequence of your choosing and letting the cell’s own somewhat error prone DNA repair system repair it. If the reparation is identical, CRISPR will cut it again, and again and again and again until a reparational error occurs. Thereby a mutation could arise; disrupting the gene, or inserting some new DNA into this cut; altering the gene.Recently further developments have been made of this technology making it more precise and allowing for insertions and deletions as well.[6,12]

So now that we know a bit more about what types of techniques are employed in the breeding of new crops we need a definition to compare these against so as to be able to distinguish what is a GMO and what isn't. Helpfully the world health organization (WHO) provides us with one: ‘‘Genetically modified organisms (GMOs) can be defined as organisms (i.e. plants, animals or microorganisms) in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating and/or natural recombination.’’.[7] This definition seems to be based on the ancient ways of breeding, i.e. ‘simple selection’ and ‘sexual crossing’ which we discussed earlier, which seems very reasonable. According to this definition then these techniques are not considered GMOs and the ‘gene cannon’ and ‘*agrobacterium*’ approach are considered GMOs. So far so good. Even ‘mutation breeding’ which has only been around for around 100 years seems to create non-GMOs since it is the same type of random mutations that arise here as do

‘‘naturally’’. However if we look at other methods included in the ‘conventional breeding techniques’ such as ‘somatic hybridization’ it seems highly unlikely for this to occur outside of a lab. Leading us to the beginning of this complexity. Recent discoveries such as CRISPR have only led to complication in the debate since with this method a point mutation can be induced at a chosen specific location, that could occur naturally, however unlikely at that specific location. Unfortunately I cannot give you a simple yes or no answer to what constitutes a GMO. I only wish to say that we have been changing the DNA of our food for thousands of years, also in ways that do not occur ‘‘naturally’’, so maybe we should be a bit more open minded in this complex issue.[6]

1.3. EMS Mutagenesis and Purpose

Because of the legal and technological situation, Olsson’s research team, who wanted to breed better oats, chose to go with mutation breeding, specifically by using the chemical mutagen EMS (Ethyl methanesulfonate).[13] EMS causes mutations in the DNA by ethylation; ethylating O-7 of the base guanine, thereby disrupting one of the hydrogen bonds with cytosine as can be seen in figure 1 below.[14] This altered base has a newfound affinity for thymine, leaving its old partner cytosine during the next replication.[15] As could be expected from this mechanism of action the vast majority of mutations are GC -> AT.[16]

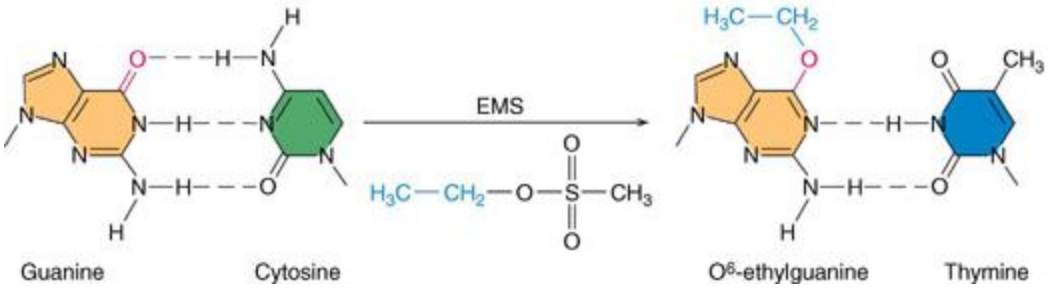

Figure 1. A graphic representation of the methylation caused by EMS and the disrupted hydrogen bond ending the GC relationship.[18].

During mutation breeding a large number of mutated organisms are created in order to have a higher probability of your desired gene(s) being altered since the mutations are random and the genome is large. Olsson’s research group created a population of 2600 mutated oat lines and therefore to screen these for the desired phenotypes of e.g. high protein or high mixed linkage beta-glucans is a massive undertaking.[13] Since oat flour is used for the assays, seeds of each variety must be ground separately and more or less simultaneously since the stability of these macromolecules has not been ascertained. Therefore the current assays are very time consuming and limited in their capacity of screening large populations quickly. Some improvements have already been made in order to increase the speed and volume, especially to the protein assay, by grinding the seeds individually in eppendorf tubes. However it would be preferable, timewise, to only be required to grind the desired variety once and be able to store

the resulting flour indefinitely and use a portion of it for the assay each time the need arises. To this end I have strived to develop and verify a method that would reach these goals.

1.4. Hypothesis

Here i present four separate yet connected hypotheses of descending importance:

1. My first hypothesis is that it is possible to store oat flour and a buffered slurry of oat flour in a chilled state without a change in the amount of protein, starch or mixed linkage beta-glucans.
2. This preparation should also be able to sustain several thawings and refreezings without degradation and be in a state that is compatible with the protein, starch and mixed linkage beta-glucan assays.
3. Furthermore, it should be possible to pipette this well mixed slurry and not compromise the accuracy of the assays.
4. Also a spectrophotometric measurement of this slurry should be an accurate replacement for a scale.

2. Materials and Methods

2.1. Materials

2.1.1. Buffers

A buffer consisting of 20 mM Na_3PO_4 was prepared by weighing 3.28 g of Na_3PO_4 and 0.2 g of sodium azide and adding these chemicals carefully to 0.9 litres of distilled water. This solution was titrated under stirring to a pH of 6.5 by addition of ≥99% acetic acid and then distilled water was added to 1 litre.

A buffer consisting of 200 mM acetic acid was prepared by pipetting 10.7 ml of ≥99% acetic acid and 0.2 g of sodium azide and adding these chemicals carefully to 0.9 litres of distilled water. This solution was titrated under stirring to a pH of 4.0 by addition of 4 M NaOH and then distilled water was added to 1 litre.

A buffer consisting of 50 mM acetic acid was prepared by pipetting 2.7 ml of ≥99% acetic acid and 0.2 g of sodium azide and adding these chemicals carefully to 0.9 litres of distilled water. This solution was titrated under stirring to a pH of 4.0 by addition of 4 M NaOH and then distilled water was added to 1 litre.

A buffer consisting of 20 mM NaOH and 2% SDS was prepared by weighing 20 g of SDS and 0.72 g of NaOH and adding these chemicals carefully to 1 litre of distilled water. This solution was stirred continuously for half an hour to ensure proper mixing before use.

2.2. Methods

2.2.1. Grinding

In order to create flour from oats, first the hull is removed by hand and are then placed 15 at a time into a Tritsch Pulverisette 23 ball mill (Fritsch GambH, Idar-Oberstein, Germany) and run at a frequency of 50 s^{-1} for 2 minutes.

Alternatively the peeled seeds were ground using a Precellys Evolution milling device (Bertin Technologies, France) at 7500 rpm for 2x 30 seconds with a 10 second pause in between.

2.2.2. Grainsense

The GrainSense is a handheld NIR absorbance spectroscopy device that is activated and the glass is checked for dirt and impurities and if necessary cleaned with an ethanol-moisted napkin before a blank is performed. 80 seeds of the specific strain are selected at random and are put unhulled in a single layer on the glass and the moisture, carbohydrates, protein and lipid content are obtained at the push of a button. The blank and any necessary cleaning is performed between every sample.

