Development of 2nd Generation Proteinous Bioplastics

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Abstract

Current environmental and economic concerns surrounding the use of petroleum-based plastics, has led to increased study of renewable natural polymers, such as proteins. Bloodmeal (BM) is a by-product of the meat industry and large volumes is sold as a low-cost fertilizer or animal feed. It contains 90 wt% proteins giving it the potential as a renewable precursor for bioplastic production. The objective of this study was to investigate the use of BM for the production of bioplastics, focusing on the use of chemical additives to facilitate thermoplastic extrusion.

Literature revealed that bioplastics formation from proteins requires denaturation and unfolding using thermal and chemical means, allowing new interactions to form between chains. Thermoplastic extrusion also requires sufficient chain mobilization, enabling flow through the barrel. The proteins physiochemical characteristics, plasticizer content and chemical additives will govern its processing behavior, structural and material properties.

Bloodmeal powder was extruded and injection moulded using water, sodium sulfite, sodium dodecyl sulfate (SDS) and urea as additives. The efficiency of these chemical additives was characterized by:

- Processability. Temperatures between 100 and 125 °C produced a successful material, above this excessive covalent cross-linking occurred which reduced chain mobilization. Sodium sulfite was essential, breaking covalent bonding which allowed chain extension. The plasticizer content also strongly influenced the processability, while water and urea were essential for improved processing.
- Consolidation, water absorption and solubility. It was found that SDS's influence on hydrophobic interactions in combination with sodium sulfites cleavage of covalent cross-links resulted in good consolidation, water absorption and solubility. Increasing sodium sulfite increased water absorption, indicative of cross-link reduction. However, high sodium sulfite at low water concentration resulted in a degraded material. The degraded polymer showed an increase in ordered structures, due to the formation of helical conformations of the short peptide chains.

- Protein conformation. It was found that BM was already highly denatured, with considerable amounts of β-structures. Successful processing with required increased chain mobilization through the reduction of inter- and intra-molecular interactions which led to less ordered structures.
- Mechanical Properties. Water was shown to be critical for processing, enhancing the action of sodium sulfite, SDS and urea. During conditioning, water would evaporate, allowing new intermolecular forces between chains, often resulting in a brittle material. SDS was essential for consolidation, but excessive amounts could restrict formation of new intermolecular forces during conditioning. The highly plasticized proteins resulted in ductile materials after conditioning. Lowering the water, sodium sulfite or urea concentration would result in a brittle material after conditioning.

Successful processability, consolidation, water absorption, solubility, and mechanical properties were achieved using 3 pph_{bm} sodium sulfite, 60 pph_{bm} water, 3 pph_{bm} SDS and 20 pph_{bm} urea. This optimal material resulted in increased unordered structures shown by Fourier transform infra-red spectroscopy. The resulting bioplastic was ductile after conditioning and had a tensile strength of 9.6 MPa and a Young's modulus of 536 MPa, comparable to low-density polyethylene.

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Chapter 1: Introduction

Society relies heavily on fossil fuels for energy and other applications such as plastics, paints, coatings and adhesives. The lack of biodegradable plastics has led to increased waste build-up in landfills and unfortunately even in the ocean. Increasing biodegradability, recycling and burning petroleum-based plastics are options for reducing waste. However, fossil fuel is a finite resource and usage needs to be minimized. These environmental and economic concerns have encouraged the study of sustainable and biodegradable biopolymers.

Leading companies, such as Mazda, have vowed to produce sustainable products using biomaterials [1], creating a strong commercial influence on the direction of research. Biopolymers used to manufacture plastics are often derived from plants or animal sources, e.g. polylactic acid from the fermentation of corn starch. One apprehension regarding bioplastics is the use of potential food resources for non-food applications. In recent years, interest has shifted to 2nd Generation Bioplastics that utilize non-food resources as raw materials.

Proteins are natural, complex hetero-polymers offering a variety of functional properties. Although edible, soybean and wheat gluten proteins are currently most popular, and are commonly processed using casting and compression moulding. Thermo-mechanical extrusion and injection moulding are generally used to process synthetic plastics. If protein based bioplastics are to be successfully commercialized, these processes should be equally applicable for processing and product shaping of bioplastics.

Bovine blood is a by-product of the meat industry and is often dried and sold as bloodmeal in large volumes. Bloodmeal is unfit for human consumption and is mostly sold as a low cost fertilizer or animal feed. Proteins account for about 90 wt% of bloodmeal, making it an attractive sustainable resource for bioplastic production. However, previous studies have claimed that blood proteins cannot be extruded due to extensive heat induced cross-linking. Covalent cross-linking is a hindrance during extrusion, preventing the required cohesion of particles.

The objective of this study was to investigate the use of bloodmeal for the production of bioplastics, focusing on the use of chemical additives to facilitate thermoplastic extrusion. More specifically the objectives of this thesis were:

- To develop an understanding of relevant processing variables and their influence on extrusion of protein-based materials.
- To identify appropriate chemical additives and assess their effect on processability and material properties.
- To develop an understanding of structure-property and processing relationships governing bloodmeal based bioplastics.
- Determine optimum process variables and chemical additives for thermoplastic processing of bloodmeal.

This study was limited to a laboratory scale process and it is recognized that some of the process variables and additive levels may require adaption for large scale production.

Chapter 2: Synthetic Polymers and Proteins

2.1 Synthetic Polymers

Polymers are macromolecules built up by the polymerization of large numbers of monomers [2]. There are many types of polymers, including synthetic and natural polymers, which can be classified according to Figure 1.

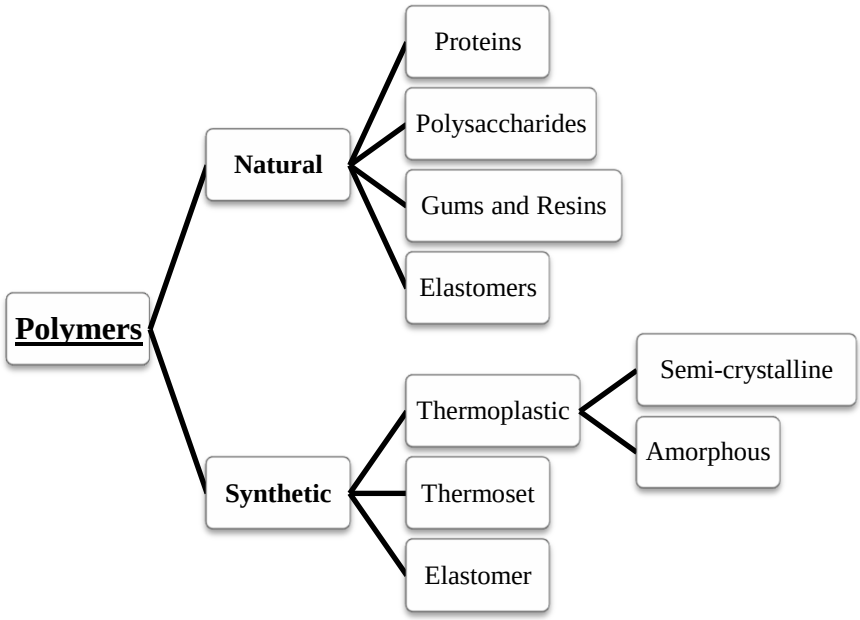
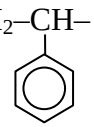
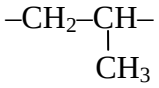


Figure 1: Polymer Classification

The development of the petrochemical industry is probably the greatest single contributing factor to the growth of the plastics industry and these two industries still have a remarkable degree of interdependence [3]. Synthetic polymers are versatile, have a high strength, low density, low price, and are easily processed [4]. Their extreme stability and lack of degradation are the main reasons they became so popular. For example, the packaging and agricultural industry rely on thermoplastic polymers that are resistant to peroxidation, water and microorganisms [5].

Synthetic polymers, are macromolecules manufactured from one or more species of monomers linked together usually by covalent bonds. The repeat unit of a polymer is the basic building block required for polymer preparation [6]. In Table 1 the repeating units of some well known synthetic polymers are listed.

Table 1: Repeat units of some well known polymers.

Polymer	Repeat Unit
Poly(vinyl chloride)	$-CH_2-CHCl-$
Polystyrene	
Polypropylene	
Nylon 66	$-(CH_2)_4CONH(CH_2)_6NHOC-$
Acetal resin	$-CH_2-O-$

There are two main commercial techniques used for the production of synthetic polymers: chain polymerization and step polymerization (condensation). For polymerization to occur the monomer must capable of bonding two (or more) other molecules of monomer by chemical reaction. Thousands or more monomer molecules linked together forms a polymer molecule, which gives it its unique properties. For example, polyethylene is a long chain consisting of repeating ethylene units (Figure 2).

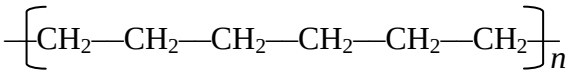


Figure 2: Polyethylene

2.1.1 Structure

The structure formed by polymers depends on their backbone arrangement. Synthetic polymers are often produced from one monomer, called a homo-polymer (e.g. polyethylene), while others may have more than one monomer, called co-polymers (e.g. poly(ethylene terephalate)). The structural complexity rises with increasing amount of monomers and functional groups.

Monomers with only 2 functional groups will produce linear polymers, while 3 or more functional groups will result in cross-linked structures (Figure 3). The skeletal structure, along with the chemical nature of the monomer, will define the final properties of the polymer, such as crystallinity.

The degree of crystallinity of a polymer can range from completely amorphous to highly crystalline. The molecular chemistry, chain configuration and rate of cooling (during processing) will influence a polymer's ability to crystallize. Chemically complex monomers do not favour crystallisation, such as the bulky benzene ring on polystyrene. Linear polymers, such as polyethylene, have nearly

no restrictions preventing chain alignment and will crystallize easily. Figure 4 illustrates the spherulitic structure of semi-crystalline polymers, such as polyethylene, polypropylene and nylon [7].

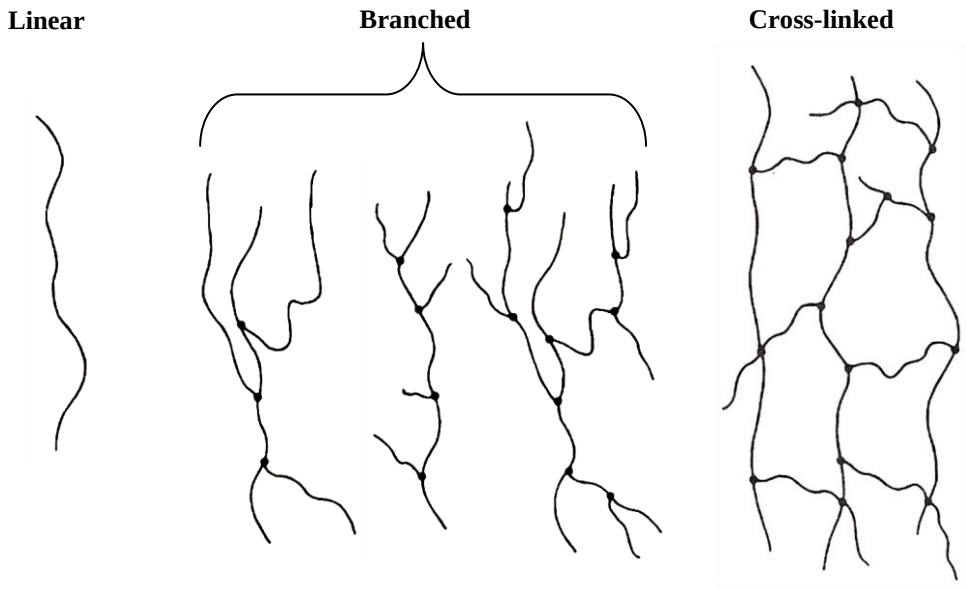


Figure 3: Structure of linear, branched, and cross-linked polymers [7].

Branched polymers have side branches of linked monomer molecules protruding from various central branch points along the main chain [2]. Short and long branches may occur, as well as branching branches (Figure 3). Branching in polymers has an effect on the polymers properties, such as a decrease in crystallinity.

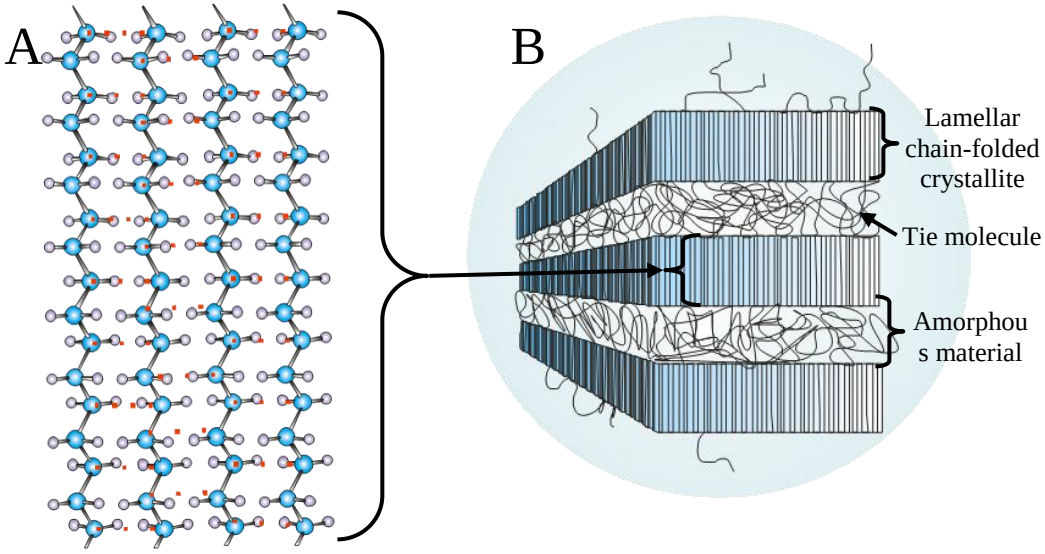


Figure 4: Arrangement of molecular chains for polyethylene. (A) Lamellar chain-folded crystallite. (B) Structure of a spherulite, with amorphous and crystalline areas [7].

When different polymer chains are linked to each other along the main chain, they are said to be cross-linked [2]. Cross-linked polymers may be the result of using

monomers with functionality greater than 2 or by the use of chemical reactions after polymerisation. When the degree of cross-linking is high, a three-dimensional or space-network polymer is produced. With polymers such as, phenol-formaldehyde and urea-formaldehyde, the high degree of cross-linking enforces high rigidity and dimensional stability under conditions of high temperature and stress. Light cross-linking is often used to impart elastic properties onto polymers, such as vulcanised rubbers.

Polymers are divided into thermoplastics, thermosets and elastomers, based on their reaction to mechanical forces with rising temperature. Table 2 describes the thermal properties and skeletal structures of each division.

Table 2: Synthetic polymer classification.

	Thermoplastic	**Thermoset**	**Elastomer**
Description	Once set, can be melted and re-molded.	Upon setting, cannot be melted and shaped again.	Cross-linked polymer, forming a three dimensional structure
Skeletal Structure	Linear or Branched	Cross-linked or Network	Cross-linked
Example	Polyvinyl chloride - insulation for electric wires, piping, signs	Phenol Formaldehyde Resin (Bakelite) – used in electrical insulators	Natural Rubber- vulcanized, used as seals, and molded flexible parts.

2.1.2 Processing

Polymer processing is concerned with the mixing and shaping of polymeric materials to form them into useful products such as, soft-drink bottles, rubber tyres, electrical plugs and sockets, paints, bags and photographic film.

Processing usually involves the application of heat and pressure. The method used to form a specific polymer depends on whether the material is thermoplastic or thermoset. If thermoplastic, the softening temperature, atmospheric stability, as well as the geometry and size of the finished product is important when considering processing means [7]. The distinction between processing thermoplastics and thermosets are summarized in Table 3 [8].

Thermoplastic processing involves melting a polymer, flowed by shaping and finally cooling the material in its new form. The heat required for melting can be supplied by radiation, conduction or mechanical work. The most important thermoplastic processing techniques can be categorized as follows: extrusion, post-die processing, thermo-forming and injection molding. The largest volume of thermoplastics is probably processed by means of extrusion.

Table 3: Comparison of thermoplastics and thermoset processing [8].

Thermoplastics	Thermosets
Molten in shaping stage	Lower molecular mass liquid or rubbery polymers at shaping stage
Harden by cooling the melt	Harden by chemical reaction, often cross-linking of chains
Liquid-solid reversible	Liquid goes irreversibly to solid
Scrap recovery possible	Scrap cannot be recovered directly
Ceiling service temperature	Often can withstand high temperatures
Processing of melt usually result in orientated polymer chains	Can be processed with low orientation

Thermosetting materials are irreversibly polymerized during processing in two stages. Firstly the linear polymer is prepared as a liquid, and secondly the linear polymer is cross-linked. The second stage is carried out in a mold having the desired shape. Curing may occur during heating and/or by the addition of a catalyst and often under pressure, forming a cross-linked or a network structure [7]. Thermoset processing is generally confined to casting and simple molding techniques, such as reaction injection molding (RIM), compression molding, and pultrusion.

Molding is the most common method for forming plastic polymers. Several techniques are used such as, casting, compression, extrusion, and injection moulding.

I Casting

Some low molecular thermoplastics and most thermoset polymers can be cast. Molten plastic material is poured into a mould and allowed to set. Thermosets harden through the polymerization process or curing at elevated temperatures, whereas thermoplastics solidify upon cooling from the molten state.

II Compression Moulding

During compression molding, the mixed polymer and required additives are placed between heated male and female mold members. Heat and pressure are applied and the material becomes viscous, conforming to the mould's shape (Figure 5). Thermosetting materials take less time to process in this way, because once formed they can be removed, where as thermoplastics need to be cooled under pressure.

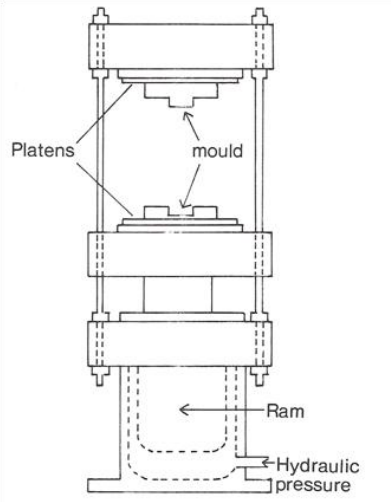


Figure 5: Compression moulding press [8].

III Extrusion

Extrusion is used to melt and pump thermoplastics through the shaping device called a die [8]. A mechanical screw pumps the palletized material, which becomes compacted, melted and formed into a continuous charge of viscous fluid. This technique can be used to produce continuous lengths of tubes, sheets and hose channels [7]. The final shape of the extrudate may be further modified by stretching before final cooling and solidification.

The two most common types of extruders are single and twin-screw extruders. The single screw extruder utilizes the frictional forces between the screw and barrel to force the material towards the die (Figure 6). The twin-screw extruder uses intermeshing screws that compound the material, and acts as a positive displacement pump (low friction), providing better mixing conditions.

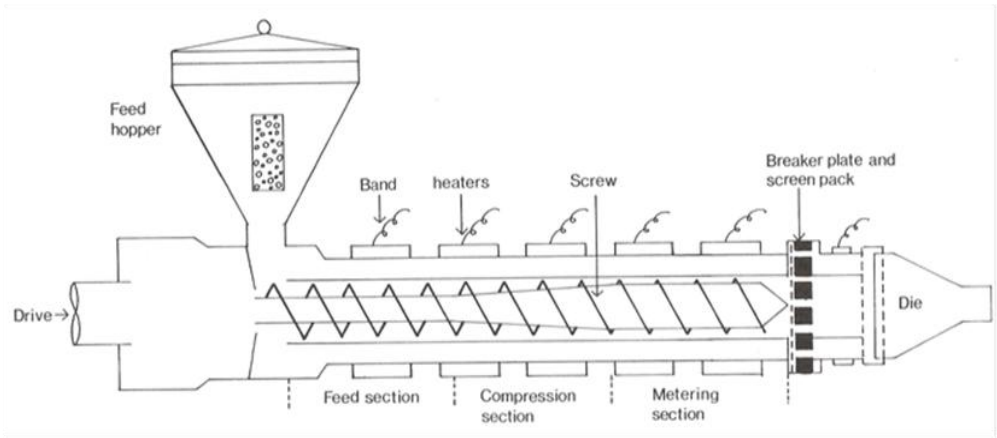


Figure 6: Main features of a single screw extruder [8].

Three zones can be identified within the extruder: the feed zone, compression zone and the metering zone (Figure 6). The feed zone is where granular material is introduced to the barrel and pre-warmed. The compression zone involves melting of the material and removal of air between the original grains. Finally the metering zone is the region where the highest temperatures and pressures are applied, in order to force the material out of the shaped die.

IV Injection Moulding

During injection molding molten polymer is forced under high pressure into a closed mold of the required shape. It is the most widely used technique for fabricating thermoplastic materials [7]. Thermosetting polymers may also be injection molded, where curing takes place while the material is under pressure in a heated mold, often termed reaction injection molding.

2.2 Proteins

Proteins are natural polymers which contribute to biological functions within a cell, along with other biological macromolecules, polysaccharides and nucleic acids. Proteins (e.g. wool and silk) are classified as condensation polymers, because their synthesis involves elimination of water to produce a polypeptide. Nearly all biological processes involve the specialized functions of one or more protein molecule.

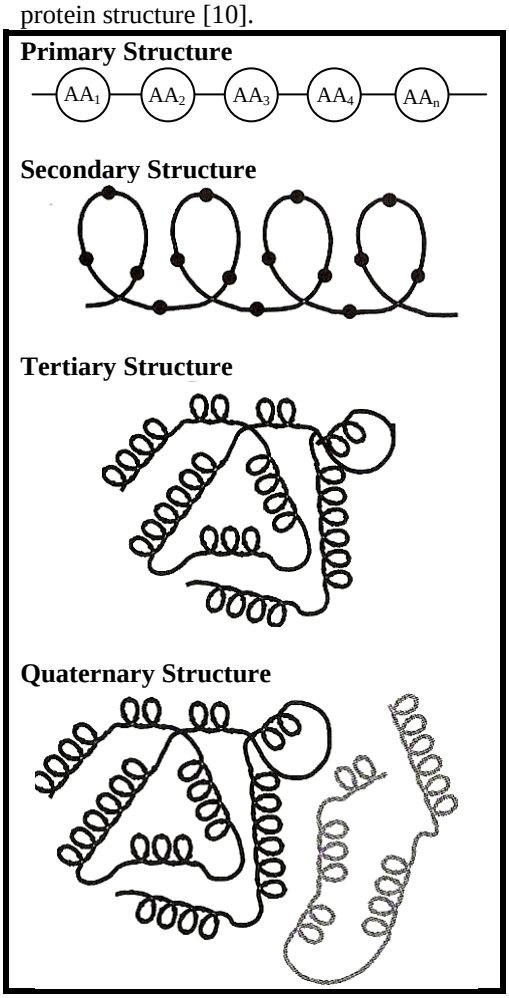
As well as being important for biological systems, proteins are also used in medicine, food and materials industries. They are fundamental and integral food components, both nutritionally (source of energy and amino acids) and functionally (physiochemical and sensory). Some examples are plant proteins, such as wheat and corn gluten meal, soy proteins, pea proteins, potato proteins, and animal proteins such as casein, whey, collagen and keratin [9].

2.2.1 Structure of Proteins

Unlike synthetic polymers, proteins are complex hetero-polymers, consisting of up to 20 amino acids joined by a peptide bond (condensation reaction), forming a polypeptide chain. The polypeptide will fold into a secondary, tertiary and quaternary structure, known as the native conformation (Table 4).

The folded conformation is a delicate balance of many interactions, meaning proteins are only marginally stable at best. A small change in environmental conditions such as, increasing temperature, pressure, change of pH or chemicals, can disrupt the folded state and is called denaturing.

Table 4: Illustration of the four levels of protein structure [10].



I Primary Structure

The primary structure is the sequence of amino acid residues in the protein. A vast number of primary structures can be constructed from the common 20 amino acids, which are classified by their side chains into polar, non-polar, acidic and basic groups. For a short polypeptide containing 25 amino acids, 20^{25} sequence combinations are possible [10]. Interactions with solvent molecules (usually water), pH and ionic content of the solvent and possible interactions between amino acids within the primary structure dictate the final folded conformation of a protein molecule.

II Secondary Structure

The amino acid sequence influences the secondary structure of a protein. Secondary structure refers to localized coiling and bending of the polypeptide chain. This coiling and bending is directed by localized hydrogen bonding between a carbonyl group of one amino acid and an amine of another. The most thermodynamically stable structures are the α-helix and the β-structures [11]. Other elements of secondary structure include β-turns and unordered structure [12].

III Tertiary and Quaternary Structure

The stable three dimensional structure of a protein is the state of minimum thermodynamic free energy, known as the native conformation of a protein.

Based on structure and solubility, proteins can be placed into three categories: fibrous proteins, globular proteins and membrane proteins.

The final structure is determined by intermolecular interactions, such as hydrophobic interactions, ionic interactions, hydrogen bonding and in some cases even stable covalent disulfide linkages between cysteine residues:

- Hydrophobic interactions (repulsion of hydrophobic groups) occur between non-polar amino acids, such as leucine, phenylalanine, tryptophan, and valine. The purpose of these interactions is to minimize interactions of the non-polar amino acids with water.
- Hydrogen bonding between NH and C=O moieties of functional groups contribute significantly to the stability of the secondary and tertiary structures. Hydrogen bonds can occur between polar amino acids, such as serine and tyrosine, and other amino acids.
- Ionic interactions can occur between positively charged side chains (lysine, arginine, and histidine) and negatively charged side chains (aspartic acid and glutamic acid).
- Covalent disulfide linkages between cysteine residues are often essential to maintain tertiary structures, by restraining the overall conformation of the polypeptide. These bonds are only broken at high temperatures, acidic pH or in the presence of reductants.

2.2.2 Comparison between Proteins and Synthetic Polymers

From the 1930's through to the 1940's the use of proteins in material applications increased significantly. With the discovery of cheaper and more versatile petrochemical-based polymers, the use of such protein polymers was overshadowed. However, increased environmental awareness has renewed interest in sustainable and biodegradable polymers, such as proteins. This has been stimulated further by the depletion of fossil fuels and its increasing cost.

Proteins from vegetables (corn, wheat gluten and soy proteins) and animals (milk proteins, collagen, and gelatin) have previously been used to manufacture bioplastics [13; 14]. Some of these proteins are readily available at low cost and are often produced as industrial waste or by-products. Examples of these include soybean meal from soybean oil production [15], and wheat-gluten from starch fabrication [16].

Proteins are natural polymers, but, are structurally much more complex, compared to synthetic polymers. The folded conformation is a delicate balance of interactions such as, covalent bonding, hydrophobic bonding, hydrogen bonding, and ionic bonding [12]. For a protein to behave like a synthetic polymer, the protein chain is required in an extended conformation enabling the formation of a three-dimensional network. In order to do this, multiple non-covalent and covalent interactions need to be reduced, allowing chains to unfold and form new interactions and entanglements. The unfolded protein offers unordered and ordered structures similar to that of semi-crystalline commodity plastics (Figure 7).

Thermal and/or chemical approaches, such as film casting, compression molding, extrusion and injection molding have been studied to produce protein-based plastics [13; 17-21]. Plastics from proteins are often brittle and water sensitive, therefore requiring much research to fully understand how to manipulate final material properties. Physiochemical properties and processing conditions are often governed by the protein's structural properties, and therefore its final material properties [13].

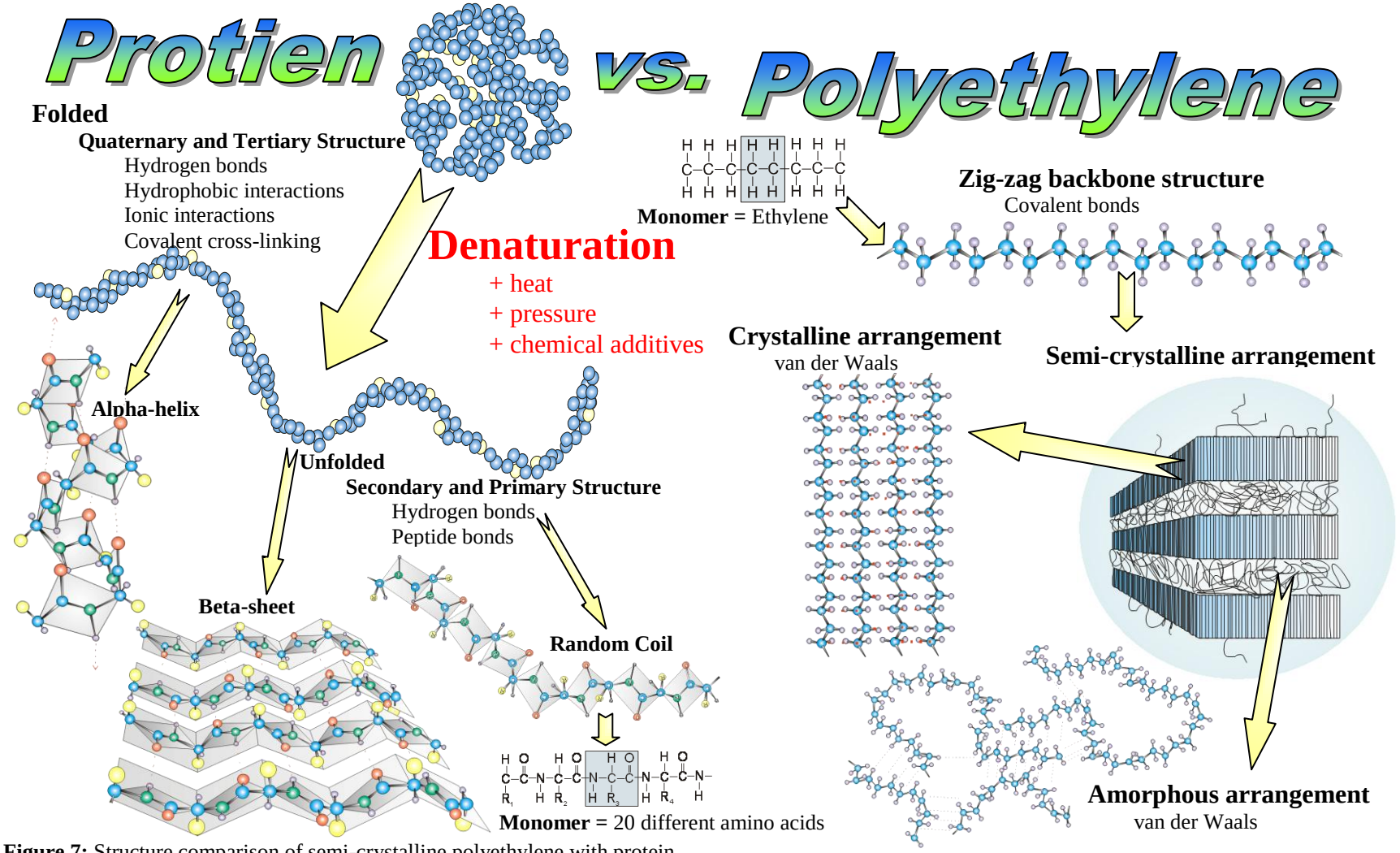


Figure 7: Structure comparison of semi-crystalline polyethylene with protein.

2.3 Bloodmeal as a Sustainable Protein Source

The agricultural industries in New Zealand produce various high quality products that are packaged locally and then exported. Plastic resins manufactured abroad are imported in granular form, and processed into various forms. The majority of shaped plastic is exported as packaging for dairy, meat and horticultural products. Exporting agricultural products is extremely important for the New Zealand economy and relies heavily on the use of plastics.

These plastics are used in great volumes and need to be disposed after use. Synthetic plastics are often not biodegradable, resulting in overfilling of landfills or the production of toxic fumes when burned. The use of sustainable and biodegradable plastics is therefore required in order to mitigate the environmental problem created by plastic packaging.

Recently, there has been growing interest in using food crops for the production of biofuels and other bio-derived products such as, corn fermentation to produce ethanol. These products have been considered to be more environmentally friendly, however, with the world's population growing, using potential food crops for energy or plastic production has been widely questioned [22]. As a result of the value added use of crops, grain price increases will be carried by consumer goods such as, dairy, meat and eggs. For economic and environmental reasons, the use of dedicated crops for energy and other non-food products in the future will be unsustainable [22].As a result, Second Generation Bioplastics manufactured from non-food sources is receiving increasing attention.

The meat industry in New Zealand is the second largest exporter, accounting for 26% of the export value for the year ending May 2008 [23]. For each live animal, approximately 60% are processed into meat products [24]. The other 40% consists of tallow, hide, blood and other inedible raw materials which are rendered into marketable products.

In New Zealand, approximately 80000 tonnes of raw blood is collected annually. Approximately 3-5 wt% of a live animal is blood, containing 80% water and 18% protein [24]. For economic and environmental reasons, blood is converted into bloodmeal and typically sold as a fertilizer. Alternatively, blood can be

fractionated into red blood cells and plasma which can be further processed into high value products [25]. Producing these high value products often require hygienic collection and can be used in food and biotechnological industries [25].

I Bloodmeal Composition

Blood is composed of cellular material and a liquid fraction, called plasma. Plasma accounts for 60-67 wt% of raw blood, consisting of 91% water and 6-8% protein. Plasma contains three types of proteins, namely: fibrinogen (23%), globulins (27%) and serum albumin (50%) (Table 5). The cellular fraction is composed of red and white blood cells, as well as platelets. Red blood cells account for 99% of the cellular fraction of which 97 wt% of the dry weight is hemoglobin.

Table 5: Protein composition of blood.

	% of total proteins	Description	α-helix content
Hemoglobin	~75 %	Not water soluble	75%
Serum Albumin	~12.5 %	Water soluble	55%
Globulins	~6.75 %	Salt soluble	3%
Fibrinogen	~5.75 %	Salt soluble	n/a

II Amino Acid Content of Bloodmeal

Knowing the amino acid content of bloodmeal may assist in understanding possible protein interactions, as pointed out earlier. In Table 6, the amino acid content of bloodmeal is listed.

Table 6: Amino acid content of bloodmeal [26].

