Decoloured Bloodmeal Based Bioplastic

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Abstract

Renewable and compostable bioplastics can be produced from biopolymers such as proteins. Animal blood is a by-product from meat processing and is rich in protein. It is dried into low value bloodmeal and is used as animal feed or fertiliser. Previous work has shown that bloodmeal can be converted into a thermoplastic using water, urea, sodium dodecyl sulphate (SDS), sodium sulphite and triethylene glycol (TEG). This material is currently being commercialised as Novatein Thermoplastic Protein (NTP) and studies are working on improving its properties through production of composites and blends. In addition further studies are working on understanding its molecular structure before and after thermoplastic processing by utilising various analytical techniques.

A specific area identified for improvement is its colour and smell. NTP is black in colour and has an offensive odour which means its current potential applications are limited to agriculture and waste disposal. Approximately 30 to 40% of plastics are used in short life span applications such as packaging and using bioplastics in these applications would be advantageous because of their compostability. To increase NTP's possible range of applications to common applications such as packaging and increase its acceptance from consumers, its colour and odour must be removed without compromising its mechanical properties.

Oxidative treatment methods for removing colour and odour from red blood cell concentrate (RBCC), modified red blood cell concentrate (mRBCC) and bloodmeal were investigated using hydrogen peroxide, peracetic acid (PAA), sodium hypochlorite, sodium chlorite, sodium chlorate and chlorine dioxide. Treatment effect on protein molecular weight, crystallinity, thermal stability, solubility, product colour and smell were investigated. Treating RBCC and mRBCC required multiple processing steps, had high water contents (67% and 93% respectively), foamed during treatment with peroxides and the protein was prone to hydrolysis. Bloodmeal contained 95% solids and was less sensitive to hydrolysis.

The best decolouring and deodorising results were obtained by treating bloodmeal with 5% PAA. Using this novel treatment method, decolouring was completed

within five minutes and produced a powder which was 67% white based on the RGB colour scale. Protein molecular weight was unaffected by PAA concentration, with a number average molecular weight ranging between 190-223 kDa for 1-5% PAA treated bloodmeal. However, its crystallinity decreased from 35% to 31-27% when treated with 1-5% PAA. Treating bloodmeal with 1-5% PAA also reduced the protein's thermal stability, glass transition temperature (225°C down to 50°C) and increased its solubility from 11% to 85% in 1% SDS solution at 100°C.

3-5% PAA treated bloodmeal powder was extruded using different combinations of water, TEG, glycerol, SDS, sodium sulphite, urea, borax, salt and sodium silicate at concentrations up to 60 parts per hundred parts bloodmeal (pph_{BM}). Partially consolidated extrudates and fully consolidated injection moulded samples were obtained using a combination of water, TEG and SDS. 4% PAA treated bloodmeal produced the best extruded and injection moulded samples and was chosen for investigating the effects of water, TEG and SDS concentration on consolidation and specific mechanical energy input (SME) as well as product colour and mechanical properties.

Analysis of variance (ANOVA) showed SDS was the most important factor influencing ability to be extruded because it detangled protein chains and allowed them to form new stabilising interactions required for consolidation. The best extruded sample, which was 98% consolidated and 49% white, contained 40 pph_{BM} water, 10 pph_{BM} TEG and 6 pph_{BM} SDS.

TEG had the greatest effect on the product's mechanical properties and colour after injection moulding because of its plasticisation effect. ANOVA showed TEG contributed 30.5% to changes in Young's modulus, 66.9% to strain, 39.7% to toughness, 0.1% to UTS and 38.1% to colour. However, SDS also contributed 8.1% to changes in Young's modulus, 13.7% to strain, 15.2% to toughness, 12.5% to UTS, 0.5% to colour. Initial water content contributed 19.7% to Young's modulus, 1.0% to strain, 0.6% to toughness, 30.0% to UTS and 29.9% to colour. The best injection moulded sample was produced using 50 pph_{BM} water, 20 pph_{BM} TEG and 3 pph_{BM} SDS. This produced a material which was 39% white, which

had an almost transparent yellow/orange colour with a tensile strength of 4.62 MPa, Young's modulus 85.48 MPa, toughness 1.75 MPa and 82.62% strain.

The mechanical properties of the product manufactured in this study were comparable to those of NTP, but the product was mostly decoloured, allowing it to be easily pigmented and without an offensive odour.

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Nomenclature

Red blood cell concentrate-RBCC

Modified red blood cell concentrate-mRBCC

Bloodmeal-BM

Novatein Thermoplastic Protein-NTP

Pre-extruded Novatein Thermoplastic Protein-PNTP

Second generation bioplastics-SGB

Peracetic acid-PAA

X-ray diffraction-XRD

Thermogravimetric analysis-TGA

Dynamic mechanical analysis-DMA

Sodium dodecyl sulphate-SDS

Triethylene glycol-TEG

Analysis of variance-ANOVA

Weight average molecular mass-MW

Number average molecular mass-MN

Polydispersity Index-PI

Ultimate tensile strength-UTS

Superoxide Dismutase-SOD

Chapter 1

Introduction

Injection moulded spoons produced from NTP.

1.1 Renewed Interest in Bioplastics

Traditional petroleum based plastics are versatile and durable materials used in many applications. Because of their durability they can persist in the environment for thousands of years after being discarded. This has led to increasing plastic pollution problems around the world. Approximately 200 million tonnes of plastic is consumed worldwide per year of which 30-45% is used as short life span products [1-4]. In New Zealand 252 thousand tonnes of plastic waste is disposed to landfills each year [5]. Plastic waste, pollution, increasing cost of petroleum products and limitations with recycling resulted in increased demand for plastic products which are both compostable and sustainable [4].

Proteins are naturally occurring biopolymers made from amino acids and have been used to produce compostable bioplastics using film casting and thermoplastic processing techniques. These products can be used in short life span applications where their biodegradability is an advantage [1, 2].

For bioplastics to become economically viable they must be processed using common plastics processing methods such as extrusion. When processing biopolymers using extrusion, there are a wide range of variables that can influence processability. These include biopolymer-biopolymer interactions, biopolymer-additive interactions, temperature and shear. A balance between reducing interactions allowing melt/flow and the formation of new interactions which stabilise the product must also be achieved. Therefore successful extrusion is influenced by many factors which mean that successful processing is a difficult task to achieve and only possible within a small window of processing conditions [6-9].

Food crops (e.g. corn) are commonly used in the bioplastics industry and there is increasing pressure on food crop supplies due to competition between the food, biofuel and bioplastics industries. This has led to increased interest in second generation bioplastics (SGB) produced from waste or by-products and do not compete with human food or biofuel production [10].

1.2 Meat Processing Industry By-Products

New Zealand has a large meat processing industry, processing approximately 7 hundred thousand pigs, 4 million cattle and 27 million sheep and lamb each year [11]. A large amount of by-products (52-68% live weight) are produced from this industry and efficient utilisation is necessary to reduce the costs of treating plant effluents, reduce impact on the environment and increase income generated [11-13]. One of these products is bloodmeal. Bloodmeal is inedible grade dried blood which has usually been unhygenically collected. Approximately 80,000 tonnes of blood is collected in New Zealand each year [7]. Bloodmeal is low value (approximately $1.3 per kg) and only used as a fertiliser or as a pet food additive in low quantities [14]. Its high protein content means it has potential to be used in higher value applications.

Bloodmeal's smell is caused by the formation of odour compounds during the storage of blood before drying. Its colour is caused by the internal bond configuration of the haem porphyrin contained in haemoglobin. To modify its colour the internal bond configuration must be modified [15, 16].

At Waikato University bloodmeal has been used to produce a second generation bioplastic using extrusion with the additives urea, sodium sulphite, sodium dodecyl sulphite, water and triethylene glycol [7, 9, 17]. The material has a tensile strength of 5.7 MPa, Young's modulus 49.4 MPa, 81% strain and degrades in 12 weeks when composted [18, 19]. The material is black in colour and has an offensive odour. To improve its range of applications and increase its acceptance by consumers its aesthetic qualities must be improved. More specifically the bloodmeal's smell and black colour must be removed or modified. The protein's molecular weight must not be significantly reduced so that its ability to be extruded and its mechanical properties are not reduced.

1.3 Project Goal

The purpose of this work is to successfully produce a deodorised and decolourised bioplastic from bloodmeal and understand the relationship between its molecular structure, processing and mechanical characteristics.

The main research questions were:

1. How can the colour and smell be removed efficiently from animal blood products (e.g. red blood cell concentrate, bloodmeal) without damaging the protein excessively?
2. How are protein properties modified after treatment and how does this affect material processability and characteristics?
3. Can the decoloured bloodmeal be processed into a bioplastic?
4. What are the mechanical properties of the new bioplastic?

Based on the questions identified in the problem statement, the objectives of this thesis were:

1. Investigate methods available for removing the colour and smell from bloodmeal while still maintaining its ability to be processed into a bioplastic.
2. Identify changes in molecular structure and molecular interactions caused by the smell and colour removal.
3. Process the decoloured bloodmeal into a bioplastic using extrusion and injection moulding.
4. Characterise the properties of the new decoloured bloodmeal based bioplastic.

Chapter 2

Meat Processing By-Products and Their Applications

Hygienic blood collection tank at a meat processing plant.

2.1 Introduction

New Zealand has a large meat processing industry processing approximately 7 hundred thousand pigs, 4 million cattle and 27 million sheep and lamb each year [11]. By-products (also known as co-products) of the meat processing industry include fractions such as offal, paunch contents, bone, skin, fat, and blood. Increasing environmental awareness and a desire to improve animal processing plant economics has led to developing strategies for exploiting by-products. This reduces the costs of treating plant effluents while also reducing the impact on the environment caused by disposal and increasing income generated by the processing plant [20]. This chapter covers the use of animal by-products, more specifically how blood is collected and processed as well as its properties and applications.

2.2 Animal Processing, By-Products and Uses

By-products represent 52%-68% (depending on type of animal) of an animal's live weight [12]. They are classified as either edible or inedible for humans. Edible by-products include organs such as livers, kidneys tongues and intestines. These are called variety meats and are good sources of nutrients [12, 21]. Bones and skin can be heated to extract gelatine which is used in a variety of applications including ice cream and lollies. Fats can be used to produce edible tallow. Hygienically collected blood can be used in specialty foods such as blood pudding as well as in laboratory applications. By-products are usually highly perishable and must be processed efficiently otherwise they degrade and become classified as inedible by-products [21].

Over 50% of by-products are not suitable for consumption [12]. Inedible by-products can include hides, bones, meat scraps, fats and blood. Hides are converted into leather, bones into bone meal, meat scraps into meat meal and fats into tallow for lubricants [21].

Approximately 80,000 tonnes of blood is collected from animal processing plants each year in New Zealand [7]. Blood contributes greatly to the total pollution from a slaughter house [22, 23]. Because of the cost and difficulty in collecting

blood hygienically it is often processed into products not suitable for human consumption such as bloodmeal. This provides a small amount of income (in New Zealand approximately $1.3 per kg) but is usually done to reduce treatment cost of plant effluents [14, 23].

2.2.1 Blood Collection

When an animal is killed, approximately 50% of its blood is released, typically to the slaughter room floor. It is collected through drains but can be contaminated with vomit, urine and excrement. The remainder of the blood is retained in the muscles, organs and capillary system [13, 21, 24]. As blood released during the slaughtering process is the largest contributor to liquid waste in the animal processing industry it is usually collected, steam coagulated and dried into bloodmeal [25]. Bloodmeal is used as fertiliser or animal feed, contains about 90% protein, but has a dark red colour and unpleasant smell.

If animal blood is used for human consumption, it must be hygienically collected. This can be done by inserting a hollow "thief" knife into the animal's jugular while the animal is still alive and allowing the blood to drain into a collection vessel [13, 26]. Some processing factories in New Zealand use the Halal kill method, which involves cutting the animals throat after the animal has been stunned, and letting the blood drain onto the floor. In this case, the blood can be collected by someone standing next to the animal with a bucket (Figure 1). Yields for collected blood from slaughtered cows and bulls range between 10-18 litres per animal [12, 13, 22, 26, 27].

The volume of blood collected depends on animal weight, bleeding time and collection method [27, 28]. Larger animals will yield more blood as will longer bleeding times, however longer bleeding times will also increase processing time.

Special precautions must be taken to collect blood hygienically for edible products [29, 30]. Released blood will clot within 10 minutes by the conversion of soluble fibrinogen into insoluble fibrin [13]. After or during blood collection anticoagulants can be mixed with blood to prevent formation of clots [13, 23, 31-34]. Sodium citrate is the most commonly used anticoagulant. Mechanical stirring, ammonium oxalate, sodium chloride, sodium fluoride and proteolytic

enzymes such as papain have also been used to prevent coagulation [22, 35]. The type of anticoagulant used must be suitable for the final application the blood will be used for.

If collected blood is destined to be used for human consumption it is stored in stainless steel tanks to prevent contamination [26]. When the animal it was collected from has passed veterinary inspection the blood can be processed [13, 26].

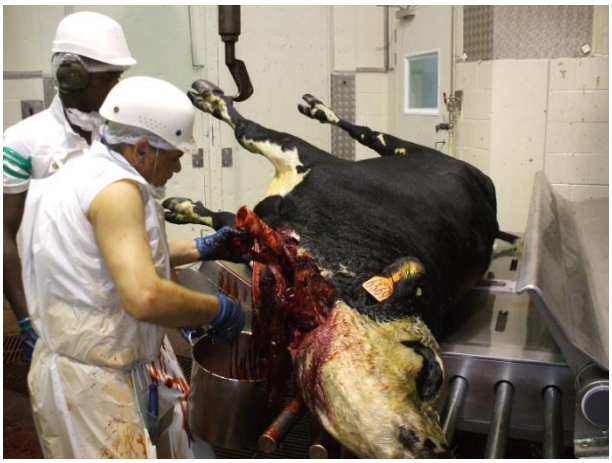


Figure 1: Blood collection during slaughter.

2.2.2 Blood Components

Blood accounts for 2-8% of an animal's live weight [13, 22, 23, 27, 36]. Its main roles are to carry nutrients, transport oxygen, remove waste products, equalise body temperature and help defend against pathogens. Blood contains high levels of protein and its composition varies slightly between species and individual animals. Bovine blood contains approximately 80% water and 17% protein. The remaining 3% is fat, carbohydrates and minerals [31].

Blood can be separated into two components by centrifugation (Figure 2). Centrifuging 100 kg of blood will yield approximately 66 kg plasma as the supernatant and 33 kg red blood cells in the heavy fraction, which are also known as erythrocytes, corpuscles and red blood cell concentrate (RBCC) (Table 1).

Figure 2: Blood being centrifuged into plasma (orange) and RBCC (red).

Plasma is 90-91% water and 8-9% protein [12]. The main proteins in plasma are albumins, immunoglobins, α and β globulins and fibrinogen [13, 31]. The majority of hygienically collected blood is used to produce plasma because of its desirable colour [12, 26, 34, 37]. Light yellow plasma has good functional properties such as emulsification, gel formation and solubility [26, 32]. Plasma can be sterilised by microfiltration and is used in various applications including food products and lab and medical applications [12, 38, 39].

The RBCC is about 33% solids (mainly haemoglobin) and contains 75% of the total protein in blood [26, 40]. However, RBCC has limited use in food because of its black colour, unpleasant metallic taste and smell when dried [30, 41-43]. It is currently only used in low-value applications such as fertiliser or as a minor additive in animal feed, but it has potential for bioplastics. To add value and increase its potential use as a bioplastic, the colour and smell must be removed.

The iron containing haem group can be separated from haemoglobin. This form of iron has high bioavailability and can be used as an iron supplement. It can also be used in foods where colour and taste are not limiting factors such as blood sausages [12, 13, 31]. The uses of blood products are summarised in Figure 3.

Table 1: Percentage composition of whole blood, plasma and RBCC [13, 31].

	Whole Blood	Plasma	RBCC
Water	80	90-91	60-65
Protein	17	8-9	30-35
Other (salts, fats, cell debris)	3	1	5

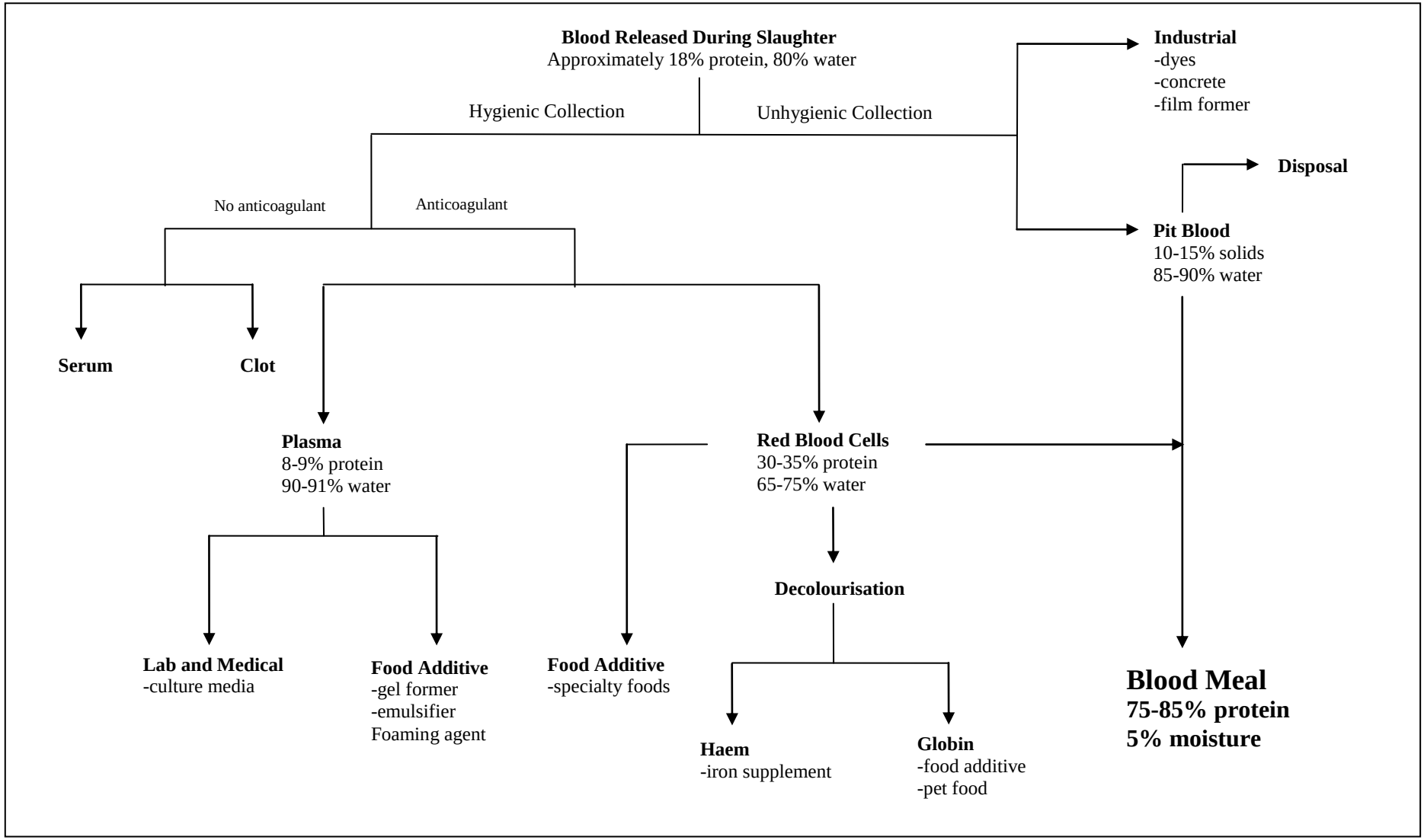


Figure 3: Uses of animal blood collected from the meat processing industry.

2.2.3 Haemoglobin

Haemoglobin is a globular protein that binds and transports oxygen in blood [44, 45]. Its molecular weight is approximately 65 kDa. It has two α and two β polypeptide chains held together by non-covalent interactions [46]. Each α chain contains 141 amino acid residues and each β chain 146 amino acid residues. Each polypeptide chain has a bound haem group which contains iron. The iron is held in the centre of the haem group by four nitrogen atoms (Figure 4). The haem group is bound to the polypeptide chain between the iron molecule and the proximal histidine on the polypeptide chain. The iron is also responsible for binding oxygen. All vertebrate haemoglobins are similar in structure and composition [44].

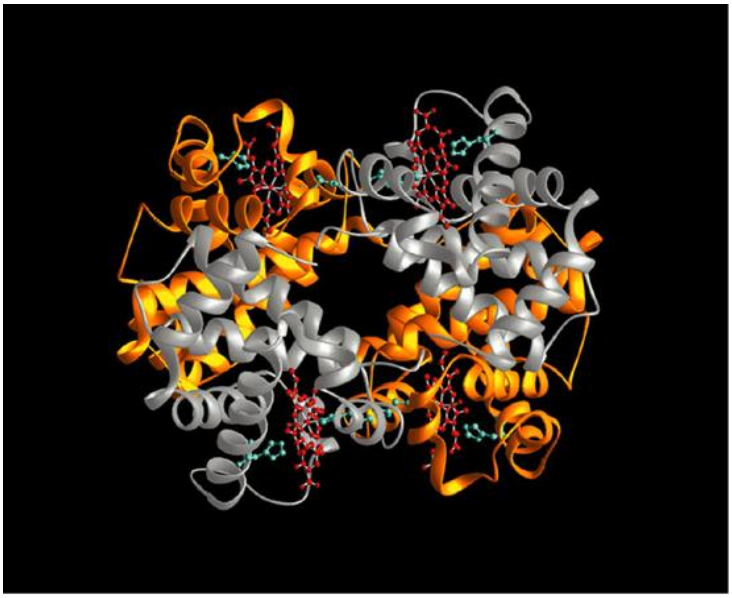


Figure 4: Structure of haemoglobin. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery [47], copyright (2004).

2.2.3.1 What Causes the Colour of Haemoglobin?

The haem group is a chromophore (part of molecule responsible for colour) and is responsible for haemoglobins dark red colour. Chromophores consist of conjugated bonds or ring systems (e.g. aromatic) which absorb light at certain wavelengths due to energy differences between orbitals. When light of a certain wavelength is absorbed by an object the objects colour will be the complementary colour of the absorbed wavelength (Table 2) [15]. Haem absorbs light between 500-550 nm (blue/green) which means its observed colour will be red [16]. To

change the colour of a molecule, the chromophore must be removed or modified by altering the bonds/structure of the chromophore.

Table 2: Colour of absorbed wavelength and observed/complementary colour [15, 48].

Wavelength (nm)	Colour	Observed/Complementary Colour
400-435	Violet	Green-yellow
435-480	Blue	Yellow
480-490	Green-Blue	Orange
490-500	Blue-Green	Red
500-560	Green	Purple
560-580	Yellow-Green	Violet
580-595	Yellow	Blue
595-605	Orange	Green-Blue
605-750	Red	Blue-Green

It has been shown using X-ray crystallography, that porphyrins such as haem (Figure 5) are planar and that the red highlighted pathway in Figure 6 is the preferred system for π-electron delocalisation because it provides the level of highest bond equalisation (the outer bonds 1-2, 3-4, 5-6 and 7-8 can be treated as normal double bonds). It has been shown that the reduction of one, two, three or four of the outer double bonds has little effect on the porphyrins absorption spectrum despite a reduction in conjugation. This showed that the highlighted centre is responsible for the colour of the porphyrin [16]. Therefore, to change the colour of haemoglobin the bonds or structure of the red highlighted centre of the haem porphyrin (Figure 6) must be altered.

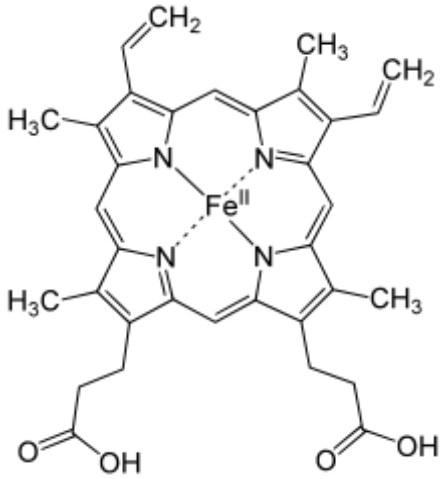


Figure 5: Structure of Haem porphyrin group [49].

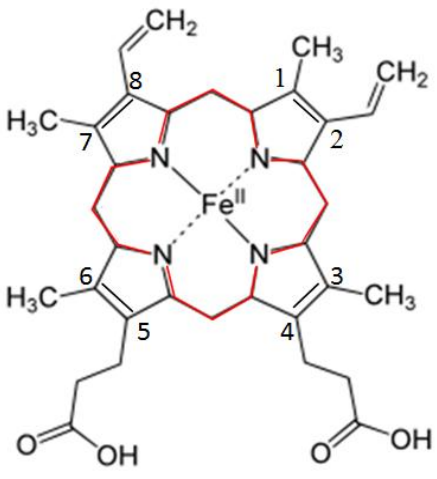


Figure 6: Preferred π-electron delocalisation pathway (highlighted in red) [16, 49].

An example where bond configuration affects colour can be seen during enzyme haem degradation into bile products. Breaking the tetrapyrrole ring into a linear molecule produces green biliverdin and removing a double bond from biliverdin produces yellow bilirubin (Figure 7).

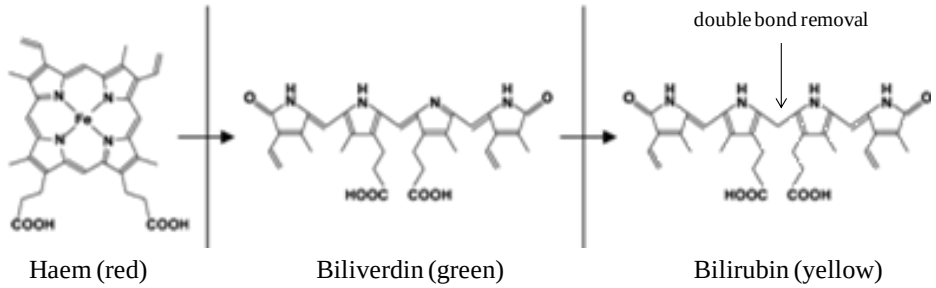


Figure 7: Enzymatic haem degradation into bilirubin [50].

2.3 Bloodmeal and Its Production

Blood not hygienically collected during slaughter is washed into an effluent holding pit below the slaughter floor and is known as “pit blood” [36]. Pit blood contains urine, hair and vomit from slaughtered animals. The blood will also be diluted with wash water and can reach dry solid concentrations as low as 10% [23, 36]. Pit blood can be combined with untreated red blood cells and dried to produce blood meal [26, 36].

Blood meal is a dark brown powder with low moisture (5-10%) and high protein content (75-85%) [13, 23, 36]. The remaining percentage contains extraneous compounds such as ash and fibres [23]. Despite the high protein content it is only used in low value products such as fertiliser and as an additive in animal feed. Because of its unpalatable taste it is usually limited to below 5% in animal feeds [51, 52].

Bloodmeal has a high percentage of hydrophobic amino acids such as valine, leucine, isoleucine, phenylalanine and methionine. This combined with heat induced disulphide and non-disulphide cross-links (e.g. lysinoalanine) means bloodmeal has poor solubility [7, 53, 54].

In many countries blood meal must be produced at high temperatures to ensure sterilisation. Covalent cross-link and hydrophobic interaction formation during heating reduces bloodmeal solubility. Blood meal production can be divided into two categories:

1. Drying whole blood: Indirect heat from a steam jacketed shell is applied to whole blood in a batch cooker to boil off water. Some processes use stirrers to increase heat transfer. Blood is dried until it reaches 2-10% moisture. Temperatures can reach up to 138°C and drying times up to 10 hours. This method is very energy inefficient and produces proteins with very low solubility due to high temperatures and long residence times. This method is usually used at plants with old equipment [23, 27, 55].

2. Solids coagulation followed by drying: Blood is first coagulated by steam injection. The optimum coagulation temperature is 90°C. Up to 92% of the solids can be coagulated using heat [28]. It is difficult to achieve uniform coagulation in large tanks because of poor steam distribution. The coagulated blood solids are separated from liquids by centrifugation. Up to half of the water can be removed before drying using coagulation and centrifugation.

The solids can be dried using a variety of drying methods e.g. rotary drum, ring and batch drying. This method requires less energy but still produces a denatured protein. Protein properties will vary depending on the type of dryer used. This method has become most commonly used. Continuous processes and different drying methods (e.g. spray dryer, spouted bed dryer) have been used to improve energy efficiency, reduce labour requirements and improve protein solubility [23, 27, 55].

Protein properties and amino acid content of blood meal will vary with processing conditions and supplier. High processing temperatures reduce the solubility of blood meal. The amino acid composition of different commercially available bloodmeals is shown in Table 3.

Table 3: Amino acid composition of commercially available bloodmeals.

	Bloodmeal 1 [55]	Bloodmeal 2 [56]	Spray Dried RBCC [31]
Lysine	10.23	7.85	10.37
Histidine	6.13	6.53	6.38
Arginine	4.23	4.18	2.07
Aspartic acid	10.42	7.17	11.03
Threonine	4.47	3.49	5.11
Serine	4.03	4.08	5.47
Glutamic acid	9.08	8.79	8.09
Proline	4.12	4.62	3.24
Glycine	4.06	4.46	4.51
Alanine	7.94	7.69	9.47
Cysteine	1.08	1.24	-
Valine	9.32	7.08	8.5
Methionine	1.01	1.10	0.36
Isoleucine	0.91	0.91	-
Leucine	12.97	11.42	13.92
Tyrosine	2.80	2.34	2.39
Phenylalanine	7.34	6.20	8.19
Tryptophan	-	1.22	-

Poor protein recovery during coagulation can be caused by wash water diluting the blood during collection [23, 27]. Excess water will also increase energy requirements and processing times [28]. Coagulation can be improved by aging the collected blood before processing. This is usually done by leaving the blood in storage tanks overnight or for several days [23, 27, 28].

Aged blood can be coagulated at lower temperatures than fresh blood. However aging blood also causes protein decomposition and odours to develop [27, 28]. 1% calcium chloride can be added to fresh blood with continuous stirring for two hours to improve coagulation without overnight aging [28]. If aging is preferred, chemical or biological methods can be used to preserve blood during aging and help prevent odour generation.

2.3.1 Blood Preservation Techniques

Blood provides an excellent environment for the growth of microorganisms and is highly susceptible to microbial spoilage [22, 57]. Unhygienic collected blood will contain large numbers of bacteria from wash water, hides and animal waste released from the intestines and stomach (e.g. vomit) [58]. *Staphylococcus aureus, Escherichia coli and Pseudomonas fluorescens* are often found in contaminated blood [59]. Blood should be processed as soon as possible to prevent degradation and odour generation [27, 51]. However blood is usually aged at room temperature to improve solids yield during coagulation. Extended storage at high temperatures allows the growth of contaminating blood degrading microorganisms. Blood can be preserved using refrigeration, chemical additives or bio-preservation.

2.3.1.1 Bio-preservation

Bio-preservation utilises lactic acid bacteria and their metabolic products to control the growth and replication of unwanted microorganisms [59-61]. LAB are also naturally present in blood and on vegetables and are considered food grade organisms [60]. They preserve blood by competing for nutrients and producing inhibitory metabolites such as lactic acid and bacteriocins [60, 61]. Inulin can be added with lactic acid bacteria to increase contaminant inhibition [62]. *Enterococcus raffinosus* is commonly used to preserve hygienically collected blood [59, 62, 63]. Bio-preservation has only been used for food grade products. It may be too expensive for inedible applications. It has yet to be tested with highly contaminated blood sources such as pit blood.

2.3.1.2 Chemical Preservation

Chemical preservation can only be used for blood if it is not intended for human consumption [13]. It is a cheap option and several different chemicals have been recommended at different strengths (Table 4).

Table 4: Chemical additives to preserve blood.

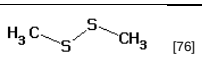
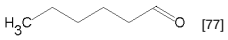
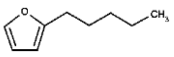
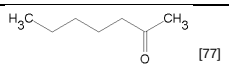
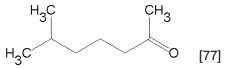
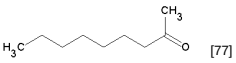
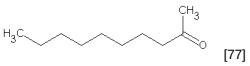
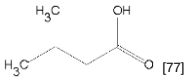
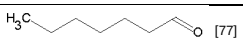
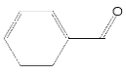
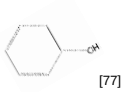
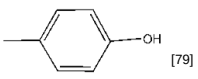
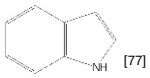
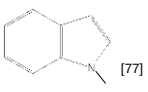
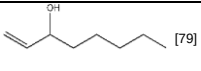
Chemical Additive	Concentration
Hydrochloric acid [13, 23, 64]	0.25 mol/L
Phosphoric acid [13, 23]	0.25 mol/L
Formic acid [13, 23]	0.25 mol/L
Sulphuric acid [13, 23, 65]	0.25-0.30 mol/L
Ammonia [13, 22, 23]	0.10-0.50%
Sodium chloride [22]	3.0%
Sodium metabisulphite [27, 28]	0.03-0.3%
Calcium Oxide [13, 66, 67]	0.125-1.5%

2.3.2 Bloodmeal and Smell

Bloodmeal is usually left to age in tanks before drying. This combined with the high probability of bacterial contamination and the high protein content means that the blood is susceptible to the formation of odour causing compounds through bacterial degradation and putrefaction (decomposition of proteins by anaerobic bacteria) leading to spoilage [68, 69]. Other odour causing compounds can also be formed during the drying process due to the degradation of thermally unstable amino acids and the oxidation of lipids.

The main compounds responsible for agricultural waste odours are ammonia, amines, sulphur containing compounds, volatile fatty acids, mercaptans, indoles, skatole, phenols, alcohols and carbonyls [70, 71]. A previous study has identified the odorous compounds in bloodmeal using gas chromatography [18]. The majority of the odour causing compounds have ring or conjugated structures which make them susceptible to oxidation (Table 5).

Table 5: Odour causing compounds identified in bloodmeal using gas chromatography [18, 72-75].

Compound	Odour	Structure/Formula	Also Found In
Common Compounds			
dimethyl disulfide	Decaying vegetables	 [76]	Meat rendering Meat meal Swine farms
hexanal	Grassy, sour, pungent	 [77]	Meat rendering Swine farms
2-pentyl furan	Earthy, Bitter	 [78]	Meat meal Swine farms
Compounds formed from thermal degradation			
2-heptanone	Dairy, cheese, mushroom	 [77]	Meat rendering Swine farms
6-methyl-2-heptanone	Leaves	 [77]	-
2-nonanone	Fruity	 [77]	-
nonanal	Waxy, fatty	 [77]	-
2-decanone	Floral	 [77]	-
Compound formed from bacterial or thermal degradation			
3-methyl butanoic acid	Body odour, sour	 [77]	Putrefaction Swine farms
Compounds formed from lipid oxidation			
heptanal	Fatty	 [77]	Meat rendering Swine farms
benzaldehyde	Almond	 [77]	Swine farms
Compounds from putrefaction			
phenol	Phenolic	 [77]	Swine farms
4-methyl phenol	Phenolic	 [79]	Swine farms
indole	Faecal	 [77]	Putrefaction Swine farms
methyl indole	Faecal	 [77]	Putrefaction Swine farms
Unclassified compound			
1-octen-3-ol	Earthy, fungal	 [79]	Mushrooms, pesticides

The study concluded that the odour compounds are caused by the storage of blood before drying. The putrefaction products 3-methyl butanoic acid, phenol, 4-methyl phenol, indole and methyl indole were found in bloodmeal. The study also suggests that other putrefaction products such as putrescine, cadaverine and methyl mercaptan could also be present, although they could not be detected due to equipment limitations. Lipid peroxidation products such as aldehydes, ketones and furans were also identified. However it was concluded that these did not impact the overall odour profile of bloodmeal significantly [18].

2.4 Proteins and Amino Acids

Proteins participate in almost every process (catalytic, contractile, structural, transport and defence) that takes place in a cell and are the most abundant biopolymer [80, 81]. Biopolymers are polymers that occur in biological systems. They are different from non-biological polymers (e.g. polyethylene) because they usually consist of a large variety of repeat units which leads to complex interactions and properties allowing them to fill different biological roles [81]. Biopolymers can be divided into nucleic acids, lipids, polysaccharides and proteins. Proteins consist of amino acid residues covalently linked in linear sequence. Proteins can be made from hundreds of amino acids (oligopeptide) or thousands/millions of amino acids (polypeptide) [82].

2.4.1 Amino Acids

Amino acids contain three parts (Figure 8):

1. Basic amino group (NH_2)
2. Acidic carboxyl group (COOH)
3. Carbon atom centre with hydrogen atom and R side group. The central carbon atom is referred to as alpha carbon. The R group can have 20 different chemical compositions (Table 6).

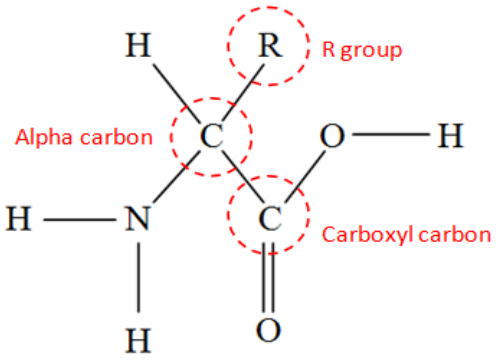


Figure 8: General amino acid structure.

R groups vary in structure, size and charge and define the type of amino acid. Different R groups interact differently with the environment such as aqueous surroundings, other parts of the protein and other substances. Physical properties such as solubility of a given protein are determined by the sequence of R groups i.e. amino acid sequence. There are two possible stereoisomers of each amino acid (left-L and right-D), however only the left configurations are found in proteins [80, 83]. Amino acids can be grouped based on the structure and properties of their R groups (Table 6).

Aliphatic amino acid side chains are hydrophobic and usually found clustered within the interior of the protein, therefore stabilising the structure. Longer side chains cause the amino acid to become more hydrophobic. The different sizes and shapes of the side chains allow them to pack together tightly [46, 82].

Phenylalanine, tyrosine and tryptophan are amino acids with aromatic side chains. These can all participate in hydrophobic interactions. These proteins absorb ultraviolet light (maximum at 280 nm) and can be used to characterise proteins. The aromatic ring of tyrosine contains a hydroxyl group making it slightly less hydrophobic. It is an important functional group in some enzymes and proteins because of its ability to form hydrogen bonds. Phenylalanine and tryptophan are highly hydrophobic and are usually contained within the core of proteins [46, 82].

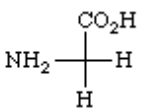
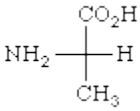
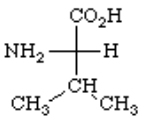
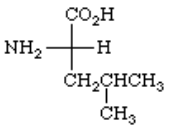
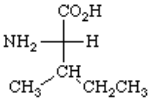
Serine and threonine have hydroxyl groups on their side chains making them hydrophilic. Amino acids with either positively or negatively charged side groups are the most hydrophilic [46]. Lysine has a second amino group on its side group. Arginine has a positively charged guanidinium group and histidine has an

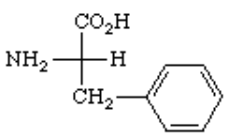
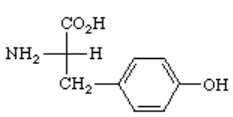
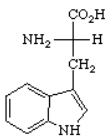
aromatic imidazole group. The side chain of histidine can be positively charged or uncharged at pH 7 and facilitates many enzyme catalysed reactions by acting as a proton donor/acceptor. Aspartate and glutamate contain side chains that are usually negatively charged at pH 7. The uncharged versions are called asparagine and glutamine which have an amine group in place of the carboxylate group [46, 82].

Cysteine and methionine both contain hydrophobic side chains. Cysteine can be oxidised to form a covalently linked dimeric amino acid called cystine. Cystine is formed when two cysteine molecules are bound by a disulphide bond. The disulphide bonds are important in many proteins as they form covalent links between parts of a polypeptide or between two different polypeptide chains [46, 82].

Unlike the other amino acids, proline has a cyclic structure. The side chain is attached to the amino acid at both the alpha carbon and amino group. The side chain provides similar properties to other aliphatic amino acids (hydrophobic, found in the interior of proteins) but has added rigidness due to the cyclic structure. Proline is usually found in locations where proteins bend [46, 82, 83].

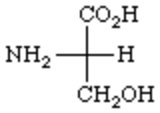
Table 6: Structure and grouping of common amino acids [46, 82-84].

Aliphatic (non-aromatic/open chain) amino acids				
Glycine (Gly, G)	**Alanine (Ala, A)**	**Valine (Val, V)**	**Leucine (Leu, L)**	**Isoleucine (Ile, I)**
				

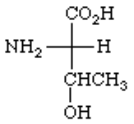
Amino acids with aromatic rings		
Phenylalanine (Phe, F)	**Tyrosine (Tyr, Y)**	**Tryptophan (Trp, W)**
		

Amino acids with hydroxyl side chains

Serine (Ser, S)

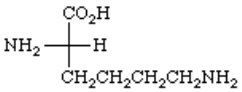


Threonine (Thr, T)

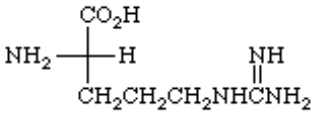


Positively charged amino acids

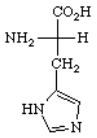
Lysine (Lys, K)



Arginine (Arg, R)

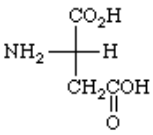


Histidine (His, H)

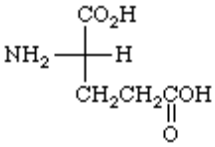


Amino acids with amido and carboxylate groups

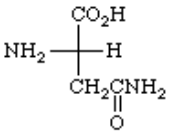
Aspartate (Asp, D)



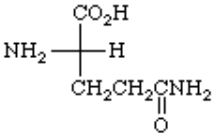
Glutamate (Glu, E)



Asparagine (Asn, N)

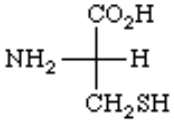


Glutamine (Gln, Q)

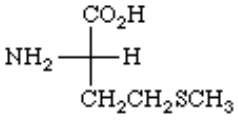


Amino acids containing sulphur

Cysteine (Cys, C)

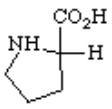


Methionine (Met, M)



Amino acid with cyclic structure

Proline (Pro, P)



Amino acids are joined together and peptide chains/proteins produced by condensation reactions [81, 82]. This involves removing hydrogen and oxygen atoms from both amino acids producing a bond between the two amino acids and water as a by-product (Figure 9). The double bond between oxygen and the carboxyl carbon is shared between the carboxyl carbon and adjacent nitrogen atom due to a resonance effect [81]. This link between amino acid residues is known as the amide link or peptide bond. Polypeptides with N number of amino acid residues will have N-1 amide links [81, 82].

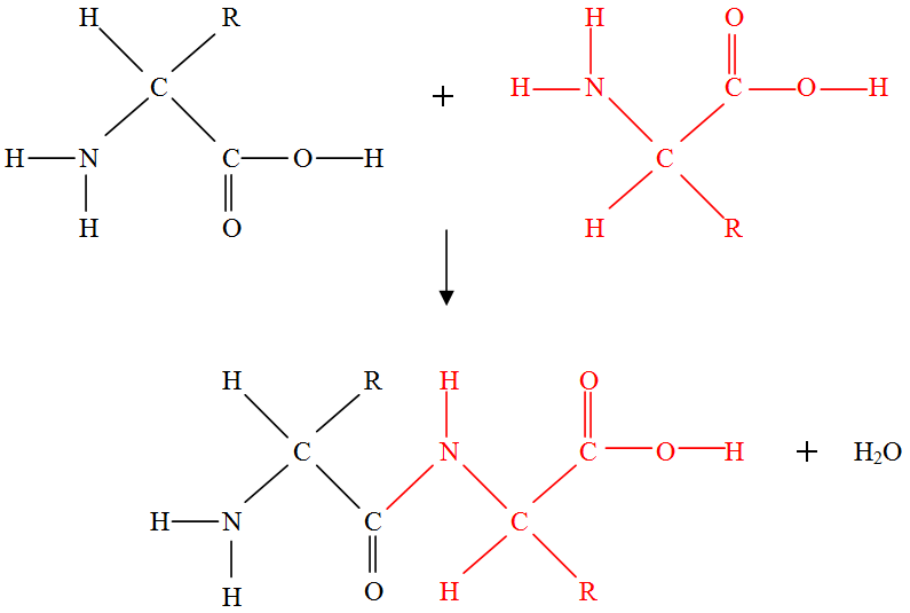


Figure 9: Peptide bond formation between amino acids.

pH is important for protein properties as it determines an amino acids state of ionisation. At low pH (high [H^+]) the amino terminal will usually acquire an extra hydrogen atom and will terminate with NH_3^+ (Figure 10). At high pH (low [H+]) the carboxyl end will usually lose a hydrogen atom and will be COO^- (Figure 11). At physiological pH (pH ≈ 7) both ends are modified as described i.e. NH_3^+ and COO^- (Figure 12). This state is known as zwitterionic (two opposing charges within the same molecule) and does not promote polymerisation because the amino end has an extra hydrogen whereas condensation requires the loss of hydrogen. At the carboxyl end the O-H bond is broken instead of the C-O bond required for condensation. This mechanism prevents spontaneous polymerisation which could have negative effects on the proteins structure and activity.

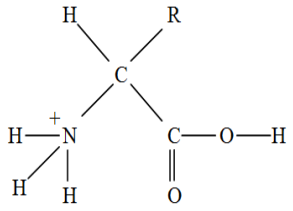


Figure 10: Amino acid at acidic pH

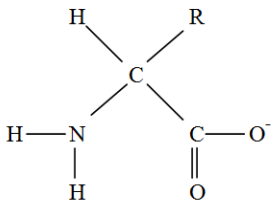


Figure 11: Amino acid at basic pH

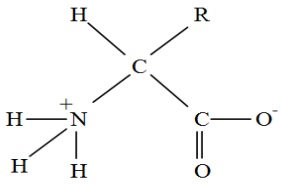


Figure 12: Amino acid at physiological pH (pH≈7)

2.4.2 Protein Structure

A protein's final three dimensional structure will be that which minimises its total free energy [81]. Protein structure is influenced by hydrogen bonding, hydrophobic interactions, electrostatic interactions and van der Waals interactions. Protein structure can be described at four different levels which are primary, secondary, tertiary and quaternary structure.

Hydrogen bonding can occur between charged and uncharged molecules. It involves sharing a hydrogen atom between two other atoms. The atom that the hydrogen is more closely associated is the hydrogen donor (usually oxygen or nitrogen covalently bonded to hydrogen) and the other atom is the hydrogen acceptor (usually oxygen or nitrogen). The acceptor has a partial negative charge which attracts the hydrogen atom [46]. Hydrogen bonding can occur between polypeptide backbone components and also between water and amino acid side chains on the proteins surface [44].

Hydrophobic interactions form because nonpolar side chains tend to cluster in a nonpolar environment rather than interact with a polar solvent like water [44]. This happens because water bonds strongly to itself and forces the nonpolar molecules together [46]. The forming of hydrophobic cores or clusters on the interior of a protein is favourable because it reduces interactions between nonpolar residues and water. The interior core of proteins are usually hydrophobic and contains nonpolar side chains whereas the surface of the protein can contain both polar and nonpolar side chains (Figure 13) [44].

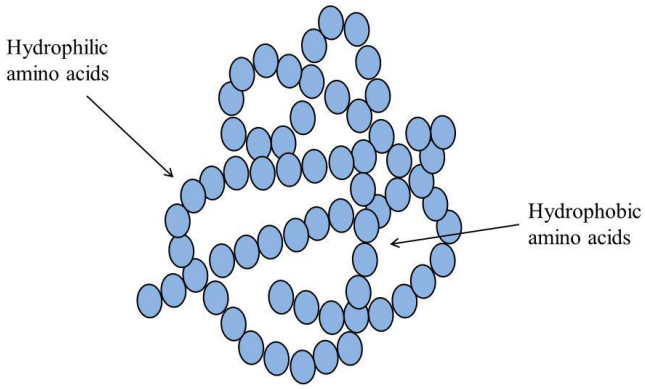


Figure 13: Hydrophobic and hydrophilic amino acid locations on proteins.

Electrostatic interactions occur between charged groups and can also be called an ionic bond, salt linkage, salt bridge or ion pair. Electrostatic interactions are weakest in water because of water's high dielectric constant [46].

Van der Waals forces are weaker than hydrogen bonding and electrostatic interactions. They are caused because at any given time the electronic charge around an atom is not distributed symmetrically. This causes the charge around neighbouring atoms to behave in a similar way. As the atoms get closer the attraction increases until they reach the van der Waals contact distance. At this distance outer electron clouds overlap and repulsive forces become more dominant causing a decrease in attractive forces [46].

The primary structure of a protein is the linear sequence of amino acid residues held together by covalent bonds. Because the backbones of all amino acid residues are the same the primary structure can be defined by the sequence of side groups i.e. R_1, R_2,...R_n [81]. The primary structure is formed by the condensation reaction described previously.

The secondary structure of a protein is the arrangement of amino acid residues that are adjacent or in close proximity to each other [46, 82]. The secondary structure is stabilised by the formation of hydrogen bonds from carboxyl group oxygens and amine hydrogens between residues of the polypeptide chain [85]. The polypeptide chain aligns so that the maximum number of hydrogen bonds are formed [80]. The most common forms of secondary structure are alpha-helices and beta-sheets. Other secondary structures include 310 helices, random coils and beta-turns. The specific organisation caused by hydrogen bonds is often called the intermolecular crystallization [80].

The alpha-helix is where the polypeptide is coiled into a helical structure where the side chains extend outwards and there is a turn every 3.6 residues [44, 46, 81]. Alpha helices are stabilised by the formation of hydrogen bonds between the NH and CO groups of the nth and the n-4 residue on the polypeptide chain [46, 81]. Each residue occupies 0.15 nm resulting in a turn every 0.54 nm. The diameter is approximately 0.6 nm if side groups are ignored [44, 81, 85]. Helix turns can be both clockwise (right handed) or anti-clockwise (left handed), but only clockwise helices are found in proteins [46]. Alpha helices are most commonly found in globular proteins such as haemoglobin [46, 85].

Beta sheets (also known as pleated sheets) are produced when hydrogen bonds are formed between carboxyl and amine groups of adjacent polypeptide chains [46, 85]. Parallel sheets form when the adjacent polypeptide chains run in the same direction (N to C or C to N) whereas antiparallel sheets are formed when the adjacent polypeptide chains run in opposite directions [44, 85]. Beta sheets can be found in both fibrous and globular proteins [85].

Beta-turns (also known as a reverse turn, hairpin, tight turn or beta-bend) are formed by the formation of a hydrogen bond between the CO and NH group three residues apart. The beta-turn allows a polypeptide chain to reverse its direction [46]. Proline has a cyclic structure and usually promotes the formation of a beta-turn. Beta-turns often cause the formation of anti-parallel beta-sheets [44].

The tertiary structure of a protein is the arrangement of amino acid residues that are far apart or the folding of a polypeptide chain [44, 46, 82]. Common tertiary structures are fibrous and globular proteins.

Fibrous proteins are formed when polypeptide chains are organized into fibres or sheets around a single axis [44]. These proteins are usually strong and resistant to solubilisation in water. Examples of fibrous proteins include alpha-Keratin (hair, nails, horns), beta-Keratin (feathers), Fibroin (silk) and collagen (tendons, skin) [44, 82].

Globular proteins are formed when different segments of a polypeptide chain folds back on itself, the structure is stabilised mostly by hydrogen bonds with some ionic interactions [82]. Globular are more common than fibrous proteins and the folding provides the diversity which allows them to carry out a wide variety of functions such as acting as catalysts, transportation and regulation [44, 46]. Globular proteins are usually folded so that hydrophobic side chains are contained within a hydrophobic core and the hydrophilic or polar side groups are on the surface [82].

The quaternary structure of a protein is the assembly of two or more polypeptide subunits. The subunits can be identical or varied. The quaternary structure is stabilised by weak forces such as hydrogen bonds, ionic interactions, hydrophobic interactions and van der Waals forces. In some cases disulphide bonds can also be involved [44]. Protein primary, secondary, tertiary and quaternary structure is summarised in Figure 14.

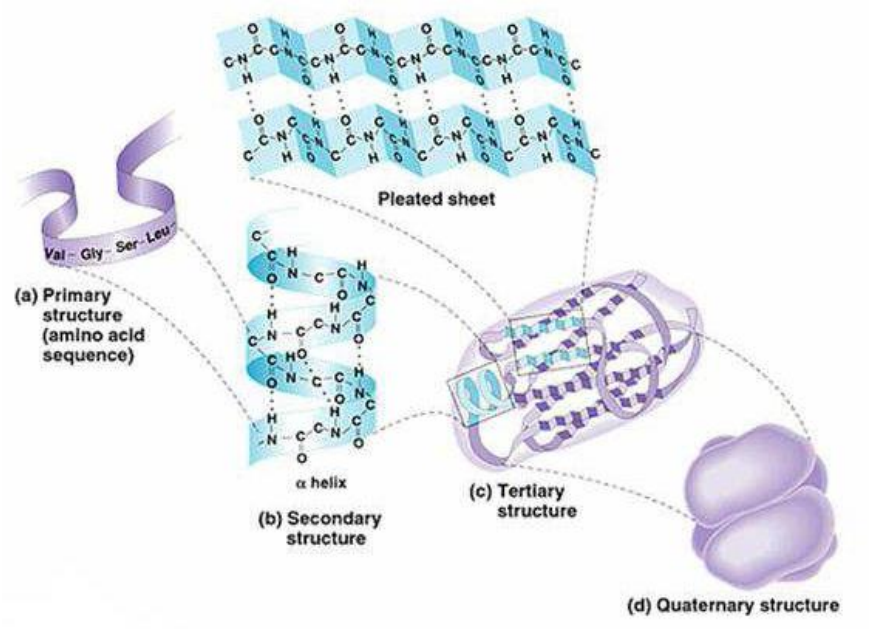


Figure 14: Primary, secondary, tertiary and quaternary structure of proteins [86].

2.5 Conclusion

New Zealand has a large meat processing industry which produces a lot of by-products. To increase income generated per animal, reduce waste and waste disposal costs by-products are used in a variety of applications. Blood is one of the major by-products but has limited edible applications because it is difficult to collect hygienically and also because of its colour, taste and smell. As a result it is usually dried into bloodmeal.

Blood colour is caused by the haem group contained in haemoglobin. The colour is caused when the bounds absorb light in the blue/green wavelength resulting in the observation of their complementary colour (red). To change the colour of haemoglobin the bonds or structure of the red highlighted centre of the haem porphyrin (Figure 6) must be altered.

Bloodmeal has an unpleasant odour which is caused by putrefaction of proteins during aging. The main odour causing compounds in bloodmeal are 3-methyl butanoic acid, phenol, 4-methyl phenol, indole and methyl indole which are formed by putrefaction during storage of blood before drying. Other putrefaction products putrescine, cadaverine and methyl mercaptan could also be present.

Chapter 3

Methods for Decolouring Blood Products

Red blood cell concentrate treated with different sanitising and bleaching chemicals.

3.1 Introduction

Current blood based bioplastics are black and have an offensive odour. To increase their acceptance by consumers the colour and smell must be removed or modified by removing or degrading the haem and odour causing compounds [26]. The objective of this chapter was to find methods available for removing colour and smell from blood products so they could be used as a feedstock to produce bioplastics. This chapter investigated existing methods for decolouring red blood cell concentrate (RBCC). It also investigated potential alternative bleaching and sanitising methods for removing the colour and smell from RBCC and bloodmeal so that they could be used to produce a decoloured bioplastic.

3.2 RBCC Decolouring Methods

The majority of methods have been developed to remove the colour from blood or RBCC for food applications (Table 7). Extraction methods using organic solvents such as methanol and acidified acetone have been used to produce a decolourised precipitated protein with high molecular weight and good foaming and emulsification properties [87, 88]. Carboxymethyl cellulose and activated carbon has been used to extract haem at low pH with protein yields between 60-100% [31, 89, 90]. However the removal of the haem group reduced the stability of the protein and makes it more susceptible to denaturants [13].

The enzymes pepsin, papain and alcalase have also been used to hydrolyse RBCC and produced decolourised peptides. These peptides were not completely colourless and required further filtration, bleaching or treatment with extraction methods [39, 40, 91-94]. Current decolourisation methods have had limited large scale success because they are expensive.

A cheaper alternative to remove colour was to degrade the haem group using an oxidant. Debuyser (1999) and Izumi et al. (1991) used hydrogen peroxide at pH 9-12.5, whereas Wismer-Pederson and Frohlich (1992) added hydrogen peroxide to RBCC at pH 2-3 [38, 42, 43]. Treatment temperatures ranged from 20°C to 140°C. The haem group was very sensitive to degradation by even small concentrations of hydrogen peroxide. Haem degradation using oxidation involved cleaving the porphyrin ring to produce yellow degradation products [95].

Table 7: Blood and RBCC decolourisation methods.

Date	Author	Material	Treatment
1975	Tybor, Dill & Landmann [88]	RBCC	Acidified acetone extraction
1980	Ranken [32]	RBCC	Chloroform and acidified acetone
1981	Hald-Christensen, Adler-Nissen, Olsen	RBCC	Alcalase and activated carbon
1981	Sato, Hayakawa & Hayakawa [89]	RBCC	SP-sephadex, Amberlite CG-120, Dowex 50W-x4, Amberlite CG-50, Carboxymethyl Cellulose exchangers
1982	Luijerink [96]	RBCC	Solid state treatment with Methanol/Ethanol solution
1982	Swan [40]	RBCC	Alcalase and activated carbon
1983	Sato & Hayakawa [97]	RBCC	Carboxymethyl Cellulose column
1984	Luijerink [87]	RBCC	Acid splitting + acidified organic solvent extraction (Methanol, Ethanol, Acetone)
1985	Tayot & Veron [90]	RBCC	Acidified methanol/ethanol and activated carbon
1986	Piot, Guillochon & Thomas [39]	RBCC	Pepsin hydrolysis and alumina column
1987	Wismer-Pedersen [98]	RBCC	Hydrogen peroxide oxidation
1990	Lee, Wang, & Nakai [99]	RBCC	Sodium alginate
1991	Izumi, Nonaka & Hayashi [43]	RBCC	Hydrogen peroxide oxidation
1992	Wismer-Pedersen [38]	RBCC	Hydrogen peroxide oxidation
1995	Aubes-Dufau, Seris, & Combes [91]	RBCC	Pepsin + various separation processes (UF, chromatography, organic solvents)
1995	Synowiecki, Jagielka & Shahidi [94]	RBCC	Alcalase hydrolysis and charcoal extraction
1996	Holm-Jensen [100]	Whole Blood/RBCC	High temperature/pressure hydrolysis
1996	Yang and Lin [101]	RBCC	Comparison between sodium carboxymethyl cellulose, propylene glycol alginate and sodium alginate viscosity enhancers

Date	Author	Material	Treatment
1998	Yang & Lin [34]	RBCC	Comparison between sodium carboxymethyl cellulose, acidified acetone, hydrogen peroxide oxidation and alcalase hydrolysis
1999	De Buyser [42]	Whole Blood/RBCC	Hydrogen peroxide oxidation
1999	Gomez-Juarez, Castellanos, Ponce-Noyola, Calderon & Figueroa [92]	RBCC	Papain hydrolysis, ultrafiltration and sodium hypochlorite bleaching
1999	Duarte, Simoes, & Sgarbieri [31]	RBCC	Carboxymethyl cellulose extraction
2003	Silva & Silvestre [35]	RBCC	Comparison between acidified acetone and CMC extraction
2007	Guo, Zhao, Wang & Cui [102]	RBCC	Protease hydrolysis

3.2.1 Globin Precipitation using Organic Solvents

Low pH (pH 2-5) causes the haem group to dissociate from globin. Dissociated haem could be separated from the globin by using organic solvents to precipitate the globin. Cold acidified acetone is the most efficient and most often used, but methylethyketone (MEK), methanol and ethanol have also been used [26, 87].

Tybor et al (1973) decoloured haemoglobin by adjusting to pH 4 using ascorbic acid prior to treatment with acidified acetone. This method required large volumes of acetone (4 L acetone for 1 L protein solution) [88]. This method has not been used on a large scale because acetone is toxic and it is difficult to remove residues from the final product [26]. In addition, acetone is a highly volatile and flammable therefore processing facilities need to be designed to contain its vapours.

3.2.2 Haem Extraction using Solid Media

Adsorption media such as activated carbon or carboxymethyl cellulose (CMC) have been used as an alternative to organic solvents to remove the haem from acidified haemoglobin solutions [26]. Sato et al (1981) used CMC chromatography to obtain a high quality product. However this method required low protein loading rates (1 g CMC to produce 70 mg globin) [89].

Tayot et al (1985) absorbed haem using activated carbon in the presence of alcohol, at pH 3 and below 20°C. Under these conditions activated carbon did not absorb globin and close to 100% protein was recovered. However a long residence time (up to 15 hours) was required for this method [90].

Lee et al (1990) developed an alternative to CMC adsorption using sodium alginate to bind haem under various conditions. They found the best conditions were pH 2.25, 0.348% sodium chloride and 0.107% sodium alginate. These conditions gave 64.9% protein yield [99].

3.2.3 Enzyme Hydrolysis

Protein hydrolysis is the process of breaking a protein down into its constituent amino acid chains. Common methods include enzyme, acidic and alkaline hydrolysis. Proteolytic enzymes have been used to degrade haemoglobin and release the haem. The released haem aggregates into micro droplets because of its hydrophobic nature [26]. The amino acids and peptides from the hydrolysed protein can then be separated from the haem by ultrafiltration or centrifugation [26].