2.2.3. Starch

This assay is largely based on Megazymes starch assay but scaled down by a factor of 5.[21] Well mixed oat flour of the interested strain is ground according to the grinding method and put in safelock 2 ml tubes with a glass ball (⌀ 6 mm). Around 15 mg is measured exactly and the weight is noted. Then 100 µl distilled water is added and the tubes are mixed with the precellys at 4500rpm for 2x15 seconds with a break of 10 seconds in between. 1600 µl refrigerated 2 M KOH is added, it is paramount to shake the tube vigorously instantly after adding the base or a lump will form and the results are lost. These tubes are put in a rotating mixer in a +5°C fridge for 20 minutes. 300 µl of this now viscous solution is then slowly pipetted into 1200 µl 1.2 M NaAc. After a thorough mixing to neutralize, 15 µl is removed and saved for later in order to serve as a blank. To the original solution, 15 µl amyloglucosidase (AMG) and 15 µl alpha amylase is added. After mixing they are incubated at 50°C for 30 minutes. Followed by a step of centrifugation at 1800g for 10 minutes. 15 µl of the supernatant is then transferred to a new tube and 1000 µl of GOPOD reagent is added. 1000 µl of GOPOD reagent is also added to the earlier mentioned blank. Also a glucose standard is prepared, consisting of 15 µl 1.0g/l glucose standard and the 1000 µl GOPOD reagent. Its corresponding blank consisting of 15 µl distilled water plus 1000 µl GOPOD reagent is also prepared. All these mentioned solutions are incubated at 50°C for 20 minutes and then the absorbance is measured at 510 nm.

From the absorption the percentage of starch in the oats is calculated as follows:

$$Starch\ (g/g\ in\ \%) = (A_{sample} - A_{blank}) * F * \frac{100}{m_{sample} * (1 - \%humidity)} * D * \frac{162}{180}$$ Equation 1.

Where A is the absorbance, F is the F factor calculated below, m is mass, D is the dilution factor calculated below and V is volume. Note that the dilution factor is constant in this experiment is equal to $1.7\ ml * \frac{1515 \mu l}{300 \mu l} * \frac{1015 \mu l}{15 \mu l} = 580.9\ ml$ so equation 1 can be simplified to:

$$Starch\ (g/g\ in\ \%) = (A_{sample} - A_{blank}) * F * \frac{100}{m_{sample} * (1 - \%humidity)} * 522.8\ ml$$ Equation 2.

The blank's absorbance is removed from the absorbance of the sample in order to receive only the contribution from the reacted glucose. Multiplication with the F factor is in order to be able to relate the absorbance to an actual concentration of glucose, obtained by a glucose standard with a known concentration. Multiplication with 100 is performed to receive an answer in percent. Humidity is removed from the sample's mass so that the dry weight is obtained and division with the sample weight is to receive not only the amount of starch in the sample but the fraction of the oat that is starch. As for the multiplication with $\frac{162}{180}$, it is because the degradation of starch results in anhydroglucose and not glucose. The molar masses of the two compounds are divided in order to compensate for the lower mass of anhydroglucose.

$$F = \frac{C_{glucose\ standard} * V_{analyzed}}{A_{glucose\ standard} - A_{glucose\ blank}} = \frac{1.0 mg/ml * 0.015 ml}{A_{glucose\ standard} - A_{glucose\ blank}} = \frac{0.015 mg}{A_{glucose\ standard} - A_{glucose\ blank}}$$ Equation 3.

The F factor is the relationship of glucose and absorbance for the particular experiment, so that the samples with unknown glucose content can be related to a known concentration and calculated.

2.2.4. ß-glucan

This assay is largely based on Megazymes ß-glucan assay but scaled down by a factor of 4.[20] Well mixed oat flour of the interested strain is ground according to the grinding method and put in safelock 2 ml tubes with a glass ball (⌀ 6 mm). Around 15 mg is measured exactly and the weight is noted. Then 800 µl of 20 mM Na_3PO_4 buffer is added and the tubes are again vortexed and also inverted a few times and placed in a 95°C heat plate that is shaking at a frequency of 1250 min^{-1} for at least 10 minutes. The tubes are then mixed using the precellys at a speed of 4500rpm for 2x15 seconds with a break of 10 seconds in between. The tubes are placed in the heat block again for an additional minimum of 10 minutes with the same settings. During this time the amount of reactant can be calculated according to equation 4:

$$V_r = (N_{samples} * 2 + 4) * 1.5\ ml$$ Equation 4.

This amount is taken out of the freezer to thaw in the absence of light in 25°C water. When removing the tubes from the heat block making sure the lids are still closed and if necessary closing them. The tubes are then allowed to cool for a few minutes. 40 µl lichinase is added and the samples are briefly vortexed and inverted a few times and put in a shaking heat block at 50°C for 20 minutes. Every 20 minutes the samples are removed from the heat block in order to be vortexed and inverted a few times and put back. This is repeated until the samples have

spent 60 minutes on the heat block. Then 1 ml of 50 mM sodium acetate buffer is added and the samples are inverted a few times before centrifugation for 8 minutes and RCF=16'000. Next the blanks are prepared by mixing 50 µl of the supernatant from the sample with 50 µl of 200 mM sodium acetate buffer so that each triplicate has a corresponding blank. A blank is also made for the glucose standard by mixing 50 µl of distilled water with 50 µl of 50 mM sodium acetate buffer . 1.5 ml well mixed reactant is then added to these blanks and they are incubated on a heat block for 20 minutes at 50°C. The blanks are inverted a few times and transferred to cuvettes and absorbance is measured at 510 nm in a spectrophotometer. The samples are then prepared by mixing 50 µl of the supernatant with 50 µl of the ß-glucosidase and are mixed well by vortexing and inverting a few times. This is also done for the glucose standard with a concentration of 1.0 g/l and also the oat standard. It is advantageous to briefly and lightly centrifuge these mixtures using a desktop centrifuge to avoid drops in the cap before incubating in a heat block at 50°C for at least 10 minutes. As with the blanks 1.5 ml of the reactant is added and incubation follows at 50°C for 20 minutes. The samples are then inverted a few times and transferred to cuvettes and absorption is measured at 510 nm.

From the absorption the percentage of beta-glucan in the oats is calculated as follows:

$$\beta - glucan\ (g/g\ in\ \%) = (A_{sample} - A_{blank}) * F * \frac{100}{m_{sample} * (1 - \%humidity)} * \frac{V_{sample,\ total}}{V_{analyzed}} * \frac{162}{180} \qquad \text{Equation 5.}$$

Where A is the absorbance, F is the F factor calculated below, m is mass and V is volume. Note that $\frac{V_{sample,\ total}}{V_{analyzed}}$ is a constant dilution factor that in this experiment is equal to: $\frac{1.88ml}{0.05ml} = 37.6$ so equation 5 can be simplified to:

$$\beta - glucan\ (g/g\ in\ \%) = (A_{sample} - A_{blank}) * F * \frac{100}{m_{sample} * (1 - \%humidity)} * 33.84 \qquad \text{Equation 6.}$$

The blank's absorbance is removed from the absorbance of the sample in order to receive only the contribution from the reacted glucose. Multiplication with the F factor is in order to be able to relate the absorbance to an actual concentration of glucose, obtained by a glucose standard with a known concentration. Multiplication with 100 is performed to receive an answer in percent. Humidity is removed from the sample's mass so that the dry weight is obtained and division with the sample weight is to receive not only the amount of beta-glucan in the sample but the fraction of the oat that is beta-glucan. The volumes that were simplified are considered because the amount of beta-glucan in the whole sample not only the analyzed fraction is desired. As for the multiplication with $\frac{162}{180}$, it is because the degradation of beta-glucan results in anhydroglucose and not glucose. The molar masses of the two compounds are divided in order to compensate for the lower mass of anhydroglucose.

$$F = \frac{C_{glucose\ standard} * V_{analyzed}}{A_{glucose\ standard} - A_{glucose\ blank}} = \frac{1.0mg/ml * 0.05ml}{A_{glucose\ standard} - A_{glucose\ blank}} = \frac{0.05mg}{A_{glucose\ standard} - A_{glucose\ blank}} \qquad \text{Equation 7.}$$

The F factor is the relationship of glucose and absorbance for the particular experiment, so that the samples with unknown glucose content can be related to a known concentration and calculated.

