Optimisation of Kafirin Extraction Parameters for Commercial Applications

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Optimisation of Kafirin Extraction Parameters for Commercial Applications

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Synopsis

Kafirin is a prolamin protein unique to sorghum grain. To make kafirin viable for commercial development there needs to be an optimised extraction process. For this purpose insight is needed into how the process is affected by the extraction temperature, grain/solvent ratio, grain/solvent contact time, and various amounts and types of reducing agents and extraction aids. Such insights are currently unavailable.

Significant insights into the extraction of kafirin were gained by performing extractions under various conditions to determine how extraction parameters affected the extraction process. Extraction temperature, grain/solvent ratio, grain/solvent contact time and various amounts and types of reducing agents/extraction aids were tested to determine the optimum extraction conditions. The extraction aids tested were acids (glacial acetic acid, citric acid and phosphoric acid) and metal bisulphite complexes (sodium metabisulphite and potassium metabisulphite) were tested as reducing agents.

Using a bench-scale extraction setup, the following important results were obtained.

- With regard to the commercialization of the process aqueous ethanol is the most promising solvent and was used throughout the study.
- The maximum amount of protein after extraction was roughly 45 g of protein for 1 000 g of grain out of a total kafirin content of roughly 62 g. This is an extraction efficiency of 70 - 75 % based on the total amount of kafirin available in the grain.

- Acids were found to have a limited role in the extraction process and can be excluded.
- The quantity of metalbisulphite complex used played a large role in the extraction process, reducing the yield by about 50 % when absent. The type of metalbisulphite used, however, did not play a role.
- The grain/solvent contact time was deemed to be the most important factor, with the highest yield occurring somewhere between 10 and 20 min.
- After 20 min the yield decreased due to increased solvent retention.

The solvent retention and increased filtering time associated with the drop in yield were investigated. The weakened hydrogen bonding in the starchy endosperm made it possible for the starchy material to bond to other starchy particles or less likely the solvent, and was most likely the mechanism of solvent retention and thickening of the slurry. This increased solvent retention resulted in approximately 50 % of the total solvent being retained; which was discarded with spent grain. At 20 min the solvent retained was roughly 12 % of the total original solvent used.

Further study is recommended with regard to:

- The mechanism of thickening to determine whether there are chemical or mechanical methods which would overcome the thickening and increased solvent retention
- The recovery, purification and subsequent reuse of ethanol
- Investigation into the mechanism that is responsible for the increased filtration time and whether this is related to the tannin-protein complex formation as the literature suggests
- The viability of the spent grain in agricultural applications such as cattle feed or fertiliser
- The effect of changes mentioned in this thesis on the end-use of the protein, e.g. film making quality

Keywords: Sorghum protein, prolamin, protein extraction, kafirin

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1 Introduction

One biomaterial that has been gaining in prominence is a prolamin protein that can be extracted from sorghum grain, commonly referred to as kafirin. Kafirin has applications such as the slow release of amino acid supplements (Pretorius, 2008), as well as the formation of micro-particles which can be cast into films with highly desirable functional properties (Taylor *et al.*, 2005b). It has also been found that kafirin has great potential as a biopolymer which could lead to even more applications where green, biodegradable and often edible polymeric substances may be useful (Di Maio, Mali & Iannace, 2010). Although kafirin has been established as a high-value material, one factor negatively affecting its commercial viability is the difficulty of large-scale extraction. This difficulty was discovered during attempts to extract large quantities of it for research purposes at the University of Pretoria.

For the commercial development of kafirin there needs to be an optimised process for extracting the protein from the grain. The problems with the extraction process are due to a lack of knowledge, especially with regard to how various extraction parameters, such as temperature, time, reducing agents and extraction aids, affect the process. This lack of knowledge is a due to the fact that the extraction process is based on the extraction of zein, a protein obtained from maize (Taylor, 2003). Because kafirin extraction is currently based directly on zein extraction, the kafirin process was very far from an optimum.

To develop an optimised process an understanding of the relationships between the extraction parameters and the final product preparation purity, yield and processing was required. The goal of this work was to determine how each extraction parameter affects the final product and process in order finally to establish the most effective extraction procedure.

To achieve these insights into the process a bench-scale extraction, with the zein process as the starting point, was used to measure and characterise the effect of the extraction parameters on the extraction. The effect of these changes on the end-use of the protein is being tested in a separate study. Unfortunately, large-scale application could not be tested due to the unavailability of equipment.

2 Theory

2.1 Limitations of Theory

There is very little information dealing directly with the extraction of kafirin from sorghum grain. For this reason the literature that gives a background to support the interpretation of the results was studied, as well as the little that does deal with the kafirin extraction process. There is also some more general background which will assist in the reading of the document.

2.2 Kafirin Compared with Zein

Zein, the prolamin protein found in maize meal, and kafirin are both unique to their respective grains, but have been found to be highly homologous (De Rose, *et al.*, 1989). Nevertheless, there are still some dissimilarities between the proteins which cause functional differences, such as the amount of each protein that is digestible (Duodo, Taylor, Belton & Hamaker, 2003).

Investigation into the properties of kafirin also led to the suggestion that kafirin is more highly cross-linked than zein (El Nour, Peruffo & Curioni, 1998). Films produced from kafirin are far stronger than those produced from zein, which could be of interest in commercial applications (da Salva & Taylor, 2005).

It is also suggested that kafirin is more hydrophobic than zein (Duodo *et al.,* 2003). This was also suggested by Wall and Paulis (1978) and could be explained by the higher cross-linking found in kafirin molecules than in zein molecules. The difference in cross-linking and higher hydrophobicity of kafirin suggests that kafirin is less soluble than zein in polar solvents. It is therefore also slightly more difficult to extract kafirin than zein (Buffo, Weller & Gennadios, 1997).

It is possible to use the same extraction method that is currently used for zein to extract kafirin from sorghum, and to achieve satisfactory extraction due to their similar structures. This involves extraction with aqueous alcohol after the removal of water soluble proteins (Osborne, 1924). However, when using this method, zein extraction is roughly three times greater than that of kafirin (Buffo *et al.,* 1997).

2.3 Extraction

Extraction involves the entire process of solubilisation, separation and isolation of a specific component from the other constituents with which it is chemically or physically in close contact (Taylor, 2003: 32).

Osborne (1924) found that prolamin proteins are soluble in aqueous alcohol mixtures. Taylor, Taylor, Dutton & de Kock (2005a) performed a study which determined the solubility of kafirin in various solvents. Nine different solvents were investigated at various temperatures. The study found that kafirin was most soluble in glacial acetic acid (25 - 70 °C) and lactic acid (25 - 70 °C) as primary solvents.

Regarding binary solvents, it was found that the highest kafirin solubility was in 55 % aqueous isopropanol (25 - 70 °C). 70 % aqueous ethanol at 70 °C also showed a high kafirin solubility, and it could potentially also be used as an effective solvent (Taylor *et al.,* 2005b).

The pH of the solution affects the nature and distribution of the net charge of the protein. Generally, the proteins are more soluble in solutions with low (acids) or high (alkaline) pH values because of the excess of charges of the same sign. This produces repulse among the molecules, and consequently contributes to their largest solubility (Pelegrine & Gasparetto, 2005).

Due to the limited research on kafirin solubility, the relation of kafirin solubility to temperature still remains unclear. It is, however, known that the solubility of kafirin increases with an increase in temperature as can be seen in Table 1 (Taylor *et al.,* 2005b).

Table 1: Solubility of kafirin at different ethanol temperatures (Taylor *et al.,* 2005b)

Solvent	Temperature (°C)	Description	Solubility (as % of protein added)
70 % Ethanol	70	Small gelled pellet, clear supernatant	90.9
	40	Very viscous clear supernatant	94.9
	25	Large white pellet, clear yellow supernatant	38.2

The extraction of a prolamin protein involves the removal of the protein from the endosperm by the addition of a solvent (most frequently an aqueous alcohol solvent) into which the protein can easily dissolve. In addition to the aqueous alcohol solvent, a reducing agent can be added to improve the extraction process (Landry & Moureaux, 1980). This reducing agent weakens and reduces the disulphide bonds of the protein matrix in which the prolamins are located as well as the intermolecular bonds of the proteins themselves. This increases the solubility of the proteins by weakening the inner molecular bonds, and increases the amount of protein that can be extracted from the grain particles due to the weakened or broken matrix (Wall & Paulis, 1978). Reducing agents such as sodium metabisulphite or extraction aids such as acetic acid are suggested (Taylor *et al*., 2005a). A non-polar solvent such as hexane or benzene is used after extraction to de-fat the extracted protein.

One set of extraction experiments that is of interest are those conducted by Xu, Reddy and Yang (2007). Their experiments involves extraction very similar to the method used by Taylor *et al.* (2005a) on which this research was based. The factors that found to be consequential to the extraction of zein were in line with many of the extraction parameters of this research.

Xu, Reddy and Yang (2007) found the following factors affect the extraction process of zein:

- extraction period
- extraction temperature
- extractant (solvent) concentration
- addition of a reducing agent
- pH adjustment
- solid-to-solvent ratio

However, the fat and moisture content of the maize does not affect the extraction process (Wu, Meyer & Johnson, 1997). Due to the structural similarities of kafirin and zein it is probable that the same factors will influence the kafirin extraction process.

As zein has highly desirable qualities when it is cast into a film, it has been used extensively (Taylor, 2003: 15). Therefore extensive research is available on the extraction of zein from maize meal. Kafirin has only recently gained a lot of attention since it was found that biofilms made with kafirin displayed similar functional properties to those of zein and that kafirin could therefore be used as an alternative to zein (Buffo *et al.,* 1997).

Research has shown that most kafirin extraction processes are adapted from the zein extraction method patented by Carter and Reck (Carter & Reck, 1970).

There is an alternative method for the extraction of zein that deviates greatly from Carter and Reck's method. This method proposes that the endosperm be dissolved in a buffer and then treated with an aqueous alcohol mixture (Wallace, Lopes, Paiva & Larkins, 1990). However, as this method is used only to extract a specific type of zein it will not be considered further.

2.4 Sorghum

Sorghum is very important and widely distributed cereal crop in several African countries (Smith & Frederiksen, 2000: 132). Sorghum consists mainly of starch but also contains proteins, fats and various other components and is classified into specific grades by the colour, cultivar and tannin content of the cultivar. Sorghum differs from maize not only because a different type of prolamin protein is present in the grain, but also because of large variances in grain composition (Rooney & Pflugfelder, 1985). This can be ascribed to different genetics (different cultivars) and the environment in which the crops are grown (FAO, 1995).

Despite the variances, the sorghum grain composition can be approximated by sorting the cultivars either by tannin content or colour. It is important to note that these two properties are not directly related although the brown/red-coloured grains have a higher probability of containing tannins (Brandt, Kuhl, Campbell, Kastner & Stroda, 1992). Tables 2 and 3 show the approximate values for the various cultivar types.

Table 2: Composition of sorghum cultivars sorted by tannin content (Brandt *et al.,* 1992).

	Cultivar	
Composition	Low Tannin (%)	High Tannin (%)
Protein	10.6 - 12.8	12.1 - 13.2
Starch	68.2 - 74.2	64.6 - 67.4
Tannin	0.2 - 0.3	1.5 - 1.7

Table 3: Approximate composition of low-tannin sorghum cultivars sorted by grain colour (Grain SA, 2012)

	Cultivar		
Composition	Red/Brown (%)	White (%)	Pale Yellow (%)
Protein	9.95	9.42	10.06
Starch	60.93	63.4	63.32
Fat	3.32	2.82	3.35
Tannin	0.35	0.16	0.23
Other/Inert	25.45	24.2	23.04

The sorghum grain is spherical in shape and has three main parts: the pericarp, the endosperm and the germ, and several smaller parts. Figure 1 shows a sorghum grain.

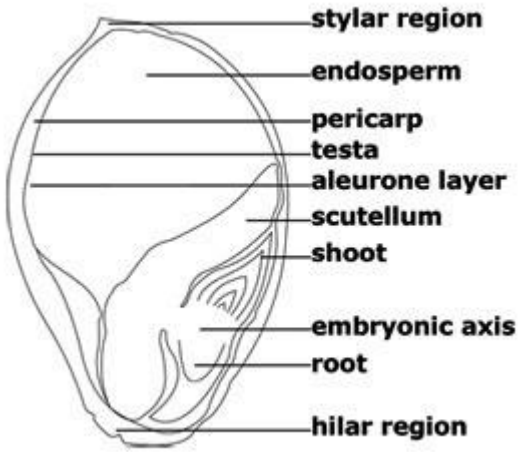

Figure 1: Sorghum grain structure (Sorghum SA, 2015)

All three of these main parts contain starch, protein and fat. The endosperm contains most of the protein in the grain. It is also suggested that the composition of the various parts of the grain is constant and not dependent on the cultivar type (Taylor & Schussler, 1986). The germ consists of approximately equal amounts of protein, starch and fat. It is in the testa that any tannins present are located within the grain particle (Taylor, 2003: 24).

2.4.1 Kafirin

Kafirin is a specific type of prolamin protein uniquely found in sorghum grain. Kafirin protein bodies are spherical in shape but the size varies depending on the specific sorghum cultivar (FAO, 1995). The prolamin proteins of sorghum (kafirin) are similar to the prolamins of maize (zein), and these are the major storage proteins of these grains. They are present in high amounts and provide a store of amino acids for use during germination and seedling growth (Shewry, Napier & Tatham, 1995). These proteins are located within the starchy endosperm and make up about 70 and 60 % of the total grain protein of sorghum and maize respectively (Paulis & Wall, 1979; Lending, *et al*., 1988). Kafirin is responsible for the storage of nitrogen within the grain which the plant requires during germination (Taylor & Schussler, 1986).

The disulphide bonds are not required to maintain the tertiary structure of the kafirin molecules, nor does the breaking of these bonds affect the monomer structures which means that the disulphide bonds can be broken to increase solubility. Kafirin displays an increase in hydrophobicity with an increase in temperature (Musigakun & Thongnam, 2007). This cannot be attributed to a change in the overall hydrophobicity of the protein since the amino acid composition does not change. This phenomenon can therefore only be explained by a change in the protein conformation, which prevents water from entering the structure itself. (Belton, Delgadillo, Halford & Shewry, 2006)

A characteristic of kafirin is that in a solution it gels upon standing for a period. Investigations led to the suggestion that this gel formation is caused by the hydrogen bonding forces. The only mechanism that prevents gelation is heating or shaking (Wall & Paulis, 1978). However, Taylor (2003: 37) suggests that an increase in temperature increases gel formation.

Kafirin denatures at a temperature of 94 °C (Mishra *et al.,* 2008). Once the kafirin is denatured it loses its functional properties and can therefore not be used. Denaturing should therefore be avoided at all costs.

Kafirin polypeptides are divided into three classes: α, β and γ-kafirin. Each class differs in solubility, molecular weight and amino acid composition (Hamaker, Mertz & Axtell, 1994). The kafirin present within the grain is comprised mainly of the α-kafirin class (Belton *et al.,* 2006). The α-kafirin is located in the centre of the protein body and the β and γ-kafirins are found at the periphery of the protein body. α-kafirin (22 000 - 28 000 g/gmol) and γ-kafirin (27 000 - 28 000 g/gmol) have the highest molecular weights, and β-kafirin (19 000 - 20 000 g/gmol) has the lowest molecular weight (Musigakun & Thongngam, 2007).

2.4.2 Polyphenols

Phenolic compounds in sorghum may be divided into three major categories: phenolic acids, flavonoids and tannins (Hahn, Rooney & Rear, 1984). Although tannins protect the grain against insects, birds and fungal attack, this agronomic advantage is accompanied by nutritional disadvantages and reduced food quality (Serna-Saldivar & Rooney, 1995). It is believed that under optimal conditions, sorghum tannin is capable of binding and precipitating at least 12 times its own weight in protein. This

tannin-protein interaction in sorghum is thought to involve hydrogen bonding and non-polar hydrophobic associations (Butler *et al*., 1984).

Lower protein yields were obtained with high-tannin (bird-resistant) sorghum than with low-tannin (condensed tannin-free) sorghum when both grains were subjected to the Landry–Moureaux protein fractionation procedure (Daiber & Taylor, 1982). This was due to interactions between tannin and the albumin, globulin and prolamin proteins, which rendered most of the proteins insoluble. Furthermore, electrophoresis indicated that the proteins extracted from high-tannin sorghum were bound to the tannins. Generally, the characteristics of proteins that bind strongly to sorghum tannin are that they are relatively large, have a loose, open structure and are rich in proline (Butler *et al*., 1984)

This binding with the proteins is a strong motivator to extract as little of the polyphenols as possible, or alternatively to use sorghum with a lower tannin content for kafirin extraction.

2.4.3 Starch

The grain is mostly comprised of starch. At certain temperatures the starch undergoes a chemical change referred to as gelatinisation. Gelatinisation is a phenomenon caused by weakened hydrogen bonds which allows starch granules to engage more water. Olkku & Rha (1978) found that the starch granules are held together by hydrogen bonds. At high temperatures the kinetic energy of the molecules causes extreme vibrations, weakening the hydrogen bonding within the structure.