Non-polar	
Valine	7.08%
Leucine	11.42%
Isoleucine	3.19%
Phenylalanine	6.20%
Methionine	1.10%
Tryptophan	1.22%
Alanine	7.69%
Proline	4.62%
Glycine	4.46%
Cysteine	1.24%
Polar	
Serine	4.08%
Threonine	3.49%
Tyrosine	2.34%
Acidic Residues	
Glutamic acid	8.79%
Aspartic acid	7.17%
Basic Residues	
Lysine	7.85%
Arginine	4.18%
Histidine	6.53%

Cysteine and lysine are the most reactive amino acids, forming covalent cross-links within a protein during heating [27; 28]. Bloodmeal is rich in the amino acid lysine [29] and has a reasonably high cysteine content. The lysine availability is reduced by the formation of non-disulfide covalent cross-linkages, such as lysinoalanine [30; 31] during drying [32; 33]. Covalent cross-link formation during heating decreases solubility of bloodmeal and stabilizes the denatured structure.

About half of the amino acid content of bloodmeal is non-polar. Of these, the most hydrophobic amino acids are, valine, leucine, isoleucine, phenylanlanine and methionine, equating to 29% of the total amino

acid content. Therefore one can expect bloodmeal to have very low solubility, in addition to the extensive heat induced cross-linking mentioned earlier.

III Bloodmeal Production

In New Zealand, blood from animal slaughtering is collected, stored, coagulated, and dried into an insoluble powder, with at least 85 wt% proteins and less than 10% moisture. Coagulation is required to avoid protein losses during dewatering. Approximately 92% of the solids can be coagulated by injecting steam at 90 °C. Aged blood is used to achieve a high degree of coagulation at lower temperatures, since fresh blood usually requires higher coagulation temperatures. Adding 1% calcium chloride and stirring continuously for two hours before processing can also improve coagulation [25]. During storage, aged blood may develop foul odors, which can be avoided by using preservatives.

Several methods are used for drying such as, batch dryers, rotary gas-fired dryers and ring dryers. During drying the proteins are subjected to temperatures over 100 °C for long periods of time in order to remove excess water and destroy any pathogenic organisms. The effect of high temperature processing leads to covalent cross-linking, producing a water-insoluble product. Figure 8 shows the insoluble bloodmeal powder produced by the drying method explained above.

Figure 8: Bloodmeal powder.

Chapter 3: A Review on Extrusion of Protein-based Plastics

Summary

Increasing interest in competitive, sustainable and biodegradable alternatives to petroleum has encouraged the development and study of protein-based plastics. Proteins are complex polymers, offering a number of different functional side groups that induce folding through a variety of strong interactions.

The formation of a homogenous protein melt during extrusion occurs through the following steps: denaturation, dissociation, unraveling and alignment of polymer chains. The presence of covalent cross-links is unfavorable before or during processing, decreasing chain mobility, increasing viscosity and preventing material homogenization.

Proteins have high softening temperatures, often above their decomposition temperatures. To avoid degradation, the required chain mobility is achieved by using compatible, low molecular mass and low volatility plasticizers. By understanding a protein's physiochemical nature, appropriate additives can be selected that, in combination with controlling temperature and the specific mechanical energy input during extrusion, would lead to a bioplastic with good processability. The final structural and functional properties are highly dependent on the protein and processing conditions, therefore requiring proper control to ensure adequate mechanical properties.

3.1 Introduction

The development of petroleum based plastics in the early 1900's was based on mimicking the structural and functional properties of natural polymers. In the past 20 years, natural polymers such as, starch, cellulose and proteins have regained attention due to economic and environmental concerns surrounding synthetic plastics. Interest in these materials is mainly because of their sustainable supply and biodegradability.

Proteins are readily available as by-products or wastes of the agricultural and horticultural industries. As a result, proteins from plants (wheat gluten, soy, sunflower and corn) and animals (gelatin, keratin, casein and whey) have been manufactured into plastics [13; 17-19; 34-36]. Many studies have been carried out using casting and compression moulding techniques. However, commercial viability of protein-based plastics is hinging on utilizing common synthetic processing techniques such as extrusion and injection moulding.

During extrusion, a considerable amount of mechanical energy is added to the material, which may affect final product properties. Proteins have a large amount of different functional groups, resulting in an array of possible chain interactions, which have to be overcome in order to have a processable material. This, combined with the fact that proteins are heat sensitive, leaves a very small window of feasible processing conditions.

The objective of this section is to review available literature on thermoplastic extrusion of protein-based plastics, identifying critical factors that influence processing and the final properties of the bioplastics.

3.2 Proteins

Proteins and petroleum-based polymers share some vital characteristics. A synthetic polymer consists of identical monomers, covalently bonded in a long chain. Proteins are also composed of repeating units, but may contain up to 20 different amino acid monomers forming a polypeptide chain. The amino acid repeat unit contains two carbon atoms as well as nitrogen, differing only in their functional side groups (Figure 9). In its natural environment, a protein will be folded into secondary, tertiary and quaternary structures stabilized through hydrophobic interactions, hydrogen bonding and electrostatic interactions between

amino acid functional groups. Once folded, the structure maybe stabilized further with strong covalent cross-links.

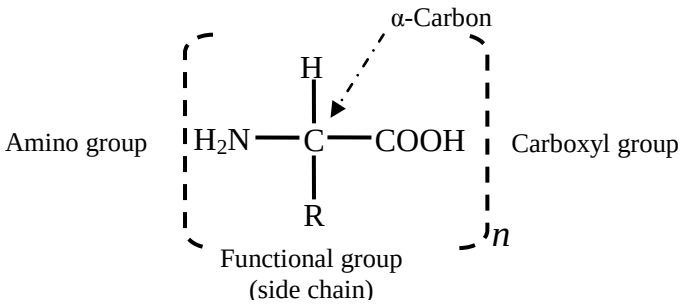


Figure 9: General structure of an amino acid [10].

The amino acid sequence will define the final properties of the polymer, such as the proportion of various secondary structures, hydrophobicity, cross-link density, and rigidity. The large number of possible amino acid sequences adds to the complex nature of these heteropolymers. Table 7 shows the amino acid contents of proteins that have been used to produce bioplastics. Each protein source has different amino acid content and will react to plastic processing conditions differently. For example, gelatin contains a large proportion proline, which is less prone to intermolecular interactions and will therefore behave more like a synthetic polymer during processing [37].

A protein based material could be defined as a three-dimensional macromolecular network stabilized and strengthened by hydrogen bonds, hydrophobic interactions and disulfide bonds [38]. Globular proteins require denaturation by unfolding and alignment before a new three-dimensional network can be formed and stabilized by new inter- and intra-molecular interactions [13; 20; 21; 39]. Protein-based bioplastics are manufactured using the process of denaturation, induced by thermal or chemical means [21]. The denaturation temperature of proteins depends on the amino acid sequence, the type of chemical additives used and processing method employed [21]. Due to the diverse building blocks of proteins and its unique structure, a large variety of biodegradable materials can be produced offering a wide range of functional properties [40].

Two processes have been developed to produce protein-based materials: wet processing and dry processing [13; 19; 21]. The wet process, or casting, involves dispersing and solublising the protein in large quantities of solvent followed by solvent removal by drying [13]. The drying process involves mixing the proteins

and appropriate additives, under low moisture conditions, followed by thermo-mechanical shaping using compression moulding, extrusion or injection moulding [13].

Table 7: Amino acid contents of some protein sources used for bioplastics.

	Egg White	Whey	Casein	Gelatin	Soy	Corn Gluten Meal	Wheat Gluten
Non-polar							
Valine	7.00%	6.00%	6.60%	2.20%	5.00%	4.60%	4.10%
Leucine	8.50%	9.50%	9.00%	3.30%	8.10%	14.50%	6.80%
Isoleucine	6.00%	6.00%	5.10%	1.50%	4.80%	3.50%	4.00%
Phenylalanine	6.00%	2.30%	5.10%	2.40%	5.20%	4.40%	4.90%
Methionine	3.60%	1.90%	2.70%	0.70%	1.30%	2.20%	1.80%
Tryptophan	1.40%	2.20%	1.30%		1.30%	0.30%	1.00%
Alanine	6.60%	5.20%	2.90%	8.90%	4.20%	11.50%	2.40%
Proline	3.80%	6.60%	10.70%	24.30%	5.10%	9.60%	13.70%
Cysteine	2.50%	2.20%	0.30%		1.30%	1.70%	2.10%
Glycine	3.60%	2.20%	2.10%	21.40%	4.10%	4.10%	3.10%
Polar							
Serine	7.30%	5.40%	5.60%	3.60%	5.20%	5.50%	5.20%
Threonine	4.40%	6.90%	4.30%	2.10%	3.80%	3.80%	2.50%
Tyrosine	2.70%	2.70%	5.60%	0.50%	3.80%	3.40%	3.80%
Acidic Residues							
Glutamic acid	13.50%	16.80%	21.50%	10.00%	19.00%	20.30%	37.30%
Aspartic acid	8.90%	10.90%	6.60%	6.00%	11.50%	5.50%	2.90%
Basic Residues							
Lysine	6.20%	8.80%	3.80%	4.50%	6.20%	1.40%	1.20%
Arginine	5.60%	2.50%	3.70%	7.80%	7.50%	2.20%	2.40%
Histidine	2.20%	2.00%	3.00%	0.80%	2.60%	1.50%	2.20%
Reference	[41]	[41]	[41]	[42]	[41]	[43]	[44]

In recent years, the majority of research has been focused on casting and thermo-moulding. However, extrusion and injection moulding are widely accepted processing techniques in the plastics industry and would favor the acceptance of protein-based bioplastics, if it can be used for bioplastic processing.

3.3 Structure-Processing Relationships

Effective and efficient extrusion technology has been widely used to process polymers. An extruder consists of a heated, fixed metal barrel that contains either one or two screws which convey the raw material from the feed end of the barrel to the die.

The screws act by conveying the material through the heated barrel, inducing shear forces and increasing pressure along the barrel. Process variables include the feed material's composition, screw speed, barrel temperature profile, feed rates and die size and shape. The degree of screw fill, specific mechanical energy

input (SME), torque, pressure at the die, residence time and product temperature are influenced by these process variables.

Both single- and twin- screw extruders have been used successfully in producing protein-based materials. However, twin screw extruders convey the material through positive displacement rather than friction forces, offering more efficient mixing and conveying which is often a requirement during bioplastic compounding.

Extrusion requires the formation of a protein melt implying processing above the protein's softening point. Proteins contain a vast range of inter-molecular interactions that reduce molecular mobility and increase viscosity, resulting in a high softening temperature, often above the decomposition temperature. To avoid degradation, additives that can alter the softening point are required for successful thermoplastic extrusion of proteins.

3.3.1 Processing Conditions

I Temperature

During extrusion equilibrium is established between protein de-aggregation due to mechanical stress and aggregation from heating [45-47]. In the metering zone, heat is supplied by external heating elements as well as by viscous heat dissipation. Excessive aggregation at high temperatures will result in physical cross-linking, reducing melt flow. The heat supplied during processing is therefore an important parameter for protein extrusion.

The effect of different barrel and die temperatures on the extrusion of feather keratin, glycerol, water and sodium sulfite blends was studied by Barone *et al.*, [48]. Barrel temperature profiles of 100-100-100 °C and 120-120-120 °C, with die temperatures of 120 °C or 140 °C were used [48]. Using lower barrel and die temperatures, resulted in polymer softening just before the die, leading to high viscosities [48]. Higher barrel temperatures had a lower apparent viscosity, and the material softened earlier inside the barrel [48].

Unfortunately, a temperature profile that induces formation of the melt early in the barrel can cause extensive aggregation before exiting the die. Thermoplastic extrusion of proteins requires the majority of reactions to occur just before, or just after exiting the die. The polymer melt is required to withstand the induced strain

upon exiting the die which is hindered by cross-linking leading to a disruption of the extrudate upon exiting.

II Specific Mechanical Energy Input

The specific mechanical energy input (SME) is a measure of the severity of extrusion conditions and is calculated using torque, screw speed and mass flow rate (Equation 1). It has a direct impact on the rheological properties of the melt, the extent of macromolecular transformations and interactions between additives, resulting in polymers that could range from partially soluble to insoluble, expanded, or even degraded [19].

$$SME\ (kJ/kg) = \frac{tourque \times screw\ speed}{mass\ flow\ rate}$$

Equation 1

Redl *et al.,* studied the effect of extrusion processing conditions on glycerol-plasticized wheat gluten [46]. The influence of feed rate, screw speed and barrel temperature was investigated. Depending on the operating conditions, smooth-surfaced extrudates with high swell or completely disrupted extrudates were obtained. The mechanical energy input (SME) and maximum temperature of the product were found to have the largest influence on the final extrudate properties. Disrupted extrudates resulted from excessive cross-linking brought about by high SME and product temperatures. It was shown that low SME input is required for effective extrusion, mainly to prevent excessive cross-linking.

Pommet *et al.*, studied the influence of mixing on the thermoplastic behavior of wheat gluten using extrusion, batch mixing and compression moulding [49]. The insoluble fraction was measured for each product. Increased insolubility was observed in disrupted extrudates, confirming that high SME and high temperatures resulted in excessive cross-linking. It was found that the activation energy for cross-linking during compression moulding and batch mixing were 170 kJ/mol and 33.7 kJ/mol respectively. It can therefore be seen that shear significantly lowers the activation energy for cross-linking and would lead to excessive cross-linking if the processing temperature is not adjusted accordingly. These results were confirmed using gluten/glycerol blends processed in a thermostated counter-rotating batch mixer [47]. They confirmed that increased cross-linking occurred above 60 °C and that the added shear reduced the activation energy of gluten cross-linking [47].

Otaigbe *et al.*, studied the shear deformation and flow behavior of soy protein isolate-corn starch plastics using torque rheometry [50]. At a typical processing temperature of 120 °C, the soy protein plastic showed shear thinning. At high shear rates, temperature and torque increased significantly, attributed to shear-induced heating. The high temperatures are believed to induce thermal degradation and/or cross-linking.

Control of protein-protein interactions that occur during extrusion is required for successful processing. Heating promotes network formations through association of polymer chains thought to be driven by hydrophobic interactions and stabilized by disulfide bonds [46; 51-55]. The formation of dense associations and cross-links inside the extruder barrel causes an increase in viscosity and a reduction in chain mobility. As a result, residence time, torque and pressure in the metering zone will increase and may result in protein degradation. Plasticizers and other chemical additives can successfully be employed to reduce macromolecular associations during extrusion, leading to improved processability and ultimate material properties.

3.3.2 Chemical Additives

The formation of covalent cross-linking during extrusion will inhibit the formation of a thermoplastic material. Instead, extensive cross-linking (>10%) can result in the formation of a thermoset material, which cannot be remolded or reshaped. A thermoset will cause the extruder to fail, rising the torque and pressure above operative maximums.

To produce a thermoplastic material from proteins, cross-linking and non-covalent interactions have to be controlled. Barone *et al.*, extruded poultry feathers (which contain keratin), with a combination of glycerol, water, and sodium sulfite as processing aids [48]. Keratins are characterized by a large amount of cysteine. Cystine-cystine cross-links (disulphide bridges) provide strength and stiffness to keratin in the solid-state. However, they are an impediment to processing in the melt-state [48]. With the use of a reducing agent, such as sodium sulfite, these cross-links can be broken, greatly improving processability. It was shown that the apparent viscosity during extrusion decreased with increasing sodium sulfite up to 3 wt%, after which it increased with the addition of more sodium sulfite. It was hypothesized that the increase in viscosity after 3 wt% was due to increased chain mobility and entanglements after the complete reduction of disulfide bonds. It

was found that protein chain mobility was sufficient above 4 wt% sodium sulfite to allow chains to be orientated into crystalline structures, as revealed by NMR spectroscopy [48].

Orliac *et al*., investigated the rheological behavior of sunflower protein isolate (SFPI) processed with water, glycerol and in some cases sodium sulfite [56]. It was shown that sodium sulfite can be used to reduce the required amount of plasticizer for thermoplastic extrusion. An initial decrease in viscosity was observed followed by an increase upon further addition of sodium sulfite. It was concluded that greater protein unfolding at higher sodium sulfite concentrations, led to a more extended structure after processing [56].

Ralston and Osswald used a screw-driven capillary rheometer to measure the viscosities of soy protein isolates extruded with cornstarch, glycerol, sodium sulfite, de-ionized water and soy oil [57]. In the absence of sodium sulfite, disulfide bonds reduced the effective chain length and led to a more globular protein conformation [57]. Soy protein does not have extensive cross-links, and processing is therefore not hugely influenced by the addition of sodium sulfite. However, it was found that the use of sodium sulfite increased consolidation by enabling chain movement. This will lead to exposure of functional groups capable of forming new interactions.

The viscosity of a polymer melt depends on protein composition. Covalent cross-links are not always the inhibiting factor. Water insoluble and hydrophobic proteins, such as zein, require the addition of surfactants to enable thermoplastic processing. Sodium dodecyl sulfate (SDS) is an amphiphilic molecule, capable of electrostatic and hydrophobic interactions, causing dissociation of protein chains. Many studies of protein-based plastics formed by cast and compression moulding have used SDS for added denaturation and dissociation, thereby improving processing [58-64].

Sessa *et al.,* studied the viscosity of zein during torque rheometry [64]. Known amounts of water, triethylene glycol (TEG) and SDS were added in order to control viscosity. The addition of water resulted in a rapid torque rise after 1 minute. Further addition of TEG and SDS delayed the rise by up to 12 minutes. They postulated that at a certain temperature and shear, zein denatures and forms entanglements and aggregates leading to a rapid increase in torque. It was

concluded that the reduction in intermolecular forces, brought about by the presence of SDS slowed the rate of aggregation induced by processing [64].

Urea can be used to denature a protein molecule, and is extensively used in proteomics [65]. It forms hydrogen bonds with amino acids thereby preventing protein-protein interactions. The majority of bioplastics research using urea has used compression moulding as a processing technique. Mo and Sun investigated the thermal and mechanical properties of plastics molded from urea-modified soy protein isolates [66]. Urea increased the degree of denaturation of soy protein and the Tg decreased with increasing urea concentration, but mechanical properties reached a maximum value at 8 mol/L. At low concentration (X<2 mol/L), urea functioned as a plasticizer, while at high concentrations (X>4 mol/L) it acted as plasticizer, cross-linking agent, and filler.

At high concentrations, urea can form a surface residue after long term storage. Pommet *et al.*, used 30 wt% urea in wheat gluten [45] and Mo and Sun investigated the effect of storage time on urea-modified soy protein, both observing the formation of urea on the surface of the material overtime [67]. This shows that protein-protein interactions are stronger than urea-protein interactions, forcing the urea out of the material overtime [67]. Compared to polyol plasticizers urea is not as flexible and the plasticized materials do not show the same extensibility. Therefore urea is good as a denaturant to increase chain mobility during processing. The use of a stronger plasticizer may further reduce strong protein-protein interactions which could force lessen urea diffusion to the surface.

3.3.3 Visco-elasticity

Denaturation and therefore chain mobility is essential for successful extrusion. Proteins are amorphous polymers and undergo a glass transition (Tg) similar to commodity plastics. The glass transition corresponds to the transformation from a glassy to rubbery material upon heating (Figure 10). Above the Tg, further heating to above the softening point, results in a material with lower viscosity, which can easily be processed. Figure 11 illustrates how plasticization and heating above a material's glass transition and softening point will allow it to become shaped into a marketable product.

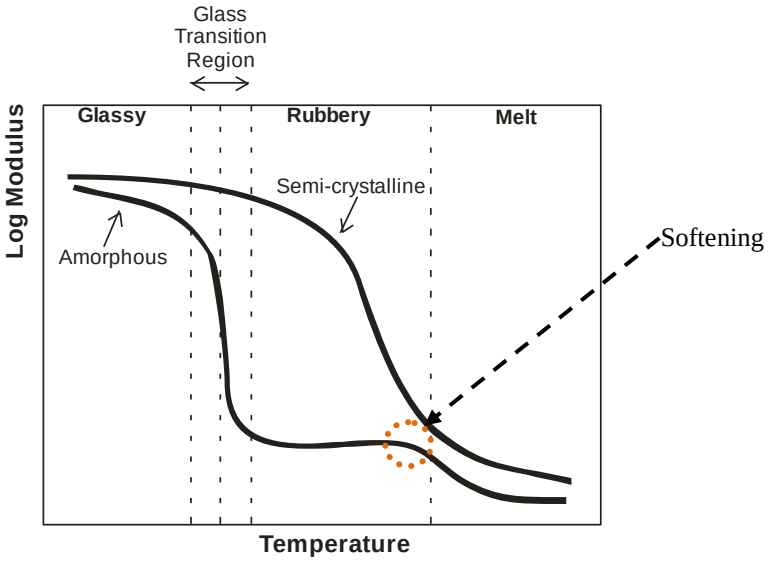


Figure 10: General change in modulus with temperature of amorphous and semi-crystalline polymer materials, showing the different regions of visco-elastic behaviour [7; 68].

The most common techniques used to measure the glass transition temperature of polymers include differential scanning calorimetry (DSC) and dynamic mechanical thermal analysis (DMTA). The glass transition appears as a step in the DSC baseline, resulting from a change in heat capacity (enthalpy). DMTA measures the change in visco-elastic properties of the polymer with changing temperature [68]. The glass transition temperature is interpreted from the storage and loss moduli data obtained from a DMTA run.

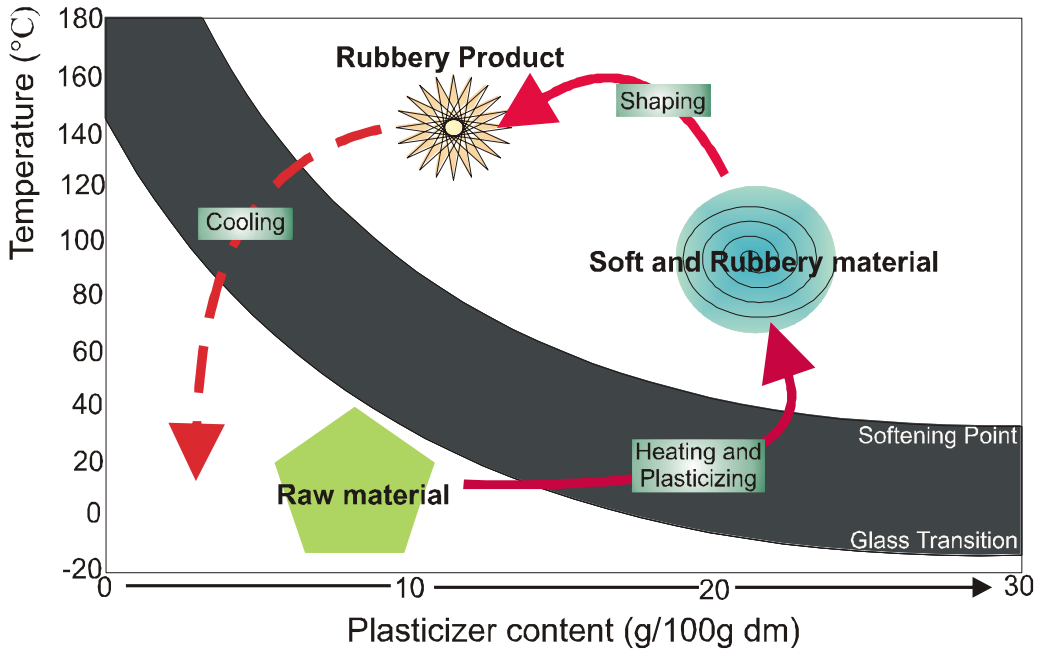


Figure 11: Schematic representation of the thermoplastic process applied to protein-based materials [13].

Processing temperature is a critical parameter for protein extrusion. Mobility of the polymer increase with increase in temperature, but hydrophobic interactions and aggregation which follows denaturation will restrict chain movement [55]. The glass transition temperature of proteins varies based on protein source, thermal history and additives. Increasing the molecular mass, chain stiffness or intermolecular forces will decrease the molecular mobility, therefore increasing the Tg. Most synthetic plastics have a Tg below 100 °C, whereas proteins with less than 5% water show glass transition temperatures close to or above their decomposition temperatures (Table 8) [69].

Table 8: Glass transition temperatures of dry proteins.

Protein Source	Tg (°C)	Reference
Feather Keratin	238	[70]
Wheat Gluten	162	[19; 37; 70; 71]
Soy	172	[37]
CGM	178	[43]

Consequently, the use of plasticizers or other additives to increase chain mobility has become essential to prevent protein degradation and increasing processability [72]. It can be seen from Figure 11 that plasticization reduces both Tg and softening point and when the softening point is below the decomposition temperature, the material should be easily processable.

Plasticizers improve processability by interposing itself between the polymer chains and alter the forces holding the chains together [43]. This occurs through two mechanisms, lubrication and increasing free volume. Small molecules are easily incorporated into the protein matrix, shown by the high plasticizing effect of water and glycerol [43; 68]. Water is considered a natural plasticizer of proteins and is used extensively in protein extrusion. Its small size allows it to easily maneuver through small openings between chains. When plasticizers are compared based on the mass fraction in a bioplastic, low molecular mass compounds such as water, will be present at larger numbers compared to high molecular mass compounds. Every plasticizer molecule can interact with a protein chain, which implies that at equal mass fractions, water is normally more efficient than other plasticizers.

Hydrophilic compounds such as polyols, carbohydrates and amines also interact with polar amino acids. Some examples are glycerol, sorbitol, starch, saccharose, urea, triethylene glycol and polyethylene glycol. Other substances such as,

amphiphilic plasticizers will interact with the hydrophobic amino acids within the protein, examples include fatty acids and phthalates and some surfactants [73].

Plasticizers are added to proteins to reduce their processing temperature, by increasing molecular mobility and decreasing viscosity. Plasticizers act by reducing hydrogen bonding, van der Waals or ionic interactions that hold polymer chains together, through forming plasticizer-polymer interactions. Sufficient plasticization will reduce the SME input, resulting in less disrupted and better quality products [19].

I Polar Compounds as Plasticizers

The effect of moisture content on the glass transition temperature has been extensively studied [21; 37; 43; 51; 71; 74-80]. Water reduces the glass transition temperature and reduces the temperature at which secondary interactions between protein chains form. During extrusion, water acts as a dispersion medium, plasticizer and solvent, influencing melting and viscosity of the extrudate and the deformation of dispersed particles [69]. Water has a low molecular mass and very low Tg (-135 °C), making it a very efficient plasticizer for proteins. In Table 9 the effect of moisture content on the Tg of various protein bioplastics are shown. It can be seen that only a small amount is required to bring about a large reduction in Tg. Water enters the protein network and interacts with protein chains by hydrogen bonding with easily accessible polar amino acid side chains, preventing protein-protein interactions and thereby leading to plasticization.

Table 9: Water effect on glass transition temperature of various protein sources.

	Tg (°C) at % Water							Analysis Technique	Reference
	0%	**5%**	**10%**	**15%**	**20%**	**25%**	**30%**		
CGM	178	100	70	55	45	40	30	MDSC	[43]
Zein	139	70	40	10	<0			DSC	[74]
Casein	210	140	90	70	50	40	25	DMTA, PTA, DSC	[37]
Soya	172	105	80	60	45	35	35		
Wheat Gluten	162	110	65	40	20	18	<18		

Another very common plasticizer for proteins is glycerol. Small and water soluble, it shows a similar plasticizing effect as water, easily penetrating the folded protein's surface to interact with polar amino acids [43]. The most important difference between glycerol and water is glycerol's higher viscosity, which has been shown to induce viscous heat dissipation during mixing of wheat gluten [45]. Hernandez-Izquierdo *et al*., also found that during extrusion of whey

protein, water and glycerol mixtures, higher glycerol contents induced viscous heat dissipation and higher SME requirements [72].

Glycerol and water are effective plasticizers, due to their low molecular mass and ability to interact with polar residues. However, studies have observed plasticizer migration during storage of protein-based plastics [81]. In dry conditions, unbound water evaporates readily over time, reducing its plasticizing effect [56; 82-84]. Low permanence of a plasticizer in the product is very important ensuring consistent properties as to when it was produced.

II Other Plasticizers

General consensus is that hydrophobic interactions govern the associations of protein chains during extrusion. Polar plasticizers, like glycerol and water, are unable to interact with the hydrophobic areas in the polypeptide chain. Hydrophobic areas will only be able to interact with each other, and not with the polar plasticizer, forming densely packed structural domains [69]. Amphiphilic plasticizers have a similar chemical makeup to proteins, containing both polar and non-polar groups.

Proteins are stabilized mainly by hydrogen bonds, but hydrophobic interactions also play an important role. Di Gioia and Guilbert studied the efficiencies of polar and amphiphilic plasticizers on reducing the Tg of corn-gluten-meal (CGM) [43]. The Tg for dry CGM was found to be above 150 °C. The plasticizing efficiency, based on decreasing the Tg, was compared on the basis of volume and molar fraction of plasticizer, theoretical hydrogen bonds supplied by the plasticizer (TH) and percentage hydrophilic groups of the plasticizer (%HG), see Table 10. CGM has approximately 37.9 %HG, distributed in an amphiphilic pattern. It was found that on a molar or TH basis, amphiphilic plasticizers were more efficient than polar plasticizers. It was also shown that Tg scaled linearly with the molecular mass and %HG of the plasticizers tested. With increasing the molecular mass, plasticizer efficiency increased, whereas with increasing %HG plasticizer efficiency decreased, except for dibutyl tartate which has a %HG very close to that of CGM (37.4 compared to 37.9), making it more compatible than the other plasticizers.

Table 10: Physiochemical characteristics of plasticisers [43; 45].

Plasticizer	Molecular mass	Hydrogen Bonds	%HG
Water	18	4	100.0
Sorbitol	182	18	56.0
Lactic acid	90	8	55.6
Glycerol	92	9	55.4
Ethylene glycol	62	6	54.8
Diethylene glycol	106	8	47.2
Propylene glycol	76	6	44.7
Triethylene glycol	150	10	44.0
1,4-butanediol	90	6	37.8
Dibutyl tartate	262	14	37.4
Dibutyl phthalate	278	4	23.5
Octanoic acid	144	5	22.9
Palmitic acid	256	5	12.9

Pommet *et al.*, tested various plasticizers for the production of thermoplastic wheat gluten materials [45]. Preliminary screening of 23 plasticizers at 30 wt% was conducted [45]. Plasticizers with few hydrophilic groups were found to be incompatible with wheat gluten, not forming a consolidated material [45]. The influence of water, glycerol, 1,4-butanediol, lactic and octanoic acid on Tg was measured using DMTA. It was found that on a molar basis, lactic acid had the highest plasticizing efficiency, whereas octanoic acid was the least efficient, mainly because of its hydrophobicity [45]. Lactic acid contains a hydroxyl and carboxyl group, favoring more interactions with different amino acid side chains compared to the other plasticizers. An additional benefit of the acidic conditions was the prevention of excessive aggregation because disulfide bond formation is favored in alkaline conditions [45]. Water, glycerol and 1,4-butanediol had the same plasticizing effect.

Orliac *et al.*, investigated a variety of plasticizers for the production of sunflower-based thermo-moulded films [85]. Polyethylene glycol, polypropylene glycol and tetraethylene glycol did not form homogenous melts with the sunflower protein (Molecular mass above 190). The smaller molecular mass plasticizers, glycerol, propylene glycol, ethylene glycol, diethylene glycol and triethylene glycol produced good homogenous melts with the sunflower protein [85]. Glycerol and triethylene glycol were the only plasticizers that did not migrate out of the sunflower plastics over 3-month aging [85]. Contrary to studies on other proteins, glycerol must have formed sufficient interactions with the sunflower protein reducing the tendency to migrate out of the material [85].

In Table 7, the amino acid content of CGM and wheat gluten is shown. CGM has ~10% more hydrophobic groups and ~10% less hydrogen bonding capacity than

wheat gluten. This affects the types of plasticizers that will be efficient for either protein source. Water and glycerol will always be most efficient when compared to higher molecular mass plasticizers based on weight fraction. However, a plasticizer that has similar TH and %HG and compared to the chosen protein, will have better plasticization efficiency. In light of this, on a molar basis, water may not always be the most efficient plasticizer.

An efficient plasticizer will have a low melting point, low volatility, and good compatibility with the protein. The plasticizing efficiency has been reported to be generally proportional to the molecular mass and inversely proportional to the percentage hydrophilic groups of the plasticizer [86]. A plasticizer that works for one protein may not necessarily be successful in another protein system because of the wide range of amino acid sequences possible (Table 7). For that reason, it is important to carry out preliminary research into compatible plasticizers, and their rheological effect on the protein network.

3.4 Material Properties

In the previous section it was shown that sufficiently plasticized proteins can be extruded above their Tg, using low specific mechanical energy (SME). Denaturation, dissociation, and unraveling, enables the alignment of protein molecules [52]. Upon cooling, new intermolecular interactions are possible due the chain alignment leading to properties different to the original protein.

The final properties of a protein bioplastics are heavily influenced by the protein source and processing conditions [87]. Solubility, water absorption, thermal decomposition and mechanical properties can be related to the structural characteristics of the protein chains in the final product [53].

3.4.1 Solubility

Solubility of protein bioplastics before and after processing is often regarded as a good indicator of cross-link formation during processing. Solubility in water can indicate the type of interactions that formed during extrusion in the following ways:

- Generally, water alone can be used to determine the total insoluble fraction.

- Protein molecules that are loose or unassociated should dissolve in buffer systems.
- Dissolution in SDS solutions could be indicative of hydrophobic or electrostatic interactions between protein chains.
- SDS insoluble fractions can be solubilised with the addition of reducing reagents that cleave cross-links.

Mohammed *et al.*, investigated the changes in solubility of heated protein systems in water as well as in SDS/ reducing reagent solutions [28]. In all cases, solubility was reduced as heating temperature was increased. Disulfide and non-disulfide covalent cross-links were believed to form upon heating, thereby reducing solubility. However, exposure to high temperatures at low moisture contents caused an increase in solubility, attributed to protein degradation [28]. It was found that proteins containing higher amounts of cysteine maintained structural integrity at higher temperatures, attributed to the formation of covalent cross-links (disulfide and lysinoalanine) [28].

Camire investigated the extent of denaturation of corn-protein during extrusion using solubility tests [54]. SDS and a reducing reagent (2-mercaptoenthanol) were used to increase the solubility of the system and characterize the type of interactions that formed during extrusion [54]. The higher solubility (at low temperature, slow screw speed and low moisture content) was attributed to increased protein denaturing [54]. High temperature, screw speed and moisture resulted in a more insoluble extrudate, indicative of cross-linking [54]. However, in light of other research it is likely that the increased solubility was due to degradation, rather than denaturing and should be assessed by other means.