Pepsin, alcalase and proteinase have been used to hydrolyse haemoglobin [39, 40, 94]. The degree of hydrolysis will effect yield and properties of the peptides [26, 94]. Peptide yields reported in literature range from 65-85%. Strategies such as using exopeptidases for controlling the extent of reaction have been included to increase yield and reduce bitterness that can result from hydrolysis [103]. The hydrolysates are not completely colourless and further treatment to remove haem using activated carbon, ultrafiltration and bentonite clay is often required.

3.2.4 Oxidative Destruction of Haem

Hydrogen peroxide (H_2O_2) has been used to destroy haem. Methods described in literature vary. De Buyser (1999) and Izumi et al. (1991) suggest adding hydrogen peroxide to haemoglobin under alkaline conditions (pH 9-12.5) [42, 43]. Wismer-Pederson and Frohlich (1992) suggest treating the haemoglobin at pH 2-2.5 [38]. Red blood cells were usually modified by diluting to approximately 7% protein concentration. The amount of hydrogen peroxide used ranged from 0.3-10% (by protein solution weight). Residence times reached up to 25 hours and temperatures ranged from 20-90°C [38, 42, 43, 98]. Care must be taken when treating haemoglobin with hydrogen peroxide because excessive hydrogen peroxide can result in the oxidation of sulphur-containing amino acids in the protein and also cause a decrease in functional properties [38]. Other oxidising agents that can be used include sodium peroxide, calcium peroxide and nitrates [42].

Hydrogen peroxide is often produced as a by-product of oxygen dependent metabolism. Catalase, a naturally occurring enzyme, prevents hydrogen peroxide damage in cells. It is present in blood and rapidly breaks down hydrogen peroxide into water and oxygen. If catalase is present during hydrogen peroxide mediated decolouring, much of the hydrogen peroxide will be consumed by the enzyme-catalysed decomposition. Successful hydrogen peroxide treatment requires catalase to be deactivated, which can be achieved by heating to above 70°C [26]. However, heat treatment will coagulate proteins making them insoluble [26, 43]. An alternative method is to adjust the pH to 2 [26].

Hydrogen peroxide treatment has the disadvantage that long times are required for decolourisation (up to 24 hours). Other potential problems with using hydrogen peroxide include modifying side groups, crosslinking, backbone fragmentation and forming protein peroxides [104, 105]. Hydrogen peroxide is also used as a cleaning agent and to bleach pulp and paper. In the wastewater treatment industry hydrogen peroxide treatment has been used to reduce smell, chemical oxygen demand and biological oxygen demand. It has also been used to treat rendering gas emissions [106]. Cysteine, methionine, histidine, glycine and lysine are all susceptible to oxidation by hydrogen peroxide [107].

3.2.5 Other Methods

Gomez-Juarez et al (1999) hydrolysed haemoglobin using papain at pH 2.5 for 2 hours. The peptides were then ultrafiltered and decolourised using sodium hypochlorite at room temperature. Low levels of sodium hypochlorite are permitted as food bleach. The decolourised protein had good emulsifying and foaming properties [93].

Piot et al (1986) hydrolysed haemoglobin using pepsin at pH 2 for three hours. The peptides were then passed through alumina columns which absorbed haem containing peptides. When attempted on a large scale they had a protein yield of 25%. A white powder was obtained and consisted of peptide chains ranging from 5-13 amino acids long [39].

3.3 Alternative Chemicals for Colour and Smell Removal from Blood Products

The previously described methods for colour removal were developed for food applications where fresh blood or RBCC were used and no degradation odour compounds were formed. Therefore they may not be able to remove the odour compounds present in bloodmeal. Possible strategies to remove the smell are extraction or oxidation of the smell causing compounds. Treating bloodmeal with steam and activated carbon to extract smell compounds has been used with limited success [18]. Therefore they were not considered during this investigation.

Chemical oxidation has been used to remove smell causing compounds from sewage treatment plants, rendering plants and swine and poultry farms. The oxidising agents used are also commonly used to bleach or remove colour from textiles, fabrics and animal by-products by oxidising the bonds of the colour causing components. This means that the colour and smell from bloodmeal could potentially be removed using one processing step using chemical oxidation. Potential oxidising chemicals include sodium hypochlorite, sodium chlorite, sodium chlorate, chlorine dioxide, hydrogen peroxide and peracetic acid.

3.3.1 Sodium Hypochlorite, Sodium Chlorite, Sodium Chlorate and Chlorine Dioxide

Sodium hypochlorite (NaClO) is a common household bleach and disinfectant, usually available in a 3-6% solution. It is used commercially for cleaning in the beer, wine and food industries, in water treatment facilities and as bleach for textiles and blood [93, 108, 109]. Sodium hypochlorite has been used to treat rendering gas emissions and was found to be effective against a wide range of odorous compounds [106]. Sodium hypochlorite solutions are able to modify proteins through amino acid side chain modification, backbone fragmentation, crosslink formation, unfolding and chloroamine formation [108, 110]. Methionine, cysteine, histidine, tryptophan, lysine, tyrosine and the alpha amino group are most susceptible to modification by sodium hypochlorite [109-112].

Sodium hypochlorite is able to form trihalomethanes (atoms replaced by halogen atoms e.g. chlorine) and other chlorinated organic compounds which are pollutants and carcinogenic [113]. As a result alternatives chemicals to sodium hypochlorite have been used for textile bleaching and water treatment.

Sodium chlorite ($NaClO_2$) and sodium chlorate ($NaClO_3$) are chemicals used for pulp and paper bleaching [114]. Sodium chlorite is also used for disinfection, sterilisation and to oxidise odour compounds [115]. Sodium chlorite and sodium chlorate can also be used to produce chlorine dioxide (ClO_2) by acidification. Chlorine dioxide has been used to bleach pulp and paper as well as to disinfect water and foods. The main advantage is that it produces less chlorinated by-products compared to sodium hypochlorite [116]. It can be used in solution or as a gas. Chlorine dioxide has been shown to oxidise SH groups into disulphide bonds [117, 118]. Oxidation of proteins with tryptophan, histidine and tyrosine with chlorine dioxide led to carbon bond breakages and low molecular mass compounds [116]

3.3.2 Peracetic Acid

Peracetic acid (PAA, CH_3COOOH) also known as peroxyacetic acid, is a clear liquid with a strong vinegar smell and low pH (less than pH 2) [119]. It is produced commercially by reacting acetic acid with hydrogen peroxide in the presence of a catalyst and sold as an equilibrium mixture containing peracetic acid, acetic acid, hydrogen peroxide and water. Non-equilibrium mixtures can be produced using distillation to remove acetic acid, hydrogen peroxide and water although this is more expensive.

Due to its effectiveness and growing concerns over the environmental impact of chlorine use, PAA has been suggested as an alternative for use in waste water treatment and pulp and textile bleaching [119]. When used to bleach linen PAA gave better brightness and less fibre damage [120]. PAA has also been used as a food and surface sanitizer as well as to remove odours from wastewater sludges [119]. PAA is a protein denaturant and is known to react quickly with proteins [109, 121]. It does not produce any harmful disinfection by-products and if discharged into the environment PAA decomposes and bioaccumulation is unlikely to occur [113, 122].

Previous studies involving PAA and proteins had variable results in terms of its reaction with amino acids. In one study PAA was used to oxidise wool and it was shown PAA exclusively oxidised disulphide bonds and tryptophan side chains while no significant oxidation of tyrosine occurred [123]. A separate study showed that PAA readily oxidised cysteine, methionine, histidine, glycine and lysine but not tryptophan [107]. While a third study involving PAA and dairy proteins suggested tryptophan and methionine were most vulnerable and lysine was not affected [109]. The variable results could be due to the different reaction conditions, proteins used and PAA mixture compositions.

No articles were identified which focused on using PAA to decolourise blood or blood products for use in bioplastics. One of the disadvantages with PAA is its relatively high cost. However as demand and global production increases its cost may decrease [119].

A patent search revealed one relevant patent where a peracid was used to treat meat industry by-products [124]. McAninch and Holzhauer (2002) developed a method for bleaching tripe using a mixture of peracids, hydrogen peroxide and water [124]. It was suggested that the peracid mixture worked better than the existing method (35% hydrogen peroxide) because hydrogen peroxide did not work well as a cleaner and the existing processes had multiple stages and was not efficient. However this method was primarily focused on mixtures involving tartaric and malic acid with the final bleached product aimed at the food industry.

The common uses for the oxidising chemicals discussed and their potential effects on bloodmeal protein are summarised in Table 8.

Table 8: Possible side effects on protein when blood products are treated with various oxidising chemicals [107, 109, 110, 123].

Chemical	Uses	Potential Effects on Bloodmeal Protein
Sodium hypochlorite	Disinfectant, cleaning agent for food and wine industry, water treatment, bleach for pulp and paper	Side chain modification, backbone fragmentation, deamination of lysine, cross linking of cysteine and methionine, tryptophan susceptible to damage, tyrosine also susceptible to forming chlorotyrosine, formation of unstable chloramines on alpha amino group and lysine side chain.
Sodium Chlorite	Disinfectant, bleach for pulp and paper, chlorine dioxide generation	Side chain modification, backbone fragmentation, cross link formation and chlorination of amino acids.
Sodium Chlorate	bleach for pulp and paper, chlorine dioxide generation	Side chain modification, backbone fragmentation, cross link formation and chlorination of amino acids.
Chlorine dioxide	Disinfectant, water treatment, bleach for pulp and paper	Denaturation by modification of tryptophan and tyrosine residues, degradation of amino acids into small molecular weight products.
Hydrogen Peroxide	Disinfectant, bleach for hair, pulp and paper, effluent treatment	Side chain modification, backbone fragmentation, cross link formation.
Peracetic Acid	Disinfectant, water and sludge treatment, tripe bleach	Backbone fragmentation if hydroxyl radicals formed during breakdown of peracetic acid, crosslink and double bond breakage, denaturation.

3.4 Protein Oxidation

Proteins and other biological molecules can be damaged or modified by oxidants. Protein oxidation can lead to side chain modification, back bone cleavage and cross link formation [125, 126]. The amount and type of damage varies depending on the type, strength and concentration of the oxidant as well as secondary reactions (e.g. repair mechanisms and antioxidants) [127]. In natural systems small amounts of some oxidants (e.g. superoxide, hydrogen peroxide, hydroxyl radical) are produced through metabolism. As a result there are antioxidant defences such as enzymes (e.g. superoxide dismutase, catalase) which can scavenge radicals and help reduce damage to biomolecules [110, 125, 127].

The strength of oxidants, stability of newly formed or intermediate oxidants, number of C-H bonds and accessibility are factors which influence where oxidation will occur. Highly reactive radicals such as the hydroxyl radical (•OH) are the least selective and cause the most damage. OH• oxidises most side chains and can also react with the polypeptide backbone causing both fragmentation and side chain modification [127, 128].

Backbone oxidation involving •OH and oxygen happens through hydrogen atom abstraction from the alpha carbon forming a carbon centred radical and can lead to fragmentation. The type of products formed by fragmentation depends on the pathway by which fragmentation occurs (Figure 15).

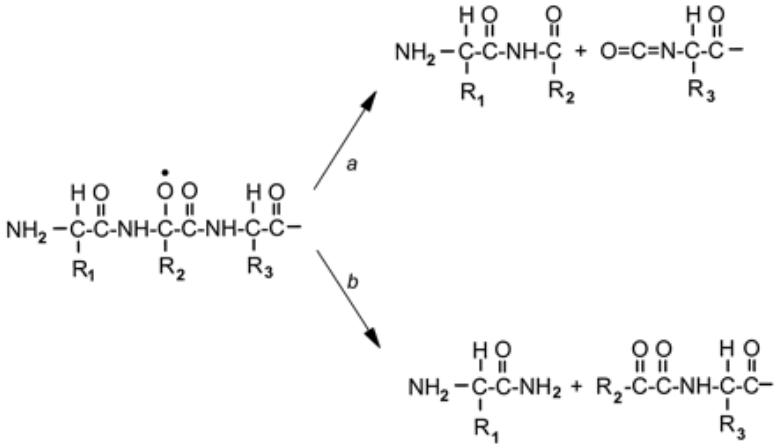


Figure 15: Peptide bond cleavage via alternative pathways. (a) diamide pathway and (b) α-amidation pathway [126].

Cysteine and methionine are the most susceptible amino acids to oxidation. Oxidation of cysteine results in the formation of disulphides and thiyl radicals. Methionine oxidation results in the formation of methionine sulfoxide. Because they are easily oxidised by a range of oxidants, repair systems exist for these two amino acids and enzymes are able to convert them back to their unmodified forms. These are the only systems known that are able to repair oxidative modification to amino acids [125, 126, 129, 130]. However these repair mechanisms are not expected to be active in RBCC or bloodmeal.

Common products from side chain oxidation are peroxides, alcohols and carbonyls. Carbonyls have been used to monitor amino oxidation using western blotting and high performance chromatography [130]. Other products used to monitor oxidative modifications include disulphides, thiyl radicals and cross-linked aggregates. Possible modifications are summarised in Table 9 and methods used for their detection in Table 10.

Table 9: Most susceptible amino acids to oxidation and their possible oxidation products [125, 126, 129].

Amino Acid	Possible modifications
Cysteine	Disulfides, glutathiolation, cysteic acid
Methionine	Methionine sulfoxide, methionine sulfone
Histidine	Oxo-histidine, asparagines, aspartic acid
Tyrosine	Dityrosine, chlorotyrosine, nitrotyrosine
Lysine	Chloramines, deamination
Arginine	Glutamic semialdehyde
Valine, Leucine, Tyrosine, Tryptophan	Hydroperoxide and hydroxide derivatives
Several	Cross links, aggregates, fragments
All	Carbonyls

Protein peroxides can also be formed which are able to damage other proteins. The amount and structure depends on the location, type of radical, oxygen concentration and the presence of other reactants [127, 131]. Transfer of the radical from the side chain to the backbone is favourable due to the stability of the alpha carbon radical, reduced steric crowding and the stability of carbonyl products formed [127].

Table 10: Methods used for detecting some oxidation products [125].

Oxidative modification	Common methods used for detection
Disulfides	SDS gel electrophoresis
Thiyl radicals	Electron spin resonance spectroscopy
Methionine sulfoxide	Amino acid analysis
Carbonyls	Western blot, high performance liquid chromatography
Crosslinks/aggregates	SDS gel electrophoresis, high performance liquid chromatography
Fragments	SDS gel electrophoresis, high performance liquid chromatography

Protein oxidation can have negative effects on the properties and processing characteristics of bioplastics (Figure 16). Side chains participate in hydrogen bonding and hydrophobic interactions which stabilise the bioplastic. If side chains are modified they may not be able to contribute to stabilising interactions which will lead to a decrease in mechanical properties such as strength, stiffness and elongation. Backbone fragmentation can also lead to reduced mechanical properties as well as poor consolidation because the smaller chains would have reduced entanglements and fewer stabilising interactions. If disulphide cross-linking between proteins occurred then processing would also be inhibited because the cross-links would prevent the protein chains from flowing past each other. This can result in processing equipment blocking or high shear rates which can lead to protein degradation.

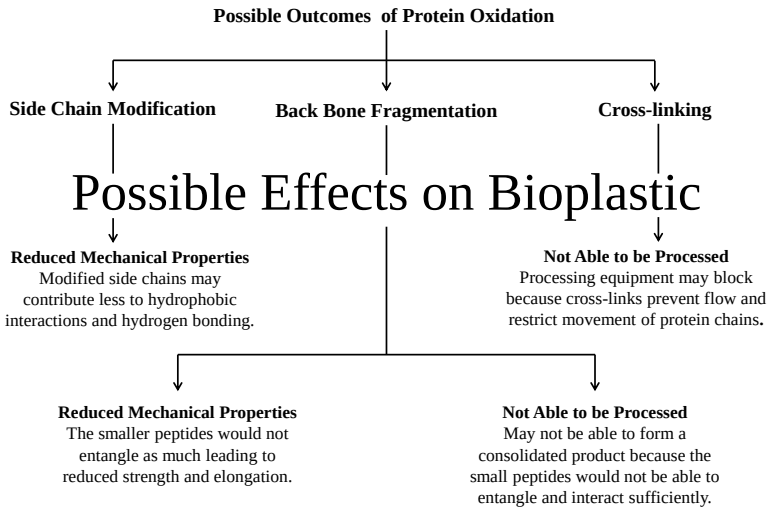


Figure 16: How protein oxidation can affect properties and processing of bioplastics.

3.5 Conclusion

Several existing methods were identified to decolour blood or RBCC with the main purpose of using them as an additive in foods. However these methods were expensive or produced peptides which were not suitable for bioplastics. In addition these methods would not be effective at removing odour compounds from bloodmeal.

From the existing methods available for decolouring RBCC for food applications, hydrogen peroxide decolourisation was identified as having the most potential for applying the decoloured RBCC powder to bioplastics. Therefore this method was selected to be investigated further in Chapter Four.

Chemical oxidation could potentially be used to remove the colour and smell from bloodmeal, but treating proteins with oxidants can cause damage to the protein. The amount and type of damage depends on the oxidant, protein and defence mechanisms. Back bone fragmentation, side chain modification and cross link formation can occur when treating proteins with oxidants. This can prevent the powder being processed or reduce mechanical properties of the bioplastic. Potential chemicals identified were sodium hypochlorite, sodium chlorite, sodium chlorate, chlorine dioxide, hydrogen peroxide and PAA. The effect of these on RBCC and bloodmeal were investigated in Chapter Five.

Chapter 4

Red Blood Cell Concentrate Decolourisation using Hydrogen Peroxide

RBCC (left) and hydrogen peroxide treated RBCC (right).

Work from this chapter was published in the journal paper: Decolouring Haemoglobin as a Feedstock for Second-Generation Bioplastics [132].

4.1 Chapter Overview

Previous studies have focused on colour removal from RBCC for food applications. Hydrogen peroxide treatment was one of these methods and was identified as having the most potential for removing the colour from RBCC for use in bioplastic applications.

RBCC concentration, pH and reaction temperature were the most important factors influencing the decolourising process. They were investigated with the aim of developing a method which could be scaled to commercial level for producing a bioplastic feedstock. Initial trials showed pH was an important factor for decolourisation and solids lost due to foaming. At pH 14 there was a 96% reduction in solution colour but 8.4% solids were lost due to foaming. There was a 76% reduction in solution colour at pH 2 and only 2.6% solids were lost due to foaming. The optimal conditions identified for the potential large scale production of decolourised protein from RBCC were: centrifuging 9% w/w RBCC aqueous solution at pH 2, then reacting with 7.5 g (30% w/w) hydrogen peroxide at 30°C. These conditions achieved a 93% reduction in solution colour after three hours and the molecular weight of the decoloured protein was not significantly reduced.

4.2 Experimental

4.2.1 Aim

The overall aim of this section was to identify optimal conditions for decolouring RBCC with hydrogen peroxide so it could be scaled to commercial production. More specifically, the objective was to investigate the effect of pH, RBCC concentration and reaction temperature on colour reduction as well as the effectiveness of the process, which was characterised by decoloured protein yield.

4.2.2 Methods and Materials

Frozen RBCC (33% solids) centrifuged from hygienically-collected bovine blood was supplied by a local meat processing facility. All other chemicals were supplied by Ajax Finechem.

The effect of pH, RBCC concentration, hydrogen peroxide concentration, reaction temperature and centrifugation on decolouring and foaming was explored. All trials were repeated at least two times.

10 g RBCC (33% solids by mass) was mixed with 100 g distilled water in 1 L glass beakers. Mass was used as RBCC was too viscous to accurately measure volumetrically. Solution pH was adjusted to 2, 5, 8, 11, 13 and 14 by adding 1 mol/L HCl or 2 mol/L NaOH. After slowly adding 15 g (30% w/w) hydrogen peroxide while mixing, the solution was left to react for three hours.

To determine the effect of RBCC concentration on decolouring and foaming, 10-30 g RBCC was added to distilled water and the total mass made up to 110 g with distilled water. Resulting solutions were adjusted to pH 2 and 15 g (30% w/w) hydrogen peroxide was added and left to react for three hours. For both pH and RBCC concentration trials, mass loss due to foaming was determined by collecting foam produced during reaction in a pre-weighed container and oven drying overnight at 60°C. Percentage mass lost due to foaming was calculated by dividing dry mass of foam by initial dry solids of the solution.

Solution colour before, during and after treatment was determined by measuring UV/Vis absorbance at 540 nm. Reduction in solution colour was reported as percentage change in absorbance from the initial absorbance.

To investigate the effect of temperature and hydrogen peroxide concentration on decolouring, 7.5, 15 or 22 g (30% w/w) hydrogen peroxide was added to 9% w/w RBCC aqueous solutions at pH 2. Solutions were kept in a preheated water bath with an oscillating platform at 20, 30 or 40°C. The absorbance of each solution at 540 nm was measured every 20-30 minutes for three hours and the percentage change calculated. Hydrogen peroxide remaining after three hours was determined by titrating liberated iodine from acidified potassium iodide solution using 0.1 M sodium thiosulfate (standardised using potassium iodate) and ammonium molybdate catalyst [133].

To investigate the effect of centrifugation, two 9% w/w RBCC aqueous solutions at pH 2 were centrifuged separately at 4000 rpm for five minutes to remove haem precipitate. Supernatants were decanted and treated with 7.5 g or 15 g hydrogen peroxide. After three hours, the final colour content was measured and percentage change calculated.

Decoloured RBCC was produced by centrifuging 9% w/w RBCC aqueous solution at pH 2, then reacting with 7.5 g (30% w/w) hydrogen peroxide at 30°C. The decoloured solution was freeze dried and ground into a powder. Protein solubility at various concentrations were tested at pH 4, 7 and 9.5 by adding 20 ml phosphate buffer to pre-weighed amounts of decolourised protein in test tubes. After one hour, solubility was investigated by visual examination.

4.2.2.1 Molecular Weight Analysis

Molecular weight was investigated by dissolving 8-9 mg decoloured powder in 0.02 M phosphate buffer at pH 7 containing 2% sodium dodecyl sulphate, 0.5% sodium sulphite and 0.1 M sodium chloride, heated to 100°C for 5 minutes. After centrifuging the solution at 11,000 rpm for three minutes to remove any remaining solid particles, a 0.5 ml aliquot was applied to a Superdex 200 gel filtration column (GE Healthcare) connected to an Akta Explorer 100 (GE Healthcare). The column was calibrated with a low molecular weight size exclusion calibration kit (GE Healthcare). Two column volumes of 0.02 M phosphate running buffer at pH 7 containing 0.1% sodium dodecyl sulphate and 0.1 M sodium chloride was applied at a flow rate of 0.5 ml/min. Protein concentration was measured at 215 nm using an inline detector.

4.3 Results and Discussion

4.3.1 Effect of pH on Decolouring and Foaming

The greatest change in colour occurred at pH 2 (76% reduction) and pH 14 (96% reduction). The change in colour decreased as pH approached pH 8, where there was only a 3% reduction in colour (Figure 17). At both pH 9 and pH 12, solution colour was reduced by 27%. Deybuyser (1999) suggested decolouring haemoglobin at pH 9-12 at 50°C. The brown protein they produced required further oxidative treatment for 1 second at high temperature (150°C) or 48 hours at low temperature (50°C). Decolouring appeared to depend on catalase activity, which was highest at pH 8. Where catalase activity was high, decolouring extent was low.

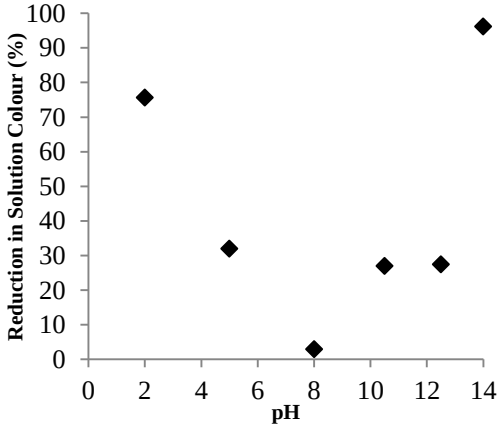


Figure 17: Reduction in solution colour at different pH.

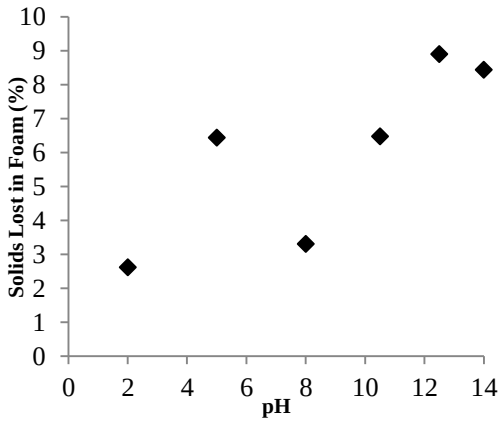


Figure 18: Solids lost in foam at different pH.

In general as pH increased, percentage solids lost increased except for pH 8 where only 3.3% solids were lost in the foam (Figure 18). The pH was clearly important for decolourisation and for controlling the amount of solids lost in the foam. Foaming was a combination of catalase action, protein denaturation and spontaneous hydrogen peroxide decomposition. As pH deviated from neutral, more protein may have been denatured and trapped in the foam. At pH 8 very little protein was denatured and foaming was mainly due to catalase action which may explain why pH 8 did not follow the general trend. At pH 14, large amounts of protein may have been denatured. Hydrogen peroxide also decomposes and readily produces oxygen in alkaline conditions. The combined effect of large amounts of oxygen and denatured protein resulted in high mass lost in the foam.

At pH 2, catalase action was reduced and hydrogen peroxide was stabilised. Therefore, there was less oxygen from hydrogen peroxide decomposition and less chance for protein to be trapped in this foam which minimised protein losses at pH 2.

Decolourisation was most effective at pH 2 and pH 14. However, pH 14 was not suitable for large scale production due to the high loss of solids in the foam. At pH 2, catalase action was reduced and hydrogen peroxide was able to degrade the haem. Haem also dissociated from the globin at this pH and formed an aggregate because of its hydrophobic nature [7]. Therefore, pH 2 was the best pH for decolourisation, which was consistent with previous work done by Wismer-Pedersen and Frohlich (1992).

4.3.2 Effect of Dilution on Foaming

It is desirable to minimise foam in a large scale process. This could be achieved by having low RBCC concentrations. At pH 2, the solids lost in foam increased linearly with RBCC concentration (Figure 19). The best concentration was found to be 9% w/w RBCC because at this concentration the least solids were lost due to foaming. Antifoam could be used as an alternative approach to reduce foaming. However this was not investigated and should be investigated in future studies.

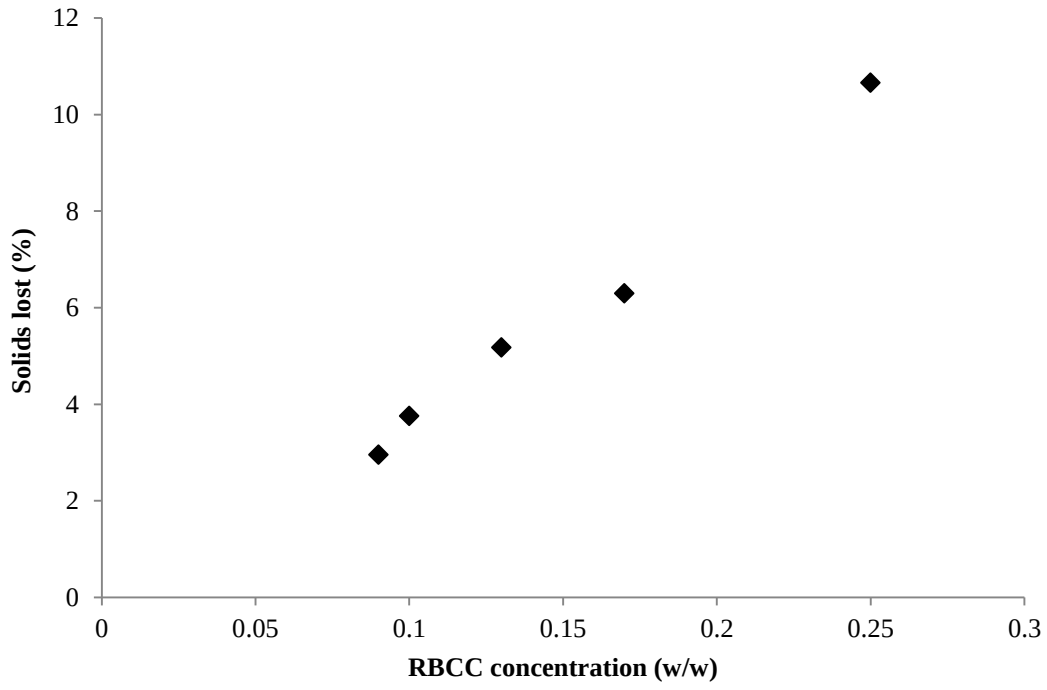


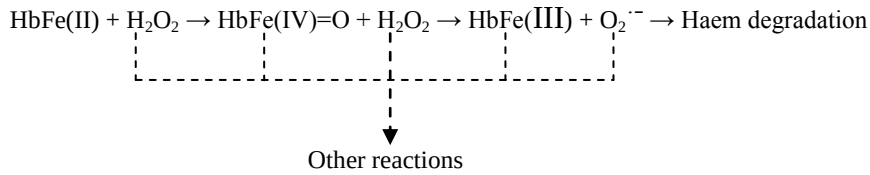
Figure 19: Percentage solids lost as foam at different RBCC concentrations.

4.3.3 Decolourisation Rate at Different Temperatures and Hydrogen Peroxide Concentrations

Rate of decolourisation increased as temperature and hydrogen peroxide added increased (Figure 20, Figure 21, Figure 22). Comparing the 20°C curve for all three hydrogen peroxide amounts shows that when the amount of hydrogen peroxide added increased from 7.5-22 g the rate of decolourisation increased. This was also consistent for 30°C and 40°C.

However, increasing the amount of hydrogen peroxide had little effect on final percentage decolourisation. Thus, regardless of how much hydrogen peroxide was used, a maximum of approximately 90% decolourisation was obtained. It would be favourable to use as little hydrogen peroxide as possible to reduce operating costs.

The reactions expected to occur during decolouring, based on the mechanism described by Nagababu and Rifkind (2000) are: Hydrogen peroxide reacts with haemoglobin to form a reactive intermediate called ferrylhaemoglobin. Ferrylhaemoglobin is a strong oxidising agent and reacts with another molecule of hydrogen peroxide to produce the superoxide free radical and water. This can occur either in the haemoglobin molecule with the haem porphyrin still attached or outside the globin if the haem has floated free due to the acidic pH. Hydrogen peroxide may also react with methaemoglobin ($HbFe^{3+}$) and produce oxygen instead of superoxide. This does not lead to haem degradation. The haemoglobin intermediates are also involved in side reactions which consume hydrogen peroxide. The overall reaction mechanism described by Nagababu and Rifkind (2000) is:



(1)

Superoxide (SO) can either react with the tetrapyrrole rings, diffuse out of the haem molecule and be removed by superoxide dismutase (an enzyme present in natural systems which degrades superoxide produced as a by-product during

metabolism), or react with the globin. It is not known how many superoxides react with a haem molecule, or are consumed by reacting with superoxide dismutase or globin.

Haem concentration was directly related to absorbance at 540 nm and reaction rates were obtained by fitting the following equations for haem degradation (2), hydrogen peroxide consumption (3), and superoxide production and consumption (4). The equations assumed two hydrogen peroxides (HP) were consumed to produce a superoxide (SO), *N* moles of SO were consumed to degrade a haem molecule, and *M* moles of SO reacted with a globin or superoxide dismutase (which will be proportional to the globin concentration). Concentration was measured in mol.g^{-1}.

$$\frac{\partial C_{Haem}}{\partial t} = -k_1 C_{Haem} C_{SO} \tag{2}$$

$$\frac{\partial C_{HP}}{\partial t} = -k_2 C_{Haem} C_{HP} \tag{3}$$

$$\frac{\partial C_{SO}}{\partial t} = 2k_2 C_{Haem} C_{HP} - k_1 C_{Haem} N C_{SO} - k_3 C_{Globin} M C_{SO} \tag{4}$$

The parameters k_1, k_2, k_3 (g.mol^{-1}.s^{-1}), *N* and *M* (Table 11) were obtained by fitting the model to experimental data. k_1, k_2 and k_3 varied with temperature and an Arrhenius equation:

$$k = Ae^{\left(\frac{-E_A}{RT}\right)} \tag{5}$$

was used to estimate the Arrhenius constants (Table 12). *A* is the frequency factor, E_A is activation energy, *R* is the ideal gas constant and *T* is temperature in Kelvin. Goodness of fit was determined by summing the error squared between model and experimental data and by regression analysis (coefficient of determination) (Table 13).

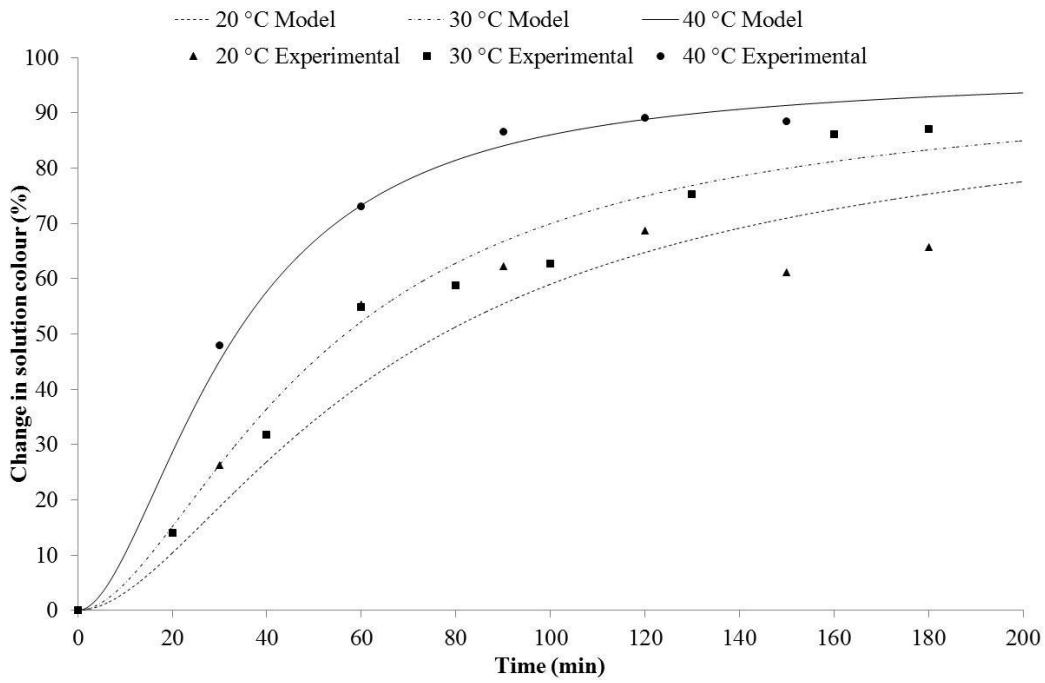


Figure 20: Change in solution colour, 10 g RBCC, 100 g distilled water, pH2, 7.5 g hydrogen peroxide at 20, 30 and 40°C.

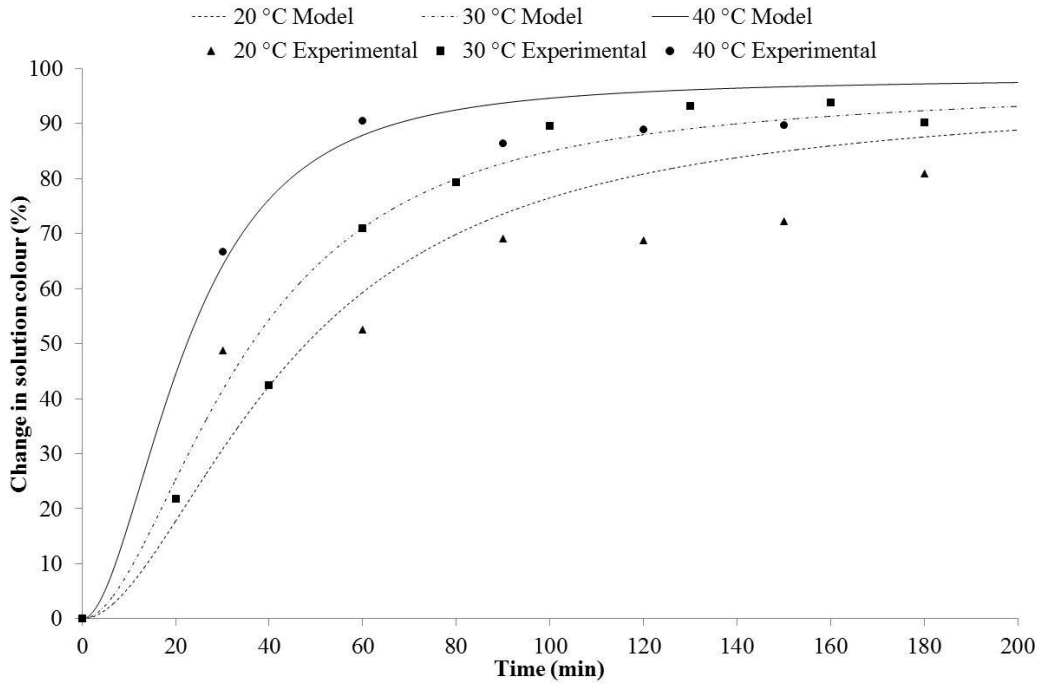


Figure 21: Change in solution colour, 10 g RBCC, 100 g distilled water, pH2, 15 g hydrogen peroxide at 20, 30 and 40°C.

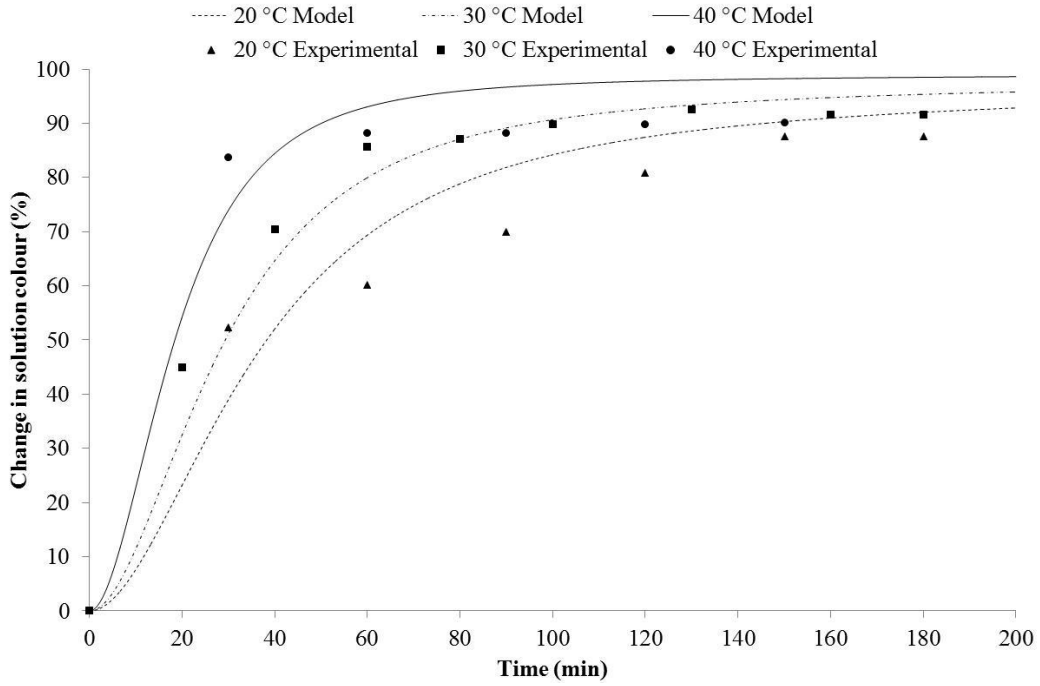


Figure 22: Change in solution colour, 10 g RBCC, 100 g distilled water, pH2, 22 g hydrogen peroxide at 20, 30 and 40 °C.

Table 11: Fitted model parameters.

Parameter	Fitted Constants		
	20°C	**30°C**	**40°C**
k_1 (g.mol^{-1}.s^{-1})	80000	100000	140000
k_2 (g.mol^{-1}.s^{-1})	20000	25000	40000
k_3 (g.mol^{-1}.s^{-1})	10000	10000	10000
N (mol)	10	10	10
M (mol)	500	500	500

Table 12: Activation energy and frequency factor for model parameters.

	k_1	k_2	k_3
A (g.mol^{-1}.s^{-1})	3.28 x 10^{8}	9.52 x 10^{8}	6.00 x 10^{5}
E_A (J.mol^{-1})	20240	26000	10330

Table 13: Goodness of fit values for fitted models.

H_2O_2 (g)	**7.5**			**15**			**22**		
Temp (°C)	**20**	**30**	**40**	**20**	**30**	**40**	**20**	**30**	**40**
R^2	0.87	0.98	0.99	0.83	0.98	0.97	0.92	0.97	0.95
SSE	520.26	139.78	22.72	742.02	205.73	162.56	476.81	244.81	319.36

The model showed a good fit with experimental data for 30 and 40°C (Table 13) but was not as good a fit at 20°C, probably due to diffusion effects that were not accounted for in the model. At the higher temperatures, diffusion was likely to be sufficiently rapid so the reaction rates were the rate limiting steps. The model was particularly sensitive to the rate of SO production (k_2), indicating that this was a limiting step. Reducing k_2 reduced overall percentage decolouring. Rate of SO reaction with superoxide dismutase and globin (k_3) appeared to be independent of temperature, but was significant because of the large *M* factor required to fit the data.

Hydrogen peroxide concentrations showed little change both experimentally (Table 14) and theoretically, showing that while initial peroxide concentration is important for reaction rates, very little is consumed. If the decoloured globin can be recovered from solution, for example by ultrafiltration, the solution could potentially be recycled to minimise hydrogen peroxide use. Alternatively, the hydrogen peroxide could be degraded by adding catalase to prevent excessive oxidation of the protein.

Table 14: Change in hydrogen peroxide concentration after three hours.

H_2O_2 (g)	**7.5**			**15**			**22**		
Temp (°C)	**20**	**30**	**40**	**20**	**30**	**40**	**20**	**30**	**40**
Initial concentration (mol/L)	0.56	0.56	0.56	1.06	1.06	1.06	1.47	1.47	1.47
Final concentration (mol/L)	0.40	0.34	0.32	0.80	0.77	0.72	1.17	1.13	1.08
Percent consumed	29	39	43	25	27	32	20	23	27

When producing decoloured protein on a large scale, it would be advantageous to reduce the time taken for decolourisation. The data (Figure 20 to Figure 22) indicated there were two options: add large amounts (22 g) of hydrogen peroxide at low temperature (20°C) or add smaller amounts (7.5 g) of hydrogen peroxide and heat to between 30 and 40°C

Adding large amounts of hydrogen peroxide is not recommended because it can oxidize amino acids in the protein or cause peptide fragmentation. Large amounts of hydrogen peroxide would also increase production costs.

If the decoloured protein is destined to be used in food applications excessive heating is not recommended as it can denature proteins causing a loss in

functional properties such as foaming, solubility and emulsification. However for bioplastics loss of functional properties is not as important, therefore a higher heating temperature could be used. The body temperature of cattle is approximately 38°C. Therefore, heating to 30°C is only a mild heat treatment and is unlikely to cause excessive damage to the protein. Adding 7.5 g (30% w/w) hydrogen peroxide and heating to 30°C was selected as the preferred method for a potential large scale process.

4.3.4 Centrifuging Before Hydrogen Peroxide Addition

When RBCC solution was reduced to pH 2, a proportion of haem dissociated from the globin and formed an aggregate, which could be removed by centrifugation. When RBCC solution was centrifuged before adding hydrogen peroxide, decolourisation was more efficient (Table 15). For example, adding 7.5 g (30% w/w) of hydrogen peroxide with no centrifugation reduced colour by 62%. If the same amount of hydrogen peroxide was added after centrifugation, there was a 92% reduction in colour. Less foam was also observed when hydrogen peroxide was added after centrifugation.

Removing the haem precipitate by centrifugation reduced the haem in solution and therefore reduced the hydrogen peroxide required for decolourisation. The haem removed by centrifugation was most probably unmodified and has the potential to be used in iron supplement products. Reducing the amount of hydrogen peroxide used was desirable so production cost would be minimised and protein molecular mass maintained.

Table 15: Percentage reduction in solution colour when different amounts of hydrogen peroxide are added without centrifuging and with centrifuging.

Conditions	No Centrifugation	With Centrifuging
RBCC	0.00%	5.52%
Control (pH 2, No H_2O_2)	0.00%	66.55%
7.5 g H_2O_2	62.70%	92.83%
15 g H_2O_2	84.68%	96.00%

4.3.5 Decoloured Powder Solubility

The decoloured protein showed good solubility at pH 4. When the pH was increased to 7 solubility decreased. The decolourised protein was sparingly soluble at pH 9.5 (Table 16). Good solubility at pH 4 and pH 7 indicated the protein was not substantially modified or damaged by lowering the pH, adding hydrogen peroxide or heat treatment.

Wismer-Pedersen and Frohlich (1992) suggested that pH of the RBCC solution be readjusted to between pH 4-5 before adding hydrogen peroxide, to protect the protein and prevent loss of functional properties. However, when this was attempted, large volumes of foam formed. Most catalases have an optimal activity between pH 7-8. Catalase activity was reduced at pH 2 but activity was restored when pH was raised causing foaming.

Table 16: Decolourised protein solubility at pH 4, pH 7 and pH 9.5.

Conc. (mg/ml)	pH 4	pH 7	pH 9.5
0.25	Dissolved	Dissolved	Dissolved
0.5	Dissolved	Dissolved	Dissolved
1.0	Dissolved	Dissolved	Not Dissolved
2.0	Dissolved	Dissolved	Not Dissolved
3.0	Dissolved	Dissolved	Not Dissolved
4.0	Dissolved	Dissolved	Not Dissolved
5.0	Dissolved	Dissolved	Not Dissolved
6.0	Dissolved	Dissolved	Not Dissolved
7.0	Dissolved	Not Dissolved	Not Dissolved
8.0	Dissolved	Not Dissolved	Not Dissolved
9.0	Dissolved	Not Dissolved	Not Dissolved
10.0	Dissolved	Not Dissolved	Not Dissolved

4.3.6 Decoloured Powder Molecular Mass

The RBCC and decoloured RBCC have similar gel filtration elution profiles (Figure 23), indicating the molecular weight of the protein was not altered significantly. This suggested that the mechanism for haem degradation described by Nagababu and Rifkind (2000) was suitable for this process [104]. Molecular mass was maintained because the iron contained within the haem group is unable to act as a Fenton reagent with hydrogen peroxide and produce the more reactive hydroxyl radical which could lead to backbone fragmentation [26, 31].

Because the molecular mass of the decoloured protein was not reduced it could be possible to process it into a bioplastic. However disulphide cross-links could also be forming during hydrogen peroxide treatment. The dissolving buffer contained sodium sulphite which removed disulphide cross-links. Therefore these would not have been detected and if present would restrict processing. This was investigated further using thermal analysis in Chapter Five.

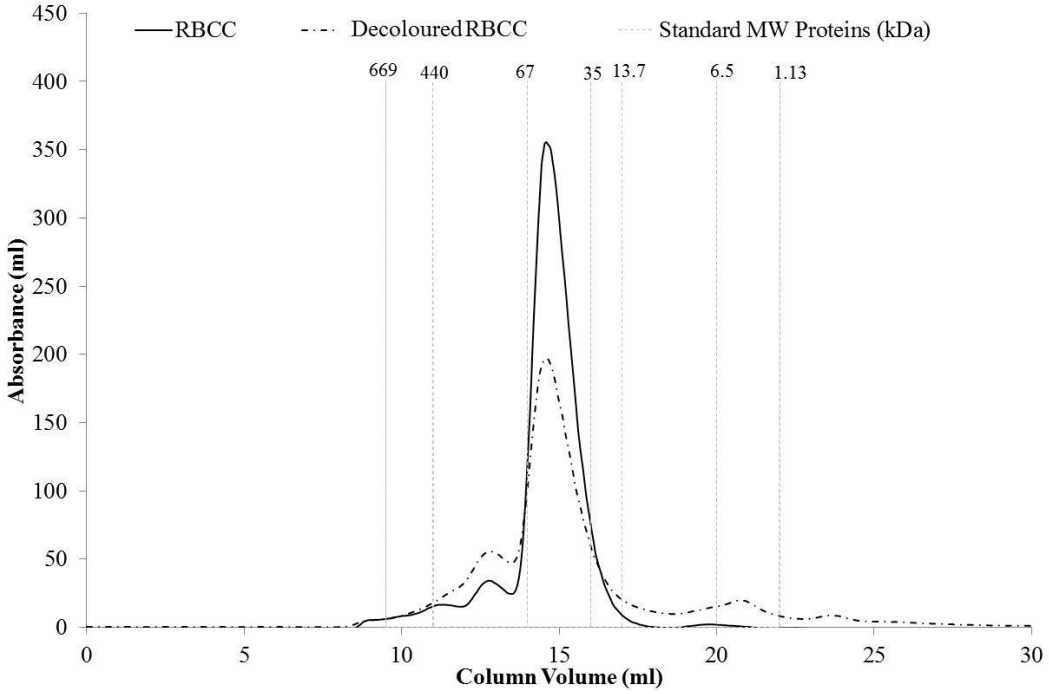


Figure 23: Elution profile for hydrogen peroxide decoloured RBCC.

4.4 Conclusion

Utilisation of the protein in RBCC in human food is limited and it could be a potential feedstock for second generation bioplastics. Published decolourisation methods utilising hydrogen peroxide have problems such as excessive foaming, long processing times and the potential for amino acid oxidation when large amounts of hydrogen peroxide are used.

The effects of RBCC concentration, pH and reaction temperature were the most important factors influencing the decolourising process. They were investigated with the aim of developing a method which could be scaled to commercial level for producing a bioplastic feedstock.

Initial trials showed pH was a very important factor and affected foaming and decolourisation. A 96% reduction in solution colour was achieved at pH 14, but 8.4% solids were lost due to foaming. At pH 8, solution colour was only reduced by 3% and 3.3% solids were lost. Solids lost in foam were thought to depend on a combination of catalase action, protein denaturation and spontaneous hydrogen peroxide decomposition. The best pH was found to be pH 2, where solution colour was reduced 76% and only 3% solids were lost in foam.

The amount of hydrogen peroxide required for decolourisation was reduced by centrifuging to remove aggregated haem after pH adjustment. The research showed that the best conditions for the potential large scale production of decolourised protein from RBCC were: centrifuging 9% w/w RBCC aqueous solution at pH 2, then reacting with 7.5 g (30% w/w) hydrogen peroxide at 30°C.

These conditions achieved a 93% reduction in solution colour after three hours and the molecular weight of the decoloured protein was not significantly reduced.

This section successfully produced decolourised protein from RBCC with a high yield. However, further investigations into removal of haem by increasing centrifugation time or speed could further reduce the treatment time and hydrogen peroxide required. Strategies for recycling or removing remaining hydrogen peroxide should also be investigated.

Although this method produced a decoloured protein from RBCC while maintaining its molecular mass, it may not be suitable for use with bioplastics because it requires large volumes of water and long residence times which can increase processing costs. Therefore it is recommended that alternative methods for decolouring RBCC and bloodmeal be investigated before selecting the most preferred method.

Chapter 5

Investigating Red Blood Cell Concentrate and Bloodmeal Decolourisation

Bloodmeal powder.

5.1 Chapter Overview

In Chapter Four, the colour from RBCC was successfully modified by diluting with water, reducing to pH 2, centrifuging and then treating with hydrogen peroxide. However, this method had several disadvantages such as long processing times, multiple processing steps and high water requirements. The objective of this chapter was to explore different methods for efficiently producing decoloured blood protein for use in bioplastics. More specifically a method was required which was simple, environmentally friendly and efficiently removed colour and odour while also avoiding significant reduction in the proteins molecular mass.

The colour is caused by the haem porphyrin and the smell by degradation odour compounds. These are susceptible to oxidation due to their conjugated or ring structures. In this chapter the oxidising chemicals sodium hypochlorite, sodium chlorite, sodium chlorate, chlorine dioxide, hydrogen peroxide and PAA were applied to RBCC, modified RBCC (mRBCC) and bloodmeal at high concentrations. Their effect on colour, smell and molecular weight were investigated. In addition crystallinity and thermal stability was investigated for the most effective methods.

When sodium hypochlorite, sodium chlorate and chlorine dioxide was used to treat RBCC, mRBCC and bloodmeal they produced treated powders which were 22-61% white. Molecular mass was not significantly reduced but they were not effective at removing the odour from bloodmeal. Therefore these methods were not suitable for producing a bioplastic feedstock.

Sodium chlorite, hydrogen peroxide and PAA treatment of RBCC and mRBCC produced treated powders which were 55-93% white. However these methods were not suitable for use with bioplastics because they significantly reduced the molecular mass of the protein, required long residence times or for the case of sodium chlorite had environmental concerns such as chlorination of the protein.

Treating bloodmeal with hydrogen peroxide, sodium chlorite and PAA produced powders which were 31-76% white and also improved the odour. PAA treatment had the greatest effect on bloodmeal colour and smell and also does not have the

same environmental problems as sodium chlorite treatments. Decolouring was completed with five minutes. Bloodmeal molecular weight was not significantly reduced which indicated it could still be possible to process it into a bioplastic and the mechanical properties may not be reduced. Therefore it was concluded that treating bloodmeal with PAA had the most potential for producing a decoloured protein for use in bioplastics.

5.2 Experimental

5.2.1 Aim

The aim of this section was to investigate the decolouring and deodorising effect of the different readily available oxidising chemicals identified in Chapter Three on RBCC, mRBCC and bloodmeal. These were used at high concentrations to reduce the time required for colour and odour removal.

5.2.2 Methods and Materials

5.2.2.1 Bloodmeal, RBCC and mRBCC Treatment Trials

Three different blood products were used in this chapter, these were bloodmeal, RBCC and mRBCC. Bloodmeal is a dried blood protein powder, RBCC is a viscous liquid produced by centrifuging whole blood and mRBCC is diluted RBCC which has been adjusted to pH 2 and centrifuged. Their properties are summarised in (Table 17).

Table 17: Summary of blood products used to produce a decoloured protein for use in bioplastics.

Material	Supplier	Grade	Solids (%)	pH
Bloodmeal	Wallace Corporation	Inedible	95	-
RBCC	Local meat processing factory	Hygienically collected	33	7
mRBCC	Produced by author from hygienically collected RBCC		6.6	2

Bloodmeal decolourisation and deodorisation was investigated by treating 5 g of bloodmeal with 20 g of decolouring solutions of varying strengths (Table 18). The contents were stirred for one hour then filtered and washed with distilled water using a Buchner funnel and Whatman grade one (11 µm cut off) filter paper.

RBCC decolourisation and deodorisation was investigated by treating 5 g of RBCC with 20 g of decolouring solutions and stirring for one hour.

mRBCC was produced by diluting 5 g of RBCC with 20 g of distilled water and lowering to pH 2 using 1 mol/L HCl. At this pH the protein becomes denatured and the haem dissociates and forms a precipitate. Solutions were centrifuged at 4000 RPM for five minutes using a Sigma 6-15 centrifuge. Supernatant was decanted and treated with 20 g of decolouring solutions. The contents were stirred for one hour. If a precipitate formed it was filtered and washed with distilled

water using a Buchner funnel and Whatman grade one (11 µm cut off) filter paper.

When decolouring mRBCC with hydrogen peroxide, the existing method from Chapter Four was used where 5 g RBCC were diluted with 50 g of distilled water, adjusted to pH 2, centrifuged and the supernatant and treated with 3.75 g of hydrogen peroxide (30%) at 30°C for three hours.

All treated samples were frozen then freeze dried overnight using a Labconco Freezone 2.5 freeze dryer to between 5-10% moisture. Improvement of smell was analysed subjectively by the author based on previous experience with blood products.

Table 18: Chemicals used for decolouring RBCC and bloodmeal.

Material	Supplier	Grade
Sodium hypochlorite (15, 10, 5 wt%)	Ajax Finechem	Analytical
Sodium chlorite (5 wt%)	Ajax Finechem	Analytical
Sodium chlorate (5 wt%)	Ajax Finechem	Analytical
Chlorine dioxide (2 wt%)	Prepared from acidified sodium chlorite	-
Hydrogen peroxide (30, 15, 5 wt%)	Ajax Finechem	Analytical
Peracetic acid (5 wt% peracetic acid, 7.5 wt% Acetic acid, 25 wt% Hydrogen peroxide)	Solvay	Industrial
Peracetic acid (15 wt% Peracetic acid, 17.5 wt% Acetic acid, 21 wt% Hydrogen peroxide)	Degussa	Industrial
Copper sulphate (5 wt%)	Allied Signal	Analytical

Note: Hydrogen peroxide and sodium hypochlorite diluted to required percentage using distilled water. Sodium chlorite, sodium chlorate and copper sulphate solutions prepared by dissolving required amount of solids in distilled water.

The blood products used and amount of decolouring chemicals used in this chapter are summarised in Figure 24.

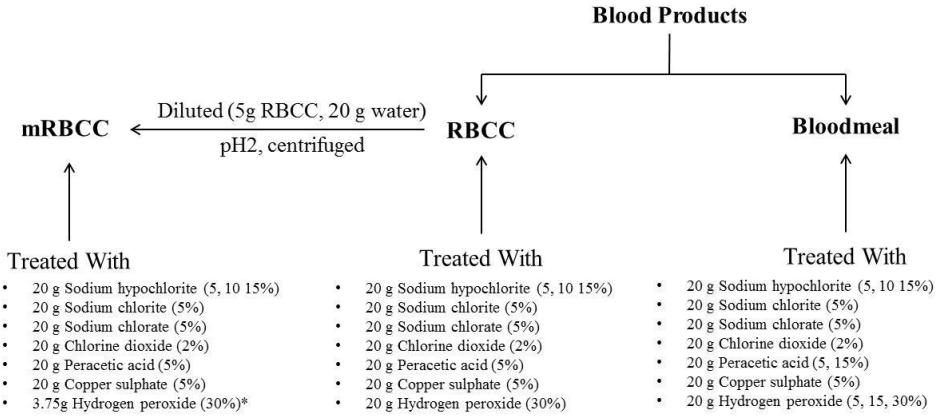


Figure 24: Summary of decolouring chemicals used on RBCC, mRBCC and bloodmeal.

*** Hydrogen peroxide method from Chapter Four.**

The applied concentrations and decolouring chemical to solids ratio varied between RBCC, mRBCC and bloodmeal because of their different solids contents. The applied concentrations and decolouring chemical to solids ratio are presented in Table 19. Applied concentration for RBCC and mRBCC was calculated by taking into account the moisture contents of RBCC and mRBCC. Because bloodmeal had low moisture content, the applied concentration of the oxidising chemicals applied to bloodmeal was assumed to be equal to the original concentration. Chemical to solids ratio (w/w) was calculated by dividing the mass of dry solids for bloodmeal, RBCC and mRBCC by the mass of the oxidising chemical (after correcting for dilution) applied to them.

Table 19: Applied concentration and chemical to solids ratio when different decolouring solutions are used on bloodmeal, RBCC and mRBCC.

		Bloodmeal		RBCC		mRBCC	
Chemical	Original Conc. (%)	Applied Conc. (%)	Chemical to solids ratio (w/w)	Applied Conc. (%)	Chemical to solids ratio (w/w)	Applied Conc. (%)	Chemical to solids ratio (w/w)
Hydrogen Peroxide	30	30	1.20	24.00	3.64	0.02*	0.70*
	15	15	0.60	Not Applied			
	5	5	0.20				
Peracetic acid	15	15	0.60				
	5	5	0.20	4.00	0.61	2.22	0.62
Sodium Hypochlorite	15	15	0.60	12.00	1.82	6.67	1.86
	10	10	0.40	8.00	1.21	4.44	1.24
	5	5	0.20	4.00	0.61	2.22	0.62
Sodium Chlorite	5	5	0.20	4.00	0.61	2.22	0.62
Sodium Chlorate	5	5	0.20	4.00	0.61	2.22	0.62
Chlorine Dioxide	2	2	0.08	1.60	0.24	0.89	0.25
Copper Sulphate	5	5	0.20	4.00	0.61	2.22	0.62

*Hydrogen peroxide method from Chapter Four.

The processability and mechanical properties of bioplastics produced from proteins can be influenced by properties such as molecular mass, crystallinity and molecular stabilising interactions such as hydrogen bonding, cross-linking and hydrophobic interactions. Therefore these properties were investigated using gel filtration chromatography, X-ray diffraction and thermal gravimetric analysis.

5.2.2.2 Molecular Mass Analysis of Decoloured Powders

Reduced molecular mass can lead to a reduction in a bioplastics mechanical properties and processability due to reduced chain entanglements. Molecular mass of freeze dried and ground treated powders were analysed using gel filtration

chromatography as previously described in Chapter Four. In addition the number average molecular weight (MN), weight average molecular weight (MW) and polydispersity index (PI) was calculated using equations 6, 7 and 8 where Ni is equal to the number of molecules with molecular weight Mi [134].

$$MN = \frac{\sum_i^\infty Ni.Mi}{\sum_i^\infty Ni} \quad (6)$$

$$MW = \frac{\sum_i^\infty Ni.Mi^2}{\sum_i^\infty NiMi} \quad (7)$$

$$PI = \frac{MW}{MN} \quad (8)$$

5.2.2.3 X-Ray Diffraction Analysis

Crystallinity can affect mechanical properties because hydrogen bonds which stabilise the regular structure of the protein can also contribute to the bioplastics strength. Processing can also be affected by crystallinity because additives may be required to disrupt hydrogen bonding to allow chains to re-entangle during processing. X-ray diffraction was used to investigate the crystallinity of treated powders which could also give an indication of changes in hydrogen bonding and how different treatments could be affecting the amino acids involved with it.