- During heating, water is first absorbed in the amorphous space of starch, which leads to a swelling phenomenon.(Jenkins & Donald, 1998)
- Water then enters via amorphous regions the tightly bound areas of double helical structures of amylopectin. At ambient temperatures these crystalline regions do not allow water to enter. Heat causes such regions to become diffuse, the amylose chains begin to dissolve, to separate into an amorphous form and the number and size of crystalline regions decreases.(Zobel, 1988)
- Penetration of water thus increases the randomness in the starch granule structure, and causes swelling, eventually soluble amylose molecules leach into the surrounding water and the granule structure disintegrates.(

The temperature at which gelatinisation starts to occur is called the initial gelatinisation temperature. After a certain temperature increase the starch molecules will be saturated and the process will stop. Udachan, Sahoo & Hend (2012: 317) found that the gelatinisation temperature of sorghum starch differed by sorghum cultivar type but ranged from approximately 66 to 68 ºC (Udachan *et al*., 2012).

The swelling power of starch, or increase in the volume of material due to the absorption of a solvent, and the water solubility of starch increases with an increase in temperature. A rapid rise in swelling power is observed between 60 and 70 °C, at which temperature it was also found that gelatinisation started to occur (Udachan *et al*., 2012).

These factors are important when discussing the increase filtration times.

2.4.4 Fat content

Fats are defined as organic compounds that are insoluble in water and soluble in organic polar solvents such as alcohols (Merriam-Webster, 2015). Fat is also one of the prevalent components in sorghum grain.

The fat within the grain affects the swelling and pasting of the starch (Udachan *et al.,* 2012). The presence of fats in solution may affect the extraction process by changing the processability of the grain/solvent slurry due to strong interactions between fats and other components (Olkku & Rha, 1978). The relationship between the solubility of fat and protein in an alcohol mixture is, however, unknown.

2.4.5 Proteins

The properties, structures and limitations of proteins stated below are responsible for many of the limitations of the process, such as the stirring rates and the acid and solvent concentrations. One such limitation is the temperature at which the solvents evaporate. It would be very easy to heat the solvent/protein mixture to evaporate the solvents, but the protein can easily be denatured, thus the application of heat is a more difficult process.

Proteins, sometimes referred to as polypeptides, are formed by a chain of amino acids linked together by covalent peptide bonds. Each protein can therefore be distinguished by the amino acid sequence unique to it (Hettiarachchy *et al.,* 2012:12). Various

weaker bonds such as hydrogen bonds, ionic bonds and van der Waals forces are present within the chain originating from the side chains of the amino acids (Alberts *et al.,* 2002). These forces may cause issues at later stages of extraction processes; however, there is nothing in the literature on the extent.

Alberts *et al.* (2002) also found that these side chains are responsible for causing hydrophobicity of the protein. The non-polar (hydrophobic) chains tend to cluster in the centre of the molecule, while the polar side chains (hydrophilic) arrange themselves around the outside of the protein where hydrogen bonding is possible. This hydrophobicity is important as this is what allows separation of the various constituent proteins of the sorghum.

The structure of a protein can be divided into four different levels. The primary structure is the linear arrangement of the amino acids. The secondary structure is the areas of folding within a protein. The tertiary structure is the final three-dimensional structure of the protein (Hettiarachchy *et al.,* 2012: 1 - 18). Lastly, the quaternary structure is determined by the non-covalent interactions between the tertiary structures to form a single large protein molecule (Bowen, Austgen & Rouge, 2002).

To aid the stability of the protein structure, covalent bonds are also formed between the side chains, cross-linking the structure. These cross-linkages are most often sulphur-sulphur bonds also referred to as disulphide bonds (Alberts *et al.,* 2002).

Prolamins are characterised by the amino acids present within the protein. Prolamins contain high amounts of proline and glutamine and low levels of lysine. These proteins are also characterised by their high hydrophobicity (Belton *et al.,* 2006). This characteristic makes them soluble in polar solvents such as alcohols. Prolamins are high molecular weight structures (Shewry & Tatham, 1990) and have no known function other than to serve as storage proteins, which are biological reserves of metal ions and amino acids used by organisms in the cell structure (Taylor & Schussler, 1986).

Certain external factors can cause the bonds of the quaternary, tertiary and secondary structures of the protein to be disrupted. This could lead to the destruction of these structures, causing the protein to lose its functionality. This process is referred to as denaturing. The solvent, temperature, pH or mechanical factors can cause the protein to denature (Shukla & Cheryan, 2000)

2.5 Rheology of Grain/Solvent Slurry

A balance between shear-induced physical disentanglement and the formation of new entanglements between polysaccharide or starch molecules will be largely affected by the temperature, and these two antagonistic processes will probably shift towards new entanglements as the temperature is increased (Ojijo & Shimoni, 2004). These entanglements and disentanglements are due to the weakened, breaking and new formation of hydrogen bonds in and between starchy endosperm materials as discussed by Olkku & Rha (1978).

Disentanglement can be observed as a decrease of viscosity, while new entanglements will be indicated by an increase in viscosity (Chamberlain & Rao, 1999).

2.6 Protein Analysis Method

To determine the yield of protein any sample of interest was tested by the Dumas method. The method consists of combusting a sample of known mass in a high temperature chamber in the presence of oxygen and CuO. The combustion products are then passed over hot Cu which converts nitrogen oxides to N_2 and CO to CO_2. Then through special columns (such as potassium hydroxide aqueous solution) that absorb the carbon dioxide and water. A column containing a thermal conductivity detector at the end is then used to separate the nitrogen from any residual carbon dioxide and water and the remaining nitrogen content is measured (Page, 1982:595)

Each sample was prepared identically as discussed in the user manual using a Gerhardt Dumatherm DT N40+ (Dumatherm, 2014) to ensure that the preparation of the samples did not play a role in the final result. The Dumatherm is shown seen in Figure 2.

Figure 2: Dumatherm used in protein analysis

The sample was prepared for analysis by thoroughly drying it in an oven at 30 °C for an hour, and was then turned into a fine powder using a coffee grinder. The coffee grinder was switched on and off at 30 s intervals to ensure that the sample did not overheat. The coffee grinder was also shaken to ensure that no clumping of the protein occurred. Grinding at these intervals was continued until a fine consistent powder was

obtained. It was very important that the sample did not get hot to the touch as this could lead to denaturing of some of the protein.

After the samples had been ground, 120 mg of each sample was accurately weighed out into a foil cup. The foil cups were then folded and crushed into tiny balls. The samples were placed in the Dumatherm auto sampler and analysed using a nitrogen factor of 6.25.

3 Study Design

3.1 Layout of this Document

This document is laid out in two parts.

Part one deals with the development of knowledge and changing all the parameters to determine how each affects the final yield. During the experimental procedure to find the most effective way of extracting kafirin, solvent retention and excessive filtration time were discovered to be the critical factors in diminishing the yield.

In Part two an attempt is made to find the mechanism by which solvent retention occurs.

3.2 Safety Considerations

Several safety considerations are laid out below which were followed to minimise any potential risks.

- Ethanol is extremely dangerous and every effort was made to reduce the risk of ignition when working with ethanol. Especially at elevated temperatures. Under vacuum filtration the vapour was passed through a cold water trap to ensure any vapour did to get released into the atmosphere.
- Metabisulphites are powerful allergens and were handled with care. Gloves were worn with all experiments.
- Acetic acid is extremely corrosive and harmful to airways and skin. Gloves and eye protection were worn when transferring liquids.
- Pouring fine flour produces fine dust which could act as an irritant to airways. A Dust mask was worn to mitigate this issue.

3.3 Flour

In the interest of being able to commercialize the extraction of kafirin the flour used was sourced off-the-shelf. It was produced by Tiger Brands and sold under the brand name King Korn Mabele. This product comes in two varieties which lend themselves to the extractions processes. These varieties are fine and course. There is no information about how the flour was produced in terms of milling. Figure 3 shows the particle size distribution for the coarse and fine flour.

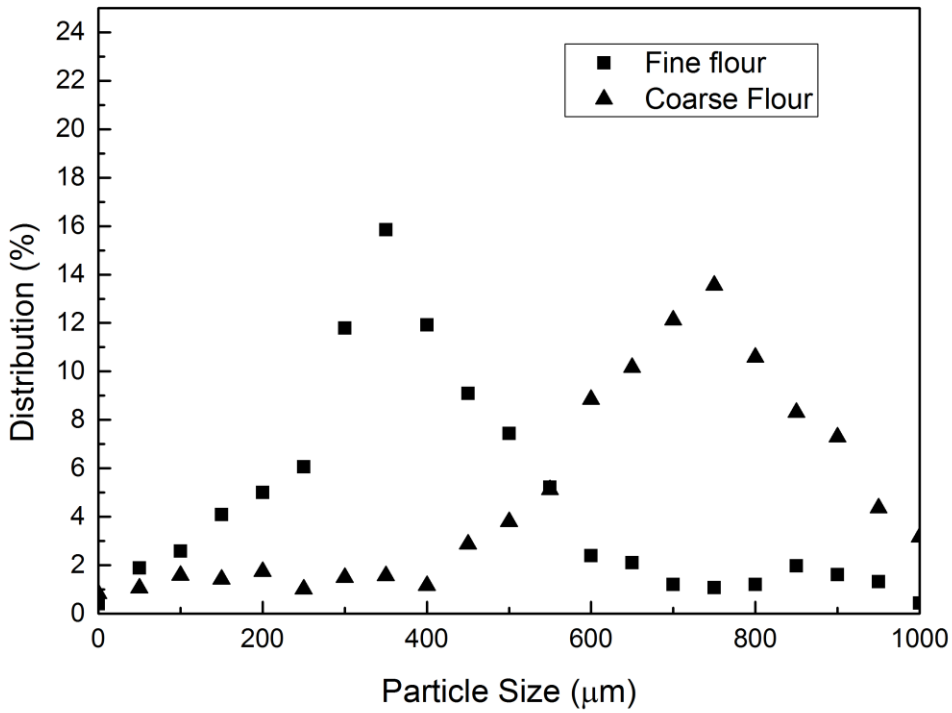

Figure 3: Particle size distribution for fine and coarse flour

When contacted about how the flour was milled Tiger Brands were unwilling to disclose this information.

3.4 Taylor Extraction Procedure

3.4.1 Method

Taylor *et al.* (2005b) described a method for the extraction of kafirin, which is detailed below.

Milled grain (500 g) was weighed into a plastic bucket (5 l) with a tight-fitting lid containing a small central hole for the rod of the stirring element. Sodium hydroxide (or glacial acetic acid) (8.75 g) and sodium metabisulphite (12.5 g) were dissolved in water (728.75 g) before being mixed with ethanol (1 750 g) and were then added to the grain. Extraction was carried for 1 h out at 70 °C in a water bath with vigorous stirring.

Directly after this the extraction mixture was either vacuum filtered through Whatman No. 4 filter paper or centrifuged at 3 000 rpm (1 000 g) at room temperature for 5 minutes. The filtrate (supernatant) was collected. The extracted grain was then washed with a further 500 g of extractant and either filtered or centrifuged. The supernatants or filtrates were combined and the ethanol allowed to evaporate overnight from a shallow open tray placed in the fume cupboard at room temperature. The tray was covered with a muslin cloth to prevent dirt falling into the extract. When the ethanol evaporated a thin yellow “taffy”-like film formed over a curd-like precipitate (the relative amounts of each type of protein varied). This protein was then washed into a beaker with a minimal amount of distilled water (at less than 10° C). The pH was adjusted to 5.0 with 1 M hydrochloric acid. The protein precipitate was filtered under vacuum before being freeze dried.

The protein was defatted with hexane (3 x 100 ml) prior to film casting.

Total protein (N x 6.25) was determined using the Dumas method.

3.4.2 Process flow diagram

Figure 4: Taylor extraction process flow diagram described in Taylor *et al.* (2005b).

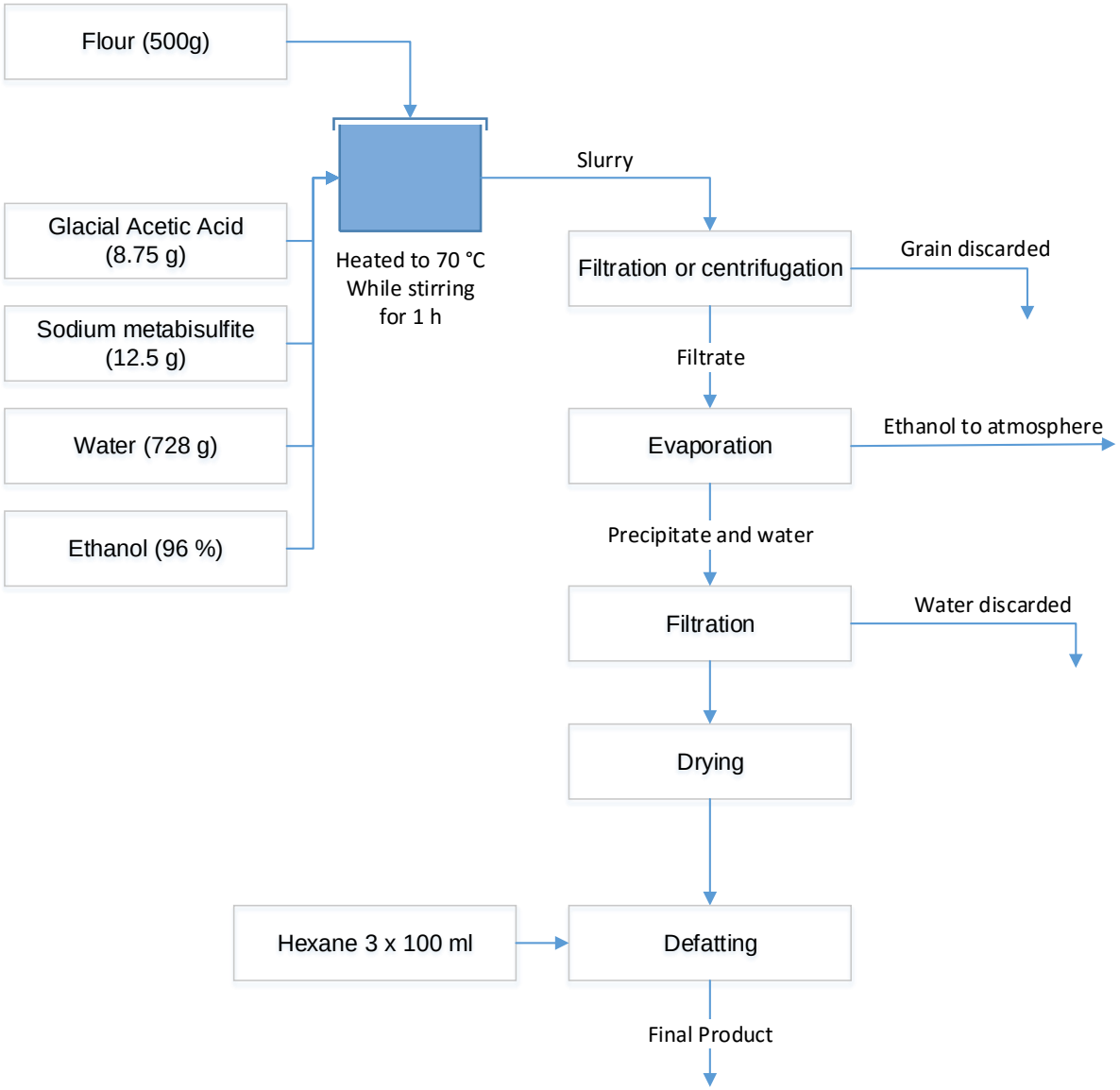

Figure 4: Taylor extraction process flow diagram (Taylor *et al.*, 2005b)

3.4.3 Points for consideration

1. The first and most important point is that the grain and solvents are mixed before heating, which means that the temperature is not constant throughout the entire extraction process. This makes it impossible to determine what effect contact temperature has on the extraction process. It also means that the time at a specific temperature is unclear, which also means that no information about the contact time can be obtained from this process.
2. The plastic bucket was not ideal as it conducts heat poorly and is less stable when placed in a water bath.

3. No difference was observed between first increasing the pH and then lowering it and just lowering it at the start. This is indicated by the method stating: “Sodium hydroxide (or glacial acetic acid) (8.75 g) and sodium metabisulphite (12.5 g) were dissolved in water…”
4. It was not necessary to de-fat the protein as it was not used to make film. Although this step was crucial for film casting, it was not important in the actual extraction of the protein.
5. Centrifugation on a large scale is a costly process and filtration is a much more attractive option.

3.5 Basic Extraction Procedure

3.5.1 Method description

The method described below was adapted from the method used by Taylor *et al.* (2005b). This procedure was altered and optimised to find a process which could be potentially commercially viable. Some changes were made to the basic procedure to increase repeatability. These changes are listed at the end of this section.

1 750 g of 99.9 % ethanol and 728 g water were added to a 5 l glass Buchner flask. The flask was placed in a water bath at the extraction temperature with an overhead stirrer to ensure thorough mixing. This setup is shown in Figure 5 A thermometer was used to verify that the temperature was at the desired level before the flour was added.