Monitoring molecular size distribution of two solubilised protein fractions is possible by using techniques such as, size-exclusion high-performance liquid chromatography (SE-HPLC). Firstly the SDS soluble fraction is analyzed, and then a reducing agent is added to the SDS insoluble fraction and analyzed. Pommet *et al.*, and Redl *et al.*, [47; 49] used this technique to assess the SDS insoluble fractions of extruded wheat gluten. They found that depolymerisation was dominant in the first stage of mixing through denaturation, and polymerization occurred when the mixing temperature reached 70 °C through covalent cross-linking. Mixing was shown to favor more protein interactions, where SDS insoluble fraction increased with time [47; 49]. The processing

temperatures were controlled efficiently, therefore no protein degradation was observed. At the same temperatures, the mixing process was more efficient at inducing cross-linking when compared to the static compression moulding technique.

3.4.2 Water Absorption

Water absorption is a qualitative test for analyzing the cross-link density of protein bioplastics, which can be related to mechanical properties. However, in some cases water absorption need to be controlled irrespective of cross-link density, depending on the intended use of the bioplastic.

Huang *et al.,* investigated the effect of injection moulding temperature on physical and mechanical properties of soy protein-starch blends [84]. Water absorption decreased and tensile properties increased with increasing injection moulding temperature, up to 130 °C. The decrease in water absorption and increase in mechanical properties was attributed to increased protein interactions after sufficient denaturing [84]. Above 130 °C the water absorption increased slightly, accompanied by a slight decrease in tensile strength and elongation at break, indicative of protein degradation [84].

The water absorption behavior of extruded wheat gluten, using various types plasticizers was investigated by Pommet *et al*., [45]. Using lactic acid as plasticizer caused disintegration after water submersion, due to the reduced aggregation and cross-link density under acidic conditions [45]. Decreased water absorption was observed for materials processed at higher temperatures, resulting from increased cross-linking [45]. However, when using octanoic acid as plasticizer, water absorption was not affected by increased processing temperatures [45]. Water absorption rate was reduced by using a hydrophobic plasticizer, and the degree of cross-linking limited the extent of water absorption [45]. Therefore, the use of hydrophobic plasticizers can be used to reduce the materials water sensitivity.

Brauer *et al.,* modified a variety of plant proteins by binding hydrophobic plasticizers to the protein chains by means of acylation reactions [73]. Palmitic acid chloride and alkenyl-substituted succinic anhydride were reacted with assessable amine and hydroxyl groups on the protein, forming new amide ester bonds. Gluten, zein, pea and soy protein were extruded between 150 and 180 °C

after modification. The modified proteins showed significant reduction in water absorption [73]. Despite the reduction in water absorption, palmitic modified soy protein was the only useful bioplastic produced, but required additional plasticization using glycerol [73]. Typically, hydrophobic plasticizers will not form homogenous protein bioplastics due to their lack of compatibility. Even after chemically linking these with the protein, the plasticizing efficiency was low, although the reduction in water absorption is a useful outcome.

Other additives can also be used to reduce the water absorption of protein bioplastics. It was shown that by adding 20 wt% bio-absorbable polyphosphate fillers to soy protein-corn starch, significantly increased water resistance [50]. It was found that plastics without the polyphosphate filler disintegrated after 24 hours of water submersion, while filled plastics remained intact. The filler also improved flexural strength and stiffness of the plastics.

In another study, soy protein was filled with ZnO_4 to produce chelating cross-links and was compared to covalent cross-links caused by epichlorohydrin and glutaric dialdehyde. It was found that the small concentration of covalent cross-linking agents used was not effective at reducing water absorption and also reduced the processability of the blends. Using, 1.5 wt% ZnO_4 did not affect processability, but did reduce water absorption and was therefore the preferred modification technique [83].

From these studies it can be concluded that water absorption is not only influenced by covalent cross-linking, but also by the proteins ability to interact with water. Exposing hydrophobic groups, either by chain rearrangement or by chemical additives may also influence water absorption. However, in the absence of these additives, water absorption could be considered proportional to cross-link density, for most protein plastics.

3.4.3 Thermal Decomposition

Thermal decomposition of protein bioplastics in air or inert gasses can be used to characterize protein interactions. In a study by Bräuer *et al.*, TGA has was used to assess the thermal decomposition of plasticized plant proteins [73]. The plasticized proteins had a reduced decomposition temperature, attributed to less protein-protein interactions in plasticized proteins. The general consensus is that a TGA curve can be divided into four parts: (1) elimination of water, (2)

plasticizer decomposition (3) weak bond cleavage leading to peptide bond cleavage and (4) stronger bond cleavage, resulting in total degradation (Figure 12) [88-91]. The temperatures at which these steps occur will depend on the protein source, plasticizer content, chemical additives and processing techniques used. The thermal decomposition temperature is shown by the inflection point of the TGA curve or the derivative peak maxima in part (3) as shown in Figure 12.

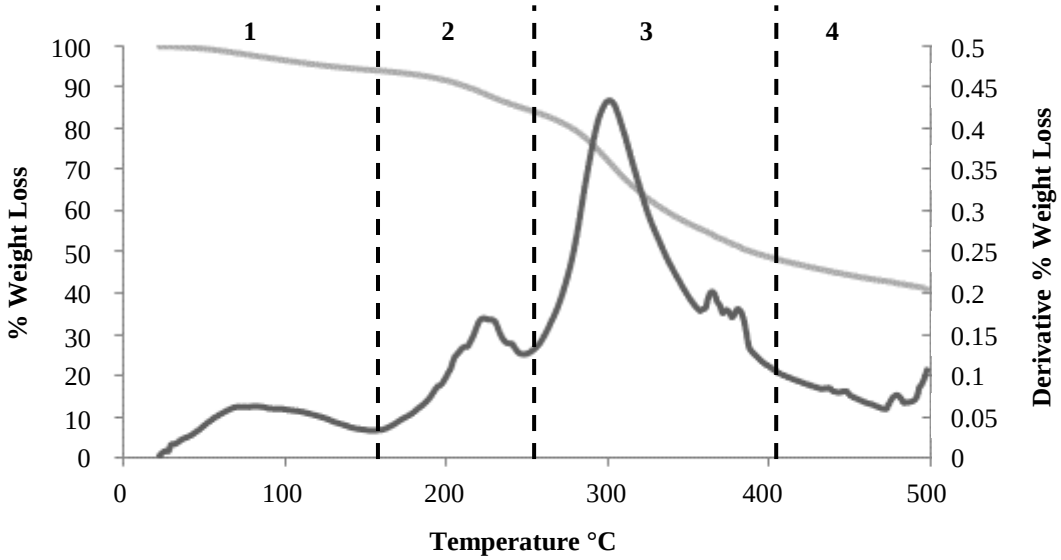


Figure 12: Typical TGA curve of a protein-based plastic.

Leblanc *et al.*, investigated the thermal stability of extruded wheat flour with water, glycerol and other additives [92]. The extruded samples were shown to have a lower thermal stability than non-plasticized bioplastics [92]. Thermal processing and additives will reduce protein-protein interactions, therefore reducing the decomposition temperature.

In general, protein modifiers that reduce intermolecular interactions, such as plasticizers or reducing reagents will reduce the thermal decomposition temperature of bioplastics [38; 63; 81; 93], whereas cross-linking agents and additives that increase intermolecular interactions will increase the thermal decomposition temperature [90; 94].

3.4.4 Protein Conformation

Proteins can form three-dimensional amorphous or semi-crystalline networks through plasticization and processing. Infra-red spectroscopy and X-ray diffraction (XRD) are often used to investigate specific molecular arrangements of protein based materials.

Fourier transform-IR (KBr disc method) and XRD analysis of compression moulded soy protein plastics revealed an amorphous structure, and weakened protein-protein hydrogen bonding with the addition of acetamide as plasticizer [81]. Leblanc *et al.,* found that extruded wheat starch (<12% protein) and wheat flour (high protein) had a reduction in crystallinity, with the starch fraction losing most of its crystallinity after extrusion. The reduced crystallinity resulted in lower thermal stability and lower Young's modulus.

In a study of extruded soy protein, it was found that β-sheet structures formed, which was not visible in the original soy protein isolate spectra [52]. Similar observations were made for extruded ovalalbumin and bovine serum albumin [95]. The β-sheet structure increased at the expense of the α-helix content due to denaturation and aggregation during extrusion.

The structural characteristics of extruded feather keratin, plasticized by glycerol and sodium sulfite was studied using Raman spectroscopy and ^{13}C NMR [48]. It was found that the native β-sheet arrangement of feather keratin was disrupted above 2 wt% sodium sulfite and a more crystalline material developed above 5 wt% sodium sulfite [48]. It was concluded that the reduction of disulfide bonds during extrusion by means of sodium sulfite, increased chain mobility allowing rearrangement, alignment and crystallization.

There is limited literature available on the secondary structure of extruded protein-based plastics. From the literature cited, it can be seen that thermal processing does indeed lead to structural changes of the polypeptide backbone. It would seem that extrusion generally favors the formation of β-sheets and leads to an increase in crystallinity from native protein structures.

3.4.5 Mechanical Properties

The mechanical properties of polymers are largely associated with distribution and concentration of inter- and intra-molecular interactions allowed by the amino acid sequence of the polypeptide chains [13]. Extrusion and other thermal processing techniques led to structural rearrangements and new interactions, which can be adjusted with the use of plasticizers and chemical additives. In general, true plasticizers will increase the flexibility of a moulded product, imparting greater extensibility. On the other hand, increasing molecular interactions will result in a material with higher tensile strength and stiffness.

Furthermore, harsh processing conditions can lead to degradation, adversely effecting mechanical properties. Table 11 summarizes some mechanical properties of various protein bioplastics plasticized with glycerol or water.

Table 11: Mechanical properties of various thermo-mechanical processed proteins.

		Extrusion (max °C)	RPM	Injection moulding (°C)	Plasticizer content	Tensile Strength (MPa) (5mm/min)	YM (MPa)	%Elgn
[84]	Soy Protein Isolate (SPI)-Corn Starch(1:1)	105	100	80	G (0.3:1), W 11.5%	2.9	29	89.5
				90		2.9	28	94.7
				100		2.9	29	94.5
				110		3.1	36	69.4
				120		3.2	34	74.1
				130		3.9	46	85.1
				140		3.7	46	74.5
[56]	SunflowerPI (100), Sodium Sulfite (SS)	120	20-200	50-120	4G, 18W	16.1	2 GPa	0.58
					22G,18W	10.6	0.5 GPa	1.8
[82]	Starch-Zein(4:1)	123	15	150-160	11.5%G, 6.6%W	22-25		5.3
					12.5%G, 8%W	6-20-25		4.7
[83]	SPI Film, 80 water (W) +Glycerol (G)	160	20 25		10	40.6	1226	3
					20	33.9	1119	74
					30	15	374	133
[55]	SunFlowerPI (100parts)	160	20		20 W 70G	3.2	17.7	73
[50]	CornStarch(100) SPI(66) , SS, soybean oil	125		120	G(46.4) W (38.7)	3.09	104.1	44.6
[72]	Whey protein, water, Glycerol	130			45.8(G)	4.1	46.5	127
					48.8	3.5	36.9	121
					51.9	3.1	30.6	132
[64]	Zein, water, Triethylene glycol SDS	90	60	Compress Mould	0 (SDS)	19.5	299.8	11.4
					2	22.4	289.5	11.4
					5	20.2	287.3	11.4
					10	17.3	186.1	12.8

Protein plastics manufactured through extrusion, produced materials with reasonable mechanical properties. Compared to synthetic plastics, they were found to be of similar tensile strength, but generally more brittle. Further research into extrusion of proteins is required to produce bioplastics with less water sensitivity, high tensile strength and good ductility.

3.5 Conclusions

Current literature concerning extrusion of proteins revealed that successful processing is only possible within a small window of operating conditions.

Extrusion uses thermal and mechanical energy to form a polymer melt, requiring sufficient chain mobility. Anhydrous proteins have high softening temperatures

due to their complex nature and the large number of macromolecular interactions. Compatible plasticizers, with a low molecular mass and low volatility are required to induce chain mobility and reduce the softening point.

The processes occurring during extrusion is considered an equilibrium reaction between temperature induced polymerization and shear induced de-polymerization. In this context, polymerization means the formation of intermolecular forces or covalent cross-links while de-polymerization may also imply protein degradation. It was found that high temperatures and high specific mechanical energy (SME) input could induce excessive cross-linking and/or degradation of protein chains. At high temperatures and low moisture or plasticizer content, viscous heat dissipation could increase the likelihood of protein degradation. Optimal processing temperature is highly dependent on internal and external factors such as, the amino-acid sequence and amount and type of plasticizer used.

The formation of a homogenous protein melt during extrusion occurs through the following steps: denaturation, dissociation, unraveling and alignment of polymer chains. The presence of covalent cross-links is unfavorable before or during processing, decreasing chain mobility, increasing viscosity and preventing material homogenization.

Preventing excessive formation of cross-links after extrusion could stabilize protein structure, leading to improved mechanical properties [53]. By understanding a protein's physiochemical nature and thermal history, appropriate additives can be selected that, in combination with controlling temperature and the SME input during extrusion, would lead to a bioplastic with good processability and adequate mechanical properties.

Chapter 4: Product Development

Summary

In contrast to the large number of studies on the production of bioplastics from soy and wheat proteins, little has been done regarding animal waste-proteins, like bloodmeal. The aim of this section was to investigate the use of bloodmeal (BM) as a precursor for thermoplastic biopolymers.

It was found that processing required water and chemical additives, mainly performing three functions. Firstly covalent cross-links have to be reduced by using sodium sulfite (SS), cleaving disulfide bridges. Secondly, inter and intra-molecular forces (denaturing) were reduced by using sodium dodecyl sulfate (SDS) and urea, allowing plasticizing. Lastly formation of new interactions to stabilize the final structure was required by evaporating some of the water (conditioning).

Processability was strongly influenced by plasticizer content, where water and urea performed a similar function in this regard. Although these would be the most important additives, processing would be impossible without sodium sulfite. It was shown that the required water for processing led to over plasticizing, evident from the low mechanical properties. However, it has been found that once water was removed the mechanical properties increased significantly, indicating the formation of new intermolecular forces. It was found that although SDS is required for processing and consolidation, it may restrict the formation of new inter-molecular forces, if used at higher levels.

The most successful material contained, 60 parts water per hundred bloodmeal (pph_{bm}), 1 pph_{bm} sodium sulfite, 1 pph_{bm} sodium dodecyl sulfate (SDS) and 20 pph_{bm} urea. The optimal material showed good consolidation and good processability. A tensile strength of 8 MPa, Young's modulus of 320 MPa and toughness 1.6 MPa.$m^{½}$ were obtained at these additive levels.

4.1 Introduction

Bioplastics have had attention since the early 1900's, but production figures were small. For example, Henry Ford tested soy plastics for automobile parts in the 1930's [96]. However, with the discovery of petroleum based polymers the use of such biopolymers was overshadowed mainly because of a relatively high price compared to petrochemical polymers [20].

Proteins can potentially be converted into bioplastics via two main routes. These are wet processing, which based on solvent casting, or dry processing, such as extrusion or compression moulding [20]. However, for successful integration into common synthetic plastic processing, dry processing is preferred.

During BM processing, proteins are subjected to temperatures over 100 °C for long periods of time to remove water and to destroy any pathogenic organisms. The effect of high temperature processing leads to cross-linking, severely reducing solubility. Previous studies have failed to produce an extrudable material from blood proteins, mainly due to excessive cross-linking [28].

The aim of this section was to investigate the use of (BM) as a precursor for thermoplastic biopolymers. More specifically the objectives were to:

- define thermoplastic processability and the attributes of a successful product
- identify inter and intra molecular interactions that inhibit or promote processability
- identify appropriate additives required to manipulate the identified interactions to promote processability
- indentify suitable process conditions for bioplastic production.

4.2 Experimental

4.2.1 Materials

Table 12: Materials used

	Supplier	**Grade**	
Bloodmeal (BM)	Taranaki Byproducts		ρ = 1300 kg/m^3 Sieved to 700 μm
Sodium dodecyl sulfate	Biolab	Technical	
Sodium sulfite	BDH Lab Supplies	Analytical	
Urea	Agrinutrients-Balance	Agricultural	

4.2.2 Equipment used

Table 13: Equipment used

	Specification
Heated press	Hydraulic press, fitted with heating elements in top and bottom plate.
Extruder	ThermoPrism TSE-16-TC twin-screw
Injection moulder	BOY15-S

4.2.3 Analysis

I Moisture Content

Moisture content was determined for extruded and injection moulded samples. Granulated samples were weighed into aluminum dishes and dried in an air-circulating oven at 100 °C for 12+ hours. The moisture content was determined by subtracting the dry weight from the initial weight. These measurements were done in triplicate for each experiment.

II Consolidation

The morphology of extruded materials was analyzed using an optical microscope and based on the physical appearance the consolidation was qualitatively scored as low, medium or high.

Samples were photographed using a microscope (model M38; Wild, Heerbrugg, Switzerland) fitted with a Nikon Digital Sight DS-SMc digital camera.

III Processability Index

Observations during extrusion and injection moulding were scaled from 0-4 where zero would correspond to a material that is not processable and four corresponding to good processability. Observations were based, in part, on the torque and pressure readings during extrusion as well as other observations such as the surface of the extrudate and whether a consistent and continuous extrudate would form. The two numbers that were obtained for extrusion (0-4) and injection moulding (0-4) were multiplied to obtain a processability index.

IV Water Absorption and Solubility

Extruded samples were granulated, weighed and immersed in distilled water for 24 hours. The surface water was dried using a paper towel and the samples were weighed in an aluminum dish. The samples were then dried at 100 °C for 12+ hours and reweighed. The difference between the initial weight and wet weight was used to calculate water absorption. The difference between the dry mass (calculated using moisture content method) and the dry mass after water immersion was used to calculate the soluble fraction of the bioplastic. The results are presented as an average of three specimens.

V Mechanical Properties

Tensile strength, elongation at break, Young's modulus and fracture toughness of injection-moulded tensile specimens were analyzed according to the ASTM D638-86 method. For each experiment five specimens were conditioned at 23 °C and 50% relative humidity for 7days, equilibrating to 10% moisture content. Five conditioned and five unconditioned injection moulded samples were tested at 5mm/min crosshead speed, using an Instron model 4204.

4.3 Chemical Product Development

Chemical product development was performed according to the steps outlined by Cussler [97] and is discussed further in subsequent sections:

- setting product requirements
- generating ideas
- selection of most promising ideas
- processing

4.3.1 Product Requirements

Extrusion and injection moulding of polymers require that a flowable melt be formed by the polymer upon the addition of heat. That implies that interactions between chains are sufficiently low to allow relative movement of chains, but some interaction is required to impart some degree of melt strength in the material.

Synthetic polymers generally satisfy these requirements and with the addition of heat polymers transform from a glassy state to a rubbery state and eventually to a

rubbery flow state [19]. In the absence of strong intermolecular forces (hydrogen bonds), chain entanglements and van der Waal's forces are the most important mechanisms that impart melt strength. However, upon solidification some polymers crystallize as a result of chain orientation and alignment, stabilized by intermolecular forces [2]. Good examples of these would be semi-crystalline polyethylene and polyamides (nylon). The addition of heat and shear is typically enough to overcome these forces during processing.

Understanding how to process bioplastics, with similar properties to synthetic polymers, requires an understanding of how these forces are manifested in proteins. The processability of proteins also depends on their transition from the glassy, rubbery and free flowing states. These transitions are achieved with judicial application of heat, pressure, shear, chemical additives and plasticizers. Specific amino acid residues (primary protein structure) and initial structure (natural protein state) of the protein will influence each of these factors.

Protein structure is described in terms of four levels of structural order. The primary structure of a protein is determined by the amino acid sequence where each amino acid has specific attributes that influence possible interactions with other amino acids. The secondary and tertiary structures of proteins are therefore determined by the order in which the amino acid residues occur in the primary structure. Folding of protein chains occur predominantly in α-helix and β-sheet conformations stabilized by hydrophobic interactions, ionic interactions, hydrogen bonding and covalent bonds, as illustrated in Figure 13 [11; 98].

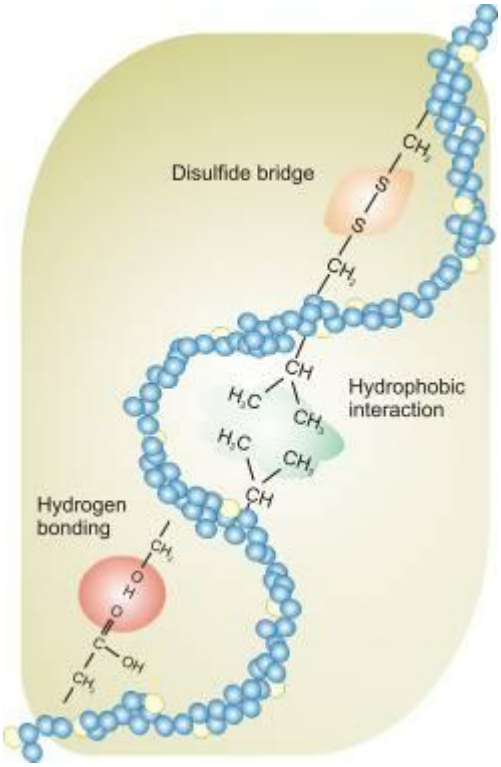


Figure 13: Protein interactions

The processing of plastic materials based on globular proteins requires three main steps:

- breaking of intermolecular bonds (non-covalent and covalent) that stabilize proteins in their native form by using chemical or physical means,

- arranging and orientating mobile chains in the desired shape
- enabling formation of new intermolecular bonds and interactions to stabilize the three-dimensional structure [21].

Formation of covalent cross-links during extrusion will decrease the free flowing state by immobilizing protein chains. Proteins containing small amounts of lysine and cysteine will be less likely to have or form covalent cross-links, and will only require temperature, pressure and a plasticizer to form a plastic [59; 62; 63; 66; 83]. Proteins, such as feather keratin and bloodmeal contain a large amount of reactive amino acids (e.g. cysteine and lysine), therefore requiring a reducing agent to break covalent cross-links [48].

To yield a thermoplastic material, it is therefore required to firstly modify bloodmeal appropriately to enable breaking of predominately disulphide bridges and preventing hydrophobic interactions and hydrogen bonding. Secondly, plasticization is required to enable flow during processing. However, excessive treatment could compromise mechanical properties to the extent that a usable product cannot be formed.

4.3.2 Generating Ideas and Selecting the most Promising Alternatives

The above mentioned processing requirements led to the identification of four inter-relating chemical requirements: a reducing agent to break covalent bonds, additives to denature the protein's structure, additives to prevent chain interactions as well as a plasticizer, many of which may perform more than one action. These are discussed below:

I Water

Water is generally required for processing firstly to facilitate the action of the additives, but also as plasticizer for proteins (decreasing the glass transition temperature). It has previously been shown that water is one of the best plasticizers for proteins.[19]. Proteins generally form brittle materials due to extensive interactions between side chains. Plasticization by water or polyhydroxy compounds is critical for controlling these interactions between protein chains and to form a continuous network from powdered raw materials [13].

Extrusion of proteins is considered a low-moisture process, usually adding less than 30wt% water. It has previously been shown that by increasing the water content from 10 to 30 wt%, led to a reduction of the denaturation temperature as well as the glass transition temperature (Tg) [21]. Water has a low molecular mass with a Tg of -135 °C making it more effective than any other plasticizer [68]. Unfortunately the low molecular mass means that it will evaporate over time, reducing its plasticizing effect [82].

II Sodium Sulfite

Sodium sulfite is a reducing agent which has been used in solution cast processing as well as thermal processing of proteins to break cystine bonds [48]. These disulfide bonds are heat resistant and an impediment to processing by locking protein chains and preventing the formation of a flowable melt.

Disulfide bonds can be reduced by sodium sulfite to form a sodium-sulfonate derivative. This reaction works optimally at alkaline pH, where the disulfide bonds are found to be less stable [27]. In oxidizing situations this reaction can be reversed, if all the available sulfite ions are consumed.

III Urea

Water molecules surround protein chains in their native state and may protect it from denaturation. Urea preferentially binds to the protein surface, disrupting the interaction between proteins and water, resulting in partially unfolded and flexible protein chains [66]. The denatured protein may form entanglements and cross-links during the moulding process, resulting in plastics with a high tensile strength, greater elongation and reduced water absorption [66].

IV Sodium Dodecyl Sulfate (SDS)

SDS is an anionic detergent known to produce considerable conformational changes in proteins at concentrations in the order of 0.02 mol/L. SDS does not cleave disulfide bonds, but prevents hydrophobic and electrostatic interactions between protein chains, leading to an ordered denatured state (not random coil) [61]. SDS has been used in many studies to modify proteins, including SDS-modified soy protein plastics [58-62]. Rhim and others has shown that the addition of SDS increased film extendibility while simultaneously improving moisture barrier properties of the films at the expense of tensile strength [61]. It

was concluded that SDS acted as a plasticizer, which is consistent with other studies [58; 59; 62].

4.3.3 Processing

Various amounts of sodium sulfite, urea and SDS were dissolved in distilled water (60°C) and subsequently mixed with sieved blood meal in a high speed mixer for at least 5 minutes. During this stage, the proteinous mass absorbed all the water and some denaturing occurred. This pre-processed blend could then be thermoformed by either compression moulding or extrusion. Processing and additive quantities are highly interactive and warrant further discussing in the sections that follow.

I Compression Moulding

Compression moulding was selected as an appropriate scouting technique to assess suitable processing conditions and additive concentrations mainly because of the inherent robustness on the process. Compression moulding is a non-continuous method for polymer processing and has shown to be effective in assessing temperature and pressure affects in protein-based materials [18; 49; 58-60; 66; 88; 90; 99-103]. During compression moulding, a visco-elastic melt is formed from the low moisture protein mixture, using a combination of high temperature and pressure. Heat and pressure will cause protein chains to unfold and once cooled allows for intermolecular bonding leading to consolidation. Although heat and pressure are effective denaturants, the addition of chemicals may also be required for improved processing [49; 58; 59; 64; 66; 102].

Temperatures between 95 and 140 °C and pressures between 0 and 9 MPa (applied for 10minutes) were used in combination with varying blends of chemical additives (Table 14). It was found that bloodmeal alone did not melt or form a consolidated material even with the addition of water as a plasticizer. This was attributed to the high degree of cross-linking formed during the production of bloodmeal. Previous authors have tried to process blood proteins, but failed mainly due to the same reason [28]. It was therefore concluded that chemical denaturants are required in addition to heat and pressure in order to form a consolidated bioplastic.

Temperatures lower than 100 °C resulted in granular materials, whereas temperatures higher than 130 °C led to excessive water evaporation causing the material to become brittle. In addition, high compression temperatures increased covalent cross-linking causing further embrittlement which has also been observed with proteins in previous studies [19]. Pressures above 3MPa were required for consolidation and it was concluded that the combination of heat, pressure and chemical additives is required to produce a consolidated bioplastic. The optimum temperature range was between 115 °C and 125 °C. The most favorable combination of chemical additives was 60 pph_{bm} water, 2 pph_{bm} sodium sulfite, 30 pph_{bm} urea and 3 pph_{bm} SDS, resulting in a flexible and consolidated sheet.

Table 14: Experimental schedule for scouting experiments using compression moulding and extrusion

Additive combination	**pph_{bm}** parts per hundred bloodmeal
Water/SS	60, 2
Water/SDS	60, 3
Water/Urea	60, 30
Water / SS / SDS	60, 2, 3
Water / Urea / SDS	60, 30, 3
Water / SS / Urea	60, 2, 30
Water / SS / Urea / SDS	60, 2, 30, 3

At 120 °C, bloodmeal mixtures were difficult to process due to excessive material being extruded through the clearances between the mould walls. Although this was seen as a negative result for successful compression moulding, it revealed the potential as an extrudable material.

II Extrusion and Injection Moulding

i *Extrusion*

Using the chemical additive formulation determined from compression moulding experiments, extrusion trials were performed to determine suitable extrusion conditions. A screw speed of 150 rpm was selected after initial trials. Temperatures between 80 °C and 120 °C for the three barrel sections and 80 °C to 140 °C at the die were tested.

It was found that a barrel temperature set at 100 °C and die temperature of 120 °C was optimal, with a relative torque of 50-60% of the maximum allowed in the extruder. The added shear led to superior consolidation and more homogeneous materials compared to corresponding compression moulded samples.

Figure 14 shows the transition from a powdery material to a consolidated thermoplastic melt. As the powder moves along the barrel, rapid heating and denaturing takes place, resulting in the material being melted just before it enters the die. These conditions correspond well with the conditions encountered during compression moulding (115-125 °C).

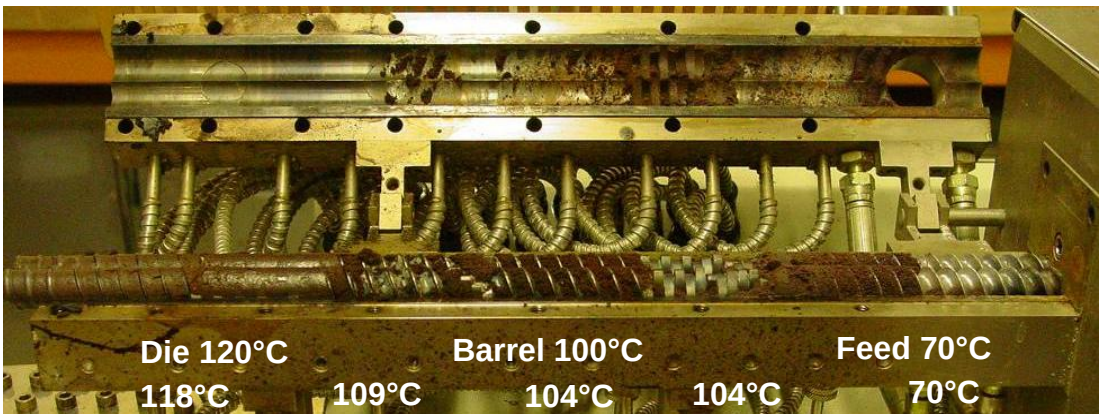


Figure 14: Photo showing the progressive consolidation of bloodmeal powder from the feed zone (70 °C) to the die (118 °C)

Various additive combinations were trialled to determine appropriate extrusion conditions leading to a consolidated material (Table 14). Combinations without sodium sulfite were granular in appearance, while the addition of sodium sulfite produced a consolidated and homogenous material. However, using sodium sulfite alone did not lead to the same result. Processing in the absence of urea proved difficult, often leading to solidification inside the barrel.

Urea and SDS act on non-covalent interactions alone, whereas sodium sulfite can break covalent cross-links and prevent further cross-link formation. It was found that combinations of sodium sulfite, urea and/or SDS were therefore required for successful processing.

Further trials were required to determine the optimal concentrations and relative effect of the selected chemical additives on the extrudability, consolidation and mechanical properties of the bioplastic. This was done in two parts: firstly the ranges of each additive were tested separately (Table 15). Secondly a combination of additives was tested according to a fractional factorial experiment designed by means of the Taguchi method [104] and is outlined in Table 16.

Urea concentrations above 30 pph_{bm} produced a white precipitate on the outer surface of the extrudate. No urea resulted in a very hard extrudate which was difficult to process. It was therefore concluded that urea also acted as a

plasticizer. Above 4 pph_{bm} sodium sulfite produced powdery, unconsolidated material and it is believed that excessive breaking of covalent bonds prohibits processing. Above 5 pph_{bm} SDS led to processing difficulties, such as excessive bubbles and water in the feed region, as well as a non-continuous extrudate. The water concentration was also assessed, in combination with the other additives. It was found that water below 30 pph_{bm} caused processing problems, failing to extrude.

Table 15: Additive ranges trailed using extrusion.

Additive	**pph_{bm}** (in addition to 60 pph_{bm} water)
Urea	0, 10, 20, 30, 40, 60
SS	1, 2, 3, 4, 6
SDS	0, 3, 5, 10, 15

Suitable additive ranges were selected based on these trials and were 30 and 60 pph_{bm} water, 10 and 30 pph_{bm} urea, 0 and 3 pph_{bm} SDS and 1 and 3 pph_{bm} sodium sulfite.

ii *Injection Moulding*

Using the formulation identified from the initial compression and extrusion trials (60 pph_{bm} water, 2 pph_{bm} sodium sulfite, 30 pph_{bm} urea and 3 pph_{bm} SDS), optimal injection moulding conditions were assessed. Temperatures between 80 and 170 °C for each heating zone in the injection moulder. Temperatures of 140 °C and above produced a shiny material containing air bubbles inside the test specimens. Temperatures below 100 °C resulted in a material with extensive flow lines. The flow lines and air bubbles are imperfections that can cause premature fractures, which is undesirable.

An optimum temperature profile of 100, 115, 120 °C starting at the feed end was chosen for future work. The screw speed was kept constant at 150 rpm.

III Taguchi Experimental Design

The Taguchi method uses orthogonal arrays in its experimental design. Following from preliminary results, it was possible to construct a L9 fractional factorial experimental design to assess the relative effect of each additive on consolidation, processability, water absorption and mechanical properties, as outlined in Table 16. The analysis was performed in triplicate for each specimen produced.

The results of the Taguchi experiment are analyzed in two steps: firstly the influence and main effects of each factor are qualitatively assessed. Secondly, analysis of variance (ANOVA) is used to quantify the relative influence of each factor. In order for this approach to be successful each experiment must be performed under the same conditions.

Table 16: Fractional Factorial design and results obtained

L3	20	3	3	60										
L2	10	1	2	45										
L1		0	1	30										
									Unconditioned			Conditioned to 10% moisture		
Exp #	Urea (pph_{bm})	SDS (pph_{bm})	Sodium Sulfite (pph_{bm})	Water (pph_{bm})	Consolidation (Low / medium / high)	Processability index	Solubility (wt %)	Water absorption (wt %)	Tensile strength (MPa)	Young's modulus (MPa)	Toughness ($MPa.m^{½}$)	Tensile strength (MPa)	Young's modulus (MPa)	Toughness ($MPa.m^{½}$)
1	2	1	1	1	Low	0	9.4	36.5	6.3	713	0.04	7.8	1497	0.05
2	2	2	2	2	med/high	6	9.6	64.7	2.8	53	0.66	22.2	1451	0.25
3	2	3	3	3	high	16	10.9	114.3	1.9	23	0.76	24.3	1324	0.41
4	2	1	2	3	med	16	8.5	40.5	3.0	92	0.68	23.5	1170	0.41
5	2	2	3	1	med	4	10.3	82.8	6.5	244	0.92	21.1	1482	0.18
6	2	3	1	2	med	12	9.1	50.3	4.7	141	1.12	22.8	1376	0.26
7	3	1	3	2	low/med	16	15.2	83.5	3.1	54	0.95	12.3	608	1.17
8	3	2	1	3	med	16	12.1	27.3	1.4	11	0.38	8.2	322	1.64
9	3	3	2	1	high	6	16.1	102.8	2.3	27	0.61	11.1	653	0.22

i *Consolidation*

Consolidation is the formation of a solid from the initial bloodmeal powder. A consolidated material has no obvious adhesive failure between particles, with the broken surface appearing smooth and not granular. A subjective technique was employed to assess the consolidation of broken sections of extruded material. Optical microscopy was used for visual inspection and samples were scored from low to high (Figure 15). A combination of 3 pph_{bm} SDS and either 2 or 3 pph_{bm} sodium sulfite resulted in the best consolidated material (Table 16).

