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Development of Food-grade Microparticles from Lignin

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

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Abstract

An opportunity presented itself to develop a new food ingredient from the wood biopolymer lignin. Lignin is set to become a high-yielding and inexpensive by-product of lignocellulosic biorefining processes. The aromatic heteropolymer is water insoluble, relatively hydrophobic, non-digestible in its native form, and also considered to be non-toxic. Retention of these properties in isolated lignin may enable a microparticulate food ingredient, with particular use as a fat mimetic, to be developed.

Lignin was extracted from shrub willow using an organosolv pulping procedure. A reactor vessel suitable for pulping under organosolv conditions was obtained, modified and commissioned. Using central composite design, an ethanol concentration of 60% (v/v), extraction time of 132 minutes and extraction temperature of 195°C were found to extract lignin of the highest yield and purity. The total lignin content of lignin extracted from chipped, untreated willow under these conditions was 96.53 ± 0.10% (w/w).

An anti-solvent precipitation technique was explored for the ability to produce lignin microparticles. An aqueous-organic solution of lignin was dispersed into a much larger volume of water, whereby leaching of organic solvent from the dispersed phase into the water resulted in progressive precipitation of the lignin solute, ultimately producing a suspension of solid microparticles. Temperature and addition of surfactant were investigated as a means of controlling particle properties. Increasing initial water temperature between 4 and 80°C produced increasingly large agglomerates of uniform sub-micron primary particles, increasingly fused into monolithic masses. Faster removal of ethanol, hydrophobic interactions or an increase in rate of diffusion limited coalescence may be responsible for the increased fusion at higher temperatures.

Incorporating the ionic surfactant sodium dodecyl sulfate (SDS) or cetyltrimethylammonium bromide (CTAB) in the water at temperatures of 20-60°C during anti-solvent precipitation produced spherical, monodisperse lignin particles in the range 0.1-0.2 μm. However, concentrations of these surfactants greater than 1% (w/v) caused aggregation of primary particles, probably due to bridging between bilayers of surfactant at high concentration. The non-ionic surfactants Tween 20 and sucrose ester were unable to prevent particle fusion at the 60°C preparation temperature, indicating that the electrostatic repulsion between particles coated with SDS and CTAB is important to stability.

The ability to manipulate the size of SDS-stabilised particles using added salt and adjustment of pH was determined. The electrostatic mechanism of stabilisation by SDS was confirmed by the ability of added potassium chloride and calcium chloride to destabilise lignin particles. Measured particle size increased with the concentration of added salt, due to either greater fusion of lignin during particle formation or to aggregation of primary particles, depending on whether the salt was added before or after particle formation. Decreasing the pH of the suspension of particles to 1.5, which is lower than the pKa of SDS, caused primary particles to aggregate. For destabilisation with salt and pH, generally only the relative volumes of particles in two modal distributions (~0.1-2 μm and ~1-100 μm) appeared to change. This indicates that the ability to control particle size may be limited by aggregation kinetics. Adjustment and control of pH throughout particle formation may enable the preparation of particles in the intermediate size range, such as narrowly distributed 1-10 μm particles. The ability to prepare particles of various and controlled size is important for maximising industry applications.

To highlight a possible application of food-grade lignin microparticles, the performance of particles as Pickering stabilisers of oil-in-water emulsions was evaluated. A literature method of emulsion formation involving the redispersion of dried lignin particles and various pH-modification steps, and a method using lignin in the form of freshly prepared microparticle suspensions were explored. Both methods produced emulsions stable to coalescence for five months of chilled storage, although the pH-modification method produced slightly smaller (D[3,2] of 2.7-11.9 μm) oil droplets than the fresh suspension method (D[3,2] of 11.6-31.2 μm). It may be possible in the future to produce smaller emulsion droplets by using a higher energy emulsification technique and by utilising sub-micron lignin particles, rather than ~10 μm particles.

Three main areas in which work could be carried out to move this project forward have been identified. These areas are:

1. Lignin characterisation – including molecular weight, chromatography to determine lignin fragment types and contact angle.
2. Control of particle size – including the investigation of more food-grade surfactants, ultrasonic mixing and controlled pH changes during anti-solvent precipitation.

3. Food applications – including further investigation into Pickering emulsions, foam stabilisation and fat mimetic applications.

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Table of Contents

List of Tables

List of Figures

List of Equations

List of Appendices

Chapter One. Introduction

Concerns regarding long-term sustainability within the food chain have seen a growing interest in the development of alternative supplies of food ingredients and materials that are usually discarded as part of waste streams processing. Materials that would normally be classed as inedible are increasingly being given consideration as potential sources of extractable nutrients, bioactive components, and functional ingredients. Furthermore, high rates of obesity, including in New Zealand, highlight the need for low or zero calorie ingredients that can replace sugar or fat in foods while still maintaining consumer acceptance. Opportunities exist for new food ingredients that can address these sustainability and health challenges simultaneously.

Lignin is an amorphous, three-dimensional, aromatic heteropolymer consisting of variously linked phenylpropanoid units (Klein, Beach, Emerson, & Zimmerman, 2010) and is found in the woody stems of hardwoods and softwoods, and in all vascular plants (Sarkanen & Ludwig, 1971; Wool, 2005). The system of 3D polymers permeates membranous polysaccharides and the spaces between plant cell walls, which strengthens the cells and allows plants to grow larger (Gellerstedt & Henriksson, 2008; Pearl, 1967). Lignin also has numerous other roles in plants, such as controlling water permeation across cell walls, providing antioxidant activity and impeding the penetration of destructive enzymes (Doherty, Mousavioun, & Fellows, 2011; Janshekar & Fiechter, 1983).

Traditionally, lignins have been obtained as by-products of the pulping and paper-making industry. The three major chemical pulping processes are the sulphite, kraft and soda processes, with each process producing lignin with different properties. Although it is estimated that over 70 million tonnes of lignin is extracted in these pulping operations every year, about 98% of this lignin is burnt as a fuel to recover energy in these processes (Lora, 2008). Thus an opportunity exists to utilise this lignin for higher value applications.

The last few decades have seen increased interest in the use of lignocellulosic biomass to produce energy and other products as an alternative to fossil fuel (González Alriols,

Garcia, Llano-ponte, & Labidi, 2010). For example, there has been increasing interest in the enzymatic digestion of starch or sucrose to produce ethanol as an alternative liquid fuel (Doherty et al., 2011). The concept of lignocellulosic biorefineries has also gained much attention. The major components of lignocellulosic biomass are cellulose, hemicellulose and lignin. Figure 1 shows that in the lignocellulosic biorefinery concept, not only is ethanol produced for use as a fuel from the cellulose component of biomass, but high quality products from lignin and hemicellulose are also generated (Kamm, Kamm, Gruber, & Kromus, 2006). The utilisation of all three major components to make high-value products makes the conversion of biomass to fuel more economic (Doherty et al., 2011). It is likely that as more biorefineries are set up, lignin will be available that could find use in new applications.

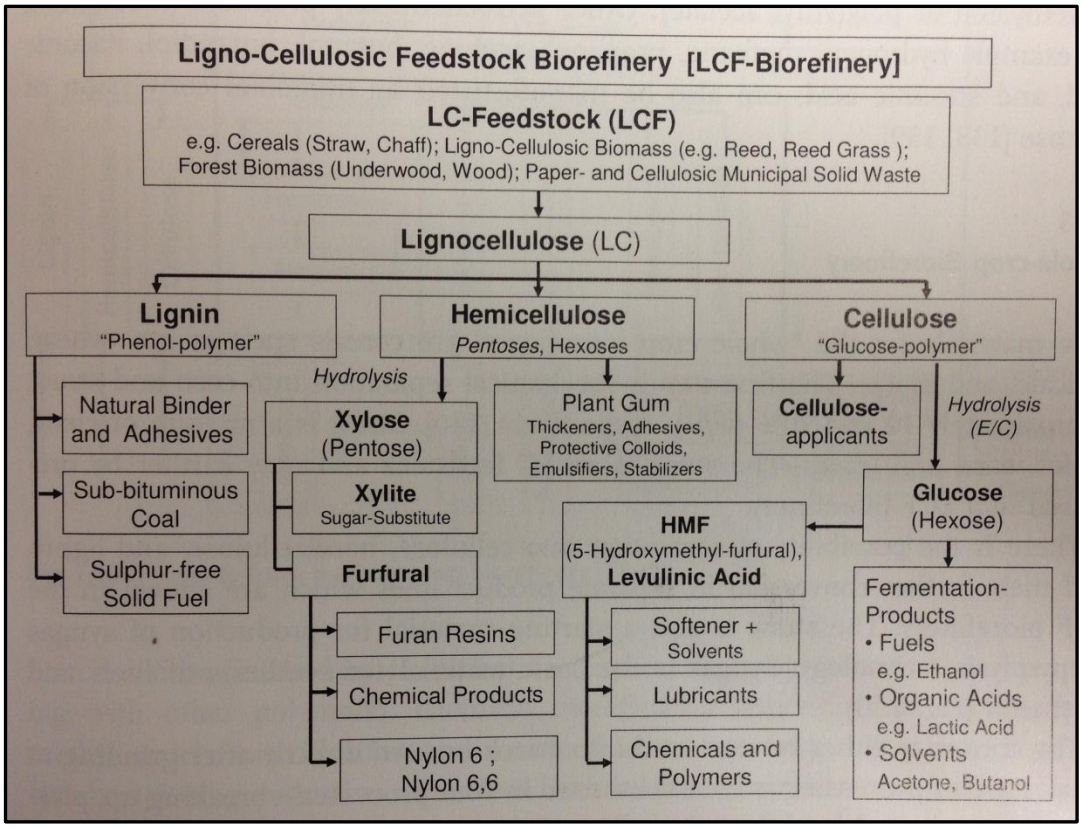


Figure 1. Products of a lignocellulosic biorefinery (Kamm et al., 2006).

Gandini & Belgacem (2008) and Doherty et al. (2011) suggest many applications for which lignin has great potential. This is not only due to large availability at modest prices, but also because of the ability of lignin to undergo chemical modifications, chain extension and polymerisation (Gandini & Belgacem, 2008). Lignins also have good rheological and viscoelastic properties, good film forming ability, small particle size and can be either hydrophilic or hydrophobic depending on origin (Doherty et al., 2011). These, among other attributes, allow lignin to be used as components of a wide range of polymer blends or as fillers in other polymers.

The properties of lignin may also enable it to be used as a food ingredient. Lignin is indigestible as part of human and animal diets (Eastwood & Kritchevsky, 2005) and also insoluble in water. Its indigestibility could present an opportunity to use lignin as an innovative bulking agent in foods. In particular, lignin could be used as a non-caloric fat mimetic, through the formation of edible microparticles that emulate the perception of emulsified fat. Microparticulate ingredients are able to emulate emulsified fats because of the similarity in size, shape and smoothness of the particles to fat globules. The microparticles must also be insoluble in water in order to be detected in the mouth. Microparticulate fat mimetics allow consumers to enjoy creamy-textured foods without the associated calories.

The relatively high hydrophobicity of lignin may also enable the microparticles to act as solid particle stabilisers of food emulsions or foams. Solid particles are able to stabilise emulsions against coalescence, often for very long periods of time, by coating the oil-water interface and providing a steric barrier to coalescence (Binks, 2002; Dickinson, 2012). Although a lot of recent research has been carried out in the area of solid particle stabilisers, it has mostly involved inorganic particles that have often been chemically modified to improve functionality as stabilisers (Dickinson, 2012). There is an opportunity for microparticles or nanoparticles that are food-grade and naturally effective as stabilisers. High performance as stabilisers without the need for surface modification is an advantage as it reduces the cost of ingredient manufacture.

In New Zealand and Australia, calcium lignosulfonates (40-65) are already approved for use as carriers of fat-soluble vitamins and carotenoids in water-based foods; 40-65

refers to the average molecular weight of this particular calcium lignosulfonate product, which falls between 40 and 65 kDa (Food Standards Australia New Zealand, 2011). Sodium lignosulfonates are also approved processing aids. The safety of calcium lignosulfonates (40-65) has also been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Panel on Food Additives and Nutrient Sources added to Food, but the additive has not yet been approved for use due to lack of longer term toxicity data. In the US, the FDA only permits the addition of lignosulfonates to animal feeds, where they have application as pelleting aids, binding aids, surfactants and sources of metabolisable energy. Although the use of lignins in human foods is not widely permitted, the approval in New Zealand and Australia indicates that the barriers for approval of a new ingredient from lignin in this work may not be so high. Therefore, with lignin poised to become an abundant by-product of bioethanol production, the time is right to investigate the development of a new ingredient or additive from lignin.

The specific aim of this thesis is:

- **To develop food-grade microparticles from lignin**

Table 1 shows the breakdown of this aim into research questions and specific objectives.

Table 1. Research questions and objectives

Research Question 1: Can food-grade lignin be extracted from wood?	
Objectives	**Milestones**
Select a suitable wood source for the extraction of lignin	- Reproducible lignin extraction method - Characterised lignin product
Process the wood into a form suitable for lignin extraction	
Develop a reproducible method of lignin extraction	
Identify techniques that can be used to determine the purity and yield of extracted lignin	
Research Question 2: Can the structure and functional properties of lignin be controlled?	
Objectives	**Milestones**
Evaluate methods of microparticle preparation	- Ability to produce lignin microparticles of controlled morphology and functional properties
Identify techniques that can be used to characterise microparticles, in particular in terms of size and shape	
Evaluate the effect of different processing conditions on the size and shape of microparticles	
Develop a reproducible process for the production of microparticles with specified properties	
Research Question 3: Can the lignin microparticles as Pickering stabilisers of emulsions?	
Objectives	**Milestones**
Determine whether existing methods for the preparation of lignin-stabilised oil-in-water emulsions can be used to prepare emulsions with the lignin microparticles produced in the current work	- Knowledge of Pickering emulsion preparation - Knowledge of ability of lignin to stabilise emulsions at various concentrations
Develop alternative methods for the preparation of lignin-stabilised emulsions	
Determine the effect of lignin-to-oil ratio on emulsion droplet size and stability	

Chapter Two. Background

2.1. Lignin occurrence and function

Lignin is found in softwoods (gymnosperms), hardwoods (dicotyledonous angiosperms) and grasses or annual plants (monocotyledonous angiosperms) (Pearl, 1967). The lignin content of these plants varies. For example, softwoods contain approximately 25-35% lignin, while hardwoods contain approximately 18-25% lignin (Wool, 2005). Table 2, adapted from Janshekar and Fiechter (1983), provides a summary of the lignin content of a range of lignocellulosic materials.

Table 2. Proportion of lignin in some lignocellulosic materials

Lignocellulosic material	Approximate proportion (% dry weight) of lignin
Coniferous wood	25 – 35
Deciduous wood	15 – 20
Cotton	0
Bagasse	20
Nut shells	30 – 40
Corn cobs	15
Corn stalks	35
Wheat straw	15

Besides lignin, the other components of lignocellulosic biomass are cellulose and hemicellulose. These three components exist in the structure shown in Figure 2, which shows cellulose in the form of microfibrils providing structure and strength, embedded within a matrix of hemicellulose and lignin, which provide bonding to the structure (Amidon & Liu, 2009; Doherty et al., 2011). There is evidence that the lignin is

chemically associated with both hemicellulose and cellulose (Gellerstedt & Henriksson, 2008; Wardrop, 1971).

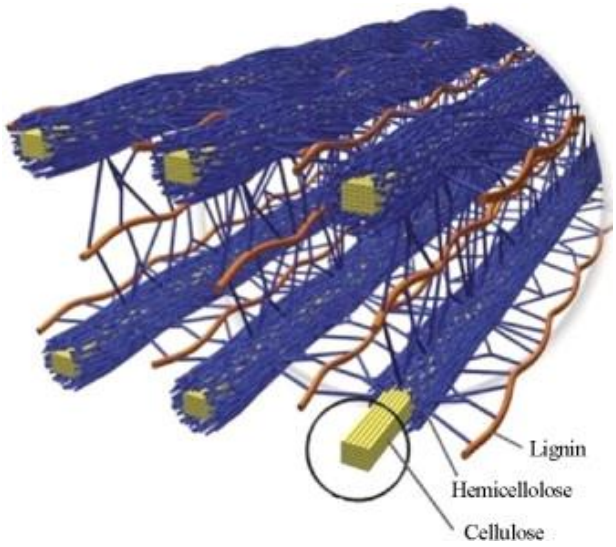


Figure 2. Cellulose strands surrounded by hemicellulose and lignin (Doherty et al., 2011).

Lignification occurs only in the cell walls of tissues with specialised functions, such as the provision of mechanical support or conduction of solutes. In fact, lignin is the major representative of encrusting cells (Wardrop, 1971). However, lignification is not only confined to cell walls, but also takes place in the roots, bark, fruit, stones, bast, pith and cork cells of wood and annual plants (Pearl, 1967). Moreover, in addition to providing structural support, lignin has many other key roles in plants, such as controlling water permeation across cell walls, providing antioxidant activity and impeding the penetration of destructive enzymes (Doherty et al., 2011; Janshekar & Fiechter, 1983).

Lignocellulosic biomass also contains up to 5% extractives. Extractives are low molecular mass compounds that are soluble in neutral organic solvents (Amidon & Liu, 2009). Cole (n.d.) suggests that wood extractives can be lipid extractive components, phenolic extractive components or other extractive components. Lipid extractive components include aliphatics, such as fatty acids, fats and waxes, and terpenoids.

Phenolic extractive components include simple phenolics, stilbenes, flavonoids and lignans. Other extractive components include alkanes, proteins, monosaccharides and derivatives. Furthermore, Cole (n.d.) suggests that although hardwood extractives contain no resin acids or monoterpenes, they contain phenolics and 60-90% fatty acids. Different solvents remove different extractives from wood. For example, dichloromethane is used to remove waxes, fats, resins, sterols and non-volatile hydrocarbons, whereas ethanol-benzene removes other dichloromethane-insoluble components, such as low-molecular-weight carbohydrates, salts, polyphenols and other water-soluble compounds (TAPPI, 2007). It may be important to remove these extractives before extracting lignin to obtain purer lignin that contains less or no extractive material.

2.2. Lignin distribution

Lignin content varies depending on a number of factors including the type of vascular plant, the type of tissue, the age of the plant and environmental factors (Janshekar & Fiechter, 1983; Wool, 2005). Available data for angiosperms (hardwoods) has shown that both lignin content and structure varies between sapwood, heartwood, tension wood, and compression wood (Sarkanen & Hergert, 1971). In many hardwood species, lignin synthesis gradually decreases during the juvenile growth period and lignin content approaches a constant in mature wood. In fact, it is suggested that most of the lignification takes place during the first year of formation (Sarkanen & Hergert, 1971). More mature trees have been found to contain more (28.3%) lignin than 1-3 year old trees (24.4-27.6%). The methoxyl content of these 1-3 year old trees is also less than normal (12.5-14.3% compared to 15% for mature wood) (Sarkanen & Hergert, 1971). Therefore, it seems that the more mature the plant selected, the higher the lignin content and the more “normal” the methoxyl content. This indicates that extracting lignin from plants of the same age is important for consistency.

It is also important to consider that lignin is more concentrated in bark than in wood (Lavoie, Capek-Menard, & Chornet, 2010; Serapiglia, Cameron, Stipanovic, & Smart, 2009). This high lignin content in bark can cause the lignin content of juvenile wood to appear higher than that of more mature wood, since the bark represents a larger volume

in young stems. Additionally, bark lignin has a lower methoxyl group content (1-2% lower) than wood species (Sarkanen & Hergert, 1971). Thus lignin extracted from wood that has not been separated from the bark may be expected to be more variable in both content and structure.

Environmental factors may also influence lignin distribution. Wardrop (1971) summarised work carried out on the effect of light on lignification, finding that light has either a direct or indirect effect on lignification. In fact, Siegel (1956) found that both too little and too much illumination reduced the lignin content of plants. Ford, Morrison and Wilson (1979) reported that lignin content increased with increasing environmental temperature in temperate grass species. However, the response to environmental temperature was variable for tropical species. Finally, Janshekar and Fiechter (1983) suggest that ecological factors also affect the chemical structure of lignin. Therefore, it seems that environmental factors can influence both lignin content and structure.