Figure 5: Extraction setup

Once the solvent mixture was at the correct temperature, 12 g sodium metabisulphite and 8 g glacial acetic acid were added. Stirring continued for a short period of time to ensure that all the components were well mixed and all the sodium metabisulphite had dissolved.

500 g of flour was placed in an oven at the extraction temperature for roughly an hour before being added to the extraction vessel. Once all the flour had been added to the vessel and the flour/solvent slurry was thoroughly mixed the timer was started.

Once the desired grain/solvent extraction time had elapsed the slurry was moved to a 5 l Buchner flask with a vacuum pump and vacuum filtered through 2 sheets of 240 mm No. 4 Wattman filter paper. A timer was started once the filtrate started running out of the filter cake. When no more filtrate was being removed the filtration time was recorded. At this point the filter cake was weighed to determine how much solvent it had retained and discarded.

The filtrate was the placed in an open 10 l glass container with an overhead stirrer to keep the liquid in motion with a fan blowing over the surface and left overnight. This was done to evaporate the ethanol and precipitate the protein which is insoluble in water. Stirring was of paramount importance as a film formed on the surface of the liquid preventing further evaporation if the liquid was not kept in motion. The fan was also important as it ensured a fresh stream of unsaturated air that aided in evaporation.

Once all the ethanol had been evaporated and the protein wholly precipitated, the water/protein slurry was filtered in a 500 ml Buchner setup through 1 sheet of 50 mm No. 4 Wattman filter paper and any protein stuck to the sides of the vessel or the surface of the stirrer was scraped into the filter. The filter cake was washed with 3 × 100 ml of water. If after the third wash it was not visibly clear another wash was done.

The filter cake, which is the desired protein at this point, was spread as thinly as possible onto a watch glass with a fan blowing air over it and left overnight to dry. Once dry the protein was weighed and stored in small polyethylene bags. The final products were not defatted as was the case in the work of Taylor (2005b) as defatting is a separate process from extraction.

3.5.2 Process flow diagram

Figure 6 shows the basic extraction procedure on which all the experiments were based.

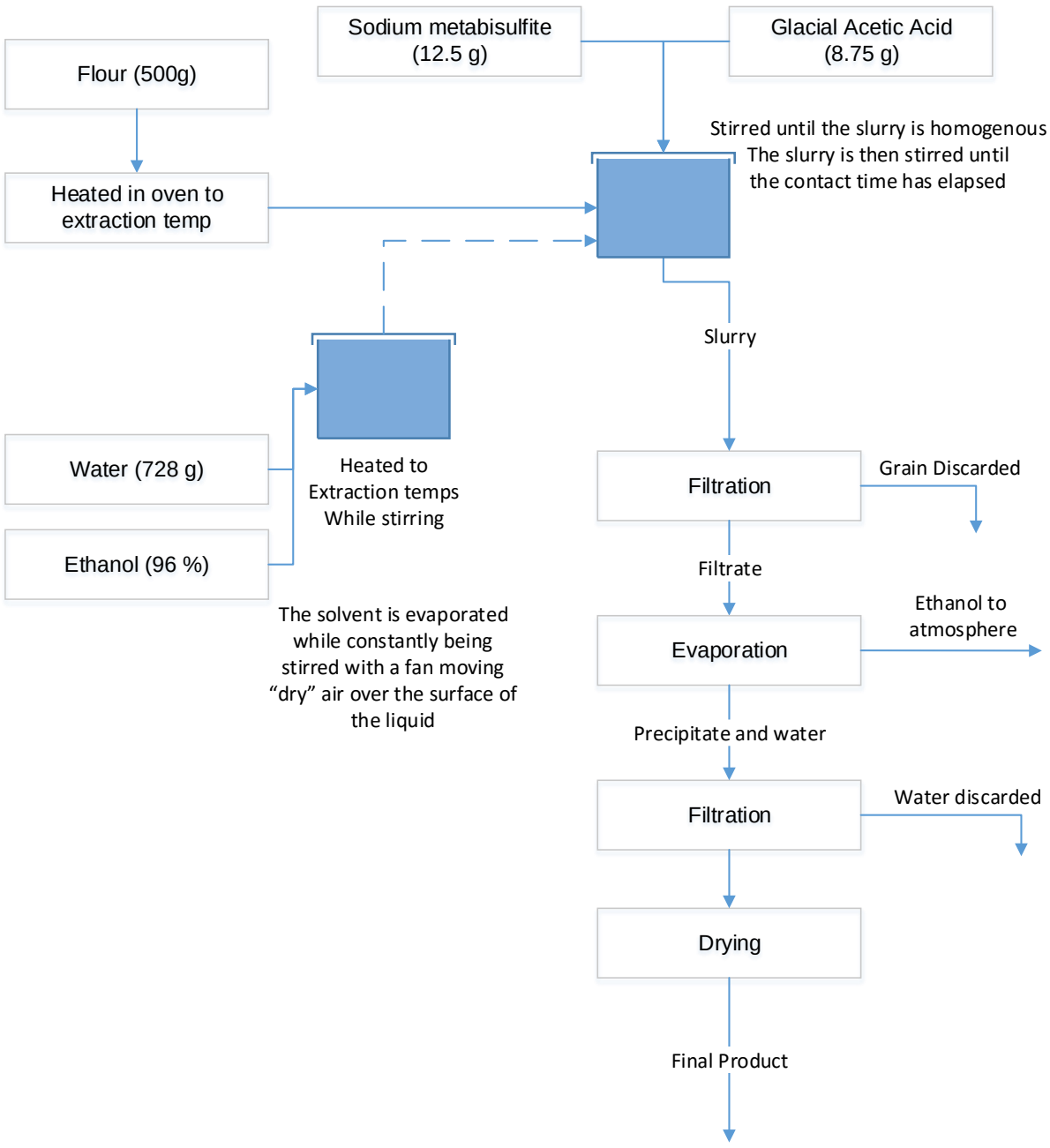

Figure 6: Basic extraction procedure process flow diagram

3.5.3 Changes made to the Taylor process

Several changes were made to the Taylor process to gain a better understanding of the process, minimise external or undesirable changes in variables and more closely reflect the capabilities of large commercial equipment.

The main changes made to the Taylor process were:

1. Heating of the grain and solvent separately before mixing to ensure the correct temperature is maintained throughout the extraction process. This also more closely reflects the capability of larger industrial equipment to maintain temperature and minimise temperature change when the flour was added to the solvent.
2. The vessel chosen was a 5 l Buchner flask which has the benefit of good heat conduction (1 - 1.4 W/m K) compared to plastic (0.12 W/m K) which will allow improved process control. It is also heavy, which makes it very stable in the water bath. Conductivity data from Cengel & Chajar (2011:877)
3. The decision not to raise and then lower the pH but instead to lower the pH before adding the grain was made to reduce the number of steps without affecting the process. It also reduces the number of hazardous chemicals to handle during a commercial process.
4. The protein was not defatted as it was outside the scope of this project.
5. The only method used to separate solids and liquids was filtration so as to reflect the probable commercial process more closely.

3.6 Choice of Optimisation Parameters

Figure 7 shows an issue tree with all the choices of parameters to change. This list conforms to MECE(mutually exclusive and collaboratively exhaustive) principals and includes all the factors that can influence the extraction process.

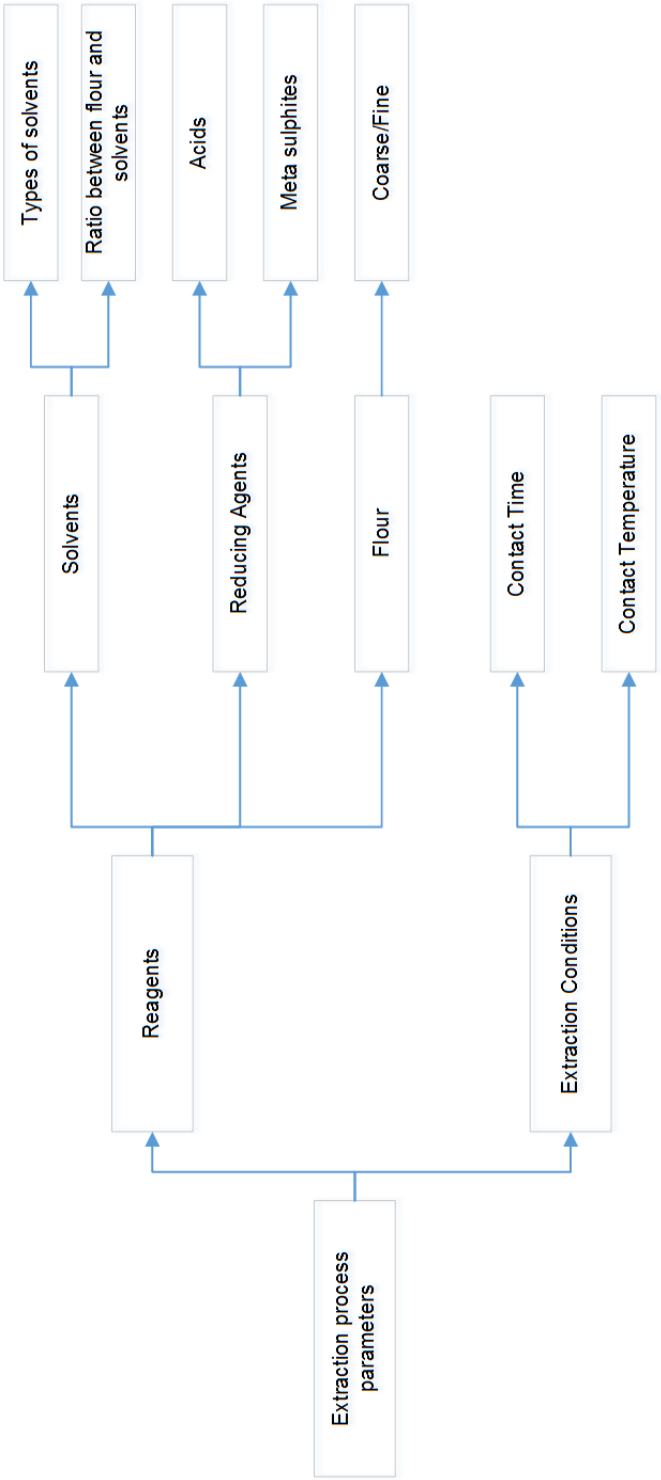

Figure 7: Issue tree with all the optimisation parameters

3.7 Order of Parameter Optimisation

The list of all the optimisation parameters in Figure 7 can be tested independently; however, it is of much more use to test each parameter sequentially and use the new optimum parameter in all subsequent tests. Figure 8 is a list of parameters and the order in which they were tested in all subsequent experiments conducted using the "new" optimum.

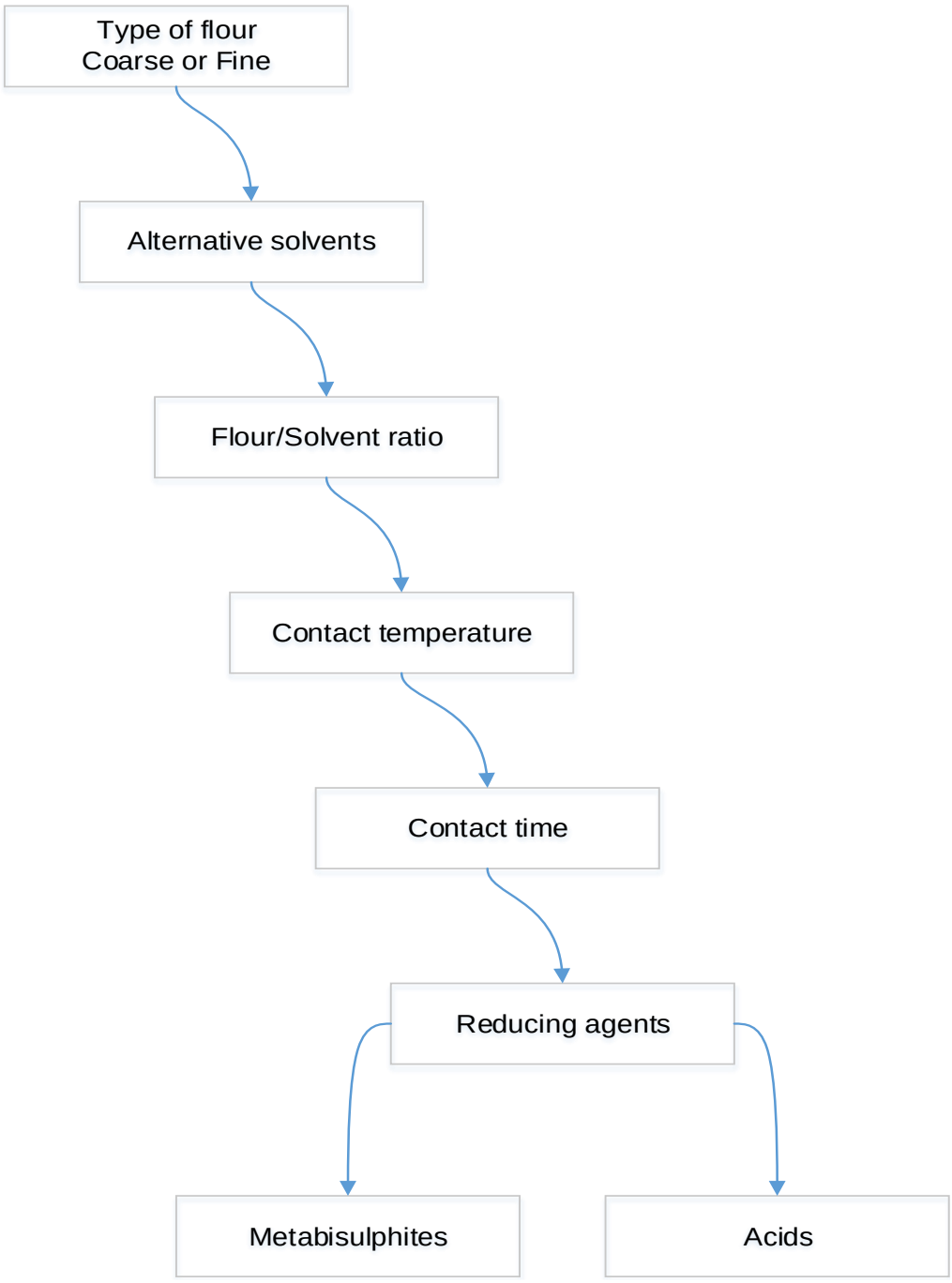

Figure 8: Order of parameters tested

PART ONE Extraction Optimisation

4 Experimental

Each of the experiments is discussed below with the motivation for the experiment as well as the plan on how to conduct the experiment. Since this was a largely exploratory study each experiment dictates the next.

4.1 Repeats of Experiments

Due to both limited time and funding it was desirable to conduct as few experiment repeats of extraction as possible. This is because each experiment takes a day to run and another day to isolate the protein by evaporating the ethanol and drying the product. Since ethanol cannot be recovered without specialised equipment it is a huge cost. Fewer repeats allowed a wider range of experiments to be done and thus provide better overall insight into the extraction procedure.

To determine whether any benefit would be gained from doing several repeats of each experiment as opposed to doing only one repeat of each experiment, the variation/variability over some extractions was tested. The extractions were done at one temperature at three contact times. If the difference was acceptably small between experiments only one repeat experiment was done. This allowed qualitative trends to be identified, which is far more interesting and useful than quantifying the process as the grains will vary naturally from season to season.

The variability associated with analytical equipment was also determined to ensure that any readings that may appear to be outliers are due to experimental phenomena and not errors in measurement.

It was arbitrarily decided that if two samples were within 10 % and a clear trend could be observed, two experiments would be sufficient. If, however, the two experiments could not be used to identify a trend due to the difference between them being too large, a third repeat would be done.

This allowed the identification any trends associated with the process and any experimental outliers, and to mark inconclusive tests for more repeats while limiting the overall number of experiments.

Method and apparatus

To determine accuracy, three extractions were done at 70 °C for extraction times of 0, 2 and 4 min. These times were chosen due to the fact that these short experiments required the greatest accuracy with regard to the precision of timing and the experimental procedure. Thus if there was not much difference between them, then other experiments were assumed to be a fair representation as well.

The flour mentioned in Section 3.2.2 was used in the basic extraction procedure with 0, 2 and 4 min contact time.

It is also important to determine what the variation/variability associated with the measurement is using three identical samples. For the purposes of these experiments a sample was randomly selected and analysed three times.

4.2 Coarse vs. Fine Flour

There are two commonly available types of sorghum flour, namely coarse (1500-1800 µm) and fine (800 - 900 µm). It is well known that solid-liquid extraction is greatly affected by the size of the solid particles, therefore the optimal type of flour should be selected by testing both. If large particles yielded sufficient protein to negate the need for more expensive milling, then both capital cost and operating costs would be reduced. The costs would be lower due to less precise milling and less energy being required to mill to a smaller particle size.