Figure 15: Cross sections showing A. Low (exp 1), B. Medium (exp 5), and C. high (exp 3) consolidation.

From initial trials it was found that sodium sulfite was required to produce a consolidated material. When using SDS in combination with sodium sulfite a material that was well consolidated and homogenous was produced. It was also found that higher amounts of water were required for effective consolidation.

Analysis of variance (ANOVA) confirmed results of the preliminary experiments. Figure 16 illustrates the main effect of each factor at the levels tested. It was found that increasing the SDS or sodium sulfite concentration, resulted in increased consolidation. Quantitative analysis revealed that SDS and sodium sulfite were the most important factors influencing consolidation, with SDS having the largest main effect (56.6%), as listed in Table 17. Significance for all measured properties was tested at a 90% confidence interval with insignificant factors pooled with the error.

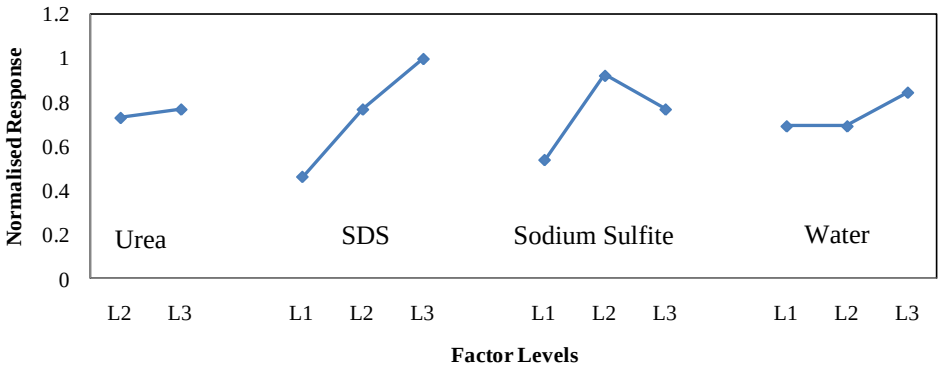
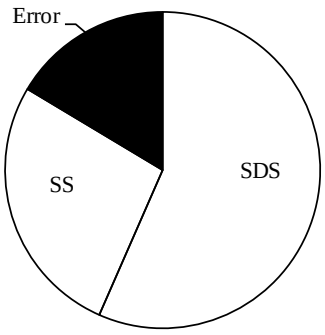


Figure 16: Main effects of urea, SDS, SS and water on consolidation.

Table 17: ANOVA of the main effects influencing consolidation.

	DOF	***F***	***Percentage Contribution***
Urea	Factor Pooled		
SDS	2	14.8	56.6%
SS	2	7.6	27.0%
Water	Factor Pooled		
Error	4		16.4%
Total	8		100%



$F_{2,4} = 4.3246$

ii *Processability*

Processability was assessed according to the method discussed earlier. It was found that water was the only statistically significant factor effecting processability according to analysis of variance (Table 18). Although this points to the importance of water as a processing aid, the results were somewhat bias

because the processability of experiment 1 was scored zero and had to be compression moulded to produce test specimens. This unsuccessful result overshadowed the importance of the other factors tested. The main effects of the other factors are shown in Figure 17. At 60 pph_{bm} water the materials were easily processed confirming water's plasticizing function.

Furthermore, water lowers the glass transition temperature and denaturation temperature of bloodmeal, enhancing the effect of urea, which is known to denature proteins. The result was that urea and water effectively plasticized the protein's leading to a visco-elastic melt. These properties were reflected in the qualitative (Figure 17) and ANOVA (Table 18), with water as the only significant factor.

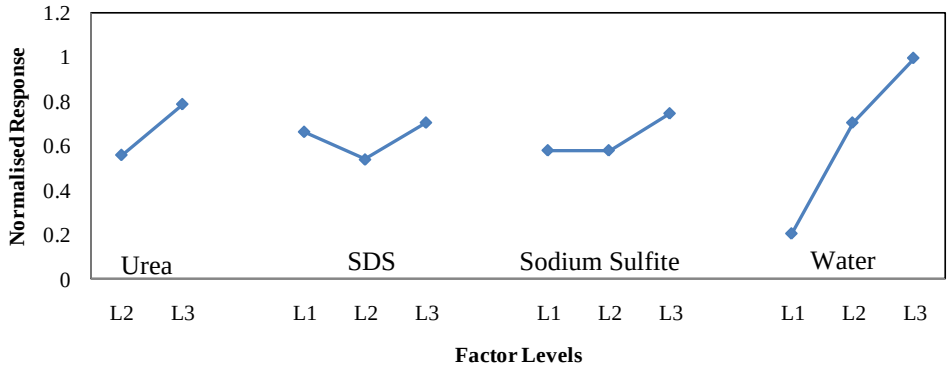
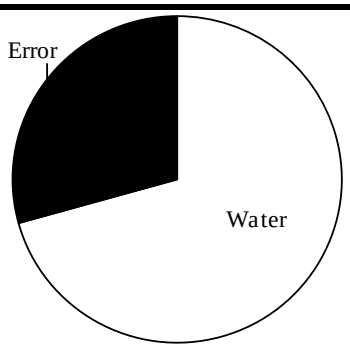


Figure 17: Main effects of urea, SDS, SS and water on the processability.

Table 18: ANOVA of the main effects influencing processability.

	DOF	*F*	*Percentage Contribution*
Urea	Factor Pooled		
SDS	Factor Pooled		
SS	Factor Pooled		
Water	2	10.65	70.7%
Error	6		29.3%
Total	8		100%



$F_{2,6} = 3.4633$

It was found that SDS affected the consolidation of the extrudate and injected material. When using 0 pph_{bm} SDS, the material had inhomogeneous properties along the length of the extrudate, showing brittle and ductile areas. This was partly attributed to inhomogeneous denaturation during the pre-denaturation stage.

A lower feed rate had to be used for compounding blends containing sodium sulfite at level 1 and water at level 1 and 2. This was necessary due to excessive

torque and pressures at the same feed rate as the other trials. The resultant material was harder to process and did not produce a well consolidated material. It was concluded that 1 pph_{bm} SS at low water levels was not sufficient to break existing disulfide cross-links, preventing consolidation. A higher concentration of water and sodium sulfite was required to form a flowable melt, in combination with the other chemical additives.

iii *Water Absorption and Solubility*

Water absorption is an effective test for cross-link density estimation. A material with increased water absorption is assumed to have less covalent cross-linking, resulting in an increased ability for the cross-linked network to swell [105]. Bloodmeal is highly cross-linked which seriously impairs processability, increasing water absorption is therefore desired as an indication of a reduction in cross-links.

Analysis of variance revealed that sodium sulfite had the largest effect on the water absorption followed by SDS (Table 19). Increasing the sodium sulfite and SDS concentration increased water absorption (Figure 18), indicating that sodium sulfite was effective in breaking covalent cross-links. Furthermore, the results also suggest that SDS was effective in reducing hydrophobic interactions which is consistent with the fact the SDS generally increases the water solubility of proteins. The effect of SDS on soy proteins has been studied and the authors came to the same conclusions [58; 59; 106].

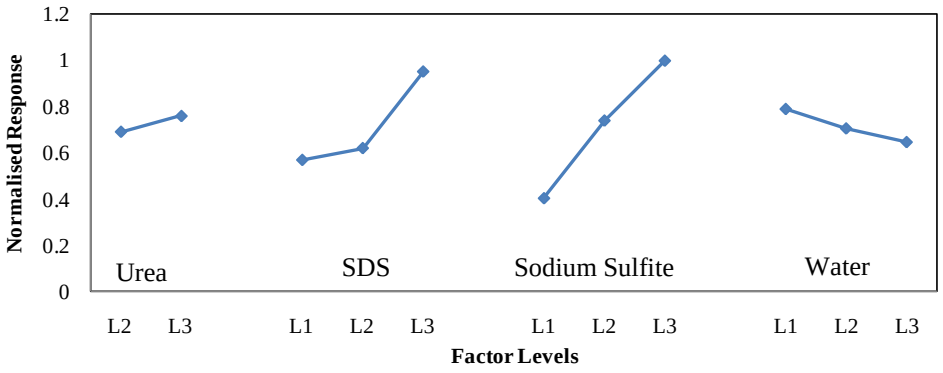
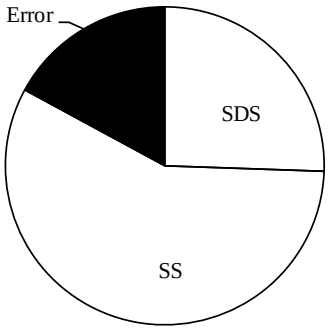


Figure 18: Main effects of urea, SDS, SS and water on water absorption, indicative of the degree of cross-linking**.**

Table 19: ANOVA of the main effects influencing Water Absorption.

	DOF	*F*	*Percentage Contribution*
Urea	Factor Pooled		
SDS	2	6.97	25.6%
SS	2	14.40	57.3%
Water	Factor Pooled		
Error	4		17.1%
Total	8		100%



$F_{2,4} = 4.3246$

Barone *et al.*, 2006 extruded poultry feathers (which contain keratin) using a combination of glycerol, water and sodium sulfite [48]. Keratins are characterized by a large amount of cysteine residues (similar to bloodmeal), which consequently implies a high degree of cross-linking. As mentioned before, disulfide cross-links are an impediment to processing in the melt-state. In their study, differential scanning calorimetry was used to assess thermal properties and it was shown that at sodium sulfite concentrations greater than 4 wt % resulted in greater chain extension compared to processing in the absence of sodium sulfite [48].

Not surprisingly, water and urea had no significant effect on reducing cross-link density, as highlighted by the ANOVA results presented in Table 19.

Due to urea's hydrophilic nature, solubility increased with increased urea concentration. It was the only statistically significant factor at 73% contributing to solubility changes (ANOVA not shown).

iv *Mechanical Properties*

To characterize the materials mechanical properties, the tensile strength, Young's modulus, percent elongation at break and toughness were assessed. For each experiment five conditioned and five unconditioned samples were tested. All samples were injection moulded into tensile test specimens except, experiment 1. It was not possible to injection mould this material and was compression moulded into sheet form which test specimens were cut to the same dimensions as injection moulded samples. The plastic material formed in this experiment was barely processable and was difficult to thermoform into a homogenous sheet. The pronounced difference between experiment 1 and the others caused a bias towards this experiment, rendering all main effects statistically insignificant at a 90% confidence interval.

Despite the overwhelming effect of experiment 1, the results did highlight some important conclusions. It was found that conditioning the materials to 10% moisture resulted in a significant improvement in mechanical properties. Also, the main effect of the variables tested was different for materials tested conditioned or unconditioned. The main effects are shown in Figure 19 and Figure 20 for unconditioned and conditioned samples, respectively.

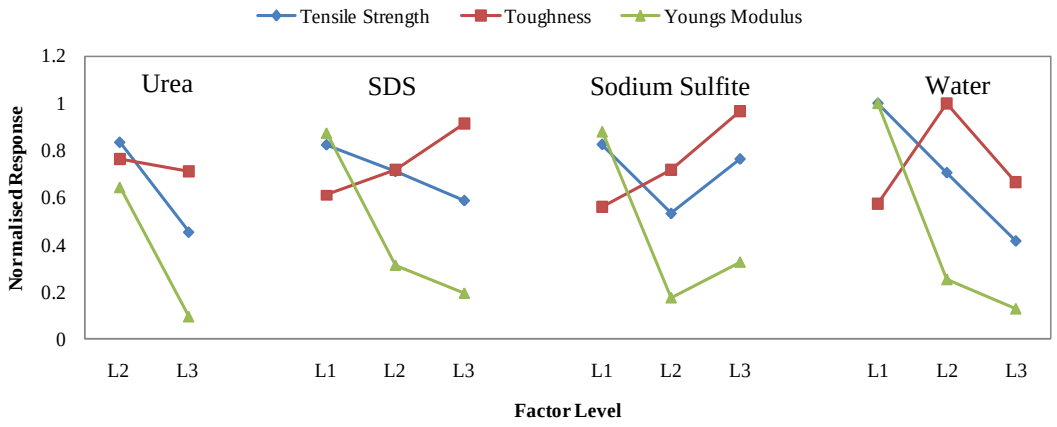


Figure 19: Main effects of Urea, SDS, SS and water of unconditioned samples tested with respect to mechanical properties.

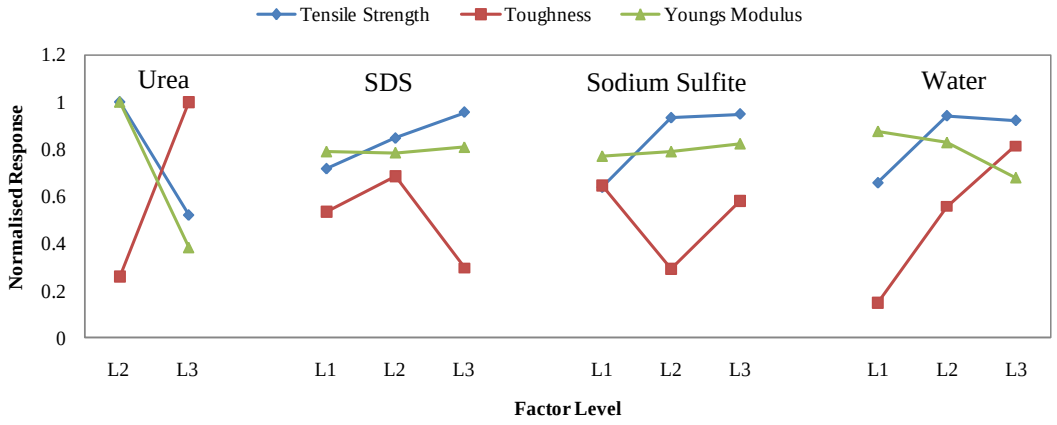


Figure 20: Main effects of Urea, SDS, SS and water of conditioned samples tested with respect the tensile strength, Young's modulus and toughness.

It was found that increasing urea consistently led to a reduction in all mechanical properties tested, except the toughness of conditioned specimens. This is consistent to what can be expected of a plasticizer in polymers. Urea was required for denaturing protein chains leading to processability, but it also led to less chain interaction and consequently reduced strength and resulted in a more flexible material. In the absence of excessive water (conditioned samples), urea had the greatest effect regarding all the mechanical properties tested (Table 20). Most

importantly, the increased ductility led to a tougher material because of the materials ability to deform more before breaking.

Table 20: ANOVA results for unconditioned and conditioned mechanical properties

	Unconditioned			Conditioned		
	Tensile Strength	**Young's Modulus**	**Toughness**	**Tensile Strength**	**Young's Modulus**	**Toughness**
Urea	32.9%	20.3%	31.9%	55.7%	91.8%	49.6%
SDS	7.6%	21.0%	14.7%	9.1%	0.11%	10.4%
SS	13.0%	22.0%	24.6%	19.3%	0.51%	9.5%
Water	46.5%	36.0%	29.3%	15.9%	7.6%	30.4%

Materials containing no SDS had irregular breakages with brittle and ductile fractures within the same treatment. For unconditioned samples, the Young's modulus and tensile strength decreased with increasing SDS content while the toughness increased. When water was removed the tensile strength was increased, suggesting that reduced interactions during processing improved processability, and enabled formation of new interactions only after conditioning. In terms of mechanical properties it can be concluded that SDS also acted as a plasticizer, by decreasing interaction between chains, most likely hydrophobic interactions, but the contribution to the main effect is relatively small compared to the other factors (Table 20).

The effect of increasing sodium sulfite was to decrease mechanical properties of unconditioned samples. However, once conditioned it can once again be concluded that sodium sulfite was required for processing and establishing chain interactions in the conditioned state. The main effect was, however, relatively small compared to the effect of urea and water in the conditioned state.

The difference between conditioned and unconditioned samples was most important when the main effect of the amount of water used during processing is considered. Although water led to a general decrease in mechanical properties of unconditioned samples, it can be seen after comparing Figure 19 and Figure 20 that a high amount of water is not only required for successful processing but also to enable the function of the other additives. Using sufficient water during processing enables chain rearrangement and therefore, after water evaporation during conditioning there was an increase in interactions between extended chains leading to superior mechanical properties.

In conclusion, an optimal formulation would result in high consolidation, processability and water absorption with a material showing good toughness, tensile strength and not being too stiff (lower Young's modulus). An optimal formulation can be determined for each measured property and is listed in Table 21. Optimal performance was calculated by the using the average effect of each statistically significant factor at its optimal level, as revealed by the main effect plots (Figure 20).

Table 21: Optimum formulation for each measured parameter. (Bolded figures denote statistically significant factors.)

					Conditioned
	Consolidation (Low / medium / high)	**Processability index**	**Water absorption (wt%)**	**Tensile strength (MPa)**	**Toughness ($MPa.m^{½}$)**
Urea	3	3	3	2	***3***
SDS	***3***	3	***3***	3	2
SS	***2***	3	***3***	3	1
Water	3	***3***	1	2	3
Value of property at optimum formulation	**High**	**16**	**127%**	**27**	**1.6**

Using urea and water at level 3 was most effective leading to consolidation, good processability and toughness (after conditioning). Both acted as plasticizers during processing, inducing a flowable melt. However, upon conditioning, water evaporated allowing further interactions between chains. It was therefore concluded that water at a higher level, was not only required for successful processing but also to enable chain rearrangement leading to interactions after conditioning.

SDS is effective at dissociating and denaturing proteins at low concentrations. It was concluded that the addition of SDS was important for improving consolidation, processability and water absorption. However, a lower level of SDS led to improved toughness (after conditioning), caused by enhanced interactions between chains after water removal. At high SDS content, a larger proportion of chains maybe prevented from interacting, leading to lower material toughness.

Finding an overall optimum for all properties is not practical and depends on the end use of the material. A material that shows good processability and toughness would contain 60 pph_{bm} water, 1 pph_{bm} sodium sulfite, 1 pph_{bm} SDS and 20 pph_{bm} urea. At these conditions, a tensile strength of 8MPa, a Young's modulus of 320MPa and toughness of 1.64 $MPa.m^{½}$ can be achieved.

4.4 Conclusions

The aim of this study was to investigate the use of bloodmeal (BM) as a precursor for thermoplastic biopolymers. It was found that low-moisture processing techniques, such as extrusion and injection moulding can successfully be used to produce a bioplastic with good mechanical properties.

It was established that processing required chemical additives, performing the following functions:

- breaking covalent cross-links, using sodium sulfite
- breaking inter and intra-molecular forces, such as hydrophobic forces by SDS, and plasticizing protein chains by using water and urea
- enabling formation of new interactions to stabilize the three-dimensional structure by evaporating some of the water.

A successful material required the plasticization of water and the combined denaturation affects of sodium sulfite, SDS and urea and that a bioplastic manufactured using bloodmeal can be characterized using:

Processability: It was shown that successful processing requires a minimum temperature of about 100 °C and that excessive cross linking occurs above 130 °C. Processability is strongly influenced by plasticizer content and it was shown that water and urea performed a similar function in this regard. Although these would be the most important factors, processing would be impossible without sodium sulfite.

Consolidation and water absorption: It was found that the consolidation of the bioplastic was strongly dependant on the amount of SDS, known to influence hydrophobic interactions in proteins. SDS had to be used in combination with SS to ensure good consolidation. Increasing the SS content also led to increased water absorption, taken as an indication of reduced cross-link density.

Mechanical properties: It was concluded that water was required for processing, enhancing the action of urea and SDS. The use of water does, however led to over-plasticization of the material, evident from the low mechanical properties. It has been found that once water evaporated during conditioning the mechanical properties increased significantly, indicating the formation of new intermolecular forces. It was found that although SDS was required for processing and consolidation, it may restrict the formation of new inter-molecular forces, if used at higher levels.

Chapter 5: Structural Changes during Thermoplastic Processing

Summary

The aim of this section was to investigate structural changes and secondary interactions after thermoplastic processing of BM, as these could directly relate to final functional properties.

Combinations of sodium sulfite, water, SDS and urea were compounded with BM and injection moulded. Processability, consolidation, water absorption, solubility, thermal stability and secondary structures were assessed for each material after extrusion and injection moulding.

Thermal stability of processed plastics were reduced when using 3 pph_{bm} sodium sulfite and 20 pph_{bm} urea, indicative of a reduction in covalent cross-linking and other secondary interactions between chains. The results showed that sufficient sodium sulfite was required to allow chain mobility during processing. However, without sufficient plasticization protein degradation will occur instead of the melt process. The addition of SDS further improved processability and consolidation leading to increased water absorption solubility, indicative of a reduction in chain interactions.

Fourier Transform Infrared Spectroscopy (FTIR) revealed that BM proteins are in a highly denatured state. Additional minor structural changes occurred during processing, resulting from reducing covalent and non-covalent interactions. Under high plasticizing conditions a less ordered structure was observed. It was concluded that the increase in processability, consolidation, water absorption and solubility was due to changes in inter- and intra-molecular interactions, rather than substantial structural changes.

5.1 Introduction

In contrast to the number of studies on the production of bioplastics from soy and wheat proteins, little has been studied regarding animal waste proteins like bloodmeal. In the previous chapter it has been shown that BM can be extruded and injection moulded using chemical additives. These were required to break covalent cross-links, inter- and intra-molecular forces, such as hydrophobic and hydrogen bonding as well as to plasticizing the protein chains.

The aim of this study was to assess changes in structural and secondary interactions as a result of thermoplastic processing of BM. More specifically the objectives were to:

- Investigate which factors influence processability and consolidation
- Understand the effects of additives and processing on the water absorption and solubility of the materials.
- Relate observed changes in secondary structures to processability, consolidation, water absorption, and solubility.

5.2 Experimental

5.2.1 Materials

Table 22: Materials used

	Supplier	**Grade**	
Bloodmeal (BM)	Taranaki Byproducts		ρ = 1300 kg/m^3 Sieved to 700 μm
Sodium dodecyl sulfate	Biolab	Technical	
Sodium sulfite (SS)	BDH Lab Supplies	Analytical	
Urea	Agrinutrients-Balance	Agricultural	

5.2.2 Sample Preparation

Various amounts of sodium sulfite (SS), urea and Sodium dodecyl sulfate (SDS) were dissolved in distilled water by mixing and heating to 60°C (see Table 23). The resulting solutions were mixed with sieved blood meal in a high speed mixer for at least 5 minutes. During this stage, the proteinous mass absorbed all the water and some denaturing occurred.

The blends were compounded in a ThermoPrism TSE-16-TC twin-screw extruder at 150 rpm with temperature settings of 120, 100, 100, 100, 70 °C starting from

the die. The extruded material was allowed to cool to room temperature (approximately 1 hour). Sections of the extrudate were collected for consolidation analysis.

The remaining extrudate was granulated and injection molded into Type 1 tensile test specimens (ASTM D638-08 [107]) using a BOY15-S injection-molding machine with temperature settings of 100, 115, 120 °C starting at the feed end. The mould itself was heated to 65 °C. Tensile test specimens (3 – 5) were conditioned for 7 days at 23°C, 50% RH.

5.2.3 Analysis

Consolidation, processability, moisture content, water absorption and solubility were measured as described in Chapter 4.

I Thermogravimetric Analysis (TGA)

Approximately 10mg of dried and ground sample was scanned using a simultaneous DTA-TGA (SDT) analyzer (SDT 2960, TA Instruments, New Castle, Delaware, USA) from room temperature to 500 °C at a rate of 10 °C/min.

II Fourier Transform Infrared Spectroscopy (FTIR)

Freeze-dried and powderised samples were prepared as KBr pellets (1 mg sample and 100 mg salt). The FTIR spectra were recorded using a Perkin Elmer spectrophotometer with 16 scans at 4 cm^{-1} resolution, from 4000 to 400 cm^{-1}. A background scan was performed before each sample scan and subtracted from each sample spectra automatically by the FTIR software.

The amide I region (1600-1700 cm^{-1}) is most sensitive for assessing protein secondary structural components, such as α-helices, β-sheets, turns and unordered structures [108]. The amide I region contours are complex composites, i.e. they consist of many overlapping component peaks that represent different structural elements. Individual component peaks cannot be resolved/or identified in the broad contours of the measured spectra [109]. Interpreting the composite profile alone may lead to misinterpretations of spectral shifts, therefore using the featureless amide I region to obtain structural parameters and their changes is limited.

Mathematical techniques such as, Fourier self-deconvolution (FSD) and second derivation are useful in visualizing overlapping peaks of the spectrum [110]. Ideally, both techniques should be used and only features in both derivative and deconvolved spectra should be assigned in order to avoid artifacts due to data processing [110].

In this study, the corresponding peaks of the FSD curve (half width = 13 cm^{-1}) and the second derivative (1.2% smoothing) were used to quantify the structural parameters, using Peak Fit v4.12 with 65% filtering between 900 to 2100 cm^{-1} [111].

Using the AutoFit II Second derivative function (Gaussian Area), corresponding component peaks between the second derivative and the FSD curve were selected. Using PeakFit v4.12, a least squares curve fitting function was used to reproduce the experimentally obtained amide I composite peak by adjusting individual component peak areas until the total is approximately the experimental area under the amide I composite profile [111]. Individual area estimates were used to manually calculate the relative amounts of helices (α-helix and 3_{10}-helix), β-sheets, β-turns and unordered structures, each corresponding to a specific wavelength.

Three specimens were prepared for each sample and the FTIR scan was repeated 3 times.

III Statistical Analysis

An L8 fractional factorial experimental design was devised using the Taguchi method [104], outlined in Table 23. Orthogonal arrays are employed in the design, allowing the relative effect of each additive and their interactions to be assessed. The results of the Taguchi experiment were analyzed in two steps: firstly, the main effect of each factor was qualitatively assessed. Secondly, analysis of variance (ANOVA) was used to quantify the relative influence of each factor and interaction, at a 90% confidence interval. In order for this approach to be successful, each experiment must be performed under the same conditions.

5.3 Results and Discussion

The experimental design and results for consolidation, processability, water absorption and solubility are displayed in Table 23. All the analysis was performed in triplicate on each specimen.

Table 23: Fractional Factorial design and results obtained.

L2	3	60	3	20						
L1	1	45	0	10						
Exp #	**Sodium Sulfite ($ppph_{bm}$)**	**Water ($ppph_{bm}$)**	**SDS ($ppph_{bm}$)**	**Urea ($ppph_{bm}$)**	**Consolidation (Low / medium / high)**	**Processability index**	**Extrusion**		**Injection**	
							Water absorption (wt %)	**Solubility (wt %)**	**Water absorption (wt %)**	**Solubility (wt %)**
1	1	1	1	1	low	1	30.6	7.7	135.6	8.7
2	1	1	2	2	low/med	9	57.8	13.6	323.7	18.7
3	1	2	1	2	med	6	20.8	12.1	197.4	13.9
4	1	2	2	1	med	6	47.2	7.8	214.0	10.2
5	2	1	1	2	med/high	6	69.7	14.5	692.3	27.1
6	2	1	2	1	high	8	102.1	10.8	717.4	25.3
7	2	2	1	1	low/med	4	71.0	9.0	335.0	13.3
8	2	2	2	2	high	16	123.4	14.9	737.5	26.0

5.3.1 Processability and Consolidation

The processability and consolidation of a material are closely related. Flow and cohesion of a powder into a solid during extrusion depends on the mobility of the protein chains. A bloodmeal particle will contain a multitude of protein chains stabilized by secondary interactions, such as van der Waals, hydrophobic, electrostatic, hydrogen, covalent disulfide bonds (S-S) and possibly lysinoalanine. Heat, shear and pressure of extrusion may break some of these interactions, apart from stable covalent bonds. These interactions can only be broken by the addition of reducing agents.

Blood proteins contain high proportions of lysine and cysteine [29], which are the two most reactive amino acids at higher pH [27]. During the drying of blood into bloodmeal (BM) the formation of covalent disulfide and lysinoalanine bonds can be induced. This phenomenon has been observed in other proteins containing lysine and cysteine residues during heating [31; 88; 94; 112-115]. These heat stable interactions will not prevent melt processing, but rather limit chain mobility thereby preventing chain unfolding and alignment during extrusion [103].

Thermoplastic processing of protein-based plastics is largely dependent on chain mobility [21]. In previous chapter it was shown that sodium sulfite was crucial for successful processing and consolidation of BM plastics. Samples that did not contain SS had obvious adhesive failure between powder particles. It was concluded that sodium sulfite cleaved disulfide bonds, allowing chains to become mobile, unfold and align during processing with the assistance of plasticizers.

I Processability

The processability index was determined for each experiment (Table 23) and subsequently used in the ANOVA (Table 24). The main effect of each variable on the processability index is shown in Figure 21.

Sodium sulfite, SDS and urea were the only statistically significant factors for processability, with no statistically significant interactions (Table 24). In the range tested, sodium sulfite did not have a large effect. However, in the absence of SS a granular material was formed, making it a required additive.

SDS was essential for good processability, the absence of which resulted in inhomogeneous extrudates with brittle as well as ductile areas. BM powder is insoluble in water and contains hydrophobic interactions. SDS reduces these interactions, enhancing chain mobility during processing.

Urea preferentially binds to protein surfaces, disrupting protein-protein and protein-water hydrogen bonding, resulting in partial unfolding and an increase in protein chain flexibility [66]. Urea therefore acted as both a denaturant and plasticizer by reducing interactions between chains and increasing chain mobility.

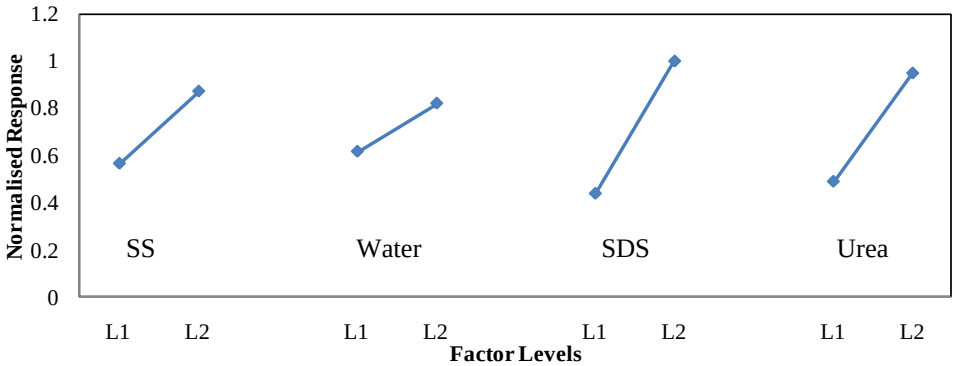
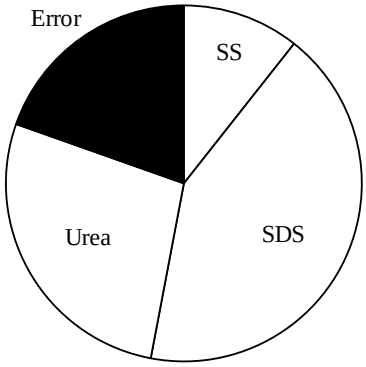


Figure 21: Main effects of SS, water, SDS and urea on processability.

Table 24: ANOVA of the main effects influencing processability.

	DOF	*F*	*Percentage Contribution*
SS	1	4.8	10.6%
Water	Factor Pooled		
SDS	1	16.1	42.4%
Urea	1	10.8	27.4%
SS x Water	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Error	4		19.6%
Total	7		100%



$F_{1,4} = 4.5448$

II Consolidation

The consolidation was determined using the morphology of broken extrudates, and scored from low to high (Table 23). The ANOVA results are presented in Table 25 and the main effect of each factor on consolidation is shown in Figure 22.

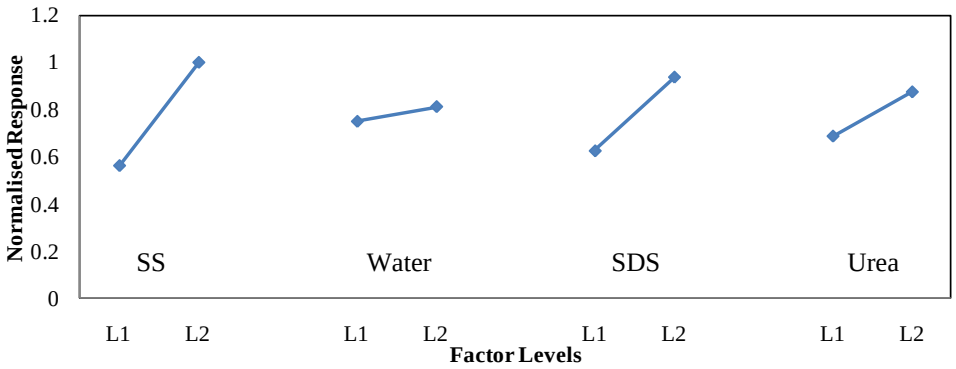
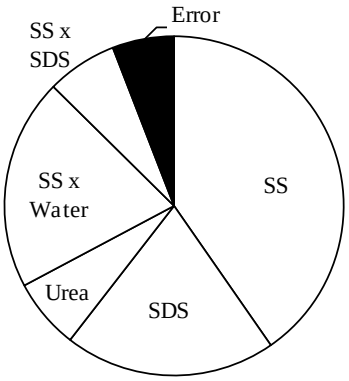


Figure 22: Main effects of SS, water, SDS and urea on consolidation.

Table 25: ANOVA of the main effects influencing consolidation.