Overall the variation in both lignin content and structure with type of plant, type of tissue, plant age and environmental factors should be taken into consideration when choosing a lignin source to extract from in this work.

2.3. Lignin synthesis and structure

2.3.1. Synthesis

Lignin originates from the enzyme-initiated dehydrogenative polymerisation of three hydroxylated cinnamyl alcohols (Janshekar & Fiechter, 1983; Pearl, 1967; Wool, 2005). These three precursor alcohols – *p*-coumaryl, coniferyl and sinapyl alcohols – are shown in Figure 3.

The polymerisation of these monolignols leads to a variety of inter-unit linkages. The linkages that can occur are “biphenyl carbon-carbon linkages between aromatic carbons, alkyl-aryl carbon-carbon linkages between an aliphatic and aromatic carbon” (Wool, 2005) and ether linkages, which are illustrated in Figure 4. Figure 5 shows the common notation used to name the different carbon atoms within different phenyl propane units.

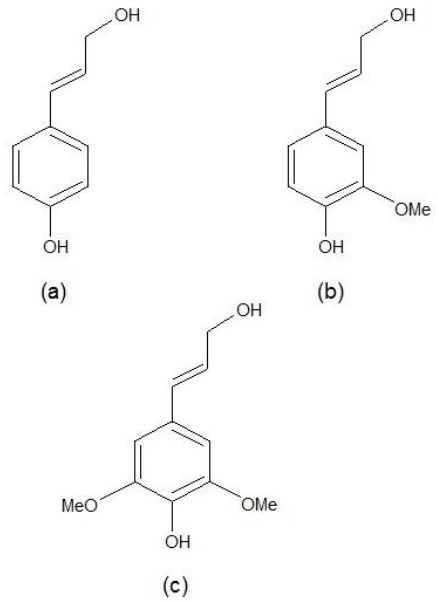


Figure 3. The primary precursors of lignin: (a) *trans-p*-coumaryl, (b) *trans*-coniferyl, and (c) *trans*-sinapyl (Wool, 2005).

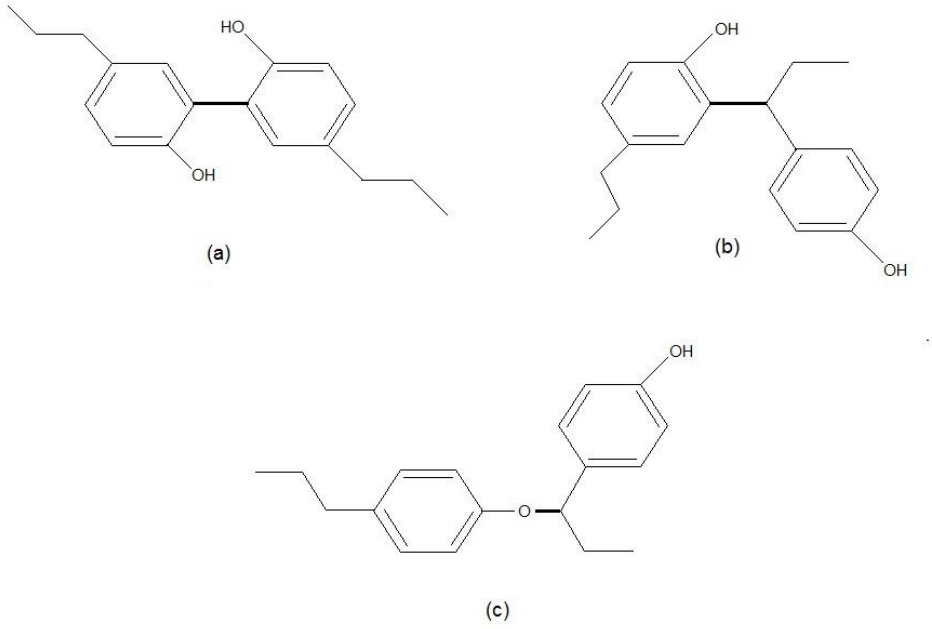


Figure 4. Representation of different linkages formed during enzymatic dehydrogenation between two trans-coniferyl units: (a) biphenyl carbon-carbon linkage, (b) alkyl-aryl carbon-carbon linkage, and (c) ether linkage (Wool, 2005).

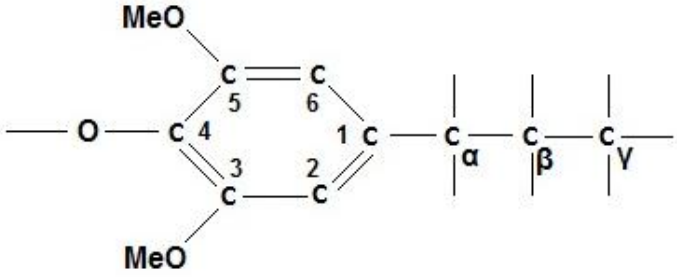


Figure 5. Common notation for denoting the different carbon atoms within each phenyl propane unit (Wool, 2005).

Boerjan, Ralph and Baucher (2003) suggest that the β-O-4 (β-aryl) linkage is the most common inter-unit linkage and that it is also the one most easily chemically cleaved. The other linkages (β-5, β- β, 5-5, 5-O-4 and β-1) are all more resistant to chemical degradation. On the other hand, Wool (2005) suggests that most of the bonds produced during lignification are exceptionally stable, and that the α-aryl ether bond is the only linkage that is relatively weak and hydrolysable. Thus although it seems unclear which bond is the weakest and most hydrolysable, it is at least evident that it is one of the ether linkages.

Janshekar and Fiechter (1983), Sarkanen (1971) and Pearl (1967) give further details of the synthesis pathways of lignin. It is interesting to note that more recent work has indicated that lignins are derived from more than just the three monolignols. In fact, many plants contain lignins derived from other monomers, "and all lignins contain traces of units from apparently incomplete monolignol biosynthesis and other (side-) reactions that occur during that biosynthesis (113)" (Boerjan et al., 2003). Recent work has also indicated that "lignin is not built up by a combinatorial polymerization of monolignols, but has a more ordered structure with a repeating unit larger than that given by the phenylpropane units" (Gellerstedt & Henriksson, 2008). It therefore seems that further work is required before the formation of lignin is fully understood.

2.3.2. Structure

Once incorporated into the lignin polymer, the p-coumaryl, coniferyl and sinapyl monolignols are known, respectively, as *p*-hydroxyphenyl (**H)**, guaiacyl (**G)**, and syringyl (**S)** phenylpropanoid units (Boerjan et al., 2003). From Figure 3, these units contain:

- *p*-hydroxyphenylpropane – no methoxy groups on the aromatic ring
- guaiacyl propane – one methoxy group on the aromatic ring
- syringyl propane – two methoxy groups on the aromatic ring (Wool, 2005)

Thus there can be wide variation in the composition of lignin polymers. However, it has been suggested that hardwood lignins generally contain mainly G and S units and traces of H units, while softwood lignins contain mostly G units with low levels of H units (Boerjan et al., 2003). This is supported by the proposed structure of Beech (hardwood) lignin presented in Figure 6, which consists mainly of G and S units, with a few H units. In fact, Gibbs (1958) divided lignins into two major classes:

1. Guaiacyl lignins – include the majority of gymnosperm lignins
2. Guaiacyl-syringyl lignins – include all angiosperm and herbaceous lignins

Therefore, the term lignin does not represent a single, well defined, uniform compound, but a group of substances that are chemically closely related, but have varying molecular weights (Janshekar & Fiechter, 1983; Pearl, 1967).

It is interesting to note that softwood lignins are a very homogeneous group; lignins extracted from different species exhibit little difference in structure and properties when isolated by similar procedures. On the other hand, hardwood lignins vary considerably between species (Pearl, 1967). This indicates that lignin should be extracted from the same species of hardwood if homogeneity is required.

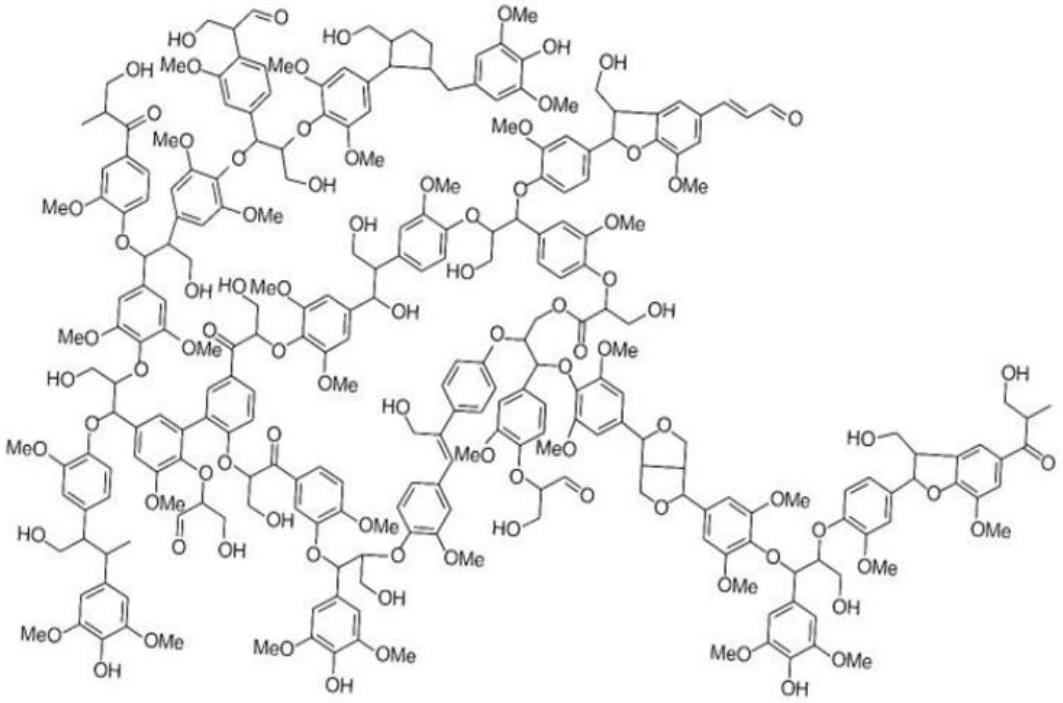


Figure 6. Proposed structure of beech lignin (Nimtz, 1973, as cited in Wool, 2005).

2.4. Effects of lignin on nutrition and health

For food grade microparticles to be developed it is critical to understand what the influence on human nutrition and health is likely to be. It is necessary to gain information on the fate of food microparticles once ingested, and both the benefits and pitfalls of incorporating lignin in the diet. Such knowledge may help to ensure that lignin microparticles with no adverse effects, and possibly even with positive effects on human health, can be developed.

2.4.1. Effects of lignin in the diet

2.4.1.1. Digestibility

Lignin is a major component of dietary fibre (Lu, Chu, & Gau, 1998) and is found in the highest concentration in foods such as fruits, vegetables and breakfast cereals (Freeman, 1979). In this native form, the lignin is associated with cell wall carbohydrates, which reduces the extent of fibre digestion (Jung & Fahey Jr, 1983). Thus lignin is considered

to be an antinutritive factor, indicating that it reduces nutrient digestibility and increases endogenous protein losses (Myrie, Bertolo, Sauer, & Ball, 2008). In fact, Ellis, Matrone and Maynard (1946) suggest that lignin provides a better indication of the indigestibility of plant material than measurement of crude fibre, and that lignin is not digested by cows, sheep or rabbits. Purified lignins, on the other hand, have different biological characteristics to native lignin and do not act as a barrier to nutrient digestion in monogastric or ruminant animals (Baurhoo, Ruiz-Feria, & Zhao, 2008).

2.4.1.2. Antimicrobial properties

It is well known that the phenolic fragments of lignin exhibit antimicrobial properties (Baurhoo et al., 2008). This is supported by the apparent resistance of lignified plant tissues to attack by alimentary bacteria (Woodman & Stewart, 1932); a characteristic that may be due to antiseptic action of lignin's phenolic nucleus (Crampton & Maynard, 1938).

Pye (2006) summarises studies showing an antidiarrhoeic effect of dietary lignin in piglets inoculated with pathogenic *E. coli*. The prophylactic use of lignin in this way may provide an alternative to the use of antibiotics in farm animal husbandry (Pye, 2006). There has also been extensive use of phenolic compounds, such as phenolic acids and flavonoids, as food preservatives (Baurhoo et al., 2008; Davidson & Branen, 1981).

2.4.1.3. Prebiotic properties

In addition to inhibiting the growth of pathogenic bacteria, lignin has also been reported to have a prebiotic effect in animals. Prebiotics are indigestible ingredients that selectively stimulate the growth or activity of a limited number of beneficial bacteria in the colon, thereby benefiting the host (Gibson, 1995). Prebiotics may also help to improve the intestinal morphology of animal species (Baurhoo et al., 2008). Thus prebiotics may have numerous beneficial effects in the gut.

Refer to Baurhoo, Letellier, Zhao and Ruiz-Feria (2007), Baurhoo, Phillip and Ruiz-Feria (2007) and Baurhoo et al. (2008) for further details of the prebiotic properties of lignin.

2.4.1.4. Adsorption and ion exchange

A lot of the work associated with the effect of lignin on digestion and metabolism has been carried out using chemically modified lignins rather than native, *in situ* lignins (Jung & Fahey Jr, 1983). Rubio et al. (1979) suggest that this lignin is slightly soluble and can be converted into powders with a range of surface areas. The polymer is generally swollen in aqueous dispersion and may adsorb organic substances and act as an ion exchanger (Jung & Fahey Jr, 1983). Furthermore, lignin is also chemically reactive, reacting with nitrite and exhibiting reducing properties. These adsorption and ion exchange properties may have an impact on nutrition and health and are further detailed here.

2.4.1.5. Ability to bind bile salts

Lignin may also have an effect on lipid metabolism due to its ability to bind bile salts (Jung & Fahey Jr, 1983). Eastwood and Hamilton (1968) and Story and Kritchevsky (1976) both reported binding of bile acids by lignin. Bile acids are reported to have a number of negative health effects, such as increasing the chemical permeability of the colon and enhancing tumour formation in the colon in carcinogen-treated animals (Gyory & Chang, 1983). Thus the binding of bile salts may help to improve intestinal health. This is supported by the finding that feeding auto-hydrolysed aspen lignin to hamsters fed a diet deficient in essential amino acids reduced the incidence of cholesterol gallstones (Rotstein, Kay, Wayman, & Strasberg, 1981).

2.4.1.6. Mineral binding

Van der Aar, Fahey Jr, Ricke, Allen and Berger (1983) studied the effect of kraft wood lignin on mineral metabolism in chicks, finding, for example, that incorporating 4% lignin in the diet increased serum zinc levels. The increase was attributed to modification of the gut microflora by lignin, decreasing competition between the host and microbes for dietary zinc. (Jung & Fahey Jr, 1983). Dietary lignin was also found to decrease liver copper levels. Thus if lignin is added to foods in high quantities, influences on mineral utilisation may need to be considered.

2.4.1.7. Antioxidant activity

Lignin has been shown to be an effective antioxidant in animal feed vitamin supplements, protecting the potency of antioxidant vitamins, such as vitamin E. Efficacy as a primary antioxidant comes from lignin being classed as a polymeric hindered phenol. Since lignin is a natural product and seems to be physiologically benign in humans, it may become a preferred antioxidant in food and feeds (Pye, 2006).

2.5. Applications of lignin in food

Only lignin sulfonates or lignosulfonates, which are lignins produced from the sulfite pulping method of paper production (refer to section 2.6.1) are currently used in foods. In New Zealand and Australia, calcium lignosulfonate (40-65) is approved as a food additive as a carrier of fat-soluble vitamins and carotenoids in water-based foods. Sodium lignosulfonate is also an approved processing aid in packaged water production. The FDA has approved lignosulfonates as binders, pelleting aids, surfactants and sources of metabolisable energy in animal feeds. Calcium lignosulfonate is also indirectly used in foods as a boiler water additive and as a raw material in vanillin production (Toledo & Kuznesof, n.d.). As mentioned in section 2.4.1.7, lignin has also been shown to be an effective antioxidant and thus may have a role as an antioxidant in feeds and human food in the future.

2.5.1. Scientific opinion on the use of calcium lignosulfonates in foods

The safety of a calcium lignosulfonate product has been recently evaluated by JECFA and the Panel on Food Additives and Nutrient Sources added to Food. This product is known as calcium lignosulfonate (40-65) because it has a weight average molecular weight range of 40,000 to 65,000, which distinguishes it from other calcium lignosulfonates in the market. The suggested application of calcium lignosulfonate (40-65) is as “a carrier (encapsulating agent) for fat-soluble vitamins, carotenoids, and other functional ingredients in e.g. fruit-based beverages, vitamin drinks, dairy products and hard candies” (Toledo & Kuznesof, n.d.).

Stability studies have shown that the weight average molecular weight and reducing sugar levels of powdered calcium lignosulfonate (40-65) remained stable when stored in

either polyethylene containers or aluminium foil bags for two years. Carotenoid preparations were also stable under both normal and accelerated conditions for 48 weeks. A β-carotene/calcium lignosulfonate (40-65) preparation in a non-pasteurised, non-carbonated soft drink retained good physical stability over a three month period. This indicates the calcium lignosulfonate (40-65) has good stability in various applications (Toledo & Kuznesof, n.d.).

It is suggested that calcium lignosulfonate would likely be consumed at low levels; for example, less than 100 mg/day when used as a carrier for carotenoids and up to less than 400 mg/day when used to add vitamins A, D and K to foods at the highest supplementation level. Hence it is suggested that although calcium lignosulfonate (40-65) would be considered to be a non-digestible dietary fibre in the applications suggested, it is unlikely to significantly contribute to overall non-digestible dietary fibre intake (Toledo & Kuznesof, n.d.).

An acceptable daily intake (ADI) of 20 mg/kg bw/day was established for calcium lignosulfonate (40-65) when evaluated by JECFA. It has also been found that this lignonsulfonate is poorly absorbed by the oral route and does not appear to have genotoxic potential. No Observed Adverse Effect Levels (NOAEL) of 1500 and 2000 mg/kg bw/day were identified after a short term toxicity study and 90-day sub-chronic toxicity study, respectively. However, this 90-day study was considered inadequate for evaluating the safety of the lignin by the EFSA Panel on Food Additives and Nutrient Sources added to Food. Hence based on the available information, safety could not be assessed (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 2010). However, in New Zealand and Australia, calcium lignosulfonate (40-65) has been approved as a food additive by Food Standards Australia New Zealand (FSANZ).

2.5.2. FDA regulatory status

The FDA has approved lignin sulfonates for use in animal feeds as binders, pelleting aids, surfactants and sources of metabolisable energy. The Code of Federal Regulations section Food Additives Permitted in Feed and Drinking Water of Animals outlines the source, form and maximum use level of lignin sulfonates in various applications. For

example, when used as a pelleting aid in liquid or dry form, the level of the lignin sulfonate must not exceed 4% of the finished pellets.

Overall, the information regarding the current food applications of lignins indicates that regulatory hurdles may need to be overcome in our work. However, the approval of calcium lignosulfonate as a food additive in New Zealand and Australia is promising.

2.6. Lignin extraction

The recovery of lignin from plant sources is known as delignification and can be achieved in a number of ways. There are two main categories of delignification:

1. Processes that remove the polysaccharide fraction of lignocellulose, and
2. Processes that selectively dissolve and recover lignin (Janshekar & Fiechter, 1983).

Delignification can also be classified as chemical or solvent delignification (Wool, 2005). Since the delignification method used influences the properties of the isolated lignin (Bhat, Khalil, & Karim, 2009), it is important to select a method based on the desired application of the lignin.

2.6.1. Traditional pulping methods

Most of the lignins extracted today are by-products of processes that produce cellulose pulp for the production of paper and some chemical derivatives (Lora, 2008). There are three chemical pulping processes used to produce cellulose pulp: the sulfite, kraft and soda processes. Most (98%) of the lignin produced by these processes is burnt as a fuel to recover energy. However, some lignins are further utilised for industrial applications. Table 3 provides a summary of these three lignin types, covering the pulping procedures, properties and applications of each lignin.