The X-ray diffraction pattern of selected decoloured powders was measured using low angle powder X-ray diffraction (XRD). XRD was carried out using a Philips X-ray diffractometer at a low angle configuration of $2\Theta=2°$ to 60°, with a scanning rate of $2\Theta=2°min^{-1}$, operating at a current of 40 mA and a voltage of 40 kV using $CuK\alpha_1$ radiation.

5.2.2.4 Thermogravimetric Analysis

Thermal stability was used as an indirect method to investigate changes in stabilising interactions such as cross-linking, hydrogen bonding and hydrophobic interactions. A reduction in thermal stability would indicate that the amount of stabilising interactions had been reduced and could suggest that the amino acids involved in these had been altered. In addition thermal stability was used as a secondary method to validate changes in molecular mass investigated using gel chromatography. If molecular mass had been significantly reduced a significant loss in thermal stability would be expected.

Thermal stability of selected decoloured powders was investigated using a thermal gravimetric analyser (SDT 2960, TA Instruments). Approximately 10 mg of sample was used. The mass loss was recorded while the sample was heated from room temperature to 800°C at a rate of 10°C per minute. The data was normalised at 150°C to account for water loss and the percentage change in mass calculated. The first derivative of percentage mass change versus temperature was also calculated to investigate temperature regions where mass loss was occurring.

5.2.2.5 Powder Colour Analysis

Dried samples were ground to a fine powder using mortar and pestle. The powder colour was analysed using a Minolta Chroma Meter CR-200b set in L*a*b* (CIE 1976) mode using D (6504K) illuminant conditions as these settings represent what is seen by the human eye the closest. The L*a*b* values were converted to RGB and percent whiteness calculated using the equation:

$$Percent\ Whiteness = \frac{R+G+B}{765} \times 100 \quad (9)$$

5.3 Results and Discussion

Chemicals were used at different concentrations for the different feedstocks because the feedstocks had different moisture contents (Table 19). Bloodmeal was treated at the highest applied concentration because of its low moisture content, but it also the lowest decolouring chemical to solids ratio because of its high solids content.

RBCC was treated at a slightly reduced applied concentration because it had higher moisture content than bloodmeal. However, because RBCC had low solids content it had a high decolouring chemical to solids ratio. mRBCC was treated at a significantly lower applied concentration which was caused by the 20 g of water used to dilute the RBCC prior to pH adjustment. The chemical to solids ratio was calculated based on previously calculated 2% solids loss after centrifugation which most probably consisted of dissociated haem and the cell membrane. The loss in solids resulted in a slightly higher decolouring chemical to solids ratio when compared to RBCC. For hydrogen peroxide treated mRBCC the applied concentration is greatly reduced because 50 g of water is used for dilution and only 3.75 g of hydrogen peroxide (30%) was used.

5.3.1 Red Blood Cell Concentrate and Modified Red Blood Cell Concentrate Treatment Analysis

5.3.1.1 Colour and Smell of Treated RBCC and mRBCC

Treating RBCC with different chemicals produced powders ranging from 26% whiteness to 91% whiteness. PAA, hydrogen peroxide and sodium chlorite treatment produced 91%, 71% and 61% white powders respectively. They also removed offensive odours (Table 20). Sodium hypochlorite (5-15% concentration) produced 55-61% white powders (Figure 26) but did not remove odour. Sodium chlorate, chlorine dioxide and copper sulphate did not alter the original colour of RBCC significantly (Figure 25) so would not be useful for producing a bioplastic feedstock.

The decolouring solutions were oxidising agents (excluding copper sulphate) and would have been altering the bond configuration of the haem porphyrin. They could also be oxidising the odour compounds. PAA, hydrogen peroxide and

sodium chlorite were the strongest oxidising agents (reduction potentials of 1.81, 1.76 and 0.681 V respectively) used which could explain why they were the most effective at removing colour and odour [18].

Because of the high concentrations, colour removal occurred very quickly. Sodium chlorite colour removal occurred within five minutes. In the case of PAA and hydrogen peroxide, colour removal was almost instantaneous.

Treating RBCC with hydrogen peroxide resulted in large volumes of foam being produced from catalase action. However, colour was still able to be removed, probably because catalase was overcome by the combination of high concentration and high ratio of hydrogen peroxide to protein. A significant increase in temperature was also observed because of exothermic decomposition of hydrogen peroxide. When RBCC was treated with PAA foaming was reduced. This was probably due to the acetic acid deactivating catalase. The foaming that did occur was probably due to PAA and hydrogen peroxide spontaneously decomposing.

Table 20: Effect of different chemicals on RBCC colour and smell.

Rank	Material	Treatment Method	Applied Conc. (%)	Chemical to solids ratio	L*	R	G	B	White (%)	Colour	Smell Improved?
1	RBCC	PAA (5%)	4	0.61	95	243	242	208	91		Yes
2	RBCC	H_2O_2 (30%)	24	3.64	77	218	184	139	71		Yes
3	RBCC	$NaClO_2$ (5%)	4	0.61	67	188	157	123	61		Yes
4	RBCC	NaClO (15%)	12	1.82	65	165	157	144	61		No
5	RBCC	NaClO (10%)	8	1.21	64	161	155	138	59		No
6	RBCC	NaClO (5%)	4	0.61	58	148	138	132	55		No
7	RBCC	ClO_2 (2%)	1.6	0.24	35	126	63	62	33		No
8	RBCC	$CuSO_4$ (5%)	4	0.61	33	86	76	67	30		No
9	RBCC	Untreated	-	-	28	106	57	58	29		No
10	RBCC	$NaClO_3$ (5%)	4	0.61	27	86	56	54	26		No

Figure 25: Freeze dried RBCC.

Figure 26: Sodium hypochlorite (15%) treated RBCC.

Sodium chlorite, hydrogen peroxide and PAA treatment were also the most effective when treating mRBCC. Hydrogen peroxide (Figure 27), PAA and sodium chlorite (Figure 28) treatment produced 55, 87 and 93% white powders respectively and also removed odour (Table 21). Sodium hypochlorite, sodium chlorate, chlorine dioxide and copper sulphate did not effectively remove colour or odour, therefore they were not suitable for producing a bioplastic feedstock which is the same result obtained when treating RBCC with these chemicals.

Table 21: Effect of different chemicals on modified RBCC colour and smell.

Rank	Material	Treatment Method	Applied Conc. (%)	Chemical to solids ratio	L*	R	G	B	White (%)	Colour	Smell Improved?
1	mRBCC	$NaClO_2$ (5%)	2.22	0.62	95	246	240	225	93		Yes
2	mRBCC	PAA (5%)	2.22	0.62	91	239	229	199	87		Yes
3*	mRBCC	H_2O_2 (30%)	0.02	0.70	64	180	147	92	55		Yes
4	mRBCC	NaClO (10%)	4.44	1.24	49	125	115	100	44		No
5	mRBCC	NaClO (5%)	2.22	0.62	47	121	110	102	44		No
6	mRBCC	NaClO (15%)	6.67	1.86	47	129	107	94	43		No
7	mRBCC	$CuSO_4$ (5%)	2.22	0.62	29	82	65	52	26		No
8	mRBCC	Untreated	-	-	28	79	62	54	25		No
9	mRBCC	ClO_2 (2%)	0.89	0.25	27	79	60	51	25		No
10	mRBCC	$NaClO_3$ (5%)	2.22	0.62	23	69	51	46	22		No

*Hydrogen peroxide method from Chapter Four.

Figure 27: Hydrogen peroxide treated mRBCC.

Figure 28: Sodium chlorite treated mRBCC.

The percent whiteness of powders for all treatments except sodium chlorite was reduced when comparing mRBCC to RBCC. This was likely caused by the decolourant being diluted by the additional 20 g of water in mRBCC. However excellent decolourisation was still achieved with much lower chemical concentrations. This was because at pH 2 the haem group dissociated from the globin and formed a precipitate which could be removed, reducing the amount of

haem that needed to be degraded. This also made haem more accessible to the chemicals so a lower concentration could still be effective at removing the colour.

Sodium chlorite has been shown to be more effective at acidic pH due to the formation of highly reactive side products such as the chlorite ion and chlorous acid [135]. mRBCC was at pH 2 whereas RBCC was at pH 7. This could explain why sodium chlorite was more effective on mRBCC than RBCC even though its concentration was lower.

When mRBCC was treated with hydrogen peroxide the dramatic decrease in colour removed was because of the 50 g diluting water and reduced amount of hydrogen peroxide used. This method produced less foam because catalase action was reduced at pH 2. There was also less temperature increase because of the reduced concentrations. However this method took three hours to achieve colour removal.

5.3.1.2 Molecular Mass of Treated RBCC and mRBCC

Although treating RBCC with sodium chlorite, hydrogen peroxide and PAA had the best colour and odour removal they also produced low molecular mass peptides (Figure 29 and Figure 30).

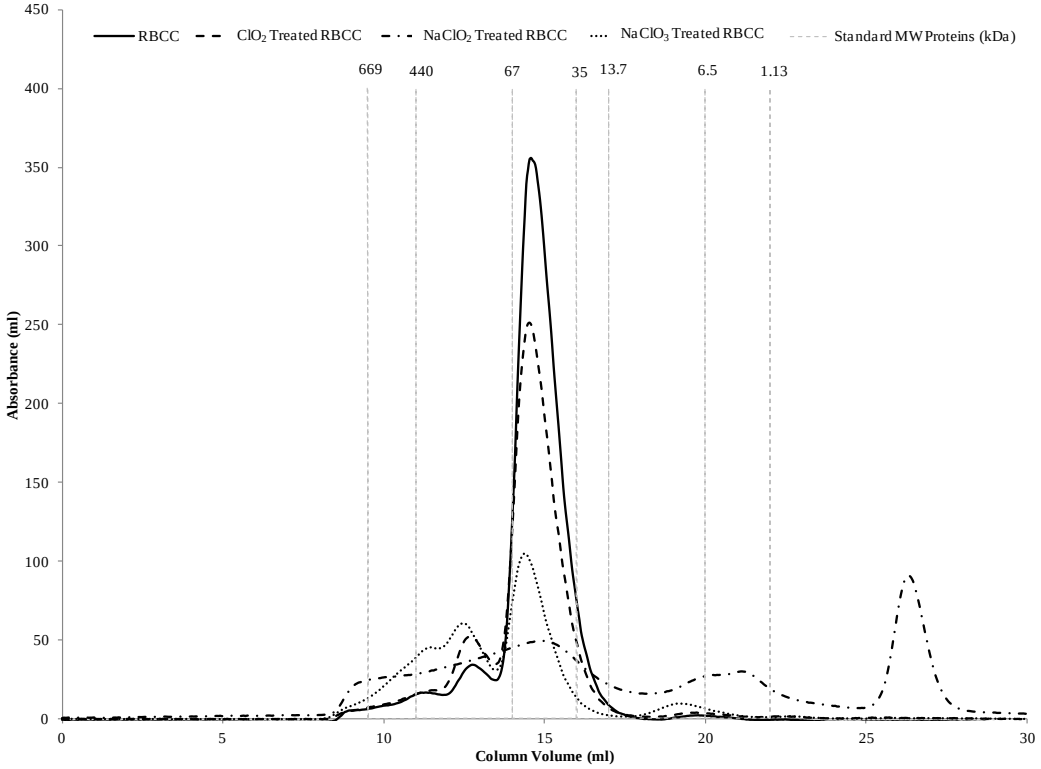


Figure 29: Elution profile for RBCC treated with sodium chlorite, sodium chlorate and chlorine dioxide.

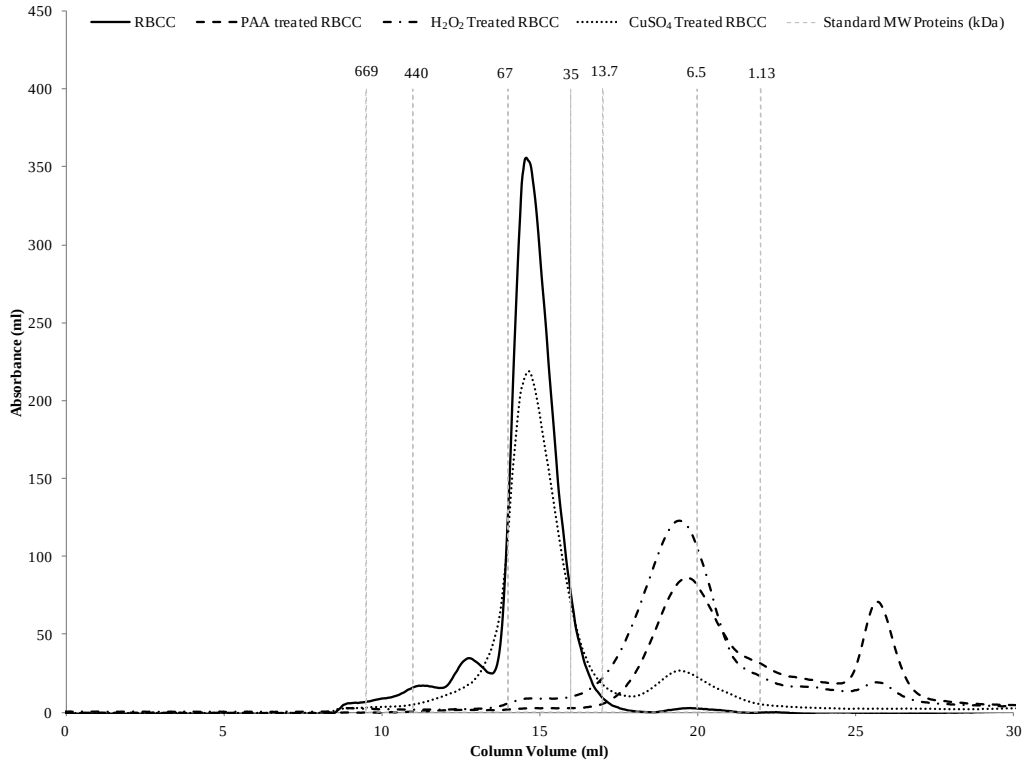


Figure 30: Elution profile for RBCC treated with peracetic acid (5%), hydrogen peroxide (30%) and copper sulphate.

Sodium chlorite can cause peptide cleavage and disulphide cross-linking by reacting with highly reactive amino acids such as tryptophan and methionine. Gel chromatography showed sodium chlorite treatment produced low molecular weight peptides because peaks at 6.5 and below 1.13 kDa were identified in addition to the original peak at 65 kDa. However, a peak was also observed at 669 kDa which suggests that high molecular weight aggregates were also formed. This indicated that some of the protein remained at its original molecular weight but a large majority was also broken into smaller peptides and some large aggregates were formed (Figure 29). This resulted in the number average molecular weight being increased from 87 kDa for untreated RBCC to 143 kDa for sodium chlorite treated RBCC. In addition 39% of sodium chlorite treated RBCC was below 30 kDa whereas only 9% of untreated RBCC is below this value (Table 22).

PAA and hydrogen peroxide had the most severe effect on RBCC molecular weight. This was expected as they have a higher oxidation potential than sodium chlorite. No peaks were identified for both at approximately 65 kDa. Instead two new peaks were formed at approximately 6.5 and below 1.13 kDa (Figure 30), indicating that they could have oxidised the protein backbone and produced small polypeptide chains. If the protein was being cut at random locations then several

random peaks would be expected. This was not observed which suggests that the protein was being cleaved at specific sites, however these were not determined. Hydrogen peroxide and PAA treatment of RBCC reduced the number average molecular weight to 19 and 13 kDa respectively. 92% of hydrogen peroxide treated RBCC was below 30 kDa whereas 96% of PAA treated RBCC was below this value. This would greatly reduce their ability to be processed into a bioplastic or if processable the bioplastic would have greatly reduced mechanical properties due to poor entanglement.

Table 22: Number average molecular weight, weight average molecular weight, polydispersity index and molecular mass distribution of decoloured RBCC powders.

				Molecular Mass Distribution (kDa)							
	MN (kDa)	MW (kDa)	PI	1490-560	560-215	215-80	80-30	30-11	11-4	4-1.6	1.6-0.6
RBCC	87	264	3.0	0.02	0.05	0.11	0.73	0.06	0.01	0.01	0.01
$NaClO_2$ (5%)	143	441	3.1	0.07	0.14	0.18	0.22	0.11	0.09	0.13	0.06
$NaClO_3$ (5%)	171	373	2.2	0.05	0.21	0.28	0.36	0.03	0.04	0.02	0.01
ClO_2 (2%)	98	264	2.7	0.02	0.06	0.18	0.65	0.05	0.02	0.01	0.01
H_2O_2 (30%)	19	372	19.8	0.01	0.01	0.02	0.04	0.12	0.48	0.24	0.08
PAA (5%)	13	288	21.6	0.01	0.01	0.02	0.02	0.06	0.40	0.33	0.15
CuSO4 (5%)	65	198	13.1	0.01	0.02	0.12	0.60	0.10	0.08	0.05	0.02

Sodium chlorite treated mRBCC (Figure 31) showed one difference to sodium chlorite treated RBCC (Figure 29). This was that no high molecular weight aggregates were detected for mRBCC treated with sodium chlorite. The acidic pH of mRBCC could be causing a greater amount of sodium chlorite to be converted to chlorite ions and chlorous acid which may lead to less disulphide cross-links forming. A small amount of these cross-links could be forming but would be removed and not detected due to sodium sulphite in the dissolving buffer. Sodium chlorite treated mRBCC had a number average molecular weight of 44 kDa and 55% was below 30 kDa.

The elution profile for PAA treated mRBCC (Figure 32) was similar to that for RBCC treated with PAA. Both showed that molecular mass was reduced significantly. PAA treated mRBCC had a number average molecular mass of 21 kDa and 91% was below 30 kDa. These values were slightly better than PAA treated RBCC. It appears that the diluting effect of the additional 20 g water (Table 19) in mRBCC slightly reduces the reduction in molecular mass but not enough to prevent excessive damage to the protein.

Hydrogen peroxide treatment of mRBCC did not reduce the molecular mass significantly as shown in Figure 32. It had a number average molecular weight of 90 kDa. 22% was below 30 kDA compared to 9% for untreated RBCC which indicated some smaller molecular mass peptides were formed. However 28% of hydrogen peroxide treated mRBCC was above 80 kDa compared to 18% for untreated RBCC indicating that some higher molecular weight aggregates were also formed which is responsible for the slightly higher number average molecular weight. Treating mRBCC with hydrogen peroxide had a significantly lower applied concentration and decolouring chemical to solids ratio than that used for RBCC because more water was used for dilution and less hydrogen peroxide was added. This was probably why molecular mass was not significantly reduced and percent whiteness was not as high as when RBCC was treated with hydrogen peroxide.

Literature has shown that sodium hypochlorite can cause high amounts of disulphide cross-links to form because it is highly reactive with cysteine [112]. Sodium hypochlorite treated RBCC and mRBCC could not be dissolved for analysis. This result suggested that sodium hypochlorite treatment caused high amounts of disulphide cross-links to form reducing its solubility. However because of the poor colour and odour removal by sodium hypochlorite it was decided not to investigate further.

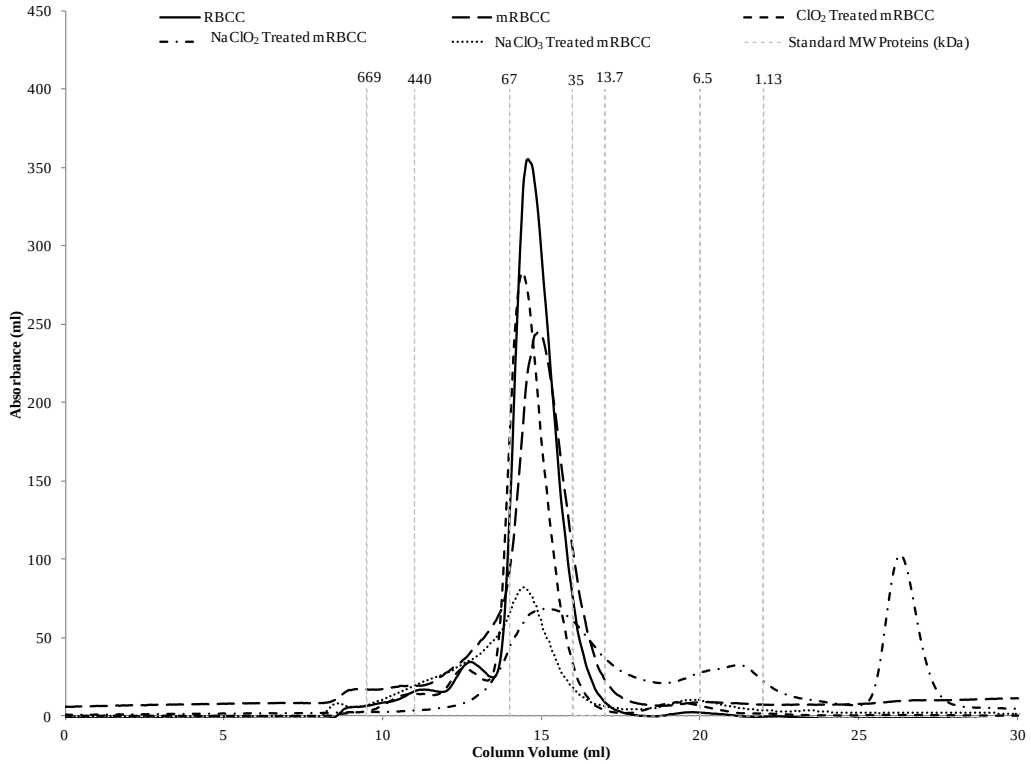


Figure 31: Elution profile for modified RBCC treated with sodium chlorite, sodium chlorate and chlorine dioxide.

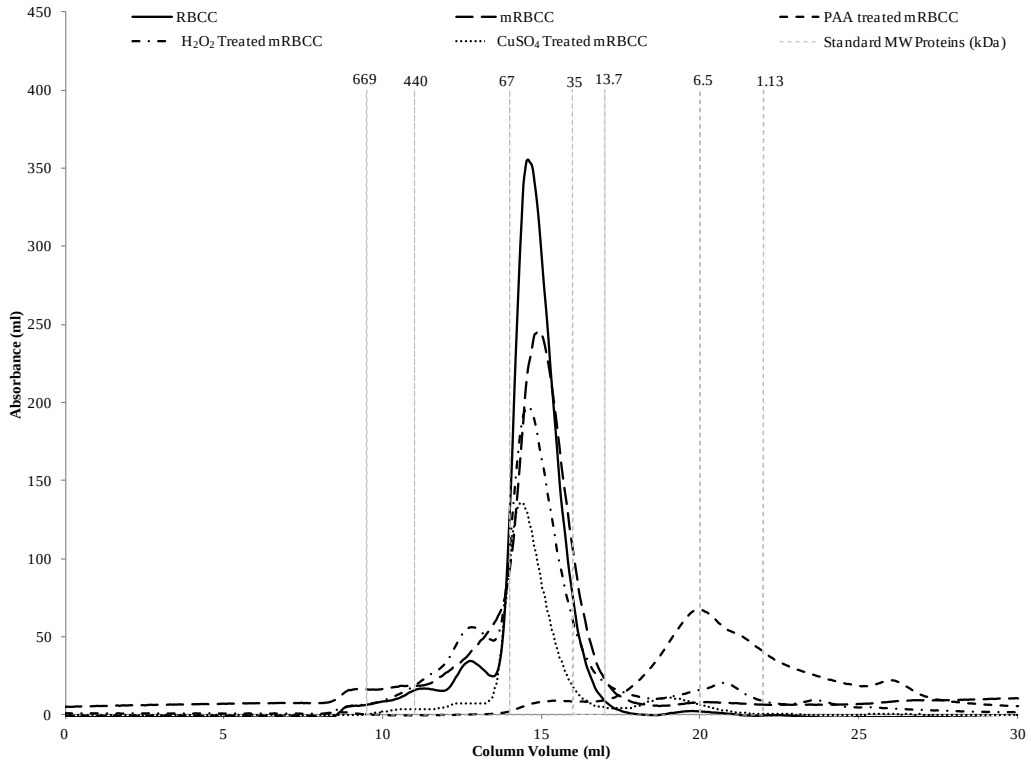


Figure 32: Elution profile for modified RBCC treated with peracetic acid (5%), hydrogen peroxide (30%) and copper sulphate.

Table 23: Number average molecular weight, weight average molecular weight, polydispersity index and molecular mass distribution of decoloured mRBCC powders.

				Molecular Mass Distribution (kDa)							
	MN (kDa)	MW (kDa)	PI	1490-560	560-215	215-80	80-30	30-11	11-4	4-1.6	1.6-0.6
mRBCC	94	316	3.3	0.03	0.05	0.15	0.64	0.10	0.01	0.02	0.00
$NaClO_2$ (5%)	44	212	4.9	0.01	0.02	0.09	0.33	0.21	0.12	0.16	0.06
$NaClO_3$ (5%)	134	373	2.8	0.05	0.13	0.27	0.37	0.06	0.05	0.05	0.02
ClO_2 (2%)	86	204	2.4	0.01	0.05	0.16	0.69	0.04	0.04	0.01	0.00
H_2O_2 (30%)	90	266	3.0	0.02	0.07	0.19	0.50	0.09	0.04	0.06	0.03
PAA (5%)	21	489	23.4	0.02	0.01	0.01	0.05	0.08	0.29	0.35	0.19
CuSO4 (5%)	72	180	2.5	0.01	0.03	0.14	0.64	0.06	0.08	0.03	0.01

Molecular mass analysis revealed that molecular mass was significantly reduced for sodium chlorite, hydrogen peroxide and PAA treated RBCC and sodium chlorite and PAA treated mRBCC. This indicated that these powders would most probably not be suitable for use in a bioplastic.

5.3.1.3 Thermal Stability of Treated RBCC and mRBCC

The thermal stability of both hydrogen peroxide and PAA treated RBCC was reduced significantly (Figure 33). Hydrogen peroxide and PAA treatment resulted in complete loss of thermal stability as shown by its rapid mass loss as temperature was increased. This was probably due to the combination of severe reduction in molecular mass as previously shown and reduction in other stabilising interactions which are investigated later.

Sodium chlorite treated RBCC and mRBCC (Figure 33 and Figure 34) also showed reduced thermal stability initially as would be expected due to its reduced molecular mass. However at approximately 350°C their rate of mass loss was reduced. This is the region where large molecules become degraded. This suggested cross-link formation occurred when RBCC and mRBCC were treated with sodium chlorite. Sodium chlorite treated RBCC appears to be more thermally stable above 350°C than sodium chlorite treated mRBCC, which suggested a greater amount of disulphide cross-linking occurred for sodium chlorite treated RBCC. There is strong evidence which supports this because high molecular mass aggregates were detected in elution profiles for sodium chlorite treated RBCC but not for sodium chlorite treated mRBCC. For sodium chlorite treated mRBCC the

cross-linked aggregates may not have been detected with gel chromatography because there were less than sodium chlorite treated RBCC and they could have been removed by the sodium sulphite in the dissolving buffer.

When the haem group is removed from the protein by producing modified RBCC (diluting RBCC and lowering to pH 2) thermal stability was also reduced slightly (Figure 34). This was because the haem group stabilises the haemoglobin molecule by filling the hydrophobic core.

Thermal degradation of untreated RBCC, untreated mRBCC and hydrogen peroxide treated mRBCC occurred in three stages. These were 0 to 150°C, 230 to 400°C and above 400°C. The first stage was attributed to the loss of bound water and the second stage to the breaking of S-S, O-N and O-O linkages. Above 400°C thermal decomposition was occurring through peptide bond reduction [7].

Thermal degradation of RBCC treated with sodium chlorite, hydrogen peroxide and PAA occurred in four stages. This was also the same for mRBCC treated with sodium chlorite and PAA. In addition to the three stages identified previously for materials such as untreated RBCC, another region was identified from 150°C to 230°C. This region can be attributed to low molecular weight compounds or oligomers [7, 136]. Gel chromatography identified small molecular weight peptides in these materials and their degradation could be responsible for mass loss in this region.

Table 24: Percentage mass loss at 0-150°C, 150-230°C and 230-400°C for decoloured RBCC powders.

Region	RBCC	H_2O_2 treated RBCC	$NaClO_2$ treated RBCC	PAA treated RBCC
0-150°C	13	7	11	15
150-230°C	-	12	9	14
230-400°C	38	33	24	29
400°C	Thermal Decomposition			

Table 25: Percentage mass loss at 0-150°C, 150-230°C and 230-400°C for decoloured mRBCC powders.

Region	mRBCC	H_2O_2 treated mRBCC	$NaClO_2$ treated mRBCC	PAA treated mRBCC
0-150°C	12	11	9	10
150-230°C	-	-	15	11
230-400°C	41	38	24	35
400°C	Thermal Decomposition			

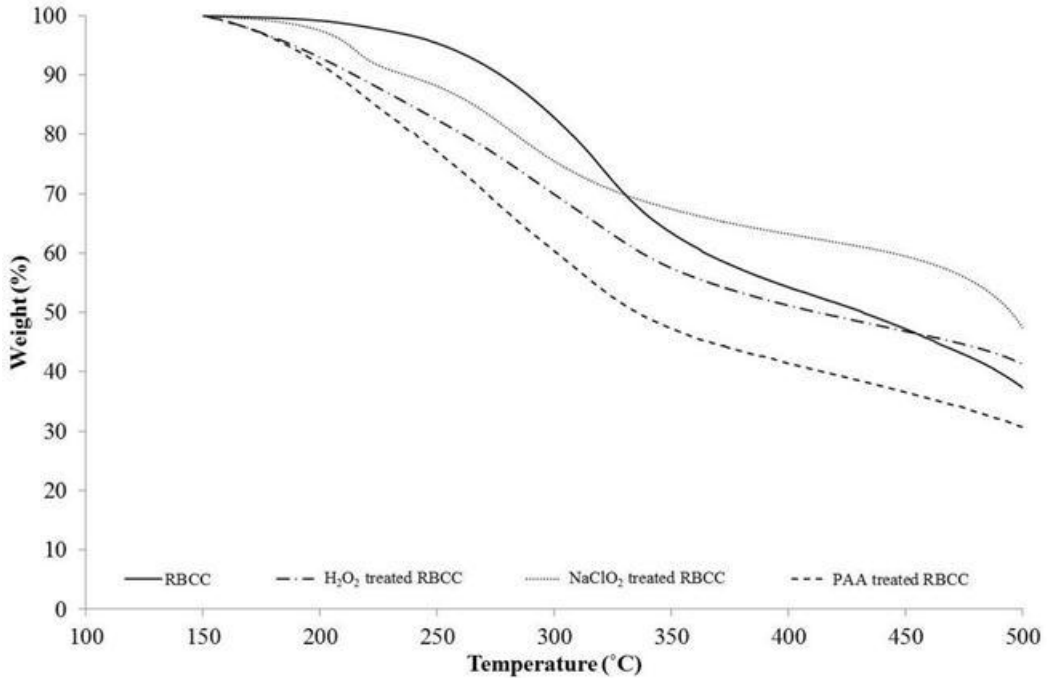


Figure 33: Thermal stability of hydrogen peroxide and sodium chlorite treated RBCC.

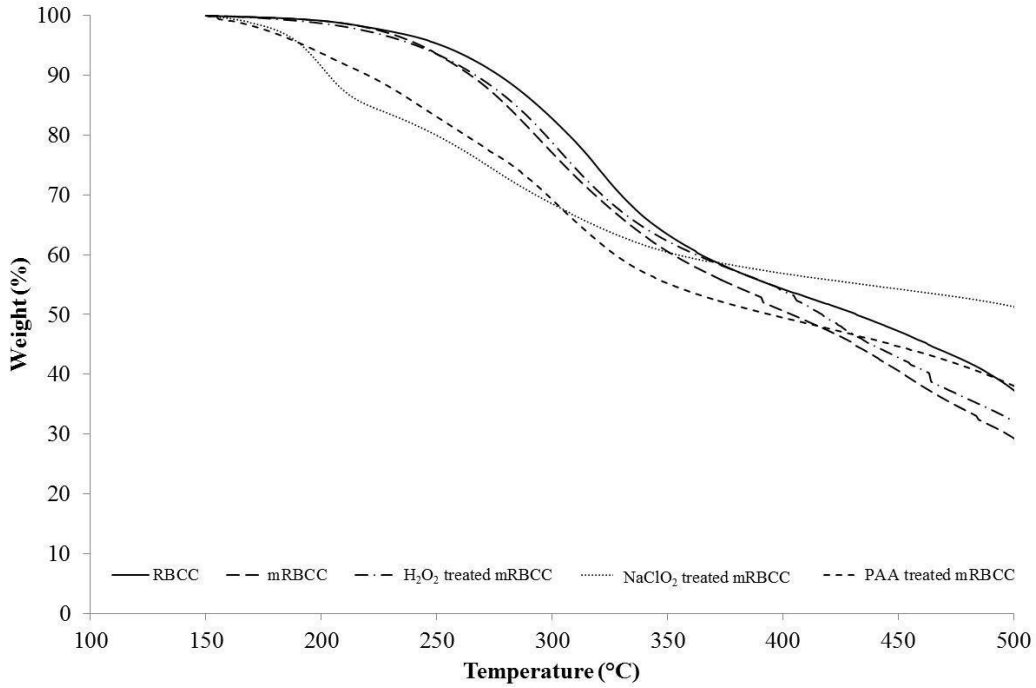


Figure 34: Thermal stability of hydrogen peroxide and sodium chlorite treated modified RBCC.

5.3.1.4 XRD Patterning of Treated RBCC and mRBCC

XRD plots of RBCC showed two peaks at 2Θ=9.5 and 2Θ=20, which correspond to inter-helix packing and the alpha helix backbone [137]. When RBCC was treated with hydrogen peroxide and sodium chlorite these peaks were reduced or disappeared completely (Figure 35), indicating loss of regular structure. This was probably caused by hydrogen peroxide reacting with highly susceptible amino acids such as tryptophan, proline and histidine which help stabilise protein structure [125, 126, 129]. Exactly what amino acids were modified could be investigated with amino acid analysis.

Chromatography analysis showed that hydrogen peroxide treatment of RBCC produced smaller peptides than sodium chlorite treatment. Whereas XRD analysis showed that hydrogen peroxide treated RBCC retained more regular structure than sodium chlorite treated RBCC. Therefore it was suggested that hydrogen peroxide and sodium chlorite acted at different locations on the protein. Hydrogen peroxide reacted preferentially with the protein back bone reducing chain size and then reduced regular structure of the protein, whereas sodium chlorite preferentially acted on the side chains reducing the regular structure of the protein then reacted with the protein backbone.

XRD analysis of mRBCC treated with sodium chlorite and hydrogen peroxide showed that regular structure was not significantly affected (Figure 36). The method used for treating mRBCC with hydrogen peroxide was much weaker than that used for RBCC which can explain why the regular structure of the protein and molecular weight was maintained when treating mRBCC but not RBCC with hydrogen peroxide. Regular structure was not reduced as significantly when mRBCC was treated with sodium chlorite as when RBCC was treated with sodium chlorite. Haem was more accessible for mRBCC and whiteness was also higher. Therefore in this case the sodium chlorite may have preferentially reacted with haem instead of structure stabilising amino acids.

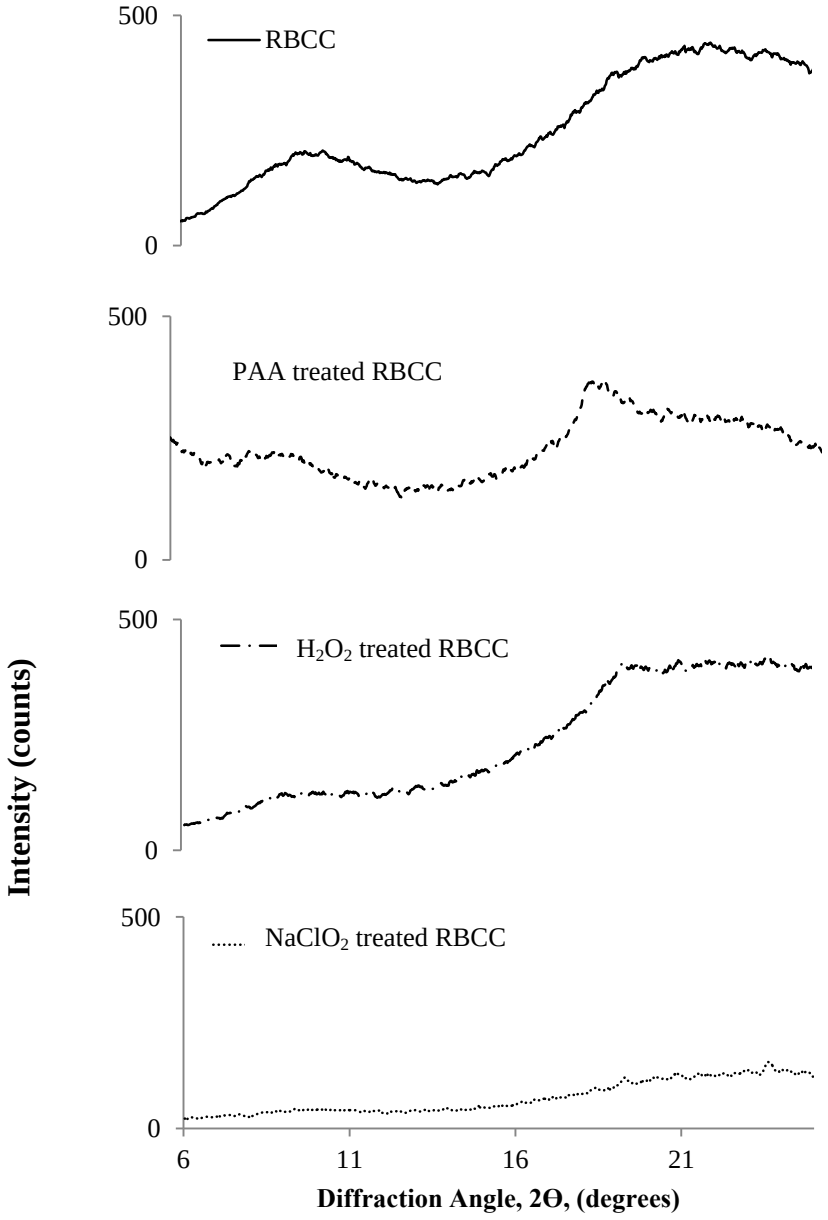


Figure 35: XRD plots of PAA, hydrogen peroxide and sodium chlorite treated RBCC.

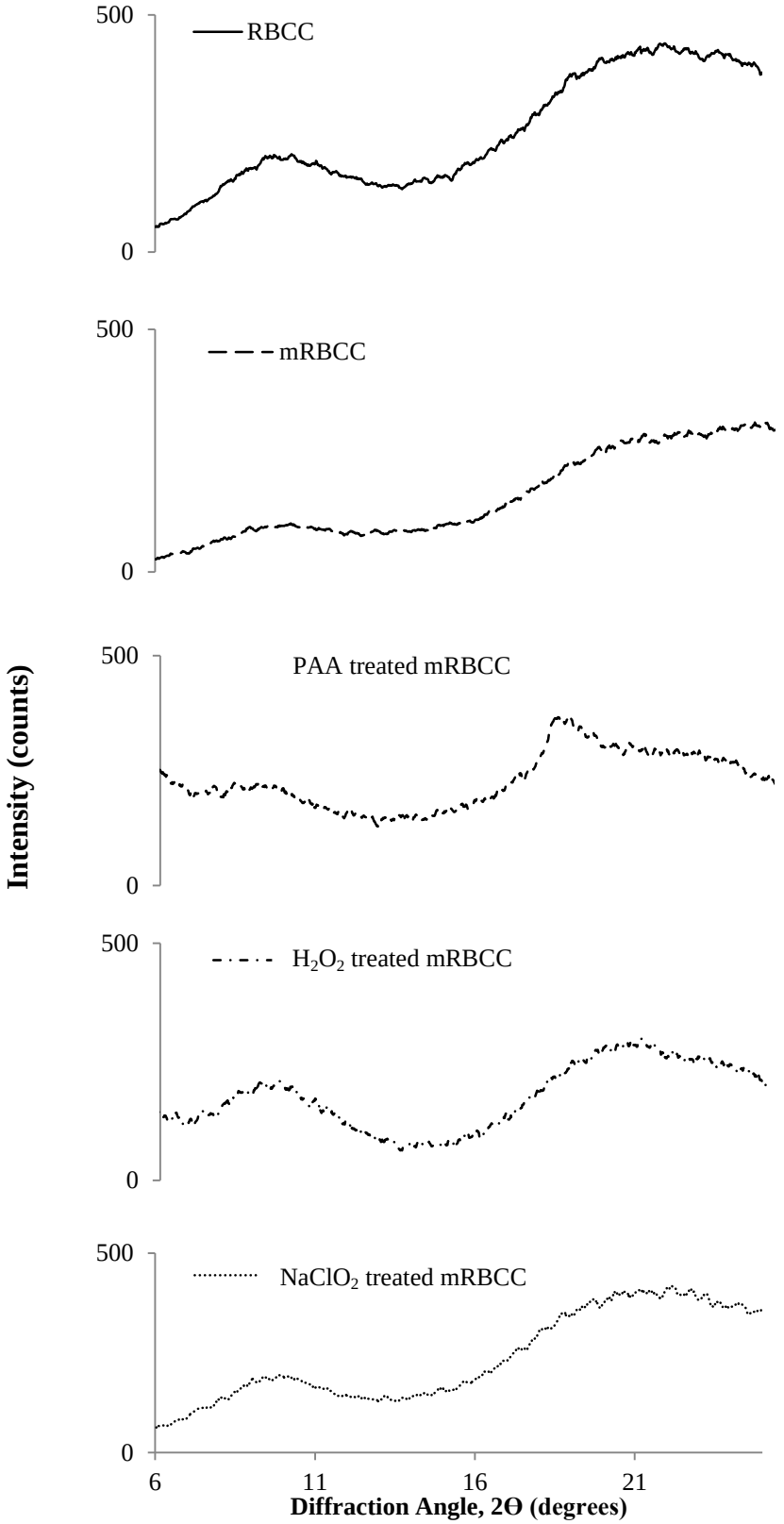


Figure 36: XRD plots of peracetic acid, hydrogen peroxide and sodium chlorite treated modified RBCC.

5.3.1.5 Suitability of Decoloured RBCC and mRBCC for Bioplastics

Treating RBCC and mRBCC with hydrogen peroxide, sodium chlorite and PAA had the best colour and odour removal. However sodium chlorite, hydrogen peroxide and PAA (except for mRBCC treated with hydrogen peroxide) treatment reduced the molecular weight of the protein. As a result any material produced would be likely to have poor mechanical properties. In addition to this there is a high possibility that chlorinated organic compounds were also formed when using sodium chlorite. All of these methods also dealt with materials with high moisture contents. This meant that high volumes of solutions had to be handled in order to produce small amounts of decoloured powders.

Treating mRBCC with hydrogen peroxide produced a decoloured and deodorised powder without significantly reducing molecular weight, molecular interactions or thermal stability. Therefore this material could be potentially processed into a bioplastic. However this method required long processing times, multiple processing steps and large volumes of water which would lead to high production costs as previously discussed in Chapter Four.

These results showed that treating RBCC and mRBCC with different chemicals prior to drying may not be an appropriate method for producing a bioplastic feedstock. Although the colour and odour could be removed using hydrogen peroxide, sodium chlorite and PAA treatment they also had undesired affects such as reduced molecular weight. This means that their ability to be processed into a bioplastic would be reduced. Even if the decoloured protein could be processed into a material its mechanical properties would most likely be significantly reduced.

5.3.2 Bloodmeal Treatment Analysis

5.3.2.1 Colour and Smell of Treated Bloodmeal

Treating bloodmeal with different chemicals produced powders ranging from 18-76% in whiteness. Hydrogen peroxide, sodium chlorite and PAA improved colour and removed odour, whereas sodium hypochlorite, sodium chlorate, chlorine dioxide and copper sulphate did not (Table 26).

Table 26: Effect of different chemicals on bloodmeal colour and smell.

Rank	Material	Treatment Method	Applied Conc. (%)	Chemical to solids ratio	L*	R	G	B	White (%)	Colour	Smell Modified?
1	BM	PAA (15%)	15	0.6	84	227	208	144	76		Yes
2	BM	PAA (5%)	5	0.2	77	215	187	111	67		Yes
3	BM	H_2O_2 (30%)	30	1.20	67	187	154	109	59		Yes
4	BM	H_2O_2 (15%)	15	0.60	66	183	153	102	57		No
5	BM	H_2O_2 (5%)	5	0.20	52	149	119	79	45		No
6	BM	$NaClO_2$ (5%)	5	0.20	35	103	77	56	31		Yes
7	BM	Untreated	-	-	20	61	45	42	19		No
8	BM	NaClO (5%)	5	0.20	20	64	43	39	19		No
9	BM	NaClO (10%)	10	0.40	20	57	46	40	19		No
10	BM	$NaClO_3$ (5%)	5	0.20	19	65	39	38	19		No
11	BM	NaClO (15%)	15	0.6	19	51	45	42	18		No
12	BM	Distilled Water	-	-	18	60	39	38	18		No
13	BM	ClO_2 (2%)	2	0.08	18	60	39	38	18		No
14	BM	$CuSO_4$ (5%)	5	0.20	19	51	45	40	18		No

Hydrogen peroxide (5-30%) treated bloodmeal produced 45-59% white powders. Odour was only able to be removed when hydrogen peroxide concentration was 30%. Sodium chlorite treatment produced a 31% white powder and also removed the odour. 5% PAA produced a 67% white powder and 15% PAA produced a 76% white powder due to its higher concentration. PAA may be more effective on colour than sodium chlorite and hydrogen peroxide because it also contained hydrogen peroxide and because of its low pH which has been shown to prevent catalase action [119]. Both PAA treatment concentrations were effective at removing the bloodmeal odour. Sodium chlorite took up to 30 minutes for a noticeable colour change whereas hydrogen peroxide and PAA colour removal occurred instantaneous and was completed within 5 minutes. These results showed a similar trend to those obtained when treating RBCC and mRBCC with these chemicals.

5.3.2.2 Molecular Mass of Treated Bloodmeal

Gel chromatography elution profiles for all treatment methods (except sodium chlorite) showed a main peak at approximately 65 kDa (Figure 37, Figure 38), indicating that the molecular mass had not been significantly reduced. Attempts to dissolve sodium chlorite treated bloodmeal were unsuccessful which could indicate cross-linking was occurring.

Sodium hypochlorite treated bloodmeal was able to be dissolved whereas sodium hypochlorite treated RBCC and mRBCC was not. This may have been due to the highly aggregated bloodmeal reducing the accessibility of reactive sites for sodium hypochlorite. RBCC would not be as aggregated and mRBCC would have been denatured at the low pH. This means accessibility to reactive amino acids would have been easier resulting in higher amounts of cross-link formation and poor solubility.

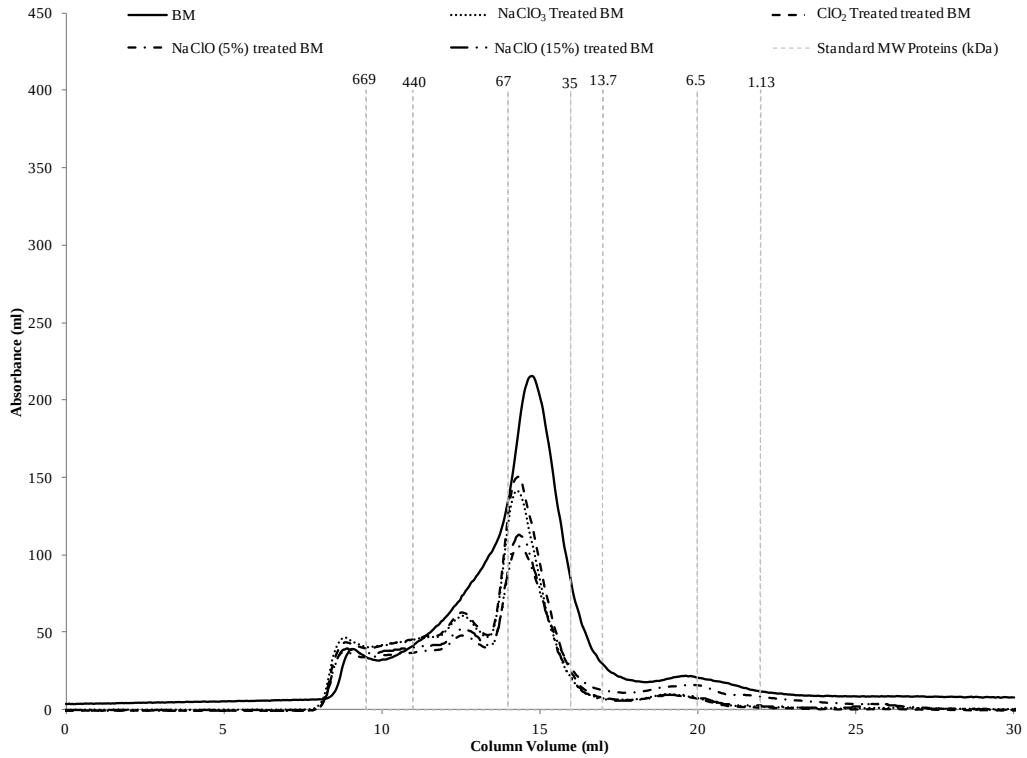


Figure 37: Elution profile for BM treated with sodium hypochlorite, sodium chlorate and chlorine dioxide.

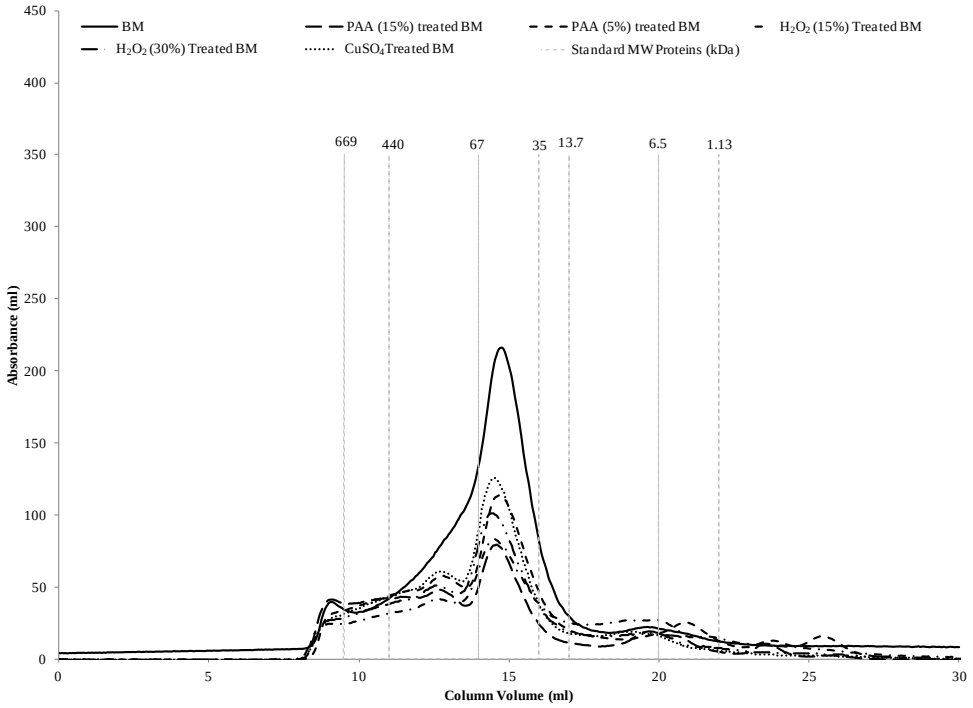


Figure 38: Elution profile for BM treated with peracetic acid, hydrogen peroxide and copper sulphate.

Previous studies suggested that treating proteins with oxidising agents could result in chain fragmentation. This was seen when treating RBCC and mRBCC with the decolouring agents. However, this did not occur when treating bloodmeal with the decolouring agents. 23% of untreated bloodmeal was below 30 kDa (Table 27). Treating bloodmeal with hydrogen peroxide (15%) increased the amount of peptides below 30 kDa to 35%. This was the only method which significantly increased the amount of peptides below 30 kDa. The lack of chain fragmentation could be due to the bloodmeal being highly aggregated which will reduce the ability of the decolouring chemicals to access the amino acid back bone.

Previous studies also suggested that treating proteins with oxidising agents could result in aggregation due to disulphide cross-linking. All treatment methods appeared to produce larger molecular weight aggregates. 39% of protein in untreated bloodmeal was larger than 80 kDa and most treatment methods increased this to between 40-50%. 48% of the protein of sodium hypochlorite (5%) treated bloodmeal had a molecular weight greater than 80 kDa. This increased to 55% when the treatment strength was increased to 15% and was most likely caused by the higher concentration. 57% of the protein in bloodmeal treated with chlorine dioxide (2%) was greater than 80 kDa and this was the highest of all the treatment methods.

Table 27: Number average molecular weight, weight average molecular weight, polydispersity index and molecular mass distribution of decoloured bloodmeal powders.

				Molecular Mass Distribution (kDa)							
	MN (kDa)	MW (kDa)	PI	1490-560	560-215	215-80	80-30	30-11	11-4	4-1.6	1.6-0.6
Bloodmeal	139	455	3.3	0.07	0.11	0.21	0.38	0.09	0.06	0.05	0.03
NaClO (5%)	199	495	2.5	0.12	0.16	0.20	0.31	0.07	0.06	0.05	0.03
NaClO (15%)	226	556	2.5	0.14	0.18	0.23	0.34	0.04	0.04	0.02	0.01
$NaClO_3$ (5%)	218	544	2.5	0.13	0.18	0.23	0.36	0.04	0.03	0.02	0.01
ClO_2 (2%)	228	556	2.4	0.15	0.18	0.24	0.34	0.03	0.03	0.02	0.01
H_2O_2 (15%)	148	497	3.4	0.08	0.13	0.17	0.27	0.11	0.11	0.08	0.05
H_2O_2 (30%)	172	496	2.9	0.09	0.16	0.21	0.31	0.09	0.06	0.05	0.03
PAA (5%)	214	555	2.6	0.13	0.19	0.19	0.26	0.06	0.07	0.06	0.04
PAA (15%)	162	477	2.9	0.08	0.15	0.19	0.31	0.09	0.06	0.08	0.04
CuSO4 (5%)	172	481	2.8	0.09	0.16	0.21	0.33	0.08	0.07	0.04	0.02

5.3.2.3 Thermal Stability of Treated Bloodmeal

Treating bloodmeal with sodium chlorite, hydrogen peroxide and PAA caused a decrease in thermal stability (Figure 39). Thermal stability can be reduced by reductions in stabilising interactions or molecular mass. Molecular mass analysis (Figure 38) showed that molecular mass was not significantly reduced for decoloured bloodmeal powders. Whereas XRD patterning (Figure 40) showed reduced peak intensities for peaks associated with alpha helices. This was seen for all treatment methods and was most significant for PAA treatment. In literature PAA has been shown to be highly reactive with the amino acid histidine, the main amino acid which stabilises alpha helices. Due to histidines ring structure it is probably easily oxidised by sodium chlorite and hydrogen peroxide as well. This could explain the reduction in those peak intensities when bloodmeal was treated with the decolouring chemicals [107]. This showed that reduction in thermal stability of treated bloodmeal powders was most likely caused by reduction of the molecular interactions that stabilise the regular structures and not by reduction in molecular mass. However other stabilising interactions such as disulphide cross-linking and hydrophobic interactions were not investigated and could also be reduced by the decolouring treatment and cause reduction in thermal stability. These can also influence processing requirements and should be investigated later.

Thermal stability was most significantly reduced for PAA treatment which also showed the greatest reduction in structure. In literature PAA has been shown to reduce disulphide cross-links which could also be a contributing factor [138]. The low pH of PAA can cause the haem to dissociate from the globin which also leads to reduced thermal stability as has previously been shown (Figure 34). These additional factors could also be contributing to the reduction in thermal stability caused by PAA treatment and are investigated further in Chapter Six. PAA contains acetic acid which can cause swelling of proteins [139]. This may increase decolouring effectiveness because of improved access to the haem porphyrin. This may also help remove odour compounds trapped between chains because it gives access to them where as hydrogen peroxide and sodium chlorite could not reach them. However this could also improve access to stabilising amino acids which could explain why PAA had a significant effect on structure whereas hydrogen peroxide and sodium chlorite did not.

Sodium chlorite treated bloodmeal initially showed a similar degradation profile to untreated bloodmeal and hydrogen peroxide treated bloodmeal. However at 350°C its rate of mass loss decreased. This was also seen for RBCC and mRBCC treated with sodium chlorite. This temperature range is usually associated with cross link degradation [7]. This confirmed that sodium chlorite treatment resulted in the formation of some disulphide cross-links.

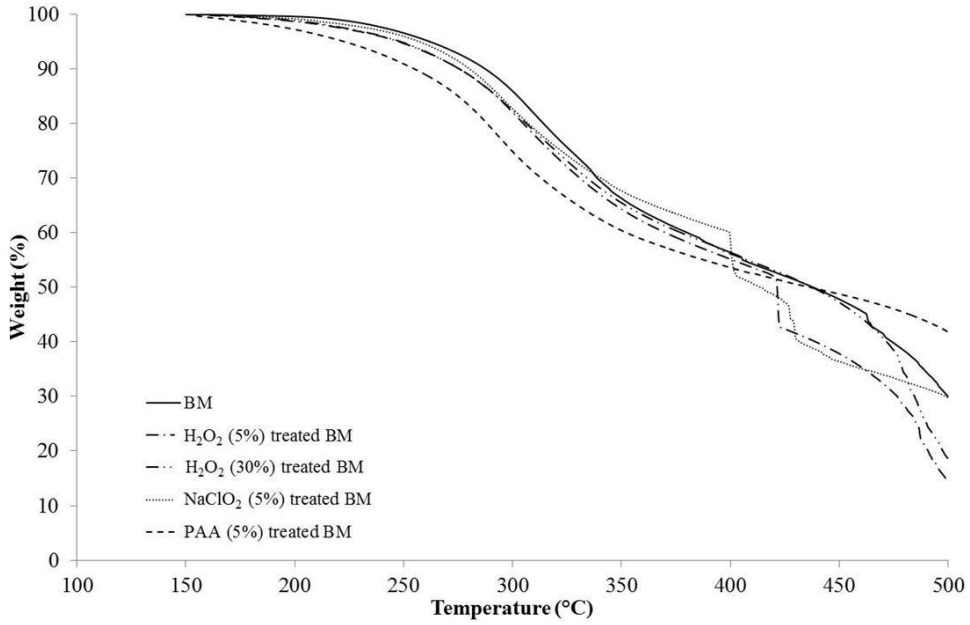


Figure 39: Thermal stability of hydrogen peroxide, sodium chlorite and peracetic acid treated bloodmeal.

Table 28: Percentage mass loss at 0-150°C and 230-400°C for decoloured bloodmeal powders.

Region	Bloodmeal	H_2O_2 (5%) treated Bloodmeal	H_2O_2 (30%) treated Bloodmeal	$NaClO_2$ (5%) treated Bloodmeal	PAA (5%) treated Bloodmeal
0-150°C	4	11	11	9	5
230-400°C	40	38	36	37	38
400°C	Thermal Decomposition				

It was interesting that sodium chlorite treated RBCC and mRBCC was still soluble whereas sodium chlorite treated bloodmeal was not. All three had similar thermal stability profiles which indicated the presence of cross-links. This suggested RBCC and mRBCC were soluble probably due to its decreased molecular mass. The other decolouring treatments had little effect on bloodmeal molecular weight. It is possible that sodium chlorite also did not reduce molecular weight but may have formed larger aggregates as shown by TGA, which would further reduce the solubility of bloodmeal after being treated with sodium chlorite.

5.3.2.4 XRD Patterning of Treated Bloodmeal

XRD analysis showed a slight reduction in the regular structure of hydrogen peroxide and sodium chlorite treated bloodmeal. PAA treatment appeared to have a more significant effect on structure than hydrogen peroxide and sodium chlorite (Figure 40). This was probably because PAA is a stronger oxidant than hydrogen peroxide and sodium chlorite. Another factor could be that bloodmeal is highly aggregated which could have prevented the sodium chlorite and hydrogen peroxide from reaching the amino acids responsible for stabilising structure, whereas PAA contains acetic acid which can cause swelling which will have increased access to the stabilising amino acids.

PAA is a strong oxidising agent capable of peptide cleavage as well as side chain modification. Chromatography results showed that molecular mass was not significantly reduced, whereas XRD analysis indicated a reduction in structure. This suggested that PAA was acting on the side groups of the protein, causing it to lose its structure but was not cleaving the protein into smaller chains.