2.2.5. Protein

A oat seed of the interested strain is ground according to the precellys grinding method and put in safelock 2 ml tubes with a glass ball. A seed of around 40 mg is selected and weighed exactly and the weight is noted. 500 µl of distilled water is added to the flour and the tubes are then mixed using the precellys at a speed of 4500rpm for 2x15 seconds with a break of 10 seconds in between. 500 µl of buffer no. 4 is added and the samples are vortexed and placed into an already shaking heat block set to 60°C and 1250 min^{-1} for 30 minutes. The samples are then centrifuged at RCF=25'000 at 20°C for 15 minutes. In order to dilute the samples 10x, 180 µl of water is pipetted into each of the wells of a 96-well plate preferably using a multi-pipette and then 20 µl of the samples' supernatant is added to the corresponding wells. BCA reagent is prepared by adding 20 µl of $CuSO_4$ to every ml of BCA and pipetting 200 µl of this well-mixed solution into the wells of another 96 well plate. 25 µl of the diluted samples are added to the corresponding wells of the reagent 96 well plate and are mixed well using the pipette. 25 µl of the 5 BSA standards of the concentrations 0, 200, 400, 600, 800 and 1000 mg/l are added in the same fashion. The 96-well plate is incubated at 37°C for 30 minutes and then allowed 5 minutes to cool. Bubbles are removed by gently blowing on the surface of the wells with a hairdryer. The 96-well plate is then checked for other disturbing elements such as dust which are removed if necessary and then absorption is measured at 562 nm.

The absorbance of the standards are used to construct a standard curve, that is the least squares method is used to calculate a linear equation that best fits these data points. There we receive the a and b values of a linear equation ($y = ax + b$) put into the following equation:

Equation 8. $$Protein\ (g/g\ in\ \%) = \frac{(A_{sample} - A_{blank}) - b}{a} * \frac{100}{m_{sample} * (1 - \%humidity)} * D$$

Where the dilution factor can be calculated as: $1\ ml * \frac{200\mu l}{20\mu l} * \frac{225\mu l}{25\mu l} = 90\ ml$ leading to the following equation:

Equation 9. $$Protein\ (g/g\ in\ \%) = \frac{(A_{sample} - A_{blank}) - b}{a} * \frac{100}{m_{sample} * (1 - \%humidity)} * 90\ ml$$

2.2.6. Optical Density and mass

The experiment was initiated by scanning from 200 nm to 900 nm in order to find a wavelength appropriate for this experiment. This was performed on a suspension of 1 seed (of mass varying between 25-45 mg) in 2 ml of distilled water pipetted into a 96 well plate with 100 µl in each well.

Then an experiment was performed in order to find the interval of concentration of suspended oat flour and the volume that would correspond to the interval of operation of a spectrophotometer, that is roughly 0.2-0.8. This was done by measuring on 100 µl of this pure suspension to a dilution of 10 times also in volumes of 100 µl in a 96 well plate. Later an experiment with the dilutions 6.66-20 times was performed with the same setup with the

exception of 1.5 ml of distilled water for the suspension. During this experiment the absorbance was measured (610 nm) on the suspended solution, during sedimentation (30s intervals for 5 minutes) and after 10 minutes when it was assumed to have completely sedimented.

The next experiment on only Belinda seeds (as all experiments so far) was performed by grinding a seed with a mass of 40.5 mg and suspending it in 1.5 ml of distilled water and pipetting 2, 3, 4, 6, 8, 10, 12 and 15 µl of this solution in octuplicates onto a 96 well plate. Thereafter adding distilled water to a volume of 100 µl in every well in the 96 well plate and measuring the absorbance at 610 nm. The final experiment was performed to ascertain the variability of different seed strains and their effect on the OD. This was done by mixing 20.2+-0.1 mg of freshly ground flour of the oat lines Belinda F, Belinda grown in Svalöv, CR1408 (high beta-glucan) grown in svalöv, and CR1200 (high protein) also grown in Svalöv. This was suspended in 1.5 ml distilled water by shaking with Precellys at 4500rpm for 2x15 seconds with a break of 10 seconds in between. Of this slurry 6, 7, 8, 9, 11, 13, 16 and 20µl were pipetted into a 96-well plate and filled up to 100µl with distilled water. This was allowed to sediment for 20 minutes and OD was measured at 610 nm with a spectrophotometer.

The other important part of correlating OD and mass is to determine if this slurry is repeatably pipettable. In order to determine this, slurries for the starch, beta-glucan, and protein assays were prepared by weighing approximately 75, 75 and 160 mg exactly and mixing this with 500, 500 and 1600 µl of distilled water respectively. After a mixing with the precellys mix program and a thorough shaking just before pipetting, 100, 100 and 400 µl of this slurry was pipetted in triplicates and compared with the regular method of these three assays involving a scale.

2.2.7. Storage

Four storage temperatures were selected: fridge (+5°C), freezer (-20°C), room temperature (+20°C) and the incubator (+37°C). Fridge and freezer were selected as reasonable storage possibilities with room temperature as a control and the incubator as a sped up control due to the limited time-scope of this project.

Four preparations were selected for storage in these four conditions: Dry flour, Flour suspended in distilled water, Dry flour heat-treated at 100°C for 20 minutes and then suspended in distilled water and Flour suspended in a 0.02% azide, sodium phosphate buffer. All samples were prepared with Belinda grown in svalöv 2017. The Flour suspended in distilled water was also prepared with a high beta-glucan strain for the beta-glucan assay and a high protein strain for the protein assay.

All three described assays were performed on freshly ground oat flour and on these preparations stored for 3 weeks and 6 weeks. Samples were also created and stored for future analysis (6 months and 12 months).

In order to ascertain the stability of the macromolecules after repeated freezing and thawing, plain flour and slurries of oat flour in distilled water and 0.02% azide, sodium phosphate buffer were prepared of the Belinda F oat variety. These slurries were then frozen and thawed by being placed alternatingly 30 minutes in the freezer (-20°C) and in room temperature. This was done once, five and ten times and then tested according to the three assays together with a freshly ground control.