2.6.2. Recovery of lignin from ethanol biorefineries

Ethanol is a renewable fuel that can be produced from various plant materials. Alternatives to petroleum are becoming increasingly important for sustainability and “studies have estimated that ethanol and other biofuels could replace 30% or more of

U.S. gasoline demand by 2030" (U. S. Department of Energy, 2014). Ethanol can be generated by the fermentation of sugars in feedstocks such as sucrose, grain and lignocellulosic materials. The use of lignocellulosic materials, particularly fast growing trees and agricultural wastes, is expected to increase since this biomass can be grown on marginal land that is unsuitable for food production and thus do not compete with food crops (Doherty et al., 2011; Lora, 2008). The availability of carbohydrates is also decreasing (Lora, 2008).

The production of ethanol from lignocellulosic biomass also generates a significant lignin-rich stream as a by-product. The production of ethanol in a lignocellulosic biorefinery requires the enzymatic hydrolysis of cellulose to glucose followed by the fermentation of glucose to produce ethanol. However, the association of cellulose with lignin and hemicellulose limits its accessibility to cellulolytic enzymes (Pronyk & Mazza, 2011; Sassner, Galbe, & Zacchi, 2005; Wyman et al., 2004). Therefore, it is necessary to pre-treat the biomass to remove lignin and hemicellulose and allow the cellulose fraction to be freely hydrolysed. The lignin that is removed amounts to greater than 60% (w/w) of the ethanol produced (Lora, 2008). Utilisation of this lignin, along with the hemicellulose and cellulose, makes the biorefinery concept economical (Pronyk & Mazza, 2011; Sassner et al., 2005). An economical process is important because the production of ethanol in this way is more expensive than production of petroleum-based fuels. Therefore, it is likely that ethanol biorefineries will be a significant source of lignin in the near future.

There are various methods available for the pre-treatment of feedstocks to remove lignin. In addition to effective breakdown of the lignocellulose structure to remove lignin, the pre-treatment should also give high yields of sugars or a very digestible pre-treated solid, avoid degradation of sugars, avoid the formation of toxic inhibitory by-products and allow lignin to be recovered and exploited to produce valuable co-products. The pre-treatment should also be inexpensive (Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010; Galletti & Antonetti, 2011; Mora-Pale, Meli, Doherty, Linhardt, & Dordick, 2011).

Table 3. Summary of lignin produced using traditional pulping methods

Lignin Type	Pulping Procedure	Properties	Applications
Sulfite	Digestion of wood at 140-170°C with an aqueous solution of a sulphite or bisulphite salt of sodium, ammonium, magnesium or calcium.	- Contains 4-8% sulfur - Contains phenolic hydroxyl, aliphatic hydroxyl and carbonyl groups - Soluble in water - Insoluble in organic solvents - Generally contaminated with sugars - Broad range of molecular weight (~ 100-90,000 Da) - No glass transition temperature	- Surfactant and dispersant applications e.g. as plasticisers in concrete admixtures (largest current application) and animal feed binders - Energy generation (most common use since other applications limited by sulfur content and high polydispersity)
Kraft	Digestion of feedstock at about 170°C with combination of sodium hydroxide and sodium sulfide (a form of alkaline hydrolysis)	- Contains 1-3% sulfur - Contains hydroxyl groups, benzyl alcohol, carboxylic acids and unsaturated structures - Decreasing solubility in water with decreasing pH - Hydrophobic - Relatively high purity - Molecular weight range of 2,500-39,000 Da - Exhibit a glass transition temperature at around 140°C	- Most burnt as a fuel to recover energy in the kraft mill. - Generally used in the form of chemical derivatives (e.g. as sulfonated and ethoxylated kraft lignins) - Surfactants, dye and agrochemical dispersants, and asphalt emulsifiers
Soda	Digestion of feedstock in a pressurised reactor at 140-170°C with an aqueous solution of sodium hydroxide. Predominantly used for the pulping of non-wood fibres (e.g. sugarcane bagasse, straw, flax and hemp)	- Similar to Kraft lignins but do not contain sulfur - Insoluble in water - Hydrophobic - Relatively high purity with low levels of sugar and ash contaminants - Molecular weight range of 6,900-8,500 Da - Exhibit a glass transition temperature in the range 158-185°C (Lora & Glasser, 2002)	- Due to lack of sulfur, used in applications in which the kraft and sulfite lignins are unsuitable - Polycarboxylate-type dispersant - Applications for which biological activity is important (e.g. in the animal feed and nutrition area)

Note: All information was obtained from Doherty et al. (2011), Lora (2008) and Pye (2006) unless otherwise referenced in Table 3.

2.6.2.1. Lignocellulosic pre-treatment processes

The pre-treatment step can be achieved using various physical, biological, thermal, or chemical methods (Pronyk & Mazza, 2011). These methods have been recently reviewed by da Costa Sousa, Chundawat, Balan and Dale (2009) and Galletti and Antonetti (2011) and include:

- Mechanical disruption (milling)
- Steam explosion
- Ammonia fibre expansion
- Acid hydrolysis
- Alkaline hydrolysis
- High temperature organic solvent pre-treatment
- Degradation by white-rot and soft-rot fungi
- Liquid hot water pre-treatment
- Supercritical CO_2 explosion
- Ionic liquids pre-treatment

For details of the advantages and disadvantages of these methods, refer to da Costa Sousa et al. (2009) and Galletti and Antonetti (2011).

The most commonly used methods have previously involved dilute acid hydrolysis. However, even dilute acid hydrolysis can result in significant polysaccharide decomposition and formation of microbial inhibitors, such as furfural and hydroxymethylfurfural, which can inhibit the downstream fermentation step (Mora-Pale et al., 2011). Acid hydrolysis also causes corrosion of reaction vessels. Furthermore, Wyman et al. (2004) suggest that fractionation using dilute acid is also very expensive. Therefore, alternative methods have been sought.

The use of organic solvents for pre-treatments has already received significant attention. High temperature organic solvent pre-treatment is known as the organosolv process and typically involves heating the biomass to high temperatures using low-boiling solvents such as acetone, ethanol, ethyl acetate or dioxane. The organosolv process has three

major advantages. Firstly, substrates pre-treated by the organosolv ethanol process are much better digested by enzymes than those pre-treated with other processes (Pan, Kadla, Ehara, Gilkes, & Saddler, 2006, p. 5806). Secondly, organosolv pulping is environmentally benign (Macfarlane, Farid, & Chen, 2009) and the solvents can be removed by distillation. Thirdly, the process not only produces a high quality cellulose pulp for ethanol production, but also produces a high quality lignin stream, a hemicellulose-derived sugar stream and other by-products. In this way, the full potential of the raw materials can be exploited. Especially if produced without the aid of acid catalysis, the lignin is relatively pure, mostly unaltered and less condensed than lignin produced from other pre-treatment processes (Quesada-Medina, López-Cremades, & Olivares-Carrillo, 2010, p. 8252). Organosolv lignins typically also have low molecular weight, low molecular weight polydispersity and are practically insoluble in water (Lora, 2008). These lignins are expected to be popular raw materials for the production of bio-products, such as adhesives, fibres, films and biodegradable polymers, and also seem suitable for use in this thesis project.

2.6.2.2. Organosolv pulping

The effect of various factors on the organosolv extraction of lignin is now summarised. Firstly, some key words are defined:

Kappa number: indicates residual lignin content – the lower the kappa number, the greater the extent of delignification that has occurred.

Extractability: the amount of starting material which has been dissolved

Delignification: the amount of Klason lignin which has been dissolved

Selectivity: the amount of Klason lignin present in the dissolved matter (Quesada-Medina et al., 2010)

2.6.2.2.1. Effect of chip size

In an investigation into the organosolv delignification of *Hesperaloe funifera* (H. *funifera*), Sánchez, Rodríguez, Navarro, Conesa and Jiménez (2010) used ground material in the range 0.25-0.40 mm for the extraction, claiming that larger material is

inefficiently acted on by chemical reagents and that smaller material interferes with filtering operations. Similarly, Quesada-Medina et al. (2010) used ground hydrolysed almond shells in the range 0.2-0.5 mm to study the organosolv extraction of lignin. However, Macfarlane, Farid and Chen (2009) found that an increase in the size of chips from 2.7 to 8.2 mm did not significantly affect the amount of lignin precipitated from *Salix schwerinii* chips. This was consistent with earlier work by Gilarranz, Oliet, Rodriguez and Tijero (1998), in which the chip size of *Eucalyptus globulus* (industrial vs matchstick size) did not significantly affect delignification or pulp yield during ethanol-water pulping. This may be due to the low viscosity of the ethanol-water solvent and the high temperatures and pressures involved, which favour the penetration of liquor into the chips. In fact, it has been suggested that only when the pulping liquor has a high salt concentration and the cooking temperature is low does chip size influence organosolv pulping. This is due to the increase in viscosity of the pulping liquor under these conditions, which reduces the penetration of the liquor into the wood chips (Benar & Schuchardt, 1994; Chiang & Sarkanen, 1983, as cited in Gilarranz et al., 1998). The consistent findings of Gilarranz et al. (1998) and Macfarlane et al. (2009) indicate that experimenting with wood chips of different size should not be an important objective of this project.

2.6.2.2.2. Effect of organic solvent type

A solvent medium requires both a nucleophilic species, to promote the cleavage of lignin into low molecular mass species, and the ability to dissolve the lignin fractions. Thus organosolvents are usually mixtures of organic solvents and water, where water is the nucleophilic agent and the organic solvent can dissolve the lignin fragments (Pasquini, Pimenta, Ferreira, & Curvelo, 2005a). Section 2.6.2.1 indicated that a range of organic solvents can be used in organosolv pulping. Results of investigations into the effect of solvent type on the extent of delignification and the properties of the lignin produced are now reported and discussed.

Sánchez et al. (2010) pulped H. *funifera* with reagents (soda and soda-anthraquinone) or organosolv solvents (ethanolamine, diethanolamine, ethylene glycol and diethylene glycol). Comparing the four organosolv solvents, the amine solvents gave the highest

quality pulps containing the least residual lignin. The amine pulp samples also produced paper with better physical properties than those of paper produced from glycol pulp samples. This provides further support for the use of amines over glycols for pulping, if paper is to be produced from the pulp.

Quesada-Medina et al. (2010) studied the effect of three different organic solvents with known lignin solubilisation capacity – acetone, ethanol and 1,4-dioxane – on the delignification of hydrolysed almond shells. It was found that for all cooking times, mixtures containing dioxane always caused a greater extent of delignification than those containing acetone. In turn, mixtures containing acetone caused greater delignification than those containing ethanol. However, it was found that ethanol mixtures were more selective than acetone mixtures, which were more selective than dioxane mixtures. Since selectivity indicates how much of the extracted material was actually lignin (Reis Machado, Sardinha, Gomes de Azevedo, & Nunes da Ponte, 1994), the usefulness of ethanol as a solvent cannot be overlooked. Statistical analysis of the differences in extractability, delignification and selectivity when different solvents were used was not performed by Quesada-Medina et al. (2010), which limits the conclusions that can be drawn from these results. However, it was concluded that pulping at 210°C with 75% mixtures of acetone, ethanol or 1,4-dioxane for a maximum cooking time of 40 minutes, without the use of catalysts, was sufficient to adequately delignify hydrolysed almond shells. This indicates that all three solvents tested were suitable for lignin extraction.

Muurinen (2000) suggests that ethanol is one of today's most promising pulping chemicals. Ethanol is also food-grade, suggesting that ethanol is a suitable solvent for this project.

2.6.2.2.3. Effect of cooking time

Cooking time is one of the main variables affecting the delignification process (Quesada-Medina et al., 2010).

Sánchez et al. (2010) included cooking time as a variable in their study on the pulping of H. *funifera*. The most lignin was removed when H. *funifera* was pulped with either 70% diethanolamine for 60 minutes at 170°C or an 80% concentration of the amine for

90 minutes at 180°C. The cooking time was increased at the same time as cooking temperature and concentration of the solvent were increased and it was therefore difficult to attribute differences in delignification to cooking time. Nonetheless, it seems that a lower cooking time of 60 minutes, or possibly even lower if a higher solvent concentration and temperature were used, could be used to provide the same extent of delignification as a longer time of 90 minutes. This indicates that it is the balance of different factors, such as temperature, solvent concentration and cooking time, that is determines the extent of delignification, rather than one factor alone.

Quesada-Medina et al. (2010) studied changes in extractability, delignification and selectivity with cooking times in the range 10-55 minutes. It was found that delignification increased slightly up to a certain cooking time, which was dependent on the solvent type (40 minutes for acetone and ethanol, 25 minutes for dioxane). Material continued to be extracted after this time, but the material extracted was not lignin. Thus an increase in extractability but decrease in selectivity was observed for longer cooking times. In fact, it was found that most of the delignification had already occurred after cooking times of only 10 minutes, indicating that for the times and solvents tested, lignin solubilisation was largely unaffected by cooking time. This suggests that the cooking time should be optimized along with other factors in the organosolv process to ensure that the shortest effective time is used.

Gilarranz et al. (1998) used a central composite design to study the effect of cooking temperature, cooking time and solvent concentration on the Kappa number and other aspects of pulp quality during ethanol-water pulping of *Eucalyptus globulus*. An important finding was that at higher pulping temperatures, shorter pulping times can achieve the same Kappa number and thus the same degree of delignification as longer pulping times at lower temperatures. This is consistent with the findings of Quesada-Medina et al. (2010), Jiménez et al. (1999) and Jiménez, Pérez, Garcia and Rodríguez (2001). However, both lignin and carbohydrates are more greatly solubilised at cooking conditions of higher severity. For example, as cooking time increases, more hemicellulose is removed. It is also important to note that as the lignin content of the pulp decreases, delignification becomes slower, since the residual lignin has a high proportion of carbon-carbon linkages (McDonough, 1993). It was found that at a

cooking temperature of 185°C and time of 90 minutes, the rate of delignification is significantly higher than the rate of carbohydrates solubilisation.

Pasquini et al. (2005a) studied the extraction of lignin from *Pinus taeda* (*P. taeda*) and sugar cane bagasse using ethanol-water mixtures and carbon dioxide under supercritical conditions. Consistent with the finding of Gilarranz et al. (1998) that lignin solubilisation increases as the severity of the pulping conditions increases, Pasquini et al. (2005a) found that delignification of *P. taeda* increased as the ethanol-water pulping time increased from 30 to 120 minutes (at 170°C, 19MPa and 50% ethanol (v/v)). However, most of the delignification occurred in the first 30 minutes, with longer times only increasing delignification from 81.3% to 84.9% as residual lignin was gradually extracted. This high delignification in only 30 minutes may have been due to the higher pressure and the expected higher acidity of the reaction medium due to the in-situ generation of carbonic acid. These results indicate the use of supercritical fluids for lignin extraction may allow shorter cooking times to be used. Less severe pulping conditions may also help to avoid the degradation and extraction of polysaccharide components.

Similarly, during the pulping of sugar cane bagasse, there were only very small changes in the extent of delignification for various cooking times between 30 and 150 minutes. Pulping for 30 minutes produced significantly higher pulp yields. This may be because the shorter cooking time allows less degradation of the polysaccharide fraction, causing more pulp to remain when lignin is removed (Pasquini et al., 2005a). This again supports using the shortest possible cooking time to avoid degradation and solubilisation of the carbohydrate fraction. Conversely, Pan et al. (2006) found that higher severity conditions generally produced higher purity lignin due to less residual carbohydrate. However, it seems to make more sense that shorter cooking times would produce higher purity lignin, since less opportunity is given for the carbohydrate fraction to degrade. Thus it is important to balance the use of severe conditions to obtain a higher yield of lignin with the use of less severe conditions to obtain a higher purity lignin fraction.

Reis Machado et al. (1994) studied the effect of cooking time on the high-pressure delignification of Eucalyptus wood by 1,4-dioxane-CO_2 mixtures. At 170°C and 170 bar, lignin extraction was found to increase with time until an extraction time of 120 minutes was reached, after which lignin extraction plateaued. For short extraction times, the extraction of hemicellulose increased drastically with time, followed by a slower rate of extraction until completely extracted at 300 minutes. This indicates that the complete extraction of hemicellulose that requires more severe extraction conditions, including longer cooking time. For this project, only lignin extraction is required and thus it seems that 120 minutes is the greatest cooking time required (Jiménez et al., 1999; Reis Machado et al., 1994).

Cooking times ranging from 10 – 300 minutes were studied in the work reviewed here. It seems that cooking time must be optimised with factors such as cooking temperature and solvent concentration to achieve suitable delignification. However, cooking times of 55-60 minutes, at a temperature of 200-210°C and solvent concentration of 75% (v/v) of ethanol have been found to provide adequate delignification.

2.6.2.2.4. Effect of cooking temperature

The cooking or extraction temperature is another of the main variables affecting delignification (Quesada-Medina et al., 2010).

Macfarlane et al. (2009) used a 2^3 full factorial design to study the effect of extraction temperature, chip size and ethanol concentration on delignification during the ethanol-water pulping of *Salix schwerinii* using a batch reactor with recycle. It was found that increasing the inlet temperature from 170 to 185°C increased the precipitable lignin concentration of the liquor. This again supports the use of higher severity pulping conditions to increase lignin solubility.

Pan et al. (2006) investigated the effect of temperatures ranging between 155 and 205°C on lignin yield and structure during the organosolv ethanol processing of hybrid poplar. As the temperature increased, the lignin yield increased, reaching a maximum yield of 20.9%, based on wood, at 195°C. It was suggested that the maximum yield was not obtained using the highest temperature of 205°C since although higher temperatures

promote delignification and lignin dissolution, they may cause excessive depolymerisation, which reduces lignin recovery. It was also found that processing at higher temperatures generated lignins with higher phenol content, due to increased cleavage of α- and β-ether linkages. This suggests that lignins recovered from different processes have different structures and thus may have different chemical reactivity.

Quesada-Medina et al. (2010) studied the effect of temperatures ranging from 150-230°C on the organosolv extraction of lignin from hydrolysed almond shells. The maximum temperature of 230°C was selected because thermogravimetric analysis has shown that lignin degradation usually begins at this temperature (Singh et al., 2005; Xiao, Sun, & Sun, 2001). Additionally, all the solvents investigated had a critical temperature below 230°C and thus the solvents would be liquid at this temperature if a suitable pressure was used. It was observed that as the temperature increased up to 210°C, the extent of delignification increased. Above 210°C, delignification decreased, to an extent dependent on the solvent used. For example, a greater decrease was observed for dioxane than for acetone or ethanol. This decrease was probably due to lignin condensation at higher temperatures. Quesada-Medina et al. (2010) summarise that Vázquez, Antorrena, González, Freire and López (1997) and Pan et al. (2006) obtained similar results. Pan et al. (2006) reported an optimum temperature of 195°C. This lower temperature may have been due to the use of sulfuric acid as a catalyst, which favoured the delignifcation process (Quesada-Medina et al., 2010). Thus a major finding of Quesada-Medina et al. (2010) was that as the cooking temperature increased from 150 to 230°C, extractability increased continuously but selectivity decreased. This means that more material was extracted when higher temperatures were used, but that less of this material was lignin. Ultimately, it was concluded that 210°C was the extraction temperature that gave the greatest degree of delignification yet maintained an acceptable selectivity. Thus it seems that temperatures of 210°C or less should be used to reduce condensation and optimise extractability and selectivity.

The cooking temperature was also found to affect the structure of lignin: Polydispersity increased from 2.1 to 2.6 and monomer content increased from 25 to 40% with an increase in temperature from 150°C to 230°C. Meanwhile, the highest weight average molecular weight of 2300 g/mol was recorded at 190°C. Cooking temperatures higher

than 190°C may cause more lignin depolymerisation and thus lower molecular weights (Quesada-Medina et al., 2010). The structure of the lignin product required will therefore be important to consider when selecting a cooking temperature.

Klein et al. (2010) found that in the ethanol-water accelerated solvent extraction (ASE) of lignin from candlenut nutshells, lignin yield increased as the extraction temperature increased from 100 to 195°C. This is consistent with the work of Macfarlane et al. (2009), Quesada-Medina et al. (2010), Vázquez et al. (1997) and Pan et al. (2006). Furthermore, it is known that an increase in delignification occurs with an increase in temperature in the industrial Alcell process, which typically operates at 195°C.