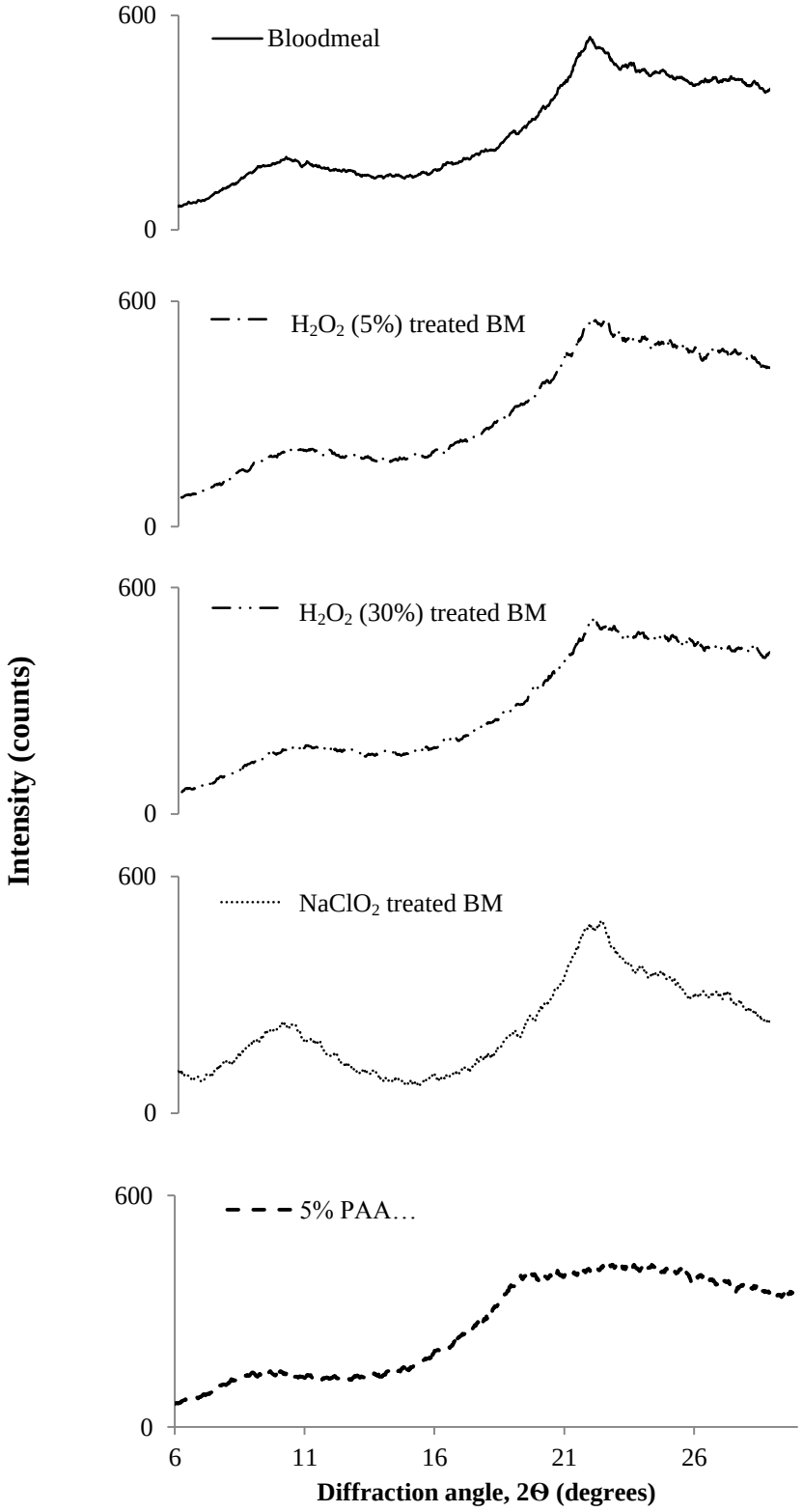


Figure 40: XRD plots of hydrogen peroxide, sodium chlorite and peracetic acid treated bloodmeal.

5.3.2.5 Suitability of Decoloured Bloodmeal for Bioplastics

Treating bloodmeal with sodium chlorite, hydrogen peroxide and PAA did not cause as severe reductions in molecular mass, interactions or thermal stability when compared to RBCC and mRBCC. This could be due to RBCC and mRBCC not being as cross-linked or aggregated as the dried bloodmeal making them more accessible to the decolouring chemicals. Also when mRBCC was lowered to pH 2 the proteins may be slightly unwound exposing more amino acids making them more susceptible to modification or damage.

Treating bloodmeal with sodium chlorite, hydrogen peroxide and PAA could potentially be used to produce a bioplastic feedstock. These methods were quick, simple and removed the colour and smell from bloodmeal.

In a previous study gas chromatography of decoloured bloodmeal powders showed that for hydrogen peroxide treated bloodmeal the odour compounds 1-octen-3-ol, phenol 4-methyl phenol, indole and methyl indole were no longer present, which suggests they were oxidised. However when prepared into a pre-extrusion mixture, 4 methyl phenol, indole, methyl indole returned. It was suggested that some of the compounds were oxidised and some were trapped between chains and became free when plasticisers were added [18].

The study also showed increased quantities of amino acid oxidation products such as hexanal, heptanone and heptanal for sodium chlorite treated bloodmeal. It also revealed the presence of esters. Ester formation can be initiated by formation of alkyl chlorides which may indicate chlorination is occurring during sodium chlorite treatment. When pre-extrusion mixtures were produced using sodium chlorite treated bloodmeal the smell returned again. This could be similar to hydrogen peroxide treated bloodmeal when some of the compounds were oxidised and some were trapped between chains and became free when plasticisers were added [18]. The study also analysed PAA treated bloodmeal and found that it removed 1-octen-3-ol, phenol, 4-methyl phenol, indole and methyl indole. PAA treatment was the most effective method at oxidising putrefaction compounds as shown by gas chromatography. In addition the bloodmeal smell didn’t return when pre-extrusion mixtures were produced, although a vinegar smell was observed most probably due to acetic acid or acetylation of the protein [18].

PAA may have been more effective at oxidising the odour compounds trapped between the chains because it causes swelling of proteins and can act as a plasticiser. This would have provided better access to the odour compounds trapped within the chains allowing them to be oxidised more efficiently (Figure 41).

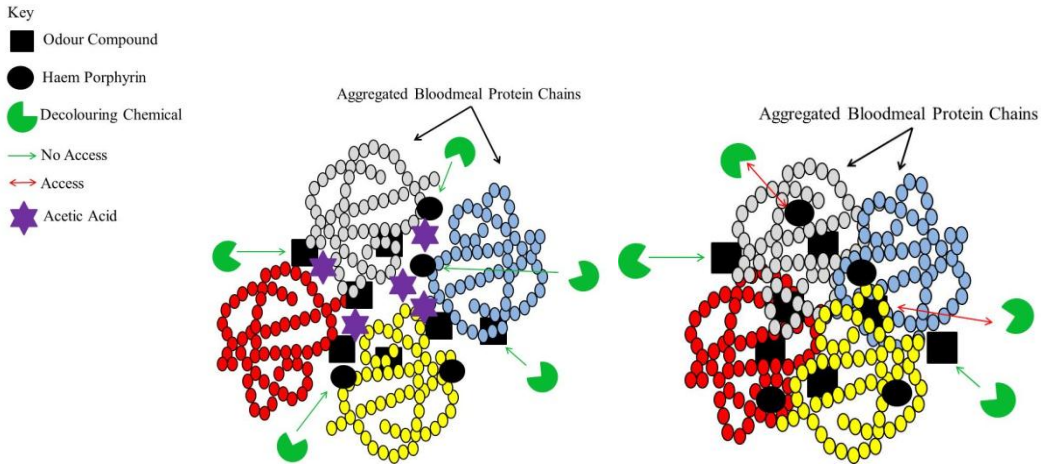


Figure 41: Acetic acid increasing access of colour and odour removing chemical to haem and odour compounds (left). Reduced access to haem and odour compounds due to aggregation (right).

5.3.3 Ranking Methods Based on Powder Properties

The methods were ranked by combining the results for colour removal, smell modification, simplicity, speed and environmental friendliness of process and effects on protein molecular weight (Table 29). Although XRD patterning and thermal stability was also investigated for some treatment methods as an indication of possible molecular interactions, these were not included in the ranking system because molecular interactions can potentially be reduced or increased during processing with the use of additives as discussed later in Chapter Seven. Points were assigned to each component and the methods were ranked based on the highest total score. The points were assigned as follows:

- Colour: Max 100
    - Points assigned were equal to percentage whiteness.
    - E.g. untreated bloodmeal is 19 % white and is assigned 19 points.
- Smell: Max 100
    - If the smell was improved-100 points.
    - If the smell was not improved-0 points.

- Simplicity of process: Max 100
    - If the process was simple i.e. low volumes of feedstocks being handled and few processing steps-100 points.
    - If the process required handling high volumes of feedstocks and many processing steps (diluting, pH adjustment, centrifuging etc.)-0 points.
- Speed: Max 100
    - If colour and odour removal occurred within 5 minutes-100 points.
    - If colour and odour removal took longer than 5 minutes-0 points.
- Environmental Friendliness: Max 100
    - If there were no environmental concerns-100 points
    - If there were environmental concerns-0points
- Molecular Weight: Max 100
    - If the molecular mass was not significantly reduced based on gel elution profiles and number average molecular weight-100 points.
    - If the molecular mass was significantly reduced based on gel elution profiles and number average molecular weight-0 points.

Table 29: RBCC and bloodmeal treatment methods ranked based on colour, smell and molecular weight.

Rank	Feedstock Treated	Treatment Method	Smell Improved ?	Colour Points	Smell Points	Simplicity Points	Speed Points	Env. Points	Molecular Weight Points	Total Points
1	**BM**	PAA (15%)	Yes	76	100	100	100	100	100	576
2	**BM**	PAA (5%)	Yes	67	100	100	100	100	100	567
3	**BM**	H_2O_2 (30%)	Yes	59	100	100	100	100	100	559
4	**BM**	H_2O_2 (15%)	No	57	0	100	100	100	100	457
5	**BM**	H_2O_2 (5%)	No	45	0	100	100	100	100	445
6	**RBCC**	PAA (5%)	Yes	91	100	0	100	100	0	391
7	**mRBCC**	PAA (5%)	Yes	87	100	0	100	100	0	387
8	**RBCC**	H_2O_2 (30%)	Yes	71	100	0	100	100	0	371
9	**mRBCC**	H_2O_2 (30%)	Yes	55	100	0	0	100	100	355
10	**BM**	Untreated	No	19	0	100	0	100	100	319
11	**BM**	Distilled Water	No	18	0	100	0	100	100	318
12	**BM**	$NaClO_2$ (5%)	Yes	31	100	100	0	0	N/A	231
13	**RBCC**	Untreated	No	29	0	0	0	100	100	229
14	**mRBCC**	Untreated	No	25	0	0	0	100	100	225
15	**BM**	ClO_2 (2%)	No	18	0	100	0	0	100	218
16	**BM**	$CuSO_4$ (5%)	No	18	0	100	0	0	100	218
17	**mRBCC**	$NaClO_2$ (5%)	Yes	93	100	0	0	0	0	193
18	**RBCC**	$NaClO_2$ (5%)	Yes	61	100	0	0	0	0	161
19	**RBCC**	ClO_2 (2%)	No	33	0	0	0	0	100	133
20	**RBCC**	$CuSO_4$ (5%)	No	30	0	0	0	0	100	130
21	**mRBCC**	$CuSO_4$ (5%)	No	26	0	0	0	0	100	126
22	**RBCC**	$NaClO_3$ (5%)	No	26	0	0	0	0	100	126
23	**mRBCC**	ClO_2 (2%)	No	25	0	0	0	0	100	125
24	**mRBCC**	$NaClO_3$ (5%)	No	22	0	0	0	0	100	122
25	**BM**	NaClO (5%)	No	19	0	100	0	0	N/A	119
26	**BM**	NaClO (10%)	No	19	0	100	0	0	N/A	119
27	**BM**	$NaClO_3$ (5%)	No	19	0	100	0	0	N/A	119
28	**BM**	NaClO (15%)	No	18	0	100	0	0	N/A	118
29	**RBCC**	NaClO (15%)	No	61	0	0	0	0	N/A	61
30	**RBCC**	NaClO (10%)	No	59	0	0	0	0	N/A	59
31	**RBCC**	NaClO (5%)	No	55	0	0	0	0	N/A	55
32	**mRBCC**	NaClO (10%)	No	44	0	0	0	0	N/A	44
33	**mRBCC**	NaClO (5%)	No	44	0	0	0	0	N/A	44
34	**mRBCC**	NaClO (15%)	No	43	0	0	0	0	N/A	43

Although sodium chlorite treatment of mRBCC produced a 93% white powder this method was not able to be used because the molecular weight of the protein had been greatly reduced (Figure 31). Sodium chlorite was also effective for treating bloodmeal. However the powder could not be dissolved using sodium sulphite SDS, suggesting cross-links had increased as a result of treatment. Also when processed into a pre-extrusion mixture the odour returned, this is discussed further in Chapter Eight. Using sodium chlorite also had environmental issues because there was the potential for chlorinated organic compounds to be produced.

Many of the RBCC, mRBCC and bloodmeal decolourisation methods had disadvantages associated with them. Hydrogen peroxide treatment of mRBCC required large volumes of water and long processing times. Sodium chlorite treatment of mRBCC reduced the proteins molecular weight and also had potential to produce chlorinated polymer chains. Treating RBCC and mRBCC with PAA resulted in the formation of small molecular weight peptides. This meant these methods were not applicable in the bioplastic process because of their high costs, reduction in protein molecular weight or formation of harmful by-products.

PAA treatment of bloodmeal was found to have the most potential for use in producing bioplastics because it removed the colour and smell from bloodmeal quickly without significantly reducing its molecular weight. Compared to other methods described in literature (organic solvents, enzymes etc.) PAA treatment was much faster with decolourisation occurring within minutes and was more environmentally friendly. Another advantage of this process was that it was relatively simple. Studies which investigated using PAA in wastewater and sanitising applications did not identify any harmful disinfection by-products and also found that PAA decomposes into water and acetic acid [113, 120].

When PAA treated bloodmeal was processed into a pre-extrusion mixture the odour did not return whereas for sodium chlorite and hydrogen peroxide treated bloodmeal it did. However a vinegar odour was observed for PAA treated bloodmeal because of the acetic acid. Structure and thermal stability were reduced

by PAA treatment and this should be further investigated with relation to its ability to be processed.

Although 15% PAA treatment was ranked first it was not considered for use in bioplastic feedstock production. This was because it would be too expensive and dangerous to use such as high concentration of PAA. There was also very little improvement in colour when compared to 5% PAA treatment. Therefore 1-5% PAA treatment was chosen to be investigated further.

PAA treatment requires cooling for the reaction vessel because heat was produced during the decolourisation treatment. The heat was caused by PAA reacting with the bloodmeal as well as PAA decomposition. The amount of heat generated increased as the amount of bloodmeal being treated increased. Temperatures up to 100°C were reached, but could be controlled with water or ice without affecting the rate of colour removal. Large batches would require sufficient cooling and stirring to maintain the temperature at safe working limits and also to protect the protein from thermal hydrolysis. On a large scale heat removed by cooling water could be cycled around the processing plant for use in other areas.

The reaction vessel must also be vented to allow the release of gases given off during the decolourisation treatment. The main gases produced are oxygen and carbon dioxide. These must be vented to prevent over pressurisation of the reaction vessel.

5.4 Conclusion

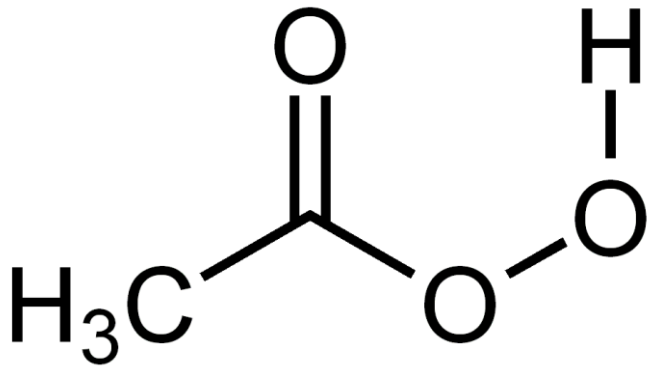
Sodium hypochlorite, sodium chlorate, chlorine dioxide and copper sulphate did not remove colour and odour from RBCC, mRBCC or bloodmeal. Whereas sodium chlorite, hydrogen peroxide and PAA removed the colour and smell from RBCC, mRBCC and bloodmeal.

Treating RBCC and mRBCC with sodium chlorite, hydrogen peroxide and PAA was not suitable for bioplastic production because they reduced molecular weight and had potential to form harmful by-products. Treating mRBCC with hydrogen peroxide did not reduce molecular weight but required long processing times and large volumes of water which would not be desirable for a large scale process.

Treating bloodmeal with sodium chlorite, hydrogen peroxide and PAA produced decoloured and deodorised powders without decreased molecular weights. Treating bloodmeal with hydrogen peroxide (30%), PAA (5%) and sodium chlorite (5%) produced 59, 67 and 31% white powders respectively and also removed the odour. Gas chromatography done in a separate study showed PAA removed the most odour causing compounds [18]. It also found that when pre-extrusion mixtures were produced some of the original odour returned for hydrogen peroxide and sodium chlorite treated bloodmeal but not for PAA treated bloodmeal. In addition, treating bloodmeal with sodium chlorite could also form chlorinated compounds. Therefore, it was concluded that PAA had the most potential for use in a bioplastics because it removed colour and odour efficiently and was also environmentally friendly. However before this was possible further research into how PAA treatment affected bloodmeal properties was required.

Chapter 6

Peracetic Acid and Bloodmeal



Peracetic acid molecule.

6.1 Chapter Overview

In Chapter Five it was found that treating bloodmeal with PAA had the most potential for producing a bioplastic feedstock. However PAA treatment could also have effects on molecular weight, structure and interactions which could influence processing, consolidation and mechanical properties. No previous work has been done involving treating bloodmeal with PAA for use in a bioplastic. Therefore this chapter investigated how PAA treatment affected the properties of bloodmeal using gel chromatography, thermogravimetric analysis, dynamic mechanical analysis, X-ray diffraction and solubility tests. The aim of this section was to understand how PAA treatment affected bloodmeal molecular weight, structure and interactions so that the information could be used later to help identify what additives and conditions were required for processing decoloured bloodmeal into a bioplastic.

It was found that PAA oxidised the haem porphyrin and odour compounds resulting in colour and odour removal. 1 and 2% PAA treatment produced 47 and 55% white powders respectively but did not remove odour completely. 3% PAA treatment produced a 63% white powder and was the lowest concentration which removed the odour from bloodmeal. 4 and 5% PAA treated bloodmeal produced 64 and 67% white powders respectively.

Gel chromatography indicated that 23% of the protein in untreated bloodmeal was below 30 kDa, whereas 22-25% of the protein in 1-5% PAA treated bloodmeal was below 30 kDa. These results showed that PAA treatment did not greatly reduce the molecular weight of bloodmeal. However, increasing PAA treatment concentration from 1-5% decreased bloodmeal crystallinity from 35-27%. Thermal stability and glass transition temperature also decreased and in addition solubility in phosphate buffer, 1% SDS and 1% sodium sulphite increased. Results indicated that PAA at low concentrations (1-2%) reacted preferentially with haem, odour compounds and disulphide cross-links due to its high affinity for conjugated compounds. When the concentration of PAA was increased above 3% the concentration was high enough to increase reactions with amino acid side chains causing a reduction in stabilising forces such as hydrogen bonding and

hydrophobic interactions which lead to decreased crystallinity, thermal stability and glass transition temperatures.

When PAA treated bloodmeal was compared to Pre-extruded Novatein Thermoplastic Protein (PNTP, bloodmeal mixed with urea, SDS, sodium sulphite, TEG, water) using XRD, TGA and DMA they had similar results. This showed that PAA treatment had a similar effect on bloodmeal structure and interactions as urea, SDS and sodium sulphite.

These results showed that 3-5% PAA treated bloodmeal could potentially be processed into a bioplastic. Because many of the stabilising interactions originally in bloodmeal were removed by PAA treatment, when processing these powders urea, SDS and sodium sulphite may be required at lower amounts or may no longer be required at all.

6.2 Experimental

6.2.1 Aim

The aim of this section was to investigate how different PAA treatment strengths affected the molecular and thermal properties of bloodmeal protein so that this information could be used to aid processing. More specifically its effect on molecular weight, crystallinity, thermal stability, glass transition temperature and solubility were investigated (Figure 42).

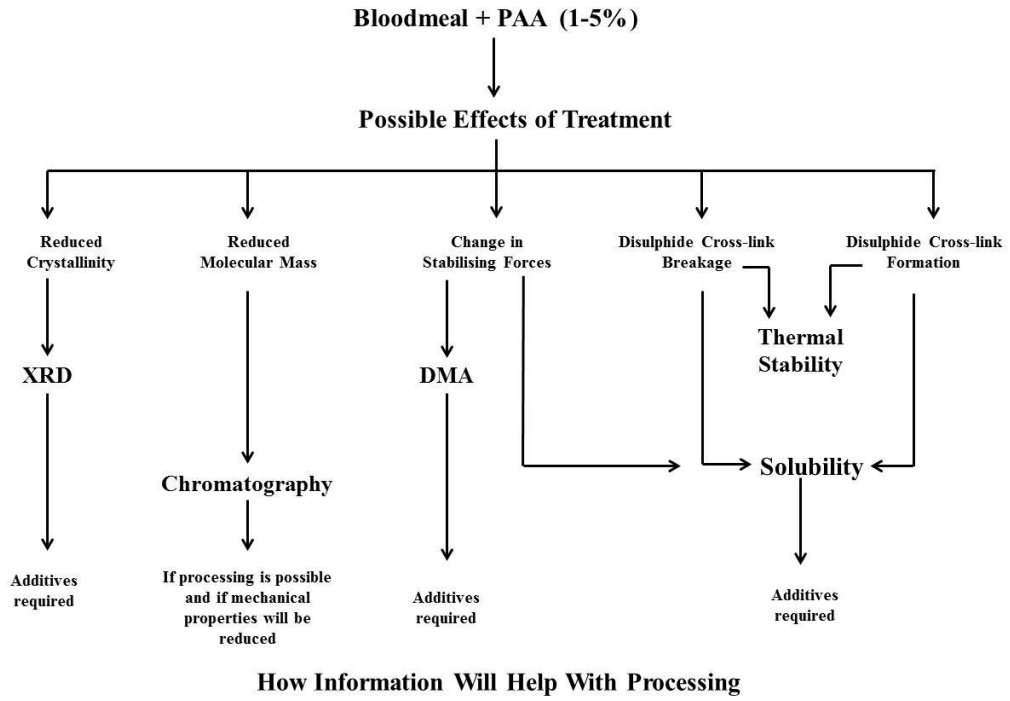


Figure 42: Experimental summary. Possible effects of PAA treatment and methods used to investigate them.

6.2.2 Methods and Materials

6.2.2.1 Peracetic Acid Treatment of Bloodmeal

Decoloured bloodmeal powders were produced for analysis by treating 100 g bloodmeal with 300 g PAA solution (1-5% concentration w/w). After 5 minutes of continuous mixing 300 g distilled water was added to form a slurry which was then filtered to remove acetic acid and unreacted PAA. Any remaining acetic acid was neutralised by submerging the filtered and treated bloodmeal in 300 g distilled water and adjusting to pH 7 by adding 1 mol/L sodium hydroxide solution. The slurry was filtered, washed, frozen and freeze dried overnight using

a Labconco Freezone 2.5 freeze dryer to 5-8% moisture. They were then ground using a bench top grinder and sieved using a 700 μm sieve.

6.2.2.2 Powder Analysis Methods

Molecular mass, powder colour, smell, and thermal stability analysis was carried out as described in Chapter Four and Chapter Five.

6.2.2.3 X-ray diffraction

Crystallinity was investigated using X-ray diffraction as described in Chapter Five. In addition the data was baseline corrected from 5-60 ° and an amorphous halo was fit to this region (Figure 43). Peaks identified at 9.5 and 20° were assigned to inter-helical packing and the alpha helical backbone based on values found in literature [137]. In addition peaks were also identified at 24 and 40°. These were assigned to beta sheets based on calculations for expected structural spacing and previous values identified in literature [46, 140]. The percent crystallinity was calculated by finding the area between the corrected XRD plot and the amorphous halo then dividing by the total area.

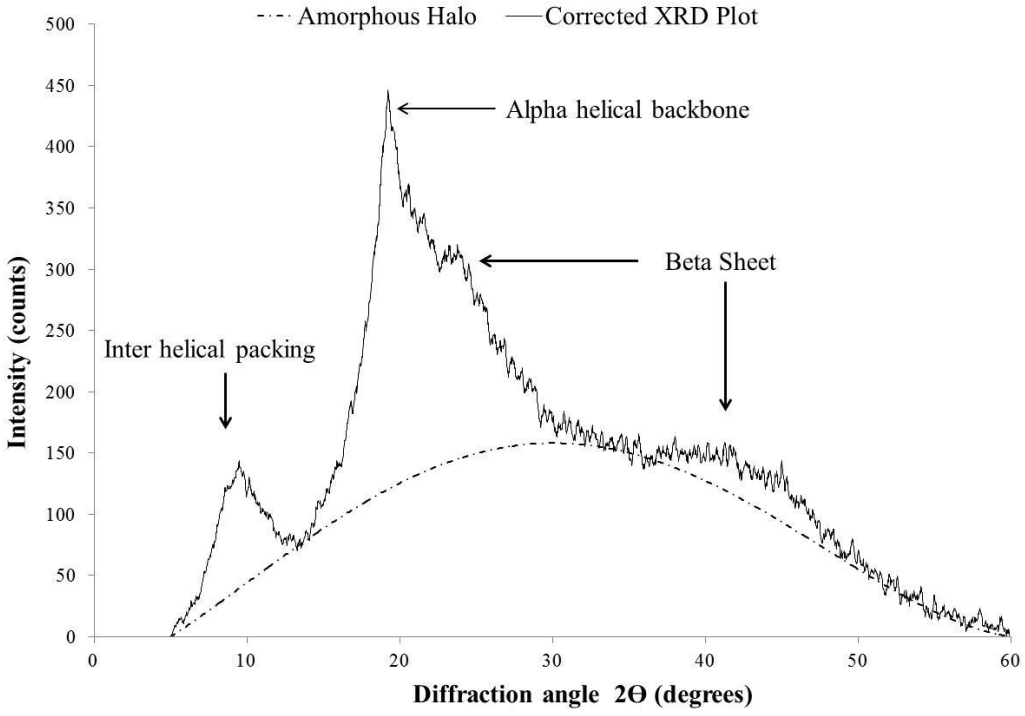


Figure 43: Example of a corrected XRD plot showing the amorphous halo and assigned peak locations.

6.2.2.4 Dynamic Mechanical Analysis

The glass transition temperature is where the amorphous regions of a polymer transition from a glassy brittle state to a rubbery flexible state. The glass transition is affected by the ability of the proteins chains to move past each other. Therefore changes in interactions cross-linking and hydrophobic interaction will alter the glass transition temperature. Glass transition temperature of PAA treated bloodmeal was investigated using dynamic mechanical analysis to investigate changes in stabilising interactions such as cross-linking, hydrogen bonding and hydrophobic interactions caused by PAA treatment.

Dynamic mechanical analysis (DMA) of PAA treated powders was carried out using a Perkin Elmer DMA8000. Approximately 50 mg of PAA treated powders were placed inside a stainless steel powder pocket (Perkin Elmer) and mounted in single cantilever mode. The sample was cooled to below -150°C using a cryo gun and liquid nitrogen, then heated from -150°C to 250°C at 2°C per minute and oscillated at frequencies of 1, 10 and 30 Hz. Glass transition temperatures were indicated by a peak in the Tan Delta (measure of mechanical damping) which was calculated based on the amount of lost and recovered energy during oscillation (Figure 44).

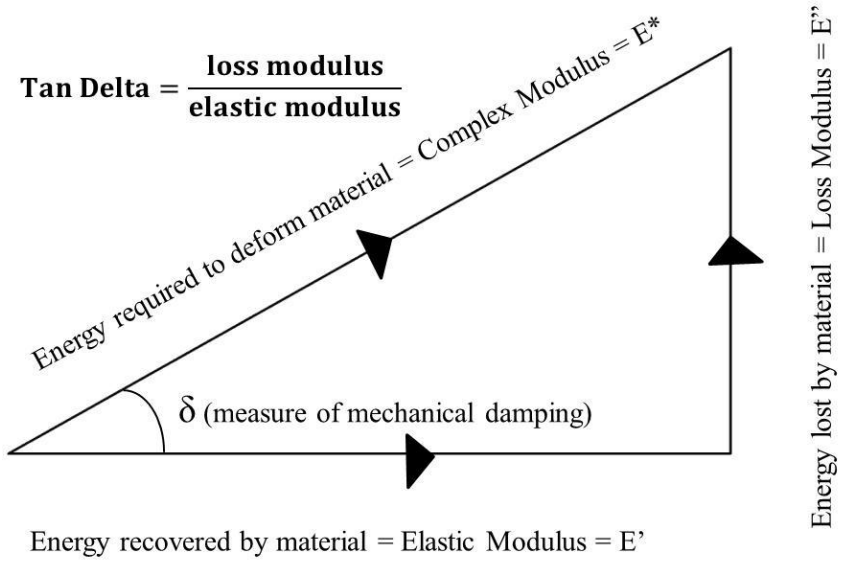


Figure 44: Calculation of Tan Delta from the loss and elastic modulus.

6.2.2.5 Solubility

The solubility of PAA treated bloodmeal was tested in phosphate buffer (pH 7), phosphate buffer with 1% SDS, phosphate buffer with 1% sodium sulphite and phosphate buffer with combined 1% SDS and 1% sodium sulphite. Solubility in phosphate buffer and phosphate buffer with 1% sodium sulphite was used to investigate changes in disulphide cross-linking. Solubility in phosphate buffer with 1% SDS was used to investigate changes in hydrophobic interactions. Solubility in phosphate buffer with 1% SDS and 1% sodium sulphite was used to investigate how SDS and sodium sulphite will affect the PAA treated bloodmeal.

1g of PAA treated bloodmeal was dissolved in 5 mL of buffer and heated to 60, 80 and 100°C for 80 minutes. The samples were centrifuged at 4000 rpm for 10 minutes and decanted. The pellets were washed with 20 mL of distilled water, centrifuged and decanted again. They were then dried at 110°C overnight and the soluble fraction calculated based on change in solids content.

6.3 Results and Discussion

6.3.1 Analysing Peracetic Acid Treated Bloodmeal

6.3.1.1 Peracetic Acid Treated Bloodmeal Colour

When bloodmeal was treated with PAA, colour removal occurred almost instantaneously. If the decoloured mixture was left for longer periods of time no increase in colour removal occurred. Therefore a reaction time of five minutes was used. When treating bloodmeal with 1 and 2% PAA the temperature of the mixture reached 60-70°C. At 3, 4 and 5% PAA concentration the temperature reached a maximum of 100°C. However it was found that this could be reduced using ice without slowing the rate of colour removal. Increased swelling of the bloodmeal protein was also observed at 5% PAA and it was difficult to filter the decoloured slurry. This was probably due to the higher acetic acid content of the 5% mixture.

PAA has been shown to remove colour by breaking the double bonds in colour compounds [141]. Its action on haem is probably similar. It would first have to break the tetrapyrole structure and then remove one of the double bonds which results in the yellow colour (Figure 6, Figure 7). Untreated bloodmeal is 19% white, treating bloodmeal with 1 and 2% PAA resulted in 47 and 55% white powders, but the odour was not removed (Table 30). Odour was removed at 3% PAA and at this concentration percent whiteness reached a plateau (Figure 45). Even 15 % PAA treatment only increased whiteness by a small amount to 76% as shown in Chapter Five. This indicated that above 3% PAA treatment most of the haem and odour compounds are degraded and is the lowest possible treatment strength able to be used for bioplastic production.

Table 30: Effect of different peracetic acid treatment strengths on bloodmeal colour and smell.

Treatment Method	L*	R	G	B	Whiteness (%)	Colour	Smell Modified?
Untreated	20	61	45	42	19		No
PAA (1%)	54	155	124	79	47		No
PAA (2%)	64	182	149	91	55		No
PAA (3%)	72	206	172	103	63		Yes
PAA (4%)	74	208	178	104	64		Yes
PAA (5%)	77	215	187	111	67		Yes

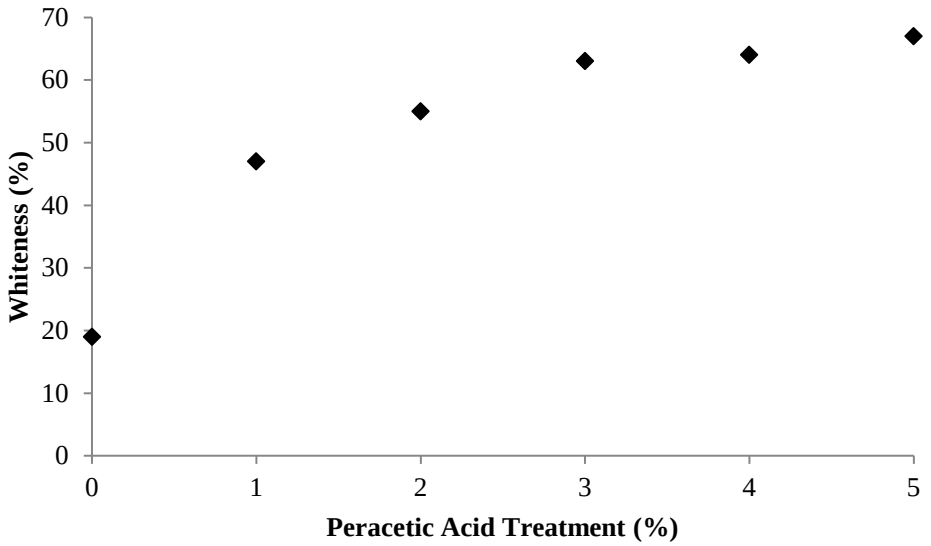


Figure 45: Percent whiteness of bloodmeal treated with 1-5% peracetic acid.

6.3.1.2 Peracetic Acid Treated Bloodmeal Molecular Mass

No significant change in peak locations were seen for PAA treated bloodmeal indicating molecular mass was not significantly reduced (Figure 46). At 4 and 5% PAA a small peak started to appear below 1.13 kDa although this was very small in comparison and outside of the column limits. Literature has shown that treating other proteins such as casein with PAA also resulted in no aggregation or protein back bone fragmentation [109]. This indicated that PAA may have preferred to react with other readily available components such as haem and odour compounds instead of the protein backbone.

Treating bloodmeal with PAA increased the number average molecular weight from 139 kDa to about 200 kDa depending on treatment strength. No trend was identified between PAA treatment strength and number average molecular weight. Most importantly PAA treatment did not result in the formation of small molecular weight compounds. 23% of untreated bloodmeal was below 30 kDa, whereas 22-25% of 1-5% PAA treated bloodmeal was below 30 kDa (Table 31). The increased number average molecular weight could be due to disulphide cross-link formation, however if this happened a decrease in solubility and increase in thermal stability would be expected. This was investigated later in this chapter. The apparent increase in molecular mass was most likely caused by PAA treatment breaking disulphide cross-links and making a higher proportion of the longer chains soluble.

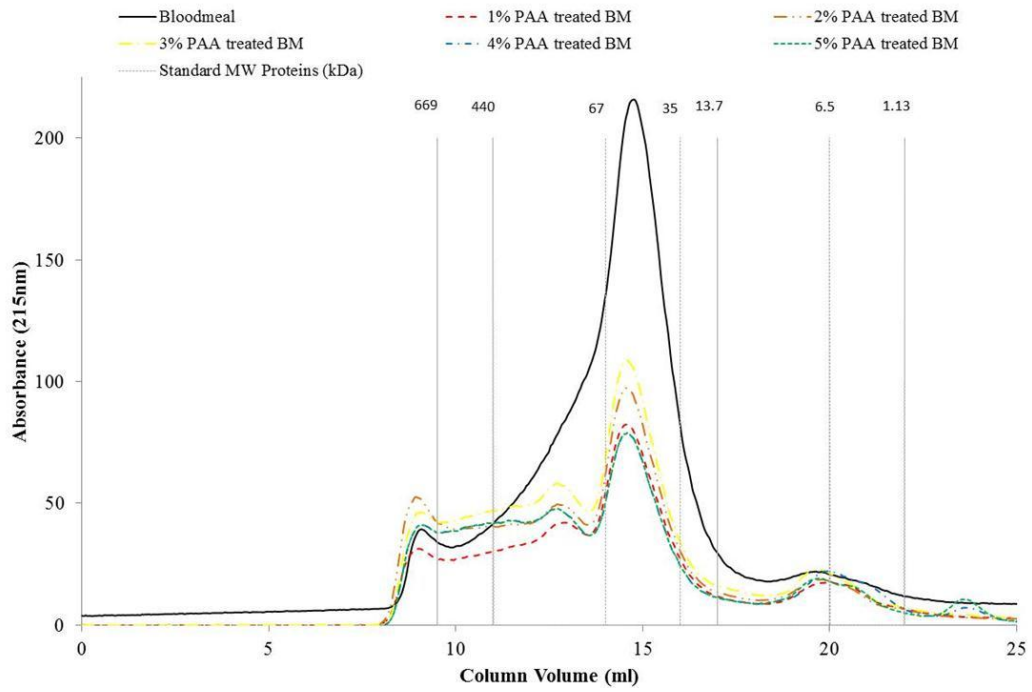


Figure 46: Elution profile for bloodmeal treated with 1-5% peracetic acid.

Table 31: Number average molecular weight, weight average molecular weight, polydispersity index and molecular mass distribution of PAA treated bloodmeal powders.

				Molecular Mass Distribution (kDa)							
	MN (kDa)	MW (kDa)	PI	1490-560	560-215	215-80	80-30	30-11	11-4	4-1.6	1.6-0.6
Bloodmeal	139	455	3.3	0.07	0.11	0.21	0.38	0.09	0.06	0.05	0.03
1% PAA	190	542	2.8	0.12	0.15	0.20	0.31	0.07	0.06	0.06	0.03
2% PAA	223	590	2.6	0.15	0.17	0.19	0.29	0.07	0.06	0.05	0.02
3% PAA	206	549	2.7	0.12	0.18	0.20	0.30	0.07	0.06	0.05	0.02
4% PAA	210	556	2.7	0.13	0.18	0.19	0.25	0.07	0.07	0.08	0.03
5% PAA	214	555	2.6	0.13	0.19	0.19	0.26	0.06	0.07	0.06	0.04

6.3.1.3 Peracetic Acid Treated Bloodmeal Crystallinity

XRD results showed that untreated bloodmeal (Figure 47) was 35% crystalline. This was reduced to 31-27% when treated with 1-5% PAA (Figure 48 to Figure 52). These results showed that when PAA treatment concentration increased crystallinity decreased (Table 32). This could indicate a reduction in stabilising interactions such as hydrogen bonding which are responsible for stabilising regular structure.

Methionine is one of the most common amino acids in alpha helices. [46]. Alpha helices are common in bloodmeal and contribute to its crystallinity. Due to its structure methionine is highly susceptible to oxidation [125, 126, 129]. Proline is usually found on the outside of alpha helices and is responsible for turns [46]. Its structure and location mean that it could also be susceptible to PAA oxidation. It contributes to the overall alpha helical structure but not to stabilisation because it cannot form hydrogen bonds. As treatment concentration increased, crystallinity decreased as expected due to the higher concentration probably modifying the methionine side groups and removing the alpha helix stabilising hydrogen bonds. Oxidation of proline may be contributing a small amount to structure loss due to loss of turns or bends.

As expected 1 and 2% PAA treatment resulted in a small reduction in crystallinity due to their weak concentration. 5% reduced the crystallinity the most which was also expected. This was probably caused by its higher concentration increasing the reactions with amino acid side chains, therefore reducing the amount of structure stabilising interactions and leading to destabilised structures and reduced crystallinity.

XRD plots for 1, 2 and 5% PAA treated bloodmeal showed a random spike at approximately 45°. These were not detected in repeat experiments and were most likely caused by the XRD equipment and should be ignored.

Table 32: Calculated crystallinity of bloodmeal and PAA (1-5%) treated bloodmeal.

	Bloodmeal	1% PAA	2% PAA	3% PAA	4% PAA	5% PAA
Crystallinity (%)	35	31	31	29	28	27

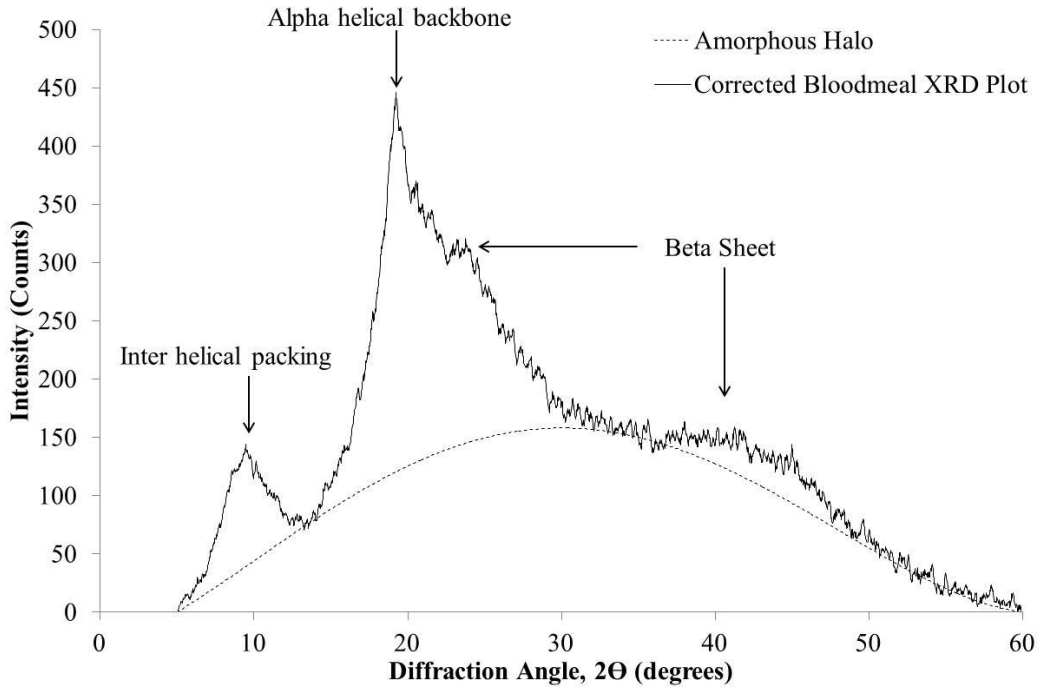


Figure 47: Corrected XRD plot for bloodmeal showing peak locations.

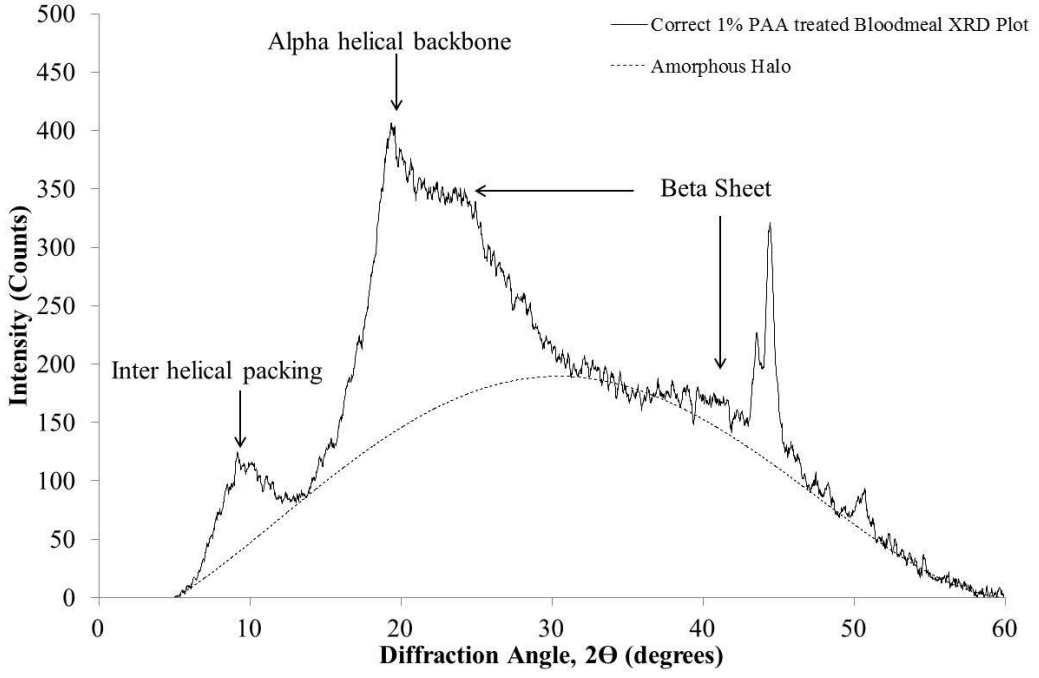


Figure 48: Corrected XRD plot for 1% PAA treated bloodmeal showing peak locations.

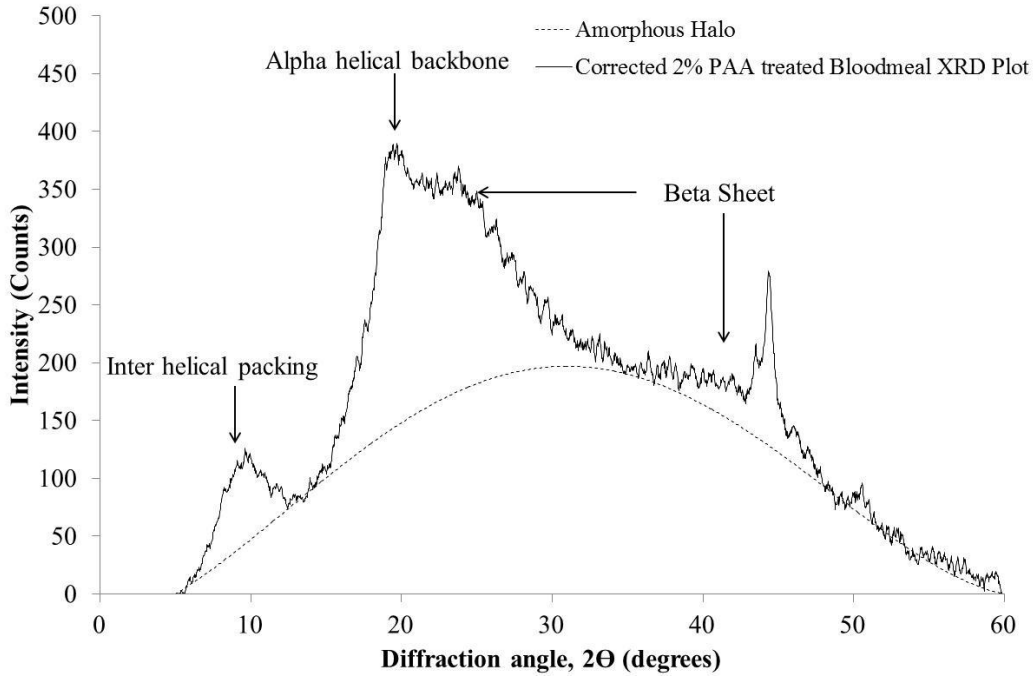


Figure 49: Corrected XRD plot for 2% PAA treated bloodmeal showing peak locations.

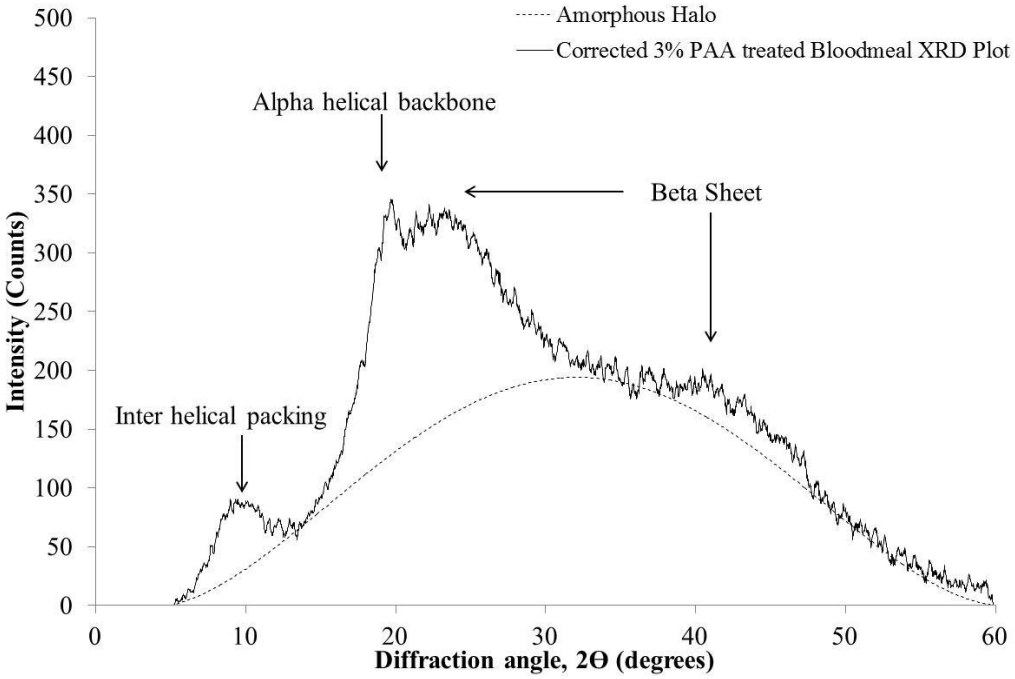


Figure 50: Corrected XRD plot for 3% PAA treated bloodmeal showing peak locations.

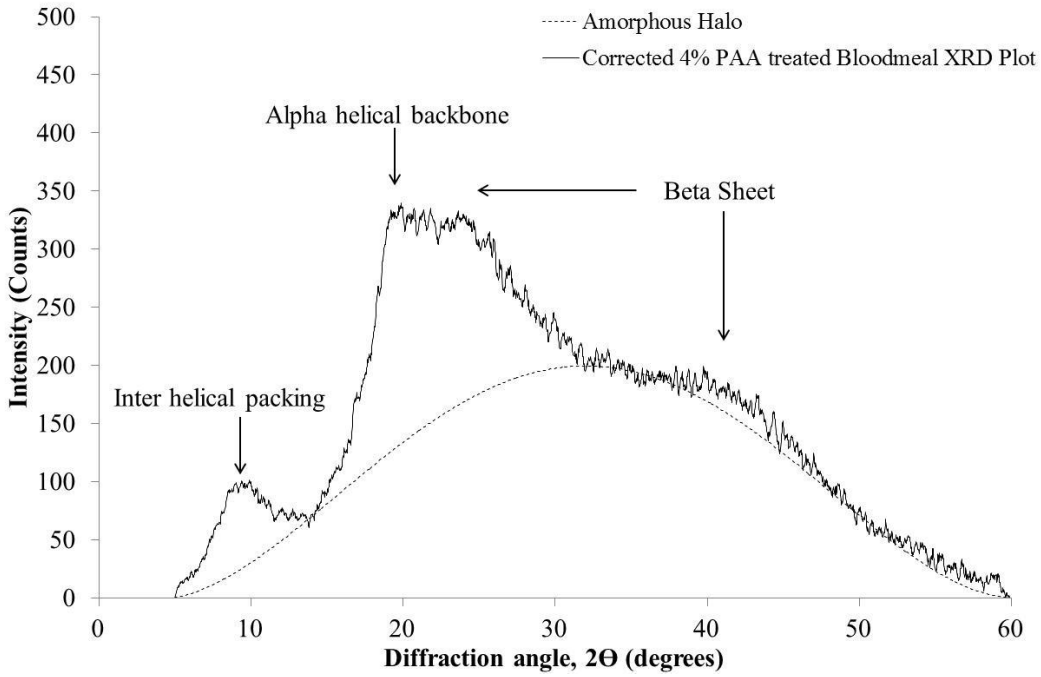


Figure 51: Corrected XRD plot for 4% PAA treated bloodmeal showing peak locations.

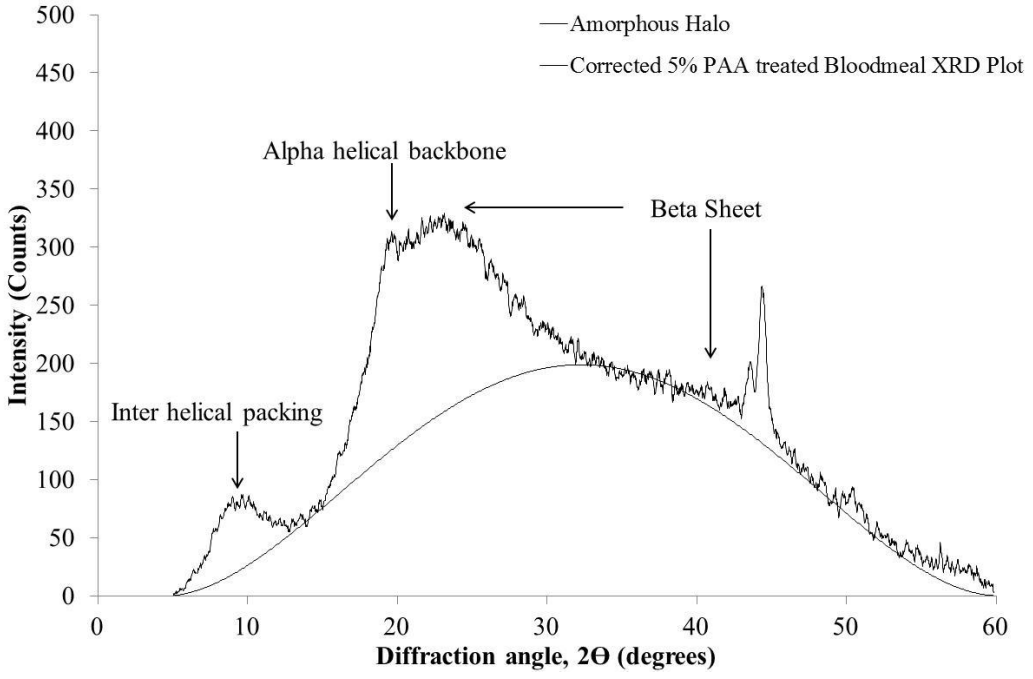


Figure 52: Corrected XRD plot for 5% PAA treated bloodmeal showing peak locations.

6.3.1.4 Peracetic Acid Treated Bloodmeal Thermal Stability

TGA results showed 1 and 2% PAA treatment had a similar mass loss profile to untreated bloodmeal (Figure 53). Oxidation of disulphide cross-links and amino acid side chains has been shown to cause loss of protein stability [107]. Above 3% PAA concentration rate of mass loss continued to increase showing loss of stabilising interactions probably due to disulphide cross-link and side chain modification.

It was previously shown in Chapter Five that thermal degradation of decoloured bloodmeal occurred in three stages. These were 0 to 150°C, 230 to 400°C and above 400°C. The first stage was attributed to the loss of bound water and the second stage to the breaking of S-S, O-N and O-O linkages. Above 400°C thermal decomposition is occurring through peptide bond reduction [7].

No significant loss in bloodmeal molecular mass was detected due to PAA treatment from TGA. If small molecular mass compounds had been produced, a complete loss of thermal stability such as that shown for sodium chlorite treated mRBCC (Chapter Five, Figure 34) would have been observed. Also if small molecular mass peptides were present an additional region of mass loss would have been observed at 150-230°C. This confirmed the results obtained using gel chromatography.

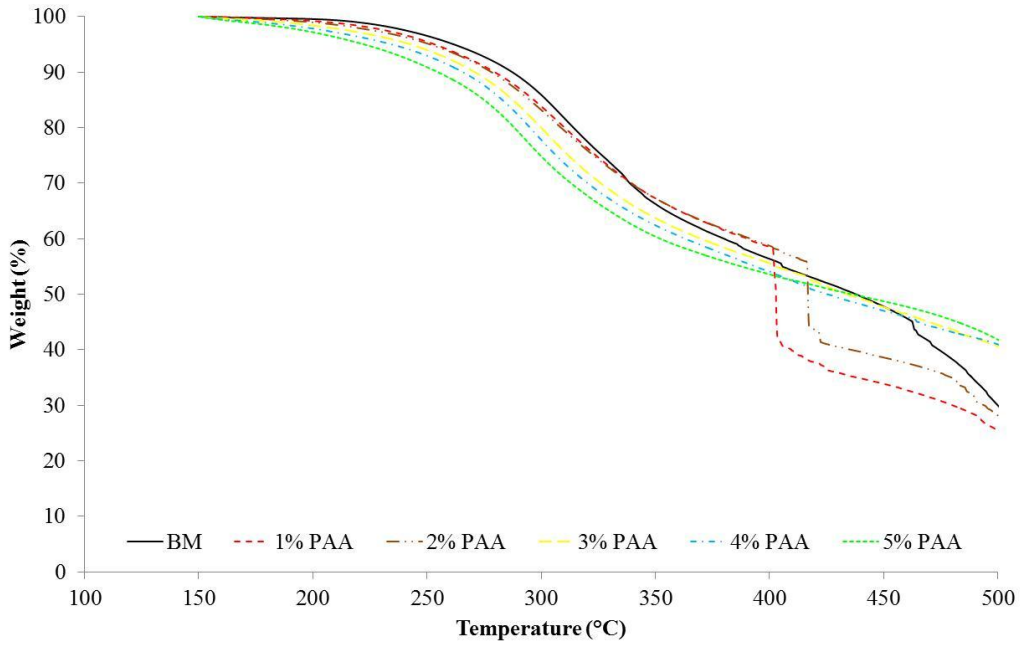


Figure 53: Thermal stability of bloodmeal treated with 1-5% peracetic acid.

Table 33: Percentage mass loss of PAA (1-5%) treated bloodmeal at different regions during thermal degradation.

Region	Bloodmeal	PAA (1%)	PAA (2%)	PAA (3%)	PAA (4%)	PAA (5%)
0-150 °C	4	7	6	7	6	5
230-400 °C	40	37	36	39	39	38
400 °C	Thermal Decomposition					

Another contributing factor to the reduction in thermal stability could be the pH. The low pH of PAA could be causing the haem group to dissociate. Removing the haem group destabilises the globin as shown in previous studies and earlier thermal stability tests in Chapter Five (Figure 34).

6.3.1.5 Peracetic Acid Treated Bloodmeal Solubility

Solubility trials were carried out to confirm disulphide cross-link and stabilising interaction reduction by PAA treatment of bloodmeal. Previous studies have used protein solubility in SDS as an indication of cross-linking [142]. Bloodmeal solubility in buffer increased linearly as PAA treatment increased (Figure 54), indicating that the amount of cross-links and stabilising interactions that were removed by PAA treatment was proportional to PAA concentration.

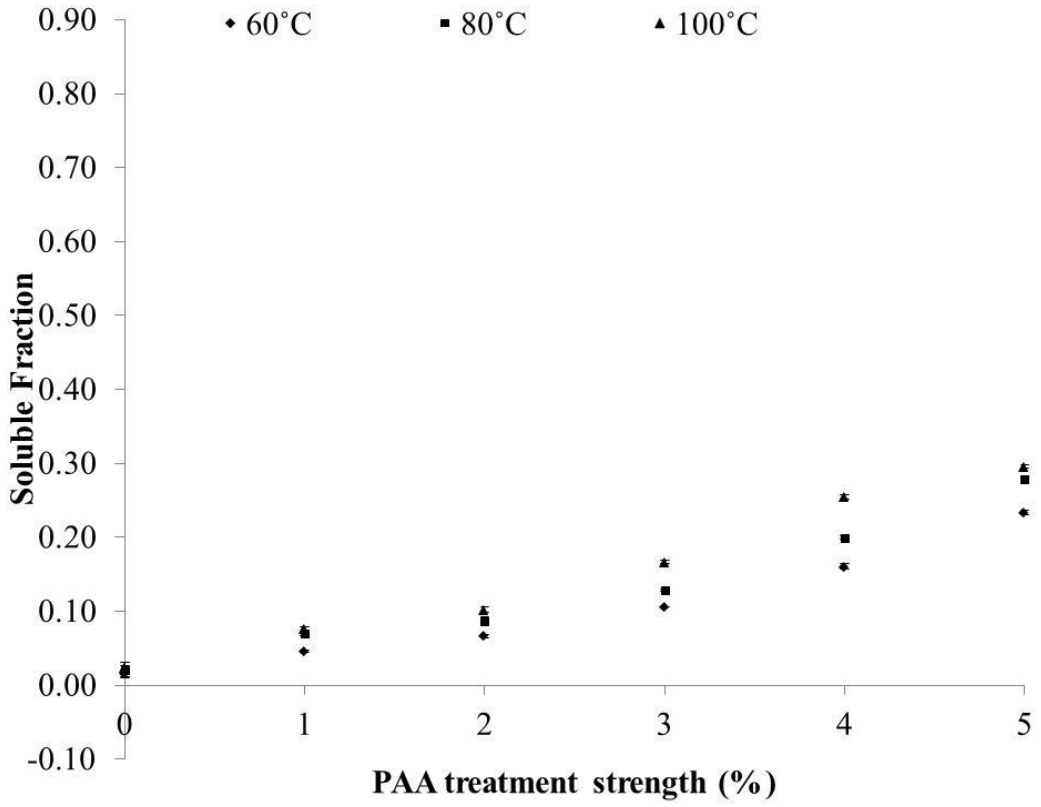


Figure 54: 1-5% peracetic acid treated bloodmeal solubility in buffer at 60, 80 and 100°C.

Sodium sulphite is a reducing agent used to break disulphide crosslinks [143]. Sodium sulphite increased solubility by approximately 5% (depending on temperature) for untreated bloodmeal, 1, 2 and 3% PAA treated bloodmeal due to its breakage of disulphide cross-links contained in these powders (comparing Figure 54 and Figure 55). Above 3% PAA a plateau was reached and solubility in sodium sulphite (Figure 55) was roughly equal to solubility in buffer (Figure 54). This indicated that 3% PAA treatment may have broken the majority of disulphide cross-links. Bloodmeal treated with PAA concentrations above 3% did not increase in solubility in sodium sulphite because there may have been no disulphide cross-links left for it to remove. No improvement in solubility in the presence of sodium sulphite for treatment concentrations above 3% PAA may show that the use of sodium sulphite is not required when processing these powders into bioplastics.