3. Results

3.1. Storage

3.1.1. Starch

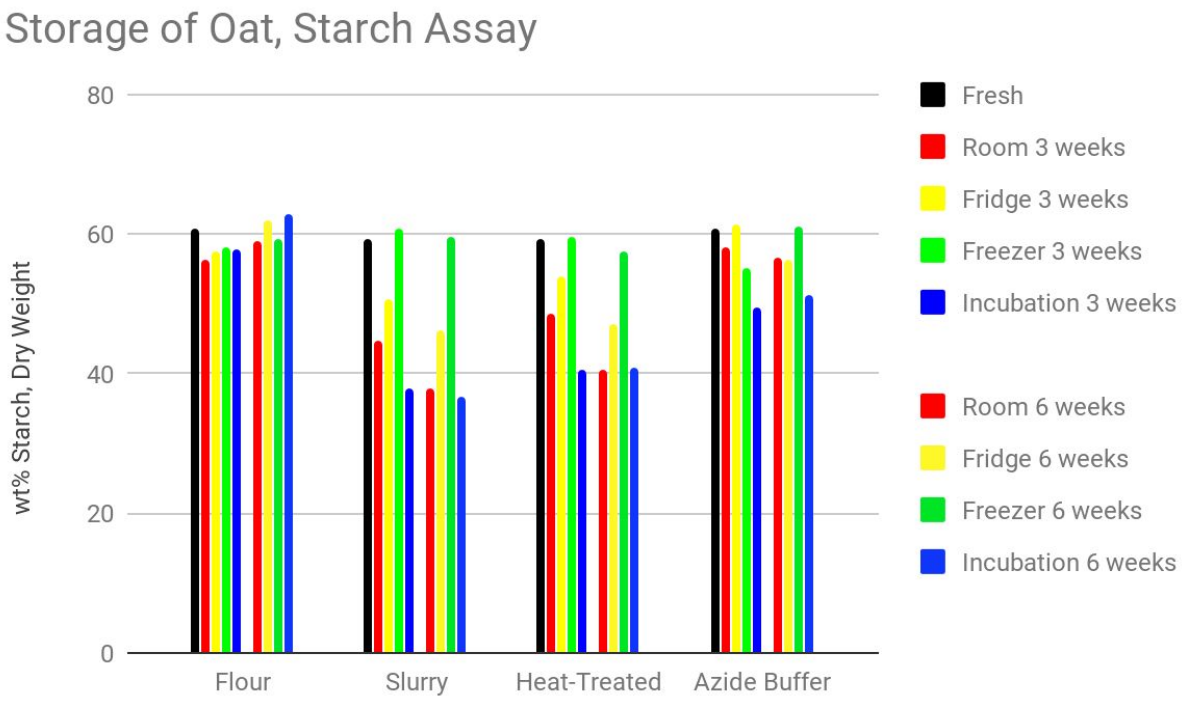

Figure 2. Results of the storage of oats in four ways in four temperatures for 3 and 6 weeks as measured by the starch assay, with the control in black and the 3 week samples to the left and 6 week samples to the right.

What cannot be seen in the above figure 2 is that of the non-frozen samples many were visibly infected by mold and the smell of several gave the same impression. Not unexpectedly, the incubated samples were most severely struck by infection. The above figure is relatively easy to

interpret; if the starch content decreases it suggests a poor storage method and if it stays constant the method is good. Flour should be storable in room temperature for several years since that is the method of storage of the standard. As soon as water is added microorganisms can start to degrade the slurry, unless it is frozen. The heat treatment seems not to have affected the slurry significantly. However the azide has inhibited the microbial growth to some extent, perhaps as well as freezing the samples.

3.1.2. Beta-Glucans

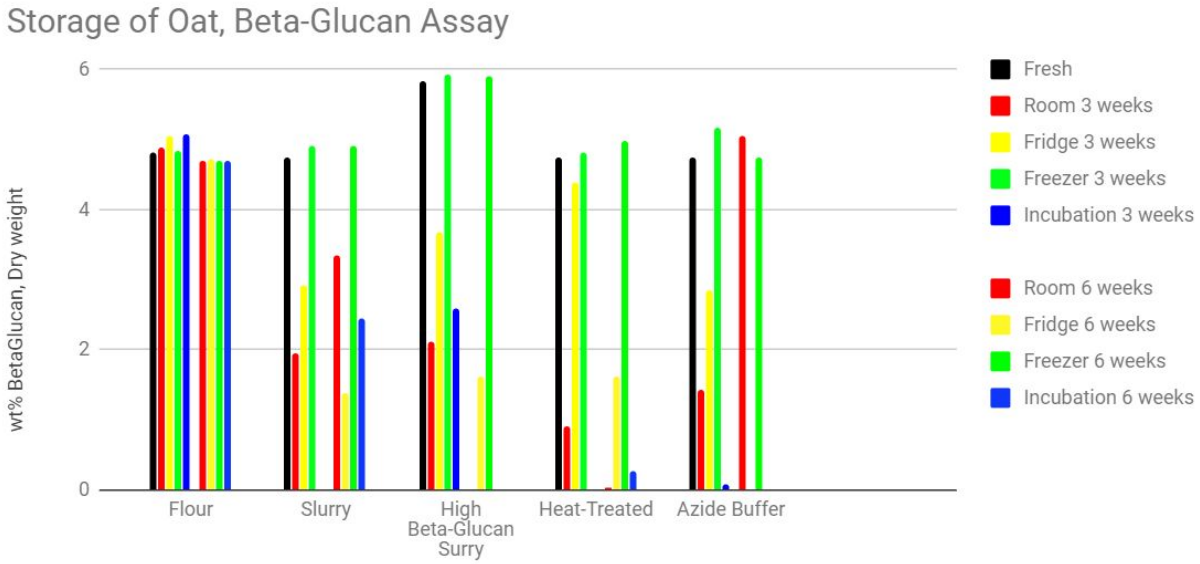

Figure 3. Results of the storage of oats in four ways in four temperatures for 3 and 6 weeks as measured by the beta-glucan assay, with the control in black and the 3 week samples to the left and 6 week samples to the righ t.

As with the starch assay many samples were clearly infected. However as is visible in the above figure 3 in many cases it was so severe that the data was emitted because of the beta-glucan content being shown as negative. This is due to that the blanks for the samples were higher than the actual sample, showing a high level of free glucose prior to enzymatic degradation.

As with the starch assay, flour is a stable method of storage, supported by the fact that the beta-glucan standard is also stored in this fashion, in room temperature and for several years. Interestingly compared with the starch, the beta-glucan is degraded very quickly in all preparations except the frozen ones. There is an oddity in the room temperature stored azide buffered sample which will be further discussed later.

3.1.3. Protein

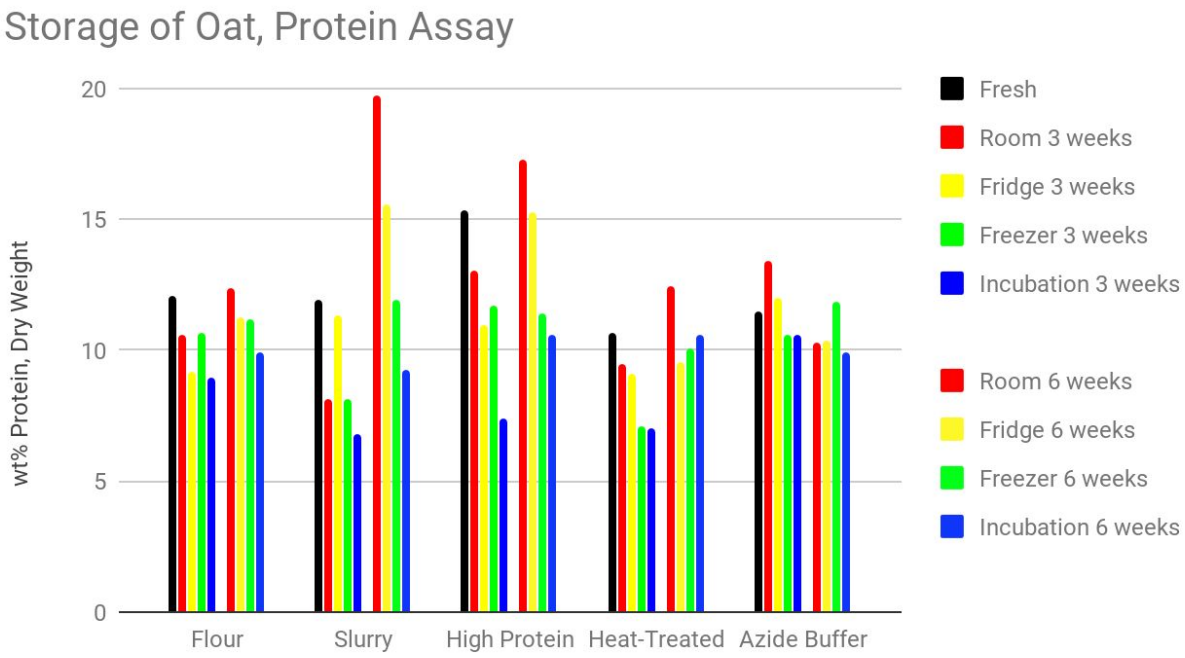

Figure 4. Results of the storage of oats in four ways in four temperatures for 3 and 6 weeks as measured by the protein assay, with the control in black and the 3 week samples to the left and 6 week samples to the right.