Each flour was used in the basic extraction procedure at 70 °C for 10 and 60 min. These temperatures and times were chosen as they would allow significant insight into the behaviour of both flours with the minimum number of experiments. After extraction was completed, each was filtered and the filtration time and the amount of solvent retained in the filter cakes were recorded. The filter cakes were tested for protein concentration and the yield was calculated.

Method and apparatus

To determine the best type of flour to use, 10 and 60 min extractions were done on both the fine and coarse flour varieties at 70 °C. The extractions were done using the standard method, and only the type of flour was changed.

The filtration time was measured using a stopwatch and recorded. The solvent retention was recorded by weighing the filter cake and calculating the difference between the wet and dry flour weight. Once the separation was complete the protein was precipitated by evaporating the ethanol, and the resulting protein was dried and weighed.

The three sets of results were then compared to determine which flour variety was most advantageous. Each set of results was individually compared, and then the total contribution of each result was compared to the process as a whole to determine the ideal flour type.

4.3 Flour Analysis

All the flour was sourced from the same supplier at the same time and was all part of the same batch. This was done to limit the variability amongst the flours and to ensure that any experiment done on the flour would give an accurate indication of the behaviour and trend of the process.

The flour was then analysed to determine how much protein it contained. This made it possible to calculate the extraction yields. This is very important when determining the efficiency of the process. Another important calculation that requires the protein content in the flour to be known is the maximum amount of protein extracted from the process.

Method and apparatus

The flour that was used in the bulk of experiments, unless otherwise stated, was decorticated red sorghum commonly sold as King Korn Mabele fine sorghum flour.

The flour was analysed to determine the amount of protein in the raw starting material.

4.4 Solvents

According to Taylor *et al. (*2005b) there are several alternative solvents that can be used to dissolve and hence extract kafirin since it will most likely dissolve in any solvent capable of dissolving zein. There was many solvents identified by Manely and Evans (1943). It is of interest to determine whether these solvents provide additional yield or affect the process in any beneficial way. When considering all the alternative solvents that could be used, it was decided that any solvent tested should allow use in large-scale extraction. For this reason any solvent selected would need to have sufficient vapour pressure to allow recovery as well as improved solubility. Acetic acid and tert-butanol were selected for testing as they both fulfil the requirements that would make them suitable for large-scale extraction.

It was not necessary to conduct all the tests with these solvents. Instead, each was tested on a 10 and 60 min extraction at 70 °C which had given results that allowed fair judgment of the performance of each solvent.

Another important aspect to consider was the ratio between the solvent and the grain. This will affect the total amount of protein that can be dissolved and will also impact heavily on the extraction process. To test this, a high, medium and low grain/solvent ratio was used to determine how the total product changes. The tests were conducted at 70 °C for 10 and 60 min.

Method and apparatus

Each solvent was tested by replacing ethanol in the basic extraction procedure with the solvent being tested. After extraction was completed, each sample was filtered and the filtration time and the total amount of protein extracted were recorded. Each solvent was tested at 70 °C, which was the maximum safe temperature for a period of 10 and 60 min.

To test the effect of the grain/solvent ratio, three ratios were tested: high, medium and low. Medium represented the ratio described in the basic extraction procedure, high was twice the ratio and low was half the ratio. Each extraction was conducted by changing the amount of solvent. The total grain used in each experiment was the same amount as used in the basic extraction procedure. Each ratio was extracted for 10 and

60 min at 70 °C. The total amount of protein extracted was recorded as well as any basic observations.

4.5 Extraction Temperature and Solubilised Protein

It was assumed that all the kafirin dissolved into the solvent was recovered. Thus the protein recovered at the end of the process would reflect the amount of protein dissolved into the solvent.

In all the previous extractions conducted during this study, an extraction temperature of 70 °C was used, which reflected the highest safe temperature to which to heat the solvent as it would boil at 78.5 °C. Boiling the solvent is a high risk as ethanol is extremely flammable at low temperatures and boiling would increase the amount of vapour present.

It was assumed that 70 °C would result in the greatest amounts of protein dissolving into the solvent. However, if this were not the case and similar amounts of protein dissolved into the solvent at lower temperatures, it would be more economical only to heat the solvent to a temperature at which the maximum or sufficient protein was dissolved.

Thus the ability to dissolve and extract the protein was observed at three different temperatures, which gave a sufficiently wide range to determine how temperature affects the amount of extracted protein. Each temperature was tested for three extractions to ensure that there was no error as a result of sufficient time at lower temperatures not being given to dissolve the maximum amount of protein.

The extraction temperature at which the highest yield was obtained would also be the extraction temperature for any subsequent extraction experiments, since only the optimum conditions were of interest.

Method and apparatus

To determine the effect of temperature on the solubility of protein, the basic extraction procedure was used at 30, 50 and 70 °C and allowed to continue for extraction times of 10, 30 and 60 min.

After extraction each product sample was dried, weighed and the protein preparation purity analysed to determine the yield.

4.6 Extraction Time vs. Yield

In extractions based on the zein extraction method from which the basic procedure was derived, the standard grain/solvent extraction time is one hour. However if this length of time is unnecessary and could be shortened, it would save heating costs as well as allow a higher production rate.

This means that a smaller plant could extract larger amounts of protein as it would be able to schedule multiple runs. A smaller plant would also require a lower initial capital cost. To determine the optimum amount of time required for an extraction, it was decided to determine how much protein is extracted at several different time intervals.

In experiments conducted during this study, regarding extraction temperature and solubilised protein, it was observed that the temperature played a significant role in the extraction procedure, with a higher temperature resulting in a higher yield. It was therefore decided to test the extraction times at the highest possible safe temperature, namely 70 °C.

The time intervals were chosen to allow a fairly high resolution of data points across the range which would allow adequate identification of the trends. With a higher resolution at shorter extraction times of 0 to10 min, a large change is expected, and a lower resolution is expected for times above 10 min. The extraction temperature of 70 °C resulted in the highest yield and was thus the only extraction temperature of interest.

Method and apparatus

It was decided to test the contact time at 70 °C for time intervals of 0, 2, 4, 7, 10, 20, 30, 40, 50, and 60 min. These time intervals were chosen to provide sufficient resolution over the extraction period to allow conclusions to be drawn and to find an optimum. This temperature was chosen as it is the hottest possible extraction before running the risk of boiling the solvent. Boiling the solvent could result in equipment failure and poses a real risk of fire, as well as possible damage to the protein which may occur at elevated temperatures.

Extraction was done using the basic procedure. The final product was then analysed for protein preparation purity and the yield calculated.

4.7 Effects of Acids

The manner and extent to which the acids effect the extraction of the protein is not well understood. It is assumed that a low or high pH would have the effect of increasing the solubility of the protein as discussed by Pelegrine and Gasparetto (2005), but this remains to be tested for this specific extraction method.

There would be some economic value if less acid could be used for adequate performance. This was tested by using varying amounts of the acid prescribed in the basic extraction procedure.

It may be possible that the prescribed acid is aids extraction. If other acids could be used, safer or cheaper acids could be considered. The only acids of interest were food-grade acids as the final product may have to conform to health and safety standards in the food, pharmaceutical and agricultural industries depending on the applications. The acids chosen were glacial acetic acid, citric acid and phosphoric acid.

From the experiments conducted in this study it was found that 10 min was the extraction time with the best yield, thus only 10 min extractions were considered. The extraction temperature of 70 °C produced the highest yield and was thus the only extraction temperature of interest.

Method and apparatus

Firstly, three different loadings of glacial acetic acid were tested to determine to what extent the amount of acid affected the extraction procedure. These loadings were substituted in the basic extraction procedures. They were 0, 8 and 15 g of glacial acetic acid and the extraction time was 10 min each at 70 °C. This extraction time was selected as it seemed to be close to the optimum determined by other experiments where the temperature was as high as possible to extract the maximum possible kafirin. The final products were analysed for protein concentration and the yield was calculated.

Secondly, to test whether the specific acid had any important consequences, two acids were considered and the glacial acetic acid used in the basic procedure was replaced by them. The acids were citric and phosphoric acid. The loading substituted was 8 g in each case, and following the basic extraction procedure they were allowed to

continue for 10 min. This extraction time was selected as it was found to be sufficiently close to the optimum determined by other experiments. The final products were analysed for protein concentration and the yield was calculated.

4.8 Effects of Sulphites

To determine how sulphites affect the extraction, the basic extraction procedure was followed with varying amounts and types of sulphites. As discussed in the literature, sulphites as reducing agents are widely known to increase the solubility of the protein and increase the amount that can be extracted from the grain (Landry and Moureaux, 1980). As sulphites are expensive and difficult or impossible to recover, it is important that the minimum amount sulphite should be used. If different forms of sulphite can be used, operational flexibility would also be increased by allowing cheaper or more readily available substitutes to be used. If less sulphite can be used operational costs would be lowered, but if it is found that more sulphite is required to achieve maximum yield this would also be beneficial by allowing higher yields.

From the experiments conducted in this study it was found that 10 min is the extraction time with the best yield, thus only 10 min extractions were considered. The extraction temperature of 70 °C produced the highest yield and was therefore the only extraction temperature of interest.

Method and apparatus

Firstly, four different loadings of sodium metabisulphite were tested to determine how the amount of sulphites affected the extraction procedure. The loadings were 0, 7, 12 and 20 g of sodium metabisulphite. The basic extraction procedure was followed for 10 min for each loading at 70 °C. The final products were analysed for protein concentration and the yield was calculated.

Secondly, to test whether the type of sulphite had an effect that could not be matched by any other metabisulphite, a different metabisulphite was used. Potassium metabisulphite was substituted in the basic extraction procedure. 12 g of potassium metabisulphite was substituted in the basic extraction procedure for 10 min at 70 °C. The final products were analysed for protein concentration and the yield was calculated.

5 Results and Discussion

All relevant data has been included in the body of the report. The complete set of data can be seen in Appendix A and B.

5.1 Repeat of Experiments

Table 4: Results of extractions at 0, 2 and 4 min at 70 °C

Extraction time	Preparation Purity (%)	Yield (g protein/ 500 g flour)
0	77.02	13.92
	76.84	14.60
	76.99	16.21
2	78.28	18.84
	78.71	18.50
	78.82	19.08
4	77.38	19.31
	78.03	19.52
	77.43	18.58

Table 4 shows that the protein preparation purity across three independent experiments is fairly constant. This indicates that the variation in the protein preparation purity between results is fairly small. The differences are negligible if considering the range in the amount of protein in the different types of grain, which as shown in Table 2 is 10.6 - 12.8 % and 12.1 - 13.2 % for low and high-tannin sorghums respectively.

From Table 4 it can see that when considering the yields there is a slightly larger variation. This is higher than that of the protein preparation purity but is still fairly small, i.e. less than 10 % in either direction, and still allows only one repeat of every experiment to sufficiently identify process behaviour while ensuring that any outliers can be identified and studied further.

To determine the variability in measurement associated with the Dumatherm, one sample was analysed three times. The results from this analysis are given in Table 5.

Table 5: Identical protein Results

Analysis	Protein (%)
1	71.48
2	71.19
3	71.77

Table 5 shows that the protein percentage falls into a fairly narrow range. The samples had an average protein preparation purity of 71.48 %, and all the data points were fairly close to this average. This fairly small variation makes it possible to identify any outliers in the results. This further supports the decision only to conduct one repeat in the interest of time and economic constraints.

5.2 Coarse vs. Fine Flour

Table 6 shows the extractions done on the coarse and fine flours to determine which flour would be used in subsequent experiments.

Table 6: Coarse vs. fine flour extraction performance

Extraction Time (min)	Flour	Filtration time (min)	Solvent retained (g)	Dried product (g)
10	Coarse	12.18	435	14.0
	Fine	4.43	257	28.7
60	Coarse	240	1864	7.0
	Fine	75	1103	19.82

From Table 6 it is clear 10 min extraction saw the coarse flour perform at a level far below that of the fine flour. The filtration time was far longer and the retained solvent was far higher, while the total extracted product was only 50 % of that of the fine flour. Thus for a shorter extraction time fine flour is far superior. The 60 min time produced similar results, with the fine flour far outperforming the coarser flour by a much larger margin than in the 10 min extraction.

Based on these results the fine flour was used for all subsequent experiments.

5.3 Flour Analysis

Table 7 gives the results of the protein analysis of three identical flour samples. The flour analysed was the fine flour, which proved to be the better material in the previous set of experiments and in all subsequent experiments.

Table 7: Protein analysis of raw flour

Run	Protein (%)
1	10.15
2	10.04
3	10.16

From the data in Table 7 it can be seen that the flour has an average protein content of 10.12 %. From the analysis it can be concluded that there is approximately 50 g of protein in 500 g of flour, and as discussed in the literature approximately 70 % of this is kafirin, which means that there is approximately 35 g of kafirin in 500 g of flour. This formed the basis of determining how efficient any extraction procedure is at removing available kafirin.

5.4 Solvents

Glacial acetic acid was excluded due its inability to separate the slurry and grain by commercially viable methods. The 60 min extraction was added to the filter, and it immediately clogged the filter with a zero flow rate. After several hours of filtration the slurry had to be discarded. It was then thought that similar to ethanol extraction a shorter contact time might prevent thickening of the slurry. This hypothesis was proven incorrect as the 10 min extraction behaved very similarly. After several hours the slurry had to be discarded. In both experiments it was then attempted separate the slurry with a centrifuge at 3 000 rpm. After an hour of centrifuging the slurry had to be discarded as it had not separated. Any benefit of acetic acid is negated by its inability to separate the solvent and the grain.

Tert-butanol was tested as both the 10 and 60 min extractions showed similar behaviour with regard to filtration.

The 10 and 60 min extractions yielded 26 and 18 g protein/ 500 g flour. This is a slightly better yield than that produced by ethanol provided, however, that tert-butanol was excluded as the cost of the solvent was too high. Since both ethanol and the grain are both fairly cheap, a small drop in yield is acceptable for a large gain in profit margin.

In Figure 9, doubling the amount of solvent resulted in a small increase in the total product extracted, which is a strong indication that the recipe are already fairly close to the optimum amount of solvent. The amount of additional protein extracted is most likely due to the retained solvent being more dilute. The additional yield, however, would not be enough to offset the additional costs.

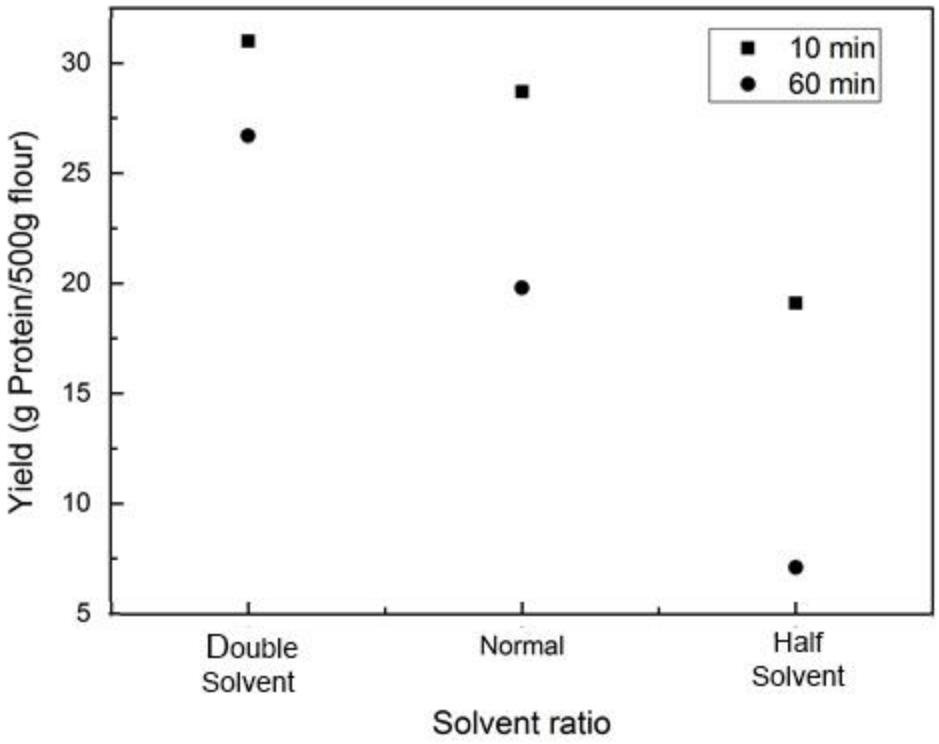

Figure 9: Effect of various grain solvent ratios on the protein yeild

Halving the amount of solvent resulted in a fairly large loss. More than half the total protein was extracted, which is a strong indication that the ratio of solvent in the basic extraction procedure is sufficient. This slurry was also extremely difficult to process and took far longer to filter remove from the reaction vessel. All subsequent experiments were conducted using the ratio as described in the basic extraction procedure.

5.5 Contact Temperature and Solubility from Grain

Figure 10 shows the average yields of extractions at three different temperatures.