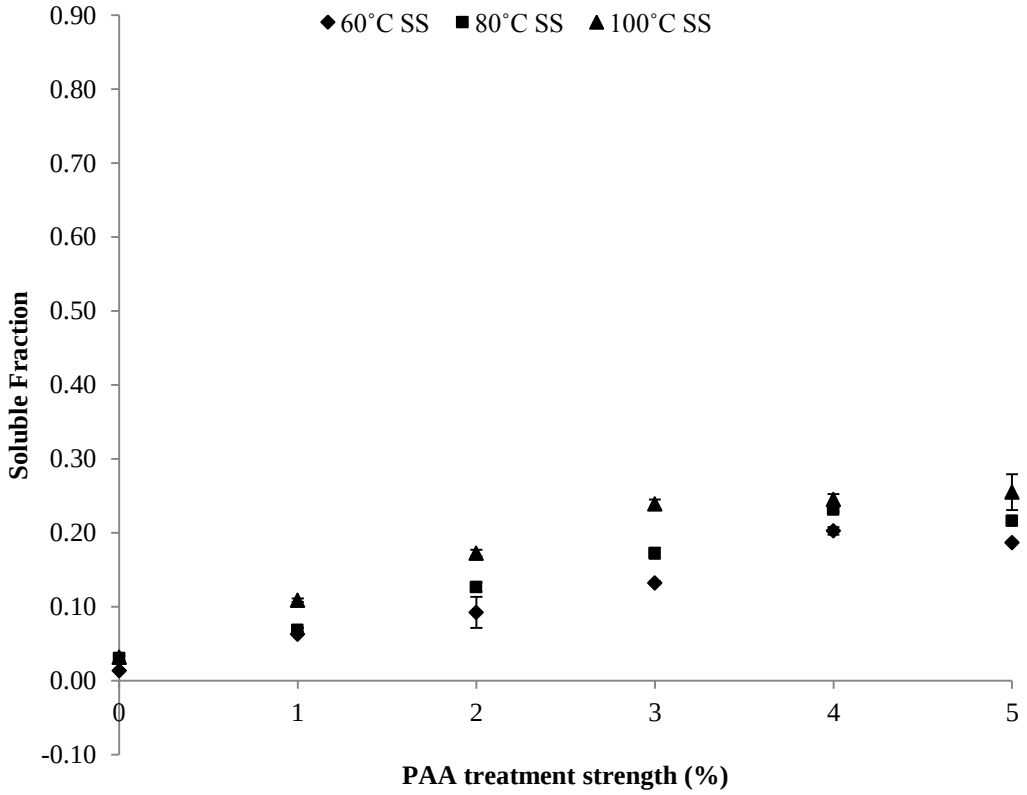


Figure 55: 1-5% peracetic acid treated bloodmeal solubility in sodium sulphite solution at 60, 80 and 100°C.

Solubility could also be affected by hydrophobic interactions. SDS is an anionic surfactant and interacts with hydrophobic regions of proteins making them soluble. Untreated bloodmeal and 1-2% PAA treated bloodmeal only showed a small increase in solubility in the presence of SDS (comparing Figure 54 and

Figure 56) which indicated that they also had other interactions such as disulphide cross-links. For untreated bloodmeal and 1% PAA treatment a small percentage mass gain was obtained and this was probably due to SDS which was not removed during the washing stages.

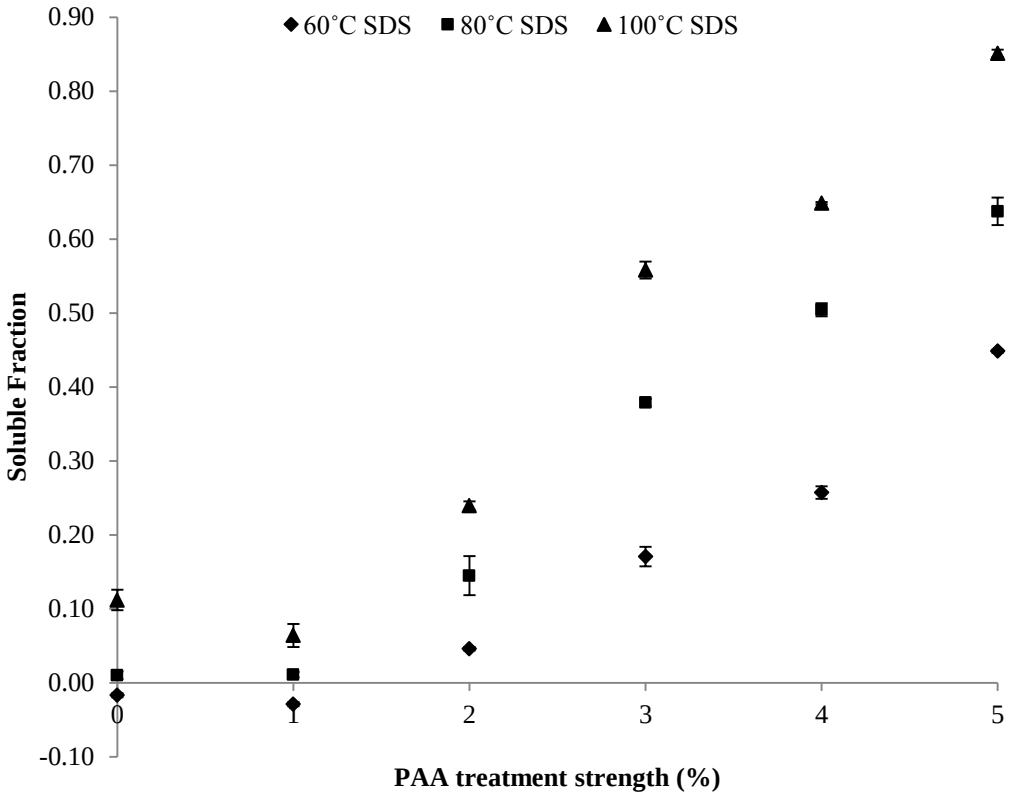


Figure 56: 1-5% peracetic acid treated bloodmeal solubility in sodium dodecyl sulphate solution at 60, 80 and 100°C.

Above 3% PAA treatment concentration, large increases in solubility were seen in the presence of SDS. This indicated that the majority of disulphide crosslinks were removed when treated with PAA concentrations above 3%. The large increase in 3, 4 and 5% PAA treated bloodmeal in the presence of SDS indicated that the remaining interactions were most likely hydrophobic interactions which could be reduced with SDS. Some of these interactions could have been formed by the drying process applied after the decolourising process.

By combining the solubility results for water, SDS and sodium sulphite it was shown that that the increase in solubility for bloodmeal treated with PAA concentrations up to 3% was mainly due to disulphide cross-link breakage. Previous studies showed that PAA reacted preferentially with conjugated systems and disulphide cross-links [109, 138]. At low concentrations it also appeared to react preferentially with the readily available haem porphyrin and odour compounds which are also conjugated or ring structures. Above 3% PAA concentration most of the cross-links, haem and odour compounds were removed and the increase in solubility in buffer is due to the PAA concentration being high enough to increase its reaction with side chains causing it to reduce other interactions such as hydrogen bonding or hydrophobic interactions.

Solubility in the presence of SDS and sodium sulphite showed large increases in solubility for untreated bloodmeal and 1-3% PAA treated bloodmeal (Figure 57). In these cases the solubility was almost equal to the solubility in SDS and sodium sulphite combined suggesting a cooperative effect between SDS and sodium sulphite at PAA concentrations up to 3%.

Above 3% PAA treatment concentration, solubility in SDS and sodium sulphite plateaued. Disulphide cross-links are broken by the action of the sulphite ion (SO_3^{2-}) [144]. If there were no disulphide cross-links for the sulphite ion to react with then it would have remained in the solution. As a result it may react electrostatically with SDS (both have negative charge) or act as a salting out agent. This result indicated that combining both SDS and sodium sulphite may be unsuitable for processing the 3-5% PAA treated bloodmeal into a bioplastic.

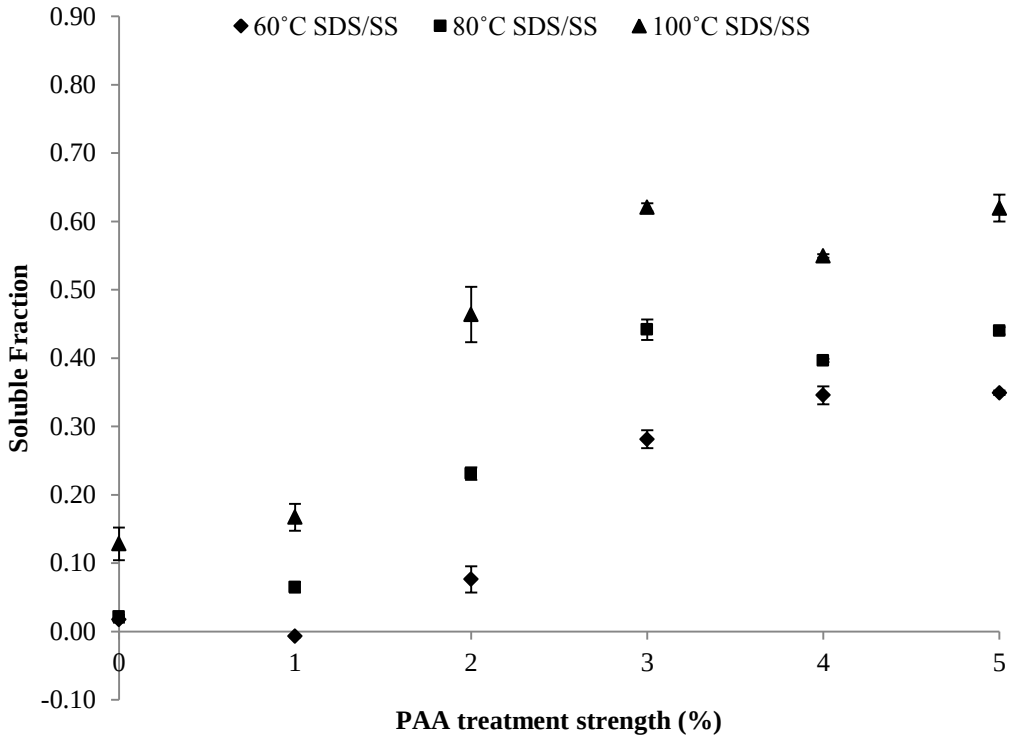


Figure 57: 1-5% peracetic acid treated bloodmeal solubility in sodium dodecyl sulphate/sodium sulphite solution at 60, 80 and 100°C.

During solubility trials, increased swelling of powders was observed for 3-5% PAA treated powders. The amount of swelling was also observed to increase proportionally as PAA treatment concentration increased. Increased swelling is also used as an indication of a reduction in disulphide cross-links and other stabilising interaction. This observation was further evidence that PAA treatment reduced disulphide cross-links and stabilising interactions.

PAA treated powders showed the highest solubility in the presence of SDS. It was also observed that the pellets and supernatants from SDS solubility trials (above 3% PAA treatment) formed a consolidated film when dried, whereas pellets from sodium sulphite solubility trials remained a powder. This may indicate that successfully processing PAA treated bloodmeal into a bioplastic could be more dependent on SDS than sodium sulphite because PAA treated bloodmeal will be stabilised by more hydrophobic interactions than disulphide cross-links.

6.3.1.6 Peracetic Acid Treated Bloodmeal Glass Transition Temperature

DMA analysis showed bloodmeal has one main peak in Tan Delta indicating a glass transition at approximately 225°C (peak 1) which is typically seen in most proteins (Figure 58). Two smaller peaks were also seen at approximately -100°C

(peak 2) and 50°C (peak 3). Peak 3 was attributed to side group motions and peak 2 to short range interactions. 1 and 2% PAA treated bloodmeal showed a similar profile to untreated bloodmeal although peak 2 became larger which indicated that the protein was becoming more mobile possibly due to reduction in stabilising forces.

At 3% PAA treatment peak 2 became larger and more distinct indicating another lower glass transition temperature. Reduction in glass transition temperature can be an indication of reduction in disulphide cross-links and other intermolecular interactions. The larger peak 2 of 3% PAA treated bloodmeal could be due to regions of low disulphide cross-linking, hydrogen bonding and hydrophobic interactions. Some stabilising interactions may still have been present and could be responsible for peak 1.

At 4% PAA treatment peak 1 began to decrease noticeably. This was probably because the higher PAA concentration increased the amount of reactions with the amino acids which were responsible for stabilising the protein which resulted in increased protein mobility and a lower glass transition temperature. At 5% PAA treatment peak 1was almost completely gone, indicating that a significant amount of the original stabilising interactions in bloodmeal could have been reduced.

These results indicated that PAA treated bloodmeal contained less stabilising interactions than untreated bloodmeal. The most interactions were removed when bloodmeal was treated with PAA concentrations above 3% as indicated by a lower glass transition temperature. Processing untreated bloodmeal into a bioplastic required that disulphide cross-links, hydrogen bonding and hydrophobic interactions be reduced using urea, SDS and sodium sulphite. It appeared that PAA treatment was completing the role of urea, SDS and sodium sulphite by reducing these interactions. The acetic acid in the PAA equilibrium mixture could also be acting as a plasticiser because it can penetrate between chains and cause swelling. This could also be contributing to the reduction in glass transition temperature. These possibilities were investigated later in this chapter by comparing PAA treated bloodmeal to untreated bloodmeal mixed with urea, SDS and sodium sulphite as well as untreated bloodmeal mixed with acetic acid.

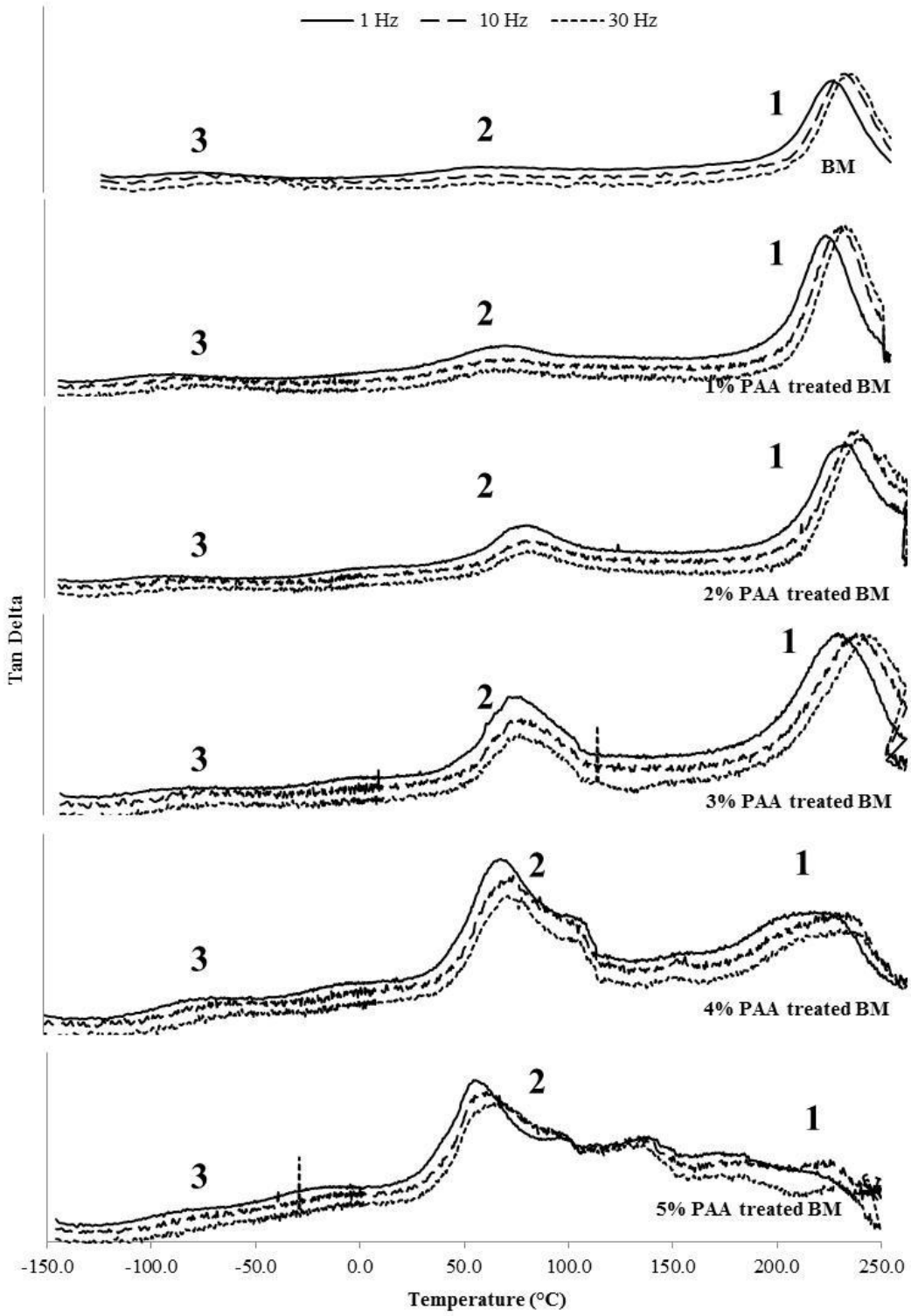


Figure 58: DMA plots for bloodmeal treated with 1-5% PAA.

6.3.1.7 Overall Effect of Peracetic Acid Treatment on Bloodmeal

1 and 2% PAA treatment did not completely remove the colour or odour from bloodmeal. 3% PAA was the minimum concentration level required for sufficient colour and odour removal (Table 30). After this no significant colour improvement was obtained as PAA concentration increased (Figure 45), which suggested that 3% PAA treatment was sufficient to remove the majority of the haem and odour causing compounds.

No significant changes in molecular weight occurred as PAA treatment concentration increased (Figure 46). As the treatment concentration increased above 3% PAA, the treated bloodmeal began to show significantly different responses when analysed with XRD, TGA and DMA. XRD showed reduced crystallinity, TGA reduced thermal stability and DMA reduced glass transition temperature. This indicated a reduction in stabilising interactions due to the higher concentrations of PAA increasing the reactions with components which were not haem or odour compounds.

Solubility analysis confirmed cross-link reduction from PAA treatment. As PAA treatment concentration increased so did the amount of disulphide cross-links and other stabilising interactions that were broken as shown by increased solubility in buffer (Figure 54). Non-disulphide interactions removed were probably hydrophobic interactions and hydrogen bonding due to the modification of proline, tryptophan and methionine. Solubility in SDS increased greatly at 4 and 5% PAA treatment (Figure 56). This showed that the majority of remaining interactions at these concentrations were most likely hydrophobic or electrostatic interactions which can be reduced by SDS.

DMA analysis showed bloodmeal had one main peak in Tan Delta at approximately 225 °C. 1 and 2% PAA treated bloodmeal showed a similar profile to untreated bloodmeal although a second peak became more distinct at approximately 50 °C. At 3% PAA both peak 1 and peak 2 were clearly visible.

At 4% PAA peak 1 was reduced and at 5% PAA it disappeared indicating that a high amount of disulphide cross-links, hydrogen bonding and hydrophobic interactions were removed at these high treatment concentrations. Peak 2 was thought to be caused by regions of low molecular stability. Solubility data

suggested that this peak which becomes distinct above 3% PAA treatment was mainly due to disulphide cross-link breakage and the remainder of peak 1 was due to the remaining interactions such as hydrogen bonds, hydrophobic interactions and electrostatic interactions. As PAA concentration increased these interactions were also reduced due to the PAA most probably reacting with tryptophan, methionine, proline and to a lesser extent tyrosine and histidine.

XRD plots and calculated crystallinity values showed 1 and 2% PAA treated bloodmeal reduced bloodmeal crystallinity from 35 to 31%. Above 3% PAA treatment concentration the XRD plots and calculated crystallinity showed a significant reduction in crystallinity (reduced from 35 to 27%) most probably though reduction in hydrogen bonding by modification of methionine and proline side chains.

By combining the results from gel chromatography, TGA, DMA, XRD and solubility analysis it was concluded that at up to 3% PAA treatment concentrations, the PAA reacted preferentially with the haem, odour causing compounds and disulphide cross-links. Above 3% PAA, the concentration was high enough to increase the amount of reactions with the amino acid side chains of proline, tryptophan, methionine, tyrosine and histidine, further reducing other stabilising interactions such as hydrogen bonding and hydrophobic and electrostatic interactions. This resulted in an overall loss of regular structure and stability. PAA showed limited reactivity with the protein backbone with even very high PAA concentrations (e.g. 15%) having limited effects on molecular mass (Figure 38). The effect of different PAA treatment concentrations on bloodmeal was summarised in Table 34.

Table 34: Effects of different PAA treatment concentrations on bloodmeal properties.

		Peracetic Acid Treatment Concentration				
		1%	**2%**	**3%**	**4%**	**5%**
	Mainly Reacted With	Haem Odour Compounds	Haem Odour Compounds	Haem Odour Compounds Disulphide Cross-links	Haem Odour Compounds Disulphide Cross-links Side Chains Peptide Backbone	Haem Odour Compounds Disulphide Cross-links Side Chains Peptide Backbone
Reduction In	**Colour**	Low	Low	High	High	High
	Odour	No	No	Yes	Yes	Yes
	Cross-link	Low	Low	High	High	High
	Crystallinity	Low	Low	Medium	High	High
	Total Stabilising Interactions	Low	Low	Medium	High	High
	Molecular Mass	Low	Low	Low	Low	Low
	Potential to be Processed?	No	No	Yes	Yes	Yes

3-5% PAA treated bloodmeal could potentially be used for producing a bioplastic. However the additives, processing conditions required and material properties for each may be different as they had different levels of stabilising interactions. The processing of bloodmeal treated with all three concentrations should be investigated because each may have different advantages and disadvantages.

6.3.2 Comparing Peracetic Acid Treated Bloodmeal to Pre-extruded Novatein Thermoplastic Protein

Pre-extruded Novatein Thermoplastic Protein (PNTP) was produced by mixing water, TEG, urea, SDS and sodium sulphite with untreated bloodmeal as described in section 8.2.2.1. PAA is an equilibrium mixture of PAA, water, hydrogen peroxide and acetic acid. It was thought that acetic acid could also be acting as a plasticiser by interfering with hydrogen bonding or by acetylating the protein and contributing to the change in thermal stability and glass transition temperature. Untreated bloodmeal was also mixed with 6% acetic acid (same amount present in 4% PAA equilibrium mixture). The bloodmeal was treated with the acetic acid for 5 minutes then washed and filtered and neutralised using the same method for producing PAA treated bloodmeal. Samples were freeze dried to 5-8% moisture. These were compared to 4% PAA treated bloodmeal to investigate how its processing requirements compared to untreated bloodmeal and also to see if acetic acid was contributing to the reduction in thermal stability and glass transition temperature.

6.3.2.1 Comparing Thermal Stability

TGA results showed 4% PAA treated bloodmeal and (PNTP) both had reduced thermal stability. However in the region between 150-230°C PNTP had greater mass loss which was attributed to the degradation of urea from the PNTP mixture. In addition the region between 350-400°C for PNTP showed greater mass loss than 4% PAA treated bloodmeal. This could be due to the presence of sodium sulphite and SDS in PNTP aiding the thermal breaking of peptide bonds.

The acetic acid treated bloodmeal displayed slightly reduced thermal stability. This could be due to the acidic pH causing some of the haem to dissociate from the protein which can reduce thermal stability as previously shown. However, the reduction in thermal stability is not as great as that seen when haem is removed from RBCC (Figure 34), probably because bloodmeal is more aggregated and has more stabilising interactions caused by the drying process. This result showed that thermal stability of bloodmeal was not significantly affected by the presence of acetic acid.

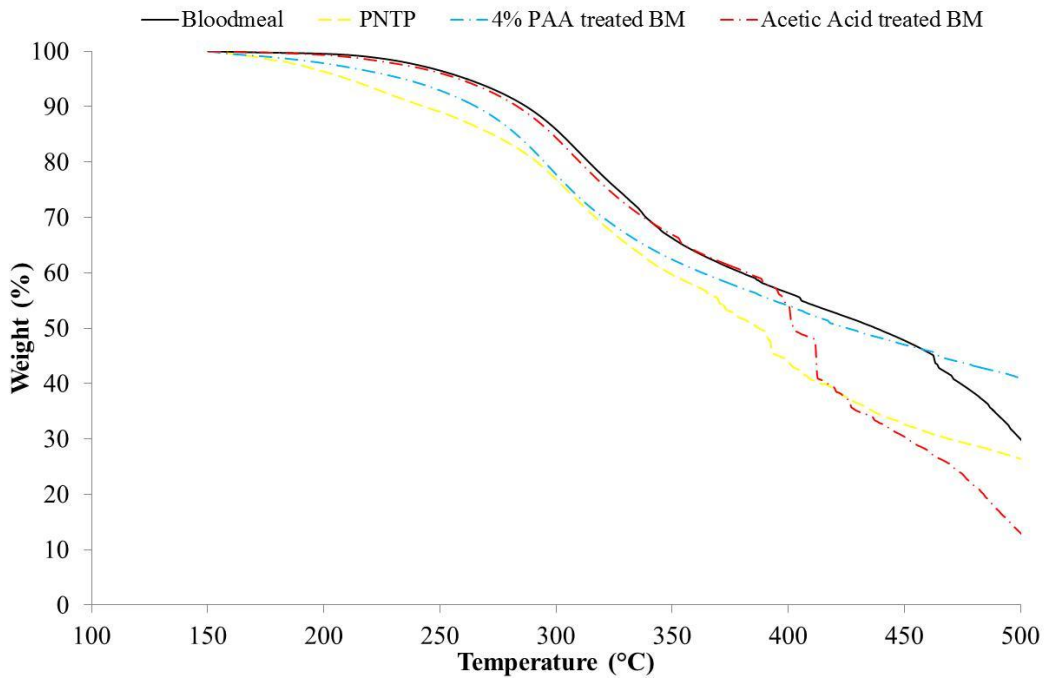


Figure 59: Thermogravimetric analysis comparison of bloodmeal, PNTP, 4% peracetic acid treated bloodmeal and acetic acid treated bloodmeal

6.3.2.2 Comparing Crystallinity

Crystallinity for PNTP and acetic acid treated bloodmeal was calculated using the same method as that for PAA treated bloodmeal. PNTP crystallinity was reduced to 33% and also showed a XRD pattern different to bloodmeal (Figure 60). Reduction in crystallinity was achieved by urea disrupting hydrogen bonding, SDS disrupting hydrophobic interactions and sodium sulphite breaking disulphide cross-links which can stabilise regular structure. Treating bloodmeal with 4% PAA resulted in a similar XRD profile to PNTP, indicating similar loss in crystallinity and stabilising interactions. This suggested that the existing combination of urea, SDS and sodium sulphite may not be suitable for processing 3-5% PAA treated bloodmeal.

Treating bloodmeal with acetic acid increased crystallinity to 65% and it had a similar XRD plot to untreated bloodmeal except the peaks were more intense. This suggested that acetic acid has plasticised the protein and weakened forces holding it in its heat denatured state allowing some of it to refold into ordered structures. Most importantly the result showed that acetic acid did not contribute to reduction in crystallinity and that the reduction in crystallinity when bloodmeal was treated with PAA was most likely caused by the action of PAA reacting with amino acid side chains as suggested previously.

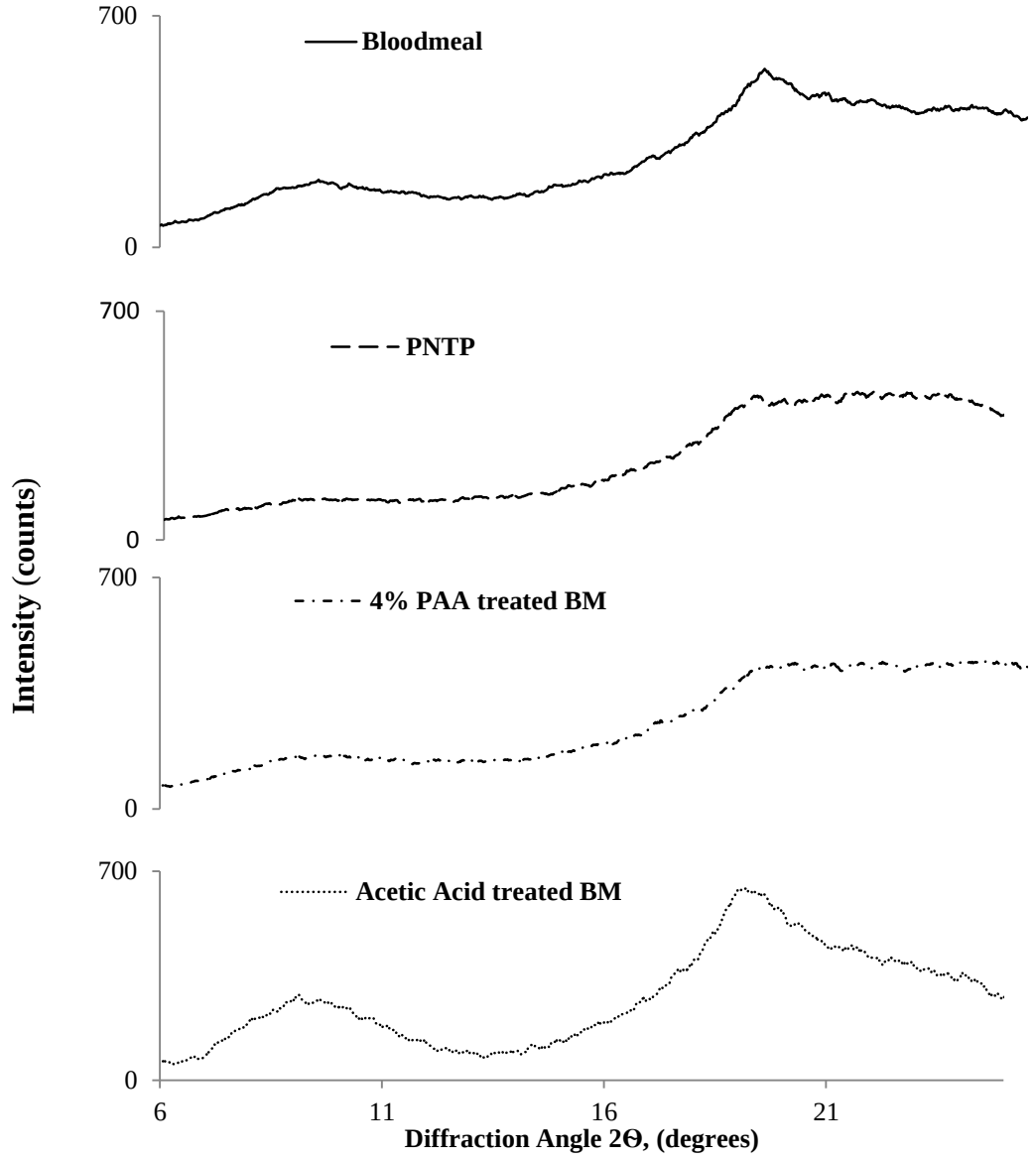


Figure 60: XRD comparison of bloodmeal, PNTP, 4% PAA treated bloodmeal and acetic acid treated bloodmeal

6.3.2.3 Comparing Glass Transition Temperature

Acetic acid treated bloodmeal formed a small peak (peak2) in the Tan Delta at approximately 50°C. This could be due to the acetic acid causing the dissociation of haem from the protein which causes a slight reduction in the proteins stability. However it is most likely due to the plasticisation effect of acetic acid. Acetic acid has been shown to cause proteins to swell or increase free volume [139, 145]. This result indicated that acetic acid could be acting as a weak plasticiser.

4% PAA treated bloodmeal had a very similar DMA profile to PNTP. Both had a large peak 2 at 50°C and a smaller peak 1 at 225 °C. This result indicated that PAA treatment was reducing the disulphide cross-links and other stabilising

interactions such as hydrophobic interactions and hydrogen bonding to a similar amount as urea, sodium sulphite and SDS. Therefore, if bloodmeal were treated with a high enough concentration of PAA it may be possible to process it into a bioplastic with none or reduced amounts of urea, sodium sulphite and SDS.

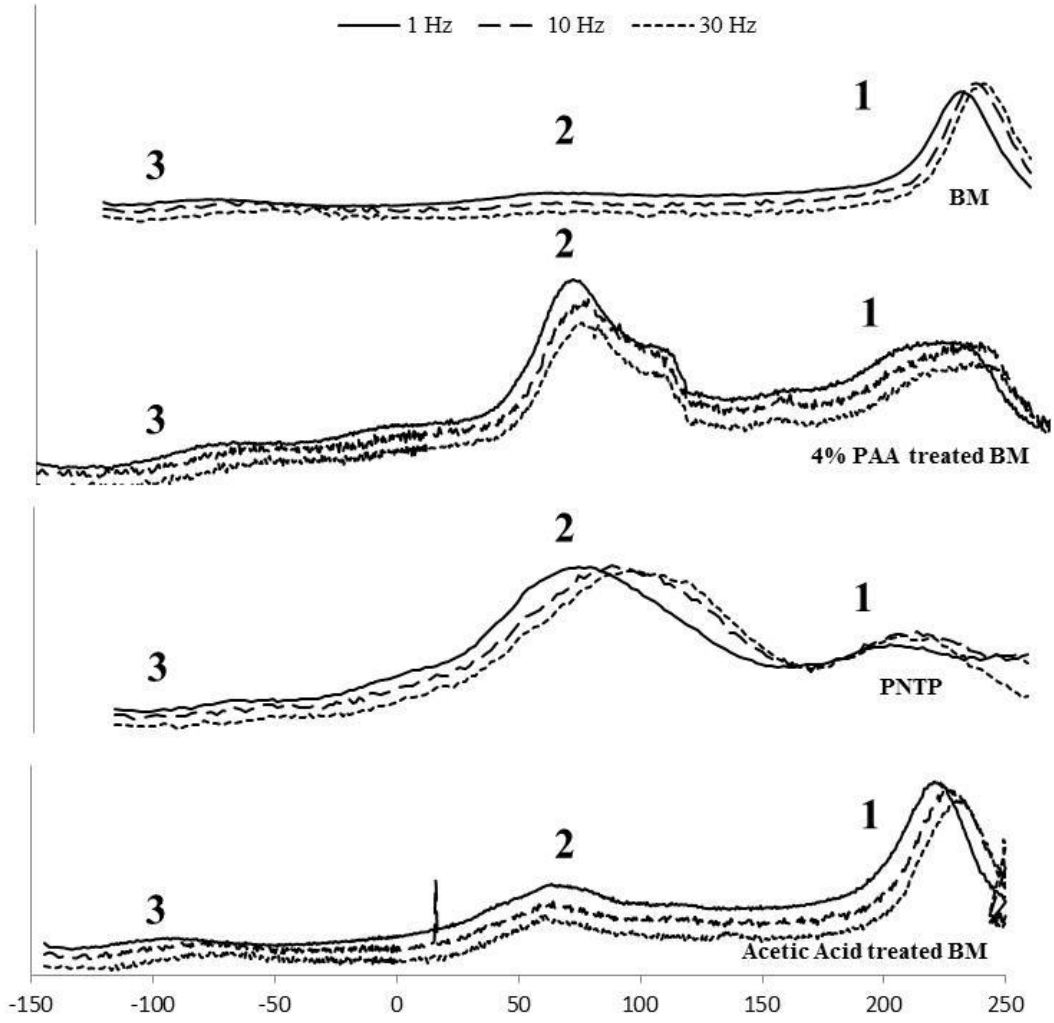


Figure 61: Dynamic mechanical analysis comparison of bloodmeal, PNTP, 4% PAA treated bloodmeal and acetic acid treated bloodmeal.

6.4 Conclusion

Treating bloodmeal with 1, 2, 3, 4 and 5% PAA resulted in 47, 55, 63, 64, 67% white powders respectively. 3% PAA was the lowest concentration required to sufficiently remove the odour from bloodmeal. PAA has a high affinity for conjugated compounds and would have oxidised the haem porphyrin and odour compounds resulting in colour and odour removal. However PAA treatment also had other effects on the bloodmeal protein.

PAA treatment reduced crystallinity, cross links, hydrogen bonding and other stabilising interactions as shown by XRD plots, reduced thermal stability, reduced glass transition temperature and increased solubility. The extent that these were reduced by was dependent on the concentration of PAA. Thermal stability, glass transition temperature and solubility of 1-2% PAA treated bloodmeal were similar to untreated bloodmeal. This indicated that at low concentrations, PAA reacted preferentially with haem, odour compounds and disulphide cross-links.

Above 3% PAA, the concentration was high enough to increase the amount of reactions with other components. These were most probably the side chains of proline, tryptophan, methionine, tyrosine and histidine as suggested in previous studies. This resulted in significantly reduced crystallinity, thermal stability, glass transition temperature and increased solubility most probably by reducing the amount of disulphide cross-links and other stabilising interactions such as hydrogen bonding.

PAA treatment increased the number average molecular mass from 139 kDa to approximately 200 kDa depending on treatment strength. From gel chromatography results it appeared that disulphide cross-linking occurred. However thermal stability, glass transition temperature were reduced and solubility increased which indicated disulphide cross-links did not form. The apparent increase in molecular mass was most likely caused by PAA treatment breaking disulphide cross-links and making a higher proportion of the longer chains soluble. Most importantly PAA treatment did not reduce molecular mass significantly even at 3-5% PAA concentration, which means consolidation may still be possible and mechanical properties may not be significantly reduced.

Solubility results also showed that combining SDS and sodium sulphite caused a reduction in solubility when compared to solubility in just SDS. This indicated that combining these two additives may have negative effects on processing PAA decoloured bloodmeal.

When bloodmeal treated with PAA at 4% was compared to PNTP using TGA, XRD and DMA they had similar results. This indicated that PAA treatment was having a similar effect as urea, sodium sulphite and SDS by reducing stabilising interactions such as disulphide cross-links and hydrogen bonding. This suggested that the processing of PAA treated bloodmeal powders may not require urea, sodium sulphite and SDS or may require them at lower amounts depending on the concentration of PAA treatment used. Alternatively additives which increase interactions between protein chains may be required because PAA treatment may have removed too many stabilising interactions which are required for consolidation into a bioplastic.

These results indicated that 3, 4 and 5% PAA could be used to produce a bioplastic, although their additives and processing requirements will most probably be different as they have different amounts of remaining stabilising interactions. All three PAA concentrations should be used to produce powders for processing trials as each powder and its bioplastic could have advantages and disadvantages.

Chapter 7

Biopolymers and Processing

Granulated Novatein ThermoPlastic Protein

7.1 Introduction

Bioplastics have been around for many years. A very early example includes Henry Ford who incorporated soy based plastics into automobile parts [4]. Casein and cellulose have also been used to produce bioplastics. However, synthetic plastics became more common because they were cheaper and easier to produce and had better properties such as strength, durability and water resistance. Recent concern over petroleum levels and plastic waste has caused renewed interest in biodegradable plastics produced from renewable biopolymers. These bioplastics can be used in applications where their biodegradability would be an advantage such as disposable items and packaging.

This chapter covers brief background information about synthetic polymers and common thermoplastic processing methods used to convert them into usable items. It then focuses more specifically on the direct conversion of biopolymers such as carbohydrates, proteins and polysaccharides into bioplastics using additives and thermoplastic processing techniques.

7.2 Synthetic Polymers and Processing

Plastics are materials made of high molecular weight molecules known as polymers [146, 147]. They are used in many applications because of their properties (low density, resistance to corrosion, low conductivities) and ability to be processed easily at relatively low temperatures [146, 147].

Polymers are long chains made from one or more types of repeating units called monomers. A polymer containing one type of monomer is a homopolymer and a polymer containing more than one monomer type is a copolymer [4]. Polymers can be linear chains, branched chains or cross-linked networks of chains (Figure 62).

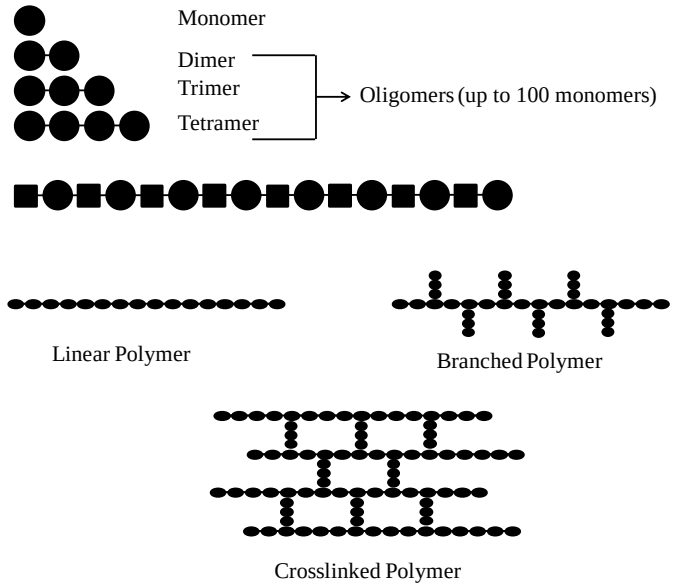


Figure 62: Summary of polymer structure [4, 147].

Synthetic polymers can be produced by addition or condensation polymerisation. During addition polymerisation monomers are added together by breaking double bonds that exist between carbon atoms. This allows them to be linked to neighbouring carbon atoms forming long chains [146]. An example is the polymerisation of polyethylene. Condensation polymerisation involves two units with end group components which react and bond with each other producing water as a molecule.

Plastics can be categorised as a thermoplastic, thermoset or rubbery. Thermoplastics solidify as they cool, but when heated again can flow as the molecules begin to slide past each other. Thermoplastics can be either amorphous (disordered, Figure 63) or semi-crystalline (some sections of order, Figure 64) [146]. When an amorphous thermoplastic cools below its glass transition temperature (T_g is the temperature when material changes from hard glassy state to a soft rubbery state), their molecules form a random molecular structure. The glass transition temperature can be recognised by a drop in shear modulus or a peak in Tan delta during a dynamic mechanical test. Amorphous polymers are transparent or clear because their bond lengths are small and absorb light smaller than visible wavelengths [146].

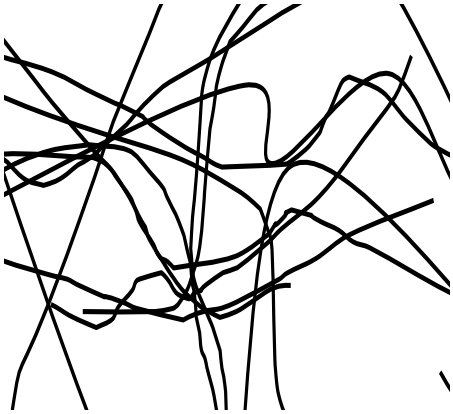


Figure 63: Amorphous structure of plastics.

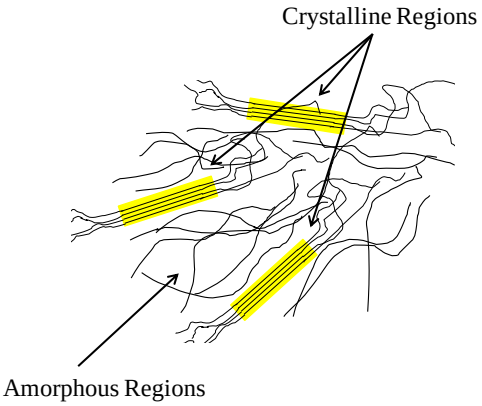


Figure 64: Structure of semi crystalline plastics.

Semi-crystalline thermoplastics have a melting temperature (T_m) as well as a glass transition temperature. Above the glass transition temperature the amorphous regions soften and above the melting temperature the crystalline regions melt (Figure 63). In semi-crystalline thermoplastics molecular arrangement of crystalline regions begin below the melting temperature and some molecules in semi-crystalline polymers which are not ordered remain in amorphous regions (Figure 64). Below the glass transition temperature the amorphous regions begin to solidify. Due to their molecular arrangement semi-crystalline polymers are translucent instead of transparent [146].

Thermosetting polymers solidify by a chemical curing process when the polymer chains form covalent crosslinks and become a network. The chains are no longer able to slide past each other even when heated. The cross-linked network causes thermosets to be brittle and have high glass transition temperatures often above their thermal degradation temperature [146]. Elastomers are rubbery materials that are slightly cross-linked. This allows them to undergo reversible deformation. Common thermoplastics, thermosets and elastomers are summarised in Table 35.

Table 35: Properties and applications of common polymers [4, 146, 148].

Polymer	Applications	Properties
Thermoplastics		
Amorphous		
Polystyrene	Transparent products, packaging, insulation	Brittle, rigid, translucent, low cost, easy to process
Polyvinyl chloride	Shoes, hoses, bottles, packaging, construction materials	Durable, rigid, good insulator, weather proof
Polycarbonate	Helmets, head lights, protective equipment	Transparent, tough, thermally stable
Semi-crystalline		
High density polyethylene (less branching-more interactions)	Milk and soap bottles, toys, electrical insulation	Flexible, translucent, low cost easy to process
Low density polyethylene (more branching-less interactions)	Household products, plastic bags, bottles	Low cost, tough, translucent, weather proof, easily processed
Polypropylene	Electrical appliance cases, automobile parts, containers	Semi rigid, translucent, good chemical resistance
Thermosets		
Phenolics	Heat resistant applications	Brittle, opaque, heat and chemical resistant
Melamine	Heat resistant surfaces	Hard, opaque, scratch resistant
Unsaturated polyester	Sinks and automotive body parts	Low weight, corrosion resistant, other properties are depending on additives used
Elastomers		
Natural rubber	Tyres and engine mounts	Flexible, undergoes reversible deformation
Polyurethane elastomer	Seats and shoe soles	Elastic, abrasion, chemical resistant
Silicone rubber	Seals, flexible hoses	Temperature and chemical resistant

The molecular weight distribution of a material is strongly related to its properties and most thermoplastic materials have a distribution similar to that shown in Figure 65. In general a low molecular weight results in poor strength and stiffness, although there are some exceptions. Increasing molecular weight increases strength and stiffness until the critical chain length is reached and no further increase in strength or stiffness occurs (Figure 66) [146].

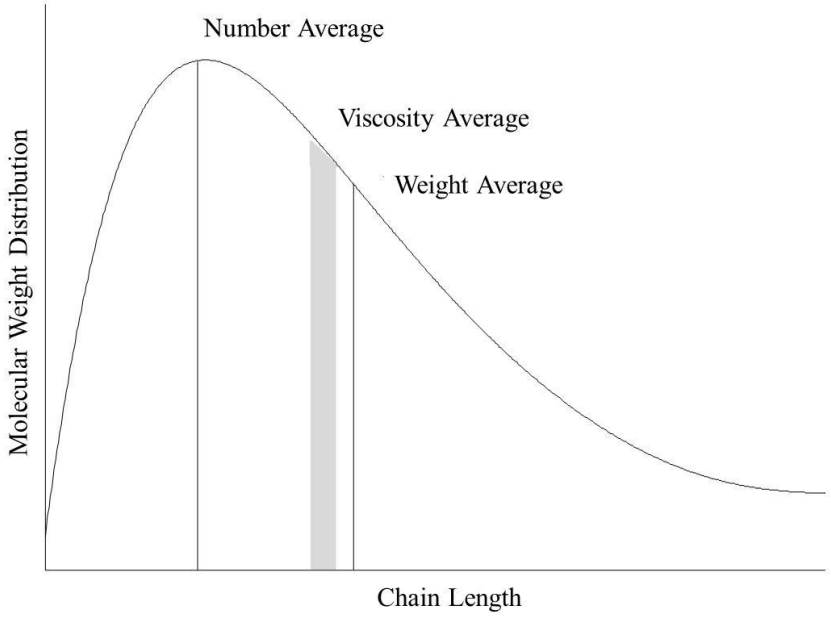


Figure 65: Molecular weight distribution of a typical thermoplastic [146].

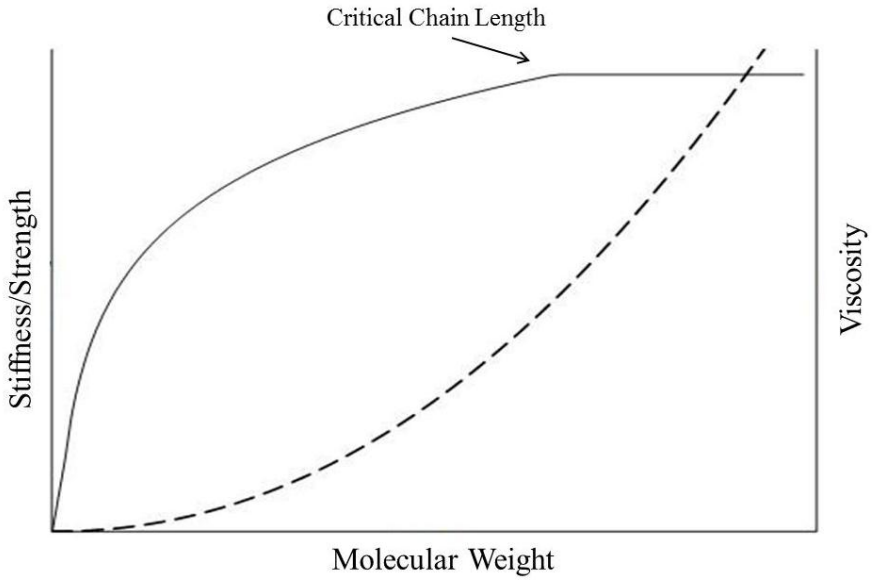


Figure 66: Influence of molecular weight on stiffness and viscosity [146].

Different additives can be added to plastics to improve the physical properties or processing. These include processing additives (lubricants, blowing agents), stabilisers (antioxidant, heat and light stabilisers), performance enhancers (fibres, pigments, fillers) and plasticisers. Plasticisers can be mixed into a polymer to change its processing and mechanical properties. They reduce stiffness and glass transition temperature of a material by loosening the molecular structure making it

easier for the polymer chains to move. They are used to aid processing because they reduce the risk of thermal degradation [4, 146].

Polymer processing involves converting raw polymer compounds into products of a desired shape. The most common methods are extrusion and injection moulding. Other processing methods include fibre spinning, film blowing, calendaring, rotational moulding and compression moulding.

7.2.1 Extrusion

Extruders are the most common and important machines in the plastic industry. Most moulding processes (e.g. injection moulding, blow moulding) also use an extruder [149]. Extrusion is widely used because it is a continuous process with high production rates. Most products are extruded twice, first to make the polymer pellets and again to form the final product [147]. During extrusion enough pressure is generated to push the molten polymer through the die so that it acquires the shape of the die. The main components of an extruder are the screw or screws, the barrel and barrel heating and cooling (Figure 67) [149]. Processing variables include the feed material composition, screw speed, barrel temperature profile, feed rates and die size/shape. These variables can influence specific mechanical energy input (SME, energy input into extruder per unit mass), torque, pressure and residence time [7].

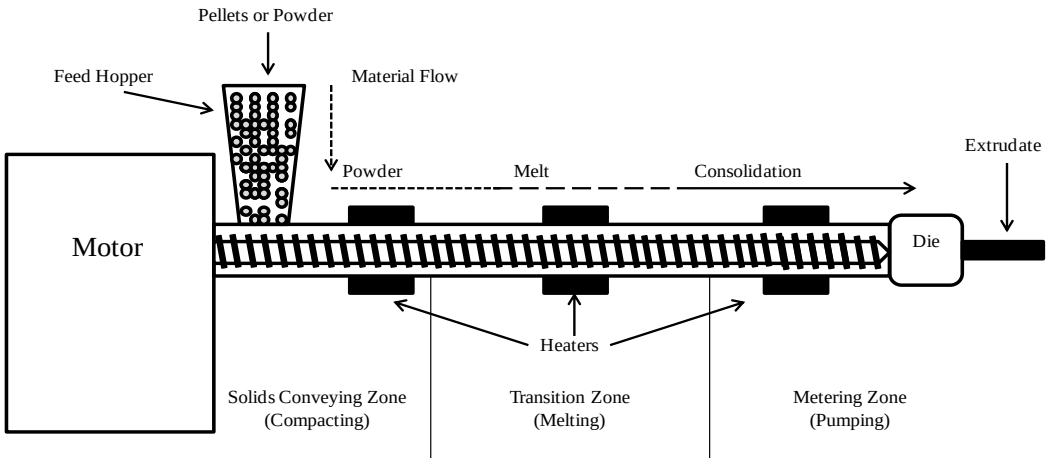


Figure 67: Main features of a single screw extruder [146, 149].

Extruders can have a single screw or twin screw configuration. Twin screw extruders can have intermeshing or non-intermeshing screws which can be co-rotating or counter rotating (Figure 68).

Single screw extruders contain a screw housed in a heated barrel. The screw is the most important feature and performance is directly related to it. The extruder acts as a volumetric pump with no positive conveying action of material. The material moves forward due to frictional differences between the screw and barrel where the screw friction is less than the barrel friction. Frictional differences and material movement can be improved by adding grooves to the barrel [146, 147].

During extrusion with twin screw extruders the majority of work is done in the screw channel and output is proportional to the screw channel volume (volume between screws). Unlike single screw extruders, twin screw extruders convey the material by positive displacement resulting in better conveying and mixing of the material. They also have better melting, faster pressure build up, shorter residence time and can be self-cleaning due to the stresses between the two screws. However twin screw extruders can be more expensive, have lower head pressures and require a metered feeder to prevent over feeding and excessive pressures.

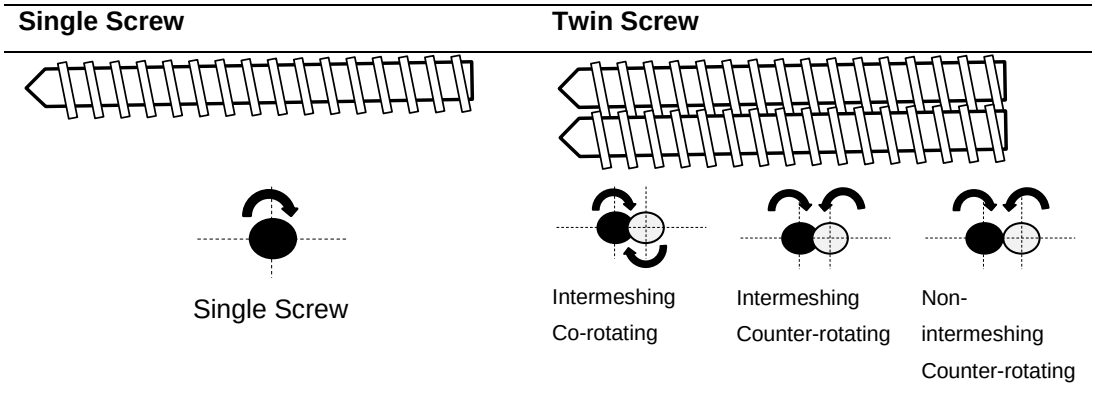


Figure 68: Single and twin screw extruder configuration [146, 147]

7.2.2 Injection Moulding

Injection moulding is another important and commonly used processing method used to manufacture plastic products into complex shapes with precise measurements. More than 30% of all thermoplastic materials are injection moulded. The main components are the plasticating unit, clamping unit and the mould (Figure 69) [146]. The plasticating unit is used to melt and inject the polymer. It is similar to a single screw extruder, except the screw is known as a

reciprocation screw because it is able to move backwards and forwards to allow accumulation and injection of the polymer melt. The clamping unit opens and closes the mould tightly to prevent flashing during filling. The mould distributes the melt and cools it into various shapes [146].

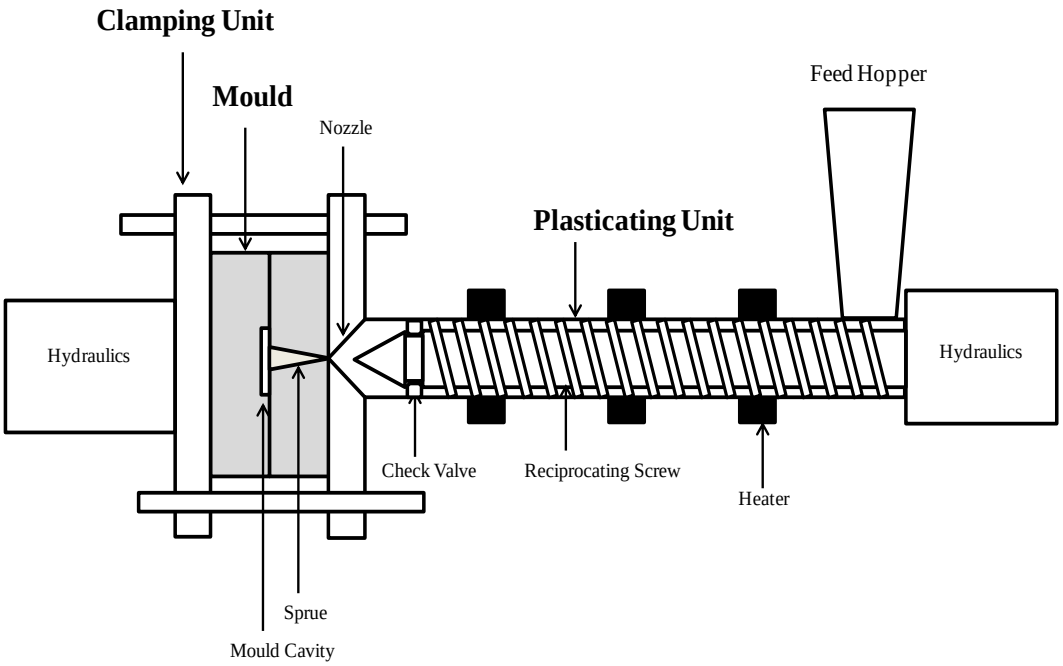


Figure 69: Basic layout of injection moulding components.

During injection moulding the polymer is melted in the plasticating unit, injected at high pressure into the mould and held at pressure while cooled. When cooled the mould is opened, the part ejected and the process repeated. This process is usually automated.

7.2.3 Compression Moulding

Compression moulding is often used to produce automotive parts and household goods [146]. Compression moulding is used for both thermoplastics and thermosets. During compression moulding the material and additives are placed in a mould and heated under pressure (Figure 70). This causes the material to melt and conform to the mould's shape. When processing thermoplastics using compression moulding they must be cooled under pressure which increases their processing time.

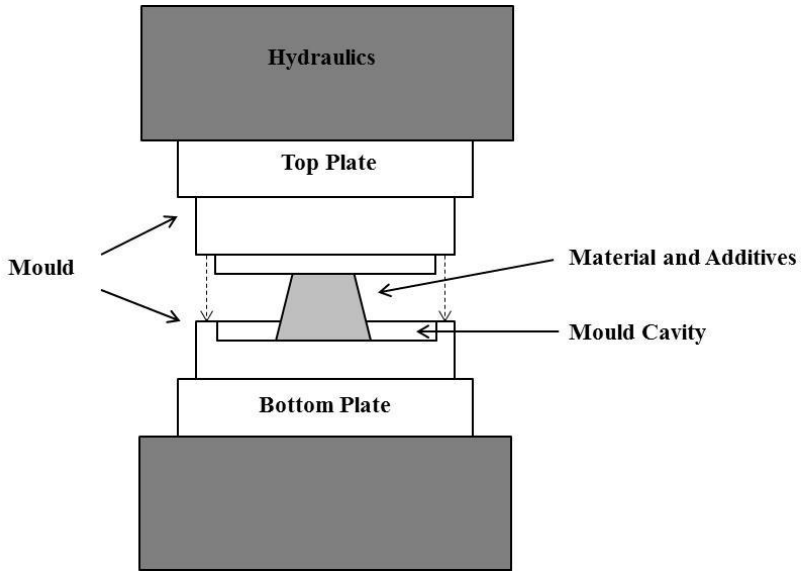


Figure 70: Compression moulding press.

7.2.4 Plastic Waste

Producing synthetic plastic feedstocks accounts for 4-5% of oil and gas production. Because plastics are durable, water resistant and not susceptible to bacterial degradation they can persist in the environment for hundreds of years once discarded. Approximately 200 million tonnes of plastic are consumed worldwide per year [1]. Approximately 30-45% of the plastic discarded worldwide is plastic used for packaging or other short life span products [2-4]. In New Zealand 252 thousand tonnes of plastic waste is disposed to landfills each year [5]. Another problem is plastic litter accumulating on land and in the sea where it is hazardous to animals [4].

Incineration and recycling are methods used to reduce plastic accumulation. However these methods both have disadvantages. Incineration can potentially release toxic pollutants and does not remove dependence on non-renewable petrochemical resources [4, 150]. Recycling is difficult because it is labour (collecting, sorting) and energy intensive. Composites and laminates can also make some types of plastics difficult to recycle, in addition material properties also deteriorate after with each cycle of recycling [4]. Sometimes recycling is not practical due to contamination or because of the plastic type [1, 2, 4].

As a result of increasing environmental and raw material supply concerns associated with petroleum based plastics, interest has increased in bioplastics

made from renewable resources. Bioplastics can potentially be used in a wide range of short life span applications such as loose fill packaging, packaging, disposable utensils, compost bags, disposable sporting equipment, controlled release systems and a variety of agricultural uses [151, 152]. Many countries are introducing initiatives and setting goals for future bioplastics use. Some estimates suggest that global bioplastics production capacity could reach 1.7 million tonnes by 2015, although this is still only a small percentage of all plastic production which is estimated to be up to 200-250 million tonnes.

Biodegradable bioplastics can be produced using two methods. The first is bacterial fermentation using energy sources (e.g. glucose) to produce intracellular polymers such as polyhydroxyalkanoates or monomers like polylactic acid which require further polymerisation [153-155]. Producing plastic using this method can be energy intensive and expensive [150, 156, 157]. However as technology improves energy use and costs may decrease [4]. The second method is direct conversion of naturally occurring biological polymers such as carbohydrates and proteins into bioplastics. The following section covers bioplastics produced using this method.

7.3 Bioplastics Produced from Biopolymers

Biopolymers such as carbohydrates and proteins are widely available, nontoxic, biodegradable and are usually sourced from agricultural operations making them available in large quantities at relatively low cost [158]. This makes them potential feedstocks for bioplastics. However they usually require plasticisers and other additives to aid processing [4]. Bioplastics are "biodegradable plastics whose components are derived entirely or almost entirely from renewable raw materials" [4].