Gilarranz et al. (1998) found that delignification was greater for an extraction temperature of 190°C than a temperature of 180°C, indicating that delignification increased with increasing temperature. It was also found that pulping at 180°C for 120 minutes is required to achieve the same extent of delignification as pulping at 194°C for 40 minutes. This indicates the importance of the temperature/time combination. In fact, Sanchez et al. (2010) found that the extent of delignification of *H. funifera* was similar when pulped with either 70% diethanolamine at 170°C for 60 minutes or 80% diethanolamine at 180°C for 90 minutes. This shows that a lower temperature of 170°C could be used to achieve sufficient delignification when the solvent concentration and cooking time were altered.

Pasquini et al. (2005a) found that temperature had a greater effect than pressure on the delignification of *P. taeda* chips, with higher temperatures causing more delignification. A reasonable extent of delignification was not achieved using temperatures lower than 150°C. A temperature of 190°C was found to cause the greatest degree of delignification (Pasquini et al., 2005a). Temperature was also found to have a greater effect than pressure on the delignification of sugarcane bagasse, with the residual lignin content of the feedstock decreasing with increasing temperature. For this feedstock, the optimum temperature and pressure, giving the best compromise between residual lignin content and pulp yield were found to be 170°C and 14.7 MPa, respectively. Thus the overall results of Pasquini et al. (2005a) support the use of more severe processing conditions to cause greater delignification. Furthermore, since it has been suggested that

temperatures below 150°C do not promote adequate delignification and that temperatures above 210 and 230°C lead to lignin condensation and degradation, respectively, it seems that a cooking temperature between 150 and 210°C should be used. Moreover, considering the reported effects of temperature on delignification and structure, temperatures between 190 and 200°C may be the most suitable for this work.

2.6.2.2.5. Effect of solvent concentration

The solvent concentration is the third main variable affecting delignification. It is important because while water controls the cleavage of lignin from the material through nucleophilic action, the organic solvent controls the dissolution of the cleaved lignin. Therefore, the balance of water and organic solvent must be carefully optimised (Pasquini et al., 2005a).

Gilarranz et al. (1998) found that at a set cooking temperature of 185°C, lower alcohol concentrations led to lower pulp Kappa numbers, indicating that delignification is greater at lower organic solvent concentrations. This may be due to higher water content and thus higher hydrogen ion concentration, which catalyses the cleavage of ether linkages in the lignin, allowing more lignin to dissolve (McDonough, 1993). However, once cleaved into fragments, the fragments must be solubilised. Sarkanen (1990) and Ni and Hu (1995) suggest that to reach a good solubility, a minimum alcohol concentration of about 50% is required. Considering this, Gilarranz et al. (1998) found that at an ethanol concentration of 50%, suitable Kappa numbers were still obtained if suitable combinations of temperature and time, such as 194°C for 40 minutes or 180°C for 120 minutes, were used. This again indicates the importance of the combination of time, temperature and solvent concentration.

Pan et al. (2006) investigated aqueous ethanol concentrations of 25-75% (v/v) and found that lignin yield increased with increasing ethanol concentration, reaching a maximum at 65% ethanol. This is illustrated in Figure 7. It has been suggested that the highest ethanol concentration did not produce the highest lignin yield because of the lower hydrogen ion concentration and also because of the poor solubility of the lignin (Pan et al., 2006), supporting the ideas of Gilarranz et al. (1998). Similar to the findings of Pan et al. (2006), Ni and Hu (1995) reported maximum lignin solubility at an ethanol

concentration of 70%. Interestingly, it was also found that lower ethanol concentrations produced more phenolic lignins. This may have been a result of the higher hydrogen ion concentration at lower ethanol concentration, which may have catalysed the cleavage of ether linkages in lignin, leading to phenol groups. Since the hindered phenol content of lignins is associated with antioxidant activity (Pye, 2006); ethanol concentration may have implications for the antioxidant activity of the lignin produced.

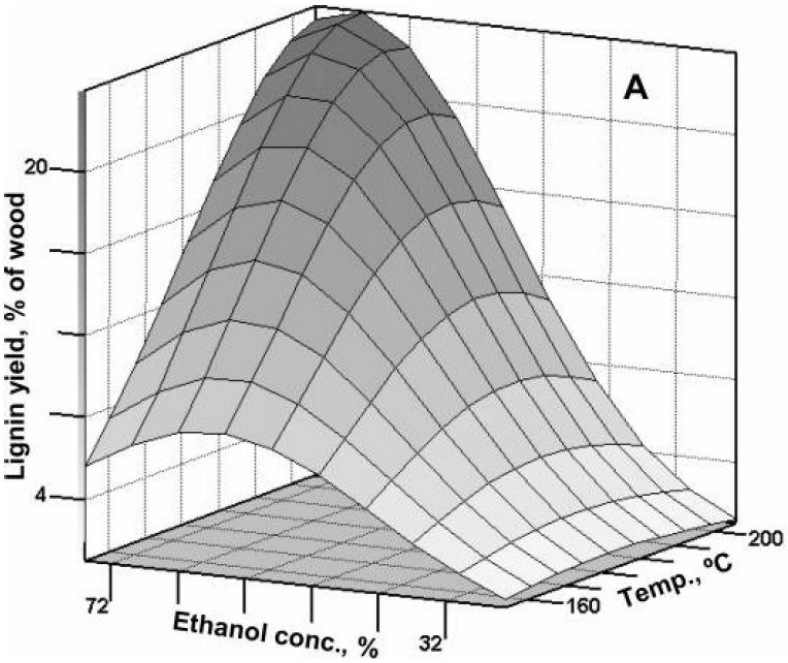


Figure 7. Effect of ethanol concentration and extraction temperature on the yield of organosolv ethanol lignin (Pan et al., 2006).

Gilarranz et al. (1998) also note the effect of solvent concentration on hemicellulose removal and solubilisation, stating that the higher hydrogen ion concentration at low alcohol concentration causes solubilisation of hemicellulose even when the cooking time is short. The acidity also causes more cellulose depolymerisation. This is important to consider if a pure lignin product is required. An alcohol concentration of 50% is suggested to provide a good compromise between hemicellulose solubilisation and minimum cellulose damage.

2.6.2.2.6. Effect of pressure

It is interesting to note the pressures employed in different organosolv pulping investigations. The Alcell® organosolv process is a proprietary ethanol-water process and operates at 2.76 MPa, 50% aqueous ethanol and 190°C. Buranov et al. (2010) used 30% aqueous ethanol at a pressure of 5.2 MPa. Meanwhile, Pasquini et al. (2005a) employed supercritical conditions, again suggesting that it is the combination of organosolv conditions selected that is critical.

2.6.2.2.7. Effect of catalysts or additives

Organosolv processing can be carried out with or without added catalysts. Quesada-Medina et al. (2010) suggest that organosolv delignification is favoured by the addition of inorganic acid catalysts. However, even without added catalysts, the reaction is also auto-catalysed, since acetic acid is released from the wood during pulping (Gilarranz et al., 1998).

Pan et al. (2006) found that lignin yield increased with increasing concentration of sulfuric acid catalyst, as shown in Figure 8. Interestingly, Figure 8 also shows that when extraction time and catalyst concentration were increased at the same time, the lignin yield tended to decrease, forming a saddle on the response surface. This may be a result of excessive degradation of the lignin when both high catalyst concentration and long extraction time are used. Excessive degradation may produce very small, highly soluble fragments of lignin that are unable to be recovered by precipitation, leading to the lower yield of lignin. This suggests that although catalysts may improve lignin yield, other conditions in the reaction must be carefully controlled to ensure that excessive degradation does not occur.

Gilarranz et al. (1998) investigated the influence of cooking additives H_2SO_4, $MgSO_4$ and $Ca(NO_3)_2$ on Kappa number and pulp yield. The results are presented in Table 4, showing that although the addition of sulfuric acid produced lower Kappa numbers and similar pulp yields compared to auto-catalysed pulping, the difference was more pronounced at 180°C than at 190°C. This indicates that when pulping at higher temperatures, additional catalysis is not really necessary. Thus, the main advantage of

catalysing with sulfuric acid may be that the reaction temperature needed to achieve a certain level of delignification can be reduced.

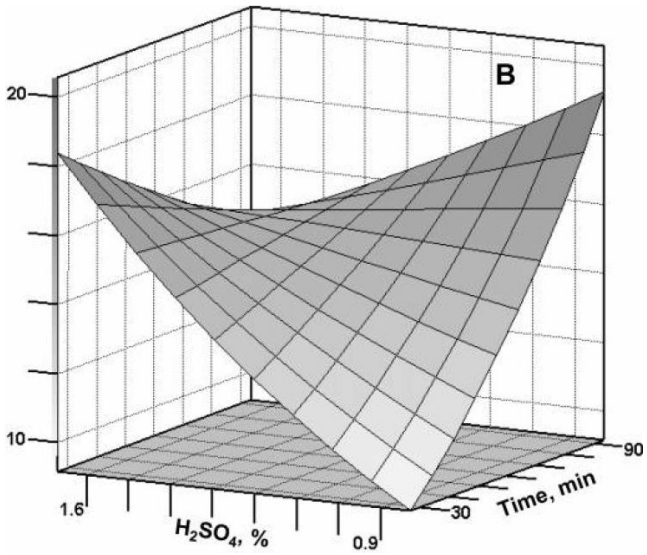


Figure 8. Effect of catalyst concentration and time on the yield of organosolv ethanol lignin (Pan et al., 2006).

Table 4 also shows that the pulping additives $MgSO_4$ and $Ca(NO_3)_2$ did not increase the extent of delignification or the pulp yield. This may have been because of the low alcohol concentration, which created an acidic environment in which colloid particles of Mg^{2+} and Ca^{2+} salts could not be formed. $MgSO_4$ and $Ca(NO_3)_2$ additives may be more useful if a higher alcohol concentration is used. However, overall the autocatalysed process was found to be the most suitable for the pulping of *Eucalyptus globulus*.

There are also some disadvantages of acid catalysis, including degradation of the lignin (Paszner & Cho, 1989; Teramoto, Tanaka, Lee, & Endo, 2008), corrosion of extraction vessels (Teramoto et al., 2008), degradation of carbohydrates (Gilarranz et al., 1998; Teramoto et al., 2008) and the formation of furfural (Teramoto et al., 2008). Finally, the addition of catalysts increases the cost of the organosolv process (Quesada-Medina et al., 2010).

Table 4. Influence of different cooking additives on Kappa number and total yield in ethanol-water pulping of Eucalyptus globulus (adapted from Gilarranz et al., 1998)

Temperature (°C)	Additive (0.01 M)	Kappa number	Pulp yield (%)
180	-	34.4	52.8
190	-	18.1	49.8
160	H_2SO_4	26.0	50.6
170	H_2SO_4	17.9	48.2
180	H_2SO_4	16.7	46.6
190	H_2SO_4	14.8	40.6
180	$MgSO_4$	32.6	53.4
190	$MgSO_4$	20.5	50.1
190	$Ca(NO_3)_2$	15.9	48.1

It seems that the extent of catalysis required depends on the conditions selected for the other pulping variables. In fact, it seems that if suitable combinations of other variables, such as extraction temperature, time and solvent concentration, are used, catalysis may not be necessary at all.

2.6.2.2.8. Effect of liquor-to-wood ratio

Young and Achmadi (1983) suggest that for ethanol-water pulping, the liquor-to-wood ratio has a large influence on the pulping results. An increase in the liquor-to-wood ratio causes greater dilution of the acid released from wood during pulping, which decreases delignification. However, the larger amount of liquor also reduces condensation and precipitation of lignin back onto the wood surface. (Gilarranz et al., 1998). Therefore, it is important to optimise the extraction conditions to obtain a balance of these effects. Young and Achmadi (1983) recommend a liquor-to-wood ratio close to 20:1 and state that measures such as diluting the pulping medium, using a two-stage cooking process or "continuous recycling of the pulping liquor" can prevent secondary lignin condensation.

Gilarranz et al. (1998) studied the effect of a range of liquor-to-wood ratios of 4, 7, 10 and 17 litres of liquor per kilogram of wood. It was found that in this range, the opposing effects of the liquor-to-wood ratio balanced each other and thus that this variable had little influence on pulping results. It was also found that the amount of acid released during pulping did not depend on the liquor-to-wood ratio. Finally, it was suggested that the weak organic acids produced act as a buffer, which reduces the effect of liquor-to-wood ratio on the pH of the reaction.

Many other investigators stated the liquor-to-wood ratio used in their investigation but did not include this ratio as a variable. Consistent with the suggestion of Young and Achmadi (1983), Sun et al. (2011) used a liquor-to-wood ratio of 20:1. However, most other investigators used lower ratios, for example, 8:1 (Sánchez et al., 2010), 6:1 (González Alriols et al., 2010) and 7:1 (Mabee et al., 2006; Pan et al., 2006), all w/w based on oven dry wood weight. Mabee et al. (2006) explained that a ratio of 7:1 was chosen to facilitate good coverage of the wood chips by the liquor.

By assuming that 1 L of 50% (v/v) aqueous ethanol has a mass of 0.895 kg, the liquor-to-wood ratios investigated by Gilarranz et al. (1998) were approximately 3.6:1, 6.3:1, 9:1 and 15.2:1. The lack of influence of changes within these ratios on pulp Kappa number indicates that ratios of 6-8:1, as used by many other investigators, are appropriate for successful ethanol-water pulping.

2.6.2.2.9. Effect of adjustments to the method

Various adjustments have been made to the organosolv process to mainly improve lignin yield. Adjustments include the use of high pressure and the separation of hemicelluloses in a prior steam explosion pre-hydrolysis step. These adjustments are discussed below.

2.6.2.2.9.1. Use of supercritical fluids

Supercritical fluids are compressed substances at a temperature and pressure above their critical point, where there is no distinction between the liquid and gas phases. These fluids can be used as solvents for extraction and are interesting because their properties can be easily adjusted by changing the temperature and pressure (Anon., n.d.).

Supercritical fluids can not only be used as an alternative to conventional solvents in some reactions, but can also achieve some reactions that are difficult or impossible in conventional solvents (Poliakoff, 2001). The use of supercritical fluids in organosolv pulping also has many economic and environmental advantages (Pasquini, Pimenta, Ferreira, & Curvelo, 2005b), including:

- Lower organic solvent requirement (Pereira et al., 2007)
- Ease of both lignin and solvent recovery through depressurisation of the reactor after pulping (Pereira et al., 2007; Santos, Kawase, & Coelho, 2011)
- The need for less severe processing conditions (e.g. lower extraction times and temperatures) (Pasquini et al., 2005a; Pereira et al., 2007)

However, there are some important factors that need to be considered in supercritical extraction. A particularly important consideration is that if the production of a high quality cellulose pulp is required in addition to delignification, there must be high selectivity for lignin while the cellulose and hemicellulose fractions are preserved (Li & Kiran, 1988; Pasquini et al., 2005b). For example, thermal degradation of carbohydrates occurs above 200°C (Li & Kiran, 1988), indicating that the critical temperature should be below this temperature. Mixtures of solvents, such as those in organosolv-CO_2 processes, can help to ensure that the critical temperature of the overall extracting fluid is less than 200°C. Mixtures of solvents have also been found to increase delignification (Li & Kiran, 1988).

However, Li and Kiran (1988) found that introducing CO_2 into the extraction fluid hindered the selectivity for lignin removal from red spruce. It was suggested that this was due to the formation of carbonic acid, leading to the hydrolysis of carbohydrates. Similarly, Reis Machado et al. (1994) found that CO_2-rich fluid mixtures extracted hemicellulose preferentially and in fact that total hemicellulose removal could be achieved. It was also found that maximum delignification was achieved when pure dioxane was used as the extracting solvent. Finally, it has been suggested that mixtures of ammonia or carbon dioxide with water are more reactive with the carbohydrate fractions of wood, whereas fluids such as methylamine are more reactive with lignin (Li

& Kiran, 1988). These results may indicate that CO_2 is not a suitable solvent for lignin extraction.

2.6.2.2.9.2. Use of a steam explosion pre-hydrolysis step

Macfarlane (2009) suggests that pre-hydrolysis removes hemicellulose, extractives and ash from wood without removing lignin, thereby increasing the speed of subsequent organosolv delignification. This could be a result of increased porosity of the wood after pre-hydrolysis (Liu, Fatehi, Jahan, & Ni, 2011). Thus the combination of pre-hydrolysis and organsolv delignifcation allows cleaner fractionation of the wood since the hemicelluloses, lignin and cellulose are obtained separately (Macfarlane, 2009). Liu et al. (2011) investigated the use of a pre-hydrolysis step to remove hemicelluloses prior to lignin extraction. The pre-hydrolysis step was carried out using steam at 170°C for 30 minutes. This step also removed some lignin, which was obtained from the pre-hydrolysis liquor by acid-induced lignin precipitation. Along with faster delignification and the fractionation of all major components, pre-hydrolysis also reduces "the bulk volume of wood requiring organosolv treatment. This in turn reduces the volume of the organosolv reactor, which typically operates at high pressure and temperature and requires corrosion resistant materials of construction" (Macfarlane, 2009). Thus in commercial processes this step may be very useful. However, for the purposes of this project, where only lignin removal is required, it may not be necessary.

2.6.2.2.10. Summary of effect of organosolv conditions

There are many different variables affecting the organosolv extraction of lignin, and a wide range over which they can be varied. The main variables affecting the delignification process are organic solvent concentration, cooking time and extraction temperature (Quesada-Medina et al., 2010). It seems that rather than individual effects, the combination of variable levels used seems to be critical. For example, the use of higher pressure may enable lower cooking time, cooking temperature or solvent concentration to be used. Similarly, variation within the three main variables above can be used to achieve the desired results. Thus it is recommended that the conditions be optimised using experimental design.

High severity operating conditions can cause degradation of carbohydrates, which has implications for the quality of the biorefinery products, and can also cause degradation of lignin. Thus there are some important ranges of conditions that should be considered when selecting organosolv extraction conditions:

- Cooking temperature – cooking temperatures between 150 and 210°C should be used to achieve adequate delignification, yet avoid lignin degradation. Temperatures between 190 and 200°C seem to work well.
- Cooking time – cooking time varies depending on the organic solvent used. It is important that the cooking time is as short as possible to ensure that the selectivity of the extraction for lignin remains high. 120 minutes may be the maximum cooking time needed for the ethanol organosolv extraction of lignin.
- Solvent concentration – it is important to remember that both water and organic solvent are needed in the solvent mixture to achieve both fragmentation of the lignin from the carbohydrate components and solubilisation of lignin in the liquor. Gilarranz et al. (1998) suggest a minimum alcohol concentration of 50% for adequate lignin solubilisation. Concentrations of 60-80% may be suitable for ethanol organosolv extraction of lignin from willow chips.

2.7. Lignin characterisation

2.7.1. Lignin yield and purity

When extracting lignin for use in any application, it is important to know the yield of lignin obtained from the extraction process and also to know the composition of the lignin. Any contaminants may influence the performance of lignin in various applications.

The lignin content in lignin samples can be determined using either National Renewable Energy Laboratory (NREL) or Technical Association of the Pulp and Paper Industry (TAPPI) standard methods. In these methods, the crude lignin sample is hydrolysed with sulfuric acid. Both acid-insoluble lignin, known as klason lignin, and acid-soluble lignin are measured and summed to give the total lignin content of the sample. The carbohydrate content of the sample can also be measured to determine what sugars are

present in the lignin. For example, Buranov, Ross and Mazza (2010) used analyses of Klason lignin, acid-soluble lignin, and constituent sugars to determine the purity of lignin samples. The NREL procedures were used.

The lignin content can also help in the yield determination. Macfarlane et al. (2009), Reis Machado et al. (1994), Quesada-Medina et al. (2010), Pasquini et al. (2005a) and Pronyk and Mazza (2011) measured lignin yield by comparing the lignin content of the feedstock before and after pulping. Klein et al. (2010) determined yield by comparing the amount of lignin recovered with the klason lignin content of the feedstock, as measured following the TAPPI standards.

For the current project, it may be important to perform lignin determination on the chips before and after extraction to determine lignin yield to ensure an optimal process, but may also be important to measure the purity of the lignin sample. This should form part of lignin characterisation protocol.