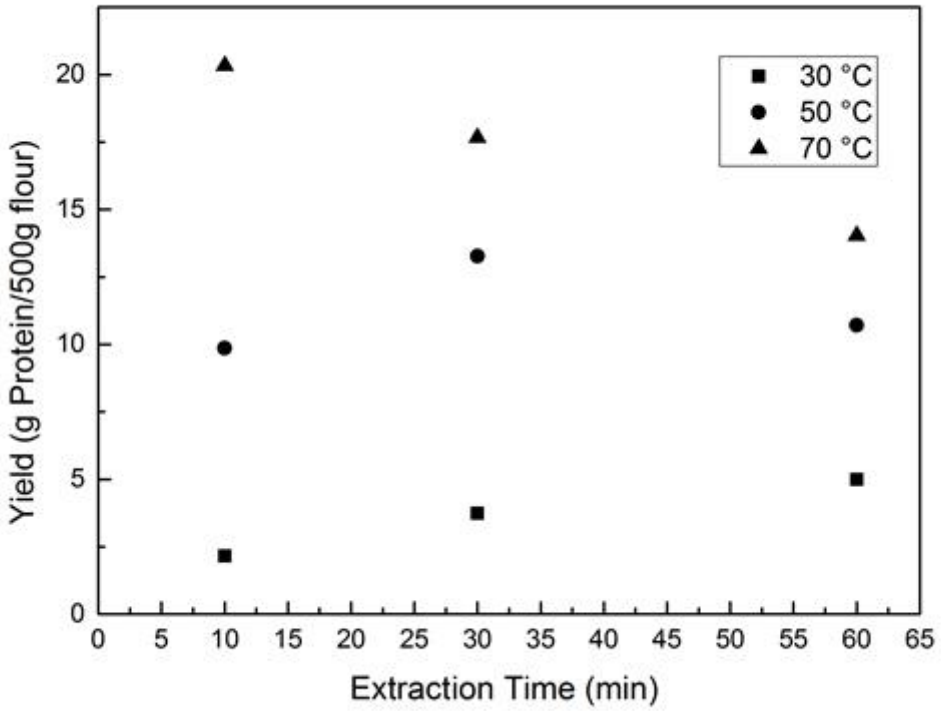

Figure 10: Protein yield at various extraction temperatures

From Figure 10 it is clear that at lower temperatures far less protein is extracted. The only aspect that could be responsible is that less protein was dissolved into the solvent. This implies that the solubility of the protein is highly dependent on the temperature at which the extraction occurs. With the optimal temperature being 70 °C in these tests, it was not possible to conclude whether this is in fact the true optimum, but for safety reasons it is undesirable to conduct experiments at higher temperatures. Thus 70 °C was considered the optimum temperature for the remainder of the experiments.

The yield for extractions at 30 °C seem increase at longer contact times. The increase occurred in a fairly steady manner to approximately 5 g of protein for 500 g of flour after an hour of extraction time. The yield appears to be approaching a limit of 5 g protein for 500 g of flour. This implies that this yield reflects the solubility limit of protein in the solvent at 30 °C.

The yield for exactions at 50 and 70 °C seems to decrease as the extraction time is increased. The yields for 50 °C extractions are much less than extractions at 70 °C thus it is of no interest doing experiments at lower temperatures.

One observation that is of interest is the decreasing yield at 70 °C over the course of the extraction. This decrease can also be seen for the 50 °C extraction, although this was not of interest. It is fair to assume that the 70 °C extraction also has a peak somewhere, although if it does occur it is not observable from this data. However, the peak has been observed in subsequent experiments.

5.6 Effect of Contact Time on Extracted Protein

Figure 11 shows the effect of extraction time on the yield of the protein. All the data points were from extractions at 70 °C. Due to the fact that the exact protein composition was unknown, a scale of g protein/500 g flour was used in lieu of a percentage yield.

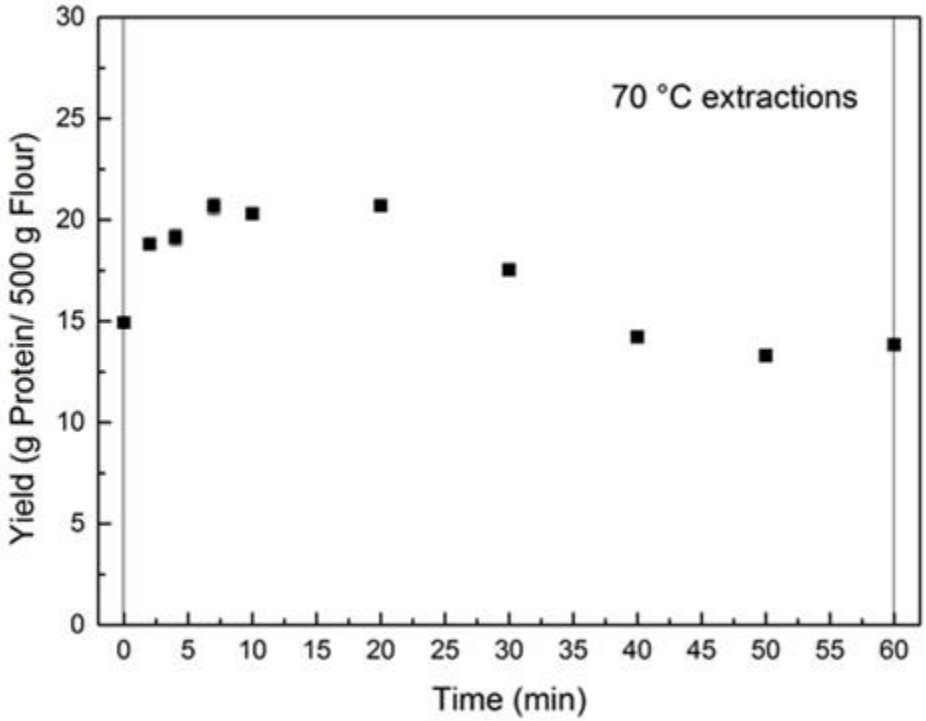

Figure 11: Protein yield at various solvent/grain contact times

Figure 11 shows that the yield of protein is initially low but very quickly increases. At the peak yield, which occurs somewhere between 7 and 20 min, seeing a yield of between 20 and 22 g of pure protein per 500 g of flour. Assuming that roughly 70 % of the proteins in the grain are kafirin, this yield conservatively represents a 60 % total kafirin extraction.

After this initial increase and subsequent plateau, however, the yield starts to drop off somewhere after 20 min and continues to drop over the duration of the extraction, and seems to plateau again somewhere between 40 and 50 min. This could be due to other material dissolving into the protein or some physical mechanism.

Figure 12 shows the protein preparation purity associated with different extraction times. These are the average purities of the dried protein from extractions at 70 °C.

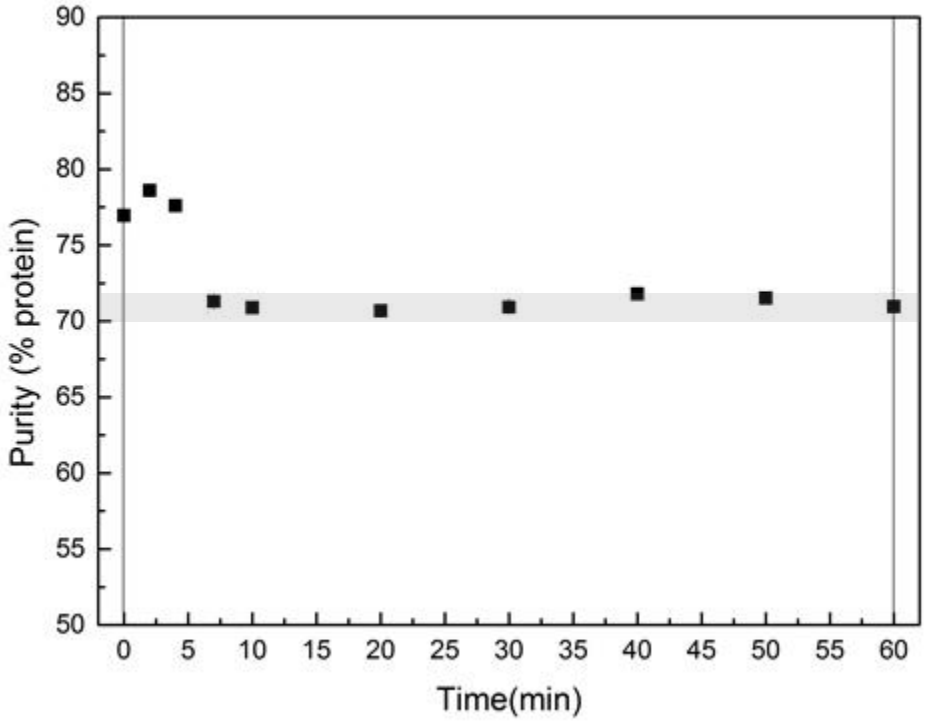

Figure 12: Protein preparation purity associated with contact times

From Figure 12 it is clear that at very short times very pure protein is extracted, which indicates that the protein readily dissolves into the solvent. This is further supported by the fact that over the course of the extraction procedure the purity does not fluctuate much after the initial dissolution. The preparation purity of the protein extracted for 10 to 60 min stays in the very narrow 70 to 72 % band.

The drop between 5 and 10 min is possibly due to other components of the grain being soluble but having a lower dissolution rate than the protein. This means that the protein is mostly dissolved after 5 min, after which other components start to dissolve into the solvent. The fact that the protein preparation purity remained fairly constant between 10 and 60 min of contact time rules out any impact that fats or other soluble materials may have had on the amount of protein extracted. This leads to the idea that a physical mechanism may be responsible.

5.7 Effects of Acid

To measure the effect of acidification on the extraction, various amounts of glacial acetic acid were used in the extraction. Results in Figure 13 show how the acetic acid loading affected the extraction and how acidification changed the final protein yield.

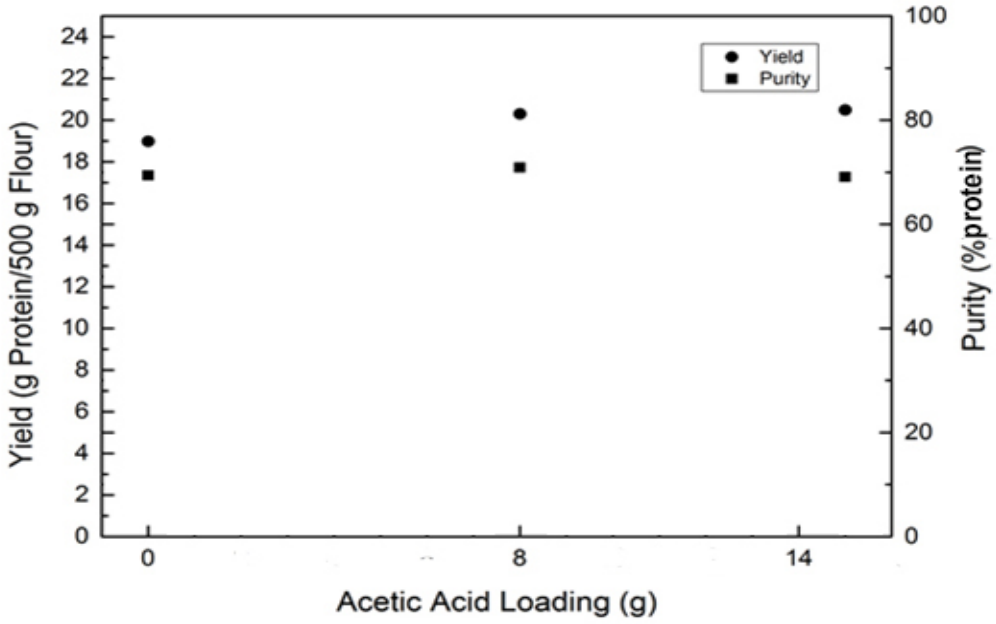

Figure 13: Protein yield and preparation purity at various acetic acid loadings

From Figure 13 it can be seen that the amount of protein extracted does not vary significantly over the range of acetic acid loadings. This leads to the idea that acid is unnecessary.

In Figure 14 shows the effect of different acids on the preparation purity and yield of the protein.

As Figure 14 shows, there is very little difference between the three types of acid and also when acids are completely excluded. This means that acetic acid does not behave in any special way and can be substituted by other food-grade acids or excluded completely while maintaining the yield. This is also of interest if one considers that glacial acetic acid is highly volatile, flammable and extremely corrosive. Replacing this with a dry acid would mean less risk to both equipment and personnel, while eliminating the acid from the process would save money.

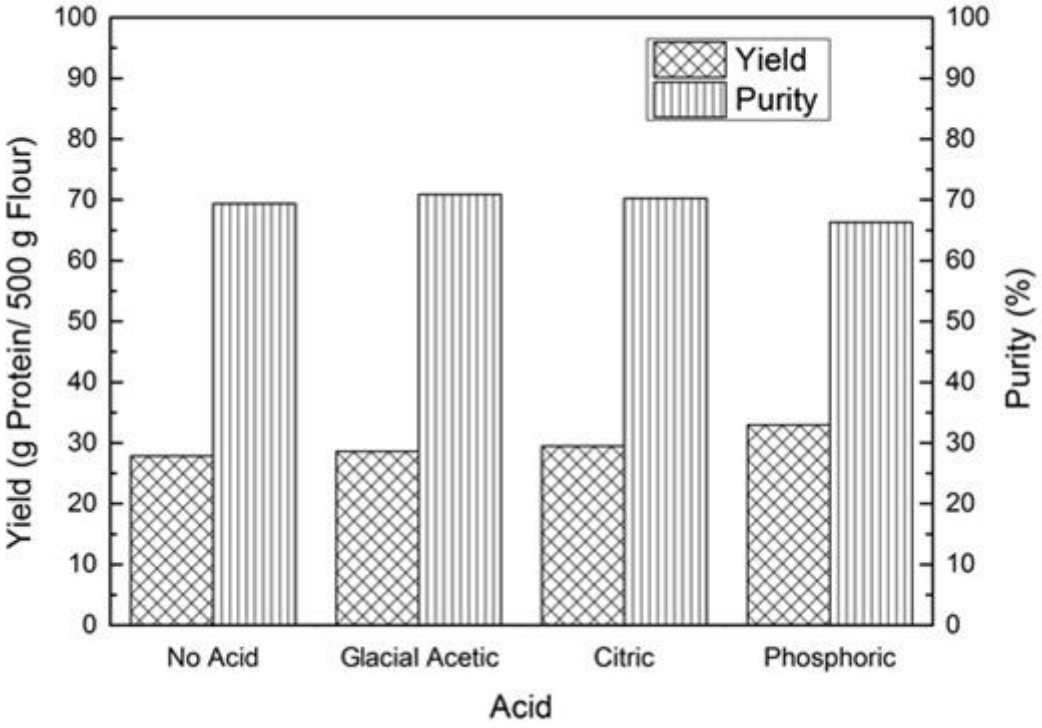

Figure 14: Preparation purity and protein yield associated with different acids

5.8 Effects of Sulphites

To measure the effect of the sulphites on the extraction, various amounts of sulphites as well as different sulphites were used to extract the protein.

In Figure 15 the effect of the type of sulphite used on both the preparation purity and the yield is shown.

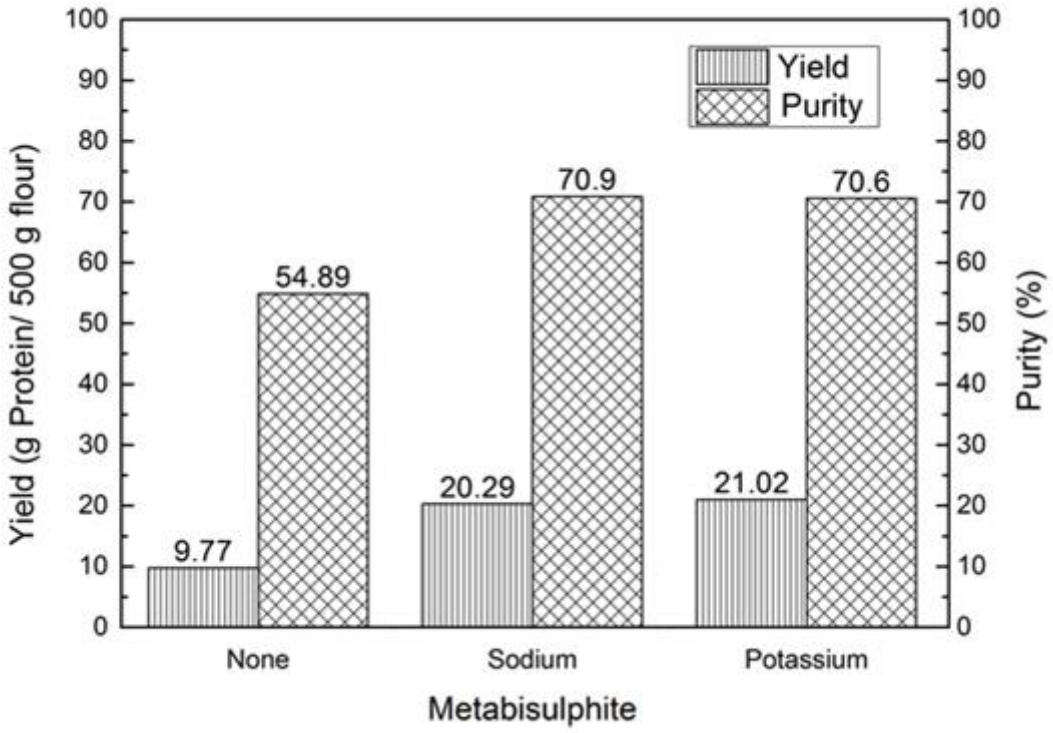

Figure 15: Protein yield and preparation purity of different metabisulphites

Figure 15 indicates that the metal in the bisulphite complex does not change the yield, and either potassium or sodium metabisulphite can be used. However, when it is not present both the preparation purity and subsequent yield are roughly 50 % less. This indicates that Wall & Paulis's (1978) conclusion is applicable here, namely that the sulphite weakens and reduces the sulphide bonds in the protein which increase solubility. It also indicates that this reducing action is of paramount importance and cannot be excluded.