	DOF	*F*	*Percentage Contribution*
SS	1	45	40.3%
Water	Factor Pooled		
SDS	1	25	20.2%
Urea	1	9	6.7%
SS x Water	1	25	20.2%
SS x SDS	1	9	6.7%
SS x Urea	Factor Pooled		
Error	2		5.9%
Total	7		100%



$F_{1,2} = 8.5263$

Sodium sulfite, SDS and urea were the main statistically significant factors, with the interaction between sodium sulfite and water also contributing. Increasing the urea concentration had a small influence on consolidation, in this case acting as a denaturant and plasticizer. Urea disrupted hydrogen bonding between protein chains within BM granules, thereby increasing chain mobility and allowing particle cohesion.

The addition of SS and SDS at level 2 increased the materials consolidation by disrupting interactions that restrict chain movement. The interaction between SS and SDS is shown in Figure 23A, revealing a slight synergy. A material containing 3 pph_{bm} sodium sulfite and 3 pph_{bm} SDS will be better consolidated (Figure 23A) as a result of the combined effect of covalent cross-link reduction and a disruption of hydrophobic interactions.

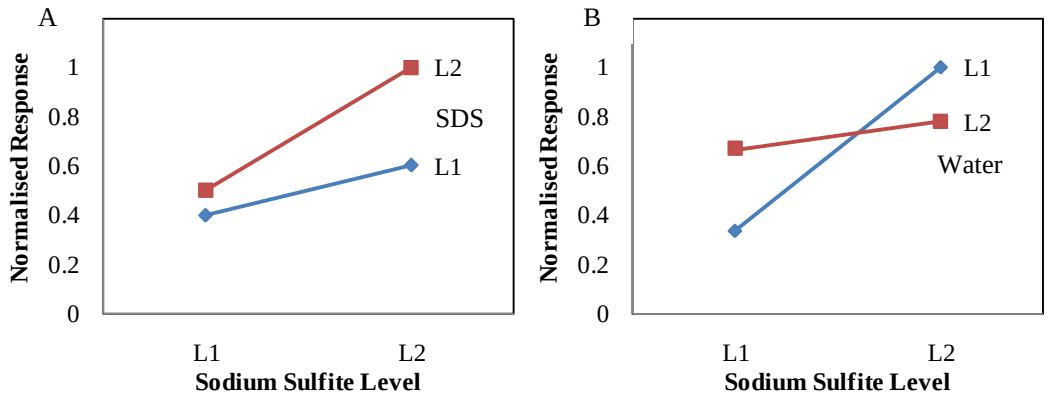


Figure 23: Main effect of the (A) SS and water interaction, (B) SS and SDS for consolidation.

Water was not statistically significant on its own, but the interaction with sodium sulfite had an effect of 20.2%. The combined effect of disulfide reduction and plasticization was important for the mobility and consolidation of BM powder during extrusion. The main effect for the interaction of SS and water is shown in Figure 23B. Sodium sulfite at 3 pph_{bm} (level 2) and water at 45 pph_{bm} (level 1) resulted in a better consolidated material. However, it was thought that this observed effect was due to protein degradation rather than unfolding and re-alignment. The addition of plasticizers facilitates melt flow without thermal degradation [19]. Low water content will result in a more viscous blend, which increases the required energy input during extrusion. At these conditions sodium sulfite may also reduce peptide bonds, resulting in protein degradation. Sodium sulfite and water at level 2 increased chain mobility, causing unfolding and re-alignment leading to true particle cohesion and consolidation.

For effective processing and consolidation of BM the following was required:

- sodium sulfite at 3 pph_{bm}, for covalent cross-link reduction
- 3 pph_{bm} SDS to break and prevent hydrophobic interactions
- reduction of hydrogen bonding by using 20 pph_{bm} urea
- plasticizers (urea and water) to reduce viscosity and prevent degradation.

5.3.2 Water Absorption and Solubility

Extruded and injection molded materials were analyzed using the water absorption and solubility methods in chapter 4 (raw data shown in Table 23). In order to assess the impact of processing on chain mobility, the difference between water absorption and solubility before and after injection moulding was calculated using Equation 2. The results are discussed further below.

$$\%\, Change \;=\; (Xi - Xo)/Xo$$

Equation 2: Relative percentage change (X_i =injection moulded, X_o= extruded).

I Water Absorption

Covalent cross-linking can hinder the mobility of chains preventing successful thermoplastic processing. Water absorption of a material can be an effective measure of the material's cross-linking and secondary interactions. A material with more cross-linking will generally have less swelling (see chapter 4).

i *Water absorption after extrusion*

From Figure 24 and the ANOVA results in Table 26, it can be seen that SDS and SS had the largest effect on water absorption. Increasing sodium sulfite or SDS from level 1 to level 2 increased the water absorption. It was concluded that the disulfide cleavage action of sodium sulfite and hydrophobic bonding restriction of SDS led to a less rigid material and reduced the insolubility of BM.

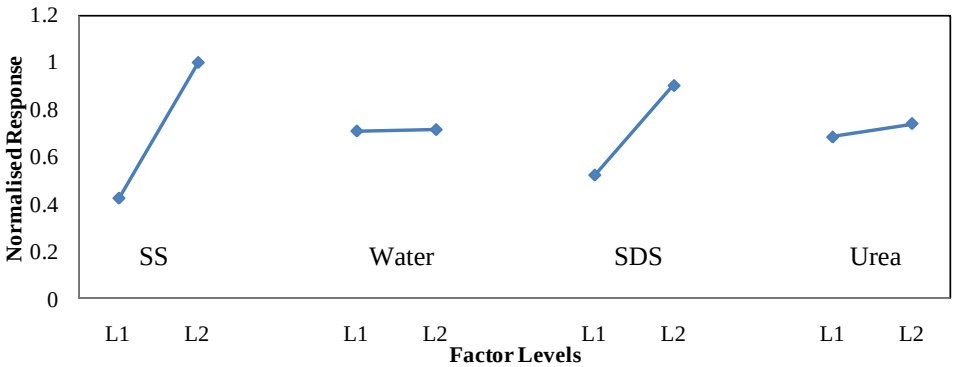
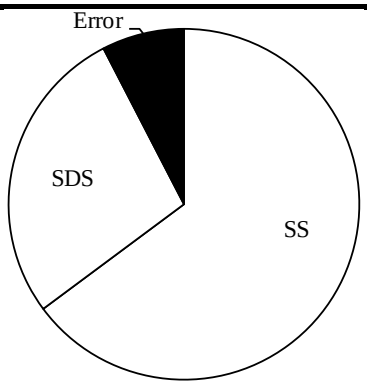


Figure 24: Main effects of SS, water, SDS and urea on extruded samples water absorption.

Table 26: ANOVA of the main effects influencing extruded water absorption.

	DOF	*F*	*Percentage Contribution*
SS	1	60.6	64.8%
Water	Factor Pooled		
SDS	1	26.4	27.6%
Urea	Factor Pooled		
SS x Water	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Error	5		7.6%
Total	7		100%



$F_{1,5} = 4.0604$

ii *Water absorption after injection moulding*

Water absorption after injection moulding was clearly higher than after extrusion (Table 23). From Table 45 it can be seen that sodium sulfite was the only statistically significant factor at 67% (results in Appendix-1A).

To investigate the effect of injection moulding, the difference between extrusion and injection moulding was calculated according to Equation 2 (X_i= water absorption after injection moulding, X_o= water absorption after extrusion). These results were analyzed using ANOVA and are presented in Table 27.

Urea and the interaction between SS and water were the most important factors. Materials containing 20 pph_{bm} urea (level 2) had higher water absorption (Figure 25) due to the increased prevention of secondary interactions between chains.

The significance of the interaction between sodium sulfite and water, again confirms the mechanism where a reducing agent is required in conjunction with a plasticizer to enable chain mobilization. The main effects of sodium sulfite and water interaction are illustrated in Figure 26.

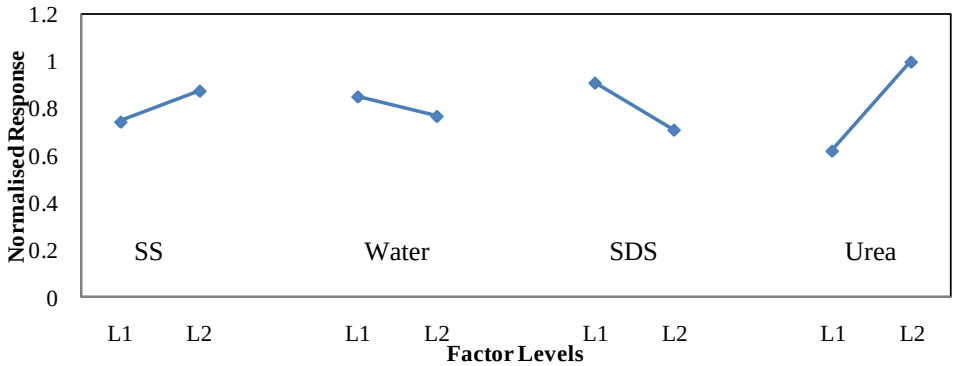
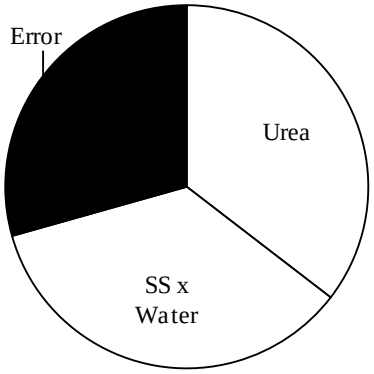


Figure 25: Main effects of SS, water, SDS and urea on difference between processing water absorption.

Table 27: ANOVA of the main effects influencing difference between processing water absorption.

	DOF	*F*	*Percentage Contribution*
SS	Factor Pooled		
Water	Factor Pooled		
SDS	Factor Pooled		
Urea	1	9.45	35.4%
SS x Water	1	9.39	35.2%
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Error	5		29.4%
Total	7		100%



$F_{1,5} = 4.0604$

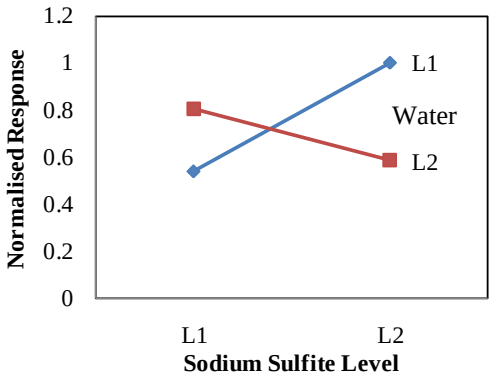


Figure 26: Main effects of interaction between sodium sulfite and water for difference between injection moulding and extrusion water absorption.

Samples containing 3 pph_{bm} sodium sulfite (level 2) and 45 pph_{bm} water (level 1) had a greater increase in water absorption after injection moulding. This was attributed to thermal degradation of the polymer at low water levels (high viscosity). 3 pph_{bm} sodium sulfite (level 2) and 60 pph_{bm} water (level 2) resulted in the smallest increase in water absorption after injection moulding, attributed to high unfolding and alignment of chains allowing increased secondary interactions. Sodium sulfite at 1 pph_{bm} was insufficient to break all covalent cross-links, resulting in reduced chain mobility, also observed by the consolidation analysis.

II Solubility

Because urea is water soluble, the measured solubility was corrected by subtracting the amount of urea from the solubility of extruded and injection moulded samples. The difference between the solubility of extruded and injection moulded samples was calculated using the corrected data, assuming that all the urea present was solubilised.

i *Solubility of extruded and Injection moulded samples*

Sodium sulfite and SDS were the most significant factors affecting solubility after extrusion (results in Figure 27 and Table 28) and injection moulding (results in Appendix-1B). The addition of SDS reduces hydrophobic interactions resulting in a more soluble material through unfolding of protein chains. Sodium sulfite at 3 pph_{bm} (level 2) resulted in higher solubility, provided sufficient plasticizer was also used.

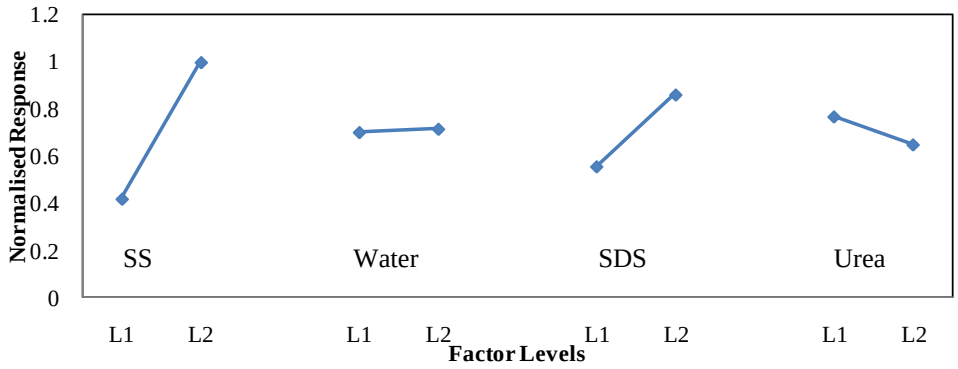
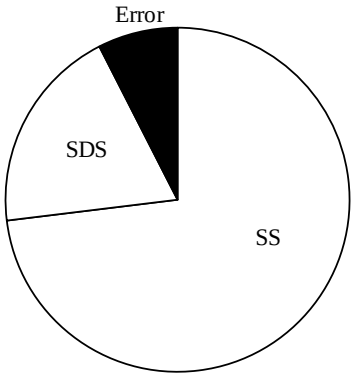


Figure 27: Main effects of SS, water, SDS and urea on extruded sample's solubility.

Table 28: ANOVA of the main effects influencing extruded sample's solubility.

	DOF	***F***	***Percentage Contribution***
SS	1	69.1	73.1%
Water	Factor Pooled		
SDS	1	19.1	19.4%
Urea	Factor Pooled		
SS x Water	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Error	5		7.51%
Total	7		100%



$F_{1,5} = 4.0604$

ii *Difference between solubility before and after injection moulding*

Generally, solubility after extrusion was less than after injection moulding. Furthermore, a clear difference in water color was observed for extruded and injection moulded samples (Figure 28). This was further investigated to determine whether the added pressure during injection moulding, or a combination of added pressure and the chemical additives was the main cause for this observation. In order to do this, the percent change between the solubility of

extruded and injection moulded samples was calculated using Equation 2 (X_i = solubility after injection moulding, X_o= solubility after extrusion). The qualitative and ANOVA is shown in Figure 29 and Table 29 respectively.

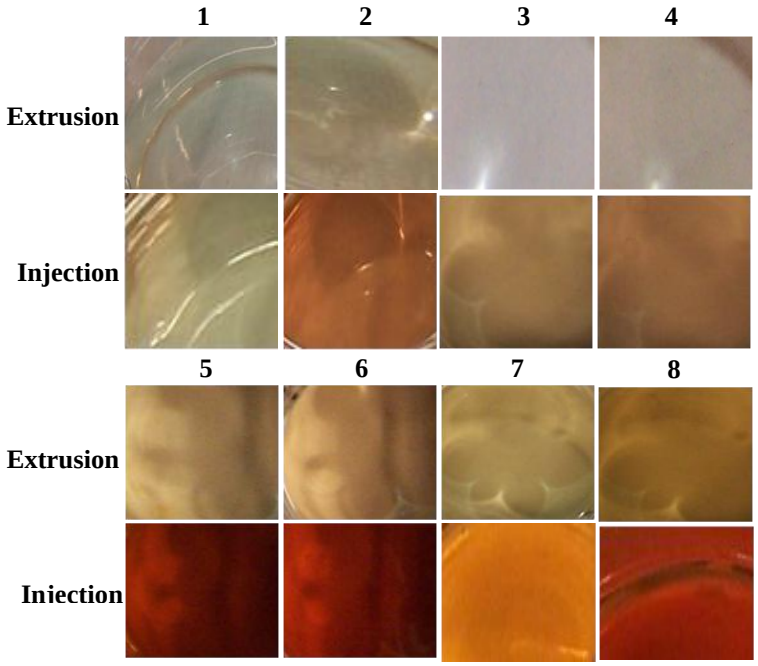


Figure 28: Colour difference in water of solubility tests of extruded and injection moulded samples for each treatment in the L8 array.

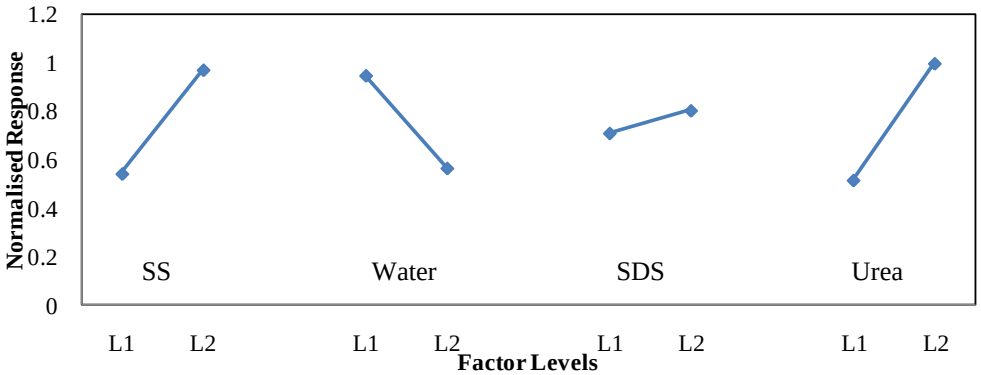
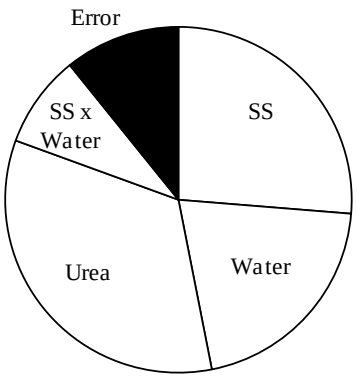


Figure 29: Main effects of SS, water, SDS and urea on the difference between processing for solubility.

Table 29: ANOVA of the main effects influencing difference between processing solubility.

	DOF	***F***	***Percentage Contribution***
SS	1	18.1	26.3%
Water	1	14.4	20.6%
SDS	Factor Pooled		
Urea	1	22.8	33.7%
SS x Water	1	6.6	8.6%
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Error	3		10.8%
Total	7		100%



$F_{1,3} = 5.5383$

Sodium sulfite, water and urea were the most influencing factors on the change in solubility after injection moulding. Increasing the concentration of sodium sulfite and urea increased the change in solubility after injection moulding. This was expected as the added pressure during injection moulding will enhance the denaturing ability of these chemicals. The opposite effect water had was the most interesting and is discussed further below.

Water content was found to be an important factor influencing the solubility difference between extruded and injection moulded samples. From Figure 29 it can be seen that increasing water content resulted in a smaller increase in solubility after injection moulding. Using water at level 1 increased the specific mechanical energy, further increasing the pressure of injection moulding resulting in protein degradation. The interaction between sodium sulfite and water (Figure 30) further supports this finding as well as color differences shown in Figure 28. Increased degradation (increased redness) was observed for samples with level 1 water (Experiments 1, 2, 5, and 6).

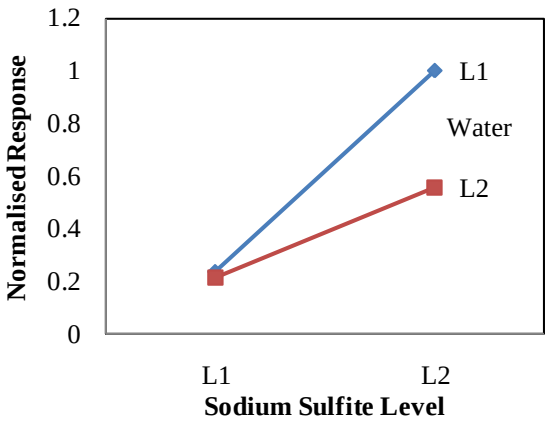


Figure 30: Main effects of sodium sulfite and water interaction on the difference between processing steps for solubility.

In conclusion the water absorption and solubility results were closely related. 3 pph_{bm} sodium sulfite and 3 pph_{bm} SDS were required for effective chain mobility during processing, by reducing secondary interactions. To avoid protein degradation during injection moulding, sufficient water should be used to allow plasticization. The addition of 20 pph_{bm} urea also increased chain mobility and prevented secondary interactions, resulting in higher water absorption and solubility. Unexpectedly, the combination of 3 pph_{bm} sodium sulfite and 60 pph_{bm} water resulted in smaller increase of water absorption and solubility after injection moulding when compared with 45 pph_{bm} water at the same SS content. This was

attributed to high chain mobilization, which allows increased secondary interactions.

5.3.3 Thermal Degradation

Generally, the onset of thermal degradation for cross-linked polymers is at higher temperatures than non-cross-linked polymers. Thermo-gravimetric analysis (TGA) is an effective method to analyze thermal stability, potentially providing information on secondary interactions.

Thermal degradation of BM-plastics occurred in four steps: 0 to 150 °C, 150 to 230 °C, 230 to 380 °C and above 380 °C (Figure 31). Up to 150 °C there was an average loss of 4.5 wt%, attributed to the evaporation of bound water. The second step was attributed to the degradation of urea. The region between 230 °C and 380 °C was credited to cleavage of S-S, O-N and O-O linkages. The final step was attributed thermal decomposition, through peptide bond reduction. Previous research on protein-based bioplastics showed similar thermal degradation behavior [38; 63; 81; 88; 92].

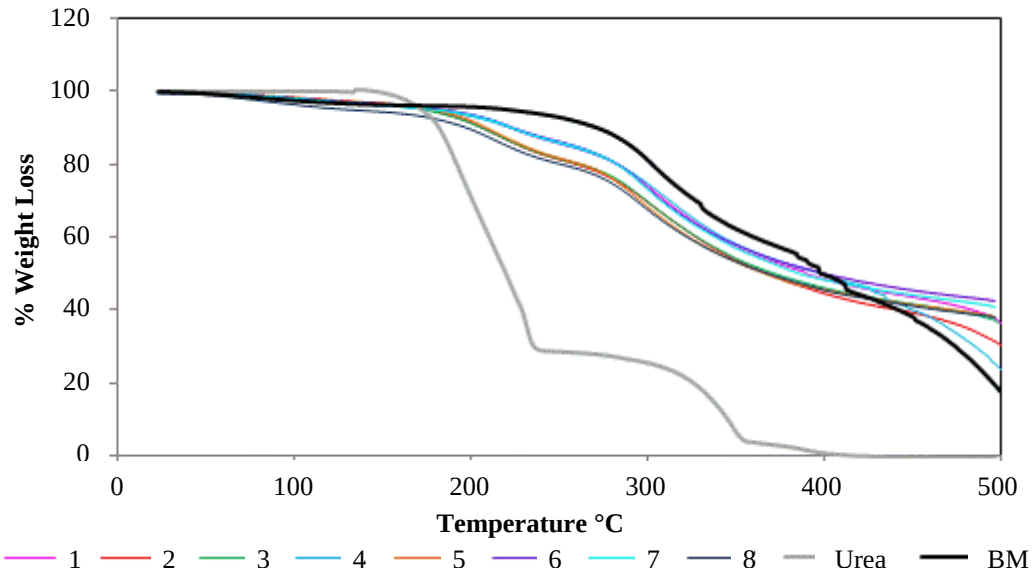


Figure 31: Mass loss curve from TGA.

It was found that bloodmeal was the most thermally stable compared to processed BM-plastics and therefore it was concluded that BM had the highest degree of cross-linking. The degradation of cross-links would occur between 230 to 380 °C and the inflection point in the percent mass loss vs. temperature curve was used for comparative processes. ANOVA (Appendix-1C) was used to determine the

importance of the chemical additives used on the thermal stability of the bioplastics.

Sodium sulfite and urea were the only statistically significant factors, contributing 34.6% and 32.8% respectively. Increasing the level of both sodium sulfite and urea would reduce the onset of thermal degradation, which implies a reduction in thermal stability. These results were consistent with earlier observations where it was concluded that sodium sulfite and urea reduced cross-links (either physical or chemical) by their combined effect on chain mobility, secondary interactions, and covalent bonds.

5.3.4 Secondary Structure Analysis

It is understood that molecular organization and structural characteristics of polymers influence the material's final properties [13]. The combination of heat, pressure, shear, and chemical additives will affect chain alignment as well as inter- and intra-molecular interactions. The objective of this section was to quantify the relative change in secondary structure due to processing.

I Background

Secondary structural patterns of proteins are characterized by periodic structures such as, helices, sheets and extended portions, as well as a variety of turns, loops and disordered coils [116]. Examples of α-helix and β-sheet are illustrated in Figure 32.

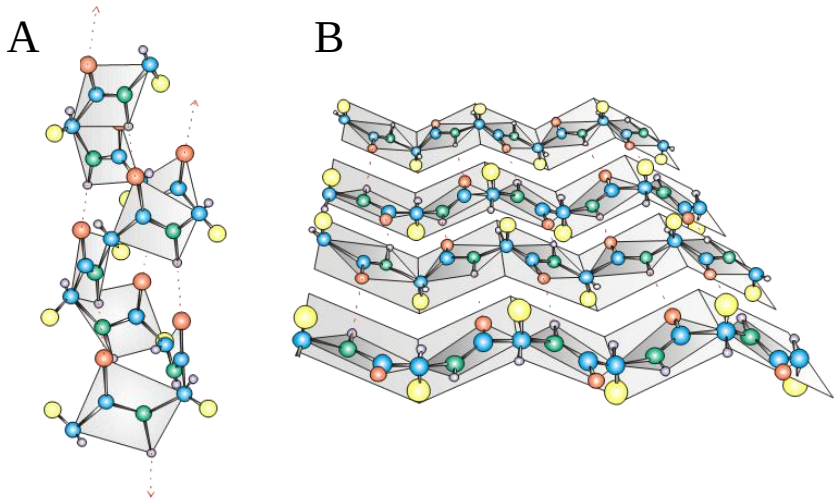


Figure 32: (A) α-helix and (B) β-sheet. (Red lines = hydrogen bonds)

In Table 30 various techniques that can be used to study secondary structures of proteins are shown. A protein's secondary structure is totally dependent on its

surrounding environment and will change with differing environments. It is important to measure secondary structures in a medium that is close to the proteins normal environment.

Table 30: Available techniques for secondary structure determination.

Technique	Sample Requirements
Nuclear magnetic resonance	<70 kDa, highly pure soluble protein (liquid sample) only
X-ray crystallography	Requires highly pure protein and crystal formation
Circular dichroism spectroscopy	Soluble protein (liquid sample) only
FTIR	Not limited by size of protein. Solid or liquid samples

The aim of this section was to investigate the secondary structure of BM and the resulting structures after processing. BM and the produced plastics are mostly insoluble. The only appropriate method available for secondary structure determination was FTIR, using the KBr pellet method.

During FTIR spectroscopy the polypeptide backbone absorbs IR radiation, which excites the vibrational modes of its constituent amide bonds [116]. There are nine characteristic IR adsorption regions, amide A, amide B, and amide I to VII (Table 31). The amide I and II are the most prominent vibrational regions of the protein backbone, with the amide I commonly used for structure analysis [11; 108-110; 116; 117].

Table 31: Characteristic infrared regions of peptide linkages [108].

Designation	Approximate Frequency (cm^{-1})	Description
Amide A	3300	NH stretching
Amide B	3100	NH stretching
Amide I	1600-1700	C=O stretching
Amide II	1480-1575	CN stretching, NH bending
Amide III	1229-1301	CN stretching, NH bending
Amide IV	625-767	OCN bending
Amide V	640-800	Out-of-plane NH bending
Amide VI	537-606	Out-of-plane C=O bending
Amide VII	200	Skeletal torsion

The amide I absorption contains contributions from the C=O stretching vibration of the amide group (about 80%) with a minor contribution from the C-N stretching vibration [110]. Each secondary structure, helix, beta-sheet, beta-turn and unordered give rise to somewhat different C=O stretching frequencies due to their unique molecular geometry and hydrogen bonding pattern [108; 110]. Stronger hydrogen bonding involving the amide C=O, results in a lower electron density in the C=O group causing amide I absorption at lower frequencies [110].

Structures involving extended sheets have shorter and stronger hydrogen bonds compared to helices and will therefore have a lower amide I absorption frequency [110].

Many researchers have assigned secondary structures to specific absorption frequencies within the amide I region. The secondary structure correlations may not apply to all proteins or solvent mediums used. The correlations used in this study are shown in Table 32, which have been suggested as guidelines for FTIR analysis of proteins by Jackson and Mantsch (1995) and also corresponds to other literature [117-119]. Above 1660 cm^{-1} the structural correlations are dipoles and longer distance interactions, making it difficult to assign an exact structure above this frequency.

Absorption peaks of various secondary structures overlap within the amide I region and often result in a featureless absorption spectra [110]. A small change in the maximum frequency of the amide I region can be caused by one or more underlying component peaks increasing in intensity or shifting frequency. To overcome this predicament, mathematical techniques have been employed to increase the resolution, revealing the overlapping peaks of the spectra. Fourier self deconvolution and second derivatives, are the most common techniques used to assign secondary structural peaks. To avoid artifacts due to data processing, only features present in both derivative and deconvolved spectra should be used for quantitative analysis [110].

Table 32: Correlations between common protein structures and amide I frequency [110].

Structure	Amide I frequency (cm^{-1})
Inter-molecular β-sheet	1610-1628
Intra-molecular β-sheet	1625-1640
Unordered	1640-1648
α-helix	1648-1658
3_{10}-helix	1660-1670
β-turns	1675-1695

Once the number and positions of structural features have been estimated using deconvolution and derivative methods, quantitative estimation can be completed using curve fitting. Curve fitting uses a least squares routine attempting to reproduce the experimentally obtained amide I profile by varying the width, height and shape of the component peaks [110]. As long as the same parameters and techniques are used in every analysis, the results can be compared to obtain possible structural changes.

II Structural changes brought about by processing

FITR spectroscopy was used to quantify the secondary structural elements of BM and plasticized BM.

FTIR spectra of proteins can be affected by substances, such as water and urea. Water absorbs strongly in the amide I region at approximately 1640 cm^{-1}, because of the strong hydrogen bonding [108]. In this study, the effect of water was reduced by freeze-drying samples prior to testing and by water vapor background subtraction. Proteins and urea both have strong hydrogen bonding capacity and urea would therefore distort quantitative structural analysis of the BM-polymers. The affect of urea on the BM spectra was further described in the following section.

i *The influence of urea on FTIR analysis*

Urea is an organic compound with a chemical formula $(NH_2)_2CO$ (Figure 33). Urea has a strong absorbance in the amide I region [120]. This and the composite nature of the FTIR spectra make it difficult to distinguish true protein structure from urea signals.

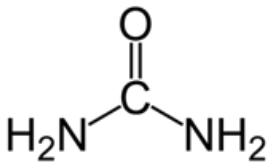


Figure 33: Structure of urea

Non-melt processed mixtures of 5 and 10% freeze-dried urea in freeze-dried BM as well as pure freeze dried urea were prepared and analyzed according to the KBr disc procedure outlined in section 5.2.3II.

The experimentally obtained FTIR spectra of urea and BM are shown in Figure 34 and Figure 35. Urea in KBr absorbs at approximately 1605, 1632 and 1685 cm^{-1} (Figure 34). In theory, these absorbencies would show up as an increase in β-sheet (1626-1640 cm^{-1}) and β-turns (1675-1695 cm^{-1}), as shown in Table 32. The spectra showing the effect of increasing the urea concentration from 0-10% in unprocessed BM can be seen in Figure 35. It can be seen that by adding dry urea to dry BM not only increases the number of peaks in the amide I region, but also widens it.

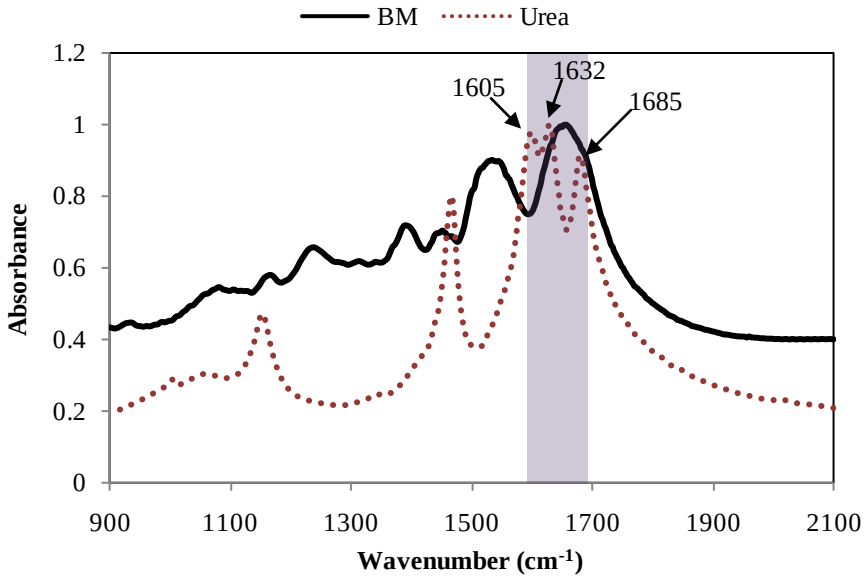


Figure 34: Absorbance spectra of BM and urea (shaded region = amide I)

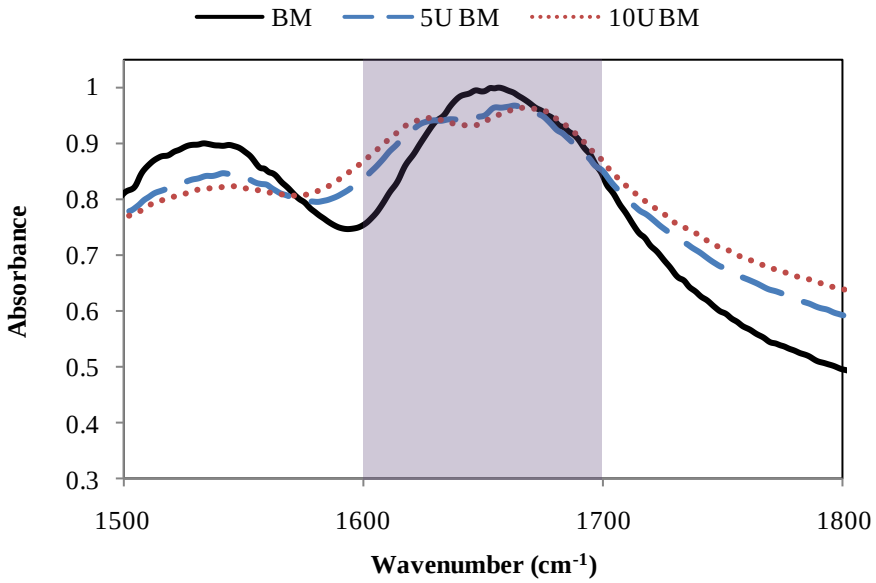


Figure 35: Absorbance spectra for BM alone and unprocessed BM and urea mixtures (shaded region = amide I)

In Table 33 results of the quantitative analysis are shown, more comprehensive tables are shown in Appendix-1D. Increasing urea content resulted in an increase in β-structure content. These results confirmed that urea does affect quantitative results by increasing the β-structures. This was taken into account when analyzing the individual component structural changes of processed samples with urea, from neat BM. However, when considering relative changes between extrusion and injection moulded samples, the urea content did not need to be corrected for.