2.7.2. Lignin structure and functional groups

To develop new applications for isolated lignin, it is important to consider the structure of the lignin produced and what functional groups it contains. Chemical structure and functional groups can be analysed using Fourier transform infrared (FTIR) spectroscopy or nuclear magnetic resonance (NMR) spectroscopy.

2.7.2.1. FTIR analysis

FTIR analysis has been used to characterise lignin samples through determination of the functional groups present and comparison of spectra for different lignin samples. FTIR spectroscopy gives an indication of the purity and structural similarity of lignin samples, with well-defined peaks that match those expected for lignin indicating that the lignin has been isolated in a form of high purity (Bhat et al., 2009; Boeriu, Bravo, Gosselink, & Van Dam, 2004; Buranov et al., 2010; Klein et al., 2010; Liu et al., 2011; Toledano, Garcia, Mondragon, & Labidi, 2010).

Hydroxyl, methoxyl, carbonyl and carboxyl groups are the most important functional groups in lignin and vary in number and proportion depending on the plant source and extraction method used (Bhat et al., 2009). Thus infrared spectra obtained for different

lignin samples can be compared, for example, to commercial lignin samples, to gain an indication of purity. This is illustrated in Figure 9, in which there is good resemblance between lignin isolated from the black liquor of oil palm waste and commercial alkali lignin. The high resemblance indicates that the extracted lignin is pure and thus could be exploited for food industry applications (Bhat et al., 2009).

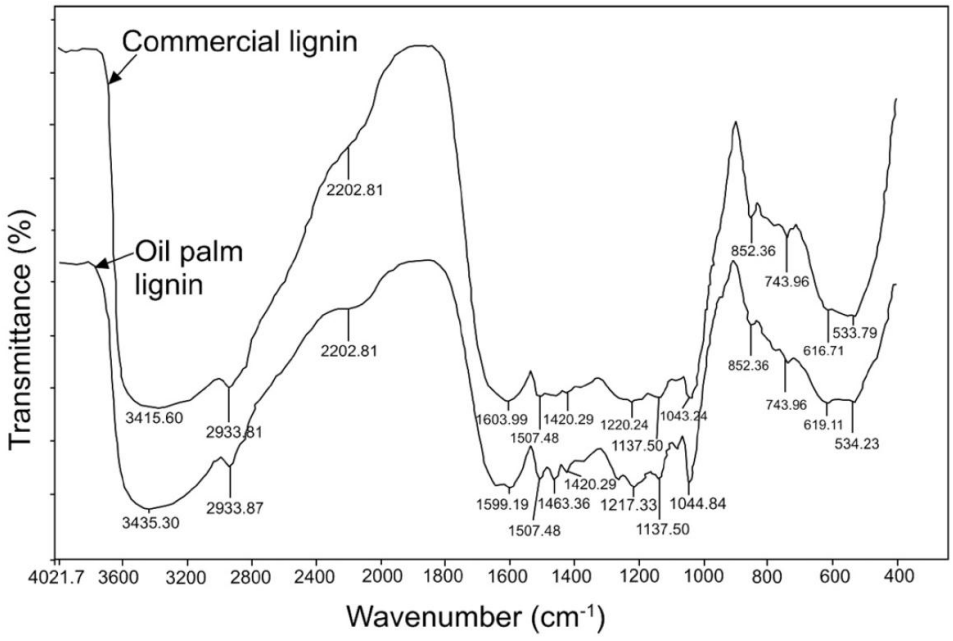


Figure 9. FTIR spectra of commercial alkali lignin and lignin isolated from oil palm waste (Bhat et al., 2009).

Table 5 outlines methods used by various researchers for FTIR analysis of lignin.

2.7.2.2. Nuclear magnetic resonance (NMR) analysis

While FTIR provides useful information on lignin structure, Klein et al. (2010) suggest that NMR spectroscopy can provide more detailed structural information. Similar to the use of FTIR analysis, NMR spectroscopy allows the whole lignin structure to be analysed. Different types of NMR can be used, including ^{13}C, ^{1}H, ^{31}P NMR and two-dimensional correlation spectra, such as heteronuclear single quantum correlation (HSQC) and heteronuclear multiple-quantum correlation spectroscopy.

Table 5. Working methods for FTIR analysis of lignin

Researcher(s)	Working method for FTIR analysis
(Klein et al., 2010)	- Analysed finely powdered lignin - Used Nicolet 6700 infrared spectrometer equipped with a Thermo Smart Orbit diamond anvil ATR probe - Sixteen scans were recorded over 1800-400cm^{-1}
(Bhat et al., 2009)	- Analysed powdered lignin - Analysis was performed in replicates (n = 5) - Used KBr pellets - Scans were recorded in the frequency range 4000-400cm^{-1}
(Buranov et al., 2010)	- Used Nicolet-380 FT-IR spectrometer using a Smart iTR ATR sampling accessory - Used a KBr disc containing 1% of finely ground samples (see method of KBr disc preparation)
(Liu et al., 2011)	- Used Perkin Elmer Spectrum 100 FTIR Spectrometer - Used a KBr disc containing about 1wt% KBr - Spectra recorded in a transmittance band mode in the range 800-4000cm^{-1}
(Toledano et al., 2010)	- Used Nicolet spectrometer of direct transmittance - Used KBR technique with 1% lignin - Twenty scans were recorded in the range 4000-400cm^{-1} with a resolution of 4cm^{-1}

Using ^{1}H NMR, Pan et al. (2006) found that long reaction time, high temperature, more catalyst and lower ethanol concentration led to lignin with increased phenolic hydroxyl groups (ArOH) and decreased aliphatic hydroxyl groups (AlkOH). This may have implications on the applications of lignins produced using different processing conditions.

Klein et al. (2010) used ^{31}P NMR to determine the hydroxyl group content of isolated organosolv lignins. The hydroxyl group values were also used to calculate the syringyl/guaiacyl/*p*-hydroxyphenyl ratio. HMQC NMR spectroscopy was used to determine composition and interunit linkages.

Sun et al. used ^{13}C and ^{1}H NMR to characterise lignin isolated from barley straw.

Capanema, Balakshin and Kadla (2004) suggest that quantitative ^{13}C NMR is the most commonly used NMR method for lignin characterisation because it is informative, reliable and relatively feasible. In addition to obtaining the same information as quantification by the HSQC technique, ^{13}C NMR can also provide information about

quaternary carbons. Therefore, ^{13}C NMR should be considered as a technique for lignin analysis in this project.

Table 6 outlines methods used by various researchers for the NMR analysis of lignin.

Table 6. Working methods for NMR analysis of lignin used by different researchers

Researcher(s)	Working method for NMR analysis
(Pan et al., 2006)	- Lignin acetylated prior to NMR analysis - Lignin acetate and p-nitrobenzaldehyde (internal standard) dissolved in 0.5mL of $CDCl_3$ - ^{1}H NMR spectrum recorded on Bruker 300 UltraShield spectrometer - Collected 128 scans - Functional group contents were calculated from integration ratios of the protons of functional groups to the protons of the internal standard (equation provided for this)
(Klein et al., 2010)	HMQC Spectroscopy - 100 mg of ASE-Alcell or dioxane-water acidolysis lignin dissolved in 0.84 mL of dimethyl-d_6 sulfoxide (DMSO) - gradient-selected heteronuclear multiple bond coherence (HMQC) spectra were recorded, with decoupling during acquisition - Spectra recorded at 25°C in an Avance 500 M Hz instrument - 12 ppm sweep width centred at 6 rpm used in ^{1}H - 200 ppm sweep width centred at 100 rpm used in ^{13}C - Spectra were required with 8 transients and a recycle delay of 1.5s over 256 increments and 2048 data points - $^{1}J_{CH}$ was 140 Hz ^{31}P NMR Spectroscopy - Lignin phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane (TMDP) - Inverse-gated decoupling - Spectra recorded at 25°C in an Avance 500 MHz instrument - 3200 data points, 256 scans, 62 ppm sweep width centred at 140 ppm, 25 s relaxation delay, and a 75° pulse - Chemical shifts calibrated from the sharp ^{31}P NMR signal at 145.1 ppm arising from the reaction product between cyclohexanol and TMDP - Cyclohexanol (4.0 mg/mL) used as internal standard
(Sun et al., 2011)	- ^{13}C and ^{1}H NMR spectra recorded on a Bruker Avance 500 MHZ spectrometer from 200 mg and 80 mg of sample dissolved in DMSO-d6 (1.0 mL), respectively - HSQC NMR spectrum acquired using 90° pulse width, 0.2 s acquisition time, 2.0 s pulse delay and $^{1}J_{C-H}$ of 150 Hz - 30,000 scans obtained for the acquisition of ^{13}C NMR spectra of hemicellulosic and lignin preparations.

2.7.3. Molecular weight analysis

The molecular weight of polymers influences the applications that they can be used in. Toledano et al. (2010) state that the high molecular weight distribution of lignin contributes to difficulties in its commercial use. Pan et al. (2006) suggest that a relationship exists between lignin molecular weight and antioxidant activity. Afanas'ev, Selyanina and Selivanova (2008) outline that molecular weight influences hydrophobicity; lower molecular weight lignin fragments are more hydrophilic because they contain a larger number of phenolic hydroxyls. Although the lignin in this project is to be microparticulated, it may still be important to characterise the molecular weight and molecular weight distribution of lignin as a raw material.

Table 7 gives details of methods used by various researchers for lignin molecular weight determination. Various size exclusion columns utilising tetrahydrofuran (THF) or aqueous NaOH as eluents have been used to determine the molecular weight distributions for pure lignin substances. For example, Table 7 shows that many investigators have used size exclusion chromatography (SEC) or gas permeation chromatography (GPC), with THF as the eluent, to determine the molecular weight of lignin samples. In these methods, lignin samples are first derivatised to improve solubility in the THF, since lignins are only sparingly soluble in the native, underivatised form (Guerra et al., 2007). A common form of derivatisation has been the acetylation of lignin with acetic anhydride/pyridine. However, Guerra et al. (2007) suggest that derivatisation in acetylbromide is an alternative that is simple and rapid, yet still allows an accurate analyses to be carried out.

Gidh, Decker, Vinzant, Himmel and Williford (2006) suggest that adjustments can be made to the basic size exclusion method to improve lignin molecular weight determination. For example, the replacement of preparative SEC columns with HPLC columns has been found to greatly reduce the reaction time. Furthermore, light scattering techniques such as multiangle laser light scattering (MALLS) and Quasi-elastic light scattering (QELS), can be integrated with SEC, allowing for direct determination of the molar mass and size distributions of polymers. In particular, high molecular weight aggregates of lignin can be detected, which cannot be detected by UV

Table 7. Working methods for molecular weight analysis of lignin used by different researchers

Researcher(s)	Working method for molecular weight analysis
(Pan et al., 2006)	- Lignin acetylated with pyridine-acetic anhydride - Analysis by GPC, using an 1100 HPLC system - 1 mg lignin acetate dissolved in 1 mL THF without stabiliser - 50 µL of solution injected onto the GPC - Column operated at 50°C and eluted with THF at 1 mL/min - Columns calibrated with polystyrene standards - Lignin samples and polystyrene standards detected by MWD at 280 and 254 nm, respectively - Determined number-average (M_n) and weight-average (M_w) molecular weights
(Liu et al., 2011)	- No mention of lignin derivatisation - Analysis by GPC on a Skodex KF-802.5 column - 10 µL samples dissolved in THF - Column operated at 30°C and eluted with THF at 1 mL/min - Column calibrated using polystyrene stanards
(Toledano et al., 2010)	- Lignins acetylated prior to analysis - Perkin-Elmer instrument equipped with an interface (PE Series 900) was used - Three Waters Styragel columns used - Flow rate of 1mL/min used - Calibration was made using polystyrene standards
(El Mansouri & Salvadó, 2006)	- Lignins acetylated first to enhance solubility in chromatographic eluent, tetrahydrofuran (THF) - Three styrene-divinylbenzene copolymer gel columns of 50, 500 and 104 Å were used - Effluent monitored at 254nm with a BECKMAN UV-detector - Calibration was made using polystyrene standards in the 92 – 66,000 g/mol range - Flow rate of 1 mL/min was used - Samples were dissolved in THF at concentration of 1 mg/mL and stored for 24 h at 5°C to avoid variations in molecular weight
(Lawther, Sun, & Banks, 1995; Sun et al., 2011)	- PL Aquagel-OH 50 column was used - Calibration was made using PL pullulan polysaccharide standards - The pump used was a Knauer HPLC Model 64 with a flow rate of 0.1 ML/min - The eluent used was 0.02 M NaCl in 0.005 M sodium phosphate buffer, pH 7.5 and the samples were dissolved in the eluent at a concentration of 0.1% - Detection was achieved using a Knauer differential refractometer - Column oven was maintained at 30°C
(Zhang, Yuan, Peng, Xu, & Sun, 2010)	- PL-gel 10mm Mixed-B 7.5mm ID column was used - 4mg lignin sample was dissolved in 2mL THF and 20µL solutions were injected - Column was operated at ambient temperature - Flow rate of 1 mL/min was used

	- Monodisperse polystyrene was used as the reference material for determining the molecular weight of lignin
(Buranov et al., 2010)	- Lignins acetobrominated first - HPLC system equipped with two Styragel columns (HR5E and HR1) connected in series was used - Eluent used was THF at flow rate of 0.55 mL/min - Analysis was carried out at 40°C - An aliquot of 120 µL of the sample dissolved in THF (approx. 2 mg/ML conc) was injected into the system - Toluene was used as an internal standard and added at a concentration of 0.05% to the samples prior to injection - Measurements were performed with a UV detector set to 280nm Calibration was made using polystyrene standards

and RI detectors. Thus, it may be useful to develop the size exclusion technique for the molecular weight analysis of lignins.

Molecular weight analyses can be used to determine the number-average (M_n) and weight average (M_w) molecular weight of lignin, and also the polydispersity (M_w/M_n). The value of the polydispersity can be used to indicate the fraction of low molecular weight lignin present in the sample. For example, a low polydispersity indicates a high fraction of low molecular weight lignin (El Mansouri & Salvadó, 2006; Toledano et al., 2010). It is interesting to note that for the pressurised aqueous ethanol extraction of flax (*Linum usitassimum L.*) shives, Buranov et al. (2010) found that lignins with high molecular weights of around 30,000 Da were produced, compared to commercial lignins, which generally have molecular weights of around 5000 Da. It is suggested that this was due to the absence of acidic or basic catalysts in the procedure used, since both acidic and basic catalysts are known to cleave bonds in lignins and hemicellulose, thus degrading the polymers and causing a reduction in molecular weight. However, the higher molecular weight may also have been due to the type of plant species and the bound hydroxycinnamic acids; refer to Buranov et al. (2010) for further details.

2.7.4. Lignin solubility

The solubility of lignin influences the possible applications it can be used in, possible chemical modifications and can also be used to separate different fractions of lignin (Buranov et al., 2010). Furthermore, Ni and Hu (1995) suggest that the solubility of

lignin in water and ethanol can indicate the functional groups present and their interactions.

Buranov et al. (2010) used the equilibrium solubility method at room temperature to determine the solubility of lignin samples in water-ethanol mixtures. The procedure involved the addition of an excess amount of lignin sample to various concentrations of aqueous ethanol solutions, mixing by sonication, centrifugation of the sample and an analysis of the lignin concentration in the supernatant/filtrate by UV-Vis spectrometry. The procedure ultimately provided solubility values in mg/L; Refer to Buranov et al. (2010) for further details of the method.

2.7.5. Differential scanning calorimetry

Diffrential Scanning Calorimetry (DSC) is used to determine the thermal behaviour associated with physical and/or chemical changes of a substance, with increases or decreases in temperature at a constant rate (Toledano et al., 2010). DSC is considered an excellent technique for the identification of phase transitions such as glass transition, cold crystallization and melting temperatures of lignin (Buranov et al., 2010; Hatakeyama, 1992; Koullas et al., 2006).

Buranov et al. (2010) suggest that the glass transition temperature (T_g) is an indicator of thermal stability and that lignins with a higher T_g are able to be used in higher temperature applications. Glass transition is also related to molecular weight. It was found that for lignin extracted from flax shives using pressurised aqueous ethanol, the T_g of the high molecular weight lignin (HML) fraction was higher than the T_g of the low molecular weight (LML) fraction. The T_g values of the low and high molecular weight fractions were 122°C and 160°C, respectively. This may indicate that higher molecular weight lignins have greater suitability for use in high temperature applications.

It must also be remembered that lignin quality varies depending on the genetic origin of the plant, the particular plant tissue and the applied extraction process (Buranov et al., 2010). Therefore, a wide range of T_g values can be expected for lignins obtained from different sources and extraction processes, as shown in Table 8.

Table 8. Glass transition temperatures of various lignins

Reference	Lignin type	Glass transition temperature (°C)
(Toledano et al., 2010)	Alkali lignin from *Miscanthus sinensis*	105 - 110
(Buranov et al., 2010)	Organosolv (Sigma-Aldrich)	106
	Lignosulfonates	105
	Bagasse lignin	142
	Steam explosion and kraft lignin	~175
	HML from flax shives	160
	LML from flax shives	122
(Fox, 2006)	Organosolv from Pine	91
	Organosolv from Aspen	97

Toledano et al. (2010) suggest that because of the strong electrostatic interactions and variation in lignin with so many factors, it is difficult to determine the glass transition temperature of lignin with reliability. However, both Toledano et al. (2010) and Buranov et al. (2010) give methods for determining the DSC analysis of lignins, as summarised in Table 9.

Table 9. Working methods for DSC analyses of lignin used by different researchers

Researcher(s)	Working method for DSC analysis for lignin
(Buranov et al., 2010)	- Performed with DSC under nitrogen atmosphere - Lignin powder dried at 50°C in vacuum oven overnight - Samples subjected to heating from -30 to 200°C at a rate of 10°C/min, cooled at a rate of 10°C/min from 200 to -30°C and heated a second time from -30 to 200°C at a rate of 10°C/min - Thermal analysis for melting point determination was carried out over a 20-300°C range at a heating rate of 10°C/min
(Toledano et al., 2010)	- Carried out in a thermal analyser DSC 821 of Mettler Toledo under nitrogen atmosphere - Dynamic scans of temperature from 30 to 250°C with a constant heating rate of 10°C/min - Sample size of approximately 5 mg used to minimise the mass and heat transference differences of the results

Table 9 shows some similarities in the DSC analysis methods used by Toledano et al. (2010) and Buranov et al. (2010), including the use of a nitrogen atmosphere, the heating rate of 10°C/min and the similar temperature ranges. More details of sample preparation and references to methods followed are given by Buranov et al. (2010).

2.8. Microparticulation

Given the insolubility and hydrophobicity of lignin, microparticulation has been identified as an appropriate means by which refined lignin can best be incorporated into food systems. Microparticles can enter food systems without imparting negative sensory or stability characteristics and may further have use as fat mimetics through the ability to mimic fat globules. Microencapsulation is a technology derived from the formation of microparticles and it is suggested that microencapsulation technologies are all based on three basic techniques – solvent extraction/evaporation, phase separation (coacervation) and spray-drying (Freitas, Merkle, & Gander, 2005). Thus microparticulation processes have not been classified since the processes are often hybrids of these three techniques or use different mechanisms simultaneously (Tran, Benoît, & Venier-Julienne, 2011). However, Table 10 summarises the key principles of various microparticulation processes, along with the advantages and disadvantages of these processes. Lignin and zein microparticles, both hydrophobic microparticles, have been produced using methods based on the liquid-liquid dispersion technique described in Table 10. Details of some of the desirable properties and production of these particles are given in Table 11.