Figure 16 shows how the metabisulphite affected the extraction and how it changed the final yield of protein.

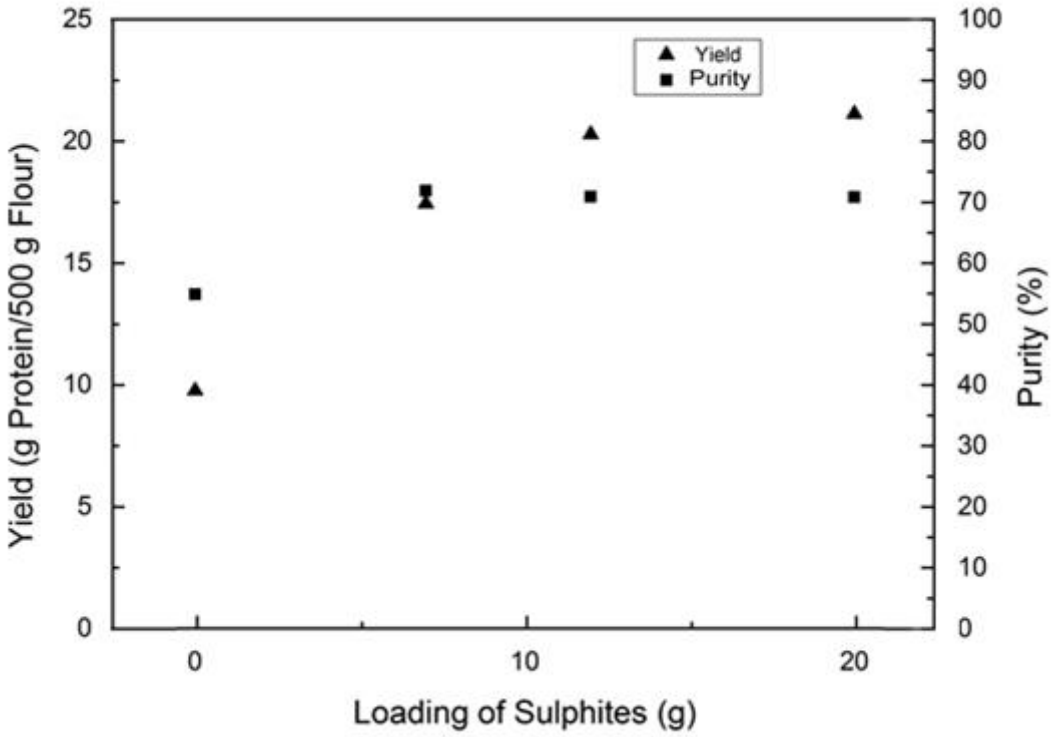

Figure 16: Protein yield and preparation purity at various sulphite loadings

From Figure 16 it can be seen that with no sodium metabisulphite the protein yield is roughly half that of the standard recipe, and as loading increases the yield increases while the preparation purity stays fairly constant. However, the difference between 12 and 20 g is not large enough to justify additional reducing agents.

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PART TWO: Filtration and Solvent Retention

6 Experimental

A discussion of each of the experiments is given below with the motivation for the experiment as well as the plan on how to conduct the experiment. Since this was largely an exploratory study each experiment dictates the next.

6.1 Contact Time vs. Filtration Time and Retained Solvent

One of the limiting factors in the upscaling of the extraction process is the time it takes to filter the slurry. In previous experiments conducted during this study it was also noticed that the filter cake retained a large amount of solvent. It was hypothesised that the solvent retention could be a mechanism that is responsible for the decrease in yield at longer contact times as well as causing filtration to be extremely slow. To test this hypothesis filter times and the retained solvent were determined for slurries for various contact times using the optimum temperature.

If it is known that the amount of time required to filter varies greatly at different contact times, this would be useful for the optimisation of any scheduling of a large-scale production. Since the expensive solvent is the component into which the protein will be dissolved and then recovered from, and flour is capable of absorbing liquid, it is very important to know how much solvent is being lost in the filter cake. The solvent retained in the filter cake will contain protein which could not be extracted due the retention.

Method and apparatus

Using the basic extraction procedure at 70 °C and contact times of 0, 2, 4, 7, 10, 20, 30, 40, 50 and 60 min, the filtration time and solvent retention were tested. The temperature of 70 °C was found to be optimal in previous experiments conducted during the course of the study, and the contact times were selected to ensure an adequate resolution of the overall process.

After each extraction the filter time and the amount of filtrate retained in the filter cake were recorded.

6.2 Use of Reducing Agents/Extraction Aids on Filtration and Retained Solvent

It was determined in previous experiments conducted during this study that the addition of reducing agents had a significant effect as with sodium metabisulphite or none as with the acids as extraction aids. It was thought that these reducing agents/extraction aids might similarly have an effect on the filtration or solvent retention.

If the reducing agents/extraction aids could either reduce the time of filtration or solvent retention, but had a limited effect on the yield, it might be more advantageous to include them if the time saved could significantly increase production capacity. If, however, the effect of reducing agent/extraction aid was, limited then it might be a more cost-effective to exclude them.

The effect of reducing agent/extraction aid was tested at the optimum temperature for 10 min extractions. Varying the amount and types of reducing agent gave an insight into the effect on the grain filtrate separation.

Method and apparatus

To test whether the sulphites had any effect on filtration, two different sulphites were used in the basic procedure. Sodium metabisulphite was used in loadings of 7, 12 and 20 g. No metabisulphite and 12 g of potassium metabisulphite were also tested. Each experiment was allowed to continue for 10 min. This extraction time was selected as it was found to be sufficiently close to the optimum found in other experiments. The time required to filter and the amount of solvent retained were recorded.

To test whether acid had any effect on filtration, three different acids were used in the basic procedure. These acids were glacial acetic acid, citric acid and phosphoric acid. The loadings substituted were 8 g in each case as well 15 g of glacial acetic acid and no acid. Following the basic extraction procedure the experiments were allowed to continue for 10 min. This extraction time was selected as this it found to be sufficiently close to the optimum found in other experiments. The time required to filter and the amount of solvent retained were recorded.

6.3 Viscosity at Different Temperatures

During experiments conducted during the course of this study it was observed that the filtration time and the retained solvent are by far the biggest concerns regarding the extraction. These two characteristics of the extraction are closely related and are highly dependent on the viscosity of the slurry. If there were a way of manipulating viscosity to increase production even at the cost of lower yields per batch, this would be beneficial in large-scale production.

Three temperatures were chosen, ranging from slightly elevated through to the optimal temperature found in previous experiments. The range of temperatures were chosen to determine whether the thickening was driven by temperature or by some grain/solvent interaction caused by the mechanical process of mixing. If a thickening action occurs at all temperatures then it can be concluded to be the result of the mechanical process of mixing, which might be manipulated by altering the stirring methods. If, however, the thickening is temperature dependant, it would allow planning to possibly identify the responsible mechanism.

It was also thought that the protein in solution might be having an effect on the viscosity which could cause some changes in the viscosity. This is difficult to test and was eventually discarded due to the fact that the isolated protein does not initially contain all the components and does not dissolve as readily in the solvent after isolation.

Method and apparatus

To gain some insight into the changing filtration rates and amount of solvent trapped in the filter cake, the viscosity was measured during extraction of the protein.

To achieve this a 20 ml cup and vane stirrer setup was used in a Physica MCR 101 from Anton Paar at three different temperatures. The stirrer was set to 900 rpm for 30 s to fully homogenise the grain/solvent slurry and then at 600 rpm for the remainder of the contact time. The runs were done isothermally at 30, 50 and 70 °C and each run was conducted for 60 min with 60 measurement points over the course of the extraction. The temperatures were chosen to determine whether the thickening was temperature driven or bought on by a mechanical process.

Due to the unique nature of the slurry, standard methods gave an unsatisfactory result, and thus it was decided to carry out the procedure in a manner closely reflecting

conditions encountered in commercial extractions. This procedure would give more useful data that would be indicative of what was actually happening.

6.4 Particle Size over Time

In an attempt to identify the mechanism causing the viscosity increase over time, the particle size at various contact times was analysed. If the mechanism were physical, it may be identified by the increase in particle size which would indicate swelling of the particle due to absorption of the solvent. If the particles became smaller, it could be assumed that the particles were breaking down, which would increase the time required to filter and reduce the effectiveness of the separation.

If there were no change in the particle size, it would be a good indicator that the increase in filtration time and solvent retention are not due to any of the phenomena mentioned above.

Method and apparatus

To test the mechanism by which the amount of solvent trapped in the filter cake changes with time, the particle size was analysed during leaching.

A 500 ml extraction setup with the appropriately scaled recipe at 70 °C was used directly on a heated stirring plate with a magnetic cross-stirrer to keep the temperature and slurry motion constant. At 5 min intervals a small sample was extracted and placed in a Melvrin Mastersizer 3000 and the d_{10}, d_{50} and d_{90} were analysed. The 5 min intervals were chosen as this is the least amount of time required to properly clean the instrument before the next run. A sample of slurry was extracted with a spoon and mixed into the water reservoir to ensure that a representative sample was taken. Slurry was added until the software indicated that the obscuration was correct. The entire procedure was conducted three times to ensure that an accurate average was found. Analysis was done according to ISO 13320: 2009.

Analysis was done at a stirrer speed of 3 000 rpm with no ultrasound and the instrument was cleaned thoroughly after every sample.

To clean the instrument the water was drained and the device rinsed with water and dishwashing detergent for 2 min, then rinsed with water at 30 s intervals before being emptied. The water rinses were continued until the obscuration was the same as the calibration. This indicated that the instrument was clean and ready for the next sample.

6.5 Visual Inspection of the Slurry

The fact that there was no significant change in the particle size over time means that another mechanism for the viscosity change must be identified. It was thought that if there were some mechanism causing the particles to agglomerate, it may be useful in identifying and mitigating the issue. Agglomeration might have been missed in the particle size analysis if the forces associated with agglomeration were easily overcome by sheer in the mixer portion of the particle size analyser.

To this end the slurry was inspected under a microscope to determine whether there was a tendency for the particle to agglomerate over the course of the extraction. If agglomeration were present then one of the possible causes could be investigated. While not the principle objective of this study, it is of interest to find the mechanism as this may inform choices such as equipment selection and operational parameters in the design of any large-scale extraction.

The slurry was observed at six time intervals over the course of an hour. Six intervals were chosen as this would allow trends to be identified.

Method and apparatus

To determine whether there is any physical change in the slurry during the extraction procedure, a Zeiss SteRO Discovery v.20 microscope was used to observe the slurry over the contact time of an hour. A 500 ml extraction setup with the appropriately scaled recipe at 70 °C was used directly on a heated stirring plate with a magnetic cross-stirrer to keep the temperature and slurry motion constant.

The extraction was done at 70 °C for 60 min with microscope photos taken every 5 min. The photos were taken at 50x magnification with a black background to allow the semi-translucent white starch globules to be seen more easily. The exposure was set to automatic while the focus was set manually to allow both smaller and larger particles to be observed.

7 Results and Discussion

7.1 Contact Time vs. Filtration Time and Retained Solvent

From Figure 11 one can see the marked difference in the yield of the final protein product from 20 to 60 min, which reflects an almost 50 % drop in yield. Figure 17 shows the filtration time associated with each of the contact times as well as the amount of solvent retained in the filter cake.

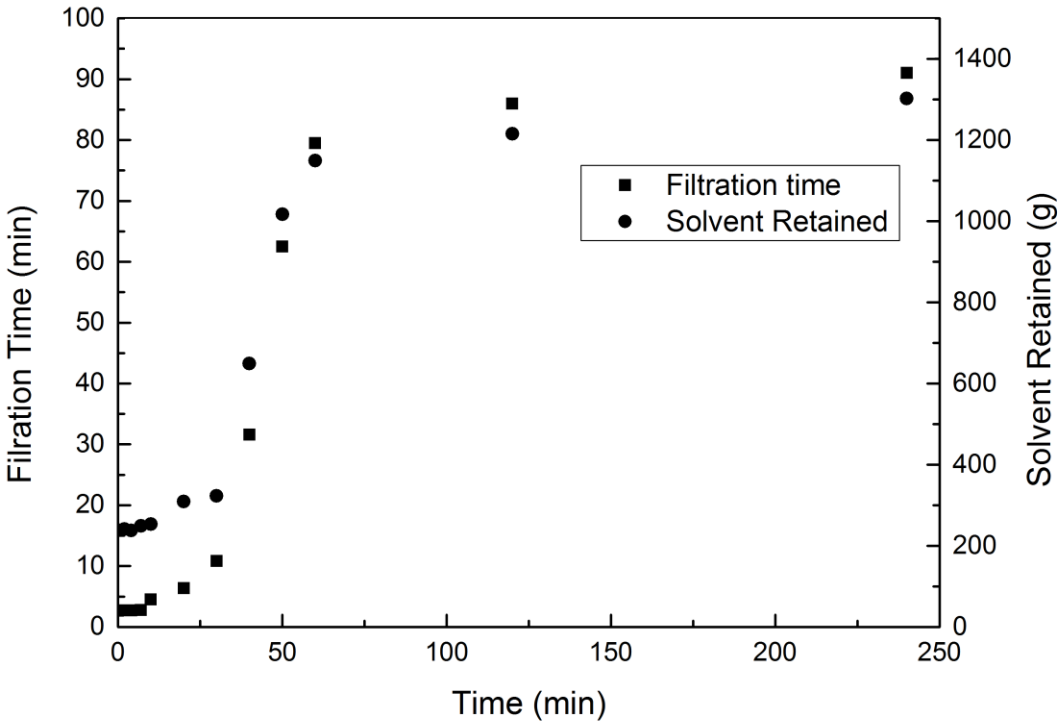

Figure 17: Filtration time and solvent retention at various contact times

In Figure 17 one can see that both the time required to filter and the amount of solvent lost closely follow an s-curve curve diverging at the 60 min mark. This suggests a plateau and some asymptotic maximum which is supported by measurements at 120 and 240 min. Even at these extended times the behaviour seems stable, deviating slightly from a true plateau. This observed solvent retention is a likely cause of the drop in yield observed in Figure 11.

To determine whether they really are closely related it is possible to observe the yield and percentage solvent recovery in Figure 18.

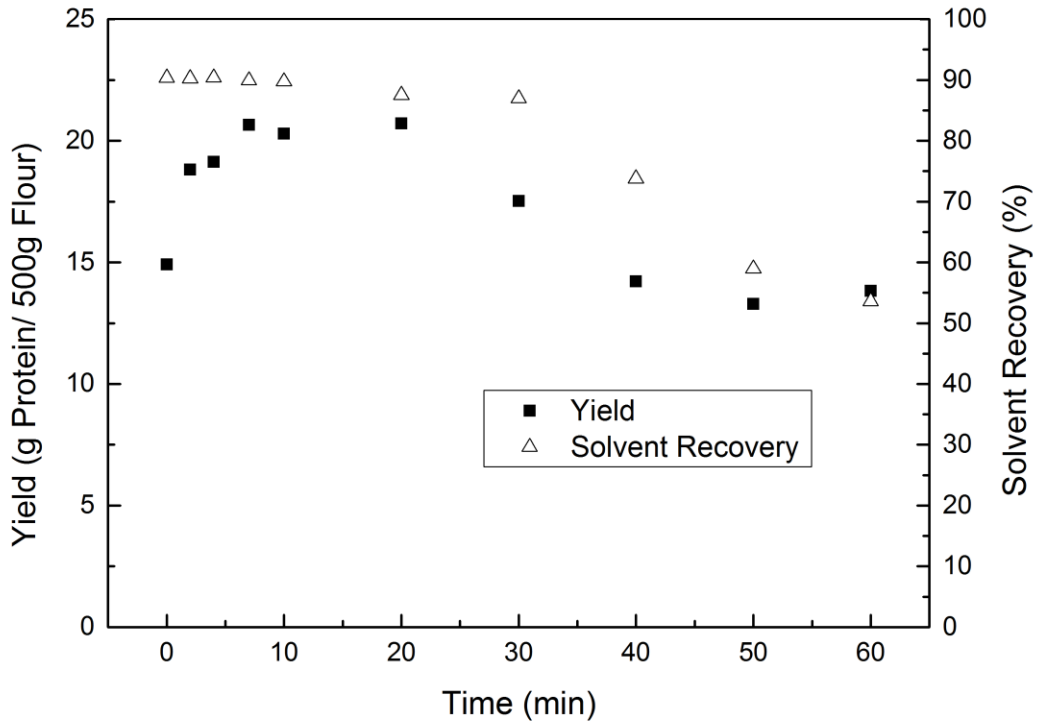

Figure 18: Protein yield with solvent recovery at different contact times

It is observed from Figure 18 that at times greater than 10 min both the yield and solvent recovery follow each other fairly closely, which is further evidence of the correlation between the two. The initial difference in trends between the yield and recovery is attributed to the rate at which the protein dissolves in the solvent as mentioned in Section 3.2.5.