Bioplastics have been produced using a range of different biopolymers (Figure 71). Various animal protein sources including egg whites, feather keratin, casein, whey, collagen and bloodmeal have been used [4, 142, 143, 159-161]. Also plant sources such as sunflower protein isolate, wheat gluten, soy protein isolate, zein, starch, corn gluten meal [8, 137, 142, 162-166]. In addition to this bioplastics from human blood plasma plasticised with water and glycerol have been produced [167].

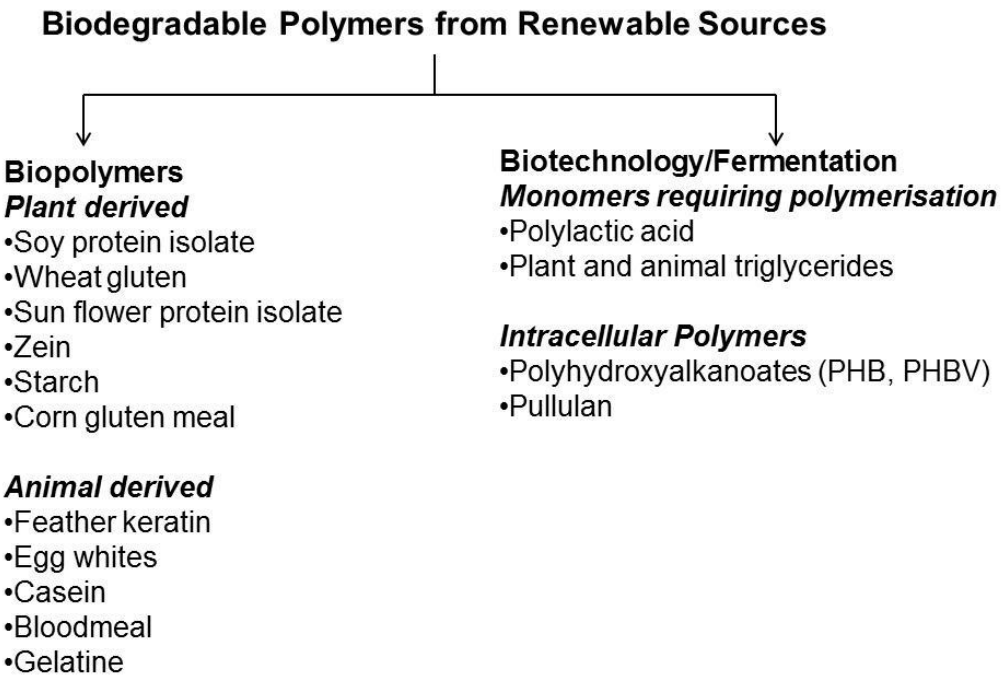


Figure 71: Examples of biodegradable polymers produced from renewable sources [4, 8].

The acceptance of bioplastic products by consumers is dependent on factors such as cost, appearance, colour, feel and smell [168]. Bioplastics can also have poor mechanical and permeability properties when compared to synthetic polymers [3, 4, 169]. However these can be improved by chemical modifications, coatings, blends and composite formation. Additives such as reactive amino acids and cross-linkers such as sodium silicate, borax, formaldehyde, glutaraldehyde have been used to increase molecular interactions [170]. Processing of bioplastics can also be difficult due to the large amount of interactions and their softening temperatures being higher than their degradation temperatures [171]. Another problem associated with bioplastics is that some feedstocks (corn, soy, wheat etc.) are also used in food applications and biofuel production. However using by-products such as bloodmeal could reduce reliance on petrochemical resources, while also avoiding competition for food and biofuel resources.

7.3.1 Bioplastic Processing and Additives

Solvent casting (wet), compression moulding, extrusion and injection moulding (thermoplastic) has been used to process bioplastics. Extrusion and injection moulding of bioplastics are most favourable and economically viable because they are already commonly used in the plastics industry [144, 172]. They also have relatively low processing temperatures and high production rates [168]. Twin

screw extruders have better mixing, conveying and shorter residence times so are often used for processing bioplastics because they are sometimes difficult to compound and have limited heat stability [150].

Proteins are heteropolymers made from up to 20 different amino acids in a specific sequence and structure of varying lengths. This means that there are a large number of different possible interactions between protein chains that can make processing difficult with only a small range of operating conditions available for successfully processing them into a bioplastic [144]. However, the range of amino acids (Table 36) and possible interactions can also be seen as an advantage as it means that proteins can be used to produce a range of unique materials.

Table 36: Amino acid profile of some proteins used to produce bioplastics.

	Egg White [173]	**Whey** [173]	**Casein** [173]	**Gelatine** [174]	**Soy** [173]	**Corn Gluten Meal** [166]	**Wheat Gluten** [175]	**Bloodmeal** [55]	**Bloodmeal** [56]
Non-polar									
Valine	7.00	6.00	6.60	2.20	5.00	4.60	4.10	9.32	7.08
Leucine	8.50	9.50	9.00	3.30	8.10	14.50	6.80	12.97	11.42
Isoleucine	6.00	6.00	5.10	1.50	4.80	3.50	4.00	0.91	3.19
Phenylalanine	6.00	2.30	5.10	2.40	5.20	4.40	4.90	7.34	6.20
Methionine	3.60	1.90	2.70	0.70	1.30	2.20	1.80	1.01	1.10
Tryptophan	1.40	2.20	1.30	-	1.30	0.30	1.00	-	1.22
Alanine	6.60	5.20	2.90	8.90	4.20	11.50	2.40	7.94	7.69
Proline	3.80	6.60	10.70	24.30	5.10	9.60	13.70	4.12	4.62
Cysteine	2.50	2.20	0.30	-	1.30	1.70	2.10	1.08	1.24
Glycine	3.60	2.20	2.10	21.40	4.10	4.10	3.10	4.06	4.46
Polar									
Serine	7.30	5.40	5.60	3.60	5.20	5.50	5.20	4.03	4.08
Threonine	4.40	6.90	4.30	2.10	3.80	3.80	2.50	4.47	3.49
Tyrosine	2.70	2.70	5.60	0.50	3.80	3.40	3.80	2.80	2.34
Acidic Residues									
Glutamic acid	13.50	16.80	21.50	10.00	19.00	20.30	37.30	9.08	8.79
Aspartic acid	8.90	10.90	6.60	6.00	11.50	5.50	2.90	10.42	7.17
Basic Residues									
Lysine	6.20	8.80	3.80	4.50	6.20	1.40	1.20	10.23	7.85
Arginine	5.60	2.50	3.70	7.80	7.50	2.20	2.40	4.23	4.18
Histidine	2.20	2.00	3.00	0.80	2.60	1.50	2.20	6.13	6.53

Consolidation of a biopolymer into a bioplastic is the detangling and flow of the original biopolymer allowing the reformation of new stabilising interactions between the biopolymer chains and the subsequent formation of a new homogenous bioplastic material. During thermoplastic processing several steps are required to form a consolidated protein network [144]. These are:

1. Disruption of stabilising intermolecular bonds and intermolecular interactions using additives and plasticisers.
2. Heating above the glass transition temperature allowing increased molecular movement.
3. Applying mechanical stress to aid molecular rearrangement and form a homogenous blend.
4. Formation of consolidated network where new stabilising bonds and interactions are formed.

In general processing requires the formation of a melt where the material flows and is able to be shaped. Large amounts of interactions between protein chains often mean the temperature where this occurs is above the proteins degradation temperature. Processing at high temperatures can also lead to excessive cross-link formation and poor quality extrudates [142, 176]. Disruption of bonds and interactions are usually achieved by using additives and plasticisers (Figure 72) [144]. This lowers the glass transition temperature and allows the proteins to be processed.

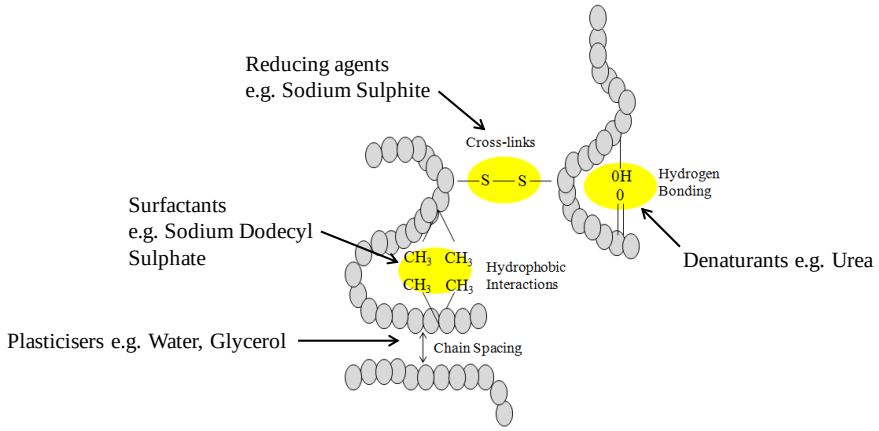


Figure 72: Protein interactions and action of additives.

Plasticisers act as lubricants by increasing chain mobility and lowering the glass transition temperature (Figure 73) [144]. Their hydrophilic parts interact with the polymer and reduce polymer-polymer interactions [177]. Plasticisers can also affect the mechanical properties of the material usually causing a decrease in resistance and stiffness but an increase in flexibility and maximum elongation [152]. They are usually used at concentration ranging from 10-50 % (w/w) [152]. Common plasticisers for bioplastics include water, glycerol, sorbitol, polyethylene glycol, sucrose, glucose, fructose, diethylene glycol and triethylene glycol. More effective plasticisers will cause a greater reduction in the glass transition temperature [152, 178]. A summary of common plasticisers used for bioplastics and their properties are given in Table 37.

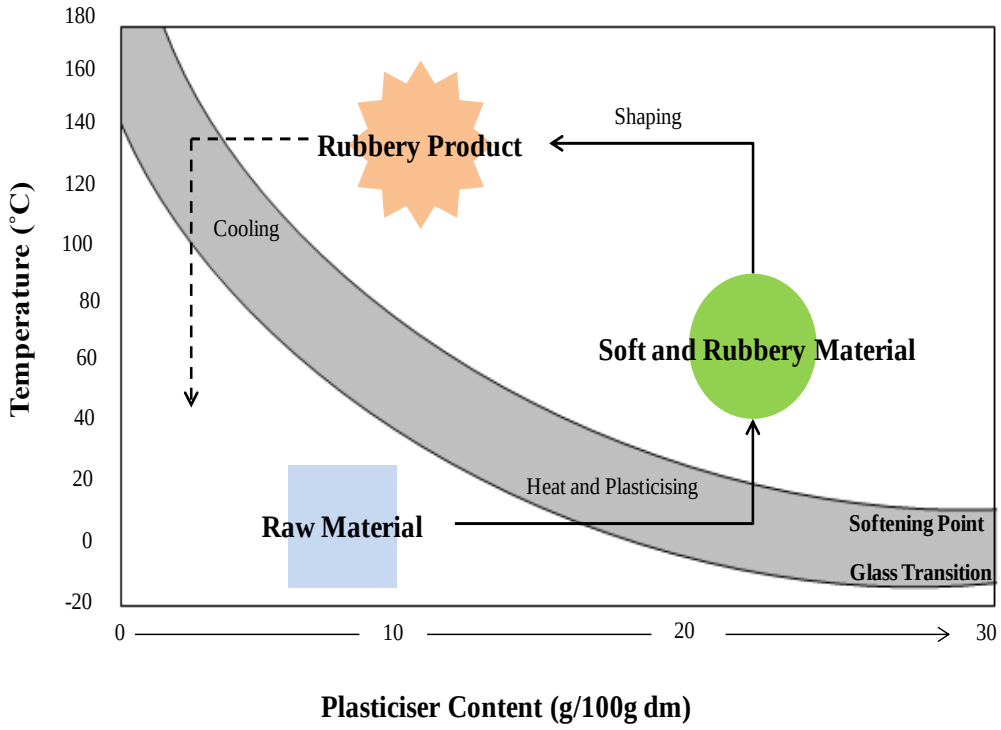


Figure 73: Schematic representation of the thermoplastic process applied to processing biopolymers in relation to glass transition and plasticiser content [179].

Plasticisers work by inserting between the polymer chains, acting as a molecular lubricant and increasing free volume [166]. Small molecules such as water and glycerol are more effective as plasticisers and often used in different processing techniques such as film blowing, extrusion, compression moulding and casting. Lower molecular mass molecules such as water and glycerol will be more effective when compared on a mass basis because each plasticiser molecule can interact with a protein chain. If a plasticiser has a lower molecular mass then there

will be more plasticiser molecules increasing plasticisation efficiency. Hydrophilic plasticisers will interact with polar amino acids while amphiphilic plasticisers will interact with hydrophobic amino acids [180].

Table 37: Characteristics of some commonly used plasticisers [142, 166]

Plasticiser	Molecular Mass	Hydrogen Bonds	Hydrophilic Groups (%)
Water	18	4	100
Sorbitol	182	18	56
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	31.80
Dibutyl Tartate	262	14	37.4
Dibutyl Phthalate	278	4	23.5
Octanic Acid	144	5	22.9
Palmitic Acid	256	5	12.9

The presence of hydrophobic interactions and cross-links prohibits processing due to reduced chain mobility. Sodium sulphite is a reducing agent used to break disulphide bonds when processing various biopolymers [9, 17, 143]. Surfactants such as sodium dodecyl sulphate have been used to denature and dissociate proteins and aid processing [178, 181]. Sodium dodecyl sulphate contains a hydrophobic tail and a negatively charged head. The tail interacts with a protein's hydrophobic regions and the head with water. This causes the protein to become detangled and increases its solubility. Urea has also been used to denature and process proteins as it prevents protein interactions by forming hydrogen bonds with amino acids [182]. Urea can be used as a plasticiser but it is not as effective as polyol plasticisers [7]. Urea can also migrate out of the bioplastic when the bioplastic is stored for long periods of time. The processing of biopolymers is summarised in Figure 74.

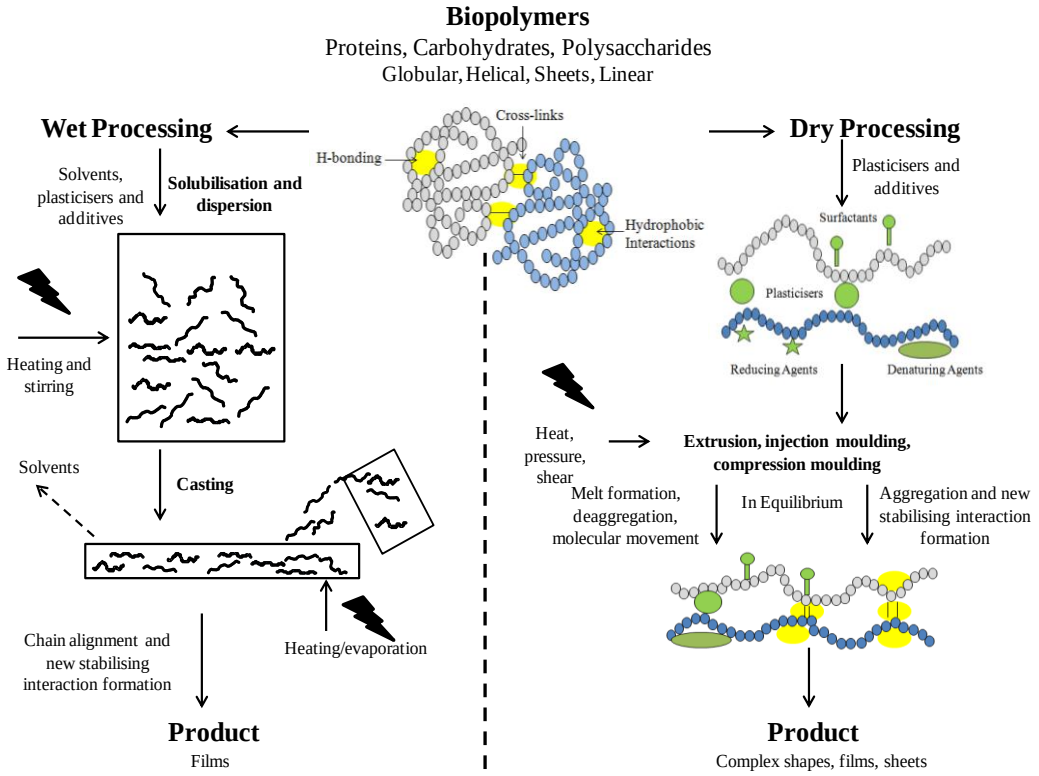


Figure 74: Summary of processing methods for direct conversion of biopolymers into film, sheet and complex shaped bioplastics.

7.3.2 Previous Studies on Processing of Biopolymers

Many studies have investigated processing biopolymers from agricultural sources into bioplastics [8]. Corn, wheat, sunflower protein isolate, soy protein isolate, casein, agar, feathers, egg white and bloodmeal are example biopolymers which have been studied [8, 9, 143, 159, 163, 183-185]. In addition one study was identified where human blood plasma was used to produce a bioplastic [167]. Selections of previous studies are outlined in this section and the mechanical properties of bioplastics produced from different biopolymer sources summarised in Table 38.

Jerez et al (2007) compression moulded egg white protein which was plasticised with glycerol at 60, 90 and 120°C under 0-12 kpsi of pressure. They compared this to wheat gluten plasticised with glycerol and concluded that egg white protein was easier to process and required less severe thermo-processing conditions. In addition they saw that at high pressures larger aggregates were formed, indicating that pressure can cause an increase in intermolecular cross-linking. They also suggested that compression moulding results could be used to help optimise other thermo-processing techniques such as extrusion [159].

Gonzalez-Gutierrez et al (2010) produced bioplastics from albumen blended with wheat gluten, potato starch and corn starch (0-30% w/w) with glycerol as the plasticiser (37.5% w/w). The material was compression moulded at 120°C, with 10 MPa pressure for 10 minutes. When compared to linear low density polyethylene the produced bioplastics had much lower tensile strengths and elongations. They also found that starch addition reduced mechanical properties of the protein bioplastics [160].

Lagrain et al (2010) reviewed the use of wheat gluten in bioplastics. They found that aggregation during thermo-processing occurs through inter and intra protein chain covalent cross-linking. They suggested that in addition to temperature, pressure and mechanical shear was important because they increased the molecular mobility of the protein chains and increased the amount of reactive sites available for cross-linking. Due to the large number of possible cross-linking reactions which will increase viscosity and restrict processing, they suggested that successfully extruding wheat gluten was only possible in a small window of processing conditions and at relatively low temperatures (80-130°C). Plasticisation with water, glycerol, polyols, oligosaccharides or lactic acid was recommended [6].

Redl et al (1999) extruded wheat gluten plasticised with glycerol using a co-rotating twin screw extruder with three heating zones which were varied between 60-140°C and screw speed between 50-200 rpm. The extruder was fitted with a converging die with diameters 19, 8 and 5 mm. They found that when the severity of the processing conditions were kept low (low rpm and temperature), extrudate surfaces were smooth. Increasing the severity of the processing conditions caused increased network formation due to covalent cross-linking. This resulted in reduced chain mobility and disrupted or poor quality extrudates [186].

Mo and Sun (2000) compression moulded (140°C, 20 MPa, 5 minutes) soy protein isolate which had been modified using SDS (0-10% w/w). They found that at up to 1% SDS, strength increased and suggested that the partially unfolded soy protein increased the amount of surface area for interactions between side groups as well as increased the amount of chain entanglements formed during thermo-processing which lead to improved strength. At high SDS levels (e.g. 10%) the

strength decreased and it was suggested that the high SDS levels disrupted the protein matrix and also reduced the interactions between the protein chains. They also found that Young's modulus decreased with increasing SDS concentration, therefore indicating that SDS was acting as a plasticiser. The plasticising effect of SDS was also observed in other studies [178].

Biopolymers sourced from agricultural rendering operations are attractive feedstocks for producing bioplastics because they are cheap, readily available in large quantities and do not compete with the human food and biofuel industries. Feathermeal and feather keratin are readily available low cost rendering products which are used as animal feed [143, 185]. Feathermeal is insoluble and stabilised by disulphide cross-links; therefore it must be processed under higher temperatures and pressures or with additives such as sodium sulphite to remove the cross-links. Sharma et al (2008) compression moulded hexane defatted feathermeal (150°C, 20 MPa, 5 minutes). They found that after processing, irreversible rearrangement of the protein occurred and suggested that heat and pressure could contribute to the formation of new structures during plastic processing [185].

The main problem with compression moulding is that it is not a continuous process and will have lower productivities when compared to extrusion. Barone et al (2006) extruded feather keratin (50-60% w/w) blended with water (8-18% w/w), sodium sulphite (2-6% w/w) and glycerol (10-30% w/w). Extrusion was carried out using a single screw extruder heated to between 100-140°C. The extruder was fitted with a 0.5 mm slit die. They found that increasing sodium sulphite concentration increased the amount of disulphide cross-links broken. They also found that processing with higher sodium sulphite concentrations resulted in increased crystallinity of the bioplastic and suggested this was due to breaking of the disulphide cross-links freeing the keratin chains and allowing them to rearrange during processing [143].

Bloodmeal is another rendering product which has been used to produce a bioplastic [7, 9, 17]. Verbeek and van den Berg (2011) extruded bloodmeal which had been blended with water, sodium sulphite, SDS and urea. Extrusion was carried out using a twin screw extruder with screw speed 150 rpm, 4 heating zones at 70, 100, 100, 100°C and a 10 mm die heated to 120°C. They found that extrusion was only possible when sodium sulphite was used to break disulphide cross-links. In addition, extrusion and consolidation was also dependent on SDS, but excessive amounts would restrict reformation of new stabilising interactions. Excessive amounts of urea also reduced mechanical properties as was expected for plasticisers. They found that a material could be produced with good consolidation and mechanical properties when 60 pph_{BM} water, 1 pph_{BM} sodium sulphite, 1 pph_{BM} SDS and 20 pph_{BM} urea were used. Subsequent studies revealed that mechanical properties could be modified by varying the levels of each additive. Addition of triethylene glycol (TEG) as a plasticiser also increased its flexibility. The optimum formulation was chosen to be 60 pph_{BM} water, 3 pph_{BM} sodium sulphite, 3 pph_{BM} SDS, 10 pph_{BM} urea and 10 pph_{BM} TEG [19].

In another example where blood proteins where used to produce bioplastics, Burgess et al (2008) produced bioplastic sheets from human blood plasma plasticised with water and glycerol by compression moulding at 9-25 kpsi and 55-150 °C. These sheets could be used for drug delivery, barrier membranes and scaffolds for tissue repair. The feed material is not available in large quantities and had a high value which makes it not applicable to lower value and high volume use applications such as single use packaging [167].

Treating bloodmeal with PAA reduced the amount of intermolecular interactions of bloodmeal (Chapter Six). Successfully extruding biopolymers requires equilibrium between removing these forces and reforming them to stabilise the new bioplastic structure. Therefore the amount of interactions between bloodmeal protein chains may need to be increased to aid processing. Cross-linkers and agents which increase intermolecular forces such as hydrogen bonding have been used to improve the mechanical properties of bioplastics and other products from biopolymers. Borax contains hydroxyl groups and can increase hydrogen bonding of polymers [187]. Coviello et al (2009) used borax, aluminium and iron to increase the molecular interactions of scleroglucan hydrogels and tablets. They

found that borax was able to increase hydrogen bonding and increased strength, whereas aluminium and iron decreased strength [187].

Sodium silicate can increase electrostatic interactions and hydrogen bonding between biopolymers [188, 189]. Lin and Gunasekaran (2010) produced an adhesive using cow blood and sodium silicate (0.5-2% v/v). They found that the adhesive had similar bonding strengths to conventionally used phenol formaldehyde in dry conditions. The presence of sodium silicate above 2% (v/v) improved water resistance but bonding in wet conditions was still weaker than when phenol formaldehyde was used [189]. Coradin et al (2003) studied the interactions between bovine serum albumin and sodium silicate. They found that at pH 4.7, the negatively charged sodium silicate caused aggregation of positively charged bovine serum albumin. They suggested that protein unfolding due to pH combined with the electrostatic effect of sodium silicate contributed to the aggregation of bovine serum albumin [190].

These studies showed that processing conditions such as temperature, pressure and additives varied greatly depending on what biopolymer was being processed into a bioplastic. Most studies showed water was required as a plasticiser, and it was often used in combination with another plasticiser such as glycerol. It was also found that extrusion was the most desirable process because it is continuous and will have higher productivity. However it was found to be a difficult processing method and extrudate quality varied greatly depending on specific mechanical energy input and additives used. It was suggested that compression moulding be used as a scouting method first then applying the best conditions to extrusion. No studies were found which attempted to process decoloured or chemically treated bloodmeal into a bioplastic.

Table 38: Examples of bioplastics made with various proteins, additives, processing methods and their properties [17, 19, 137, 143, 160, 162-165, 169, 181, 183, 191-196]

Protein	Plasticiser/Additives (wt %)	Processing Method	Tensile Strength (MPa)	Young's Modulus (MPa)	Elongation (%)
Agar [197]	Water (96), Glycerol (1.3)	Solvent Casting	29.7	-	45.3
Casein [198]	Glycerol (30), Aldehyde Cross-linkers (2)	Solvent Casting	49-52	1107-1391	-
Casein and Maillard Modified Casein [199]	Glycerol (20)	Solvent Casting	-	-	13.4-42.5
Whey Protein Isolate [200]	Glycerol (30)	Solvent Casting	8	275	20
Starch [165]	Glycerol (0-20)	Extrusion, Compression Moulding	-	710-1380	
Wheat Gluten [160]	Glycerol (37.5)	Compression Moulding	0.52	-	235
Wheat Gluten [172]	Glycerol (25)	Extrusion	2-5.1	4-26	85-165
Wheat Gluten + Corn Starch (20 wt %) [160]	Glycerol (37.5)	Compression Moulding	0.13	-	70.8
Wheat Gluten + Potato Starch (20 wt %) [160]	Glycerol (37.5)	Compression Moulding	0.32	-	163.5
Soy Protein Isolate + Corn Starch (50 wt %) [194]	Water, Glycerol (11.5 total)	Extrusion, Injection Moulding	2.9-3.9	28-46	69.4-94.7
Soy Protein Isolate + Polyester Amide (33 wt %) [196]	Glycerol (0-20), Sorbitol (0-20)	Extrusion, Injection Moulding	9-13	200-800	-

Protein	Plasticiser/Additives (wt %)	Processing Method	Tensile Strength (MPa)	Young's Modulus (MPa)	Elongation (%)
Soy Protein Isolate [181]	Sodium Dodecyl Sulphate (0,0.5, 1, 5, 10)	Compression Moulding	5.38-26.5	758-1667	1.4-2.4
Soy Protein Isolate [163]	Sodium Dodecyl Sulphate, Polycaprolacton-triol (various)	Solvent Casting	3-23	436-1718	1-6.9
Soy Protein Isolate [169]	Glycerol (0-30), Stearic Acid (0-30)	Solvent Casting	6.2-18.1	120.2-1121.7	3.6-168.4
Sunflower Protein Isolate [162]	Water (18), Glycerol (4-22)	Extrusion, Injection Moulding	10.6-16.1	500-2000	0.58-1.8
Sunflower Protein Isolate [193]	Water (10.5), Glycerol (37)	Film Extrusion	3.2	18	73
Corn Gluten Meal and Starch (25, 50, 75, 100 wt %) [184]	Glycerol (20)	Compression Moulding	2.5-3	100-250	20
Zein [192]	Sodium Dodecyl Sulphate (0-10)	Extrusion, Compression Moulding	17.3-22.4	186-299	11-13
Zein (various sources) [162]	Polyethylene glycol (25)	Compression Moulding	-	4.1-383	42-270
Zein [164]	Oleic Acid (30-50), Linoleic Acid (37.5-44.4)	Hot Press	2-9.4	136-557	5.9-46.9
Starch-Zein (0-100 wt %) [183]	Glycerol (22, 30, 40)	Compression Moulding	1.5-12	10-1150	2-66
Bloodmeal [17]	Water (32), Urea (11), Sodium Sulphite (1.6), Sodium Dodecyl Sulphite (1.6)	Extrusion, Injection Moulding	9.6	534.9	12.1

Protein	Plasticiser/Additives (wt %)	Processing Method	Tensile Strength (MPa)	Young's Modulus (MPa)	Elongation (%)
Bloodmeal	Water (33), Urea (11), Sodium Sulphite (0.5), Sodium Dodecyl Sulphite (0.5)	Extrusion, Injection Moulding	8	320	-
Bloodmeal [19]	Water (22.7), Urea (5.7), Sodium Sulphite (1.7), Sodium Dodecyl Sulphite (1.7), Triethylene Glycol (11.36)	Extrusion, Injection Moulding	5.7	49.4	81
Feather Keratin [143]	Glycerol (30), Water (8), Sodium Sulphite (2)	Extrusion	-	25	8-50
Feathermeal + Whey or Albumen (50 wt %) [185]	Water (25)	Compression Moulding	9-13	2000-3500	1.4-1.8
Gelatine [195]	Glycerol (30)	Compression Moulding	3.9	3.7	96.9
Human Blood Plasma [167]	Glycerol, Water	Extrusion, Compression Moulding	0.8	9	-
Egg White [159]	Glycerol (37.5)	Compression Moulding	7.1	-	130

7.4 Conclusion

Renewed attention in bioplastics emerged because of concerns with petroleum levels and pollution caused by synthetic polymers. Different methods for producing biodegradable bioplastics include biotechnology/fermentation and direct conversion of biopolymers into products. Direct conversion involves solvent casting and thermoplastic processing.

Due to the complex nature of biopolymers and their many different stabilising interactions, thermoplastic processing is only possible within a small window of processing conditions. Another problem is that the glass transition temperature is usually higher than the degradation temperature. To reduce the glass transition temperature and enable processing required the use of surfactants, reducing agents, surfactants and plasticisers to reduce interactions and increase chain mobility enabling the formation of a melt. The type and amount of additives required for processing is dependent on the type of biopolymer, its structure and amino acid content. After or during the end of processing new interactions are formed which stabilise the final structure.

For bioplastics to become economically viable they must be processed using common plastics processing methods such as extrusion. Successfully extruding bioplastics requires equilibrium between the loss of molecular interactions (enabling melt and flow) and the reformation of molecular interactions which stabilise the extrudate. High temperatures can lead to excessive stabilising interactions (e.g. cross-links) forming during extrusion causing the extruder to fail, poor quality extrudate or degradation. Whereas insufficient temperatures prevent melt formation.

When processing biopolymers using extrusion, there are a wide range of variables such as biopolymer-biopolymer interactions, biopolymer-additive interactions, temperature and shear. A balance between reducing interactions allowing melt/flow and the formation of new interactions which stabilise the product must also be achieved. Therefore successful extrusion is influenced by many factors and it is difficult to achieve and only possible within a small window of conditions.

Chapter 8

Processing Decoloured Bloodmeal Powders

Twin screw extruder at Waikato University.

8.1 Chapter Overview

Peracetic acid has been used to decolour and deodorise bloodmeal. This chapter investigated the use of decoloured bloodmeal powders for bioplastic production. The aim of this section was to identify the additives and processing conditions required to produce a consolidated bioplastic from PAA decoloured bloodmeal powders. This was achieved by investigating many combinations of water, plasticisers (TEG, glycerol), SDS, sodium sulphite, urea and additives which aid the formation of stabilising interactions (sodium chloride, sodium silicate and borax).

Initial extrusion trials using PAA, hydrogen peroxide and sodium chlorite treated bloodmeal were carried out using the standard formula (10 pph_{BM} urea, 3 pph_{BM} sodium sulphite, 3 pph_{BM} SDS, 60 pph_{BM} water, 10 pph_{BM} TEG) for processing untreated bloodmeal into Novatein Thermoplastic Protein (NTP). Hydrogen peroxide and sodium chlorite treated bloodmeal extrusions were unsuccessful and resulted in the extruder blocking, as well as the return of the bloodmeal smell. This result further justified the decision not to use these methods. PAA treated bloodmeal extrusion had limited success, the extruder did not block but instead the extrudate was small sections of compressed powder.

Subsequent extrusions revealed that if acetic acid was not neutralised the extrudate would darken possibly due to the acetic acid degrading the protein through deamidation. PAA treatment reduced stabilising interactions of bloodmeal and because successful extrusion required equilibrium between the removal of forces in the extruder barrel and the formation of new stabilising forces as the material exits the die it was initially thought that additives were required to help reform the stabilising interactions. However, when these were used extrudates were sections of compressed powders, indicating increased aggregation but no melt formation.

It was found that extrudate quality improved when only water, TEG and SDS was used. However the extrudates were still not completely consolidated and still contained sections of compressed powder. Extrusion was a difficult technique to use as a scoping method due to the wide range of variables (feed rate, barrel

temperatures, die size, screw speed) and a switch to compression moulding was made. Compression moulding achieved complete consolidation of PAA treated bloodmeal using 25 pph_{BM} water, 3 pph_{BM} SDS and 20 pph_{BM} TEG. This showed that it was possible to produce a consolidated bioplastic using PAA treated bloodmeal but high pressure was required.

A return to extrusion was made using 3, 4 and 5% PAA treated bloodmeal. In addition the extrudate was granulated and injection moulded to obtain the high pressures required. After injection moulding, consolidated and flexible samples were obtained and their colours were brown (3% PAA treated bloodmeal), yellow/orange (4% PAA treated bloodmeal) and yellow (5% PAA treated bloodmeal). Bioplastics produced using 3% PAA treated bloodmeal were not chosen to be investigated further because of its dark brown colour and bioplastics produced from 5% PAA treated bloodmeal were not investigated further because they broke apart easily when stretched. 4 % PAA treated bloodmeal was chosen to be optimised because it produced the best extruded and injection moulded samples.

8.2 Experimental

8.2.1 Aim

The aim of this section was to investigate the processing of decoloured and deodorised bloodmeal powders into a bioplastic using extrusion, compression moulding and injection moulding.

8.2.2 Methods and Materials

8.2.2.1 Standard Method for Producing Novatein ThermoPlastic Protein

Processing bloodmeal into Novatein ThermoPlastic Protein (NTP) requires water, urea, sodium sulphite and sodium dodecyl sulphate. Sodium sulphite is required to break covalent crosslinks. While urea and sodium dodecyl sulphate act as processing aids by denaturing the protein. After processing water content was reduced allowing new intermolecular interactions to form. The materials properties vary depending on the amount of additives used [9, 17, 19].

The standard method for producing NTP involves dissolving urea (10 pphBM), sodium sulphite (3 pphBM, sodium dodecyl sulphite (3 pphBM) in water (60 pphBM) and heating the solution to 60 °C while stirring. The hot solution is added to the bloodmeal in a high speed mixer and blended for 5 minutes. Triethylene glycol (10 pphBM) is then added and the mixture blended for a further 5 minutes. The pre-extrusion mixture (PNTP) is left for at least one hour to equilibrate. Extrusion is carried out using a twin screw extruder with temperature profile 70, 100, 100, 100, 120°C from the feed zone to the die. The extrudate is granulated and injection moulded with temperature profile 100, 100, 100, 115, 120°C from feed to exit. The mould was heated to 65 °C [7, 18]. The final material is black and has an offensive odour.

8.2.2.2 Producing Decoloured Bloodmeal Powders

Several decoloured bloodmeal powders were produced for processing trials by treating 100 g bloodmeal with 300 g of sodium chlorite, hydrogen peroxide or PAA solution (Table 39). After 5 minutes of continuous mixing, 300 g distilled water was then added to create a slurry which was then filtered. The treated bloodmeal was washed again with 200 g distilled water. It was frozen and freeze

dried overnight using a Labconco Freezone 2.5 freeze dryer to 5-8% moisture. They were then ground with a bench top grinder and sieved using a 700 μm sieve.

In some cases when using PAA, acetic acid was neutralised by submerging the filtered and treated bloodmeal in 300 g distilled water and adjusting to pH 7 by adding 1 mol/L sodium hydroxide solution. The slurry was then filtered, washed, dried, ground and sieved as normal.

Table 39: Decoloured bloodmeal powders used for extrusion, injection moulding and compression moulding.

Type of Treated Bloodmeal Powder
Sodium Chlorite (5%) treated bloodmeal
Hydrogen Peroxide (30%) treated bloodmeal
Peracetic Acid (3%) treated bloodmeal
Peracetic Acid (4%) treated bloodmeal
Peracetic Acid (5%) treated bloodmeal
Peracetic Acid (3%) treated bloodmeal (Acetic Acid Neutralised)
Peracetic Acid (4%) treated bloodmeal (Acetic Acid Neutralised)
Peracetic Acid (5%) treated bloodmeal (Acetic Acid Neutralised)

8.2.2.3 Extrusion

Decoloured bloodmeal powder was processed into pre-extrusion mixtures by dissolving various pre-weighed amounts of additives in different volumes of distilled water heated to 60°C while stirring (Table 40). The hot mixture was added to the decoloured bloodmeal powder and mixed in a high speed mixer for five minutes. Plasticiser (TEG or glycerol) was then added and the pre-extrusion mixture mixed for a further 5 minutes. The mixture was transferred to an air tight bag and left in the fridge at 2°C overnight to equilibrate.

Table 40: Additives used for processing.

Material	Supplier	Grade	Purpose	Amount (pph_{BM})
Sodium Dodecyl Sulphate (SDS)	Biolab	Technical	Surfactant	0-6
Sodium Sulphite	BDH Lab Supplies	Analytical	Remove cross-links	0-6
Urea	Agrinutrients-Balance	Agricultural	Denaturant	0-10
Distilled Water	-	-	Plasticiser, Dissolve Additives	0-60
Triethylene Glycol	BDH Lab Supplies	Analytical	Plasticiser	0-40
Glycerol	BDH Lab Supplies	Analytical	Plasticiser	0-45
Sodium Chloride	Ajax Finechem	Analytical	Increase Aggregation	0-10
Sodium Silicate	Ajax Finechem	Analytical	Increase Electrostatic Interactions	0-15
Borax	Ajax Finechem	Analytical	Increase Hydrogen Bonding	0-15

Extrusion was carried out using a ThermoPrism TSE-16-TC twin screw extruder fed with a rotating auger. The feed rate was adjusted to 30-35 g/min. Feed zone temperature was varied between 70-100°C and barrel temperature (zones 2, 3 and4) between 100-140°C. The exit die temperature was varied between 120-155°C. The screws were operated between 100-150 RPM and die size was either 5 mm or 10 mm in diameter (Figure 75).

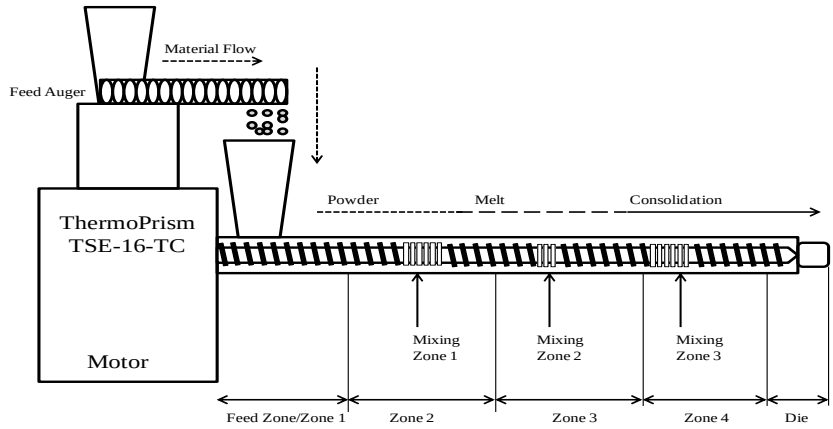


Figure 75: Extrusion screw configuration and heating zones.

8.2.2.4 Compression Moulding

3pph$_{BM}$ SDS was dissolved in 25 pph$_{BM}$ distilled water. The solution was heated and stirred to 60°C. The hot solution was added to 1-5% PAA decoloured bloodmeal (acetic acid neutralised) and mixed in a high speed mixer for 5 minutes. 20 pph$_{BM}$ TEG was added and mixed for further 5 minutes. These conditions were chosen after several initial compression moulding trials.

50 g of the mixture was compression moulded using a hydraulic press with top and bottom heated plates. The mixture was placed in the preheated mould (size 150 x 220 x 2 mm) and compression moulded at 110°C (top and bottom plate) under 2.2 MPa of pressure for 5 minutes. Heating was turned off after 5 minutes and the mould was left under pressure for a further 5 minutes. The pressure was released, the sheet removed and left to cool.

Compression moulded sheet colour was measured at multiple randomly selected locations using a Chroma Meter set in L*a*b* (CIE 1976) mode using D (6504K) illuminant conditions. L*a*b* values were converted to RGB and average percent whiteness calculated.

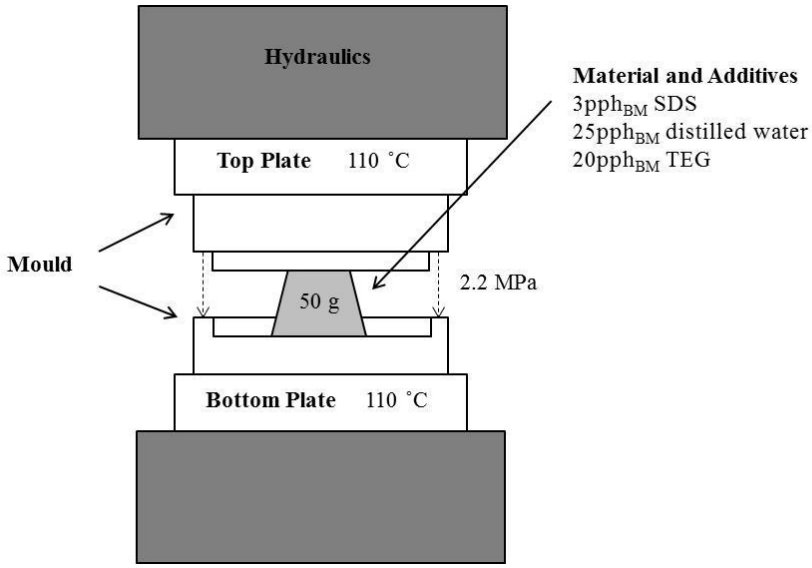


Figure 76: Compression moulding conditions.

8.2.2.5 Injection Moulding

After extrusion, some samples were granulated using a tri-blade granulator from Castin Machinery Manufacturer Ltd and injection moulded into tensile test specimens using a Boy 35A injection moulder. Injection pressure was 160 bar and back pressure 10 bar. The mould was water heated to 70°C and the barrel temperature profile from feed to exit was 100, 100, 100, 115, 120°C. The clamp pressure was 270 kN and the total cycle time was 45 seconds.

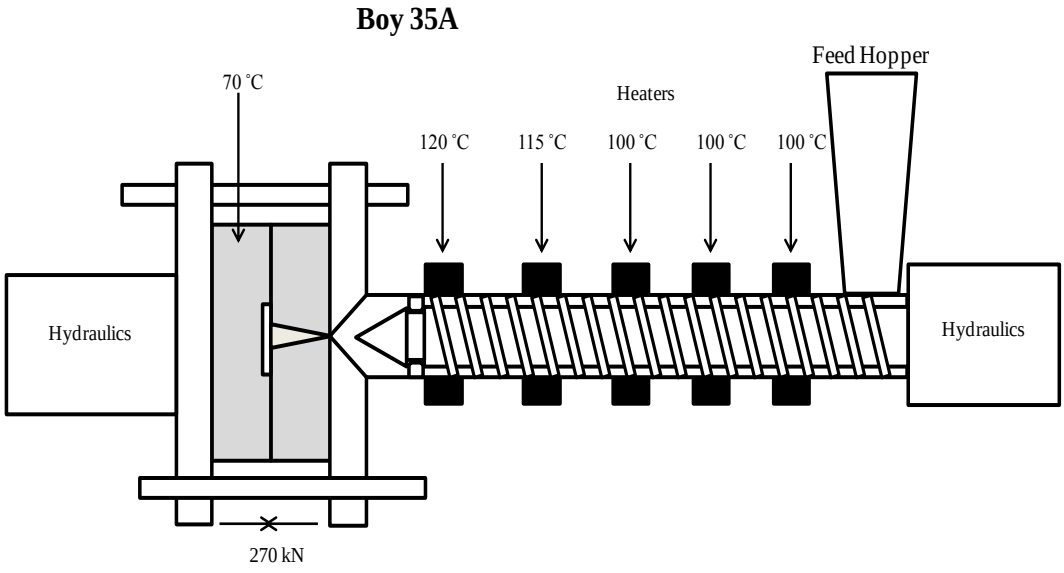


Figure 77: Injection moulder configuration and temperature settings.

8.3 Results and Discussion

8.3.1 Extrusions with Sodium Chlorite, Hydrogen Treated Bloodmeal

Sodium chlorite, hydrogen peroxide and PAA treated bloodmeal were extruded using the existing NTP (10 pph_{BM} urea, 3 pph_{BM} SDS, 3 pph_{BM} sodium sulphite, 60 pph_{BM} water, 10 pph_{BM} TEG) formulation. Initial attempts to extrude sodium chlorite and hydrogen peroxide decoloured bloodmeal resulted in excessive pressure and torque readings and the extruder blocking. This could have been caused by aggregates and disulphide cross-links formed during the decolouring treatment. These would not have been detected during gel chromatography because the sodium sulphite in the dissolving buffer would have removed them. No consolidation or melt formation was observed when the extruder was opened for cleaning (Figure 78). In future studies, problems with the extruder blocking could be avoided by using a mixing chamber to investigate processing prior to extrusion. However this piece of equipment was not available during this study.

When the pre-extrusion mixture for sodium chlorite and hydrogen peroxide treated bloodmeal was prepared the original bloodmeal odour returned when the denaturants were added. In addition a chlorine smell was also observed for sodium chlorite treated bloodmeal and a burnt hair smell for hydrogen peroxide treated bloodmeal. This justified the previous decision made in Chapter Five of not utilising these two treatment methods.

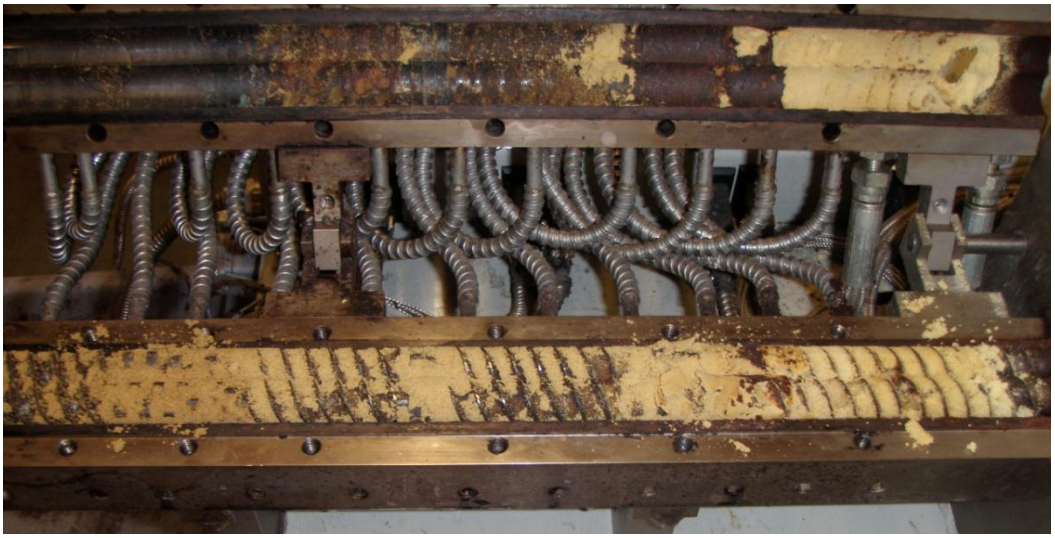


Figure 78: Failed attempt at extruding sodium chlorite treated BM.

8.3.2 Extrusions with Peracetic Acid Treated Bloodmeal

Initial attempts to extrude 3% PAA treated bloodmeal using the existing formulation for producing NTP were also unsuccessful. However the extruder did not block as was the case for sodium chlorite and hydrogen peroxide treated bloodmeal. Instead extrusions resulted in the extrudate exiting the extruder as either a powder or small sections of compressed powder. When pre-extrusion mixtures where being produced a strong acetic acid smell was observed but the characteristic bloodmeal smell did not return.

3% PAA treated bloodmeal was used because this was the lowest treatment strength which removed the smell from bloodmeal. Below this strength smell and colour removal was not sufficient. Choosing the lowest possible PAA treatment strength also meant that the processing behaviour of the decoloured powder could be as close as possible to the untreated bloodmeal making results easier to understand.

Extrusion trials with PAA treated bloodmeal resulted in the formation of four general types of extrudate as shown in Table 41. Additives, processing combinations and the resultant extrudate type are summarised in Table 42 and a full photo summary can be found in Appendix 1.

Table 41: Types of extrudate formed when extruding three percent PAA treated bloodmeal with different additives under various processing conditions.

Type 1. Powder/Compressed Powder
 
There was no melt formation and extrudate exited the extruder as powder or small sections of compressed powder. The sections of compressed powder had high amounts of surface defects and broke apart easily if bent or compressed.
Type 2. Dark Poorly Consolidated/Compressed Powder Extrudate
 
No melt formation and extrudate flowed poorly out of the extruder causing high pressures and torque. Extrudate was not flexible or rubbery and broke when bent. Surface defects such as voids, cracks and shark skinning were also present.
Type 3. Semi-Consolidated with Rough Surface and Powdery Sections
 
Extrudate flowed well out of the extruder with moderate pressures and torque. Extrudate was flexible and rubbery but had large sections with defects such as voids, cracks, compressed powder sections and shark skinning. They also broke easily when bent or compressed.
Type 4. Semi-Consolidated with Semi Smooth Surface
 
Extrudate flowed well out of extruder with moderate pressures and torque. Extrudate was flexible and rubbery and its surface was reasonably smooth. Some surface defects such as small cracks, powder and shark skinning were present.

Table 42: Summary of extrudates produced using various additive and processing combinations. Red=Poor, Yellow=Average, Green=Good.

	Additives (pph_{BM})												
	Additives to Decrease Interactions			Additives to Increase Interactions			Plasticisers			Processing Conditions			
Treatment Type	**Urea**	**SDS**	**Sodium Sulphite**	**Salt**	**Borax**	**Sodium Silicate**	**Water**	**TEG**	**Glycerol**	**Screw Speed (RPM)**	**Temperature Profile (feed to die °C)**	**Die Diameter (mm)**	**Extrudate Type**
Initial Trials													
Sodium Chlorite (5%)	10	3	3	-	-	-	60	10	-	150	70,100, 100, 100, 120	10	Blocked
Hydrogen Peroxide (30%)	10	3	3	-	-	-	60	10	-	150	70,100, 100, 100, 120	10	Blocked
PAA (3%) AA not Neutralised	10	3	3	-	-	-	60	10	-	150	70,100, 100, 100, 120	10	1
Extrusion Trials with Peracetic Acid Treated Bloodmeal (Acetic Acid Not Neutralised)													
PAA (3%)	10	3	3	-	-	-	60	10	-	150	80,110, 110, 110, 130	10	1
PAA (3%)	10	3	3	-	-	-	60	10	-	150	70,100, 100, 100, 120	5	1
PAA (3%)	10	3	3	-	-	-	60	10	-	150	80, 110, 110, 110, 130	5	1
PAA (3%)	10	3	3	-	-	-	60	10	-	150	90, 110, 110, 110, 120	5	1
PAA (3%)	10	3	3	-	-	-	60	10	-	150	90, 110, 115, 120, 120	5	2
PAA (3%)	10	6	3	-	-	-	60	10	-	150	70, 105, 110, 110, 120	5	1
PAA (3%)	10	6	3	-	-	-	60	10	-	150	70, 110, 110, 110, 130	5	2
PAA (3%)	10	6	3	-	-	-	60	10	-	125	70, 110, 110, 110, 130	5	2
Extrusion Trials with Peracetic Acid Treated Bloodmeal (Acetic Acid Neutralised)													
Standard Additives													
PAA (3%)	10	3	3	-	-	-	60	10	-	150	70, 100, 100, 100, 120	10	1
PAA (3%)	-	-	-	-	-	-	60	-	-	150	70, 100, 100, 100,120	5	1
PAA (3%)	10	-	-	-	-	-	60	-	-	150	70, 100, 100, 100, 120	5	1
Additives to Increase Aggregation, Electrostatic Interactions and Hydrogen Bonding													
PAA (3%)	10	-	-	10	-	-	60	-	-	150	70, 100, 100, 100, 120	5	1
PAA (3%)	10	-	-	10	-	-	60	-	-	150	95, 105, 105, 105, 120	5	1
PAA (3%)	10	-	-	-	-	20	60	-	-	150	80, 100, 100, 100, 120	10	2
PAA (3%)	-	-	-	-	5	-	60	10	-	150	70, 100, 105, 110, 130	10	2

Treatment Type	Urea	SDS	Sodium Sulphite	Salt	Borax	Sodium Silicate	Water	TEG	Glycerol	Screw Speed (RPM)	Temperature Profile (feed to die °C)	Die Diameter (mm)	Extrudate Type
Extrusion Trials with Peracetic Acid Treated Bloodmeal (Acetic Acid Neutralised) Continued													
Additives to Increase Aggregation, Electrostatic Interactions and Hydrogen Bonding Continued													
PAA (3%)	10	-	-	-	-	20	60	10	-	140	75, 100, 100, 100, 120	10	2
PAA (3%)	-	-	-	-	-	5	60	-	-	140	70, 100, 100, 100, 120	10	2
PAA (3%)	-	-	-	-	-	10	60	-	-	140	70, 100, 100, 100, 120	10	2
PAA (3%)	-	-	-	-	-	15	60	-	-	140	70, 100, 100, 100, 120	10	2
PAA (3%)	10	1.5	-	-	-	10	60	10	-	150	70, 100, 100, 100, 120	10	2
PAA (3%)	-	-	-	-	-	15	60	5	-	150	70, 100, 100, 100, 120	10	2
PAA (3%)	-	-	-	-	-	15	60	10	-	150	70, 100, 100, 100, 120	10	2
PAA (3%)	-	-	-	-	-	15	60	15	-	150	70, 100, 100, 100, 120	10	2
PAA (3%)	10	-	-	-	5	-	60	10	-	150	70, 105, 110, 110, 125	10	2
PAA (3%)	-	-	-	-	10	-	60	-	-	150	70, 100, 100, 100, 120	5	2
PAA (3%)	-	-	-	-	10	-	60	10	-	150	70, 105, 110, 110, 130	5	2
PAA (3%)	-	3	-	-	5	-	60	10	-	150	70, 105, 110, 110, 130	5	2
PAA (3%)	-	3	-	-	5	5	60	10	-	150	70, 100, 100, 100, 120	10	2
PAA (3%)	-	-	-	-	5	-	60	10	-	150	70, 100, 100, 100, 120	10	2
PAA (3%)	-	-	-	-	5	-	60	10	-	150	70, 100, 105, 110, 125	5	2
PAA (3%)	-	-	-	-	5	-	-	-	45	80	70, 100, 105, 110, 125	5	2
PAA (3%)	-	-	-	-	5	-	-	-	45	150	70, 100, 105, 110, 125	5	2
PAA (3%)	-	-	-	-	5	10	60	15	-	150	70, 100, 100, 100, 120	5	2
PAA (3%)	-	-	-	-	5	10	60	15	-	150	70, 100, 110, 115, 125	5	2
PAA (3%)	-	-	-	-	5	10	60	15	-	150	70, 100, 110, 120, 130	5	2
PAA (3%)	-	-	-	-	5	-	60	15	-	150	70, 100, 110, 120, 140	10	2
PAA (3%)	-	-	-	-	10	-	60	15	-	150	70, 100, 110, 130, 140	10	2
PAA (3%)	-	-	-	-	5	2.5	60	15	-	150	70, 100, 110, 130, 140	10	2
PAA (3%)	-	-	-	-	5	5	60	15	-	150	70, 100, 110, 130, 140	10	2
PAA (3%)	-	-	-	-	10	2.5	60	15	-	150	70, 100, 110, 130, 140	10	2
PAA (3%)	-	-	-	-	10	5	60	15	-	150	70, 100, 110, 130, 140	10	2
PAA (3%)	5	-	-	-	10	-	60	15	-	150	70, 100, 110, 130, 140	10	2
PAA (3%)	-	-	-	-	15	-	60	15	-	150	70, 100, 110, 130, 140	10	2

Treatment Type	Urea	SDS	Sodium Sulphite	Salt	Borax	Sodium Silicate	Water	TEG	Glycerol	Screw Speed (RPM)	Temperature Profile (feed to die °C)	Die Diameter (mm)	Extrudate Type
Extrusion Trials with Peracetic Acid Treated Bloodmeal (Acetic Acid Neutralised) Continued													
Urea and SDS													
PAA (3%)	10	3	-	-	-	-	30	30	-	150	80, 100, 100, 105, 125	10	3
PAA (3%)	10	3	-	-	-	-	40	30	-	150	70, 110, 115, 130, 140	10	2
PAA (3%)	10	3	-	-	-	-	30	30	-	150	80, 100, 120, 140, 155	5	2
SDS													
PAA (3%)	-	6	-	-	-	--	25	30	-	100	80, 100, 100, 105, 130	10	3
PAA (3%)	-	6	-	-	-	-	25	-	-	150	80, 100, 100, 105, 125	10	3
PAA (3%)	-	6	-	-	-	-	25	-	-	100	80, 100, 100, 105, 125	10	3
PAA (3%)	-	6	-	-	-	-	25	15	-	150	80, 100, 100, 105, 125	10	3
PAA (3%)	-	6	-	-	-	-	40	30	-	150	70, 100, 100, 100, 120	10	3
PAA (3%)	-	4.5	-	-	-	-	20	30	-	150	80, 100, 100, 105, 125	10	3
PAA (3%)	-	3	-	-	-	-	30	30	-	150	80, 100, 100, 105, 125	5	3
PAA (3%)	-	6	-	-	-	-	20	30	-	150	80, 100, 100, 105, 125	10	3
PAA (3%)	-	6	-	-	-	-	30	25	-	100	80, 100, 100, 105, 125	5	3
PAA (3%)	-	6	-	-	-	-	30	25	-	75	80, 100, 100, 105, 125	5	3
PAA (3%)	-	6	-	-	-	-	25	30	-	120	80, 100, 100, 110, 125	5	4
PAA (3%)	-	6	-	-	-	-	25	30	-	150	80, 100, 100, 100, 120	10	4
PAA (3%)	-	6	-	-	-	-	50	40	-	125	80, 100, 100, 110, 120	10	4
PAA (4%)	-	3	-	-	-	-	50	30	-	150	80, 100, 100, 110, 125	10	4
PAA (4%)	-	6	-	-	-	-	25	30	-	150	80, 100, 100, 110, 125	10	4
PAA (4%)	-	6	-	-	-	-	50	40	-	150	80, 100, 100, 110, 125	10	4
PAA (5%)	-	6	-	-	-	-	50	20	-	150	80, 100, 100, 115, 135	5	3

8.3.3 Extruding Peracetic Acid Treated Bloodmeal

8.3.3.1 Extrusions without Neutralising Acetic Acid

PAA treated bloodmeal (acetic acid not neutralised) was extruded using the NTP formulation. These extrusions resulted in compressed powders and no melt formation (type one). A strong acetic acid smell was also observed during mixing and extrusion. It appeared that a higher temperature was required to form a melt, but when this was done the extrudate darkened and material clumped in the feed zone, most probably due to water evaporating in the barrel because of the high temperatures and accumulating there. Previous studies have shown that successful extrusion requires equilibrium between the loss of stabilising interactions which allows the material to flow and entangle during extrusion and the reformation of stabilising interactions as the extrudate exits the extruder which leads to consolidation. Literature has shown that acetic acid can denature and degrade proteins through deamidation [139, 201]. This could explain why no consolidation occurs and the extrudate darkens when PAA treated bloodmeal is extruded without neutralising the acetic acid. If it was not neutralised it could have caused excessive denaturation and prevented the formation of the stabilising forces required for consolidation. It could have also caused degradation which resulted in the dark coloured extrudate.

The presence of urea, sodium sulphite and SDS may not be necessary because PAA treatment has already reduced some of the interactions. The reduction of interactions by PAA treatment combined with the presence of acetic acid and the other denaturants may also be preventing the reformation of chain interactions during extrusion. When acetic acid was neutralised and the temperature increased no darkening occurred which confirmed that acetic acid was responsible for the dark extrudate. It was decided that acetic acid should be neutralised before extrusion to prevent excessive denaturation and degradation. It was also decided to carry out extrusion trials with additives which increase interactions as well as less/different combinations of urea, sodium sulphite, SDS, water and TEG.

8.3.3.2 Extrusions with Additives to Increase Interactions

It appeared that PAA treated bloodmeal required more interactions to stabilise the extrudate and help aid consolidation. This could be achieved by removing denaturants or adding additives which increased interactions. Extrusion with just water and no denaturants resulted in compressed powder extrudates (type one). Sodium chloride, sodium silicate and borax have been used to increase aggregation, cross-linking and hydrogen bonds in proteins [187-190, 202, 203]. These were added to try and improve consolidation.

Sodium chloride has been used to increase protein aggregation. When it was added to extrusion mixtures it produced type one extrudates which showed increased aggregation when exiting the extruder but no melt formation. Sodium silicate and borax have been used to increase electrostatic interactions and hydrogen bonding between biopolymers. When these were used in extrusion, type two and three extrudates were produced. Interactions were increased as shown by increased extrudate length, aggregation and semi-consolidation, but melt formation was poor and extrudate surfaces were rough. These extrusions often had high pressures and torque due to the increased interactions. They were also often dark which could be due to the high pressures and torques which have been shown to cause protein degradation [142, 176]. It was decided that denaturants were still required to reduce interactions but not at the levels or combinations that were required for untreated bloodmeal.