The above figure 4 depicting the results of the protein assay is a bit more complicated to interpret than the previous two. Firstly all of the week 3 samples (except the Azide buffered samples that were spilled and therefore tested together with the week 6 samples) are much lower than the control. Also samples far higher in protein than the control have been measured.

The flour preparation seems to not degrade, perhaps only the incubated samples. As for the slurry, the frozen samples are the only ones close to the control. For the high protein slurry the fridge seems to be the best storage option. The heat treatment has had good preserving

capabilities for all except perhaps the room temperature sample. The azide buffer has worked well to inhibit degradation as well, perhaps slightly better in a frozen state.

3.2. 5 Year Flour in Fridge Samples

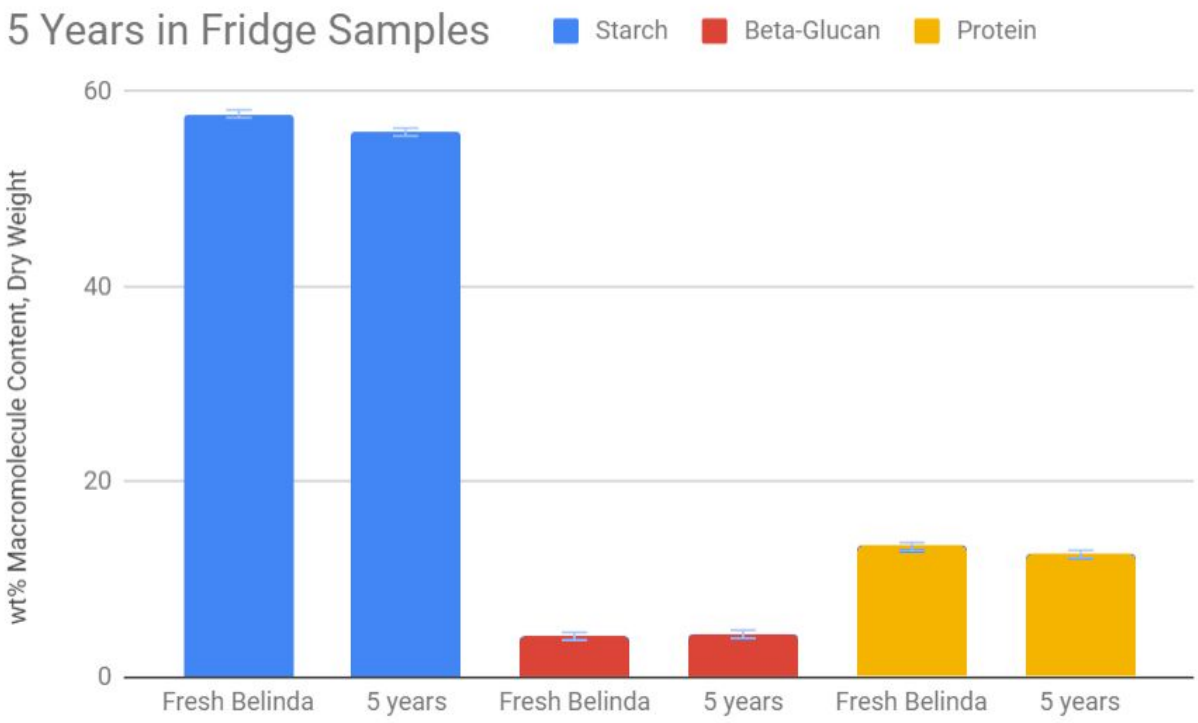

Figure 5. A comparison of freshly ground Belinda F with flour stored in a fridge for around 5 years for all three macromolecules.

As detailed by the above figure 5, the changes in the content of macromolecules is very small. An unpaired t-test was performed and the two-tailed p values were 0.013, 0.03 and 0.06 for starch, beta-glucan and protein respectively.

3.3. Repeated Thawing and Refreezing

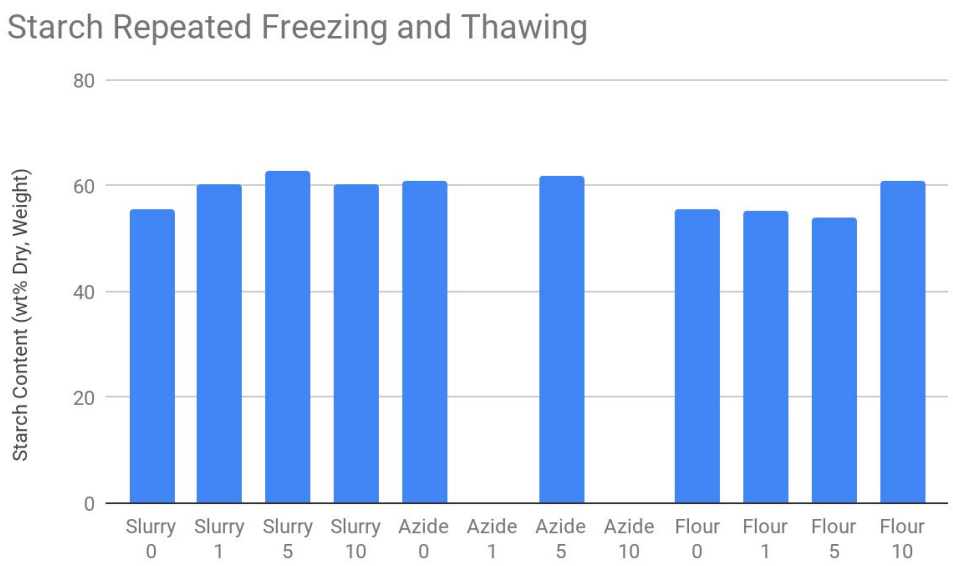

Figure 6. Effect of repeated thawing and refreezing on the starch content of pure oat flour, suspended in distilled water, and azide buffer.

As the above figure 6 demonstrates, there is some variability, yet there does not seem to be a clear trend of degradation after multiple thawings. Unfortunately some of the azide buffered samples gave an absorbance equal to the blank, most likely by human error during the assay.

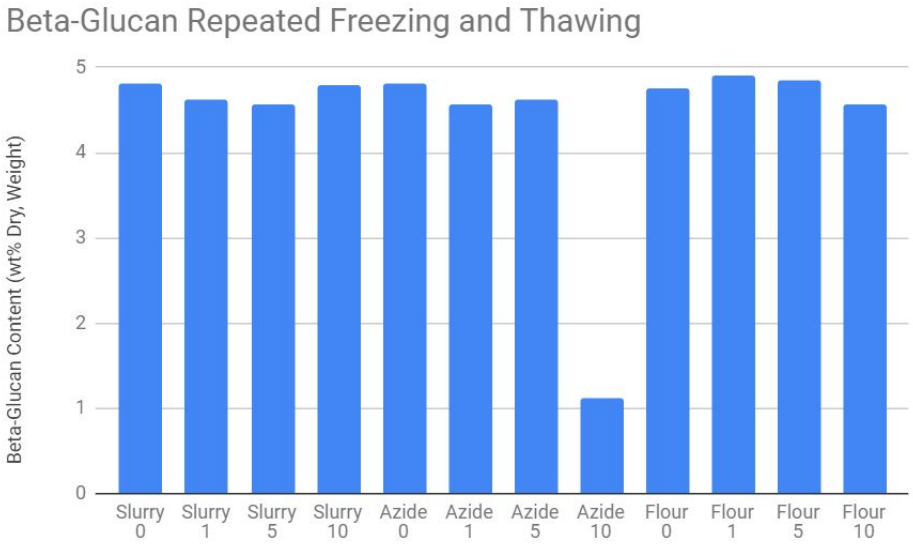

Figure 7. Effect of repeated thawing and refreezing on the beta-glucan content of pure oat flour, suspended in distilled water, and azide buffer.