Table 33: Relative comparison of urea's effect on quantitative secondary structure results.

BM	*Urea*	Helix	Beta Structures	Unordered
100%	0%	29.0%	55.2%%	15.8%
95%	5%	27.9%	56.8%	15.3%
90%	10%	27.3%	57.6%	15.1%

ii *Relative comparison of secondary structure*

The average α-helix content of blood proteins (serum albumin, immunoglobulin and hemoglobin) is about 62%. Where hemoglobin, consist of approximately 75% α-helices [121]. From Table 33 it can be seen that BM contains 29% α-helices indicative of a highly denatured structure. When BM is produced, heating results in protein denaturation and water is evaporated leaving dehydrated polymer chains. The removal of protein-water interactions increases the protein-protein interactions, allowing close packing of protein chains in the beta sheet conformation. Previous studies have investigated the conformational change of native proteins due to thermal energy. In most cases, increases in β-sheet structures at the expense of α-helices were observed [95; 122; 123].

Table 34: Percent difference in grouped ordered structures between extrusion and injection moulding.

	L2	3	60	3	20
	L1	1	45	0	10
Exp #		**Sodium Sulfite (pph_{bm})**	**Water (pph_{bm})**	**SDS (pph_{bm})**	**Urea (pph_{bm})**	**Δ Ordered**
1		1	1	1	1	4.1%
2		1	1	2	2	2.6%
3		1	2	1	2	1.6%
4		1	2	2	1	4.1%
5		2	1	1	2	0.1%
6		2	1	2	1	1.8%
7		2	2	1	1	-2.8%
8		2	2	2	2	-3.0%

Initially each secondary structure type was quantified individually, but changes as a result of varying additive levels were too small to allow an assessment of their effect on structure. However, it was found that compared to neat BM there was a decrease in helix and β-sheet accompanied by an increase in β-turns and unordered structures when using the additives, as outlined in Appendix-1E Table 51. Because of these small changes and uncertainty of structural assignments above 1660 cm^{-1}, the ordered structures were considered together, as helices, β-sheets and β-turns (Table 34).

It can be seen from Table 34 that generally there was an increase in ordered structure after injection moulding. The only exception to this observation was experiments 7 and 8 where sodium sulfite and water were used at level 2.

The difference in ordered structure between injection moulded and extruded samples was calculated using Equation 2 (X_i = injection moulded ordered structure, X_o= extruded ordered structure). The main effects of the additives used are presented in Figure 36 while the ANOVA results are presented in Table 35.

From Table 35 it can be seen that sodium sulfite was the most significant factor. Ordered structures were reduced by the combined action of sodium sulfite at level 2 and the more aggressive conditions of injection moulding. When excessive covalent disulfide bonds are present with SS at level 1, chain mobilization is restricted, forcing chains to form more thermally stable localized interactions leading to a more ordered structure.

From Figure 36 it can be seen that by increasing urea concentrations the amount of ordered structure is also reduced. Urea preferentially binds to protein chains preventing protein-protein or protein-water hydrogen bonding. Helix and β-sheet structures rely on hydrogen bonding to hold them together. Urea may break these bonds resulting in more unordered structure.

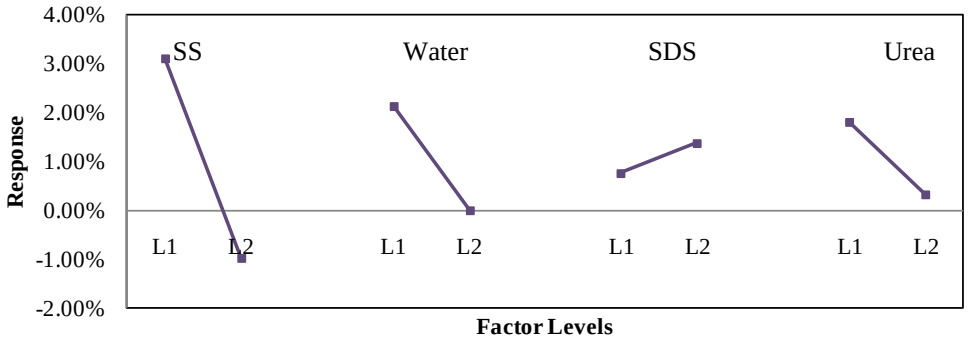
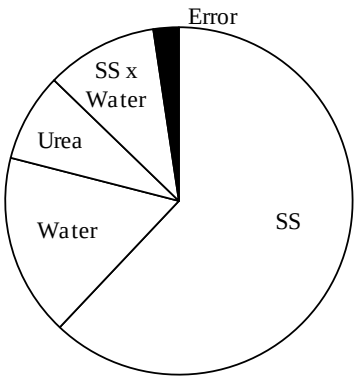


Figure 36: Main effects of SS, water, SDS, urea on the change in ordered structure from extrusion to injection moulding.

Table 35: ANOVA of the main effects influencing change in ordered structure from extrusion to injection moulding.

	DOF	***F***	***Percentage Contribution***
SS	1	78.8	62.0%
Water	1	21.5	17.0%
SDS	Factor Pooled		
Urea	1	10.5	8.2%
SS x Water	1	13.2	10.4%
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Error	3		2.4%
Total	7		100%



$F_{1,3} = 5.5383$

Results concerning water absorption and solubility pointed out the importance of the combination of sodium sulfite and water. Sodium sulfite at level 2 and water at level 1 led to degradation as opposed to chain mobilization. This was further supported by the trends shown in Figure 37. When using sodium sulfite at level 2 and water at level 1, materials had a highly ordered structure compared to using sodium sulfite and water both at level 2. Furthermore, at higher water content, the plasticization effect of water will increase free volume and chain mobility leading to a more unordered structure after injection moulding.

When water was used at level 1 in combination with SS at level 2, degradation was observed as opposed to chain rearrangement. Helical conformations are one of the most thermodynamically stable structures. Short peptides, as a result of degradation, can also form local hydrogen bonds, resulting in the thermodynamically stable helix structure. The overall result was an observed increase in ordered structure at level 1 water (Figure 37).

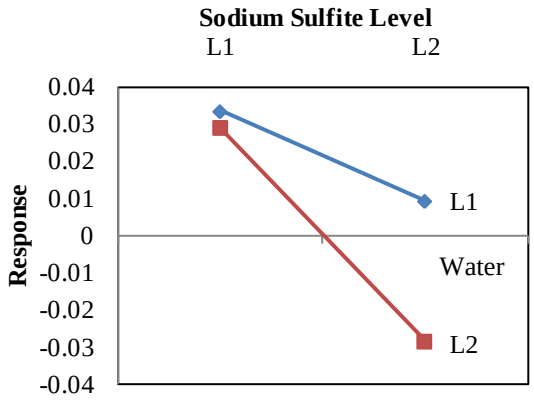


Figure 37: Main effects of sodium sulfite and water interaction on the difference in ordered structure from extruded to injection moulded materials.

The formation of ordered structures requires stabilization by secondary interactions, like hydrogen bonding. Increased chain mobility and decreased secondary interactions will therefore favor unordered structures. The increase in unordered structure when using sodium sulfite and water at level 2, may explain the increase in water absorption, because less chain entanglements and interactions can occur between chains.

Structural changes due to processing were small compared to change in secondary structure of native blood proteins upon drying. The chemical additives had an influence on reducing ordered structures by altering the inter- and intra-molecular interactions. Injection moulded samples with sodium sulfite and water at level 2

was highly plasticized and was the only samples with decreased ordered structure after injection moulding. Increased plasticization resulted in an increase in chain mobility and reduction in protein-protein interactions, therefore reducing the amount of ordered structures. This change in macromolecular interactions increased the processability, consolidation, water absorption and solubility of the BM-plastics as observed in this study.

5.4 Conclusions

The aim of this study was to investigate structural changes and inter/intra molecular interactions as a result of thermoplastic processing of BM. Extrusion and injection moulding were used to produce biopolymers based on BM and appropriate chemical additives.

Processability and consolidation was highly dependent on chain mobility which was required for new interactions and chain alignment during processing. For effective processing and consolidation of BM the following additives were required: 3 pph_{bm} sodium sulfite, 3 pph_{bm} SDS, 20 pph_{bm} urea and 60 pph_{bm} water.

Sodium sulfite and SDS were the most important factors leading to increased water absorption and solubility after processing. Increased water redness was observed after injection moulding which was attributed to the interaction between sodium sulfite and water. Protein degradation occurred at the combination of high sodium sulfite and low water levels compared to chain mobilization at higher water contents.

Plasticized polymers had a less ordered structure compared to BM. However, an increase in order was observed between injection moulded samples and extruded samples, except when SS and water were both at higher levels. Sodium sulfite, water and their interaction were the most important factors affecting structural changes. In the absence of sufficient plasticizers, the addition of SS may also of led to degradation during processing, as opposed to melt formation. It was concluded that the increase in processability, consolidation, water absorption and solubility was due to changes in inter- and intra-molecular interactions, rather than substantial structural changes.

Successful processing of proteins using extrusion requires increased chain mobility. This was achieved with BM by using sodium sulfite, water, SDS and urea at level 2. At this composition inter- and intra-molecular interactions were

reduced shown by the increase in processability, consolidation, and water absorption. This reduction in macromolecular interactions resulted in decreasing the amount of ordered structures.

Chapter 6: Mechanical Properties

Summary

Bloodmeal mixtures containing sodium sulfite (SS), water, sodium dodecyl sulfate (SDS) and urea were extruded and injection moulded. Tensile strength, Young's modulus, elongation at break, toughness and impact strength of moulded specimens were measured before and after 1 week of conditioning at 23 °C, and 50% relative humidity.

During conditioning the water content was reduced to around 10%. Increased chain mobility with sufficient plasticization during processing increased the amount of available amino acids for strong water-protein interactions. The remaining water in the material after conditioning had no obvious plasticizing effect, therefore relying on the secondary plasticizers urea and SDS. However the original water content and its interaction with SS during processing had an adverse effect on the conditioned elongation, toughness and impact results.

Low water content resulted in low chain mobilization ultimately leading to protein degradation during processing. Materials containing low water and increased SS had reduced tensile strength and elongation, compared to higher water at the same SS content.

Materials containing 3 pph_{bm} sodium sulfite, 60 pph_{bm} water and 20 pph_{bm} urea, were the only ductile materials after conditioning. Changing any one of these factors to a lower level will result in a brittle material. This mixture in combination with 3 pph_{bm} SDS resulted in optimal mechanical properties (tensile strength of 9.6 MPa and 536 MPa for Young's modulus) comparable with low density polyethylene.

6.1 Introduction

In the previous section it was revealed that non-covalent and covalent bonds must be reduced to improve melt flow and chain rearrangements during extrusion and injection moulding. This was achieved by judicial use of sodium sulfite, water, urea and SDS.

Structural characteristics of polymers are known to influence its mechanical properties [19]. A single protein chain may contain up to 20 different monomer units leading to large differences in properties between protein-based materials. Heat, pressure, shear and chemical additives used for processing protein-based materials will affect molecular interactions and thereby its mechanical properties.

The aim of this section was to investigate the effects of chemical additives on the mechanical properties of bloodmeal-based bioplastics. Samples were compounded using twin screw extrusion and injection moulded into appropriate test specimens. Specimens were conditioned for 7 days at 23 °C and 50% relative humidity and the mechanical properties were measured before and after conditioning.

6.2 Experimental

6.2.1 Materials

Table 36: Materials used

	Supplier	**Grade**	
Bloodmeal (BM)	Taranaki Byproducts		ρ = 1300 kg/m^3 Sieved to 700 μm
Sodium dodecyl sulfate	Biolab	Technical	
Sodium sulfite	BDH Lab Supplies	Analytical	
Urea	Agrinutrients-Balance	Agricultural	

6.2.2 Sample Preparation

Various amounts of sodium sulfite, urea and SDS were dissolved in distilled water by mixing and heating to 60°C. Table 37 lists the individual experiments where the compositions specified are based on parts per hundred bloodmeal. The resulting solutions were mixed with sieved blood meal in a high speed mixer for at least 5 minutes. During this stage, the proteinous mass absorbed all the water and some denaturing occurred.

The blends were compounded in a ThermoPrism TSE-16-TC twin-screw extruder at 150 rpm with temperature settings of 120, 100, 100, 100, 70 °C starting from the die. The extruded material was allowed to cool to room temperature (approximately 1 hour).

The extrudate was granulated and injection molded into Type 1 tensile test specimens using a BOY15-S injection-molding machine with temperature settings of 100, 115, 120 °C starting at the feed end. The mould itself was heated to 65 °C. Tensile test specimens were conditioned for 7 days at 23 °C, 50% RH.

6.2.3 Analysis

I Moisture Content

Moisture content was determined for extruded and injection moulded samples, before and after conditioning. Granulated samples were weighed into aluminum dishes and dried in an air-circulating oven at 100 °C for 12+ hours. The moisture content was determined by subtracting the dry weight from the initial weight. These measurements were done in triplicate for each experiment.

II Mechanical Properties

Tensile strength, elongation at break, Young's modulus and fracture toughness of injection-moulded tensile specimens were analyzed according to the ASTM D638-86 method. For each experiment five specimens were conditioned at 23 °C and 50% relative humidity for 7 days. Five conditioned and five unconditioned samples were tested at 5mm/min crosshead speed, using an Instron model 4204.

III Statistical Analysis

A L16 full factorial experimental design was devised using the Taguchi method [104] outlined in Table 37. Orthogonal arrays were used as described in previous chapters. Analysis of variance (ANOVA) was used to quantify the relative influence of each factor and interaction, at a 95% confidence interval.

Table 37: Factorial Design of Experiments.

Factor and Level			**Experiment Number**															
L1	**L2**		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
1	3	**Sodium sulfite (pph_{bm})**	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2
45	60	**Water (pph_{bm})**	1	1	2	2	1	1	2	2	1	1	2	2	1	1	2	2
0	3	**SDS (pph_{bm})**	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
10	20	**Urea (pph_{bm})**	1	2	1	2	2	1	2	1	2	1	2	1	1	2	1	2

6.3 Results and Discussion

Table 38 lists the results for tensile strength, Young's modulus, elongation at break, toughness, and impact strength before and after conditioning. For comparative reasons the processability, consolidation, water absorption and solubility results for all 16 experiments are listed in Appendix 2A, Table 52.

Table 38: Results obtained for experiments 1 through 16.

Exp #	Unconditioned						Conditioned						
	Moisture Content (wt %)	**Young's Modulus (MPa)**	**Tensile Strength (MPa)**	**Elongation at Break (%)**	**Toughness ($MPa.m^{½}$)**	**Impact Strength (kJ/m^2)**	**Moisture Content (wt %)**	**% Water Change***	**Young's Modulus (MPa)**	**Tensile Strength (MPa)**	**Elongation at Break (%)**	**Toughness ($MPa.m^{½}$)**	**Impact Strength (kJ/m^2)**
1	23.6	167	3.7	11.3	0.32	25.9	6.8	-71.1	1847	27.4	1.8	0.27	0.74
2	23.5	51	2.7	39.2	0.81	33.1	7.5	-68.0	705	12.4	2.8	0.22	0.73
3	26.5	58	2.5	29.0	0.55	29.0	11.7	-55.8	1548	24.3	2.1	0.30	0.91
4	24.2	18	1.7	43.1	0.50	20.9	12.7	-47.6	515	8.9	3.0	0.19	0.55
5	22.0	33	2.1	38.8	0.58	31.5	8.1	-63.2	719	12.1	3.4	0.30	0.71
6	23.1	89	3.1	26.0	0.64	34.0	7.2	-68.6	1543	19.7	1.5	0.16	0.84
7	27.9	19	1.8	42.6	0.50	19.8	12.6	-54.6	652	11.5	2.8	0.21	0.54
8	31.3	46	1.9	27.0	0.40	30.2	11.8	-62.4	1259	19.0	1.9	0.21	0.64
9	17.8	60	2.9	33.7	0.74	36.5	9.7	-45.7	530	7.0	1.7	0.07	0.45
10	21.2	172	4.1	15.2	0.49	41.8	9.0	-57.7	1354	17.3	1.7	0.16	0.50
11	26.6	16	1.7	45.3	0.54	16.1	11.6	-56.3	673	12.3	9.9	0.89	0.91
12	27.3	40	2.3	46.7	0.83	31.5	10.9	-60.0	1539	26.3	2.3	0.37	0.77
13	22.7	230	4.8	14.1	0.55	4.1	8.6	-62.1	1469	19.5	1.7	0.19	0.57
14	21.3	59	2.5	28.4	0.55	37.9	9.7	-54.3	508	5.8	1.4	0.05	0.45
15	26.7	56	2.8	46.0	1.01	31.3	11.5	-56.9	1693	26.9	2.1	0.33	0.97
16	26.3	17	1.6	49.3	0.54	13.1	12.0	-54.4	536	9.6	12.1	0.94	0.87

*Calculated using Equation 3 discussed in next section.

6.3.1 Moisture Content

Plasticizers are critical for biopolymer processing of which water is one of the most effective plasticizers [19; 83]. The low molecular mass of water enables plasticization, but will evaporate overtime losing its effectiveness [82]. Under ambient conditions, only water molecules that have strong interactions with protein side-chains will remain in the material as bound water.

From Table 38 it observed that the chemical additives influenced water content during conditioning. Before conditioning moisture contents ranged from 17.8% to 31.3% of the materials total weight (Table 38). After conditioning water content was reduced to approximately 10%.

Analysis of variance was performed on the water content after conditioning and the results are shown in Appendix-2B (Figure 52 and Table 53). Water and the

interaction between sodium sulfite (SS) and water were the most influencing factors, 81.5% and 9.96% respectively. As expected, materials containing water at level 2 had more water after conditioning than level 1.

Figure 38 illustrates the interaction between SS and water on the water content of conditioned materials. This supports the fact that samples with water at level 2 will contain more bound water after conditioning. However, increasing the sodium sulfite content, with water at level 1 caused an increase in bound water. From the previous chapter it was concluded that using water at level 1 and SS at level 2 resulted in possible protein degradation. It was concluded that protein degradation led to a reduction in average chain length which resulted in more mobile chains (Chapter 5). The increased mobility enables the formation of the thermally stable helical conformation, exposing side-chains that may form strong interactions with water.

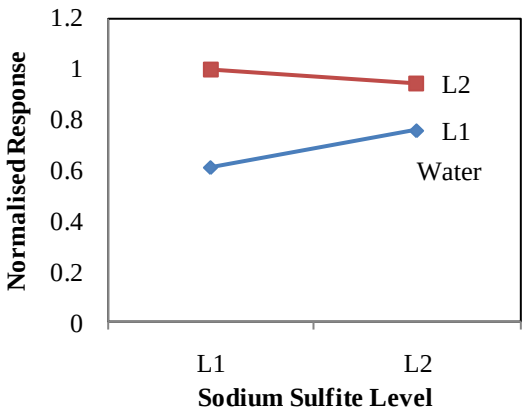


Figure 38: Main effects of sodium sulfite and water interaction on water content of conditioned materials.

The water loss during conditioning was different for each experiment. The % water loss was calculated using Equation 3 (X_i=the conditioned water content and X_o=original water content). The ANOVA results are shown in Figure 39, Table 39 and Figure 40.

$$\%\, Change \;=\; (Xi - Xo)/Xo$$

Equation 3: % water loss during conditioning.

From Table 38 it can be seen that unconditioned specimens containing water at level 2 had higher moisture content, which is to be expected. The rate of diffusion is driven by concentration gradients. Under ambient conditions a material with more water has a higher concentration gradient. Therefore samples containing water at level 2 were expected to have a higher percentage water loss. However,

the percentage water loss during conditioning was reduced when more water was used in the formulation and is explained further below.

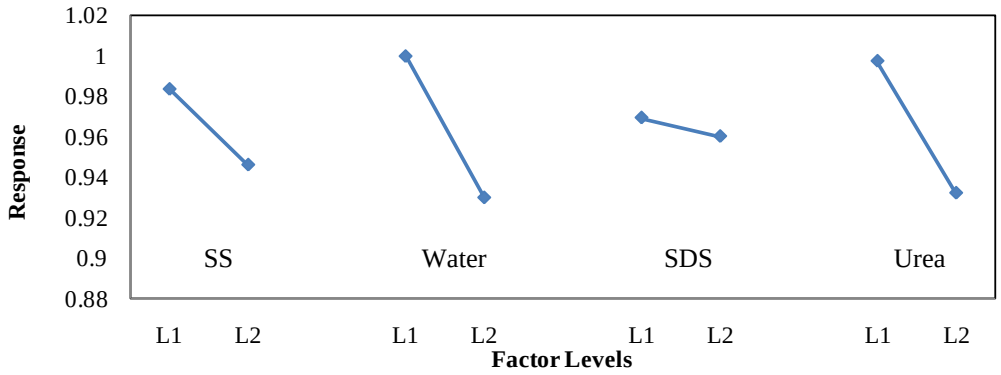
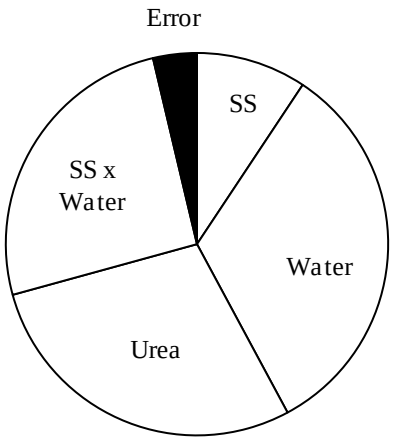


Figure 39: Main effects of SS, water, SDS, urea on the % water loss between original to conditioned materials.

The effect of sodium sulfite, water, urea, and the interaction between sodium sulfite and water were found to be statistically significant factors affecting water loss during conditioning. Less water was lost with materials containing sodium sulfite, water and urea at level 2. At this level a higher degree of chain mobilization occurs, which increases the amount amino acid side chains available to form strong interactions with water, thereby increasing the amount of bound water.

Table 39: ANOVA results of the main effects influencing percentage water loss during conditioning.

	DOF	***F***	***Percentage Contribution***	
SS	1	32.1	9.3%	
Water	1	112	32.8%	
SDS	Factor Pooled			
Urea	1	97.7	28.6%	
SS x SDS x Urea	Factor Pooled			
SDS x Urea	Factor Pooled			
SS x Water	1	87.4	25.6%	
Water x SDS x Urea	Factor Pooled			
SS x SDS	Factor Pooled			
SS x Urea	Factor Pooled			
Water x SDS	Factor Pooled			
SS x Water x SDS	Factor Pooled			
SS x Water x Urea	Factor Pooled			
Water x Urea	Factor Pooled			
Error	11		3.7%	
Total	15		100%	$F_{1,11} = 4.8443$



Sodium sulfite alone did not have as such a large influence compared to its interaction with water (Table 39). From Figure 40 it can be seen that water loss was larger in the materials containing sodium sulfite at level 1 and water at level 1 (-73%). This confirmed that water and sodium sulfite at level 2 allowed more chain mobility during processing, unraveling and exposing amino acids to strong protein-water interactions. However, only when the sodium sulfite level was increased at low water content, there was a relative decrease in water loss due to increased bound water from protein degradation.

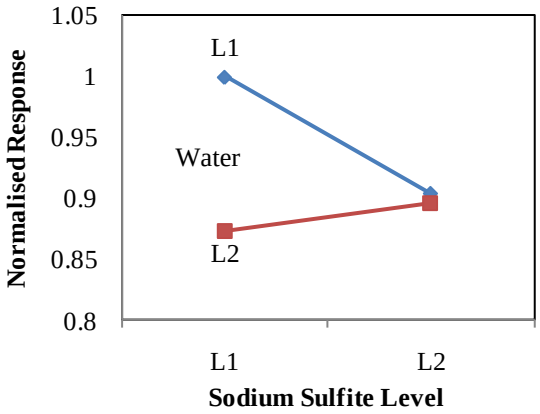


Figure 40: Main effects of sodium sulfite and water interaction in the % water loss from original to conditioned materials.

Water was essential for increasing processability through promoting chain mobility, but was only possible in the presence of sodium sulfite. Acting as a plasticizer, it will also reduce the tensile strength and Young's modulus. Therefore, samples with water at level 1 were expected to have lower elongation and higher tensile strengths than samples processed with water at level 2. After conditioning the water content for all samples was approximately 10%. Therefore, samples only differing in the original water content should have the same mechanical properties. This is explored in more depth in the next section.

6.3.2 Mechanical Properties

Tensile strength, Young's modulus, elongation at break, toughness and impact strength were analyzed for unconditioned and conditioned materials using ANOVA. The main effects and percentage contributions of the various factors are shown in Appendix-2D, and Appendix-2E.

I Unconditioned

Figure 41 shows the main effects for statistically significant factors on the mechanical properties tested. The percentage contribution of the factors is summarized in Table 40. For unconditioned materials, it was found that water and urea were the most important factors influencing tensile strength, Young's modulus and elongation. Increasing either urea or water, decreased tensile strength and Young's modulus, but increased the elongation at break (Figure 41). Therefore, both water and urea acted as plasticizers in the unconditioned materials.

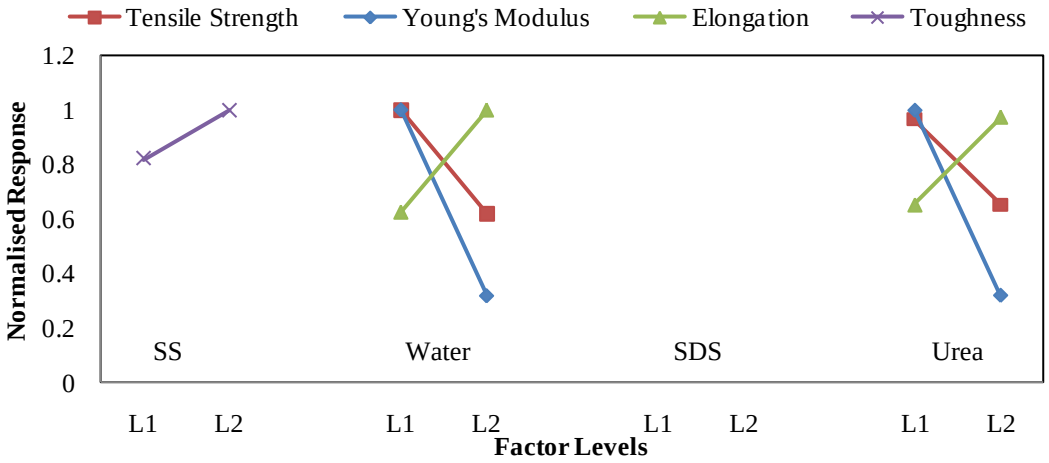


Figure 41: Main effect of SS, water, SDS, urea for unconditioned tensile strength, Young's modulus, elongation and toughness.

Table 40: Summary of the percentage contribution of the statistically significant factors influencing mechanical properties.

	Percentage Contribution				
Factor	**Tensile Strength**	**Young's Modulus**	**Elongation at Break**	**Toughness**	**Impact Strength**
SS				11.7%	
Water	45.6%	34.4%	39.2%		
SDS					
Urea	30.5%	34.0%	28.6%		
SS x Water			11.7%	13.0%	
SS x SDS				8.2%	
SS x Urea				13.0%	
Water x SDS				4.7%	
SS x Water x SDS				7.5%	
SS x Water x Urea				5.3%	
Water x Urea		9.4%		26.1%	25.0%
Error	24.0%	22.2%	20.5%	10.5%	75.0%%

Good impact strength occurs at a degree of intermolecular interaction where sufficient chain slippage can occur in order to absorb energy during an impact. The individual influence of urea and water on impact strength was not significant, whereas their interactive effect was. Figure 42 illustrates the interaction between water and urea on the impact strength of unconditioned materials. Three combinations of urea and water were possible: both factors at level 1, one at level 2 or both at level 2. It is known that urea and water influence hydrogen bonding between proteins, therefore, by using both these factors at level 2 led to excessive reduction in protein-protein interactions resulting in lower impact strength. When both factors were at level 1, excessive hydrogen bonding between chains led to embrittlement and low impact strength. Using urea at level 1 and water at level 2 was preferred to avoid protein degradation and to optimize impact strength. This was also observed in the unconditioned toughness, discussed further below.

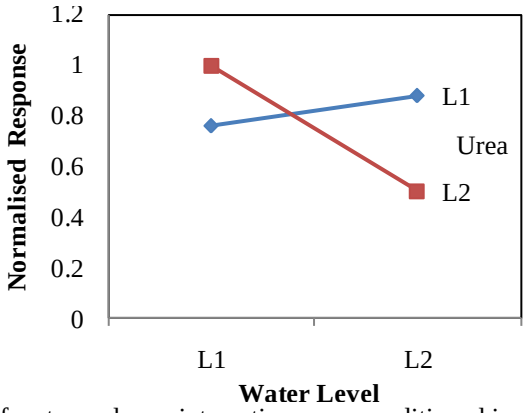


Figure 42: Main effects of water and urea interaction on unconditioned impact strength.

It was found that the interaction between sodium sulfite and water significantly influenced the elongation at break of the specimens tested. It was said earlier that protein degradation may occur when SS is used at level 2 and water at level 1. It can be seen from Figure 43 that, at low water content, elongation was reduced when increasing sodium sulfite. If protein degradation occurred, the shorter average chain length would therefore led to less physical chain entanglements required for high elongations seen in polymers. More disulfide bonds were reduced when using SS and water at level 2. Chain slippage was now not restricted by cross-links, thereby enabling relative chain movement upon the application of stress, the result of which was an increase in elongation.

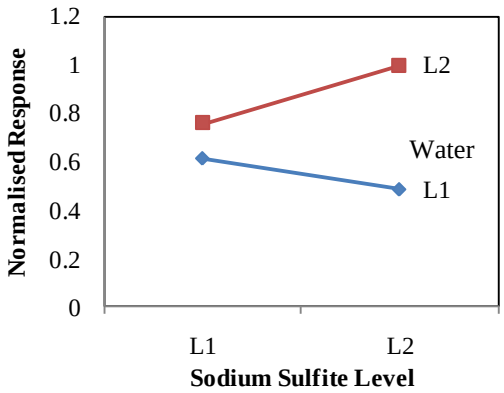


Figure 43: Main effects of sodium sulfite and water interaction on unconditioned elongation.

Toughness of a material, similar to impact strength, is the amount of energy absorbed up to the point of rupture. Figure 41 shows that water and urea had opposite effects on tensile strength and elongation, therefore the effect of these factors on toughness was very small. However, the toughness of unconditioned material was reliant on plasticizer content, as evident by the importance of the numerous interactions between urea, water and SS (Table 40). Because urea competes for hydrogen bonding with protein chains, urea at level 1 in combination with SS or water at level 2 resulted in a tougher material, due to the increased hydrogen bonding between chains at the lower urea level (Appendix-2D, Figure 58).

Sodium sulfite was also important by itself and in interactions with the other additives. Materials containing sodium sulfite at level 2 had a higher toughness. Previous structural analysis of the BM-plastics showed that sodium sulfite at level 2 in addition to level 2 of water and urea, resulted in high chain mobility and flow, allowing unfolding and alignment of protein chains during processing. The opportunity for more secondary interactions to occur between chains was increased at level 2 SS, resulting in a tougher material.

In the previous chapter it was evident that samples containing sodium sulfite and SDS at level 2 had the most favorable consolidation and water absorption results. Figure 44 illustrates the stress-strain graphs for materials containing SS and SDS at level 2, with varying amounts of water and urea. This allows one to compare the effect of the plasticizers on the mechanical properties.

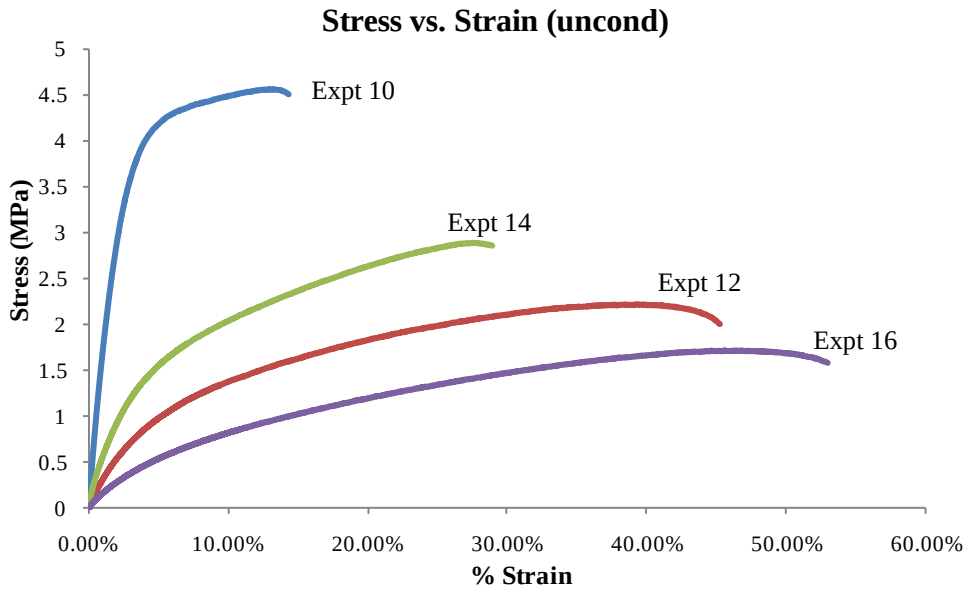


Figure 44: Stress vs. Strain graph for a selected specimens of unconditioned materials.
Exp 10: 3 SS, 45 W, 3 SDS, 10 U
Exp 12: 3 SS, 60 W, 3 SDS, 10 U
Exp 14: 3 SS, 45 W, 3 SDS, 20 U
Exp 16: 3 SS, 60 W, 3 SDS, 20 U

It can been seen from the stress-strain graph that increasing water or urea led to a reduction in tensile strength, but increased elongation due to the plasticizing effect of both these additives. At low water and urea contents (exp 10) some strain hardening occurred, while highly plasticized-protein bioplastics showed a non-linear stress-strain behavior very similar to synthetic polymers.