Table 10. Details of various microparticulation methods – how the method works, implications, examples of use and reference sources

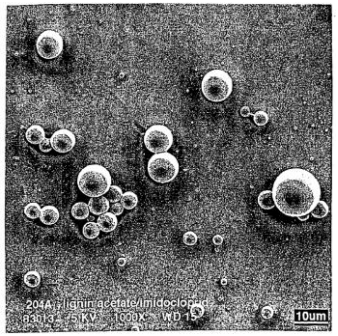
Method	How it works	Advantages and disadvantages	Examples of use	References
Liquid-liquid dispersion / Solvent evaporation / extraction	- Polymer is dissolved in a solvent. An emulsion of solvent droplets in a continuous phase is formed. The polymer is not soluble in the continuous phase, therefore, microparticles form by solidification/precipitation when the solvent is removed by either evaporation or extraction. - Solvent evaporation – disperse phase solvent is unable to be completely dissolved in continuous phase, therefore solvent must evaporate from the surface of the dispersion to yield sufficiently hardened microparticles. - Solvent extraction – disperse phase solvent can be completely dissolved in the continuous phase	- Solvent evaporation requires relatively high temperatures and reduced pressures - Solvent extraction uses large amounts of second solvent and therefore creates problem of solvent mixtures recovery - Both processes require long processing times, which may promote particle aggregation and therefore high polydispersity - Can be performed in either batch or continuous mode	Zein, lignin microparticles for controlled release of agricultural actives, poly(lactic-*co*-glycolic acid) (PLGA) microparticles	(Della Porta, Falco, & Reverchon, 2011; Freitas et al., 2005)
Rapid expansion of supercritical solutions (RESS)	- Based on expansion of supercritical solution through a narrow nozzle - Rapid expansion favours solute nucleation leading to particles of narrow size distribution - Factors influencing particle size include temperature, pressure and solute concentration	- Small particles with narrow size distribution are formed - But product morphology can vary greatly depending on solute concentration, pre-expansion and expansion temperature and pressure of extraction - Some polymers may not be soluble in the supercritical solvent	Development of fine caffeine powders	(Ksibi, Subra, & Garrabos, 1995)

Method	How it works	Advantages and disadvantages	Examples of use	References
Supercritical emulsions extraction (SEE)	- SC-CO2 used as the extracting agent for the "oily" phase of oil-in-water emulsions. Leads to solvent-free microparticles	- The presence of an external water phase, immiscible with SC-CO_2 prevents the aggregation of particles - Very fast process, which enables narrow particle size distribution - Major limitation is that it is intrinsically a batch process. However, SEE can now be operated in continuous mode	PLGA microparticles	(Della Porta et al., 2011)
Jet break-up processes	- Polymeric solution pushed through nozzle at constant flow rate, forming a laminar jet, which is then broken into a chain of monodispersed droplets - Different processes: - Electrostatic droplet generation - Jet-cutter technology - Jet excitation - Flow focusing - Precision particles fabrication	- Refer to (Tran et al., 2011) - Some advantages include high reproducibility of microparticle production, time saving, ability to be carried out under aseptic conditions and ability to be scaled up		(Tran et al., 2011)
High pressure homogenisation	- Suspensions heated above T_g of polymer to lower the viscosity of the dispersed polymer phase, then dispersion by high pressure homogenisation	- Use of organic solvents can be avoided - Scale-up is simple - Can run process continuously if use special equipment	PLGA microparticles	(Calvör & McIller, 1998)

Method	How it works	Advantages and disadvantages	Examples of use	References
Microfluidisation	- Dispersed phase pushed through micro-channel and broken into droplets at the end of the channel - Microporous membrane emulsification – either the dispersed phase droplets are created at the membrane surface as the dispersed phase is pushed through membrane pores into the continuous phase or use a 'premix-membrane,' where a coarse O/W emulsion is prepared first and then pushed through the membrane many times to obtain particles with a fairly narrow size distribution. - Terrace-like microchannel system – droplet formation is governed only by interfacial tension, which leads to monodispersed droplets.	- Produces monodisperse particles - Membrane process allows narrow droplet size distribution with low mechanical stress and low energy input compared to conventional mechanical methods - Membrane processes are limited by low production rates and coalescence of microparticles - Membrane and microchannel systems require low viscosity solutions - Process conditions for microchannel emulsification are relatively easy	Gelatin and alginate microparticles (terrace-like microchannel system)	(Tran et al., 2011)

Method	How it works	Advantages and disadvantages	Examples of use	References
Spray drying	- Transforms a feed solution, suspension or emulsion from a fluid state into a dried particulate form by spraying into a hot drying medium	- One step, continuous process that does not require a second drying step - Size of the powder can be controlled in a single step and particle morphology and density can be controlled - Highly reproducible, easy to scale up and offers narrow particle size distribution - High throughput - Cannot be used for temperature sensitive materials	PLGA microparticles,	(Della Porta et al., 2011; Elversson & Millqvist-Fureby, 2005; Elversson, Millqvist-Fureby, Alderborn, & Elofsson, 2003; Freitas et al., 2005; Rizi, Green, Donaldson, & Williams, 2011)
Powder production via printing	- Process: High viscosity printing head (inkjet type) ↓ Generate monodisperse droplets ↓ Droplets are dried in a tower ↓ Monodisperse powder is obtained - Can dry with mediums other than air, such as nitrogen , CO_2, zeolites and superheated steam	Advantages: - Optimal powders are produced (monodisperse with tunable size, homogeneous, no fines, high density powders possible) - Other drying gases can be used (e.g. N_2) – can dry sensitive products or change the powder properties produced - Can be combined with existing processes (e.g. spray-drying) Disadvantages - Need new equipment	None, but a wide range of product streams can be used	(TNO, n.d.; van Bommel, 2010)

Table 11. Applications, important characteristics and production methods of relevant microparticulate systems

Microparticle Type	Application(s)	Important characteristics	Production
Lignin matrix microparticles	Controlled release of agricultural actives (Asrar & Ding, 2006, 2008)	Size – influences release of agricultural active. Preferred average diameter < 100 µm, more preferred < 25 µm and even more preferred < 10 µm. The microparticles do not have to be the same size, but may be of different sizes within the *same range*. Shape – regular spherical shape is advantageous because it ensures steady and more predictable release of the active from the particle	Range of methods used previously presented by Asrar and Ding (2006) – e.g. *in situ* polymerization of a polymeric barrier wall at the surface of droplets in emulsions, melting lignin with an active agent and subsequently cooling the melt to form a glass that can take any size or shape, for example, granules and, finally, the formation of matrix microparticles. Method patented by Asrar and Ding (2006) comprises the steps of: "forming an emulsion… of an organic solution in an aqueous solution, wherein the organic solution contains a lignin derivative which is soluble in the organic solvent in an amount of at least about 1% by weight at 20°C, and an agricultural active in a volatile organic solvent and the aqueous solution contains an emulsifier which is different from the lignin derivative and the agricultural active; and removing the organic solvent, thereby producing microparticles having a matrix comprising the lignin derivative within which the agricultural active is distributed." Using this emulsion / liquid-liquid dispersion technique, it is easy to produce microparticles using conventional equipment. 

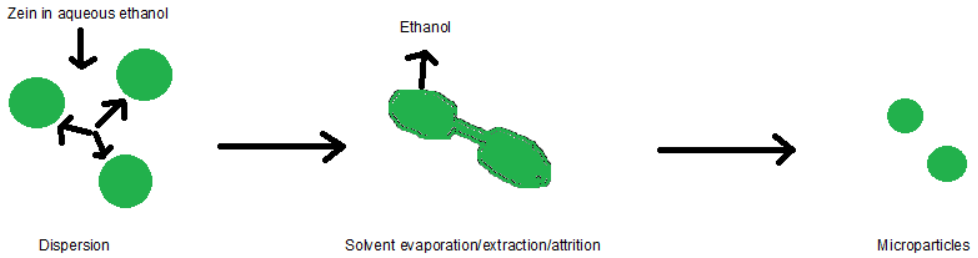
Microparticle Type	Application(s)	Important characteristics	Production
Zein microparticles	Fat substitute (Stark & Gross, 1994) Carrier materials / delivery systems in foods (nanoparticles) (Patel, Bouwens, & Velikov, 2010; Zhong & Jin, 2009)	Isoelectric point – influences stability in intestine and aggregation during drying	Based on liquid-liquid dispersion (Patel et al., 2010; Zhong & Jin, 2009), which "generally involves solubilizing the hydrophobic protein in an organic solvent or aqueous mixture thereof, a salt solution, or a solution having an extreme acidic or basic pH, and adding the resulting protein solution to an aqueous medium under conditions appropriate to precipitate the protein, and thereby to form a suspension of the protein microparticles. Microparticles produced by the method are uniform, spherical, water-insoluble, water-dispersible particles of non-denatured hydrophobic protein" (Stark & Gross, 1994). 
Whey protein microparticles	Fat substitute	Uniform, spherical, water insoluble particles 0.1-4.0 µm in diameter	Gelled whey protein microparticles formed by simultaneous homogenisation at pasteurisation temperatures. Heating causes proteins to denature and then aggregate. The shearing action caused by homogenisation ensures that the aggregation stops in the micrometer range (Singer, 1996). Microfluidisation and high intensity ultrasounds have also been used.

Table 11 indicates that liquid-liquid dispersion is likely to be a useful technique for the production of lignin microparticles. Therefore, it is important to understand the parameters influencing liquid-liquid dispersion and how these can be controlled to produce microparticles with certain properties.

2.8.1. Factors influencing microparticulation by liquid-liquid dispersion

There are a range of factors affecting the properties of microparticles produced by the liquid-liquid dispersion technique. These include mixer type and speed, polymer concentration in the dispersed phase, the volume of the dispersed phase, the viscosity of the continuous phase, the rate of solvent removal and the type and concentration of added surface materials. The purpose of this section is to discuss the effects of each of these factors on microparticle properties. However, before considering the effects of different factors on microparticulation, it is first important to look more closely at the events that take place during liquid-liquid dispersion.

2.8.1.1. Liquid-liquid dispersion

The dimensionless capillary number characterises the break-up of droplets in liquid-liquid dispersion, with droplet break-up occurring when the capillary number reaches a certain critical value. Equation 1 defines the relationship between the capillary number, Ca, the viscosity of the continuous medium, μ_o, the shear rate, G, the droplet radius before deformation, a, and the interfacial tension, γ.

$$Ca = \mu_o Ga/\gamma$$

Equation 1

Any factors that influence the parameters in Equation 1 are important factors in the control of particle properties. For example, any change that reduces γ, such as the addition of surfactants or the choice of more miscible phases for the dispersed and continuous phase, will cause droplet deformation and break-up to occur more easily (Alargova, Paunov, & Velev, 2006; Wege, Kim, Paunov, Zhong, & Velev, 2008).

Zhong and Jin (2009) suggest that there are three competing mechanisms involved in zein particle formation during liquid-liquid dispersion:

- Break-up of stock solution droplets by the shear force
- Solidification of zein during solvent attrition
- "Coalescence" or "partial coalescence" of droplets

It is suggested that if droplets collide and the zein is still soluble at the point of collision due to high ethanol concentration of the stock solution, the droplets may coalesce and thus precipitate in irregular shapes or structures. Precipitation of zein into irregular structures can also occur if the zein precipitates before the stock solution is broken up into droplets, which may occur if the ethanol concentration of the zein stock solution is low. Thus solvent concentration and also the time-scale of droplet break-up compared to polymer solidification are important for the formation of discrete particles (Zhong & Jin, 2009).

It is also important to note that particles of different shape can be produced. Campbell, Holt, Stoyanov and Paunov (2008) report that anisotropic micro-rods can be produced by dispersing a viscous polymer solution under high shear into a viscous continuous phase solution, in which the dispersed phase solvent, but not the polymer itself, is soluble. Rods are then formed if the dispersed phase droplets precipitate while in an elongated state. Alargova, Bhatt, Paunov and Velev (2004) suggest that the viscosity of both phases and the capillary number are critical determinants for the successful formation of microrods. Lens and donut shaped microparticles have also been produced (Wege, Dyab, Velev, & Paunov, 2007). The preparation of a variety of particle shapes could be very useful for food industry applications; however, it is likely to fall outside the scope of the current work.

2.8.1.2. Effect of mixer type and speed of mixing

Mixing is important during both initial emulsion or droplet formation and droplet breakup. The type and speed of mixing influences the initial droplet radius, which according to the capillary number equation, influences the capillary number and thus the

final particle size. Mixing type and speed also directly influences droplet deformation and break-up through control of the shear rate.

2.8.1.2.1. Type of mixing

O'Donnell and McGinity (1997) suggest that the most common method of dispersion of the "oil" phase into the continuous phase is the use of a propeller style blade attached to a variable speed motor. As the speed of the motor increases, the shear induced by the propeller increases and the size of the dispersed droplets decreases. Smaller droplets should correspond to smaller particles after solidification (Zhong & Jin, 2009). Other methods for producing the initial emulsion include homogenisation, microfluidisation, sonication and potentiometric dispersion. Homogenisation applies very high shear to produce the emulsion, allowing the production of much smaller particle sizes compared to conventional agitation (O'Donnell & McGinity, 1997).

Stark and Gross (1994) describe both batch and continuous methods for the preparation of zein microspheres. Figure 10a shows the batch method, in which an organic phase containing zein is pumped into an aqueous phase undergoing rapid stirring. In addition to the speed of mixing, conditions in the reactor such as pH, temperature, residence time, protein concentration and presence of additives determines the size of the resulting particles. Figure 10b shows a continuous method, in which the aqueous and organic solutions are pumped separately into a mixing cell and the final mixture flows to a collection tank. The formation of microparticles and control of microparticle properties is the same as for the batch process, except that because the protein, solvent and particle concentration are constant over the whole time of mixing, final particle properties can be better controlled. Finally, Figure 10c shows a "plug flow precipitation" method, which is a continuous method with recycle of the microsphere suspension back through the homogeniser. Thus there is a variety of mixing techniques available for the formation of microparticles. It appears that batch methods are simple to set up, but the changing conditions in the mixing tank indicate less control of final particle size compared to the continuous techniques.

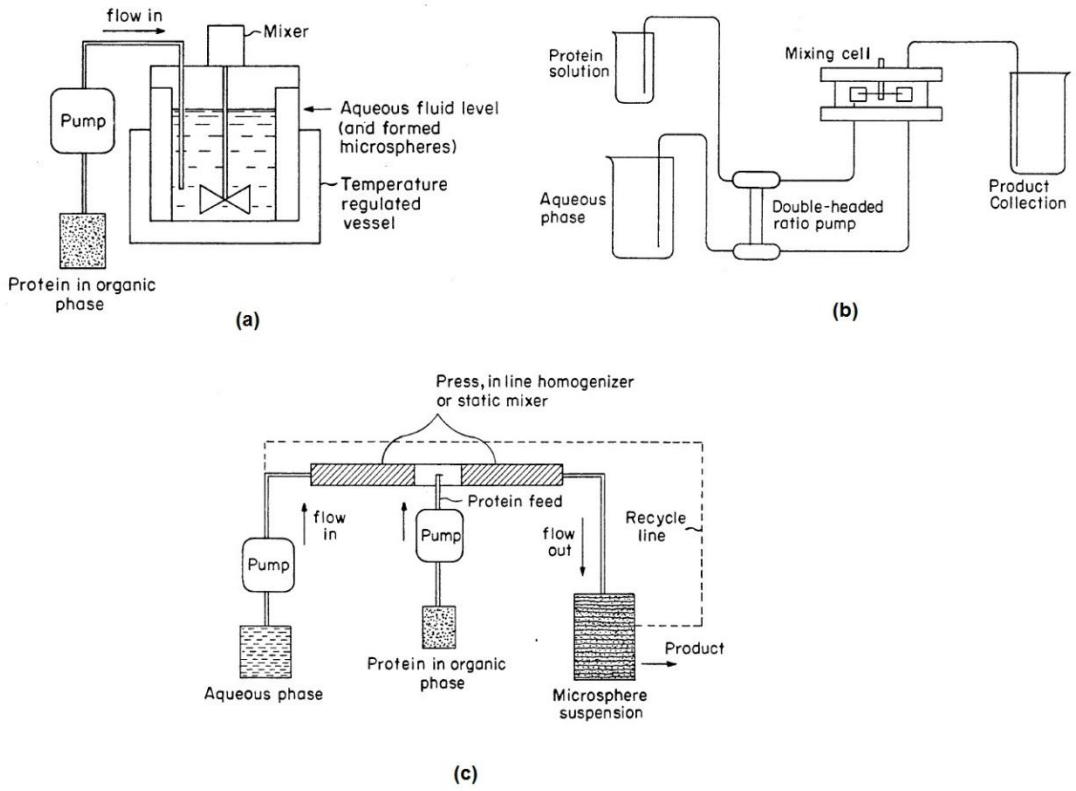


Figure 10. Examples of methods used for the preparation of microspheres: (a) the "fed batch" method, (b) the flow through mixing system and (c) the "plug flow precipitation" method (Stark & Gross, 1994).

2.8.1.2.2. Speed of mixing

The speed of mixing is also important. Consistent with the suggestion of O' Donnell and McGinity (1997) that droplet size decreases with increasing shear rate, Sansdrap and Moes (1993) found that an increase in stirring rate caused a decrease in both particle size and particle size distribution. Similarly, Alargova et al. (2006) found that both rod length and diameter decreased with increasing shear stress, which was increased via an increase in shear rate. Stark and Gross (1994) also suggest that faster mixing reduces the chance of aggregation and that the most efficient formation of homogeneous spheres can be achieved through fast mechanical stirring.

Zhong and Jin (2009) found that smaller zein microparticles were prepared using a rotor speed of 10,000 rpm than using a rotor speed of 5,000 rpm. However, particles prepared

at 15,000 rpm were larger than those prepared at 10,000 rpm. Scanning electron microscopy (SEM) analysis revealed that particles prepared at all three speeds were spherical and had diameters < 200 nm. Nonetheless, the SEM images also showed that particles produced at 15,000 rpm were less discrete than those prepared at either 5,000 or 10,000 rpm (Figure 11). Since the dynamic light scattering (DLS) used to measure particle size in this work measures the hydrodynamic diameters of structures, the "less discrete" nature of the particles prepared at 15,000 rpm may explain why the measured size of these particles was larger. This indicates that there is a limit to the decrease in particle size that occurs with increase in mixing speed and that larger particles can even be formed when the speed is excessive.

Ellis and Jacquier (2009) obtained similar results in the preparation of κ-carrageenan microspheres. The microspheres were produced by forming an emulsion of κ-carrageenan solution in hot rapeseed oil and subsequently tempering the emulsion under mild agitation to produce gelled microspheres. When both the emulsion formation step and the quenching stage were carried out using the Silverson at 7000 rpm, it was found that although small microspheres had been produced, they had largely aggregated in clumps, leading to larger measured size. Collisions of microspheres due to the high shear force applied during the quenching stage may have caused the aggregation. Hence the preparation process was altered to prevent aggregation: Silverson mixing at 3000 rpm was used for emulsion formation, followed by mixing with a Heidolph mixer at 400 rpm for the quenching step. It is necessary to carefully optimise mixing speed to ensure that particles of the desired size are produced and do not coalesce or aggregate as they solidify.

Asrar and Ding (2006) also utilised a two-stage mixing regime for the preparation of lignin microparticles. A Silverson mixer was used for the initial mixing of the dispersed and aqueous phases, which lasted for a period of three minutes. The emulsion was then mechanically stirred for 20 hours, allowing the organic solvent to slowly evaporate. This indicates that strong mixing is important when the two phases are initially mixed, but subsequent mixing should be gentle to avoid droplet coalescence and/or aggregation of solidified particles.

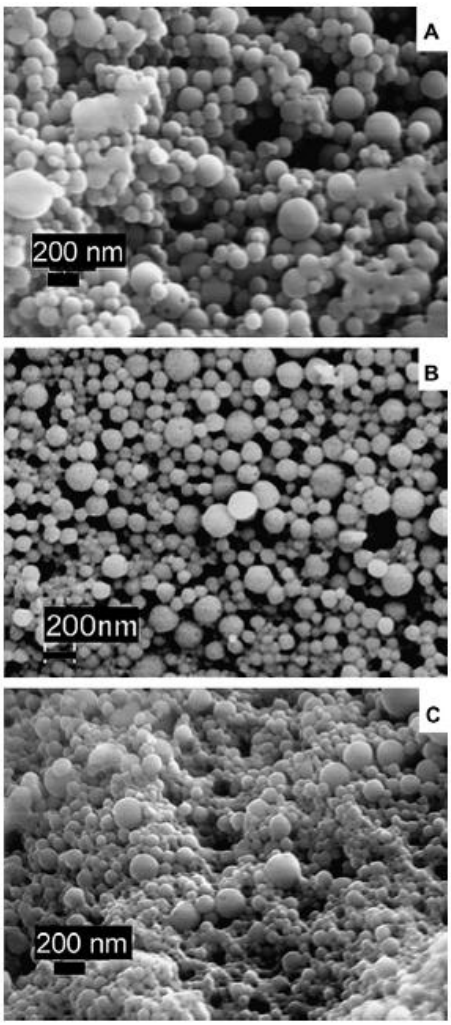


Figure 11. SEM images of zein nanoparticles prepared by shearing a zein stock solution (1g of zein in 85% aqueous ethanol) into deionised water at a homogenisation speed of (A) 5000, (B) 10,000, and (C) 15,000 rpm (Zhong & Jin, 2009).