Much like the previous figure, figure 7 shows no visible signs of degradation. However all three of the triplicates of the azide buffered oat flour did show a low content of beta-glucan.

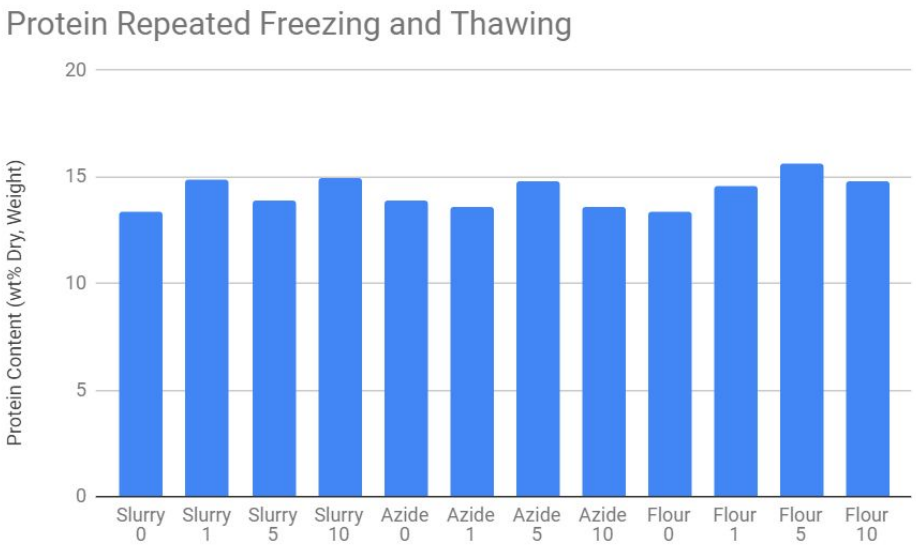

Figure 8. Effect of repeated thawing and refreezing on the Protein content of pure oat flour, suspended in distilled water, and azide buffer.

As mentioned previously some variability exists and is unavoidable, but no trends can be observed for any of the preparations in figure 8 above.

3.4. Volume vs Mass

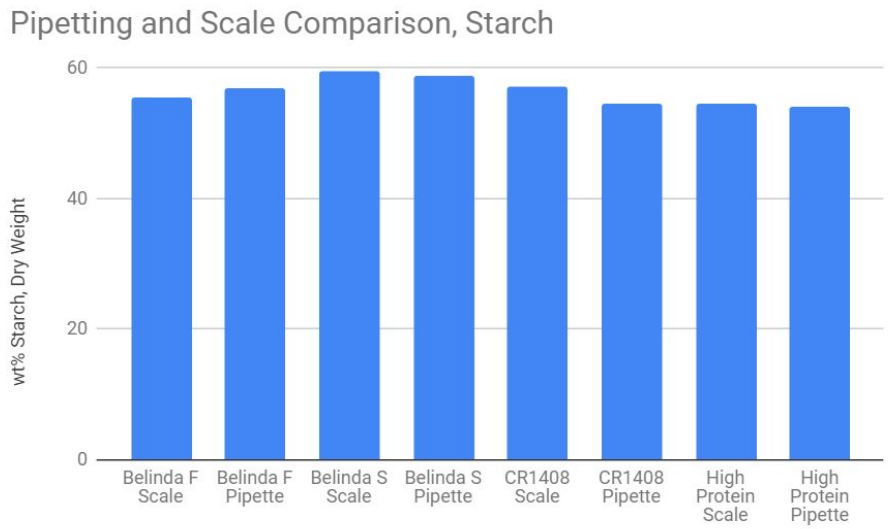

Figure 9. A comparison of measuring the flour by weight or by pipetting a slurry of known concentration, starch assay.

The differences between the pipetted and scale measured samples are slight in figure 9 above and the pipetted samples show no trends of either being always higher or lower than their scale measured counterparts.

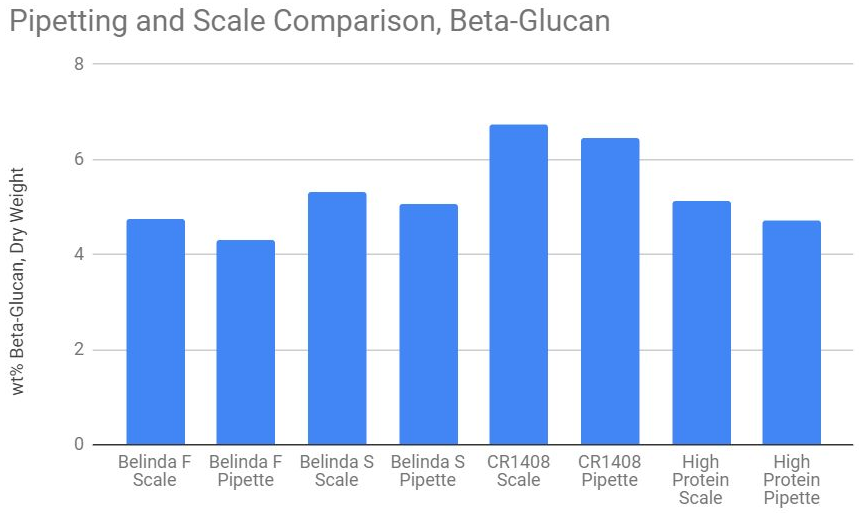

Figure 10. A comparison of measuring the flour by weight or by pipetting a slurry of known concentration, beta-glucan assay.

In contrast with the starch assay, here as shown in figure 10 above, the pipetted samples constantly show a lower content of beta-glucan than if the same sample were measured by scale.

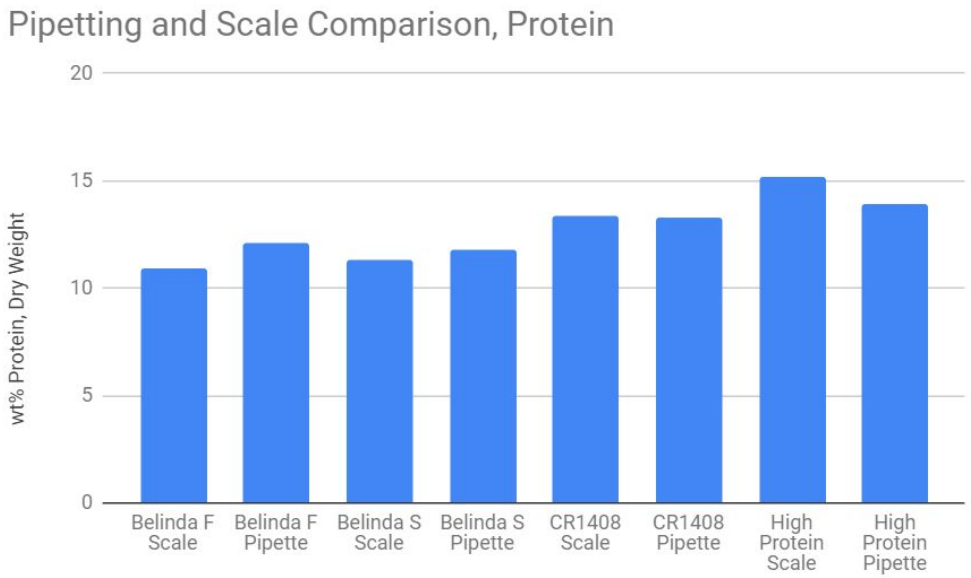

Figure 11. A comparison of measuring the flour by weight or by pipetting a slurry of known concentration, Protein assay.