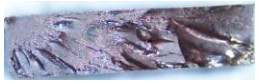
For unconditioned materials both plasticizers strongly influenced the mechanical properties of the bioplastics. Proteins have many different amino acid side chains available to form secondary interactions, leading to brittle materials in the solid state. Therefore plasticization was critical to form a continuous, cohesive network from powdered raw materials [13]. Water will evaporate overtime negatively influencing some of the mechanical properties [81].Conditioning the materials could therefore reveal more of the structure-property relationships and is discussed in the next section.

II Conditioned

During conditioning, water evaporates from the BM materials, allowing new secondary interactions to occur between chains. Most of the plastics tested (Table 38) became brittle after conditioning, except for experiments 11 and 16, which contained sodium sulfite, water and urea at level 2. The only difference between these samples was the amount of SDS used. Facture surfaces for a selection of specimens are shown in Table 41, while a more comprehensive list can be found

in and Table 54 (Appendix-2C). From these images the dramatic change from ductile to brittle after conditioning is clearly visible, evident from the typical topography observed for brittle and ductile fractures. Ductile fractures showed plastic deformation, with observed dimples or tearing on the fracture surface, evidence of microvoid coalescence. Whereas brittle materials had flat, faceted fracture surfaces, with radial fan ridges originating from a notch. It was concluded that SDS contributed to the retention of ductile properties by inhibiting hydrophobic interactions, even after water has evaporated, enabling some degree of chain mobility.

Table 41: Facture surface of processed and conditioned tensile specimens

	10	12	14	16
UC*				
C**				

*UC = unconditoned ; **C = conditioned

Figure 45 and Table 42 summarize the ANOVA and main effects of statistically significant factors influencing the mechanical properties of conditioned materials (more detail is shown in Appendix-2E).

After conditioning, the average equilibrium moisture content was found to be about 10 wt%. At this level, urea and SDS can be considered to be the primary plasticizers. By increasing either the SDS or urea content, both tensile strength and Young's modulus were reduced. However, elongation was strongly influenced by the plasticization effect of urea.

Although it was shown that higher levels of SDS led to more ductile materials, it has to be acknowledged that SDS was present at only low concentration (3 pph_{bm}). Its effect on tensile properties was therefore small only because the brittle-ductile transition was not directly measured. The highest toughness was achieved in materials that showed a ductile fracture surface (exp 11 and 16). However, ANOVA revealed that water and SS were the two most important factors. It can therefore be concluded that water and SS are required for the reduction of cross-links and for the initial chain rearrangement. SDS acted as a secondary plasticizer, promoting the effect of urea by inhibiting hydrophobic interactions between protein chains. Used together they acted like an amphiphilic plasticizer.

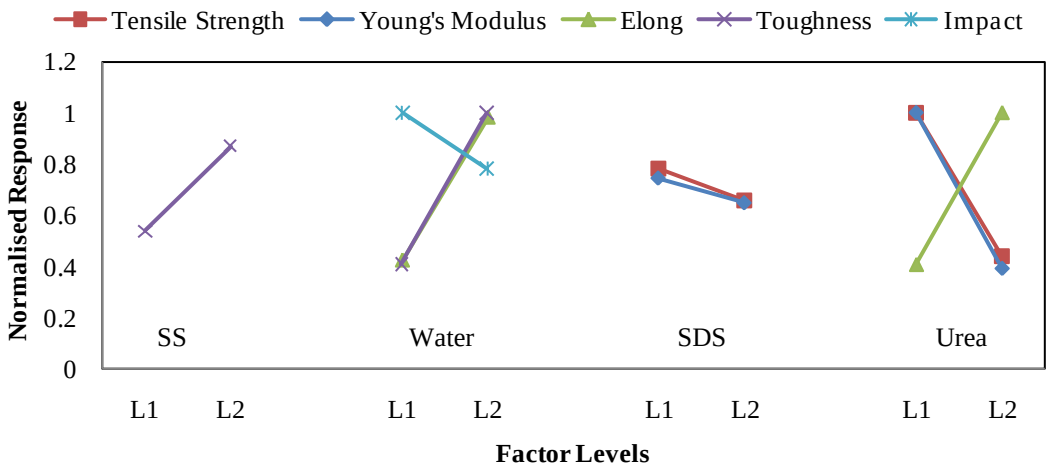


Figure 45: Main effect of SS, water, SDS, urea for conditioned tensile strength, Young's modulus, elongation and toughness.

Table 42: ANOVA of the main effects influencing mechanical properties of conditioned materials.

	Percentage Contribution				
Factor	**Tensile Strength**	**Young's Modulus**	**Elongation at Break**	**Toughness**	**Impact Strength**
SS				7.3%	
Water			18.2%	25.5%	17.1%
SDS	3.8%	2.3%			
Urea	79.6%	92.1%	20.6%		
SS x Water	8.7%	3.4%	16.6%	27.1%	50.0%
SS x Water x Urea			15.6%	14.6%	
Water x Urea			11.9%	7.5%	
Error	8.0%	2.2%	17.1%	2.2%	32.9%

Extensive secondary interactions between chains will exclude energy absorption by means of chain slipping, resulting in brittle fracture. At these conditions, a higher tensile strength and Young's modulus was observed, but was accompanied by a decrease in elongation. Experiments 12 and 16 are good examples. These materials contained SS, water and SDS at level 2, and urea at level 1 and 2 respectively. At level 1 urea, melt flow and chain re-arrangement will occur to a lesser extent and more hydrogen bonds are possible between chains. It was found that experiment 12, with urea at level 1, had a lower elongation at break, but a higher tensile strength in both unconditioned and conditioned materials.

From the ANOVA water was shown not to have an influencing affect on the tensile strength or Young's modulus. This was expected, since conditioning resulted in very similar water contents. However, it did have a significant influence on elongation, toughness and impact strength. Increasing the original

water content increased the conditioned materials elongation, toughness and impact strength. The interaction between sodium sulfite and water also resulted in improved properties with sodium sulfite and water at level 2. These results are discussed in more detail in the following discussion of Figure 46.

Figure 46 illustrates the stress-strain graphs for materials containing SS and SDS at level 2, with varying amounts of water and urea. Unlike the unconditioned materials, the actual water content in these samples only varies by a small amount. It is assumed the conditioned water content formed strong water-protein interactions and will have little plasticizing effect on the mechanical properties. Experiments 10 and 12 contain urea at level 1 whereas experiment 14 and 16 contain urea at level 2. Therefore implying urea is the only varying factor after conditioning. Yet there is a dramatic difference in properties within each similar pair (Figure 46).

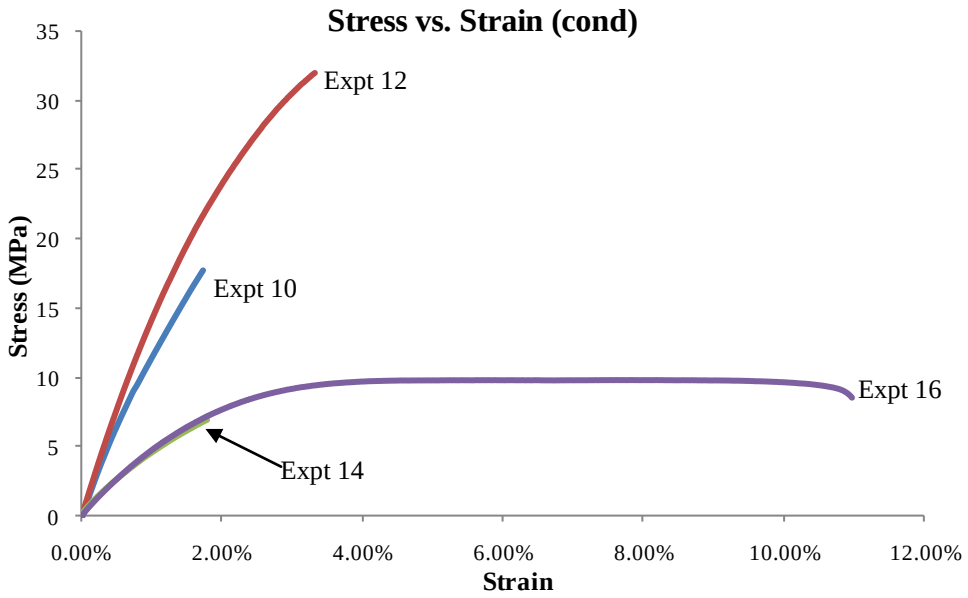


Figure 46: Stress vs. Strain graph for selected conditioned materials.
Expt 10 = 3 SS, 45 W, 3 SDS, 10 U
Expt 12 = 3 SS, 60 W, 3 SDS, 10 U
Expt 14 = 3 SS, 45 W, 3 SDS, 20 U
Expt 16 = 3 SS, 60 W, 3 SDS, 20 U

Both experiments 10 and 14, which were processed with water at level 1, had a reduced tensile strength and elongation at break when compared to the corresponding treatments (experiment 12 and 16). This opposes the theory that at lower plasticizer content there should have been an increase in tensile strength. It can be seen from Table 41 and Figure 46 that the conditioned specimens of experiment 14 resulted in brittle fracture, lower tensile strength and elongation compared to that of experiment 16. There was only 2% difference in water

content after conditioning between these materials and all the other additives were the same. This further highlights the interaction between sodium sulfite and water during processing.

The previous chapter water absorption and solubility tests revealed that SS at level 2 and water at level 1 caused protein degradation during processing. Table 43 and Figure 47 show the water absorption and solubility results for these experiments. The deep red color, high water absorption and solubility of experiments 10 and 14, compared to 12 and 16 indicate protein degradation. As mentioned earlier, protein degradation led to decreased chain lengths which are unable to absorb energy, resulting in lower tensile strengths and elongations (Table 44).

Table 43: Water absorption and solubility results for experiments 10, 12, 14 and 16.

Expt #	Water Absorption %	% Solubility
10	717.4	25.3
12	582.8	20.1
14	805.3	29.6
16	737.5	26.0

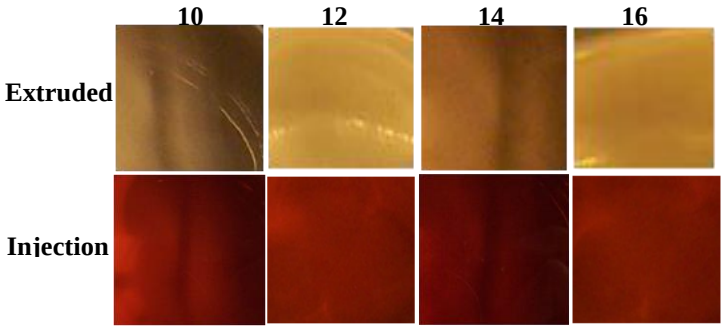


Figure 47: Change in redness of water absorption and solubility tests for extruded and injection moulded samples.

The results of the conditioned materials have further confirmed that water at level 2 was essential for processability, and was required for the correct action of sodium sulfite at level 2. This, in combination with urea and SDS at level 2, resulted in a material that was ductile after conditioning. The ductility resulted in mechanical properties comparable to commodity plastics, such as low density polyethylene and other agro-polymers (Table 44).

Table 44: Mechanical properties of experiment 16, low density polyethylene (LDPE), Injection moulded soy protein isolate (SPI), and poly-lactic acid (PLA).

		Tensile Strength (MPa)	Elongation (%)	Young's Modulus (MPa)	
Experiment 16		9.6	12.1	536	
LDPE		8-12	600-650	200-400	[124]
Soy Protein Isolate	100:33 (SPI:U)	4	13	131	[66]
Poly-Lactic Acid		10-60	1.5-380	350-2800	[124]

Overall, experiment 16 was considered to be the optimal material. Under tension protein chains will slide past each other until microvoid coalescence occurs (breaking). Urea will preferentially bind to the protein functional groups, disrupting interactions between protein chains and water, resulting in partially unfolded and flexible protein chains [66]. However, to form a ductile material, sodium sulfite and water were also required at level 2, without which melt flow would not occur. Figure 48 reveals the synergistic effect of SS, water and urea, changing anyone of them to level 1, resulted in a brittle material after conditioning.

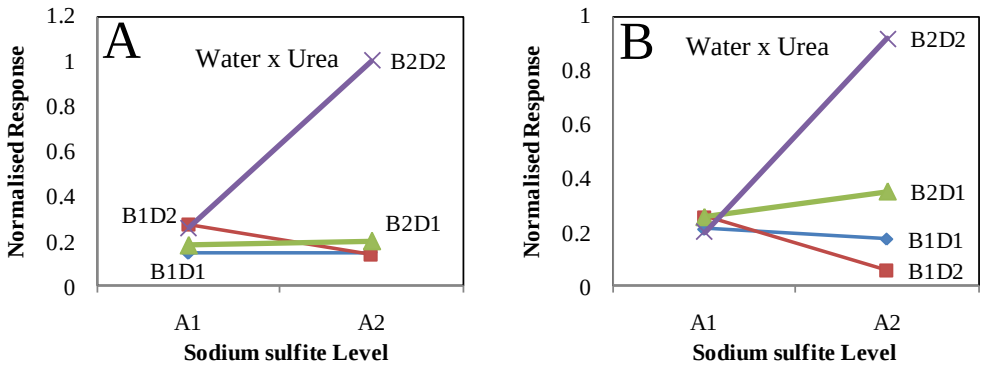


Figure 48: Main effects of the interaction between sodium sulfite, water and urea on the (A) elongation and (B) toughness of conditioned materials.

Ductility after conditioning is not the only important property. Processablitily, consolidation and other mechanical properties should also be taken into consideration. It was found that the addition of SDS was important for the good processability, consolidation, water absorption and solubility, in combination with the other additives. Under these conditions the material's secondary structure will become more unordered than bloodmeal powder, which was required for ductility after conditioning.

6.4 Conclusions

The aim of this study was to investigate the mechanical properties of thermoplastically processed BM treated with different concentrations of sodium sulfite, water, SDS and urea.

Water and sodium sulfite at level 2 resulted in increased chain mobility exposing side chains allowing strong water-protein interactions, resulting in more bound water after conditioning. Water at level 1 and sodium sulfite at level 2 resulted in increased bound water compared to SS at level one. However, this was indicative

of protein degradation during processing, as confirmed through the resulting mechanical properties.

In unconditioned materials, water and urea had the most significant effect, acting as plasticizers. Increasing their content decreased the tensile strength and Young's modulus but increased elongation. Protein degradation led to reduced elongation and toughness of the unconditioned materials, as opposed to chain mobilization with SS and water at level 2. However, urea at level 1 in combination with water and SS at level 2 resulted in a material with higher toughness, due to the greater amount of protein-protein interactions at the lower urea level.

Conditioning of the materials reduced the water content to approximately 10%, resulting in brittle materials due to reduction in plasticizer content. The conditioned water content had no effect on the mechanical properties. However, the original water content had a noticeable effect. The protein degradation that occurred during processing with water at level 1 and sodium sulfite at level 2 resulted in a reduced tensile strength and elongation. The resulting shorter chains were unable to absorb energy and broke more readily than the same treatment with water at level 2.

Ductile materials after conditioning contained sodium sulfite, water and urea at level 2. Changing anyone of these chemical additives to level 1 resulted in brittle materials. In the previous chapter, increased disorder occurred at this level and was therefore required for a ductile response after conditioning.

A material with all the additives at level 2 was found to be ductile after conditioning, and had the best mechanical properties, comparable to low density polyethylene which has a tensile strength of 9.6 MPa and a Young's modulus of 536 MPa.

Chapter 7: Conclusions and Recommendations

The objective of this study was to investigate the use of bloodmeal for the production of bioplastics, focusing on the use of chemical additives to facilitate thermoplastic extrusion.

From literature it was shown that the formation of a homogenous protein melt during extrusion occurs through the following steps: denaturation, dissociation, unraveling and alignment of polymer chains. Processing temperature, specific mechanical energy and plasticization are the most influencing factors during extrusion of protein based materials. High temperatures, high SME and low chain mobility will result in protein degradation. Proteins have high softening temperatures, often above their decomposition temperatures. To avoid degradation, the required chain mobility is achieved by using compatible, low molecular mass and low volatility plasticizers. Depending on the protein's amino acid content, other chemical additives may be required to further increase chain mobility. The final structural and functional properties are highly dependent on the protein and processing conditions requiring proper control to ensure adequate mechanical properties.

Using bloodmeal powder as protein source, product development was undertaken using compression moulding, extrusion and injection moulding to form a suitable bioplastic. It was concluded that extruding bloodmeal bioplastic required plasticization of water and the combined denaturation affects of sodium sulfite, SDS and urea. More specifically, the additives performed the following functions:

- breaking covalent cross-links by using sodium sulfite
- breaking inter and intra-molecular forces, such as hydrophobic forces by SDS, and plasticizing protein chains by using water and urea
- formation of new interactions to stabilize the final structure by evaporating some of the water.

Furthermore, it was shown that the efficiency of these additives could be characterized by:

- Processability. It was shown that successful extrusion required a minimum temperature of about 100 °C and that excessive cross-linking occurred above 130 °C. Extrusion was strongly influenced by plasticizer content and it was shown that water and urea performed a similar function in this regard. Although these would be the most important factors, processing would be impossible without sodium sulfite.
- Consolidation, water absorption and solubility. It was found that the consolidation of the bioplastic was strongly dependant on the amount of SDS which is known to influence hydrophobic interactions in proteins. SDS had to be used in combination with SS to ensure good consolidation. Increasing the SS content also led to increased water absorption, taken as an indication of reduced cross-link density. However without effective water plasticization protein degradation would occur, evident by an increase in ordered structures, due to the formation of helical conformations of the short degraded peptide chains.
- Mechanical properties. It was concluded that water was required for processing, enhancing the action of urea and SDS. The use of water did, however, led to over plasticization, evident from poor mechanical properties. It was found that chain rearrangement resulted in an increase in bound water after conditioning and that after conditioning the mechanical properties increased significantly, indicating the formation of new intermolecular forces. It was found that SDS was also required for processing and consolidation, but, excessive amounts may restrict the formation of new inter-molecular forces.
- Protein conformation. It was found that BM was already highly denatured with a majority of the conformation in β-structures. Successful processing of proteins with thermoplastic extrusion requires increased chain mobility brought about by a reduction in inter- and intra-molecular interactions leading to less ordered protein conformation.

An optimal material formulation contained 3 pph_{bm} sodium sulfite, 60 pph_{bm} water, 3 pph_{bm} SDS and 20 pph_{bm} urea and was shown to have similar mechanical properties to low-density polyethylene. After conditioning the bioplastic had a tensile strength of 9.6 MPa and a Young's modulus of 536 MPa. Depending on the intended application, the additive levels could be adjusted leading to materials that may be, for instance, stronger, but more brittle.

It was found that the evaporation of water from the bioplastic led to embrittlement over time. It is recommended that further study is undertaken to assess various alternative plasticizers to maintain or improve the mechanical properties after conditioning. Viscosity and glass transition measurements along with biodegradability of the plasticized BM should also be a priority in order to prepare this for a marketable product.

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Appendix 1

Appendix-1A

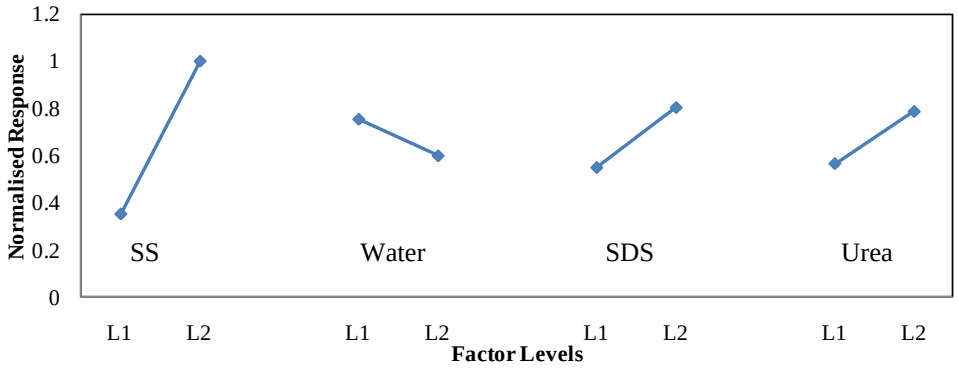
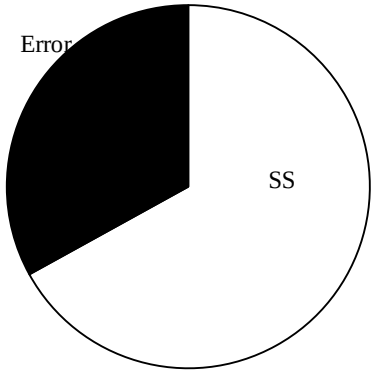


Figure 49: Main effects of SS, water, SDS and urea on injection moulded samples water absorption.

Table 45: ANOVA of the main effects influencing injection moulded water absorption.

	DOF	***F***	***Percentage Contribution***
SS	1	15.20	67.0%
Water	Factor Pooled		
SDS	Factor Pooled		
Urea	Factor Pooled		
SS x Water	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Error	6		33.0%
Total	7		100%



$F_{1,6} = 3.7760$

Appendix-1B

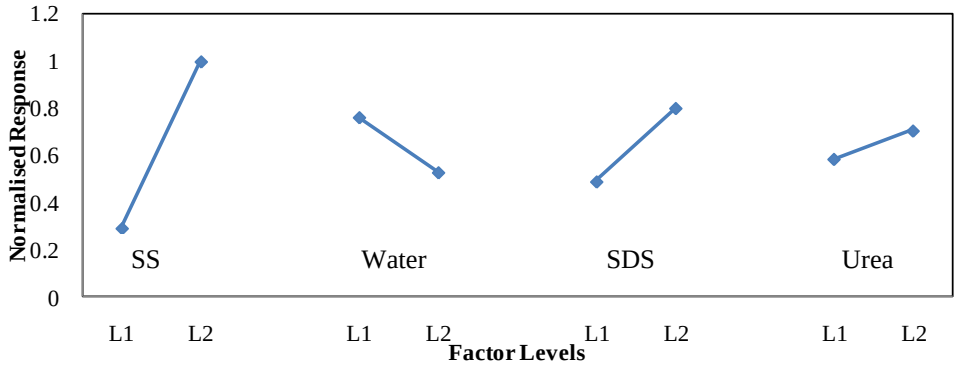
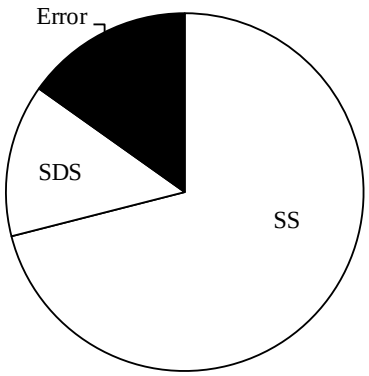


Figure 50: Main effects of SS, water, SDS and urea on injection moulded samples solubility.

Table 46: ANOVA of the main effects influencing injection moulded solubility.

	DOF	***F***	***Percentage Contribution***
SS	1	40.9	71.0%
Water	Factor Pooled		
SDS	1	7.6	13.8%
Urea	Factor Pooled		
SS x Water	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Error	5		15.2%
Total	7		100%



$F_{1,5} = 4.0604$

Appendix-1C

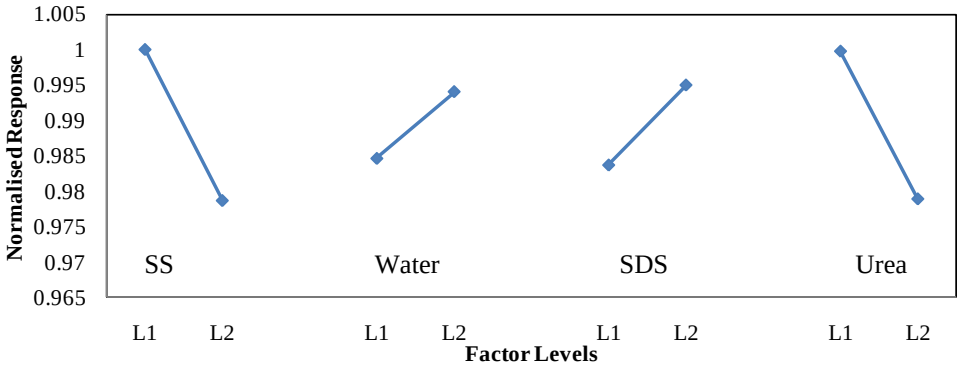
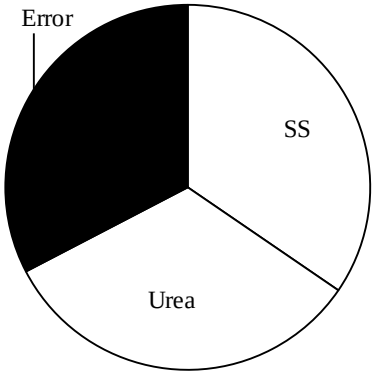


Figure 51: Main effects of SS, water, SDS and urea on position of TGA derivative maxima between 230 and 380°C.

Table 47: ANOVA of the main effects influencing TGA derivative maxima between 230 and 380 °C.

	DOF	***F***	***Percentage Contribution***
SS	1	8.41	34.6%
Water	Factor Pooled		
SDS	Factor Pooled		
Urea	1	8.04	32.8%
SS x Water	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Error	5		32.6%
Total	7		100%



$F_{1,5} = 4.0604$

Appendix-1D

Table 48: Urea effect on quantitative individual secondary structures results.

BM	*Urea*	Helix	Beta Sheet	Beta Turns	Unordered
100%	0%	29.0%	27.5%	27.7%	15.8%
95%	5%	27.9%	28.5%	28.3%	15.3%
90%	10%	27.3%	30.2%	27.4%	15.1%

Table 49: Grouped as ordered structure results for urea analysis.

BM	*Urea*	Ordered	Unordered
95%	5%	84.7%	15.3%
90%	10%	84.9%	15.1%

Appendix-1E

Table 50: Fractional factorial design and results obtained for individual quantitative secondary structures.

L2	3	60	3	20								
L1	1	45	0	10								
Exp #	**Sodium Sulfite (pph_{bm})**	**Water (pph_{bm})**	**SDS (pph_{bm})**	**Urea (pph_{bm})**	**Extruded**				**Injection Moulded**			
					Helix	**β-sheet**	**β-Turns**	**Unordered**	**Helix**	**β-sheet**	**β-Turns**	**Unordered**
1	1	1	1	1	23.1%	25.1%	31.6%	20.2%	25.3%	27.4%	30.4%	16.9%
2	1	1	2	2	24.9%	24.5%	32.3%	18.3%	26.8%	26.7%	30.4%	16.2%
3	1	2	1	2	24.2%	26.5%	32.5%	16.9%	29.5%	26.1%	28.9%	15.5%
4	1	2	2	1	24.6%	23.7%	31.7%	20.0%	26.5%	25.8%	30.9%	16.7%
5	2	1	1	2	26.6%	27.5%	30.5%	15.4%	29.0%	26.5%	29.1%	15.4%
6	2	1	2	1	21.2%	27.6%	33.7%	17.5%	28.4%	26.9%	28.7%	16.0%
7	2	2	1	1	25.5%	24.7%	31.3%	18.4%	25.8%	23.7%	30.3%	20.7%
8	2	2	2	2	29.8%	27.1%	27.9%	15.1%	18.8%	29.6%	33.8%	17.7%

Table 51: Fractional factorial design and results obtained for quantitative secondary structure grouped as ordered and unordered.

L2	3	60	3	20				
L1	1	45	0	10				
Exp #	**Sodium Sulfite (pph_{bm})**	**Water (pph_{bm})**	**SDS (pph_{bm})**	**Urea (pph_{bm})**	**Extruded**		**Injection Moulded**	
					Ordered	**Unordered**	**Ordered**	**Unordered**
1	1	1	1	1	79.8%	20.2%	83.1%	16.9%
2	1	1	2	2	81.7%	18.3%	83.8%	16.2%
3	1	2	1	2	83.1%	16.9%	84.5%	15.5%
4	1	2	2	1	80.0%	20.0%	83.3%	16.7%
5	2	1	1	2	84.6%	15.4%	84.6%	15.4%
6	2	1	2	1	82.5%	17.5%	84.0%	16.0%
7	2	2	1	1	81.6%	18.4%	79.3%	20.7%
8	2	2	2	2	84.9%	15.1%	82.3%	17.7%

Appendix 2

Appendix-2A

Table 52: Processability results for experiments 1 through 16.

L2	3	60	3	20						
L1	1	45	0	10						
							Extrusion		**Injection**	
Exp #	**Sodium Sulfite (pph_{bm})**	**Water (pph_{bm})**	**SDS (pph_{bm})**	**Urea (pph_{bm})**	**Consolidation (Low / medium / high)**	**Processability index**	**Water absorption (wt %)**	**Solubility (wt %)**	**Water absorption (wt %)**	**Solubility (wt %)**
1	1	1	1	1	low	1	30.6	7.7	135.6	8.7
2	1	1	2	2	low/med	9	57.8	13.6	323.7	18.7
3	1	2	1	1	low	4	24.3	7.3	146.0	8.5
4	1	2	2	2	low/med	12	41.3	12.8	270.3	17.4
5	1	1	1	2	low/med	6	28.6	12.3	220.5	14.5
6	1	1	2	1	med	6	49.3	8.4	200.0	9.2
7	1	2	1	2	med	6	20.8	12.1	197.4	13.9
8	1	2	2	1	med	6	47.2	7.8	214.0	10.2
9	2	1	1	2	med/high	6	69.7	14.5	692.3	27.1
10	2	1	2	1	high	8	102.1	10.9	717.4	25.3
11	2	2	1	2	med	12	88.6	10.4	554.6	21.1
12	2	2	2	1	med/high	8	119.5	10.0	582.8	20.1
13	2	1	1	1	low/med	2	60.3	8.6	410.2	16.5
14	2	1	2	2	med/high	12	136.26	15.8	805.3	29.6
15	2	2	1	1	low/med	4	71.0	9.0	335.0	13.3
16	2	2	2	2	high	16	123.4	14.9	737.5	26.0

Appendix-2B

Conditioned % Water Content

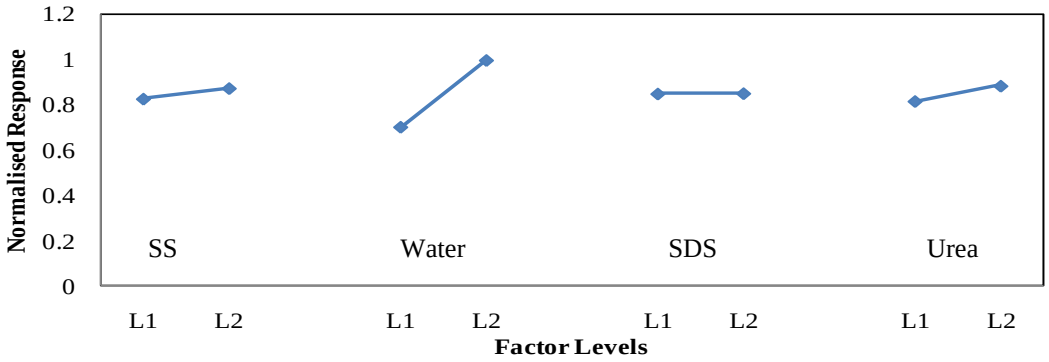
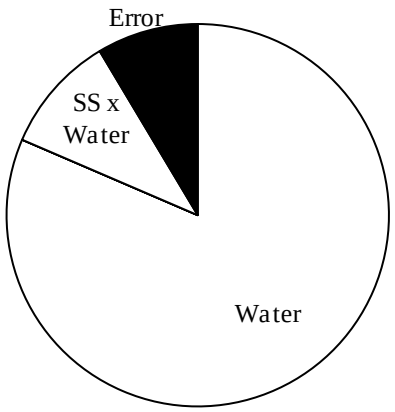


Figure 52: Main effect of factors SS, water, SDS and urea on the water content of conditioned materials.

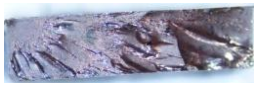
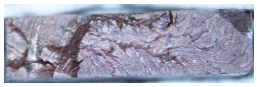
Table 53: ANOVA of the main effects influencing water content of conditioned materials.

	DOF	*F*	*Percentage Contribution*	
SS	Factor Pooled			
Water	1	143	81.5%	
SDS	Factor Pooled			
Urea	Factor Pooled			
SS x SDS x Urea	Factor Pooled			
SDS x Urea	Factor Pooled			
SS x Water	1	18.4	9.96%	
Water x SDS x Urea	Factor Pooled			
SS x SDS	Factor Pooled			
SS x Urea	Factor Pooled			
Water x SDS	Factor Pooled			
SS x Water x SDS	Factor Pooled			
SS x Water x Urea	Factor Pooled			
Water x Urea	Factor Pooled			
Error	13		8.58%	
Total	15		100%	$F_{1,13} = 4.6672$



Appendix-2C

Table 54: Tensile fracture surfaces for unconditioned and conditioned materials.

	1	2	3	4
UC*				
C**				
	5	**6**	**7**	**8**
UC*				
C**				
	9	**10**	**11**	**12**
UC*				
C**				
	13	**14**	**15**	**16**
UC*				
C**				

*UC = unconditoned ; **C = conditioned

Appendix-2D

Unconditioned Mechanical Properties

Tensile Strength

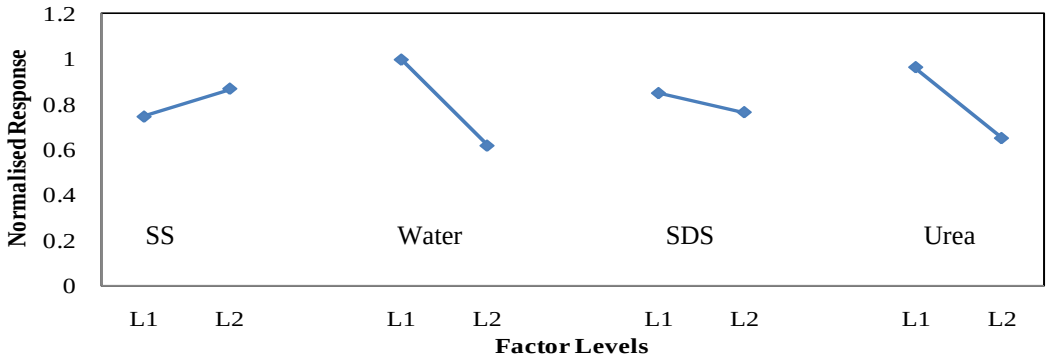
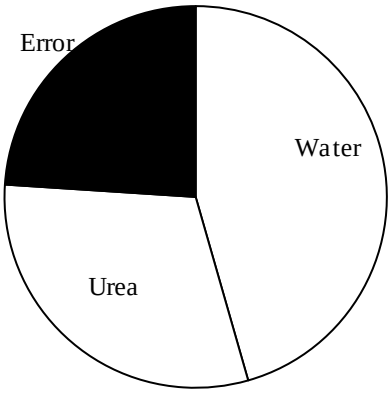


Figure 53: Main effects of SS, water, SDS, urea on unconditioned Tensile Strength.