Zein particles have been produced using both magnetic stirring and high shear mixing (Parris, Cooke, & Hicks, 2005; Patel et al., 2010; Zhong & Jin, 2009). Notably, Patel et al. (2010) mixed the dispersed and continuous phases using magnetic stirring at 1000 rpm and obtained particles 120-150 nm in size, indicating that very high shear rates are not always necessary for the production of small particles. The solvent was removed using rotary evaporation, which perhaps allowed a more gentle agitation of the particles during solvent removal, thereby preventing coalescence and aggregation. However, the

particles were also stabilised by sodium caseinate and thus the speed of mixing during solvent removal may have been less critical. Ultimately, this indicates that mixing types are open for investigation in this thesis project, and also that factors other than shear may have an important influence on particle properties.

2.8.1.3. Effect of polymer concentration in the dispersed phase

As the concentration of polymer in the dispersed phase increases, the size of the resultant microspheres also increases. This may be due to the effect of polymer concentration on dispersed phase viscosity; as the polymer concentration increases, the viscosity of the dispersed phase increases and higher shear forces are required for droplet break-up. Hence larger droplets and, ultimately, larger final particles, are produced (Freitas et al., 2005; Wege et al., 2008; Zhong & Jin, 2009). Stark and Gross (1994) found that reducing zein concentration in the dispersed phase from 9 to 7% (w/v) reduced the median particle size from 1.10 µm to 0.42 µm. Zhong and Jin (2009) reported no increase in particle size with increase in dispersed phase zein concentration for concentrations up to 6.7% (w/v). However, for concentrations greater than 13.3% (w/v), large agglomerates of zein were visible after homogenisation, indicating that the stock solution was not dispersed effectively at these higher concentrations. This suggests that there is an upper limit for polymer concentration that cannot be exceeded if discrete particles are to be formed.

Alargova et al. (2006) claim that the increase in dispersed phase viscosity with increase in polymer concentration also influences the rate of solvent attrition. Higher viscosity droplets will retain their shape while the solvent is partitioning into the continuous phase or evaporating. This may be important in the formation of non-spherical shapes such as rods or discs, where the deformed shape must be maintained during solidification of the polymer.

The precipitation of polymers into particles can also be described in terms of classical crystallisation theory (Dixon, Johnston, & Bodmeier, 1993). At a higher polymer concentration, the ratio of overall polymer to uncrystallised polymer after reaching equilibrium is higher. This higher ratio or degree of supersaturation means that there is a larger number of nuclei present (Kashchiev & Van Rosmalen, 2003). The presence of

more nuclei favours faster polymer precipitation since there are more nuclei for polymers to precipitate on. Faster precipitation may lead to larger particles since there is less time available for droplet size to be reduced (Zhong & Jin, 2009).

For the preparation of lignin microparticles, there is an opportunity to use the lignin-containing solvent liquor immediately after the lignin extraction stage of the process. In this case, the lignin concentration will be dependent on the amount of lignin extracted and solubilised in the extraction liquor. Therefore, it will be important to quantify the concentration of lignin in the extraction liquor and to determine whether this concentration is consistent between runs.

2.8.1.4. Effect of solvent type and concentration

The solvent type and concentration chosen depends on the polymer solubility characteristics and the method of solvent removal.

2.8.1.4.1. Effect of solvent type

The solubility of the polymer in the solvent solution impacts the final size of the microparticles. If the polymer has high solubility in the solvent solution, it is less likely to aggregate in the solvent and more likely to have a smaller final particle size once the solvent is removed. For example, Hurtado-Lopez and Murdan (2005) found that during the preparation of ovalbumin-loaded zein microspheres, the addition of sodium hydroxide to the ovalbumin-zein-ethanol mixture resulted in a decrease in particle size and in the mass of protein precipitated. It was suggested that this was due to the solubility of zein in alkaline solutions (Hurtado-Lopez & Murdan, 2005).

Freitas et al. (2005) state that regardless of whether solvent evaporation or solvent extraction is used for the removal of solvent in microparticle formation, the dispersed phase solvent must be at least slightly miscible with the continuous phase. This allows the solvent to partition into the continuous phase, allowing the polymer to precipitate. On the other hand, O'Donnell and McGinity (1997) suggest that for solvent evaporation processes, the dispersed phase solvent should be immiscible with the continuous phase. However, it is agreed that for microparticles to form, the organic solvent must diffuse into the continuous phase to evaporate at the air-water interface. Perhaps slight

miscibility ensures that the polymer remains soluble for long enough for dispersion of the solvent solution into small droplets, before the solvent slowly diffuses into the continuous phase.

Baimark, Srihanam, Srisuwan and Phinyocheep (2010) found that, using a water-in-oil emulsification method for the preparation of silk fibroin (SF) microparticles, organic phase solvents with higher water solubility led to more rapid diffusion of water from the droplet to the continuous organic phase. The rapid solidification influenced both particle size and shape. For example, using ethyl acetate or diethyl ether as the continuous phase caused more rapid solidification of SF particles than dichloromethane or chloroform. Faster solidification resulted in larger particle sizes, which was likely to have been due to the reduced time available for the SF stock solution to be sheared into smaller droplets before solidification. Hollow or bowl-shaped particles were also produced when the solidification was rapid. This may have been caused by movement of the organic continuous phase solvent into the SF solution droplets at the same time as droplet solidification, leading to distorted final particle shape (Baimark et al., 2010). The same effect on size and shape may occur when microparticles are prepared using the oil-in-water emulsification method.

For the preparation of lignin microparticles by solvent evaporation with water as the continuous phase, Asrar and Ding (2006) suggest that the organic solvent should have low solubility in water and a normal boiling point lower than that of water. Considering these preferences, methylene chloride, chloroform and ethyl acetate are suggested to be preferred solvents, with methylene chloride found to be the most preferred solvent. The suggestion of low water solubility may support that high solubility can cause solvent diffusion to occur too rapidly, yet some degree of miscibility with the continuous phase is still important to allow diffusion to occur. In this thesis project, the organic solvent is likely to be limited to the solvent used for lignin extraction. The likely solvent is aqueous ethanol. This solvent would ensure good solubility of the lignin since it is the extracting medium, but is also completely miscible with water. This miscibility may be something to consider if only large particles or irregularly shaped particles are formed in this work.

2.8.1.4.2. Effect of solvent concentration

The higher the solvent concentration, the smaller the particle size produced. Zhong and Jin (2009) suggest that this is due to the effect on the rate of polymer solidification. When the solvent concentration is high, it takes longer for the solvent to be removed, allowing more time for the droplets to be sheared into smaller sizes. For example, Figure 12 shows the effect of increasing ethanol concentration on the size of zein nanoparticles.

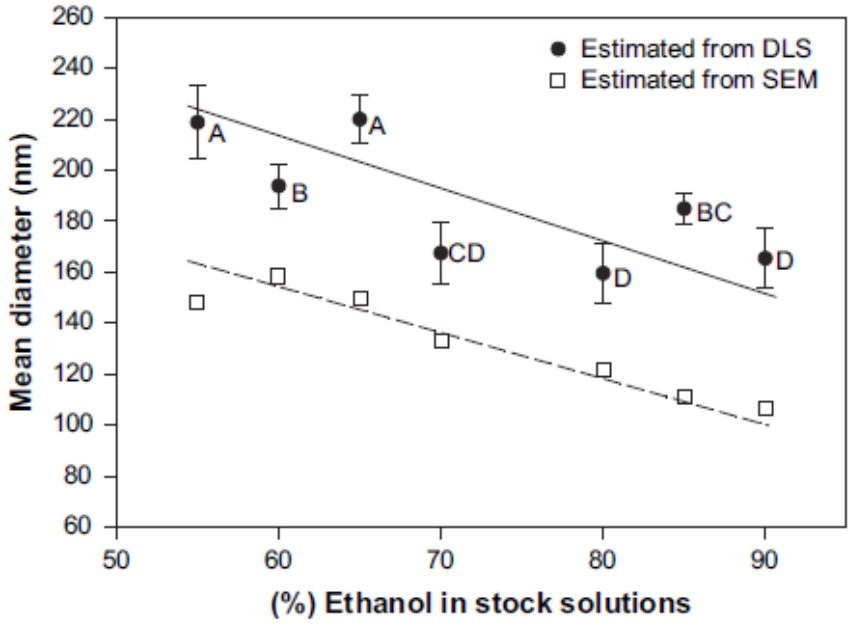


Figure 12. Effect concentration of ethanol in stock solutions on the average diameters of zein nanoparticles (Zhong & Jin, 2009).

It is interesting to note the larger sizes estimated by dynamic light scattering (DLS), suggested to be due to some connected or aggregated particles. However, Figure 12 ultimately supports that higher solvent concentration leads to smaller particles.

2.8.1.5. Effect of continuous phase type

The importance of the miscibility of the dispersed and continuous phase was outlined in section 2.8.1.4.1 above. Selective miscibility ensures that upon addition of the dispersed polymer phase to the continuous phase, the solvent can partition into the aqueous

medium, allowing the polymer to precipitate and harden into microparticles. For example, zein is soluble in mixtures of ethanol and water, but insoluble in pure water. Therefore, ethanol-based polymer solvents and water continuous phases were used by Hurtado-Lopez and Murdan (2005), Parris, Cooke and Hicks (2005), Patel et al. (2010), Stark and Gross (1994) and Zhong and Jin (2009) to produce zein microparticles. Similarly, for the production of lignin microparticles, Asrar and Ding (2006) used methylene chloride as the solvent and water as the continuous phase.

Other properties of the continuous phase can also be important factors in the formation of microparticles. For example, control of continuous phase viscosity is important for the formation of certain microparticle shapes, such as rods, in which the deformation of the droplet from a sphere to a rod shape is controlled by the capillary number. Considering the dimensionless capillary number (Equation 1), a higher continuous phase viscosity leads to a higher capillary number and thus the easier occurrence of deformation (Alargova et al., 2006). The viscosity ratio between the dispersed and continuous phases also has a critical effect on particle size, morphology and quality (Campbell et al., 2008). Therefore, controlling the continuous phase viscosity may be useful if non-spherical shapes are to be produced in the future.

2.8.1.6. Effect of volume ratio of polymer dispersion and continuous phase

Freitas et al. (2005) state that there is conflicting information regarding the effect of the volume ratio between the dispersed and continuous phase on microsphere size, with some investigators reporting a decrease in mean microsphere size when the continuous phase volume is reduced, while others report no effect. Thus volume ratio may be an interesting parameter to study in this work.

2.8.1.7. Effect of temperature of precipitation

Stark and Gross (1994) suggest that smaller microparticles are formed when higher temperatures are used during the precipitation process. However, the boiling point of the system, along with the denaturation and solubility properties of the material to be microparticulated limit the temperatures that can be reached.

2.8.1.8. Effect of rate of solvent removal

The importance of the rate of solvent removal in the formation of microparticles has been highlighted throughout section 2.8.1, with the main idea being that the faster the rate of solvent removal, the larger the microparticles formed. However, Freitas et al. (2005) suggest that the ideal rate of solvent removal depends on a variety of factors, including the type of material to be microparticulated, the type of solvent and the desired porosity of the microparticles. There are two methods of solvent removal, evaporation and extraction, which are defined in Table 10. These methods are now further discussed.

2.8.1.8.1. Solvent evaporation

Solvent evaporation can be achieved using either temperature control or reduced pressure. Using temperature control, it has been suggested that at higher temperatures, such as close to or above the boiling point of the solvent, the solvent evaporates faster from the surface of the dispersion. Thus the time available for droplet breakup is reduced and larger particles, with wider particle size distributions and greater porosity are likely to be precipitated (Yang, Chia, & Chung, 2000; Yang, Chung, Bai, & Chan, 2000). For example, during the encapsulation of salmon calcitonin in PLGA, a rapid temperature increase of the dispersion from 15 to 40°C in 30 minutes produced hollow particles with a thin wall. Conversely, a gradual or stepwise increase over 200 minutes produced particles with increased wall thickness. The preparation of particles of varying porosity may be important for different food applications.

Freitas et al. (2005) suggest that the use of reduced pressure evaporation decreases the size of microparticles. This may be controlled by the Laplace equation (Equation 2), in which if the interfacial tension (γ) is assumed to remain constant for a given system, an increase in the pressure difference between the inside and outside of a liquid film of a droplet (dP) as occurs using reduced pressure evaporation, yields a smaller mean spherical droplet radius (r). Additionally, the lower the pressure used for the evaporation, the shorter the time required for evaporation (Chung, Huang, & Liu, 2001). Similar to evaporation using temperature control, the pressure of the extraction can also influence microparticle structure. This indicates that both temperature control and

reduced pressure could be investigated as factors for controlling microparticle size and structure.

$$dP = \frac{2\gamma}{r}$$

Equation 2

2.8.1.8.2. Solvent extraction

Solvent removal by extraction is commonly performed as a two-step process. In this process, the polymer dispersion is first mixed with a small amount of the continuous phase to form an emulsion with droplets of the desired size. In the second step, further continuous phase or additional extraction agents are added in an amount sufficient to absorb the entire solvent leaching from the solidifying microspheres. One-step extraction processes, in which the quantity of continuous phase is able to dissolve the entire amount of the dispersed phase solvent at once, have also been used (Vuaridel & Orsolini, 2000). However, such processes require careful control of the physicochemical parameters during the homogenisation step to obtain homogeneously dispersed particles.

There are a number of other possible types of solvent extraction, including:

- The addition of the continuous phase continuously over an extended period of time
- A combination of solvent extraction and evaporation
- Reprocessing and recycling of the continuous phase to minimise waste

Refer to Freitas et al. (2005) for further information on solvent extraction.

2.8.1.9. Effect of changes to the particle surface

Emulsifiers and stabilisers make it easier to form a stable emulsion, enable better control of droplet or particle size and can prevent aggregation during drying. Freitas et al. (2005) and O'Donnell and McGinity (1997) suggest that stabilisers can be used to control particle size; increase in stabiliser concentration generally causes a decrease in

particle size. For example, in the preparation of nifedipine microspheres, increasing the amount of hydroxypropyl methyl cellulose in the continuous phase from 0.4 to 2.4% decreased the mean particle size from 28.5 to 12.9 μm (Sansdrap & Moes, 1993). Asrar and Ding (2006) found anionic, cationic, nonionic and amphoteric emulsifiers to be useful in the preparation of lignin microparticles, with methylcellulose found to be the best emulsifier for this system. Polysaccharides such as gum Arabic and carboxymethyl cellulose (CMC) have been found to be effective stabilisers of zein microspheres. These polysaccharides stabilise the microspheres by interacting with positively charged regions on the surface of the protein molecules. Lecithin, polysorbates, pyrophosphates and sodium dodecyl sulfate (SDS), among other stabilisers, are also effective (Stark & Gross, 1994). However, surfactants are unnecessary if the pH of the aqueous phase is below 6 or above 7 (Stark & Gross, 1994), likely because the isoelectric point of zein is 6.2 and therefore it is unstable near this pH (Patel et al., 2010). Patel et al. (2010) used sodium caseinate to stabilise zein colloidal particles and found that a minimum concentration of 0.3% (w/v) of sodium caseinate was required for stability, while 1.25% caseinate was found to be optimum for stability and redispersibility. Zhong and Jin (2009) prepared zein microparticles without using any stabilisers.

2.8.1.10. Effect of microparticle harvest and drying conditions

If solid microparticles are required, the microparticles must be recovered from the dispersion and dried. Freitas et al. (2005) suggest that there are three steps involved in this process – separation, rinsing and drying. Each step can be performed in a different way depending on the available equipment and the desired particle properties. Furthermore, some steps may be unnecessary in some cases and therefore can be omitted.

2.8.1.10.1. Separation

Solid microspheres are usually separated from the continuous phase by either filtration or centrifugation (2005). Hurtado-Lopez and Murdan (2005) collected zein microspheres from suspension by centrifugation at 10,000 rpm for five minutes. Patel et al. (2010) used centrifugation at 4000 rpm for ten minutes to remove large aggregates from the dispersion. Ultracentrifugation at 28,000 rpm for one hour, followed by air-

drying overnight in a 25°C oven was then used to obtain solid powder samples. This indicates that multiple separation steps can be used to obtain microparticles with suitable characteristics. Interestingly, Zhong and Jin (2009) did not utilise a separation step in their microparticulate production procedure and simply freeze-dried the microparticle dispersion to obtain solid powder.

2.8.1.10.2. Rinsing

A rinsing step may be used to remove dispersion stabilisers or non-encapsulated drugs from particle surfaces or to reduce the amount of residual solvent in the microparticles (Freitas et al., 2005). O'Donnell and McGinity (1997) explain that limits exist for some residual solvents, such as methylene chloride and chloroform. However, residual solvents appear to be more of an issue for microspheres containing encapsulated drugs, since the solvent can be trapped inside the microspheres.

2.8.1.10.3. Drying

Microparticles can be dried using a number of different methods. These methods include drying in ambient conditions, under reduced pressure, or by heat or lyophilisation (Freitas et al., 2005). For example, Patel et al. (2010) used air-drying overnight in an oven set to 25°C to obtain solid zein powder, while Zhong and Jin (2009), Parris, Cooke and Hicks (2005) and Stark and Gross (1994) used lyophilisation. Perhaps the method used depends on the equipment available.

2.9. Particle characterisation

It will be important to characterise any lignin particles produced to determine their suitability for use in various applications. Table 12 gives details of techniques used to characterise various particles.