As with the starch assay no clear trends are visible here in figure 11 above, only the natural variability.

Table 1. The p-values of an unpaired two tailed t-test with assumed equal variance of the pipetted and scale measured sample populations with.values of above 95% significance in green and below said significance in red.

	Starch	Beta-glucan	Protein
Belinda F	0.53	0.00020	0.012
Belinda S	0.71	0.12	0.37
CR1408	0.20	0.11	0.80
High Protein	0.84	0.022	0.015

The p values seen in table 1 above, especially those of the starch assay are too high to definitively call these two populations the same. However for the beta-glucan and protein assay it seems promising.

3.5. Optical Density

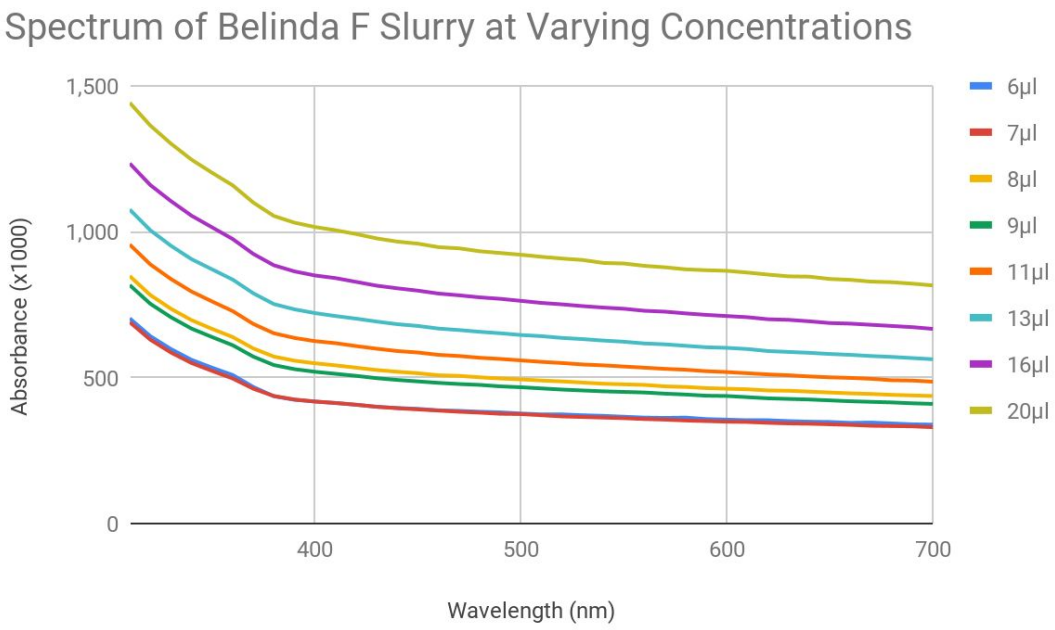

Figure 12. Clear depiction of the peakless, linear nature of the slurry's absorbance after 400 nm regardless of concentration.

In order to ascertain which wavelength to measure the OD of the slurry, a spectral scan was performed, the results of which are presented in figure 12 above. Because of the linear nature of the curve, any wavelength above 400 nm appears acceptable. Yet according to the literature starch has an absorbance peak at 610 nm, leading me to choose this wavelength since the oat slurry consists of mostly starch.[9]

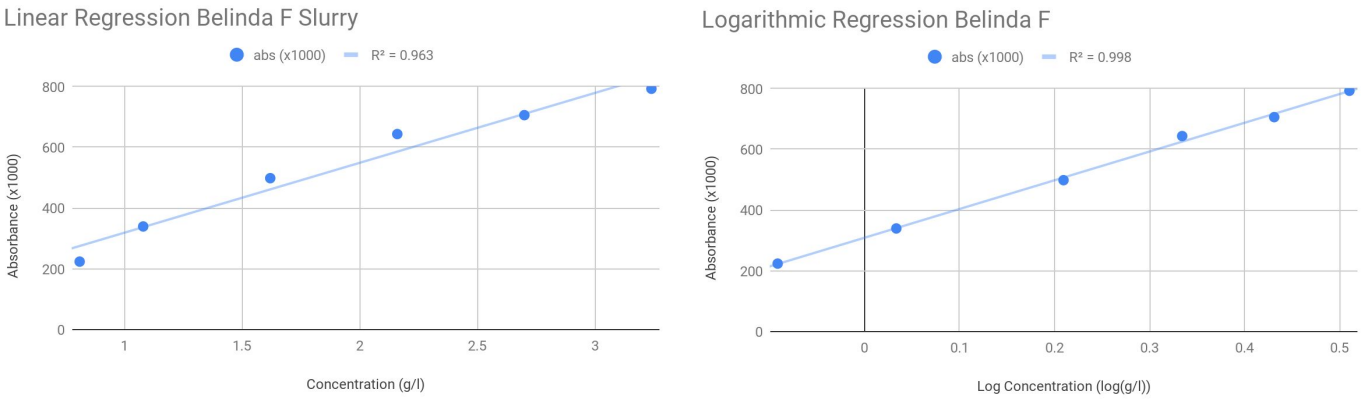

Figure 13. Comparison of a linear (left) and logarithmic, base 10, (right) regression of the OD measurements.

In order to find the concentrations matching the range of the spectrophotometer (0.2-0.8) a wide variety of concentrations were tried (data not shown). This knowledge was used to construct an experiment, the results of which are presented above in figure 13. As can be seen in this graph the interval is approximately from 0.8 g/l to 3.2 g/l. The shape of the left curve and the better fit of the logarithmic regression, shown by the higher R^2-value, led me to believe that there was a logarithmic correlation between the concentration and OD. However as can be seen by the later experiments, performed on various seed strains with more data, it seems to have been a coincidence. The figure 14 below shows a good correlation with a linear regression and also the average R^2-values of a linear and a logarithmic regression were 0.989 and 0.991 respectively. Furthermore some R^2-values increased while some decreased with a switch to a logarithmic regression as is visible in the figure. The linear regression R^2-value of all the seeds together was 0.932.