8.3.3.3 Extrusions with Urea, SDS, SS, TEG and Water

Using various combinations of water, urea, sodium sulphite, SDS and TEG resulted in type three extrudates (Table 42). The presence of sodium sulphite and urea resulted in non-continuous extrudates which showed some consolidation but had a lot of defects and broke apart easily. When sodium sulphite was excluded it was still possible to process the PAA decoloured powder and extrudate quality was reasonable (type four). Processing without urea was also possible and extrudate quality was reasonable (type four). Processing without SDS resulted in poor extrudates (type three). Insufficient water and TEG (below 40 pph_{BM} total) produced poor flow, high pressures and torques, as well as brittle extrudates with various surface defects (type three and type four). This suggested that successful

processing may be possible with just water, SDS and TEG if their concentrations and processing conditions could be optimised.

Extrusion had limited success and this was caused by the wide range of variables such as the die size, screw speed, feed rate and five temperature zones making it difficult to understand the processing requirements of the decoloured bloodmeal powders. A simpler method such as compression moulding with fewer variables could help to identify what conditions and additives were required for consolidation of PAA treated bloodmeal powders. These conditions and additives could then be applied to extrusion. Compression moulding was used with the aim of investigating what processing conditions and additives were required for consolidation of PAA decoloured bloodmeal powders. After these were found, they could then be applied to extrusion.

8.3.4 Compression Moulding Peracetic Acid Treated Bloodmeal

Compression moulding is a simpler technique with fewer variables and has been used previously to process bioplastics into sheets as previously described in Chapter Seven.

1-5% PAA treated bloodmeal was compression moulded using only water (25 pph_{BM}), SDS (3 pph_{BM}) and TEG (20 pph_{BM}). These additives were used for compression moulding as they gave the most promising results from extrusions. Using a smaller number of additives also reduced the amount of variables making the processing easier to understand.

Initial trials involved varying temperature and pressure until a temperature of 110°C (top and bottom plate) and 2.2 MPa pressure was selected. It was found that at temperatures above this the sheets would be brittle due to the evaporation of water and below this the material would not form a melt. Excessive pressures (above 3 MPa) would cause the material to flow out the sides of the mould. This was not good for compression moulding but was a good sign that melting and consolidation was possible during extrusion if high pressures could be achieved.

Compression moulding results showed that consolidation was possible for 3-5% PAA treated bloodmeal. Compression moulded sheets of 1 and 2% PAA treated

bloodmeal were slightly granular and brittle. This could possibly be caused by the remaining disulphide cross-links as the treatment strength may not have been high enough to break a significant number of them. These sheets were also dark brown to black in colour (Table 43, Figure 79-Figure 81).

The incomplete consolidation of sheets made from 1 and 2% PAA treated bloodmeal could also be caused by the absence of urea and sodium sulphite. Results from analysis carried out in Chapter Six showed that 1 and 2% PAA treated bloodmeal showed similar thermal stability, glass transition temperatures and solubility to untreated bloodmeal. This means that its processing requirements (i.e. additives) would also be similar to untreated bloodmeal. Therefore 1 and 2% PAA treated bloodmeal may also require some urea and sodium sulphite to aid processing and consolidation. However because colour and smell was not completely removed at these treatment concentrations, their processing was not investigated further.

Table 43: Colour of 1-5% PAA treated bloodmeal compression moulded sheets.

	L*	R	G	B	Whiteness (%)	Colour
Untreated BM (standard formula)	23	59	60	61	24	
1% PAA BM	26	62	62	62	24	
2% PAA BM	28	86	59	58	27	
3% PAA BM	57	182	123	43	45	
4% PAA BM	71	199	171	72	58	
5% PAA BM	72	203	173	99	62	

Compression moulded sheets from 3-5% PAA treated bloodmeal produced flexible, consolidated and translucent sheets (Figure 79, Figure 80). Results from Chapter Six showed that treatment above 3% PAA concentration caused the decoloured powder to begin to act similarly to PNTP. This could explain why it is possible to compression mould these powders without the use of urea and sodium sulphite.

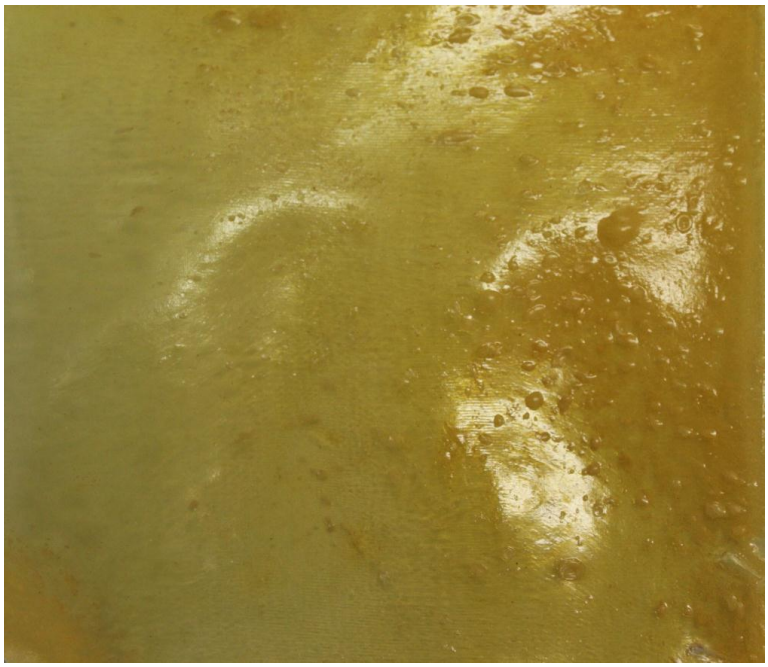


Figure 79: Compression moulded sheet produced using 4% PAA treated bloodmeal.

Figure 80: Compression moulded 3 % PAA treated bloodmeal cut into dog bone tensile bar samples.

One of the problems identified with compression moulding was the presence of bubbles in some of the sheets and samples (Figure 80). This could have been caused the water evaporating (due to insufficient cooling) when the pressure was released. If compression moulding were to be used to produce these sheets for use or applications then the method should be adjusted to allow sufficient time for the moulds to cool below 100°C while still under pressure to prevent the evaporation of water.

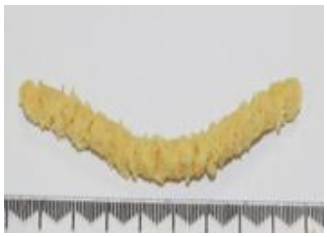
Figure 81: Compression moulded 1 % PAA treated bloodmeal cut into dog bone tensile bar samples.

Compression moulding showed that processing PAA decoloured bloodmeal into a bioplastic was possible using water (25 pph_{BM}), SDS (3 pph_{BM}) and TEG (20 pph_{BM}). Observations from compression moulding showed that at 110°C and high pressures the decoloured powders formed a melt and squirted out the edges of the mould as consolidated ribbons. This showed that processing temperatures around 110-120°C were sufficient but high pressures were required. From these results it was decided to return to extrusion but follow with injection moulding to achieve the high pressures required. The water and TEG levels were increased to improve material flow and help prevent degradation due to the increased shear rates of these processing methods.

8.3.5 Extruding and Injection Moulding Peracetic Acid Treated Bloodmeal

Extruding 3 and 4% PAA treated bloodmeal with water (40-50 pph_{BM}), SDS (3pph_{BM}) and TEG (20-40 pph_{BM}) produced type four extrudates. Extrudates flowed well out of the extruder and excessive pressures, torques or blocking did not occur. Insufficient TEG resulted in shark skinning on the extrudate surface. Extruding 5% PAA treated bloodmeal with the same additives resulted in a semi-consolidated extrudate with a rough and powdery surface. The extrudate was rubbery and could be stretched but broke apart easily. This could possibly be due to very little remaining interactions available to stabilise the extrudate (Table 44). The extrudates were then granulated and injection moulded.

Table 44: Summary of extruding and injection moulding 3-5 % PAA treated bloodmeal.

Extrusion and Injection Moulding of Peracetic Acid Treated Bloodemal		
3% PAA Treated BM Extrusion and Injection Moulding		
Type 4		
Formulation (pph_{bm}) 40 water, 30 TEG, 6 SDS,		
Type 4		
Formulation (pph_{bm}) 50 water, 40 TEG, 6 sds		
4 % PAA Treated BM Extrusion and Injection Moulding		
Type 4		
Formulation (pph_{bm}) 50 water, 40 TEG, 6 sds		
Type 3		
Formulation (pph_{bm}) 50 water, 20 TEG, 6 sds		
5 % PAA Treated BM Extrusion and Injection Moulding		
Type 3		
Formulation (pph_{bm}) 50 water, 20 TEG, 6 sds		

Note: Extrusion conditions were, temperature profile (feed to exit °C): 70, 100, 100, 100, 120, screw speed: 150rpm.

Injection moulding 3% PAA treated bloodmeal required sufficient levels of water and TEG (above 60 pph_{BM} total) to prevent blocking the injection moulder components and protein degradation (see full results in Appendix 1). When these conditions were met a brown, flexible and consolidated sample was produced. Injection moulding 5% PAA treated bloodmeal produced a consolidated translucent sample. However this sample was very flexible and easy to break, possibly due to very little stabilising interactions. 4% PAA treated bloodmeal injection moulded well and produced an orange, translucent sample which appeared to have acceptable mechanical properties.

Because 4% PAA treated bloodmeal performed the best when extruded and injection moulded it was chosen to be investigated further. Additive levels and processing should be optimised using this material. The mechanical properties of the material should also be investigated.

8.4 Conclusion

Successful extrusion without neutralising acetic acid could not be achieved because of its denaturing and degradation effects on bloodmeal protein. Additives to increase chain interactions were unsuccessful and some caused the extrudate to darken, possibly due to increased interactions causing an increase in shear stresses which caused the protein to degrade. Excluding urea and sodium sulphite produced better quality extrudates, although these still showed poor consolidation and a rough surface. Successful extrusion required equilibrium between removing interactions and reforming them to stabilise the structure. Peracetic acid treatment reduced interactions as shown in Chapter Six. Therefore including urea and sodium sulphite further reduced interactions and prevented the reformation of enough stabilising interactions during extrusion.

Extrusion was a difficult scouting technique to use due to its wide range of parameters and limited success was achieved. As a result, compression moulding was utilised to investigate if consolidation was possible using decoloured bloodmeal powders. Compression moulding with water, SDS and TEG showed consolidation was possible using all PAA treatment strengths without urea and sodium sulphite. This showed that a higher pressure was required during processing. Therefore it was decided to extrude 3, 4, and 5% PAA treated bloodmeal, granulate the extrudate and injection mould them.

After returning to extrusion with water, SDS and TEG, better quality extrudates were obtained for 3 and 4% PAA treated bloodmeal. 5% PAA treated bloodmeal produced a poor extrudate with a powdery surface, possibly because of insufficient chain interactions caused by the strong decolouring treatment. Successfully injection moulding 3% PAA treated bloodmeal required at least 60 pph_{BM} total of water and TEG to enable chain mobility, otherwise the injection moulder die would block and protein degradation would occur. When injection moulded, 3% PAA treated bloodmeal produced a brown sample, whereas 4 and 5% PAA treatment produced orange/yellow translucent samples. The 5% PAA treated bloodmeal sample was very flexible and broke easily when stretched.

4% PAA treated bloodmeal was chosen to be optimised because it produced the best extruded and injection moulded samples. The work done in this chapter is summarised in Figure 82.

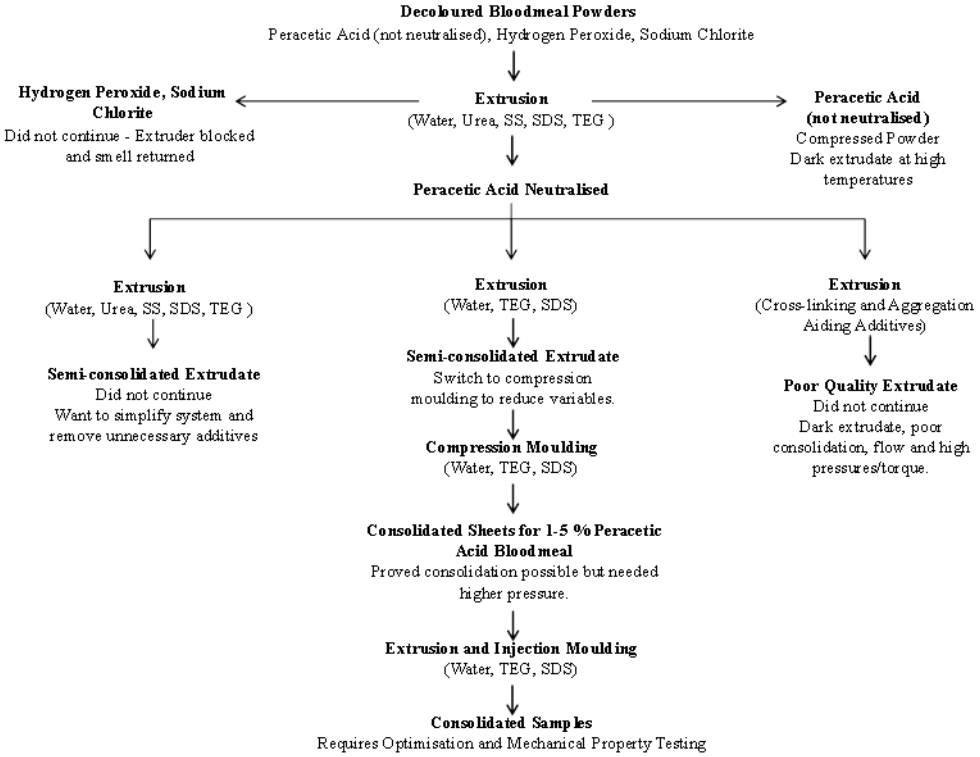


Figure 82: Summary of extruding, compression moulding and injection moulding decoloured bloodmeal powders done in Chapter Eight.

Chapter 9

Extrusion, Injection Moulding and Mechanical Properties of Decoloured Bloodmeal Bioplastics

Injection moulded samples produced using peracetic acid treated bloodmeal.

9.1 Chapter Overview

In Chapter Eight, 4% PAA treated bloodmeal was chosen to be optimised for extrusion and injection moulding. Water, TEG and SDS were identified as the main additives required for processing. This section aimed to find the optimum levels of water, TEG and SDS and to investigate how these additives affected extrusion, injection moulding and material properties.

It was found that the optimum combination of water, TEG and SDS differed for extrusion and injection moulding. SDS was the most important factor for whiteness, consolidation and specific mechanical energy (SME) input during extrusion. The best extruded sample based on consolidation and whiteness contained 40 pph_{BM} water, 10 TEG pph_{BM} and 6 pph_{BM} SDS. This produced a sample which was 98% consolidated and 49% white.

TEG and SDS were the most important factors for injection moulded sample colour, processing and material mechanical properties. Water also contributed to colour of injection moulded samples and mechanical properties but its contribution was reduced due to its evaporation during processing and conditioning. Increasing TEG resulted in decreased Young's modulus and UTS, but higher strain at break and toughness. The best injection moulded sample based on colour and mechanical properties was obtained using 3 pph_{BM} SDS, 50 pph_{BM} water and 20 pph_{BM} TEG. This produced a material with tensile strength 4.62 MPa, Young's modulus 85.48 MPa, toughness 1.75 MPa and 82.62% strain at break. These mechanical properties were comparable to Novatein Thermoplastic Protein produced from untreated bloodmeal.

9.2 Experimental

9.2.1 Aim

The aim of this section was to find the optimum levels of water, TEG and SDS for processing 4% PAA decoloured bloodmeal and investigate how these additives affected extrusion, injection moulding and mechanical properties.

9.2.2 Methods and Materials

9.2.2.1 Extrusion and Injection Moulding

Large quantities of bloodmeal were decoloured with 4% PAA and the acetic acid was neutralised as previously described in Chapter Eight. The decoloured bloodmeal powder was processed into different pre-extrusion mixtures at compositions shown in Table 45. SDS was dissolved in distilled water heated to 60°C while stirring. The hot mixture was added to the decoloured bloodmeal powder and mixed in a high speed mixer for 5 minutes. TEG was added and the pre-extrusion mixture mixed for a further 5 minutes. The mixture was transferred to an air tight bag and left in the fridge at 2°C overnight to equilibrate.

Table 45: Different combinations of water, SDS and TEG used to extrude four percent PAA treated bloodmeal.

		SDS (pph_{BM})								
		6			3			0		
		Water (pph_{BM})								
		30	40	50	30	40	50	30	40	50
TEG (pph_{BM})	10	S1	S2	S3	S10	S11	S12	S19	S20	S21
	20	S4	S5	S6	S13	S14	S15	S22	S23	S24
	30	S7	S8	S9	S16	S17	S18	S25	S26	S27

Extrusion was carried out using a ThermoPrism TSE-16-TC twin screw extruder fed with a rotating auger. The feed rate was adjusted to approximately 35 g/min. The extruder temperature profile was 80, 100, 100, 110, 125°C from the feed zone to the die. The screws were operated at 150 rpm and the die was 10 mm in diameter (Figure 83). Pressure, torque and mass flow rates were recorded during extrusion and used to calculate specific mechanical energy input using equation 10 [7].

$$SME\ \left(\frac{kJ}{kg}\right) = \frac{Torque \times Screw\ Speed}{Mass\ Flow\ Rate} \quad (10)$$

Multiple cross sections were cut for consolidation analysis. Injection moulding into tensile bar specimens was then carried out using a Boy 35A injection moulder with injection pressure 160 bar and back pressure 10 bar. The mould was water heated to 70°C and the barrel temperature profile from feed to exit was 100, 100, 100, 115, 120°C. The clamp force was 270 kN and the total cycle time was 45 seconds (Figure 77).

The colour of extruded and injection moulded samples were analysed using the same method as described for measuring the colour of compression moulded samples in Chapter Eight.

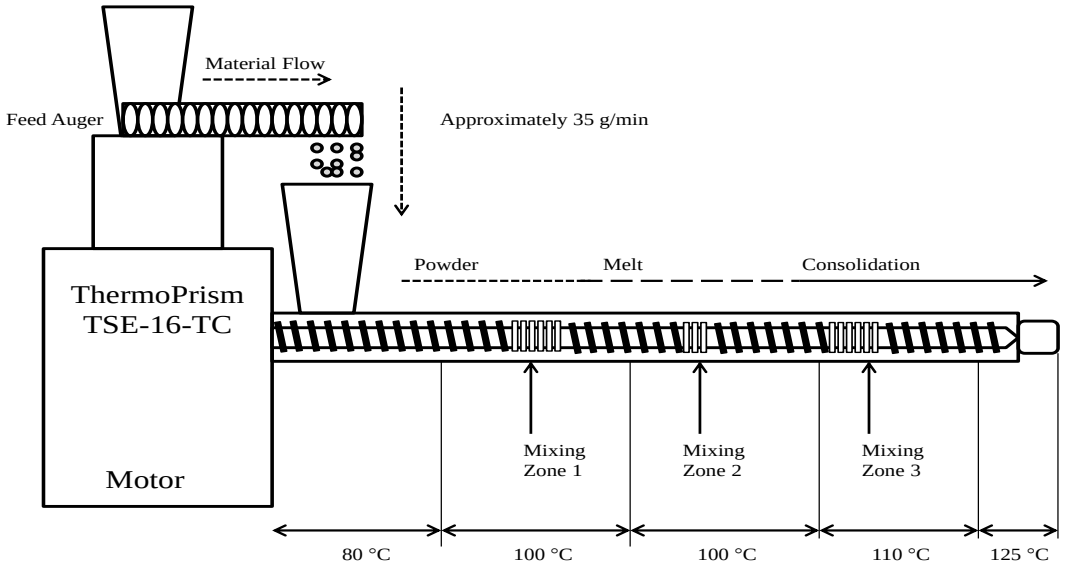
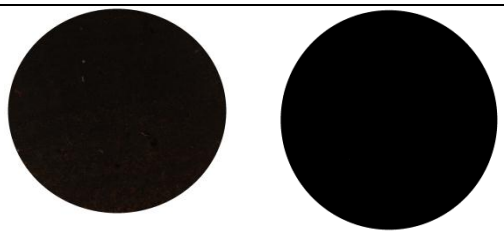
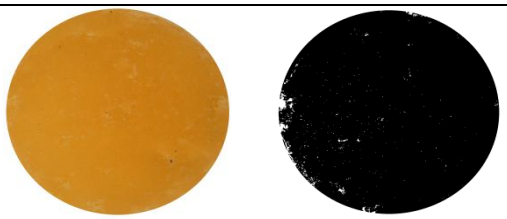
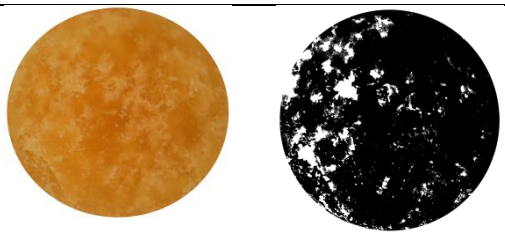
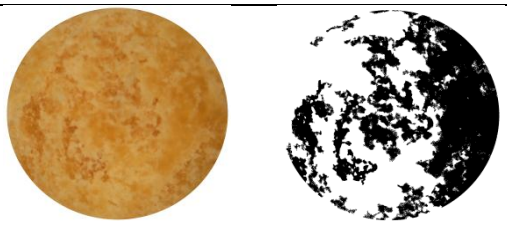


Figure 83: Extuder conditions used to process 4% PAA treated bloodmeal.

9.2.2.2 Measuring Consolidation

Extrudate cross sections were examined using a Heerbrugg Wild 38 microscope and photographed with a Nikon DS 5MC digital sight camera attached to the microscope. The images were converted into black and white using ImageJ image processing software. Consolidated regions appeared black and unconsolidated regions appeared white. Percent consolidation was calculated by dividing the black area by the total area. Example conversions are shown in Table 46.

Table 46: Example black and white cross sections showing complete, high, average and poorly consolidated samples.

Cross Sections Converted into Black and White Images	
Complete Consolidation (NTP 100%)	*High Consolidation (Above 95%)*
	
Average Consolidation (80-95%)	*Poor Consolidation (Less than 80%)*
	

9.2.2.3 Mechanical Testing

Injection moulded samples were conditioned to approximately 10% moisture at 23°C and 50% relative humidity for 7 days. The tensile strength, strain at break, Young's modulus and toughness of injection moulded samples were analysed according to ASTM D638-86 method using an Instron model 4204 fitted with a 5 kN load cell, extensometer with a gauge length 50 mm and with a cross head speed of 5 mm/min. Five samples were analysed for each additive combination and the results presented as averages. The standard error was calculated by dividing the standard deviation by the square root of the number of samples. The mechanical properties of unconditioned samples were also analysed and the results can be found in Appendix 2.

9.2.2.4 Percentage Contribution of Additives to Product Properties

The contribution of SDS, water and TEG to the average of SME, extrudate whiteness, consolidation, injection moulded sample whiteness, tensile strength, elongation at break, Young's modulus and toughness was quantified by carrying out analysis of variance using Minitab statistical processing software.

9.3 Results and Discussion

9.3.1 General Observations

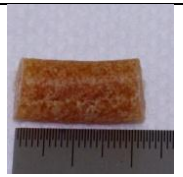
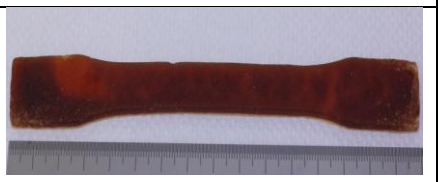
During extrusion several general observations were made. Samples with SDS showed better consolidation than samples without SDS. At high water and TEG content consolidation was reduced. In addition these samples also had difficulty feeding and water was also seen in the feed zone. The powders would clump and block in the feed zone leading to build up in the feed hopper. Water in the feed zone was probably due to water being evaporated in the extruder barrel.

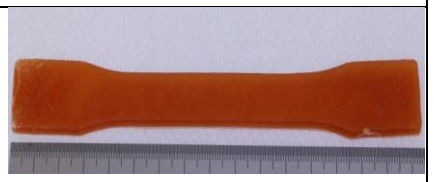
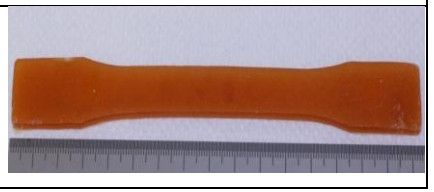
Water and TEG was observed to be the most important factors for injection moulding processing. Samples with low water and TEG content could not be injection moulded. The material became stuck in the mould and sprue or formed incomplete tensile test samples. Low water and TEG also resulted in darkening of the material once injection moulded which could indicate thermal degradation of the protein. These results are summarised and pictures of extruded and injection moulded samples presented in Table 47.

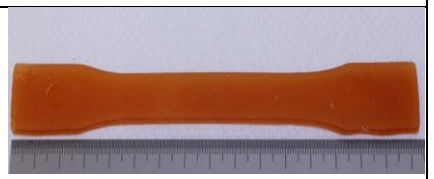
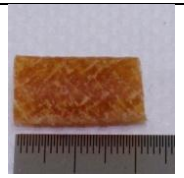
These observations suggested that SDS was the most important component for consolidation during extrusion, whereas injection moulding is more dependent on water and TEG. The primary role of SDS is to detangle the bloodmeal protein. During injection moulding the bloodmeal protein would have experience greater pressure than it would during extrusion. The high pressures of injection moulding could also have detangled the bloodmeal protein which could be why formulations without SDS were able to be consolidated with injection moulding but showed poor consolidation after extrusion. The higher shear stresses and mechanical energy input during injection moulding could be what makes the role of plasticisers more important as these have been shown to reduce thermal and mechanical degradation [8].

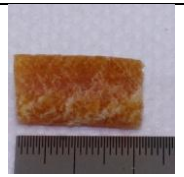
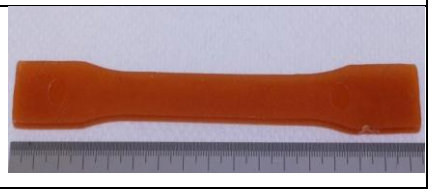
The majority of extruded samples were a transparent yellow/orange colour. Injection moulding produced transparent yellow/orange or brown tensile test samples depending on plasticiser content. The lighter coloured samples could be modified with the use of pigments if desired.

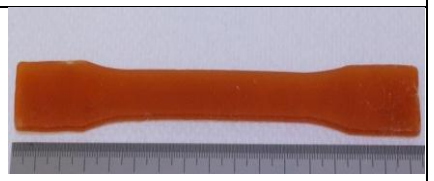
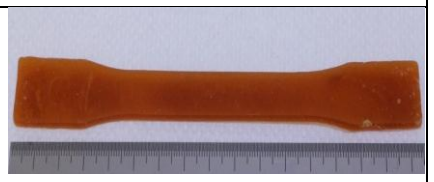
Table 47: Extrusion and injection moulding of 4% PAA treated bloodmeal with different amounts of water, SDS and TEG.

Formulation (pph_{BM})				Observations						Pictures		
				Extrusion					Injection Mouldable			
Sample	SDS	Water	TEG	Flowed Well	Continuous	Extrudate Surface	Cross Section Consolidation	Other		Extrusion	Cross Section	Injection Moulded Test Piece
1	6	30	10	Yes	Yes	Smooth	Good		No			
2	6	40	10	Yes	Yes	Smooth	Good		Yes			
3	6	50	10	Yes	Yes	Small Cracks	Good	Extrudate broke easily.	Yes			
4	6	30	20	Yes	Yes	Smooth	Good	Extrudate broke easily.	No			

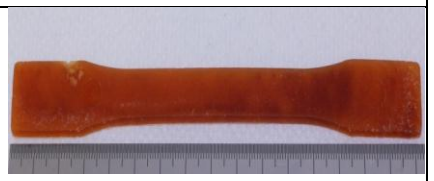
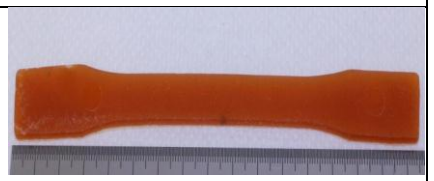
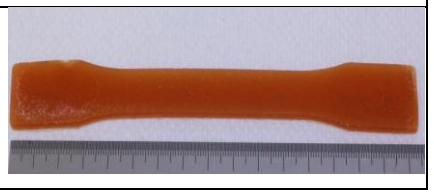
Formulation (pph_{BM})				Observations						Pictures		
				Extrusion					Injection Mouldable			
Sample	SDS	Water	TEG	Flowed Well	Continuous	Extrudate Surface	Cross Section Consolidation	Other		Extrusion	Cross Section	Injection Moulded Test Piece
5	6	40	20	Yes	Yes	Smooth	Good	Extrudate broke easily.	Yes			
6	6	50	20	Yes	Yes	Cracks Powder	Good	Extrudate broke easily.	Yes			
7	6	30	30	Yes	No	Cracks Powder	Reasonable	Extrudate broke easily.	Yes			
8	6	40	30	Yes	No	Cracks Powder	Reasonable	Extrudate broke easily.	Yes			

Formulation (pph_{BM})				Observations						Pictures		
				Extrusion								
Sample	SDS	Water	TEG	Flowed Well	Continuous	Extrudate Surface	Cross Section Consolidation	Other	Injection Mouldable	Extrusion	Cross Section	Injection Moulded Test Piece
9	6	50	30	No	No	Cracks Powder	Poor	Powder clumped in extruder feedzone.	Yes			
10	3	30	10	Yes	Yes	Smooth	Good		No			
11	3	40	10	Yes	Yes	Smooth	Good		No			
12	3	50	10	Yes	Yes	Smooth	Good		Yes			

Formulation (pph_{BM})				Observations						Pictures		
				Extrusion								
Sample	SDS	Water	TEG	Flowed Well	Continuous	Extrudate Surface	Cross Section Consolidation	Other	Injection Mouldable	Extrusion	Cross Section	Injection Moulded Test Piece
13	3	30	20	Yes	Yes	Smooth	Good		Yes			
14	3	40	20	Yes	Yes	Smooth	Good	Extrudate broke easily.	Yes			
15	3	50	20	No	No	Cracks	Reasonable	Extrudate broke easily.	Yes			
16	3	30	30	Yes	Yes	Small Cracks	Good	Extrudate broke easily.	Yes			

Formulation (pph_{BM})				Observations						Pictures		
				Extrusion								
Sample	SDS	Water	TEG	Flowed Well	Continuous	Extrudate Surface	Cross Section Consolidation	Other	Injection Mouldable	Extrusion	Cross Section	Injection Moulded Test Piece
17	3	40	30	No	No	Cracks	Reasonable	Powder c;umped in feed and extrudate broke easily.	Yes			
18	3	50	30	No	No	Cracks Powder	Poor	Powder c;umped in feed and extrudate broke easily.	Yes			
19	0	30	10	No	No	Cracks Powder	Reasonable		No			
20	0	40	10	No	No	Cracks Powder	Reasonable	Extrudate broke easily.	Yes			

Formulation (pph_{BM})				Observations						Pictures		
				Extrusion					Injection Mouldable			
Sample	SDS	Water	TEG	Flowed Well	Continuous	Extrudate Surface	Cross Section Consolidation	Other		Extrusion	Cross Section	Injection Moulded Test Piece
21	0	50	10	No	No	Cracks Powder	Poor	Extrudate broke easily.	Yes			
22	0	30	20	No	No	Cracks Powder	Reasonable	Extrudate broke easily.	No			
23	0	40	20	No	No	Cracks Powder	Poor	Extrudate broke easily.	Yes			
24	0	50	20	No	No	Cracks Powder	Poor	Extrudate broke easily.	Yes			

Formulation (pph_{BM})				Observations						Pictures		
				Extrusion								
Sample	SDS	Water	TEG	Flowed Well	Continuous	Extrudate Surface	Cross Section Consolidation	Other	Injection Mouldable	Extrusion	Cross Section	Injection Moulded Test Piece
25	0	30	30	No	No	Cracks Powder	Poor	Extrudate broke easily.	Yes			
26	0	40	30	No	No	Cracks Powder	Poor	Powder clumped in feed and extrudate broke easily.	Yes			
27	0	50	30	No	No	Cracks Powder	Poor	Powder clumped in feed and extrudate broke easily.	Yes			

9.3.2 Effect of SDS, Water and TEG on Consolidation, Colour and Mechanical Properties

9.3.2.1 Extrusion Properties

The best consolidation results were achieved using 6 pph_{BM} combined with 40 pph_{BM} water and 10 pph_{BM} TEG or 30 pph_{BM} water and 20 pph_{BM} TEG. These samples were 98% consolidated. 97% was also achieved using 3 pph_{BM} SDS with 40 pph_{BM} water and 10 pph_{BM} TEG or 30 pph_{BM} water and 20 pph_{BM} TEG. The highest consolidation at 0 pph_{BM} SDS (86%) was also achieved using this combination of water and TEG. This suggested that 50 pph_{BM} total plasticiser is the optimum level for consolidation during extrusion. These extrusion trials were summarised in Table 48.

Table 48: Percent consolidation and whiteness after extrusion for different combinations of SDS, water and TEG.

Formulation	SDS (pph_{BM})	Water (pph_{BM})	TEG (pph_{BM})	SME (kJ/kg)	Standard Error	Extrusion Consolidation (%)	Standard Error	Whiteness (%)
1	6	30	10	30.39	0.87	96	0.5	43
2	6	40	10	23.68	0.09	98	0.6	49
3	6	50	10	23.37	0.12	96	0.2	48
4	6	30	20	20.97	0.15	98	0.4	44
5	6	40	20	18.36	0.02	96	1.4	49
6	6	50	20	17.63	0.34	96	0.6	52
7	6	30	30	17.02	0.17	93	0.3	51
8	6	40	30	16.16	0.26	93	0.4	52
9	6	50	30	16.88	0.56	92	0.0	53
10	3	30	10	29.30	0.26	95	0.2	43
11	3	40	10	26.12	0.72	97	1.1	38
12	3	50	10	20.52	0.23	94	1.7	37
13	3	30	20	19.22	0.05	97	0.3	37
14	3	40	20	18.36	0.37	95	1.2	42
15	3	50	20	18.81	0.85	87	2.3	50
16	3	30	30	18.31	0.58	95	1.2	42
17	3	40	30	22.27	1.50	90	1.1	53
18	3	50	30	23.32	2.11	83	1.5	58
19	0	30	10	11.81	0.18	83	5.6	52
20	0	40	10	10.07	0.10	86	2.1	49
21	0	50	10	10.29	0.05	62	6.7	63
22	0	30	20	8.74	0.12	74	6.9	58
23	0	40	20	10.63	1.67	68	3.5	63
24	0	50	20	9.40	0.15	63	5.8	65
25	0	30	30	11.09	0.92	62	9.3	62
26	0	40	30	10.19	2.29	38	8.3	67
27	0	50	30	8.39	0.49	54	11.0	64

Main effects graphs (Figure 84 to Figure 86) and observations suggested SDS had the greatest impact on consolidation, extrudate colour and SME. This was

confirmed using analysis of variance (Table 49). Analysis of variance revealed that SDS contributed 71.2% to SME input, 71.9% to whiteness and 58.5% to consolidation. This confirmed the observations made during extrusion where extruding formulations with no SDS resulted in poorly consolidated samples, which were powdery, had many surface defects and were easy to break. In some cases consolidation reached a plateau at 3 pph_{BM} SDS which suggested that this amount of SDS was sufficient to reduce hydrophobic interactions allowing the protein chains to interact and reform new stabilising interactions so the plastic could consolidate.

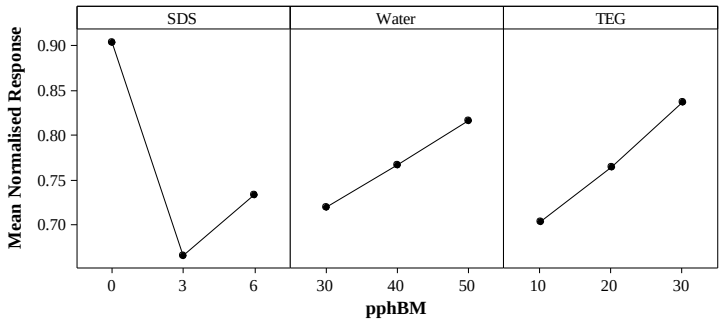


Figure 84: Main effects of SDS, water and TEG on extrudate whiteness.

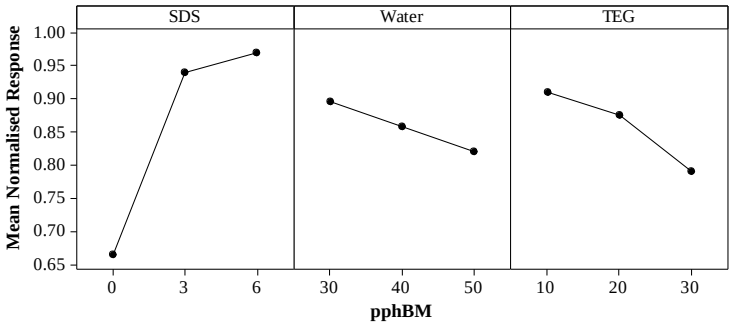


Figure 85: Main effects of SDS, water and TEG on extrusion consolidation.

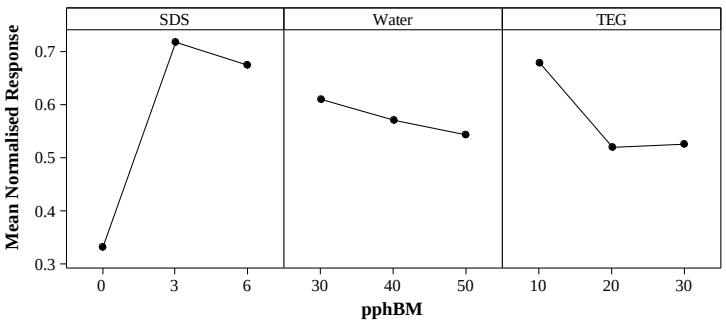


Figure 86: Main effects plot of SDS, water and TEG on SME input during extrusion.

Table 49: Contribution of each additive to the average extruded properties, as determined using analysis of variance.

	SME (%)	Extrudate Whiteness (%)	Consolidation (%)
SDS	71.2	71.9	58.5
Water	1.8	3.5	9.1
TEG	12.9	9.6	17.4
SDS*Water	1.1	2.0	0.6
SDS*TEG	6.4	6.1	2.8
Water*TEG	3.4	2.2	2.5
SDS*Water*TEG	3.2	4.6	9.2

SDS was required to detangle the protein chains in the bloodmeal aggregates by reducing the hydrophobic interactions. The detangled proteins were then able to re-entangle and form new stabilising interactions during extrusion resulting in consolidation. Previous studies have suggested that when proteins are detangled or unwound the amount of surface area for new stabilising interactions and entanglements increases during processing [181]. If this was true then at 0 pph_{BM} SDS the bloodmeal protein would not be detangled sufficiently resulting in low amounts of entanglement and few molecular interactions due to the low contact area between protein chains. This could explain the poor consolidation at 0 pph_{BM} SDS. At 3 and 6 pph_{BM} the protein was unwound enough to allow sufficient entanglements and the formation of new stabilising interactions during extrusion resulting in good consolidation.

When biopolymers entangle and form new stabilising interactions during extrusion, their viscosity increases and cause an increase in required SME input. At 0 pph_{BM} SDS, low SME input was required during extrusion. This was probably due to low chain entanglements and chain interactions as the protein was not detangled sufficiently enough to allow a high amount of these to new entanglements and stabilising interactions to form.

At 3 pph_{BM} SDS, the protein was detangled sufficiently and the chains were able to entangle and interact during extrusion resulting in a high SME input. No increase in SME occurred at 6 pph_{BM} SDS and in some cases SME decreased slightly. This suggested that 3 pph_{BM} SDS was sufficient to detangle enough of the protein chains and as SDS content increased the excess SDS could have been acting as a plasticiser as has been shown previously in other studies where SDS was used at high concentrations [181].

9.3.2.2 Injection Moulded Sample Properties

Injection moulded mechanical properties and sample colour varied greatly depending on what combinations of water, TEG and SDS were used. SDS and water appeared to have a small effect on colour whereas TEG appeared to have a significant effect. Water was evaporated during extrusion, injection moulding and conditioning as shown in Table 50, which may be why its effect on injection moulded sample whiteness and mechanical properties was not as great as TEG and SDS.

Table 50: Moisture contents of samples produced using different combinations of SDS, water and TEG after extrusion, injection moulding and conditioning.

Sample	SDS (pph_{BM})	Water (pph_{BM})	TEG (pph_{BM})	Initial Moisture Content (%)	Moisture Content After Extrusion (%)	Moisture Content After Injection Moulding (%)	Moisture Content After Conditioning (%)
1	6	30	10	25	20	18	N/A
2	6	40	10	30	25	21	11
3	6	50	10	34	23	22	11
4	6	30	20	24	20	17	N/A
5	6	40	20	28	24	20	12
6	6	50	20	32	27	23	11
7	6	30	30	22	22	18	13
8	6	40	30	27	24	19	12
9	6	50	30	31	30	23	13
10	3	30	10	26	21	18	N/A
11	3	40	10	31	22	19	N/A
12	3	50	10	35	27	23	9
13	3	30	20	24	18	16	10
14	3	40	20	29	22	18	10
15	3	50	20	33	28	24	11
16	3	30	30	23	19	16	12
17	3	40	30	27	23	19	13
18	3	50	30	31	28	23	13
19	0	30	10	26	20	16	N/A
20	0	40	10	31	25	19	8
21	0	50	10	36	29	22	8
22	0	30	20	25	20	16	N/A
23	0	40	20	29	26	19	9
24	0	50	20	34	30	22	9
25	0	30	30	23	24	16	10
26	0	40	30	28	28	19	10
27	0	50	30	32	32	22	10

Note: dried 4% PAA treated bloodmeal contained 7% moisture.

Table 51: Mechanical properties of conditioned injection moulded samples produced from four percent PAA treated bloodmeal with different water, TEG and SDS combinations.

Formulation	SDS (pph_{BM})	Water (pph_{BM})	TEG (pph_{BM})	Young's Modulus (MPa)	Standard Error	UTS (MPa)	Standard Error	Strain at Break (%)	Standard Error	Toughness (Mpa)	Standard Error	Whiteness (%)
1	6	30	10	Not Injection Mouldable								
2	6	40	10	795.66	27.57	4.60	0.17	1.78	0.05	0.02	0.00	23
3	6	50	10	687.17	27.65	4.51	0.27	1.82	0.06	0.02	0.00	31
4	6	30	20	Not Injection Mouldable								
5	6	40	20	223.36	15.21	2.99	0.33	5.06	0.73	0.05	0.02	37
6	6	50	20	252.81	7.95	3.32	0.08	4.20	0.11	0.04	0.00	38
7	6	30	30	51.26	1.34	3.50	0.06	71.44	1.01	1.13	0.05	41
8	6	40	30	51.33	1.72	3.43	0.03	80.34	2.02	1.18	0.08	39
9	6	50	30	46.11	1.75	3.27	0.06	88.10	1.81	1.29	0.08	40
10	3	30	10	Not Injection Mouldable								
11	3	40	10	Not Injection Mouldable								
12	3	50	10	848.11	26.11	5.50	0.44	2.28	0.13	0.03	0.01	29
13	3	30	20	90.85	1.90	4.65	0.10	59.12	2.10	1.21	0.10	28
14	3	40	20	88.98	1.49	4.89	0.10	75.78	1.70	1.70	0.08	29
15	3	50	20	85.48	2.40	4.62	0.10	82.62	1.33	1.75	0.09	37
16	3	30	30	37.82	1.16	3.05	0.07	76.50	2.72	0.92	0.13	33
17	3	40	30	32.23	1.62	2.81	0.05	81.84	2.60	0.91	0.12	38
18	3	50	30	40.58	4.99	2.63	0.12	84.86	3.46	0.75	0.15	36
19	0	30	10	Not Injection Mouldable								
20	0	40	10	1217.17	48.45	8.78	0.76	2.44	0.12	0.04	0.01	26
21	0	50	10	1133.66	85.92	8.42	0.63	2.42	0.09	0.04	0.01	26
22	0	30	20	Not Injection Mouldable								
23	0	40	20	352.60	17.46	6.31	0.11	14.44	1.01	0.37	0.08	36
24	0	50	20	418.26	17.13	6.45	0.38	7.06	0.36	0.14	0.03	33
25	0	30	30	125.67	11.16	4.47	0.15	49.70	4.31	0.81	0.18	35
26	0	40	30	95.73	3.12	4.43	0.06	58.14	1.72	0.12	0.07	37
27	0	50	30	89.36	5.26	4.13	0.03	52.30	0.99	0.96	0.04	37

Main effects graphs (Figure 87 to Figure 91) and observations made during processing and testing suggested TEG to be most important for both colour and mechanical properties overall. High TEG produced injection moulded samples with whiteness values between 35-41% whereas low TEG resulted in 23-31% white samples. Analysis of variance showed that TEG contributed the 38.1% to colour of injection moulded samples while water contributed 29.9% and SDS contributed 0.5% (Table 52).

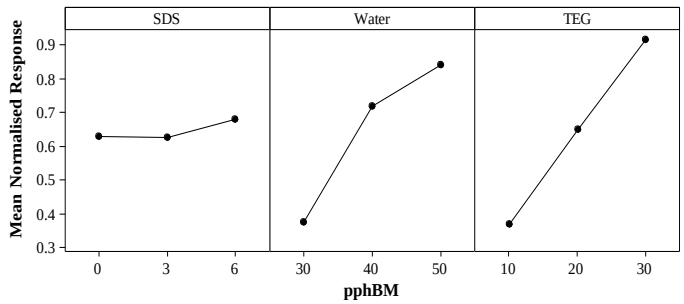


Figure 87: Main effects of SDS, water and TEG on whiteness of injection moulded sample.

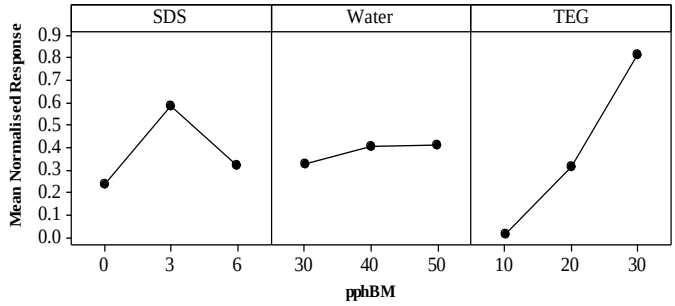


Figure 88: Main effects of SDS, water and TEG on strain at break.

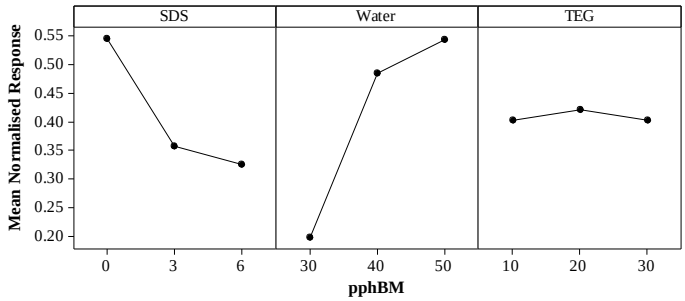


Figure 89: Main effects of SDS, water and TEG on UTS.

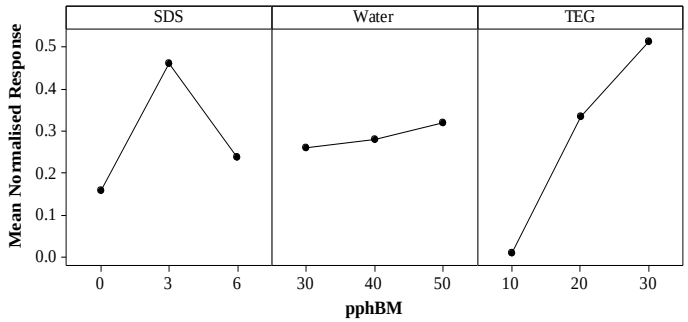


Figure 90: Main effects of SDS, water and TEG on toughness.

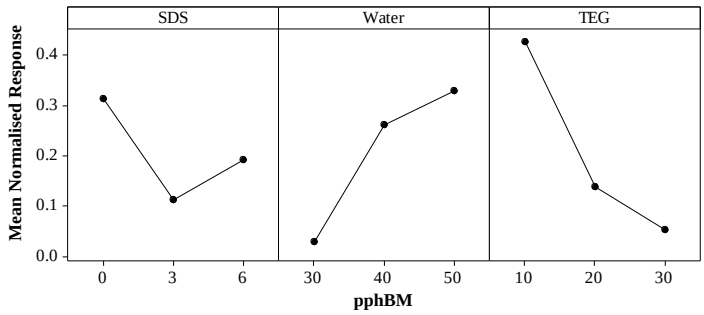


Figure 91: Main effects of SDS, water and TEG on Young's modulus.

Table 52: Contribution of each additive to the average mechanical properties of injection moulded samples, as determined using analysis of variance.

	Young's Modulus (%)	UTS (%)	Strain (%)	Toughness (%)	Injection Moulded Sample Whiteness (%)
SDS	8.1	12.5	13.7	15.2	0.5
Initial Water	19.7	30	1.0	0.6	29.9
TEG	30.5	0.1	66.9	39.7	38.1
SDS*Water	6.8	12.8	0.2	0.7	6.3
SDS*TEG	4.6	12.5	17.6	37.4	4.2
Water*TEG	21.1	21.9	0.3	2.5	14.1
SDS*Water*TEG	9.2	10.3	0.4	3.9	6.9

Polymers can form carbonyl compounds when exposed to high temperatures during processing resulting in a brown discolouration of the product [146]. This combined with high pressures and shear could be what caused darkening during injection moulding. Plasticisers have been shown to prevent thermal and mechanical degradation by increasing workability through molecular lubrication [164]. Thermal degradation is more likely to occur at low plasticiser contents [8]. The higher TEG content could reduce thermal and mechanical degradation by improving lubrication and heat dissipation between the chains resulting in a lighter colour. Oxidation could also be a cause of discolouration during processing. Using antioxidants to prevent this should be investigated in future studies.

The whiteness of injection moulded samples was slightly higher at 6 pph_{BM} SDS than 0 and 3 pph_{BM}. At 6 pph_{BM} SDS the excess SDS could be acting as a plasticiser and help prevent darkening of the injection moulded sample.

It is interesting that SDS contributed 71.9% and TEG contributed 9.6% to extrudate colour. Whereas SDS only contributed 0.5% and TEG 38.1% to injection moulded sample colour. This was probably because after extrusion a consolidated sample darkens slightly and there was a clear relationship between consolidation and whiteness as shown in Figure 92. This could be why SDS had a high contribution to the colour of extrudates. After extrusion consolidation has most probably reached a maximum for most cases so any change in colour during injection moulding was most probably due to degradation. TEG had an increased contribution to injection moulded sample colour, because it can help prevent thermal and mechanical degradation of proteins during thermoplastic processing.

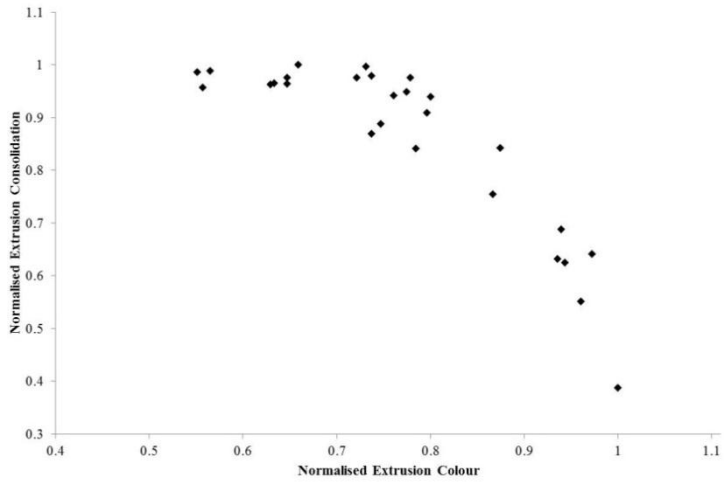


Figure 92: Relationship between extruded colour and consolidation.

A similar result was found for mechanical properties. SDS and water appeared to have a small effect while TEG had a strong effect. In general it appeared that as TEG content increased Young's modulus and UTS decreased whereas extension and toughness increased. Analysis of variance (Table 52) confirmed that TEG had a 30.5% contribution to Young's modulus, 66.9% contribution to extension and 39.7% contribution to toughness. TEG on its own only contributed 0.1% to UTS which was not expected, however its combined effect with water contributed 21.9% to UTS. Plasticisers can greatly affect mechanical properties due to their lubricating effects [164]. High TEG content would have reduced bloodmeal protein-protein interactions and increased free volume between molecules making movement easier which resulted in the low Young's modulus and UTS. However the increased free volume increased the material's ability to be stretched without breaking which led to greater percentage strain at break and increased toughness.

Initial water content had a greater effect on unconditioned sample properties than conditioned samples (Appendix 2). In general as water content increased Young's modulus and UTS decreased whereas extension and toughness increased as expected. Unconditioned samples had lower mechanical properties overall than conditioned samples because of the high water content.

Initial water had a 30% contribution to UTS and 19.7% contribution to Young's modulus of conditioned samples. However it had only a minor effect on strain at break and toughness. These results suggest that even though water was removed during conditioning, initial water content was still relatively important to the

formation of stabilising interactions. This has also been found in other studies [7]. The initial water content may aid the action of SDS during processing which may explain why it still made a contribution to mechanical properties after conditioning.

Initial water was the second highest single contributor injection moulded sample whiteness. Its lower contribution effect is probably because plasticisation with water can be affected by evaporation [164]. Its effect on mechanical properties could be reduced when compared to unconditioned samples because after conditioning, water levels have been equilibrated to approximately 10% and new interactions were formed which override the initial contribution of water.

SDS can increase mechanical properties because detangled proteins are able to entangle and their side groups able to interact. However SDS can also reduce mechanical properties by removing too many hydrophobic interactions between protein chains or by disrupting the protein matrix [181]. SDS had a slight effect on UTS (12.5%) and Young's modulus (8.1%). Generally as SDS increased, UTS and Young's modulus decreased indicating SDS also acted as a plasticiser.

TEG was found to have the greatest effect on mechanical properties because of its plasticising effect. As TEG increased, Young's modulus and UTS decreased This is typical behaviour observed with plasticisers, due to the plasticiser increasing free volume between chains and making molecular movements easier which causes a reduction in Young's modulus and UTS.

The combination of SDS and TEG contributed 17.6% to strain at break and 37.4% to toughness. Strain at break and toughness were closely related and showed similar trends. This was because toughness is the amount of energy absorbed before break which is proportional to the amount of strain.

In general low SDS and TEG caused low strain at break and toughness due to poor entanglement and protein chain interactions from a lack of aggregate unwinding. Low plasticisation resulting in restricted molecular movement was probably another contributing factor. As SDS and TEG increased so did strain at break and toughness because of greater entanglement and plasticisation. When

data was analysed it was found that 0 and 6 pph_{BM} SDS showed similar trends for strain at break and toughness, although this was due to different reasons.

When 0 pph_{BM} SDS and low TEG was used there was low strain at break, probably due to the combination of low amounts of entanglement and plasticisation. Although there was no SDS, pressure and heat could also be acting to detangle the aggregated protein and this would have resulted in some entanglement. When TEG was increased so did extension due to the increased plasticiser making the entangled proteins able to move past each other more easily. When 6 pph_{BM} SDS was used, very low strain at break was obtained at 10 and 20 pph_{BM} TEG. Strain at break was lower than when 0 pph_{BM} SDS was used. However when TEG was increased to 30 pph_{BM}, strain at break increased greatly. High SDS content can put an overall negative charge on the protein which could lead to repulsion effects. This could explain the low strain at break and toughness at 10 and 20 pph_{BM} TEG when 6 pph_{BM} SDS was used. At 30 pph_{BM} TEG the free volume between chains could be increased sufficiently to overcome these repulsion effects leading to improved strain at break and toughness.

When 3 pph_{BM} SDS was used a different trend was observed for percentage strain at break and toughness. Low percentage strain at break occurred when 10 pph_{BM} TEG was used which was similar for the other SDS contents. However at 20 pph_{BM} TEG the percentage strain at break increased greatly and reached a plateau. When increased to 30 pph_{BM} TEG very little increase in percentage strain at break occurred and toughness was reduced. This is the usual trend observed with plasticiser use, where above a critical plasticiser content intermolecular interactions become too weak resulting in reduced percentage strain at break and toughness [177]. These results suggested that the optimum combination was 3 pph_{BM} SDS and 20 pph_{BM} TEG. This combination provided the optimum level of entanglement, stabilising forces and plasticisation as shown by the best mechanical properties. Plasticiser content was at the right level so to allow sufficient chain movement for flexibility without causing too much loss in strength. If plasticiser was increased above this level then excessive plasticisation caused a loss in Young's modulus and UTS without significant increases in percentage strain at break and caused reduced toughness.

9.3.3 Ranking Formulations Based on Extruded Sample Properties

Formulations were ranked for extrusion by adding the normalised values for consolidation and colour multiplied by an importance factor. Consolidation was decided to be most important; therefore its normalised value was multiplied by 2.5. When formulations were ranked based the best formulations were, formulation 2 and 6 (Table 53). These produced samples which were 98% consolidated and 49% white using 6 pph_{BM} SDS combined with 40 pph_{BM} water and 10 pph_{BM} TEG. The worst formulation was formulation 26 which achieved 38% consolidation and 67% whiteness using 0 pph_{BM} SDS, 40 pph_{BM} water and 30 pph_{BM} TEG. If producing an extruded product formulation 2 and 6 would be the preferred formulations to use. However reasonable results could still be obtained when 3-6 pph_{BM} SDS were combined with a total plasticiser content between 50 and 60 pph_{BM}.

Table 53: Ranking samples produced from 4% PAA treated bloodmeal with different water, TEG and SDS combinations based on consolidation and whiteness after extrusion.

Ranking Formulations Based on Extrusion							
Rank	**Formulation**	**SDS (pph_{BM})**	**Water (pph_{BM})**	**TEG (pph_{BM})**	**Consolidation Points (max 2.5)**	**Extrusion Colour Points (max 1)**	**Total (max 3.5)**
1 equal	2	6	40	10	2.49	0.73	3.22
1 equal	6	6	50	20	2.44	0.78	3.22
2	5	6	40	20	2.45	0.74	3.19
3	3	6	50	10	2.44	0.72	3.16
4	4	6	30	20	2.50	0.66	3.16
5	9	6	50	30	2.35	0.80	3.15
6	8	6	40	30	2.37	0.77	3.15
7	7	6	30	30	2.36	0.76	3.12
8	1	6	30	10	2.44	0.65	3.09
9	17	3	40	30	2.27	0.80	3.07
10	10	3	30	10	2.41	0.65	3.06
11	16	3	30	30	2.41	0.63	3.05
12	14	3	40	20	2.41	0.63	3.04
13	11	3	40	10	2.47	0.56	3.04
14	13	3	30	20	2.47	0.55	3.02
15	18	3	50	30	2.11	0.87	2.98
16	15	3	50	20	2.22	0.75	2.97
17	12	3	50	10	2.39	0.56	2.95
17	20	0	40	10	2.17	0.74	2.91
19	19	0	30	10	2.10	0.78	2.89
20	22	0	30	20	1.89	0.87	2.75
21	23	0	40	20	1.72	0.94	2.66
22	24	0	50	20	1.60	0.97	2.58
23	25	0	30	30	1.58	0.94	2.51
24	21	0	50	10	1.56	0.94	2.51
25	27	0	50	30	1.38	0.96	2.34
26	26	0	40	30	0.97	1.00	1.97

When samples were ranked based on extrusion the most obvious trend was that as SDS increased so did the quality of the extrudate. This was also clearly observed during extrusion trials as previously described. However SDS along with water and TEG can affect injection moulded sample mechanical properties. Therefore samples were ranked based on injection moulded sample properties as well to find the optimum levels of SDS, water and TEG which produced a light coloured sample with acceptable mechanical properties.

9.3.4 Ranking Formulations Based on Injection Moulded Sample Properties

Formulations were ranked based on colour and mechanical properties of injection moulded samples by summing the normalised colour and mechanical property responses for each formulation (Table 54). Formulation 15 was found to be the best combination of water (50 pph_{BM}), TEG (20 pph_{BM}) and SDS (3pph_{BM}). This produced a sample with tensile strength 4.62 MPa, young's modulus 85.48 MPa, toughness 1.75 MPa and 82.62% strain at break. However the combination of these can be altered depending on the desired properties of the final material. For example, more water or TEG could be used to produce a lighter more flexible and flexible sample or less water and TEG could be used to produce a darker sample with higher stiffness and strength.

Table 54: Ranking samples produced from 4% PAA treated bloodmeal with different water, TEG and SDS combinations based on whiteness and mechanical properties after injection moulding.