7.2 Use of Reducing Agents/Extraction aids on Filtration and Retained Solvent

Figure 19 shows the time taken to filter and the solvent retained by the slurry associated with different metabisulphite loadings.

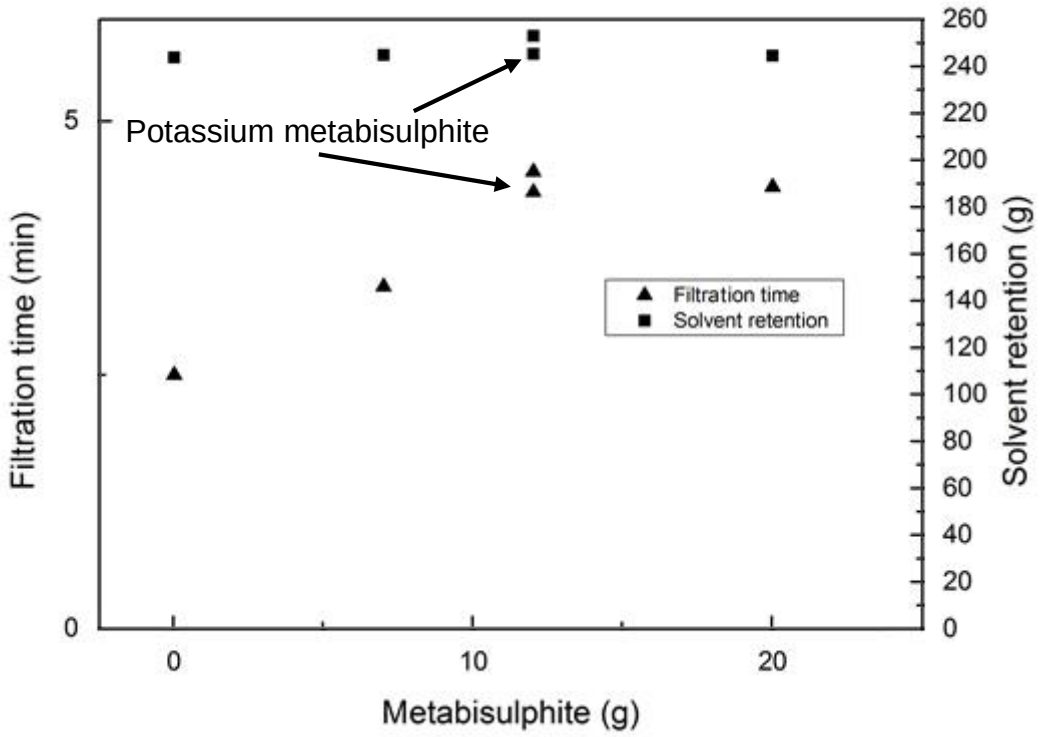

Figure 19: Filtration time and solvent recovery associated with metabisulphite loadings

Figure 19 shows that the amount of solvent retained after 10 min seems to be unaffected by the presence of the metabisulphite or the type of metabisulphite. When looking at the time required to filter the slurry, there is a trend for it to increase with the amount of metabisulphite very similar to the trend in the Figure 15. This does suggest that the addition of metabisulphite has a fundamental effect on the physical behaviour of the slurry.

Figure 20 shows the filtration time and retained solvent for various acid loadings.

From Figure 20 it is clear that there is no effect on the physical operation of separating the liquids and solids by the acids. The type and load of the acids plays no role in the separation.

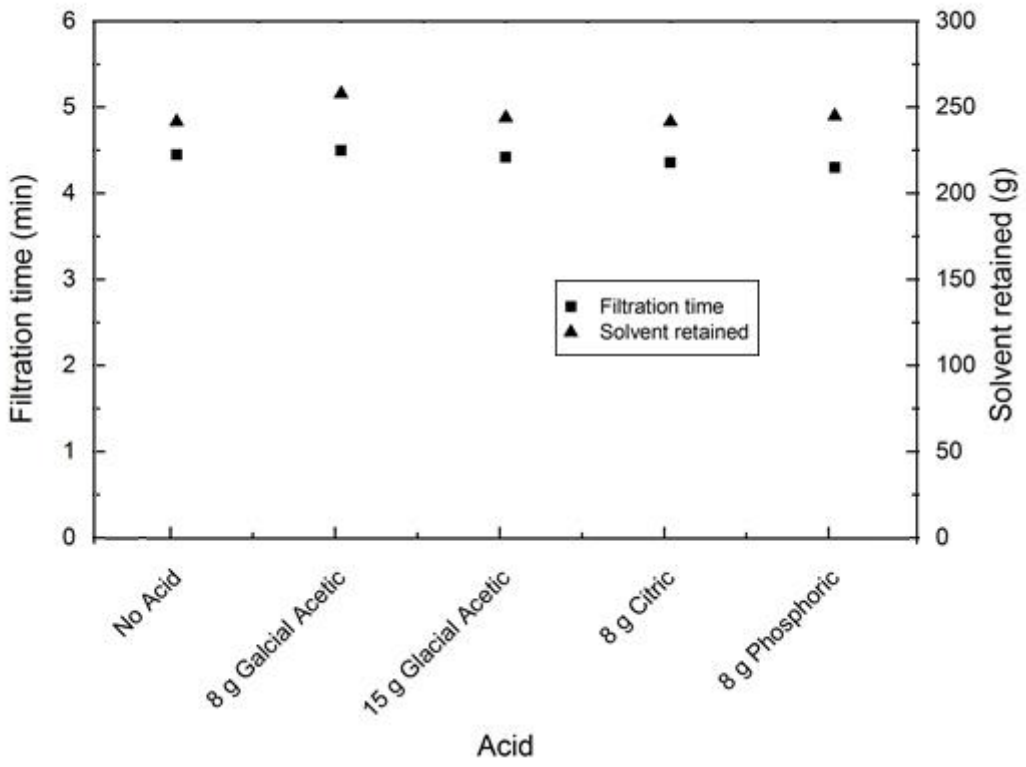

Figure 20: Filtration time and retained solvent for various acid loadings

7.3 Viscosity at Different Temperatures

Viscosity tests were conducted at three temperatures 30, 50 and 70 °C to determine the extent and possibly identify the mechanism which causes the slurry to become thicker and cause to retention, which results in loss of efficiency Each run was conducted 3 times to ensure a representative data set. The viscosities can be seen in Figure 21.

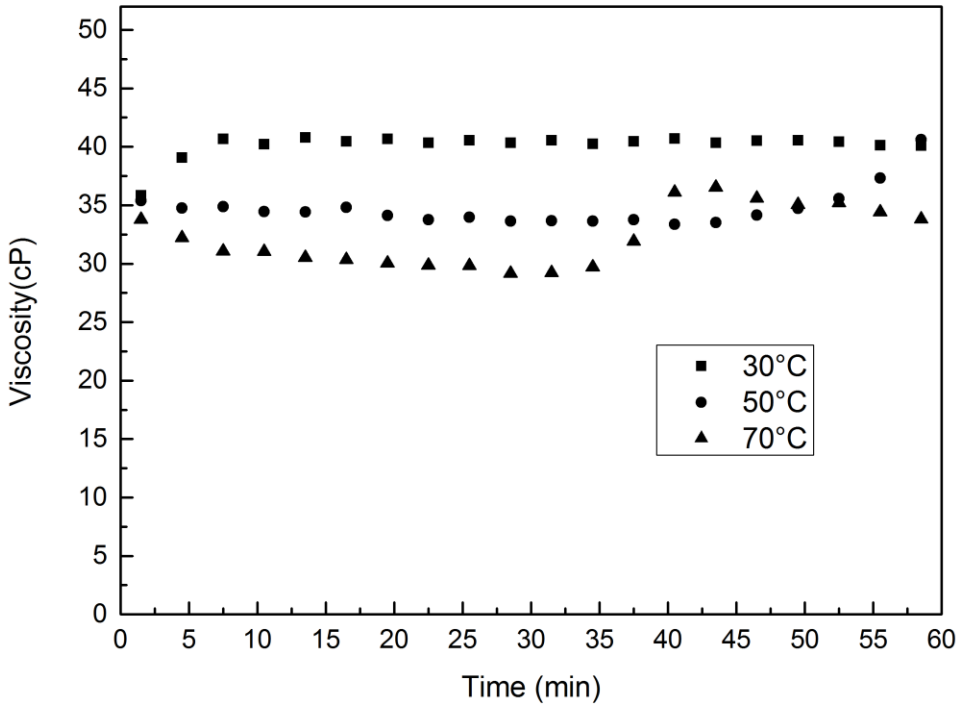

Figure 21: Viscosity over time at various temperatures

Figure 21 shows that for the extraction at 30 °C the viscosity rises slightly and then stays constant over the course of the extraction. The viscosity of the 50 °C extraction stays fairly constant over the first 45 to 50 min, and after 50 min the viscosity increases possibly due to some gelling behaviour. However, on the 60 min scale it is hard to determine.

Figure 22 shows the viscosity of the 50 °C extraction after the time up to 120 min.

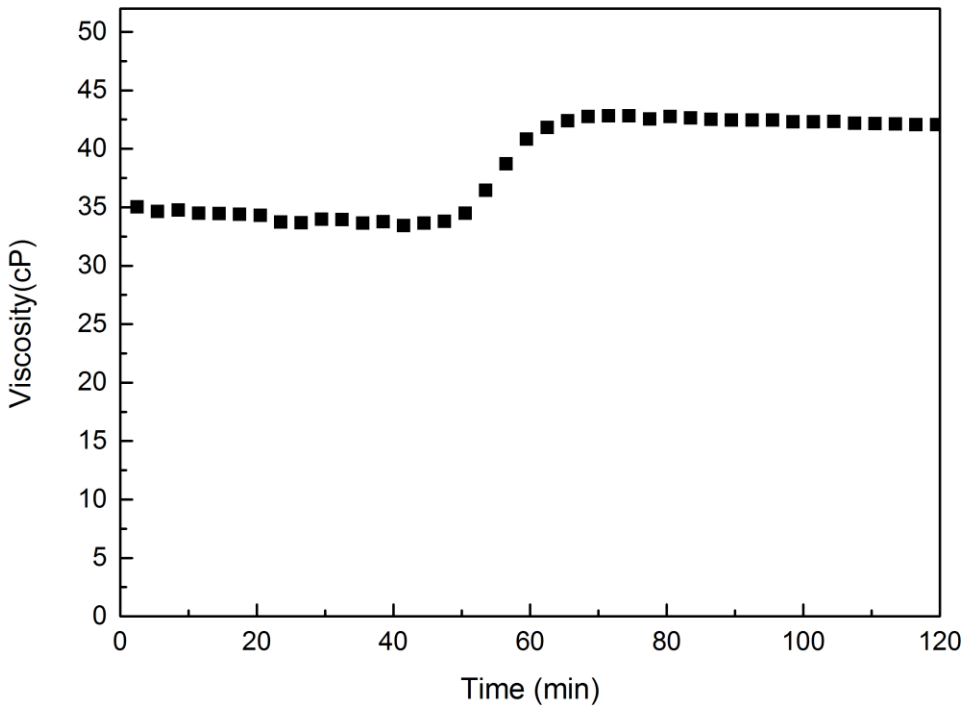

Figure 22: Viscosity of slurry over 120 min at 50 °C

In Figure 22 the viscosity of the slurry at 50 °C showed an increase followed by a new stable region with a slightly decreasing trend over the second 60 min.

With the slurry at 70 °C there was some disentanglement behaviour, dropping almost 5 cP or 14 % over the course of the first 30 min, which should see a decrease in filtration time but this was not observed. If, however, the particles become disentangled or break down and there are smaller particles, this may impede the filtration by blinding the filter medium, which would explain the increasing filtration time.

Between 35 and 42 min there seems to be thickening behaviour as the viscosity increases dramatically. This correlates very accurately with both the increased filtration time and the dramatic increase in the amount of solvent trapped in the filter cake. After this thickening there is continued disentanglement.

At 70 °C the thickening occurs roughly 15 min earlier than at 50 °C, which implies that the temperature is the factor playing the largest role in the thickening. It is, however, impossible to conclude what mechanism is responsible for the thickening.

There are two possible mechanisms that may explain the thickening:

1. Weakened hydrogen bonding in the starchy endosperm: due to the fact that the material is sufficiently gelatinized to allow bonding between the particles as well as solvent, increased energy would be required to move the stirrer through them.
2. Protein-tannin complex forms and causes a gelation to occur.

The first mechanism is more likely as a higher temperature is expected to increase the gelatinisation and subsequent hydrogen bond degradation/reformation rate which explains the thicken occurring more readily at higher temperatures. This is supported by the evidence of disentanglement. However, in the literature it is suspected that higher temperatures are more likely to cause a gel to form as the protein reacts with the tannins.

As for the disentanglement after the period of thickening, this is again most likely due to the hydrogen bonds weakening.

As the protein did not readily dissolve into the solvent after isolation, one can assume that isolating it does in fact change some of the physical properties of the protein. This also makes it difficult to determine the exact mechanism; however, it is most likely a combination of the two mechanisms mentioned above.

It is unlikely that the particles bonding to the solvents is the cause of the thickening. If there was significant hydrogen bonding between the starchy endosperm and the the solvent we would have expected the results in 5.2 to favour the coarse material. The increased surface area of the finer particles would favour solvent bonding.

7.4 Particle Size over Time

Figure 23 shows the d_{10}, d_{50} and d_{90} of the particles in the slurry over the course of the extraction.

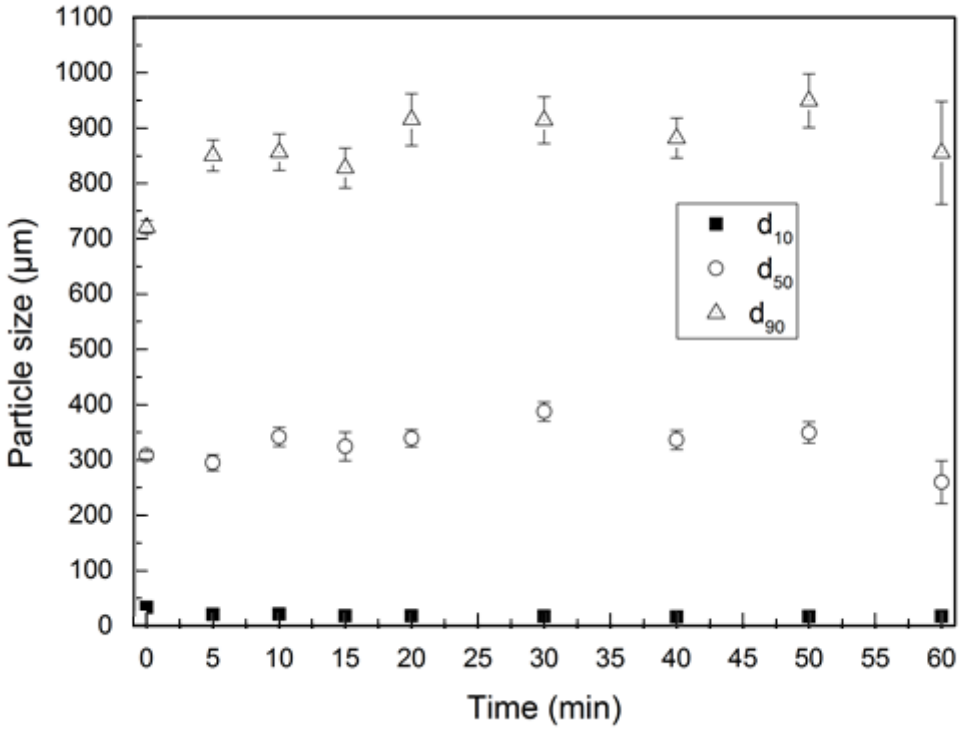

Figure 23: Particle size analysis over the extraction period

Figure 23 shows that the particle sizes remain fairly constant over the extraction period. There seems to be a large amount of variability in the particle size distribution with no specific trend, but instead it stays fairly constant within a region. Figure 24 shows the d_{10} size distribution to determine whether there is an increase in the number of smaller particles which may clog the filter and retard the filtration rate.