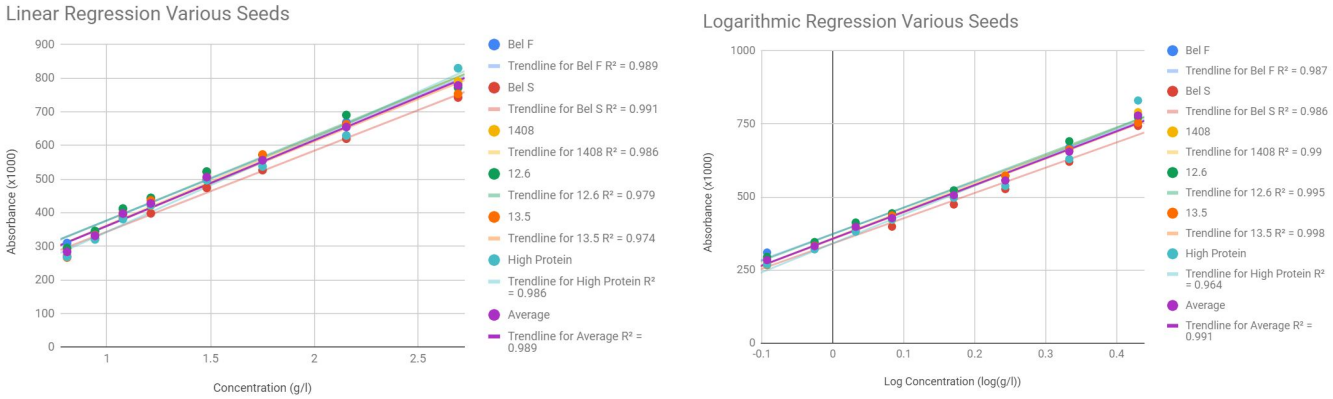

Figure 14.The linear and logarithmic regressions of the OD measurements of the various seed strains and their R^2-values.

4. Discussion

4.1. Storage

The results of the storage experiment for both beta-glucan and starch degradation is very clear. To avoid degradation the storage has to be performed dry, in any temperature, or if a slurry is desired, it has to be frozen. Yet, the results of the protein assay is a bit trickier to interpret, mostly due to the large variance and the constantly lower protein content of the 3 week samples. I have therefore chosen to disregard the 3 week samples and base my conclusions solely on the week 6 samples. With a quick glance dry flour, heat treated slurry and azide buffered slurry look the most stable. The water-based slurries have either increased or decreased significantly in protein content. A decrease seems reasonable, just as the other macromolecules are degraded by bacteria and fungi, however protein is also created by these

organisms, complicating the matter. So as can be seen in figure 2 the protein content can deviate in both directions. What further complicates the protein assay is that the other two assays have blanks to ascertain the level of free glucose prior to enzymatic degradation of the carbohydrates. Therefore, the infections presence is visible by a high blank value, yet the protein assay has no such control.

Beyond the data presented in figures 2, 3 and 4 the smell and appearance of several of the slurries, both with and without azide, suggested a bacterial or fungal infection. This lead me to believe that if these samples are not frozen it is a matter of chance whether or not it will be infected since they have not been sterilized. And the likeliness of infection will increase with time and with increased incubation temperature. Unfortunately the heat treatment, that was designed to prevent infection by sterilization, was flawed in its method. Since to avoid solubilization and gelatinization of the starch and fibres the water or buffer, if any, was added after the heat treatment step. Naturally this is a source of contamination, additionally the addition by pipetting was not performed under sterile conditions, adding further possibilities for the bacteria to sneak in. However an important result of the heat treatment experiment is that the actual heating of the oat flour did not degrade any of the macromolecules. With a p- value of 0.056 for the t-test it is not quite significantly the same population, but with a larger sample size I would estimate that it could be proven to be no significant difference. Therefore pointing to the possibility of storing properly sterilized oat slurry for longer periods of time without degradation.

The azide buffer interestingly, seems to have protective effects on the starch and protein, having significantly better values compared to a slurry in distilled water. However azide has several drawbacks, namely, the acute toxicity in humans and the resistance of gram-positive bacteria to the bacteriostatic effects.[19] This leads me to believe that a physical sterilization step is superior to a chemical step. Yet perhaps they could be combined for an improved effect.

The oat flour that had been refrigerated for approximately 5 years showed no significant change in starch or beta-glucan content, however the protein content might have slightly decreased. With a larger sample size this could be determined with more reliability.

The higher protein content of some of the stored samples lead me to think that perhaps a better way of producing a high protein food could be by fermenting regular oats instead of finding a high protein oat variety. However on closer inspection of the method, glucose was found to have interactions with BCA (the colour-forming reactant) and could perhaps therefore have increased the results without increasing the protein level. This is a likely, but unfulfilling explanation of these results since glucose was found in large quantities in many of these stored samples.

4.2. Repeated Thawing and Refreezing

As expected some variation between the samples is present yet no clear trends are visible. No increase or decrease in the macromolecules after several thawings and refreezings seems to occur. This indicates that the preparations are stable and that samples can be retrieved from the freezer with no concern for degradation. However some peculiarities exist in the data, for example the very low level of beta-glucans in the 10 times refrozen azide-buffered sample which I have no explanation for. Also the loss of most of the azide-buffered samples for the starch assay were lost, most likely due to human error.

4.3. Volume vs Mass

As with all other experiments, this one too shows variability between seemingly same samples. The standard deviation of the pipetted samples was often higher yet not always, and sometimes lower than the scale measures samples. The averages were both higher and lower than the scale measured samples, yet for the beta-glucan assay, the pipetted samples are constantly lower. My interpretation of this is that the fibres may be less soluble and perhaps denser than the other macromolecules, leading to them sedimenting at a higher rate than the other macromolecules. A rate so high that within the few seconds a pipetting requires the beta-glucan fibres have already sedimented. Yet, perhaps it is just a coincidence and more research would disprove this. A student t-test was also performed to determine if pipetting and scale measuring belong to the same population. However, probably due to the very small population size of three samples, the p values vary quite a lot, from extremely significant ($p=0.0002$) to almost significantly different populations ($p=0.84$). Therefore I conclude that the data is not the most reliable, so in order to prove this a larger sample size would be essential. Although, at least for the protein and beta-glucan assays it seems plausible that enough accuracy is retained for the purposes of screening.

4.4. Optical density

Firstly I would like to say that this experiment taught me a lot about the scientific method, since the first results showed a clear pattern that was later disproved by more research. By getting a logarithmic regression to fit to the data unlikely well I was convinced that this was the true nature of correlation between the OD and concentration. However this hypothesis was easily disproved by further experimentation, whereby I obtained more data and realized that this correlation was a mere coincidence. Because in science if the significance level is at least 95% the results are seen as true, however this also means that these experiments will present false conclusions as true every 20th experiment just by coincidence. So therefore the need of repeating experiments is paramount to the scientific method.

The further analysis of several seed varieties revealed that a linear and logarithmic regression yields very similar R^2-values for the lines leading me to think that the most simple model is

closer to the truth. And with all of these values above 0.95, with the lowest at 0.974 I would conclude that this is a good model. What also has to be considered is that this method is designed to be able to measure hundreds of seeds quickly in order to find the outliers in macromolecule content. Therefore the accuracy is not the highest priority, the method only needs to retain some degree of accuracy to be able to find the desired traits. This is exactly what this data shows.

4.5. Conclusions

My conclusions regarding the storage of assay-ready oats is the following: a refrigerated dry oat flour can be stored for several years without noticeable degradation. Aslo a frozen slurry of oat flour in water, preferably heat sterilized in a proper fashion and a universal biostatic added as precautionary methods, can likely be stored for longer periods of time without degradation. Based on these conclusions my recommendation in storing the pre-ground oat is this: after grinding, the flour is refrigerated in a sealed tube until the macromolecule content is wished to be ascertained. At that time water can be added, the assay performed and thereafter the sample will be stored in a frozen state. If further stability is desired the flour can be heat sterilized prior to refrigeration and water can be substituted with a biostatic solution that will not interact with the assays. Naturally this second method required the extraction of the sample to be done in sterile conditions.

As for the scale replacement method, pipetting a suspension of oat flour in water, and measuring the concentration of said suspension by spectrophotometry both seem to have sufficient accuracy for the screening of oat lines in order to find the high and low macromolecule-containing oats.

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