Ranking Formulations Based on Injection Moulding										
Rank	Formulation	SDS (pph_{BM})	Water (pph_{BM})	TEG (pph_{BM})	Injection Moulded Colour Points (max 1)	Young's Modulus Points (max 1)	UTS Points (max 1)	Strain Points (max 1)	Toughness Points (max 1)	Injection Mould Total (max 5)
1	15	3	50	20	0.90	0.07	0.53	0.94	1.00	3.44
2	14	3	40	20	0.71	0.07	0.56	0.86	0.97	3.17
3	9	6	50	30	0.98	0.04	0.37	1.00	0.73	3.13
4	8	6	40	30	0.95	0.04	0.39	0.91	0.67	2.97
5	7	6	30	30	1.00	0.04	0.40	0.81	0.65	2.90
6	17	3	40	30	0.92	0.03	0.32	0.93	0.52	2.71
7	20	0	40	10	0.64	1.00	1.00	0.03	0.02	2.69
8	13	3	30	20	0.69	0.07	0.53	0.67	0.69	2.66
9	27	0	50	30	0.92	0.07	0.47	0.59	0.55	2.60
10	18	3	50	30	0.88	0.03	0.30	0.96	0.43	2.60
11	16	3	30	30	0.82	0.03	0.35	0.87	0.52	2.59
12	21	0	50	10	0.64	0.93	0.96	0.03	0.02	2.58
13	25	0	30	30	0.86	0.10	0.51	0.56	0.46	2.49
14	23	0	40	20	0.88	0.29	0.72	0.16	0.21	2.26
15	26	0	40	30	0.90	0.08	0.50	0.66	0.07	2.22
16	12	3	50	10	0.71	0.70	0.63	0.03	0.02	2.07
17	24	0	50	20	0.82	0.34	0.73	0.08	0.08	2.06
18	3	6	50	10	0.77	0.56	0.51	0.02	0.01	1.88
19	2	6	40	10	0.57	0.65	0.52	0.02	0.01	1.78
20	6	6	50	20	0.94	0.21	0.38	0.05	0.02	1.60
21	5	6	40	20	0.90	0.18	0.34	0.06	0.03	1.51
22	10*	3	30	10	0.83	0.00	0.00	0.00	0.00	0.83
23	19*	0	30	10	0.81	0.00	0.00	0.00	0.00	0.81
24	22*	0	30	20	0.77	0.00	0.00	0.00	0.00	0.77
25	1*	6	30	10	0.68	0.00	0.00	0.00	0.00	0.68
26	11*	3	40	10	0.68	0.00	0.00	0.00	0.00	0.68
27	4*	6	30	20	0.67	0.00	0.00	0.00	0.00	0.67

* Samples not injection mouldable.

9.3.5 Ranking Formulations Based on Extruded and Injection Moulded Sample Properties

When formulations were ranked based on extruded and injection moulded sample properties using the previously described method, formulation 15 was also found to be the optimum combination of water, TEG and SDS. However during extrusion this sample only produced an 87.4% consolidated sample with many surface defects which broke easily and when assessed based solely on extrusion properties, formulation 15 was ranked 16th. Other combinations of water, TEG and SDS could be used to produce better quality extrudates. This showed that different combinations of water, TEG and SDS must be chosen to obtain the best quality sample depending if extrusion or injection moulding will be the final processing step. If extruding is the final processing technique used to produce the

product then 3-6 pph_{BM} combined with 50-60 pph_{BM} total plasticiser content will produce a good quality sample. Whereas if injection moulding is the final processing technique used then a good quality sample can be obtained using 3-6 pph_{BM} with 70-80 pph_{BM} total plasticiser where at least 20 pph_{BM} is TEG.

Table 55: Ranking formulations produced from 4% PAA treated bloodmeal with different water, TEG and SDS combinations based on consolidation, whiteness after extrusion and injection moulding and mechanical properties after injection moulding.

Ranking Formulations Based on Extrusion and Injection Moulding							
Rank	**Formulation**	**SDS**	**Water**	**TEG**	**Extrusion Total (max 3.5)**	**Injection Mould Total (max 5)**	**Extruder and Injection Mould Total (max 8.5)**
1	15	3	50	20	2.97	3.44	6.40
2	9	6	50	30	3.15	3.13	6.28
3	14	3	40	20	3.04	3.17	6.20
4	8	6	40	30	3.15	2.97	6.12
5	7	6	30	30	3.12	2.90	6.01
6	17	3	40	30	3.07	2.71	5.78
7	13	3	30	20	3.02	2.66	5.68
8	16	3	30	30	3.05	2.59	5.64
9	20	0	40	10	2.91	2.69	5.60
10	18	3	50	30	2.98	2.60	5.58
11	21	0	50	10	2.51	2.58	5.09
12	3	6	50	10	3.16	1.88	5.04
13	12	3	50	10	2.95	2.07	5.02
14	25	0	30	30	2.51	2.49	5.01
15	2	6	40	10	3.22	1.78	5.00
16	27	0	50	30	2.34	2.60	4.94
17	23	0	40	20	2.66	2.26	4.92
18	6	6	50	20	3.22	1.60	4.81
19	5	6	40	20	3.19	1.51	4.69
20	24	0	50	20	2.58	2.06	4.63
21	26	0	40	30	1.97	2.22	4.19
22	10*	3	30	10	3.06	0.83	3.89
23	4*	6	30	20	3.16	0.67	3.83
24	1*	6	30	10	3.09	0.68	3.77
25	11*	3	40	10	3.04	0.68	3.72
26	19*	0	30	10	2.89	0.81	3.70
27	22*	0	30	20	2.75	0.77	3.52

* Samples not injection mouldable.

9.4 Conclusion

It was found that the optimum combination of water, TEG and SDS differed for extrusion and injection moulding. SDS was found to be the most important factor for consolidation during extrusion and SME due to it unwinding the aggregated protein and allowing new protein entanglements and new protein interactions to form. The best extruded sample based on consolidation and whiteness contained 40 pph_{BM} water, 10 TEG pph_{BM} and 6 pph_{BM} SDS. This produced a extrudate which was 98% consolidated and 49% white.

TEG was most important for injection moulded sample whiteness with a 38.1% contribution. TEG reduced thermal and mechanical stresses which contributed to degradation and darkening of the sample. Water contributed 29.9% and SDS also had a small 0.5% contribution. Main effects plots and experimental observations revealed that high SDS helped prevent darkening which suggested it acted as a plasticiser at high concentrations.

TEG also had the greatest impact on injection moulded sample mechanical properties with 30.5% contribution to Young's modulus, 66.9% contribution to extension and 39.1% contribution to toughness. It only contributed 0.1% to UTS which was much lower than expected, but its combined effect with water contributed 21.9% to UTS. Increasing TEG resulted in increased free volume and easier molecular movements which led to decreased Young's modulus and UTS but higher percentage strain at break and toughness.

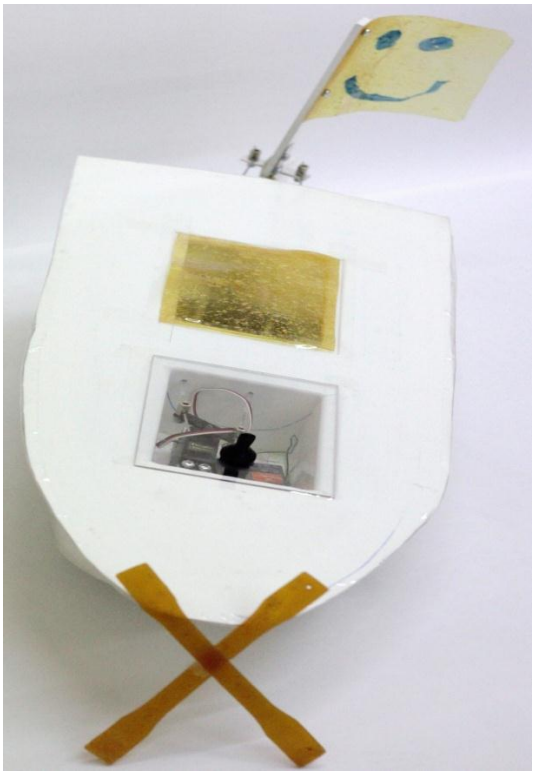
SDS was the second highest single contributor to mechanical properties. It contributed 8.1% to Young's modulus, 12.5% to UTS, 13.7% to extension and 15.2% to toughness. 0 pph_{BM} SDS had low extension and toughness which was probably caused by a lack of entanglement from low protein aggregate unwinding. 6 pph_{BM} SDS had poor mechanical properties at 10-20 pph_{BM} TEG because of electrostatic repulsion. When TEG reached 30 pph_{BM} free volume was increased sufficiently to overcome the repulsion effect.

At 3 pph_{BM} SDS, 50 pph_{BM} water and 20 pph_{BM} TEG an optimum level of entanglement, stabilising interactions and plasticisation occurred which resulted in an injection moulded sample with tensile strength 4.62 MPa, Young's modulus

85.48 MPa, toughness 1.75 MPa and 82.62% strain at break. These mechanical properties were comparable to Novatein Thermoplastic Protein produced from untreated bloodmeal and bioplastics produced from other agricultural biopolymer sources.

Chapter 10

Conclusions and Recommendations



Bioplastic components produced from peracetic acid treated bloodmeal incorporated into remote controlled boat.

10.1 Conclusions

It was shown that a yellow/orange and odourless thermoplastic material could be made from peracetic acid (PAA) treated bloodmeal. The most significant findings were that bloodmeal's molecular weight could be presevered by using PAA as an oxidant and odour causing compounds were effectively oxidised while degrading the iron containing haem group in haemoglobin to reduce the material's colour. This will lead to bloodmeal being used more widely in bioplastics as it can be pigmented and converted into a thermoplastic by extrusion and injection moulding. This could potentially add value to what was traditionally a low value meat processing industry by-product. The overall research programme is summarised in Figure 93 for easy reference.

Haem consists of a highly conjugated structure while the odorous compounds also contain double bonds and ring structures. In both cases, these can be readily removed by oxidation. The haem group can be dissociated from haemoglobin by dilution and reducing solution pH to 2. In this study hydrogen peroxide, PAA, sodium hypochlorite, sodium chlorite, sodium chlorate, and chlorine dioxide were used to reduce colour and smell in red blood cell concentrate (RBCC), modified RBCC (mRBCC), and bloodmeal.

Treatment of RBCC and mRBCC with peroxides resulted in extensive foaming of soluble proteins by oxygen released during peroxide degradation by catalase present in blood. Dilution reduced foaming but increased processing volume. Hydrogen peroxide treatment of mRBCC resulted in a powder which was 55% white, whereas hydrogen peroxide treatment of RBCC produced a powder which was 71% white. Treating mRBCC and RBCC with PAA produced powders which were 87 and 91% white respectively. Both treatments for RBCC were rapid and were completed within 5 minutes. Treatment time and extent of decolouring were dependent on protein and oxidant concentration as was shown when treating mRBCC with hydrogen peroxide. Sodium chlorite was the most successful oxidant, producing a powder which was 61% white from RBCC and a powder which was 93% white from mRBCC within 30 minutes. In most cases, where decolouring was successful, protein average molecular weight was reduced from 87-94 kDa to 13-44 kDa. The exception was sodium chlorite treated RBCC

where average molecular weight increased to 143 kDa. This method produced small molecular weight peptides but also caused aggregation which was responsible for the increase in average molecular weight.

Treating bloodmeal with hydrogen peroxide, PAA and sodium chlorite produced powders which were 59%, 67% and 31% white respectively. The molecular weight for hydrogen peroxide and PAA treated bloodmeal was unaffected, but sodium chlorite treated powder was unable to be dissolved in SDS and sodium sulphite solutions for molecular weight analysis. Significant foaming occurred during hydrogen peroxide treatment, but not for PAA treatment of bloodmeal.

Bloodmeal was selected as the best feedstock for decolouring as it had a high solids content of 95%, it did not need to be diluted for successful treatment and protein molecular weight was not reduced. In addition, using bloodmeal avoided handling large processing volumes associated with RBCC and mRBCC and prevented microbial degradation because bloodmeal is a dried, stable feedstock.

PAA preferentially reacted with the haem group, odour compounds and disulphide bonds. At higher concentrations it also reacted with amino acid side groups reducing hydrophobic interactions and hydrogen bonding between protein chains. This reduced protein crystallinity, thermal stability, and glass transition temperature, while increasing protein solubility in phosphate buffer, SDS and sodium sulphite solutions heated to 100°C. Powder X-ray diffraction showed bloodmeal crystallinity decreased from 35% to 31-27% when treated with 1-5% PAA accompanied by a decrease in glass transition temperature from 225°C to 50°C. The protein solubility increased from 1% to 25% in phosphate buffer, 11% to 85% in 1% SDS and 3% to 26% in 1% sodium sulphite.

Protein chains have a wide range of interactions such as electrostatic and hydrophobic interactions, hydrogen bonding and disulphide bonding that are responsible for stabilising their structure. For successful extrusion, these interactions must be controlled using a combination of denaturants, surfactants, reducing agents and plasticisers. It was shown that PAA treated bloodmeal could not be extruded using the same operating conditions and additives as those required for untreated bloodmeal.

Initial attempts at extruding PAA treated bloodmeal using water (60 pph_{BM}), urea (10 pph_{BM}), TEG (10 pph_{BM}), sodium sulphite (3 pph_{BM}) and SDS (3 pph_{BM}) were unsuccessful and resulted in an unconsolidated powder. In further trials, 3-5% PAA treated bloodmeal powder was extruded using different combinations of water (0-60 pph_{BM}), TEG (0-40 pph_{BM}), glycerol (0-45 pph_{BM}), SDS (0-6pph_{BM}), sodium sulphite (0-6 pph_{BM}), urea (0-10 pph_{BM}), borax (0-15 pph_{BM}), salt (0-10 pph_{BM}) and sodium silicate (0-15 pph_{BM}).

Urea and sodium sulphite which were required to produce NTP were found to be unnecessary for PAA treated bloodmeal as the disulphide cross-links and hydrogen bonds had been reduced by PAA treatment. Using sodium silicate, borax, and salt were also unsuccessful and gave dark poorly consolidated extrudates. The most important additives for successful extrusion and injection moulding were water, TEG and SDS.

3% PAA treated bloodmeal produced dark brown injection moulded samples while 5% PAA treated bloodmeal resulted in yellow but easily broken test specimens. 4% PAA treated bloodmeal produced the best extruded and injection moulded samples and was chosen for investigating the effects of water, TEG and SDS concentration on consolidation, specific mechanical energy input, extrudate and injection moulded colour as well as mechanical properties.

ANOVA showed that SDS was the most important variable for extrusion. The best extruded sample which was 98% consolidated and 49% white contained 40 pph_{BM} water, 10 pph_{BM} TEG and 6 pph_{BM} SDS.

TEG had the greatest effect on colour after injection moulding and mechanical properties because of its plasticisation effect. ANOVA showed that TEG contributed 30.5% to Young's modulus, 66.9% to strain, 39.7% to toughness, 0.1% to UTS and 38.1% to colour, while SDS contributed 8.1% to Young's modulus, 13.7% to strain, 15.2% to toughness, 12.5% to UTS, 0.5% to colour. Initial water contributed 19.7% to Young's modulus, 1% to strain, 0.6% to toughness, 30% to UTS and 29.9% to colour.

The best injection moulded sample was produced using 50 pph_{BM} water, 20 pph_{BM} TEG and 3 pph_{BM} SDS. This produced an injection moulded sample which was 39% white, had an almost transparent yellow/orange colour with a tensile strength

of 4.62 MPa, Young's modulus 85.48 MPa, toughness 1.75 MPa and 82.62% strain. These mechanical properties were comparable to NTP and the resulting product can be easily pigmented.

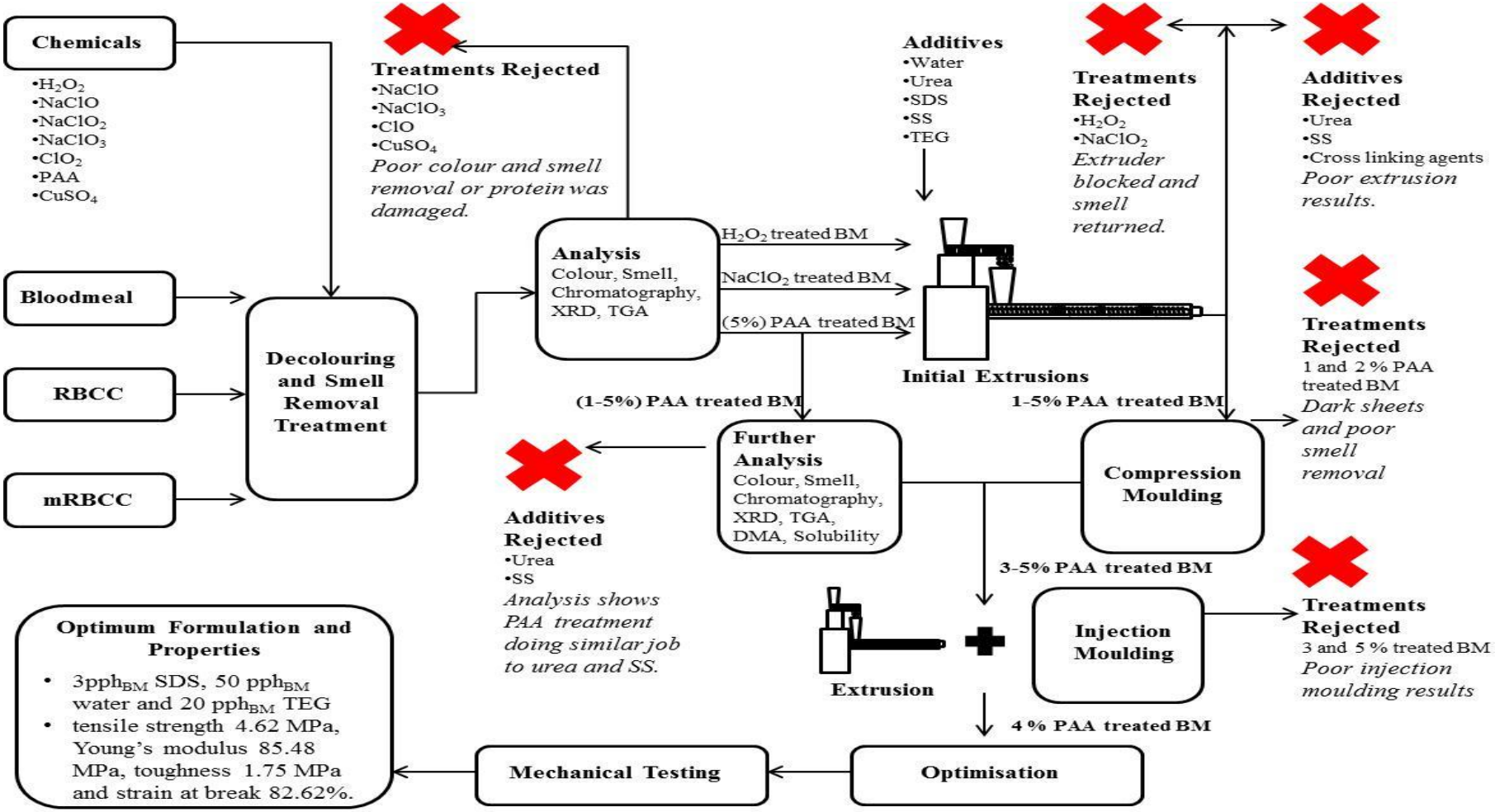


Figure 93: Flow diagram summarising work carried out and conclusions made during the production of decoloured bioplastic from bloodmeal

10.2 Recommendations for Future Work

Recommendations for further research cover three areas: The first is fully characterising the reaction kinetics of PAA treatment, secondly processing decoloured bloodmeal and thirdly biodegradation.

Further research could be carried out on the PAA bleaching process. This should focus on developing the decolouring processing and looking at recycling unreacted PAA. This could reduce treatment costs and also reduce waste. Work could also be done to investigate the time required for any PAA, acetic acid and hydrogen peroxide to decompose when it is discarded. The effect of PAA treatment on the amino acid composition of bloodmeal should also be investigated as these may help to understand protein-protein interactions.

The effect of processing on the molecular structure and protein chain interactions should be explored further, including processing using alternative plasticisers. Mechanical properties of PAA decoloured bloodmeal were highly dependent on TEG content. Using a different plasticiser could produce a material with different advantages. Attempts could also be made to improve the mechanical properties and water resistance of PAA decoloured bioplastics by incorporating fibres, clays and additives. Initial trials using compression moulding suggested that this may be feasible (Appendix 3). However, this needs to be confirmed with extrusion, injection moulding and more accurate tensile testing. Low strength, stiffness and water resistance are the main disadvantages associated with bioplastics when compared to synthetic plastics. If these could be improved then bioplastics could be used in a wider range of applications where synthetic plastics are conventionally used.

Life cycle assessment studies on NTP have been completed [204, 205]. However these should be updated to include treating bloodmeal with peracetic acid. Finally investigations into how PAA treatment affects the bioplastics compostability should be investigated. NTP degrades within twelve weeks when composted and it would be interesting to see if the sanitising effect of PAA treatment had any impact on degradation time.

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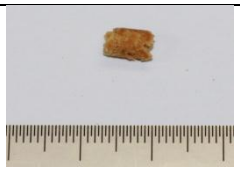
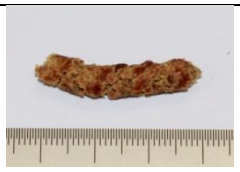
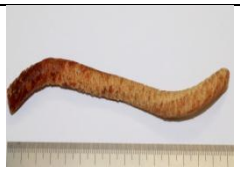
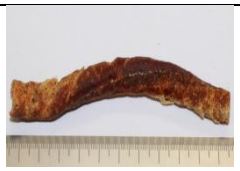
Appendix 1: Summary of Extrusion and Injection Moulding Trials

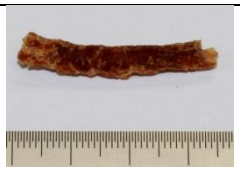
Table 56: Full extrusion and injection moulding summary.

Additives (pph_{bm})	**Conditions (extruder temperature profile °C, screw speed RPM)**	**Observations Comments**	**Photo (scale: cm)**
60 water, 10 TEG, 10 urea, 3 sds, 3 sodium sulphite	Temp: 120, 100, 100, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM Acetic acid not neutralised	
60 water, 10 TEG, 10 urea, 3 sds, 3 sodium sulphite	Temp: 130, 110, 110, 110, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM Acetic acid not neutralised	
60 water, 10 TEG, 10 urea, 3 sds, 3 sodium sulphite	Temp: 120, 100, 100, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM Acetic acid not neutralised	
60 water, 10 TEG, 10 urea, 3 sds, 3 sodium sulphite	Temp: 130, 110, 110, 110, 80 Screw speed: 150	5mm diameter die 3% PAA treated BM Acetic acid not neutralised	

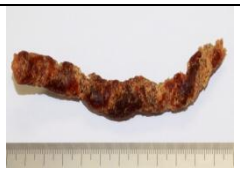
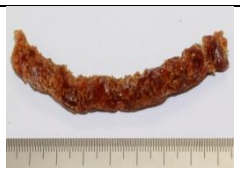
60 water, 10 TEG, 10 urea, 3 sds, 3 sodium sulphite	Temp: 120, 110, 110, 110, 90 Screw speed: 150	5mm diameter die 3% PAA treated BM Acetic acid not neutralised	
60 water, 10 TEG, 10 urea, 3 sds, 3 sodium sulphite	Temp: 120, 120, 115, 110, 90 Screw speed: 150	5mm diameter die 3% PAA treated BM Acetic acid not neutralised	
60 water, 10 TEG, 10 urea, 6 sds, 3ss	Temp: 120, 110, 110, 105, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM Acetic acid not neutralised	
60 water, 10 TEG, 10 urea, 6 sds, 3ss	Temp: 130, 110, 110, 110, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM Acetic acid not neutralised	
60 water, 10 TEG, 10 urea, 6 sds, 3ss	Temp: 130, 110, 110, 110, 70 Screw speed: 125	5mm diameter die 3% PAA treated BM Acetic acid not neutralised	
60 water	Temp: 120, 100, 100, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM Acetic acid neutralised for all samples from here	

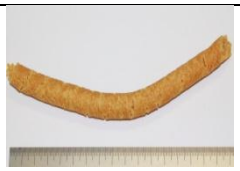
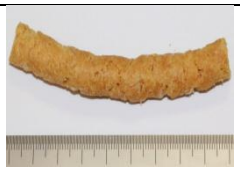
60 water, 10 urea	Temp: 120, 100, 100, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 10 urea, 10 salt	Temp: 120, 100, 100, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 10 urea, 10 salt	Temp: 120, 105, 105, 105, 95 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 10 urea, 20 sodium silicate	Temp: 120, 100, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 5 borax	Temp: 130, 110, 105, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 urea, 15 sds, 20 sodium silicate, 20 polyethylene glycol, blue pigment	Temp: 120, 100, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 10 urea, 20 sodium silicate	Temp: 120, 100, 100, 100, 75 Screw speed: 100	10mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 10 urea, 20 sodium silicate	Temp: 120, 100, 100, 100, 75 Screw speed: 140	10mm diameter die 3% PAA treated BM	
60 water, 5 sodium silicate	Temp: 120, 100, 100, 100, 70 Screw speed: 140	10mm diameter die 3% PAA treated BM	

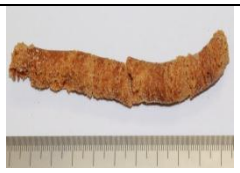
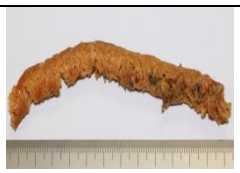
60 water, 10 sodium silicate	Temp: 120, 100, 100, 100, 70 Screw speed: 140	10mm diameter die 3% PAA treated BM	
60 water, 15 sodium silicate	Temp: 120, 100, 100, 100, 70 Screw speed: 140	10mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 10 urea, 1.5 sds, 10 sodium silicate	Temp: 120, 100, 100, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 5 TEG, 15 sodium silicate	Temp: 120, 100, 100, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 15 sodium silicate	Temp: 120, 100, 100, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 15 sodium silicate	Temp: 120, 100, 100, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 10 urea, 5 borax	Temp: 125, 110, 110, 105, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 10 urea, 5 borax	Temp: 125, 110, 110, 105, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM extruded, granulated and re-extruded	

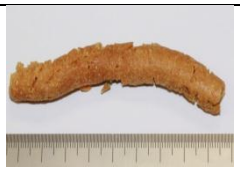
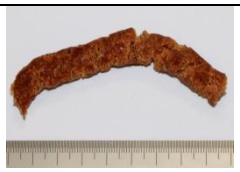
60 water, 10 borax	Temp: 120, 100, 100, 100, 70 Screw speed: 150	5 mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 10 borax	Temp: 130, 110, 110, 105, 70 Screw speed: 150	5 mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 3 sds, 5 borax	Temp: 130, 110, 110, 105, 70 Screw speed: 150	5 mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 5 borax, 5 silicate	Temp: 120, 100, 100, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 5 borax	Temp: 120, 100, 100, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 5 borax	Temp: 125, 110, 105, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
45 glycerol, 5 borax	Temp: 125, 110, 105, 100, 70 Screw speed: 80	5mm diameter die 3% PAA treated BM	
45 glycerol, 5 borax	Temp: 125, 110, 105, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 5 borax, 20 guar gum	Temp: 125, 110, 105, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	

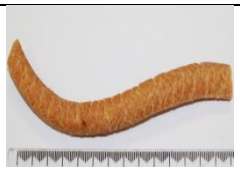
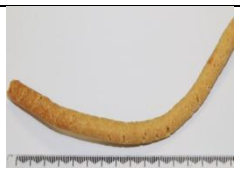
60 water, 15 TEG, 5 borax, 20 guar gum	Temp: 125, 120, 110, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 5 borax, 10 silicate	Temp: 120, 100, 100, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 5 borax, 10 silicate	Temp: 125, 115, 110, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 5 borax, 10 silicate	Temp: 130, 120, 110, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 10 silicate, 3 centrimonium chloride	Temp: 130, 120, 110, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 5 borax, 10 silicate, 20 guar gum	Temp: 130, 120, 110, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 5 borax, 20 guar gum	Temp: 140, 130, 110, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 5 borax, 20 guar gum	Temp: 140, 120, 110, 100, 70 Screw speed: 150	5mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 5 borax	Temp: 140, 120, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	

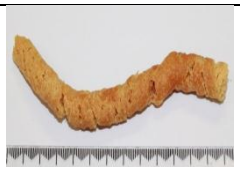
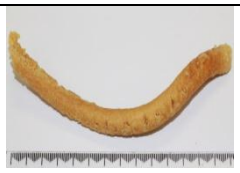
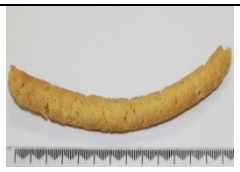
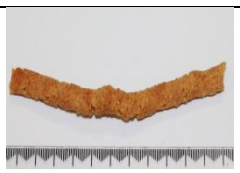
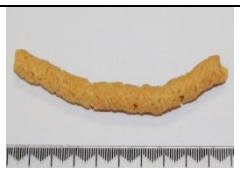
60 water, 15 TEG, 10 borax	Temp: 140, 130, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 5 borax, 2.5 silicate	Temp: 140, 130, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 5 borax, 5 silicate	Temp: 140, 130, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 10 borax, 2.5 silicate	Temp: 140, 130, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 10 borax, 5 guar gum	Temp: 140, 130, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 10 borax, 5 sodium silicate	Temp: 140, 130, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 5 urea, 10 borax	Temp: 140, 130, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 15 borax	Temp: 140, 130, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	

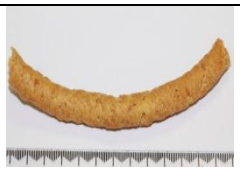
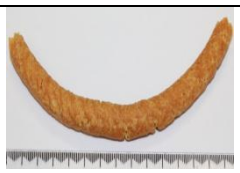
60 water, 15 TEG, 15 borax	Temp: 140, 130, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM extruded, granulated and re-extruded	
30 water, 30 TEG, 10 urea, 3 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 10 urea, 3 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM Extruded, granulated and injection moulded Injection temp: 125, 105, 100, 100, 80	
25 water, 30 TEG, 6 sds	Temp: 130, 105, 100, 100, 80 Screw speed: 100	10mm diameter die 3% PAA treated BM	
25 water, 6 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
25 water, 6 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 100	10mm diameter die 3% PAA treated BM	
25 water, 15 TEG, 6 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	

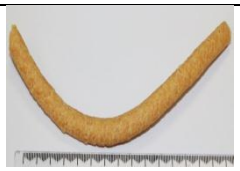
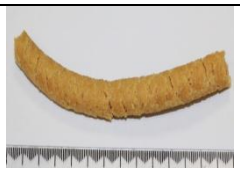
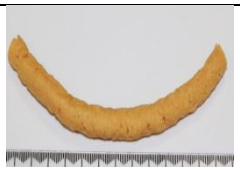
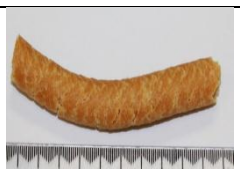
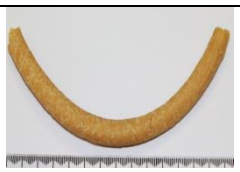
40 water, 10 TEG, 10 borax, 1.5 silicate	Temp: 140, 135, 115, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 triacetine, 3ss, 10 borax	Temp: 125, 120, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
50 water, 10 TEG, 5 borax, 1 sodium silicate	Temp: 125, 120, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
10 water, 10 TEG, 3 sodium sulphite, 10 borax	Temp: 125, 120, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
50 water, 10 TEG, 5 borax	Temp: 125, 125, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
40 water, 10 TEG, 10 borax, 1.5 sodium silicate	Temp: 135, 130, 115, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
40 water, 10 TEG, 10 borax, 1.5 sodium silicate	Temp: 130, 125, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 5 borax	Temp: 130, 125, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 TEG, 10 borax	Temp: 130, 120, 105, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	

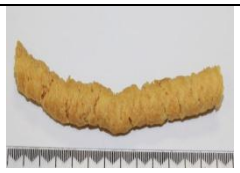
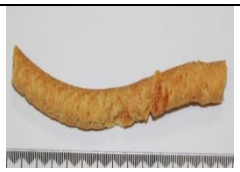
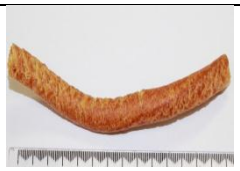
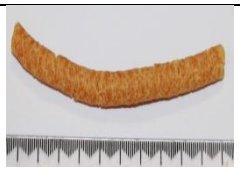
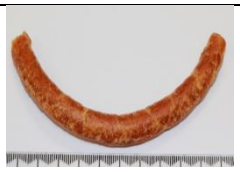
60 water, 10 triacetine, 10 borax, 3 sodium sulphite	Temp: 130, 125, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
50 water, 5 borax, 1 sodium silicate	Temp: 125, 125, 115, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 triacetine, 10 borax, 3 sodium sulphite	Temp: 135, 125, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 10 triacetine, 10 borax, 3 sodium sulphite	Temp: 120, 100, 100, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 10 borax, 2.5 sodium silicate	Temp: 145, 140, 135, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 10 borax, 2.5 sodium silicate	Temp: 140, 140, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 10 borax, 2.5 sodium silicate	Temp: 145, 140, 120, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 15 TEG, 10 borax, 2.5 sodium silicate	Temp: 145, 140, 130, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
60 water, 5 TEG, 5 borax	Temp: 140, 130, 110, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	

25 water, 30 TEG, 6 sds, 5 borax	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
25 water, 30 TEG, 6 sds	Temp: 120, 100, 100, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
40 water, 30 TEG, 6 sds	Temp: 120, 100, 100, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 10 urea, 3 sds, 3 sodium sulphite	Temp: 120, 100, 100, 100, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 10 urea, 3 sds, 3 sodium sulphite	Temp: 130, 110, 105, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 3 sds, 3 sodium sulphite, 3 borax	Temp: 130, 115, 105, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 3 sds, 3 sodium sulphite, 3 borax	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 30 triacetine, 10 urea, 3 sds, 3 sodium sulphite	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
40 water, 30 TEG, 5 borax	Temp: 140, 130, 115, 110, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	

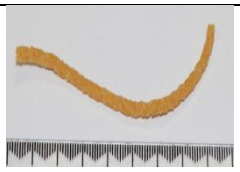
40 water, 30 TEG, 10 urea, 3 sds	Temp: 140, 130, 115, 110, 70 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 10 TEG, 10 urea, 3 sds	Temp: 140, 130, 115, 110, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 10 urea, 3 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 10 urea, 3 sds	Temp: 155, 140, 120, 100, 80 Screw speed: 150	5mm diameter die 3% PAA treated BM	
40 TEG, 10 urea, 3 sds	Temp: 155, 140, 120, 100, 80 Screw speed: 150	5mm diameter die 3% PAA treated BM	
30 TEG, 10 urea, 3 sds	Temp: 155, 140, 120, 100, 80 Screw speed: 150	5mm diameter die 3% PAA treated BM	
20 water, 30 TEG, 5 borax	Temp: 130, 110, 105, 100, 80 Screw speed: 150	5mm diameter die 3% PAA treated BM	
20 water, 30 TEG, 10 urea, 3 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	5mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 10 urea, 4.5 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 120	5mm diameter die 3% PAA treated BM	

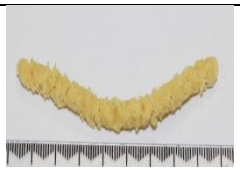
30 water, 30 TEG, 4.5 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
20 water, 30 TEG, 4.5 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 10 urea, 3 sds	Temp: 140, 130, 115, 110, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
40 TEG, 5 borax	Temp: 155, 140, 120, 100, 80 Screw speed: 150	5mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 3 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	5mm diameter die 3% PAA treated BM	
20 water, 30 TEG, 5 borax	Temp: 145, 130, 110, 100, 80 Screw speed: 150	5mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 10 urea	Temp: 125, 105, 100, 100, 80 Screw speed: 150	5mm diameter die 3% PAA treated BM	
20 water, 30 TEG, 4.5 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
20 water, 30 TEG, 6 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	

20 water, 30 TEG, 4.5 sds, 5 stearic acid	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 4.5 sds, 5 stearic acid	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
30 water, 30 TEG, 4.5 sds, 5 stearic acid	Temp: 140, 115, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
25 water, 30 TEG, 6 sds, 5 borax	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
25 water, 30 TEG, 6 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 100	5mm diameter die 3% PAA treated BM	
25 water, 30 TEG, 6 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 75	5mm diameter die 3% PAA treated BM	
25 water, 30 TEG, 6 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 80	5mm diameter die 3% PAA treated BM	
25 water, 30 TEG, 10 urea, 3 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 100	10mm diameter die 3% PAA treated BM	
25 water, 30 TEG, 10 urea, 3 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	

25 water, 5 urea, 6 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 100	10mm diameter die 3% PAA treated BM	
25 water, 5 urea, 6 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	
25 water, 15 TEG, 6 sds	Temp: 125, 105, 100, 100, 80 Screw speed: 100	10mm diameter die 3% PAA treated BM	
25 water, 30 TEG, 6 sds	Temp: 125, 110, 100, 100, 80 Screw speed: 120	10mm diameter die 3% PAA treated BM	
25 water, 30 TEG, 6 sds	Temp: 125, 110, 100, 100, 80 Screw speed: 120	10mm diameter die 3% PAA treated BM extruded, granulated and re-extruded	
25 water, 30 TEG, 6 sds	Temp: 125, 110, 100, 100, 80 Screw speed: 120	5mm diameter die 3% PAA treated BM	
25 water, 30 TEG, 6 sds	Temp: 120, 100, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM	

40 water, 30 TEG, 6 sds	Temp: 120, 100, 100, 100, 80 Screw speed: 150	10mm diameter die 3% PAA treated BM Extruded, granulated and injection moulded Injection temp: 120, 100, 100, 100, 70	
50 water, 30 TEG, 3 sds	Temp: 125, 110, 100, 100, 80 Screw speed: 150	10mm diameter die 4% PAA treated BM	
25 water, 30 TEG, 6 sds	Temp: 125, 110, 100, 100, 80 Screw speed: 150	10mm diameter die 4% PAA treated BM	
50 water, 40 TEG, 6 sds	Temp: 125, 110, 100, 100, 80 Screw speed: 150	10mm diameter die 4% PAA treated BM	
50 water, 40 TEG, 6 sds	Temp: 125, 110, 100, 100, 80 Screw speed: 150	10mm diameter die 4% PAA treated BM Extrude, granulate and injection mould Injection temp: 120, 115, 100, 100, 100	
50 water, 40 TEG, 6 sds	Temp: 120, 110, 100, 100, 80 Screw speed: 125	10mm diameter die 3% PAA treated BM	

50 water, 40 TEG, 6 sds	Temp: 120, 110, 100, 100, 80 Screw speed: 125	10mm diameter die 3% PAA treated BM Extrude, granulate and injection mould Injection temp: 120, 115, 100, 100, 100	
50 water, 40 TEG, 6 sds	Temp: 120, 110, 100, 100, 80 Screw speed: 125	10mm diameter die 3% PAA treated BM Extrude, granulate and injection mould (straight from nozzle) Injection temp: 120, 115, 100, 100, 100	
50 water, 20 TEG, 6 sds	Temp: 135, 115, 100, 100, 80 Screw speed: 150	5mm diameter die 4% PAA treated BM	
50 water, 20 TEG, 6 sds	Temp: 135, 115, 100, 100, 80 Screw speed: 150	Capillary tube (approx. 3mm diameter) 4% PAA treated BM	

50 water, 20 TEG, 6 sds	Temp: 135, 115, 100, 100, 80 Screw speed: 150	5mm diameter die 4% PAA treated BM Extrude, granulate and injection mould Injection temp: 120, 115, 100, 100, 10	
50 water, 20 TEG, 6 sds	Temp: 135, 115, 100, 100, 80 Screw speed: 150	5mm diameter die 5% PAA treated BM	
50 water, 20 TEG, 6 sds	Temp: 135, 115, 100, 100, 80 Screw speed: 150	5mm diameter die 5% PAA treated BM Extrude, granulate and injection mould Injection temp: 120, 115, 100, 100, 100	
50 water, 20 TEG, 6 sds	Temp: 135, 115, 100, 100, 80 Screw speed: 150	5mm diameter die 5% PAA treated BM Extrude, granulate and injection mould (straight from nozzle) Injection temp: 120, 115, 100, 100, 100	

Appendix 2: Properties of Unconditioned Samples Produced from 4% PAA Treated Bloodmeal

Table 57: Mechanical properties of unconditioned injection moulded samples produced from four percent PAA treated bloodmeal with different water, TEG and SDS combinations.

Formulation	SDS (pph_{BM})	Water (pph_{BM})	TEG (pph_{BM})	Young's Modulus (MPa)	Standard Error	UTS (Mpa)	Standard Error	Strain at Break (%)	Standard Error	Toughness (Mpa)	Standard Error
1	6	30	10	#N/A							
2	6	40	10	20.77	0.29	2.12	0.03	61.44	1.85	0.45	0.04
3	6	50	10	20.72	0.60	2.10	0.05	76.70	1.30	0.60	0.02
4	6	30	20	#N/A							
5	6	40	20	13.74	0.52	1.69	0.03	80.82	2.77	0.49	0.05
6	6	50	20	9.74	0.16	1.25	0.05	66.78	2.29	0.26	0.03
7	6	30	30	12.89	0.27	1.47	0.05	55.54	1.72	0.30	0.03
8	6	40	30	9.49	0.07	1.26	0.02	60.90	1.24	0.25	0.02
9	6	50	30	7.91	0.10	0.94	0.02	48.38	0.75	0.13	0.01
10	3	30	10	#N/A							
11	3	40	10	#N/A							
12	3	50	10	39.08	4.34	2.41	0.05	58.90	2.67	0.54	0.09
13	3	30	20	27.89	1.85	2.33	0.10	56.12	1.90	0.46	0.05
14	3	40	20	24.16	0.57	2.30	0.02	73.68	0.62	0.61	0.02
15	3	50	20	17.84	0.75	1.70	0.02	69.80	2.61	0.38	0.04
16	3	30	30	20.20	0.73	2.09	0.03	67.38	1.43	0.49	0.03
17	3	40	30	15.19	0.59	1.50	0.05	60.76	2.99	0.31	0.05
18	3	50	30	11.24	0.47	1.25	0.04	61.34	1.22	0.24	0.01
19	0	30	10	#N/A							
20	0	40	10	70.56	4.71	3.02	0.11	43.14	2.51	0.56	0.10
21	0	50	10	48.52	3.94	2.29	0.06	46.46	2.06	0.43	0.06
22	0	30	20	#N/A							
23	0	40	20	26.98	1.44	1.75	0.13	46.06	3.44	0.30	0.06
24	0	50	20	20.09	0.88	1.64	0.06	61.70	2.15	0.37	0.05
25	0	30	30	21.54	1.39	1.45	0.08	38.50	2.01	0.16	0.04
26	0	40	30	16.55	0.37	1.30	0.04	43.62	2.56	0.20	0.03
27	0	50	30	14.08	0.40	1.09	0.03	35.42	0.57	0.14	0.01

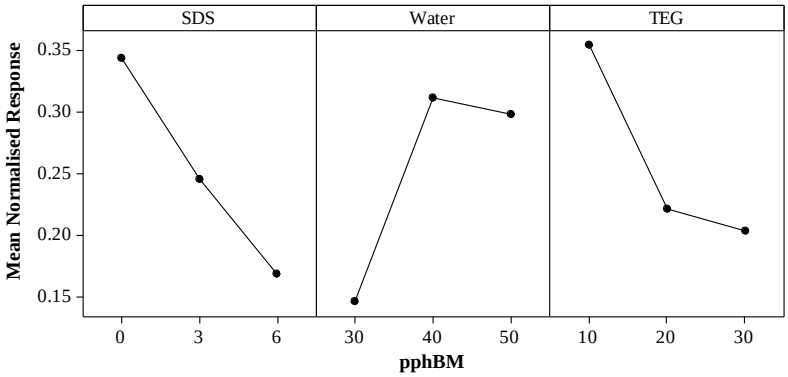


Figure 94: Main effects of SDS, water and TEG on Young's modulus of unconditioned injection moulded samples.

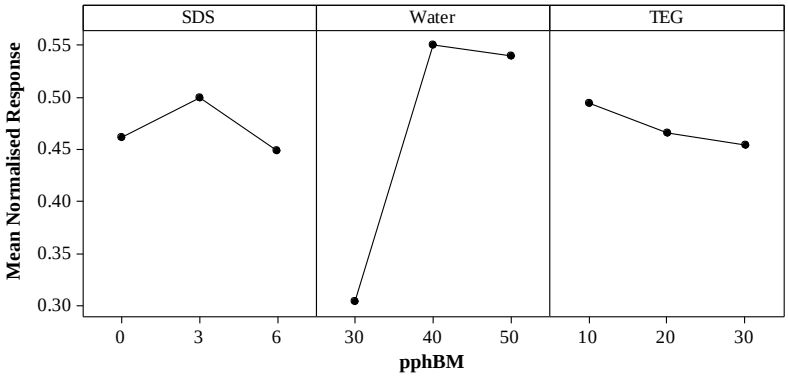


Figure 95: Main effects of SDS, water and TEG on UTS of unconditioned injection moulded samples.

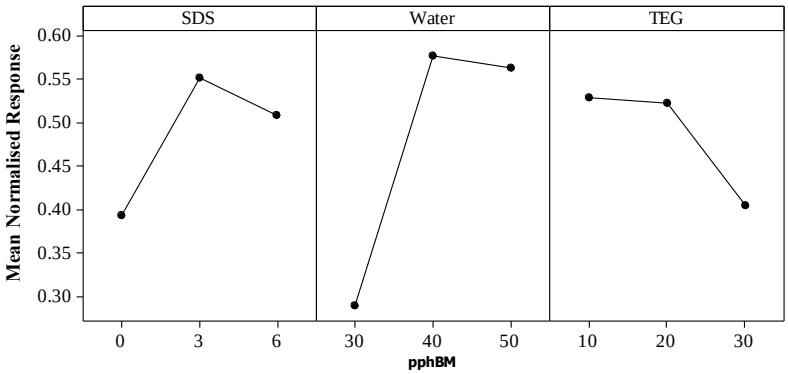


Figure 96: Main effects of SDS, water and TEG on toughness of unconditioned injection moulded samples.

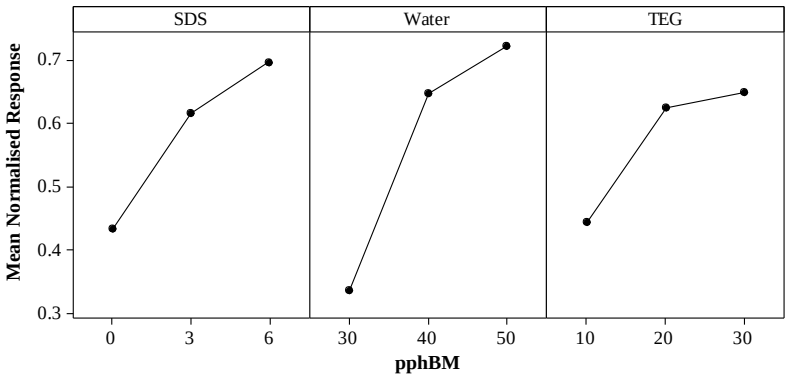


Figure 97: Main effects of SDS, water and TEG on strain at break of unconditioned injection moulded samples.

Appendix 3: Mechanical Properties of 4% PAA Treated Bloodmeal with Clay, Borax and Sodium Silicate

Method

Sodium dodecyl sulphate (SDS) and clay were premixed with water before adding to 4% PAA treated bloodmeal. Reagents were mixed in the following proportions: 3 g SDS and 1, 3 or 5 g additive (borax, sodium silicate, sodium bentonite or octodecylamine bentonite) was mixed with 15 g distilled water, heated to 60°C, and added to 100 g treated bloodmeal. This was mixed using a high speed mixer for 5 minutes, before 20 g of triethylene glycol (TEG) was added and mixed for a further five minutes. The mixture was transferred to a sealable plastic bag and stored overnight.

Compression molding

Bioplastic sheets were formed by compression moulding 50 g of the mixture at 110°C (top and bottom plate). 10 tonnes of pressure was applied for five minutes. Heating was turned off after five minutes and the mould was left under 10 tonnes of pressure for a further five minutes. The pressure was released, the sheet removed and left to cool.

Tensile test

Compression moulded sheets were laser cut into type 1 dog bone tensile test specimens. The tensile test specimens were conditioned for seven days in a conditioning chamber at 50% humidity. The mechanical properties were analysed using a Lloyd Tensile Tester LR 30K with a 500 N load cell at 5 mm/min.

X-ray diffraction

Sections of compression moulded sheets were powderised using an IKA MF10 basic Microfine grinder and clay-protein or additive-protein interactions analysed using a Phillip's X-ray diffractometer at low angle configuration of $2\theta = 2^{o}$ to 12^{o}, a scanning rate of $2\theta = 2^{o}min^{-1}$, operating at a current of 40 mA and a voltage of 40 kV using $CuK\alpha_1$ radiation.

Results

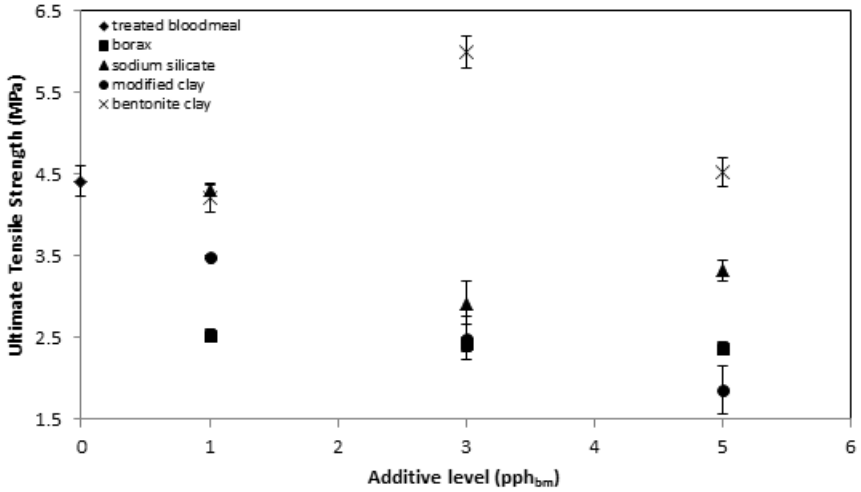


Figure 98: UTS at different clay/additive levels.

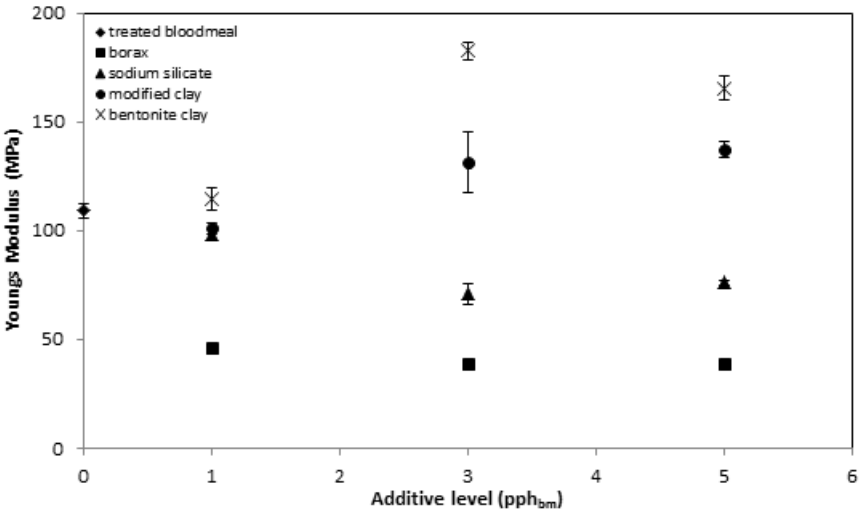


Figure 99: Young's modulus at different clay/additive levels.

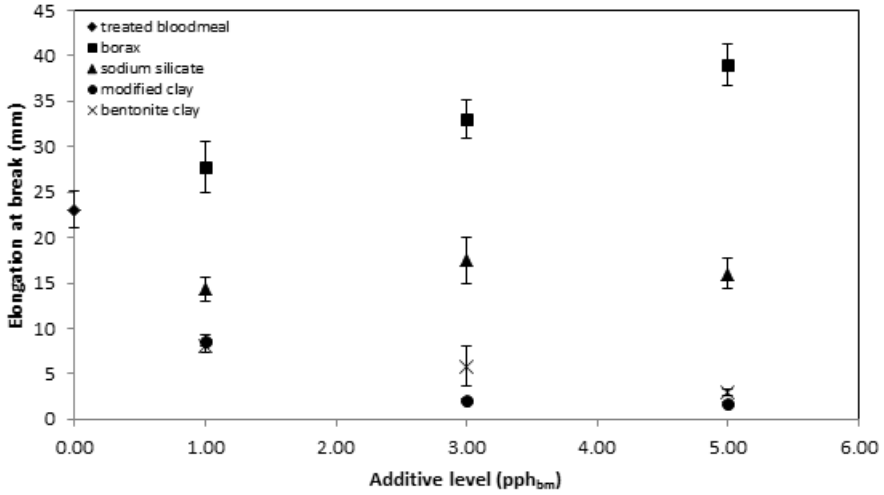


Figure 100: Extension at break at different clay/additive levels.

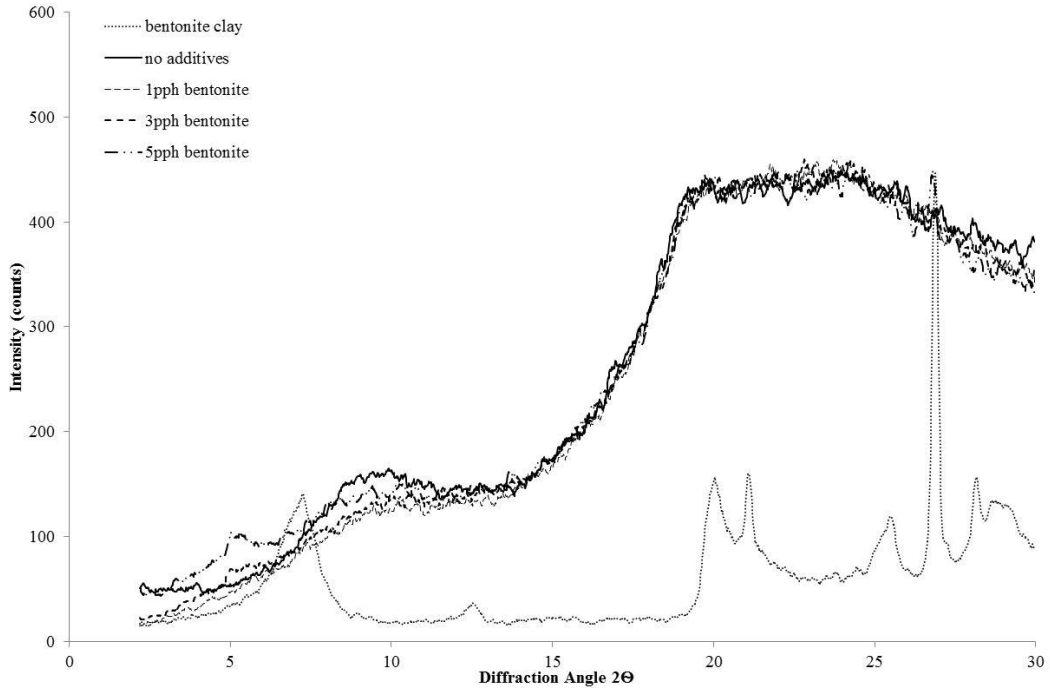


Figure 101: x-ray diffraction pattern for bentonite clay and 4% PAA treated bloodmeal composite.

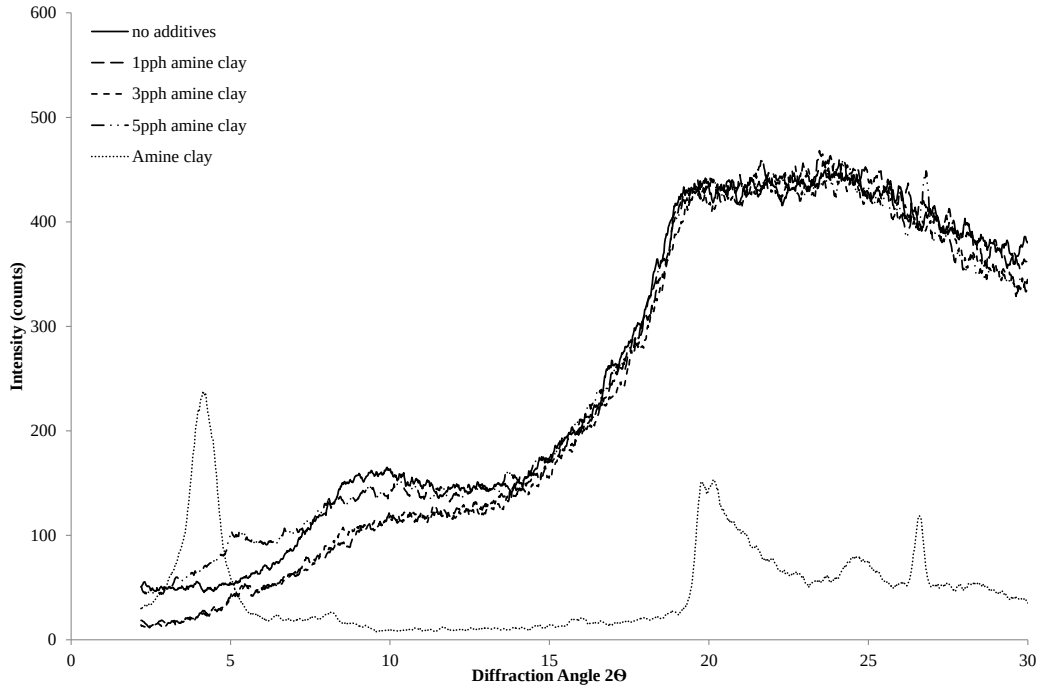


Figure 102: x-ray diffraction pattern for amine modified clay and 4% PAA treated bloodmeal composite.

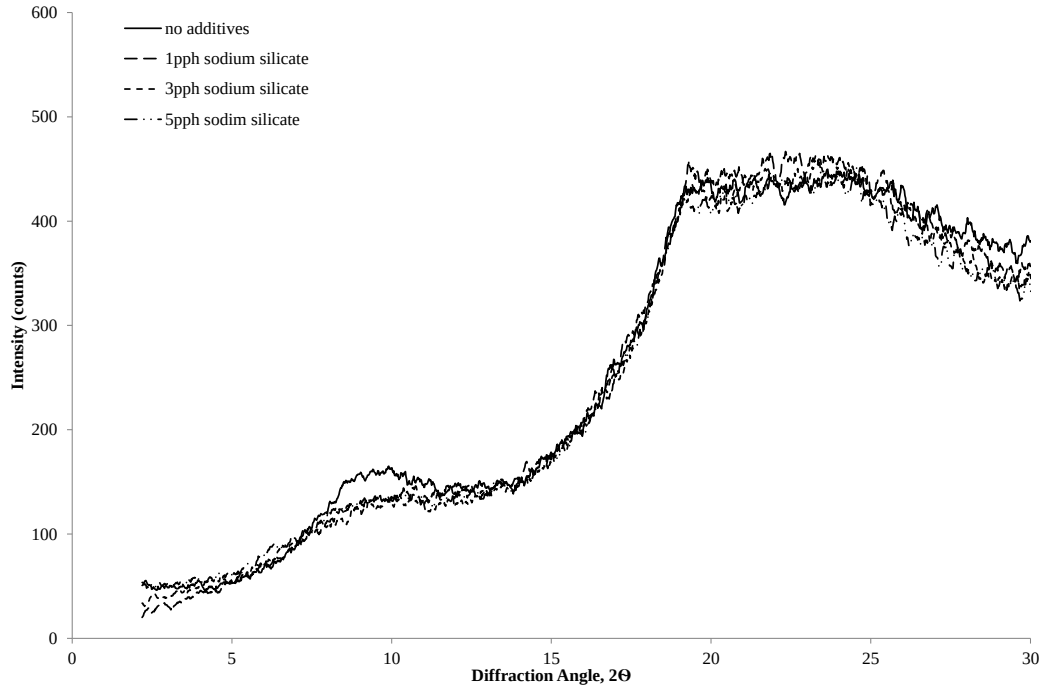


Figure 103: x-ray diffraction pattern for sodium silicate and 4% PAA treated bloodmeal bioplastic.

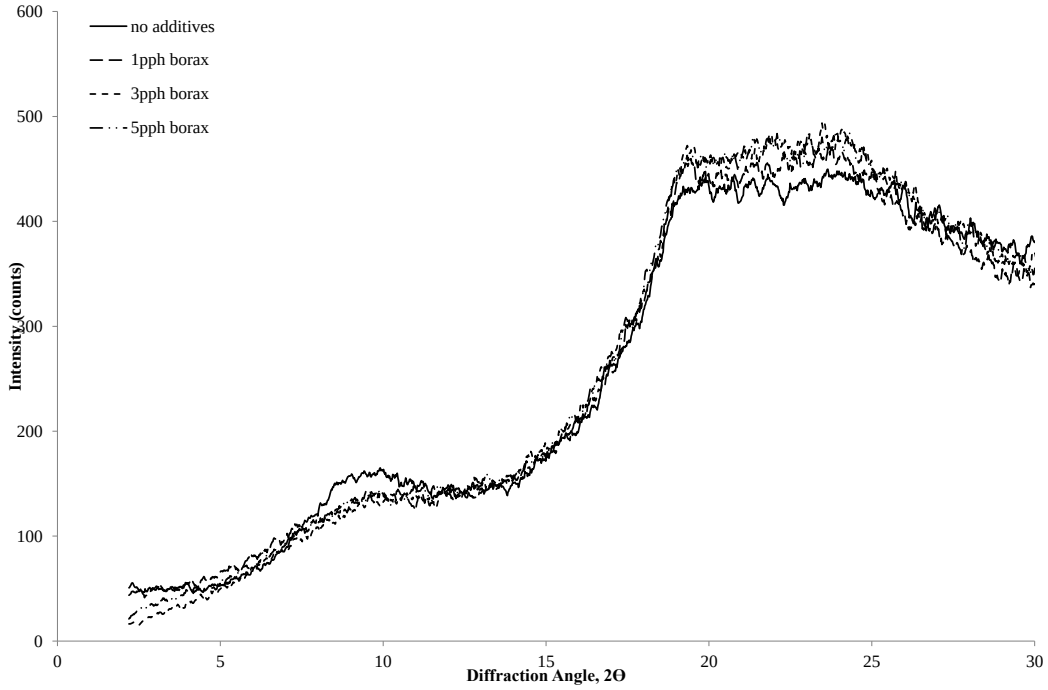


Figure 104: x-ray diffraction pattern for borax and 4% PAA treated bloodmeal bioplastic.