Table 55: ANOVA of the main effects influencing tensile strength of unconditioned materials.

	DOF	*F*	*Percentage Contribution*
SS	Factor Pooled		
Water	1	29.5	45.6%
SDS	Factor Pooled		
Urea	1	20.1	30.5%
SS x SDS x Urea	Factor Pooled		
SDS x Urea	Factor Pooled		
SS x Water	Factor Pooled		
Water x SDS x Urea	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Water x SDS	Factor Pooled		
SS x Water x SDS	Factor Pooled		
SS x Water x Urea	Factor Pooled		
Water x Urea	Factor Pooled		
Error	13		24.0%
Total	15		100%



$F_{1,13} = 4.6672$

Young's Modulus

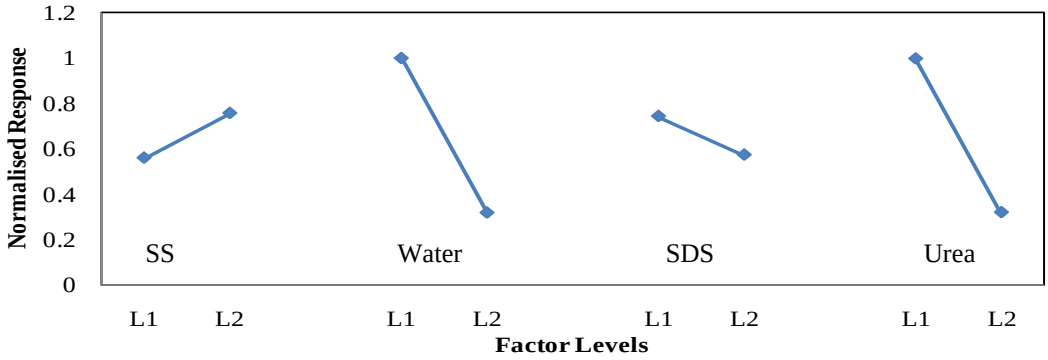
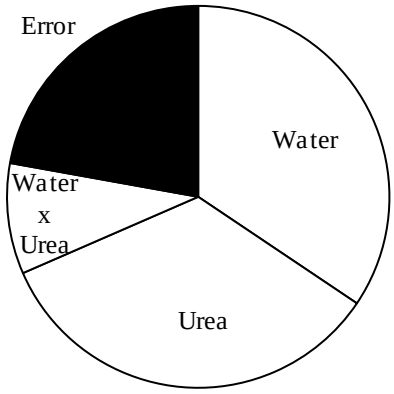


Figure 54: Main effects of SS, water, SDS, urea on unconditioned Young's Modulus.

Table 56: ANOVA of the main effects influencing Young's Modulus of unconditioned materials.

	DOF	*F*	*Percentage Contribution*	
SS	Factor Pooled			
Water	1	24.3	34.4%	
SDS	Factor Pooled			
Urea	1	24.0	34.0%	
SS x SDS x Urea	Factor Pooled			
SDS x Urea	Factor Pooled			
SS x Water	Factor Pooled			
Water x SDS x Urea	Factor Pooled			
SS x SDS	Factor Pooled			
SS x Urea	Factor Pooled			
Water x SDS	Factor Pooled			
SS x Water x SDS	Factor Pooled			
SS x Water x Urea	Factor Pooled			
Water x Urea	1	7.3	9.4%	
Error	12		22.2%	
Total	15		100%	$F_{1,12} = 4.7472$



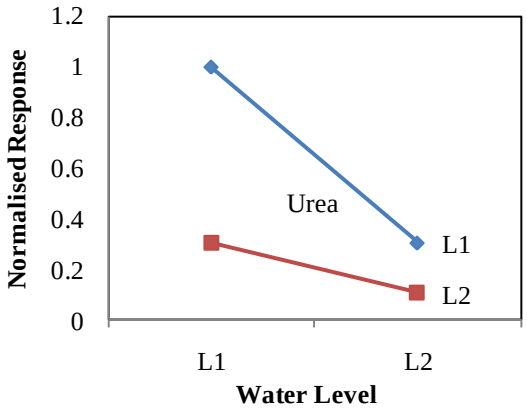


Figure 55: Main effects of water and urea interaction on the unconditioned Young's Modulus.

Elongation at Break

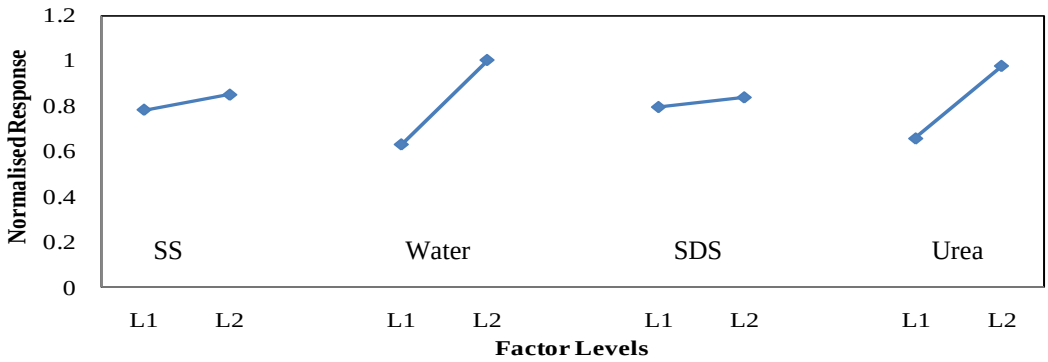
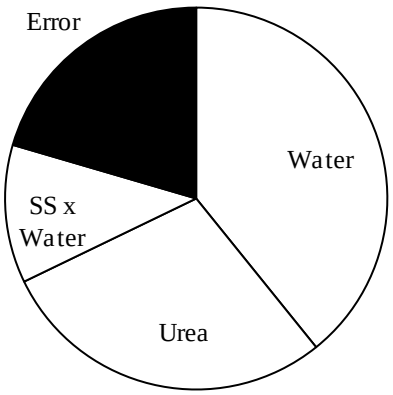


Figure 56: Main effects of SS, water, SDS, urea on unconditioned elongation.

Table 57: ANOVA of the main effects influencing elongation of unconditioned materials.

	DOF	*F*	*Percentage Contribution*
SS	Factor Pooled		
Water	1	29.7	39.2%
SDS	Factor Pooled		
Urea	1	22.0	28.6%
SS x SDS x Urea	Factor Pooled		
SDS x Urea	Factor Pooled		
SS x Water	1	9.6	11.7%
Water x SDS x Urea	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Water x SDS	Factor Pooled		
SS x Water x SDS	Factor Pooled		
SS x Water x Urea	Factor Pooled		
Water x Urea	Factor Pooled		
Error	12		20.5%
Total	15		100%



$F_{1,12} = 4.7472$

Toughness

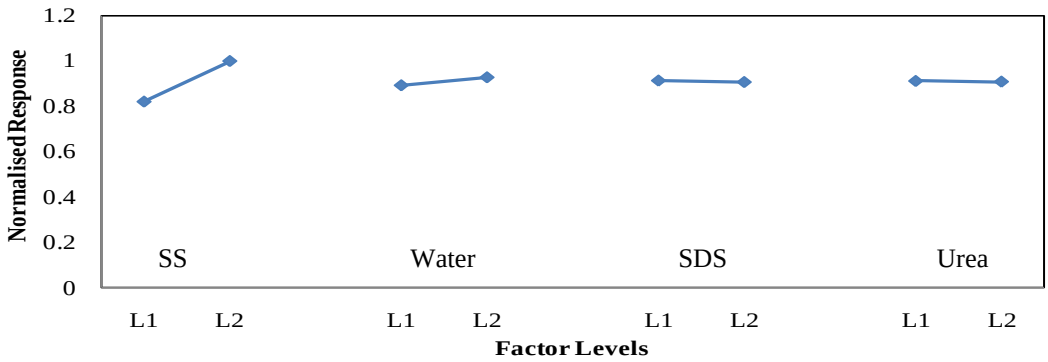
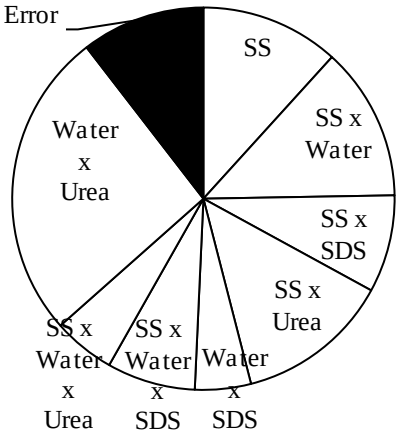


Figure 57: Main effects of SS, water, SDS, urea on unconditioned toughness.

Table 58: ANOVA of the main effects influencing toughness of unconditioned materials.

	DOF	*F*	*Percentage Contribution*
SS	1	17.8	11.7%
Water	Factor Pooled		
SDS	Factor Pooled		
Urea	Factor Pooled		
SS x Water	1	19.6	13.0%
SS x SDS	1	12.8	8.2%
SS x Urea	1	19.7	13.0%
Water x SDS	1	7.8	4.7%
SS x Water x SDS	1	11.7	7.5%
SS x Water x Urea	1	8.5	5.3%
Water x Urea	1	38.4	26.1%
Error	7		10.5%
Total	15		100%



$F_{1,7} = 5.5914$

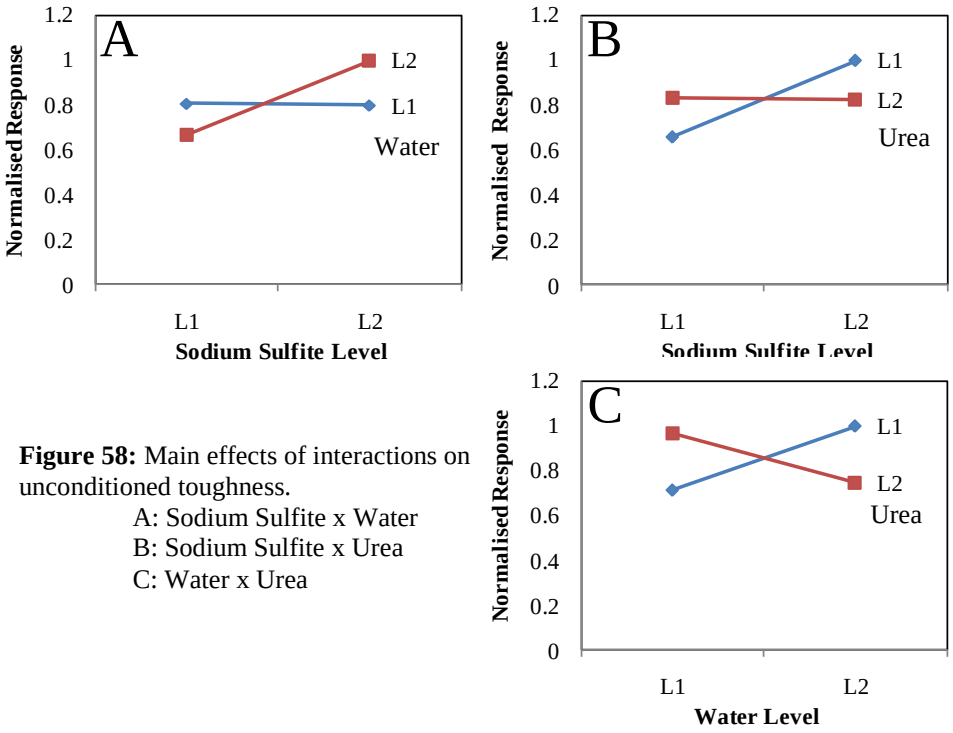


Figure 58: Main effects of interactions on unconditioned toughness.
A: Sodium Sulfite x Water
B: Sodium Sulfite x Urea
C: Water x Urea

Impact Strength

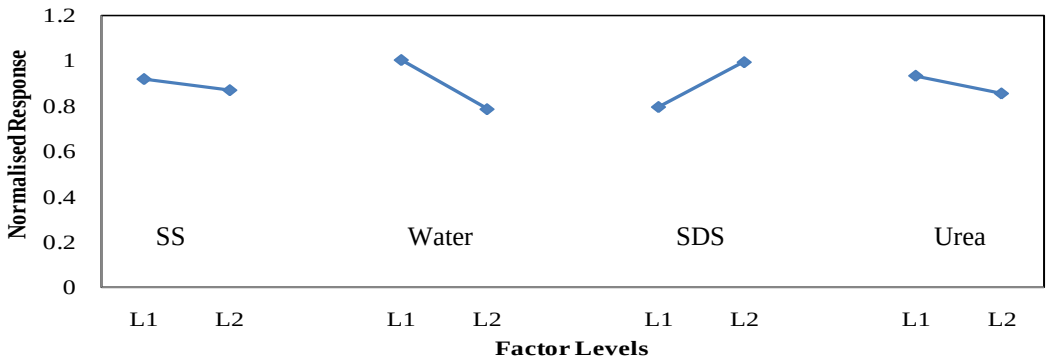
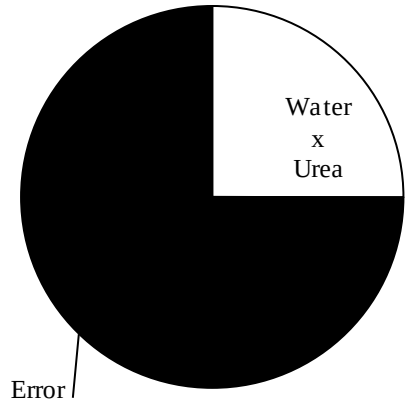


Figure 59: Main effects of SS, water, SDS, urea on unconditioned impact strength.

Table 59: ANOVA of the main effects influencing impact strength of unconditioned materials.

	DOF	*F*	*Percentage Contribution*
SS	Factor Pooled		
Water	Factor Pooled		
SDS	Factor Pooled		
Urea	Factor Pooled		
SS x SDS x Urea	Factor Pooled		
SDS x Urea	Factor Pooled		
SS x Water	Factor Pooled		
Water x SDS x Urea	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Water x SDS	Factor Pooled		
SS x Water x SDS	Factor Pooled		
SS x Water x Urea	Factor Pooled		
Water x Urea	1	6.0	25.0%
Error	14		75.0%
Total	15		100%



$F_{1,14} = 4.6001$

Appendix-2E

Conditioned Mechanical Properties

Tensile Strength

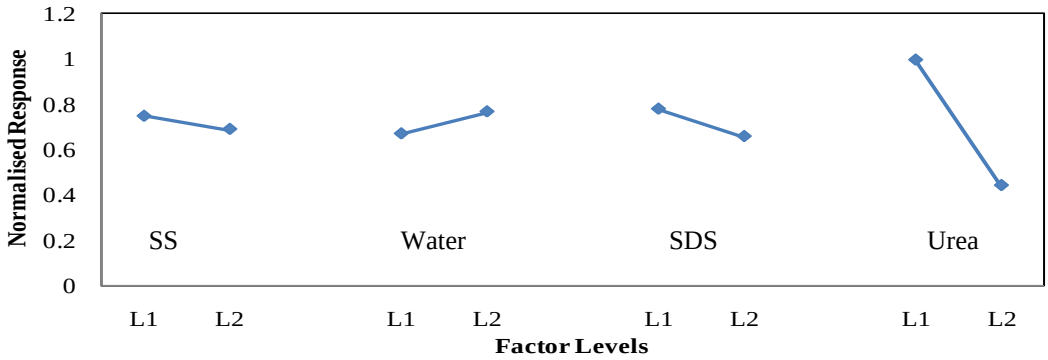
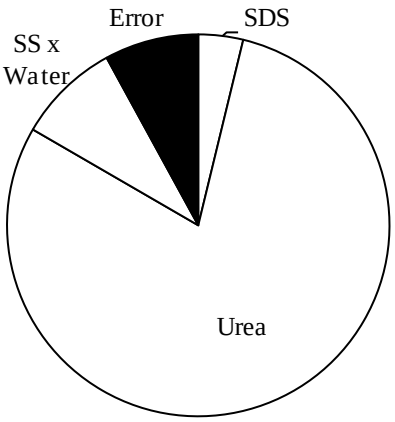


Figure 60: Main effects of SS, water, SDS, urea on conditioned Tensile Strength.

Table 60: ANOVA of the main effects influencing tensile strength of conditioned materials.

	DOF	*F*	*Percentage Contribution*
SS	Factor Pooled		
Water	Factor Pooled		
SDS	1	5.7	3.8%
Urea	1	120	79.6%
SS x SDS x Urea	Factor Pooled		
SDS x Urea	Factor Pooled		
SS x Water	1	13.1	8.7%
Water x SDS x Urea	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Water x SDS	Factor Pooled		
SS x Water x SDS	Factor Pooled		
SS x Water x Urea	Factor Pooled		
Water x Urea	Factor Pooled		
Error	12		8.0%
Total	15		100%



$F_{1,12} = 4.7472$

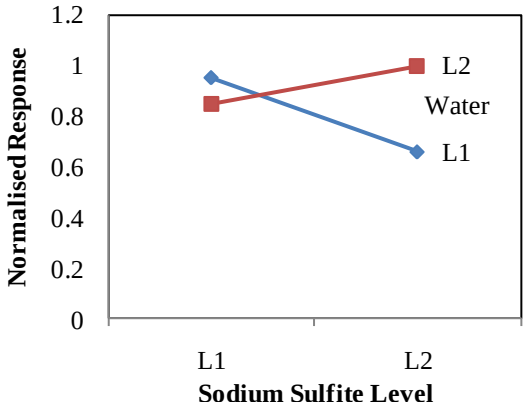


Figure 61: Main effects of sodium sulfite and water interaction on tensile strength of conditioned materials.

Young's Modulus

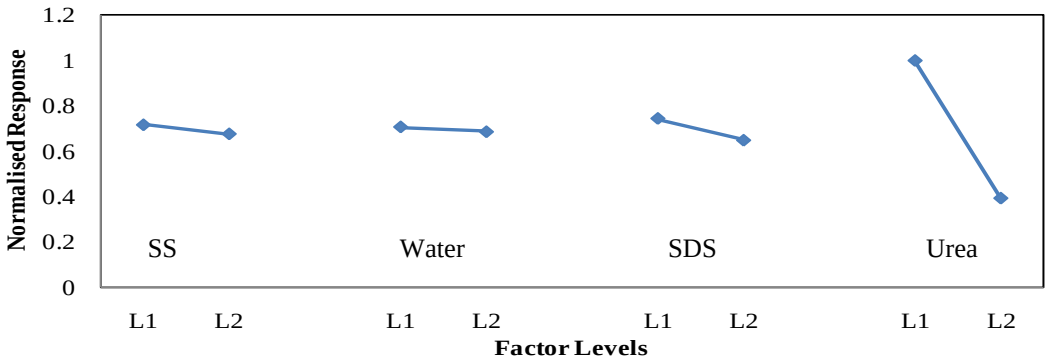
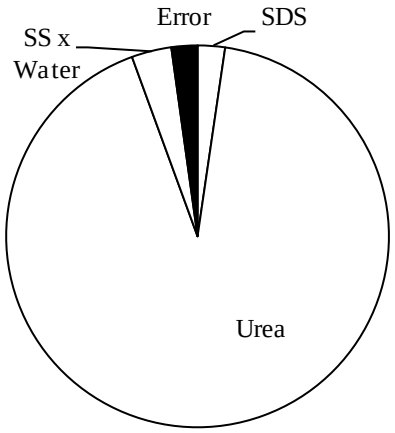


Figure 62: Main effects of SS, water, SDS, urea on Young's modulus of conditioned materials.

Table 61: ANOVA of the main effects influencing Young's modulus of conditioned materials.

	DOF	*F*	*Percentage Contribution*
SS	Factor Pooled		
Water	Factor Pooled		
SDS	1	12.6	2.3%
Urea	1	504	92.1%
SS x SDS x Urea	Factor Pooled		
SDS x Urea	Factor Pooled		
SS x Water	1	18.4	3.4%
Water x SDS x Urea	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Water x SDS	Factor Pooled		
SS x Water x SDS	Factor Pooled		
SS x Water x Urea	Factor Pooled		
Water x Urea	Factor Pooled		
Error	12		2.2%
Total	15		100%



$F_{1,12} = 4.7472$

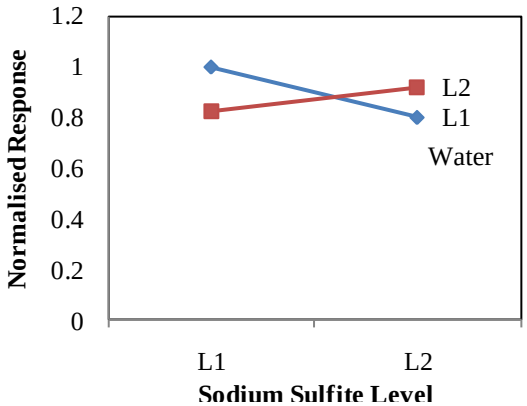


Figure 63: Main effects of sodium sulfite and water interaction on Young's modulus of conditioned materials.

Elongation at Break

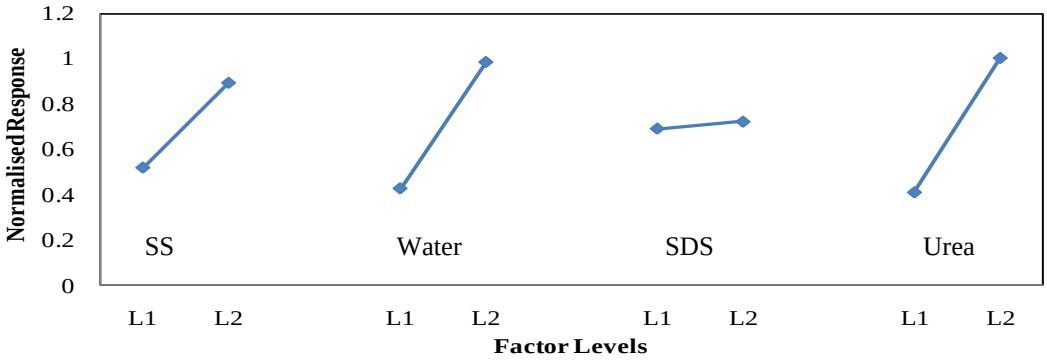
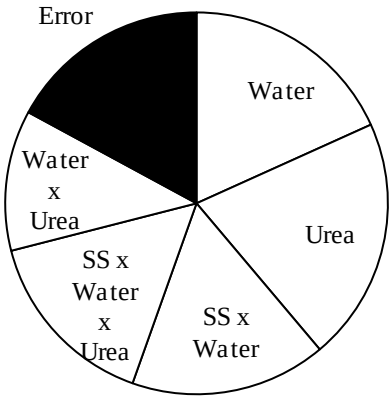


Figure 64: Main effects of SS, water, SDS, urea on elongation of conditioned materials.

Table 62: ANOVA of the main effects influencing elongation of conditioned materials.

	DOF	*F*	*Percentage Contribution*
SS	Factor Pooled		
Water	1	10.7	18.2%
SDS	Factor Pooled		
Urea	1	12.1	20.6%
SS x Water	1	9.7	16.6%
SS x Water x Urea	1	9.1	15.6%
Water x Urea	1	7.0	11.9%
Error	10		17.1%
Total	15		100%



$F_{1,10} = 4.9646$

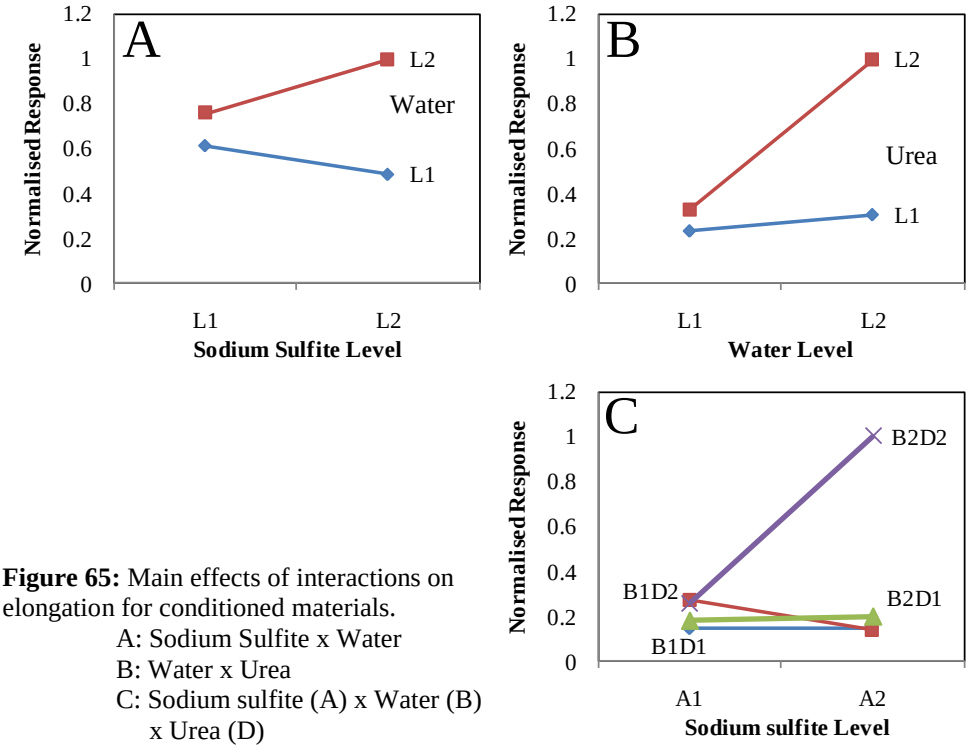


Figure 65: Main effects of interactions on elongation for conditioned materials.
A: Sodium Sulfite x Water
B: Water x Urea
C: Sodium sulfite (A) x Water (B) x Urea (D)

Toughness

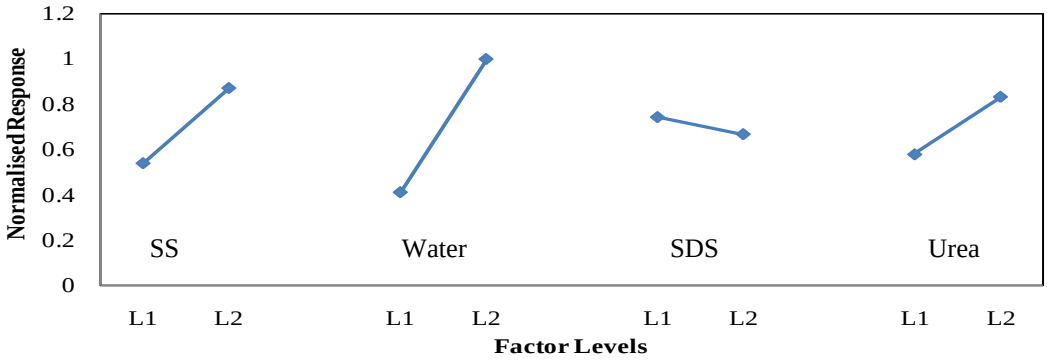
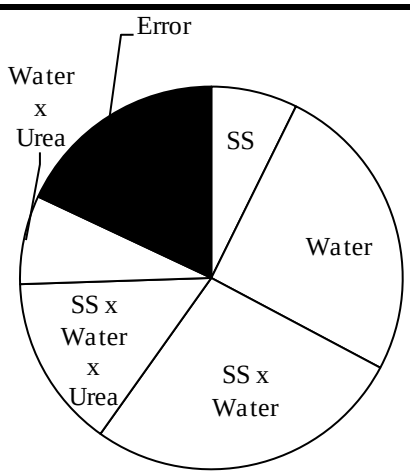


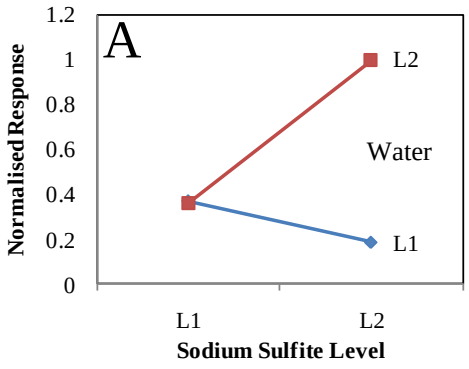
Figure 66: Main effects of SS, water, SDS, urea on toughness of conditioned materials.

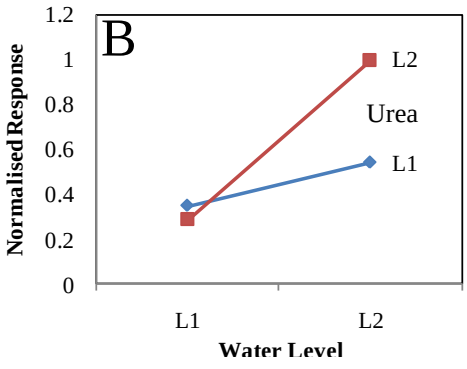
Table 63: ANOVA of the main effects influencing toughness of conditioned materials.

	DOF	*F*	*Percentage Contribution*
SS	1	7.1	7.3%
Water	1	22.2	25.5%
SDS	Factor Pooled		
Urea	Factor Pooled		
SS x Water	1	23.5	27.1%
Water x SDS x Urea	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Water x SDS	Factor Pooled		
SS x Water x SDS	Factor Pooled		
SS x Water x Urea	1	13.2	14.6%
Water x Urea	1	7.3	7.5%
Error	10		2.2%
Total	15		100%



$F_{1,10} = 4.9646$





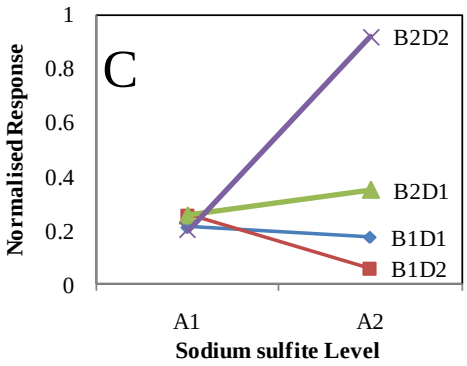


Figure 67: Main effects of interactions on toughness for conditioned materials.
A: Sodium Sulfite x Water
B: Water x Urea
C: Sodium sulfite (A) x Water (B) x Urea (D)

Impact Strength

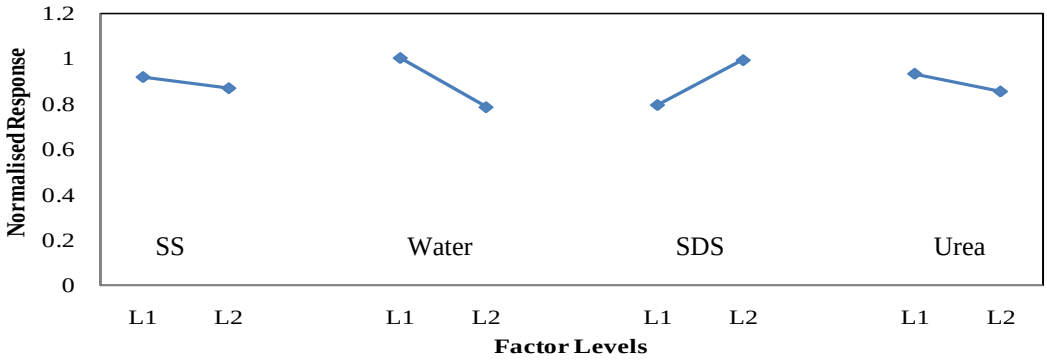
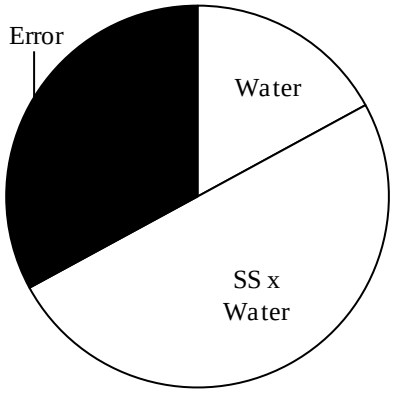


Figure 68: Main effects of SS, water, SDS, urea on impact strength for conditioned materials.

Table 64: ANOVA of the main effects influencing impact strength of conditioned materials.

	DOF	*F*	*Percentage Contribution*
SS	Factor Pooled		
Water	1	8.8	17.1%
SDS	Factor Pooled		
Urea	Factor Pooled		
SS x SDS x Urea	Factor Pooled		
SDS x Urea	Factor Pooled		
SS x Water	1	23.8	50.0%
Water x SDS x Urea	Factor Pooled		
SS x SDS	Factor Pooled		
SS x Urea	Factor Pooled		
Water x SDS	Factor Pooled		
SS x Water x SDS	Factor Pooled		
SS x Water x Urea	Factor Pooled		
Water x Urea	Factor Pooled		
Error	13		32.9%
Total	15		100%



$F_{1,13}$ = 4.6672

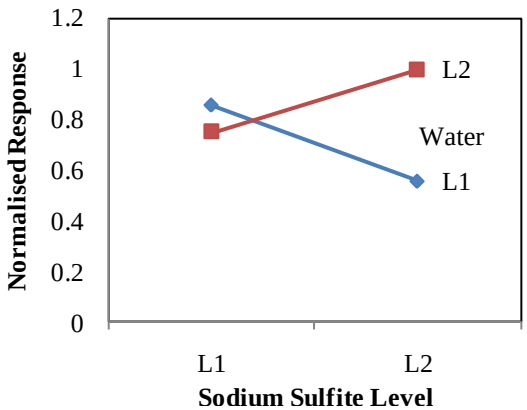


Figure 69: Main effects of sodium sulfite and water interaction on impact strength of conditioned materials.