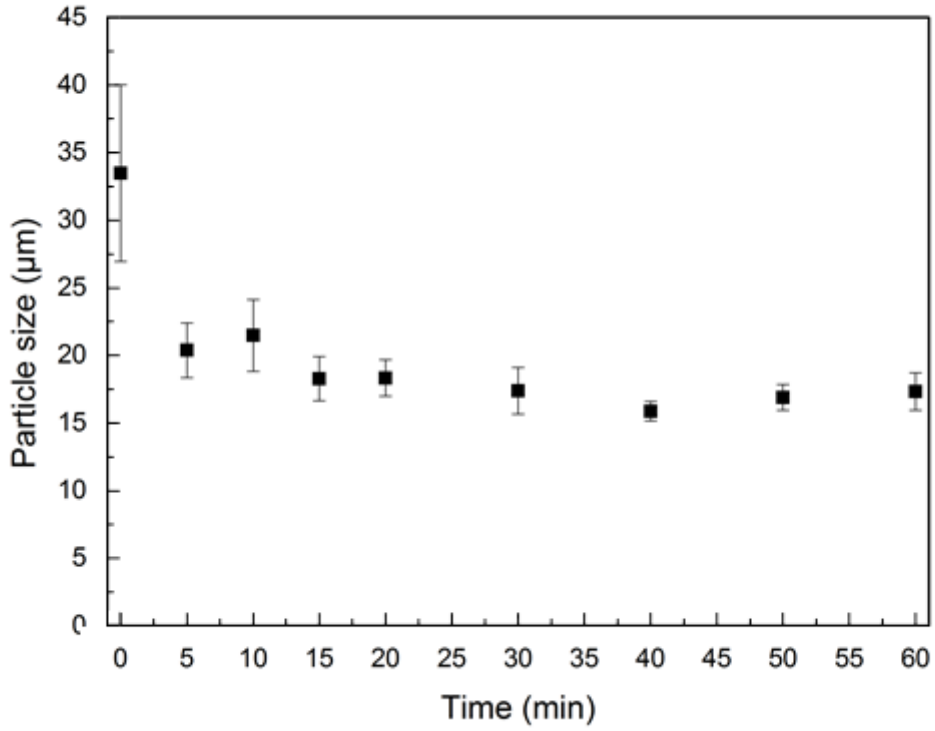

Figure 24: d_{10} particle size distribution over the course of extraction

From Figure 24 it can be seen that there is a decrease after the first sample. This is most likely due to dust and other fine particulates on the flour which have been bought into solution while the slurry was being mixed. Subsequent samples are constant and there is no breakdown of particles.

From Figure 23 and Figure 24 one can conclude that the particles do not break down over the course of extraction or swell by absorbing solvent. However, as the particle size analysis is done by agitating the particles it cannot be concluded that there is no agglomeration.

7.5 Visual Inspection of Slurry

Figure 25 shows 6 photos taken at various intervals at 50x magnification. The photos are representative of the slurry.

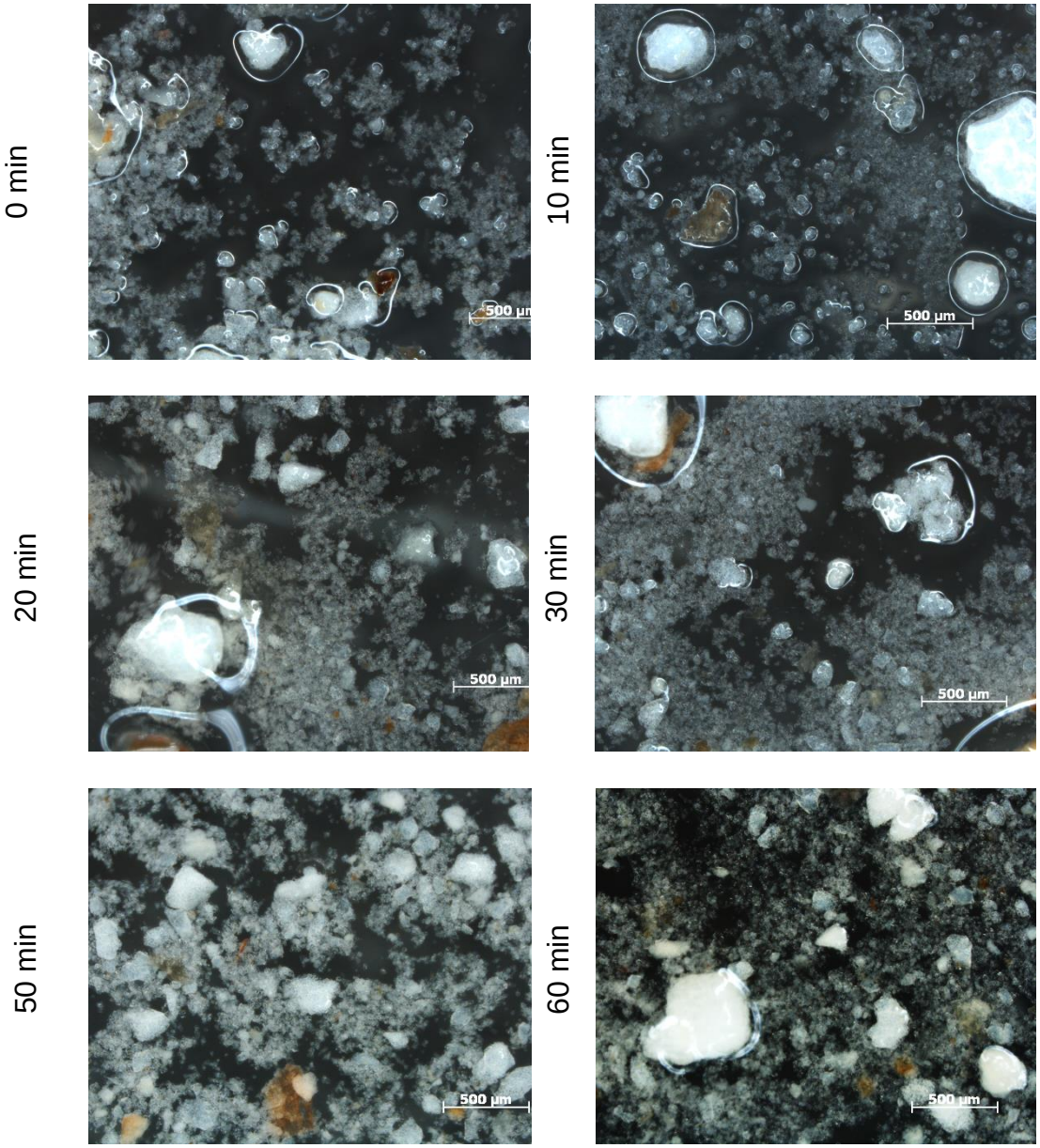

Figure 25: Visual inspection of the grain solvent slurry

From the magnified photos one can see that as time progresses the smaller grain particles which are recognisable as endosperm are far more prone to agglomeration. This also supports the first mechanism proposed, because if the particles were more likely to bond to each other through hydrogen bonding one would expect to see an increase in the agglomeration.

We can see this behaviour peaking around 50 min where there are almost no loose starchy segments. This indicates that agglomeration is occurring, which is exactly what one would expect to see if there were an increase in the amount of hydrogen bonding between loose pieces of endosperm.

8 Conclusions and Recommendations

8.1 Part One – Extraction Optimisation

The coarser flour product compared unfavourably to the finer product and for that reason the continued use of fine flour is recommended.

During the testing of alternative solvents and alternative solvent ratios there was no significant improvement. The continued use of ethanol is recommended as it is far less expensive than alternative solvents. It is also recommended to continue using the ratio in the basic extraction procedure. It is recommended that the ethanol procured be as pure as possible to limit transport costs associated with transporting the water component of the ethanol shipping large amounts of solvent. Further study into the recovery of the ethanol is also recommended as this would drive costs down further.

The amount or type of acid does not have a significant effect on the yield. Even with no acidification the loss in yield is not affected, which means that the solubility of kafirin is not greatly affected by the presence of acids. The amount or type of acid does not have a significant effect on the extraction process either with regard to filtration as extraction with all three of the food-grade acids and with no acid performed very similarly. It is recommended that acid be excluded pending further investigation into whether acidification plays any other role in the protein structure, or has any effect on the different types of kafirin which are extracted or on the shelf life of the extracted proteins.

The type of metabisulphite does not affect the extraction; however, the amount of metabisulphite is important. With no sulphite complex the yield is approximately half of what it otherwise was, but adding double the original amount of sulphite did not have an effect which would justify the additional cost. It is recommended that 12 g of sodium metabisulphite per 1 750 g of ethanol be used. The use of sodium metabisulphite is recommended over any alternative metal sulphite complex as there was no significant difference in the performance of potassium metabisulphite. The recommendation to continue using sodium metabisulphite is based on cost as potassium metabisulphite is significantly more expensive.

The rate at which the protein is initially dissolved into the solvent is higher than the rate of any other soluble component. After 10 min the protein preparation purity does

not change significantly and is concluded to be constant. This indicates that the only reason for the yield to decrease is the solvent trapped in the flour.

The yield of the extracted product in terms of pure protein is at a peak sometime between 10 and 20 min. After the 20 min mark there is a substantial drop in the yield due to increased solvent being retained. If one were to consider the trend of solvent retention and the drop in yield, one find that they are very similar in shape and the solvent retained by the flour is what causes the loss of extracted protein.

8.2 Part Two – Filtration and Solvent Retention

The time required to filter as well as the amount of solvent retained in the filter cake increases dramatically after 20 minutes. The contact time between grain and solvent should be kept to 10 min at 70 °C to avoid these occurrences. This would minimise the time lost filtering the slurry while maximising the amount of solvent recovered as well as maximising the amount of protein extracted. The filtration equipment should be able to filter the slurry as quickly as possible as any filtration time should be included in the determination of contact time.

The viscosity of the slurry increases dramatically over the course of the 70 °C extraction and shows both disentanglement behaviour as well as thickening. The weakening of the hydrogen bonding inside the starchy endosperm is most likely the cause of the increased solvent retention and thickening. This thickening is responsible for the increased time required to filter the slurry.

At 50 °C one sees a much later onset of the thickening which then remains stable, while at 30°C the viscosity increase over the course of the 10 min extraction and after that no change in viscosity. This implies that weakening of the hydrogen bonding is only significant at higher temperatures. The increased propensity of the starchy material to bond with other starchy particles is most likely the cause of the thickening. Further research into the mechanism of thickening is recommended, but this is only expected to provide academic insights and have limited practical application.

From the particle size analysis one can conclude there is no mechanical breakdown or swelling of the particles. The mechanical force of measurement and low concentration will stop the particles from agglomerating, which explains the lack of large particles forming as would be expected if the conclusion about the thickening mechanism is correct.

Visual inspection shows an increase in the number of particles that are sticking together, which indicates that the weakened internal hydrogen bonding is now free to form external hydrogen bonds and stick together. Without mechanical agitation the force between the particles is enough to cause them to stick together, which is similar to what would be experienced on the surface of the filter medium. Thus it is possible to conclude that this agglomeration behaviour is at least partially responsible for the increased solvent retention and filtration time.

From the visual inspection and particle size data one can conclude that formation of a gel by the protein-tannin complexes is unlikely to be the main mechanism for the thickening as this would not explain the agglomeration. This theory however cannot be completely dismissed.

9 General discussion

The process used by Taylor *et al.* (2005b) was the most successful kafirin extraction found and achieved protein yield of roughly 75 % using "… for kafirin extraction were 70% (w/w) ethanol containing 0.5% (w/w) sodium metabisulphite and 0.35% (w/w) NaOH at 70°C" and "…clean, whole grain milled using a laboratory hammer mill (Falling Number, Huddinge, Sweden) fitted with an 800-µm opening screen". The four was extracted for 1 hr and centrifuged at 1000 x g. Another method discussed by them, includes a pre-soak in reducing agents for 16 hr before extraction. These process worked well on the laboratory scale however is not feasible for the commercial applications on which the work in the document was aimed.

Using off-the-shelf flour, minimal time and reagents a comparable amount of protein was extracted in 16 % of the grain/solvent contact time as the Taylor. With ordinary vacuum filtration instead on centrifugation and limited processing after extraction with regards to pH adjustments and washing. The aim of this work was to find a bench scale process which would be easily adapted to be a commercially viable, large scale process and in that aim, it was successful.

When comparing the results of the this study to that of Xu, Reddy and Yang (2007) the results are for the most part similar, which is expected due to the similarities between zein and kafirin. The only two differences in the behaviour of the processes were that absence of the dip in kafirin yield for longer grain/solvent contact times and the low pH giving an increased yield of zein extraction.

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11 Appendix

11A Complete Protein Results

Sample	Protein (%)	Total weight(g)	Yield (g protein/500 g flour)
0 min 70 °C	77.019	18.07	13.92
0 min 70 °C	76.844	19.00	14.60
0 min 70 °C	76.994	21.06	16.21
2 min 70 °C	78.275	24.07	18.84
2 min70 °C	78.70 6	23.50	18.50
2 min 70 °C	78.819	24.21	19.08
4 min 70 °C	77.381	24.96	19.31
4 min 70 °C	78.031	25.01	19.52
4 min 70 °C	77.425	24.00	18.58
7 min 70 °C	70.525	28.72	20.25
7 min 70 °C	72.106	29.21	21.06
10 min 70 °C	70.844	28.70	20.33
10 min 70 °C	70.956	28.55	20.26
20 min 70 °C	70.544	29.43	20.76
20 min 70 °C	70.837	29.16	20.66
30 min 70 °C	71.70 6	24.64	17.67
30 min 70 °C	70.112	24.80	17.39
40 min 70 °C	72.075	19.59	14.12
40 min 70 °C	71.537	20.01	14.31
50 min 70 °C	71.544	18.67	13.36

Sample	Protein (%)	Total weight(g)	Yield (g protein/500 g flour)
50 min 70 °C	71.481	18.50	13.22
60 min 70 °C	70.831	19.82	14.04
60 min 70 °C	71.094	19.17	13.63
120 min 70 °C	70.056	19.83	13.89
240 min 70 °C	70.811	18.95	13.42
Potassium metabisulphite	70.394	30.14	21.22
Potassium metabisulphite	70.806	29.40	20.82
10 min 60°C	65.706	25.70	16.89
10 min 60°C	68.013	24.89	16.93
8 g Phos 70 °C	66.894	32.96	22.05
8 g Phos 70 °C	65.756	31.81	20.92
8 g Citric 70 °C	70.031	29.49	20.65
8 g Citric 70 °C	70.444	29.82	21.01
0 Na 70 °C	55.046	17.50	9.63
0 Na 70 °C	54.743	18.10	9.91
7 Na 70 °C	72.356	24.10	17.44
7 Na 70 °C	71.431	24.47	17.48
20 Na 70 °C	70.835	29.45	20.86
20 Na 70 °C	70.875	30.19	21.40
0 Acetic 70 °C	69.075	27.86	19.24
0 Acetic 70 °C	69.713	26.84	18.71
15 Acetic 70 °C	67.506	29.14	19.67
15 Acetic 70 °C	70.737	30.12	21.31

11B Complete Filtration and Solvent Retention Data

Sample	Filtration time (h:min:s)	Solvent trapped (g)
0 min 70 °C	00:02:38	238.56
0 min 70 °C	00:02:42	244.97
0 min 70 °C	00:02:41	231.26
2 min 70 °C	00:02:40	240.86
2 min70 °C	00:02:45	235.42
2 min 70 °C	00:02:39	247.69
4 min 70 °C	00:02:44	231.28
4 min 70 °C	00:02:42	237.39
4 min 70 °C	00:02:37	244.89
7 min 70 °C	00:02:46	250.52
7 min 70 °C	00:02:48	248.29
10 min 70 °C	00:04:26	257.93
10 min 70 °C	00:04:34	248.73
20 min 70 °C	00:06:15	310.98
20 min 70 °C	00:06:27	306.19
30 min 70 °C	00:10:42	320.92
30 min 70 °C	00:10:57	324.31
40 min 70 °C	00:30:48	651.21
40 min 70 °C	00:32:27	647.08
50 min 70 °C	01:04:00	1 024.45
50 min 70 °C	01:01:00	1 009.27
60 min 70 °C	01:15:00	1 103.69

Sample	Filtration time (h:min:s)	Solvent trapped (g)
60 min 70 °C	01:24:00	1195.35
120 min 70 °C	01:26:00	1215.00
240 min 70 °C	01:31:00	1302.00
Potassium metabisulphite	00:04:21	241.86
Potassium metabisulphite	00:04:16	248.91
10 min 60°C	00:02:11	231.83
10 min 60°C	00:02:16	223.54
8 g Phos 70 °C	00:04:22	241.07
8 g Phos 70 °C	00:04:24	248.97
8 g Citric 70 °C	00:04:17	243.39
8 g Citric 70 °C	00:04:23	239.79
0 Na 70 °C	00:02:36	241.02
0 Na 70 °C	00:02:24	246.38
7 Na 70 °C	00:03:20	247.13
7 Na 70 °C	00:03:24	242.34
20 Na 70 °C	00:04:24	241.90
20 Na 70 °C	00:04:21	246.64
0 Acetic 70 °C	00:04:28	238.06
0 Acetic 70 °C	00:04:21	245.24
15 Acetic 70 °C	00:04:24	240.33
15 Acetic 70 °C	00:04:23	247.55