Table 12. Details of characterisation techniques used for various microparticulate systems

Reference	Technique	Details
(Zhong & Jin, 2009) (Zein nanoparticles)	Dynamic light scattering (DLS)	- Size and PSD of fresh dispersions - Laser operated at 658nm - Scattering angles of 15, 30 and 160°C - Two samples drawn from each dispersion and two measurements of each sample
	SEM	- Characterised morphology of powdered samples - Also analysed images for dimensions of spherical structures using Image J
	Rheological measurements	- Used freeze-dried nanoparticles dispersed at a concentration of 2% (w/v) in deionized water for rheological tests (refer to Zhong and Jin (2009) for sample preparation details) - Prepared solutions of 1% CMC in deionized water - Mixed zein dispersions and CMC solutions before rheological tests (used CMC because it is used extensively in food and non-food products) (refer to Zhong and Jin (2009) for sample preparation details) - Used rheometer with Searle set-up (bob ID = 28mm and cup ID = 30mm) - Layer of mineral oil applied to top of sample to minimise moisture loss during measurements - 2 step viscosity test: 1. Shear rate ramp from 1 to 1000 s^{-1} at 20°C 2. Heating ramp from 20 to 90°C at 2°C/min, with a shear rate of $1s^{-1}$ - Each sample measured in triplicate
(Patel et al., 2010) (zein colloidal particles)	DLS	- Size and zeta potential of dispersions - Used a Zetasizer Nano - MPT-2 multipurpose autotitrator (ZEN10001) was used in combination with the Zetasizer to study the effect of pH on zeta potential - Measurements carried out at 25°C - Three readings taken for each sample, average reported
	TEM	- Particle shape (spherical shape was confirmed) - Particles dispersed in medium (i.e. Milli-Q water at appropriate pH - One drop of diluted dispersion placed on a 200-mesh carbon-coated copper grid - Photographs taken at various magnifications and 100 kV voltage
	DSC	- 10mg samples analysed in flat-bottom aluminium pan using Perkin-Elmer Pyris I DSC - Samples heated from 30 to 230°C at 10°C/min - Inert atmosphere maintained by purging with nitrogen at 20mL/min

Reference	Technique	Details
	FTIR	- Compare peaks for colloidal zein and colloidal zein stabilised with sodium caseinate to determine if chemical interaction between zein and sodium caseinate occurs - Spectra obtained in 400-4000 cm^{-1} range - Used Bio-Rad FTS-6000 spectrometer
	X-ray diffraction	- Crystalline / amorphous nature of particles - Used Bruker D8-Discover diffractometer with a copper target, voltage of 40 kV, current of 40 mA and scanning rate of 3°/min

	Stability evaluation	- Aggregation
		  - Added sample to phosphate buffer and visually noted aggregated particles
		  - Added sample to solutions of varying ionic strength to determine effect of ionic strength on colloidal stability (maintained constant pH)
		- Stability against drying
		  - Samples freeze-dried then redispersed using Milli-Q water and the time taken for redispersion was noted
		  - PSD measured before and after drying
		- Long-term physical stability
		  - Dispersions stored at 4°C and at ambient temperature for 3 months
		  - Visually inspected for aggregation or sedimentation at the end of the storage period

(Hurtado-Lopez & Murdan, 2005) (Zein microspheres)	SEM	- Shapes and surfaces of particles - Zein microspheres collected from a suspension by centrifugation (10,000 rpm, 5 min), a sample of the pellet was dried, sputter-coated with a thin layer of gold and observed microscopically
	PCS	- Particle size and size distribution of zein microsphere suspension suspended in purified water - Measured three samples three times each - Calculated the standard error of the means for each measurement
	Zeta potential	- Relative measure of surface charge - Used LDV, Malvern Mastersizer - Used 0.1mM phosphate buffered saline pH 7.4 as the dispersing medium - Measured three samples three times each - Calculated the standard error of the means for each measurement
(Parris et al., 2005) (Zein nanospheres)	SEM	- To determine particle size distributions from topographical images - Thin layers of powder particles glued to carbon adhesive tabs, mounted on specimen stubs and sputter-coated with gold - Digital images of particle topographies collect

Reference	Technique	Details
		using SEM operated in secondary electron imaging mode
		- PSDs from topographical images processed and analysed using Fovea Pro v.3 plug-ins and PhotoShop v. 7 (see details of image analysis). Equivalent diameters were computed and plotted by grouping 25 measurements.
	TEM	- Not very clear about reason for use
		- Applied 10µL aliquots of dispersions of powder particles in 24% aqueous ethanol solutions to Formvar coated specimen screens and negatively stained with a solution of 2% uranyl acetate (see details)
		- Samples air-dried before viewed with TEM operated in bright field mode
		- Photographic images recorded and digitized using a SprintScan Ultra 45 film scanner
(Asrar & Ding, 2006) (Lignin microparticles)	Electron microscopy	- Particle morphology – to relate to release rate curves for the agricultural active - Obtained electron micrographs at magnifications of 100X, 500X, 1000X and 2000X
(Asrar & Ding, 2008) (Lignin-based microparticles)	Electron microscopy	- Particle size and shape - Obtained electron micrographs at magnifications of 100X, 500X, 1000X and 2000X
	Coulter Counter	- Particle size (average) - Found average particle size to be 7.3 µm
(Wege et al., 2008) (Microparticles from Hyrdophobic cellulose)	Optical microscopy	- Structure and morphology of HP particles - HP size and PSD obtained by measuring lengths and widths of > 100 particles from digital microscopy images
	SEM	- Particle morphology - Performed at 5kV with vacuum-dried HP particle samples - 200 Å thin layer predeposited on sample surface to improve imaging quality
	Contact angle measurements	- To indicate hydrophobicity and suitability as foam/emulsion stabilisers (can compare / use in conjunction with zeta potential measurements) - Used Rame-Hart goniometer - HP films prepared by spin-coating HP stock solutions with different solvents, followed by drying in vacuum oven at 90°C for 6h - Measured the advancing contact angles of droplets of 10 µL deionised water at room temperature onto these films - Contact angle measurements were made at 3 different sample positions and an average taken
	Viscosity measurements	- To determine the viscosity of stock solutions of different HP concentrations and the viscosity ratio - Used Searle set-up (bob OD = 28 mm, cup ID =

Reference	Technique	Details
		30mm) - Shear rate ramps from 0.01 to 100s^{-1} at 20°C - The average viscosities were determined from the measurements at the shear rate higher than 1 s-1 (because of the difficulty in quantifying the exact shear rate during blending)
	Zeta potential	- To determine the hydrophilicity of aged particles - Used zeta potential meter to estimate surface charges of HP particles - Zeta potentials of the HP particles prepared from different stock solutions were determined from average values of three measurements
(Gordon & Pilosof, 2010) (Whey protein particles)	Light scattering	- Mean particle diameter and PSD of dispersions - For particles 0.1 – 1000 μm, a Mastersizer 2000E (He-Ne (633nm) laser and fixed scattering angle of 90°) was used. - Used refractive index of dispersed phase of 1.450 and absorption parameter of 0.001. - Reported D[3,2] - For particles 0.3 – 6000 nm, a ZS Zetasizer Nano (He-Ne laser (633 nm), digital correlator model ZEN 3600 and scattering angle 173°) was used - Samples placed in disposable polystyrene cuvette - Two approaches utilised to obtain size information: - (a) Contin's algorithm - (b) Cumulant method
	Confocal microscopy	- Particle size and morphology - Looked at polydispersity, shape and smoothness of particles that had been sonicated on non-sonicated - Used He-Ne laser (543 nm) and objective PLAN 60X and 100X to obtain images - Protein was stained with Rhodamine

Table 12 reveals that a range of techniques can be used for microparticle characterisation. SEM and light scattering seem to be the most commonly used techniques, probably because they provide information on particle size and morphology, which are two of the most basic particle characteristics. For example, size and shape would be very important to characterise during the development of microparticulate fat mimetics. These microparticulates are small, spherical particles 0.1-3 μm in diameter that can physically mimic fat droplets in oil-in-water emulsions (Jones, 1996). Singer (1996) suggests that particles larger than about 0.5 μm impart a feeling of 'substantialness,' whereas particles with sizes below 0.1 μm give a watery feeling. Meanwhile, particles with sizes greater than about 3 μm are perceived as powdery,

chalky or gritty. Thus particles must fall into a narrow range of sizes to impart a feeling of 'substantialness' yet avoid grittiness.

Other techniques can provide additional information depending on the likely application of the microparticles and thus the desired particle characteristics. For example, for microparticulate fat mimetics, in addition to particle size, similarity of physical properties to those of fats is also important. Hydrophobic particles may more closely mimic fat droplets than hydrophilic particles, since fat is hydrophobic. Furthermore, hydrophilic particles often require an increase in water content in the food when replacing fat, leading to physical, chemical and sensory changes in the food (Jones, 1996).

For all food applications, a certain palatability is required to ensure that the consumer acceptance of products is maintained when fat is replaced with microparticles. Hence characterisation of odour, flavour and colour is likely to be important for the development of food-grade lignin microparticles.

2.10. Pickering emulsions

2.10.1. Types of emulsions

An emulsion is defined as a dispersion of one phase in another in which it is immiscible (Dalgleish, 2003). Emulsions are formed by mixing the two immiscible liquids together in a process known as homogenisation. There are three main types of emulsions that are important in the food industry:

1) Oil-in-water (o/w) emulsions – the most versatile type of emulsion, in which oil droplets are dispersed in an aqueous continuous phase. Examples include milk, mayonnaises, cream liqueurs, creamers, whippable toppings, ice cream mixes, soups and sauces.
2) Water-in-oil (w/o) emulsions – water droplets dispersed in oil continuous phase. Examples include butter, margarines and other fat-based spreads.

3) Water-in-oil-in-water (w/o/w) – water droplets contained in oil droplets that are suspended in water. These emulsions are the most difficult to produce and control (Dalgleish, 2003; McClements, 2005).

2.10.2. Types of instability for oil-in-water emulsions

O/w emulsions can suffer three main types of instability:

1) Coalescence – caused by thermodynamic instability and poor interfacial layers. To reach the most thermodynamically stable state emulsions seek to minimise the interfacial tension between oil and water. If no emulsifiers are present, the interfacial tension is reduced by coalescence of the oil droplets, which ultimately results in separate oil and water layers (Dalgleish, 2003). Coalescence can only be reversed by re-homogenising the product and is therefore the most undesirable type of instability.
2) Creaming – a form of gravitational separation in which emulsion droplets retain their identity but gather at the top of the emulsion. This happens because the density of oil is lower than the density of the surrounding water and the oil droplets are of big size, namely above 1 μm.
3) Flocculation – a type of droplet aggregation in which the droplets come together but still retain their individual integrity. Both creaming and flocculation can promote coalescence so should be avoided (Dalgleish, 2003; McClements, 2005).

2.10.3. Emulsifiers

Emulsifiers allow kinetically stable emulsions to form. Emulsifiers are surface-active and adsorb at the oil-water interface, lowering interfacial tension and reducing the driving force for coalescence. Emulsifiers can also prevent coalescence by altering the viscoelastic properties of the interface, conferring charge to the droplet surface to provide electrostatic stabilisation or by creating a barrier the droplet to physically prevent coalescence (Dalgleish, 2003). Most emulsifiers are also amphiphilic molecules. McClements (2005) suggests that small molecule surfactants, phospholipids, proteins and polysaccharides are the most commonly used emulsifiers in the food industry.

Stabilisation can also be provided by texture modifiers such as thickening and gelling agents. These agents either increase the viscosity of the continuous phase or form a gel in the continuous phase, thus improving emulsion stability by slowing the movement of emulsion droplets (McClements, 2005).

2.10.4. Pickering emulsifiers

Solid particles can also be used to stabilise emulsions. Emulsions stabilised by the adsorption of solid particles to the oil-water interface are known as Pickering emulsions. The underlying principle of Pickering stabilisation is very simple: "the dispersed particles accumulate at the oil-water interface to form a mechanical (steric) barrier that protects the emulsion droplets against coalescence" (Dickinson, 2012). Stabilisation with solid particles is favourable in situations where surfactants cause negative side effects such as air entrapment, foaming and irritancy (Frelichowska, Bolzinger, & Chevalier, 2010). Furthermore, Pickering emulsions have generated a lot of interest in recent years due to their long term stability against Ostwald ripening and coalescence, and also the availability of many new types of nanoparticles that could act as Pickering stabilisers (Gould, Vieira, & Wolf, 2013; Li, Li, Sun, & Yang, 2013).

Some natural examples of stabilisation by solid particles are known to exist, for example, ice crystals in ice cream and fat particles in whipping cream (Binks, 2002). Otherwise, a range of particle materials have been investigated, including inorganics such as silica, latex, alumina and clay, and organics, such as modified starch, cellulose derivatives, chitin, chitosan, zein, solid lipids, some flavonoids, wax, proteins and carbon nanotubes (Gould et al., 2013; Kurukji, Pichot, Spyropoulos, & Norton, 2013; Li et al., 2013; Liu & Tang, 2013). Liu and Tang (2013) state that particles are often too polydisperse, require surface modification to achieve surface activity, are poorly characterised, non-food-grade or ineffective in real food systems. Thus finding cheap and effective food-grade particles that are able to stabilise emulsions by the Pickering mechanism is a significant challenge for the food industry.

2.10.5. Examining the potential of particles to act as Pickering emulsifiers

An important first step when investigating the effectiveness of potential new Pickering particles is to consider the particle properties. The critical factors thought to determine the performance of particles as Pickering stabilisers are:

- Particle size
- Particle shape
- Particle concentration
- Particle wettability
- Interactions between particles
  (Binks, 2002)

The importance of each of these factors is outlined below.

2.10.5.1. Effect of particle contact angle

The particle contact angle gives an indication of wettability for spherical particles and has been suggested to be the major determinant of emulsion stability (Binks & Clint, 2002). The contact angle is similar to the hydrophile-lipophile balance (HLB) of surfactants, determining whether the surfactant resides in the water, oil or some other phase and the type of emulsion that is formed. Hydrophobic particles generally have a contact angle greater than 90° and a greater portion of the particle resides in the oil or air phase than in the water phase. The opposite is true for hydrophilic particles (Binks, 2002). The particle layer at the interface curves so that the larger area of the particle surface stays on the external side. Therefore, air or oil in water emulsions are formed for more hydrophilic particles and water in air or oil emulsions are formed for more hydrophobic particles (Figure 13).

The contact angle also affects the energy required to attach or remove a particle from the interface between the two fluids, which influences the stability of an emulsion. Particles that are strongly adsorbed can be considered to be irreversibly held at the interface and can provide long-term stability against changes in droplet size (i.e. stable to Ostwald ripening and coalescence). Equation 3 defines the relationship between the

energy of attachment, E, of particles to an interface and the particle size, contact angle and the interfacial tension between the two fluids in an emulsion.

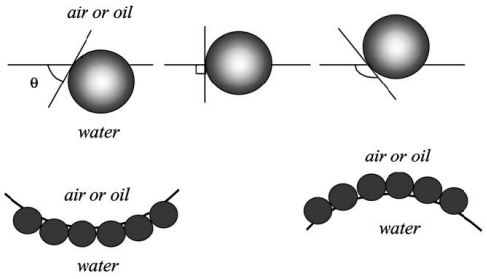


Figure 13. Top: Positioning of hydrophilic (left), intermediate hydrophobicity (centre) and hydrophobic (right) particles at an air or oil/water interface, showing contact angles < 90°, equal to 90° and > 90°, respectively. Bottom: The curvature of the interface depending on the particle contact angle; aqueous foams or o/w emulsions formed when θ < 90° and aerosols or w/o emulsions formed when θ > 90° (Binks, 2002).

$$E = \pi r^2 \gamma_{\alpha\beta} (1 \pm \cos\theta)^2$$

Equation 3

Where r = particle radius (m), $\gamma_{\alpha\beta}$ = interfacial tension between fluids α and β (Nm^{-1}), θ = contact angle that the particle makes with the interface (degrees) and the sign inside the bracket is negative for particles that are removed into the water phase, and positive for particles that are removed into the oil phase (Binks, 2002).

Equation 3 shows that particles with contact angles close to 90° are held strongly at the interface, whereas above and below 90°, E decreases rapidly. For example, E for a 10nm particle with a contact angle of 90° is several orders of magnitude higher than that of a surfactant adsorbed at the interface. Surfactants "are considered to be in a state of dynamic equilibrium constantly undergoing adsorption and desorption," which is known to enhance coalescence and Ostwald ripening in droplets. Conversely, particles

are considered to be irreversibly adsorbed at the interface and therefore confer extreme stability against changes in droplet size distribution (Gould et al., 2013). This has even been found to be true for millimetre-sized droplets, with the large droplets rolling over each other without deformation in the same manner as we would expect for true solid spheres (Binks, 2002). This extreme stability is one of the major advantages of Pickering stabilisation.

Another significant advantage of particles with intermediate hydrophobicity is that they can sometimes be used to form both o/w and w/o emulsions depending on the oil:water ratio. However, more knowledge still needs to be gained as to how one type of particle can stabilise both types of emulsions (Binks, 2002).

The wettability of particles can be altered by coating with various reagents, such as alkylsilanes and fluorocarbon (Binks, 2002; Binks & Lumsdon, 2001). For example, Frelichowska et al. (2010) investigated the ability of two types of silica that had been grafted with dichloromethylsilane to make their surfaces more hydrophobic to act as Pickering stabilisers.

However, although coatings make the particles surface active, the particles do not necessarily become amphiphilic. Amphilicity influences the strength of attachment of particles to the interface; Binks and Fletcher (2001) suggest that amphiphilic particles remain strongly surface active even for particles with contact angles approaching either 0 or 180°C. Particles can be made amphiphilic by applying the coating to only certain areas of the particle such that it has specific areas that are oil-liking and specific areas that are water-liking (Binks, 2002).

2.10.5.2. Effect of particle size

It has been suggested that smaller particles pack more efficiently at an interface than larger particles, thus producing a more homogeneous layer around the droplets that makes them more stable (Gould et al., 2013; Kurukji et al., 2013). Monodisperse particles may also be expected to pack more efficiently at the interface. It is also known that to effectively stabilise an emulsion, the size of the stabilising particle "should be at least an order of magnitude smaller than the emulsion droplet" (Gould et al., 2013). For

example, to stabilise emulsion droplets of 0.5-10 μm, the particles must be in the sub-micron/nanometre size range (Dickinson, 2012).

Equation 3 indicates that large particles would be held most strongly at the interface. However, the equation does not hold true for particles with diameters greater than a few microns (Binks, 2002). Furthermore, it has been found that as long as the contact angle is close to 90°, even particles as small as 5-10 nm can adsorb essentially irreversibly to the interface, supporting the importance of the contact angle (Dickinson, 2012).

2.10.5.3. Effect of particle shape

Spherical particles are often investigated as Pickering stabilisers. However, other particle shapes such as rods and discs can also be used. Other shapes offer various advantages, for example, rods can out-perform equivalent spherical particles and create "super-stable" foams and emulsions. This is because of the high surface area-to-volume ratio and the ability of rods to intertwine at interfaces, resulting in enhanced steric stabilisation (Campbell et al., 2008; Campbell, Stoyanov, & Paunov, 2009; Wege et al., 2008). Using this logic, Li et al. (2013) hypothesised that wheat and potato starch granules, which are oval-shaped, would provide more effective emulsification than polygonal-shaped rice and waxy corn starch granules. However, the rice and waxy corn starch granules were found to be the best emulsifiers, indicating that particle shape is not the critical factor controlling the effectiveness of native starch granules to act as emulsifiers. The rice and waxy corn starch granules were smaller than the wheat and potato starch granules, suggesting that in this case the difference in size has had more effect than the difference in shape. Perhaps it is the combination of particle properties that is important, rather than individual properties.

2.10.5.4. Effect of particle concentration

Particle concentration determines how much of an interfacial area can be stabilised and therefore the size of resulting emulsion droplets. Equation 4 shows how the mean emulsion droplet diameter (D) is related to the volume fraction of the dispersed phase (ϕ_v) and the interfacial area per unit volume of emulsion (A/V).

$$D = \frac{6\varphi_v \mathrm{V}}{A}$$

Equation 4

The particle-to-oil ratio is an important parameter, since both the particle concentration and the amount of oil influence the interfacial area per unit volume of emulsion and thus the droplet diameter (Frelichowska et al., 2010).

2.10.5.5. Effect of particle-particle interactions

Many studies have shown that particles must be weakly flocculated or aggregated to enable stable emulsions to form (Briggs, 1921; Lucassen-Reynders & Van Den Tempel, 1963; Wei, Yang, Yang, & Wang, 2012). Briggs (1921) suggests that if solids are in suspension in one of the liquid emulsion components, these solids may not be sufficiently surface active enough to act as emulsifiers. Weak flocculating agents can be added to force the particles to go to the interface, thus improving emulsifying ability. Ashby and Binks (2000) also suggest that due to their larger size, flocs adsorbed to an interface have a greater energy of attachment compared to single droplets and are therefore less likely to desorb from the surface and destabilise the emulsion. However, flocculating agents that are too powerful may cause the solid to agglomerate into large masses or flocs that are unable to form a stabilising film around emulsion droplets (Briggs, 1921; Lucassen-Reynders & Van Den Tempel, 1963). Therefore, weak flocculating agents that cause moderately prevalent attraction between the particles are required (Lucassen-Reynders & Van Den Tempel, 1963). It is suggested that flocculation or aggregation can be induced by the addition of salt, change in pH or addition of surfactant, depending on the type of emulsion (Binks, 2002; Frelichowska et al., 2010; Gould et al., 2013; Liu & Tang, 2013).

Gould et al. (2013) found that larger emulsion droplets were produced when flocculated cocoa particles were used to stabilise the emulsions than when non-flocculated particles were used. It was mentioned that this may have been due to the effect of the acid conditions that were used to induce the flocculation on the hydrophobicity of the particles. However, this result may suggest that it is the overall suite of particle

properties that are important in particle performance, rather than the domination of one certain factor.

Interactions between aggregates or single particles are also thought to be of importance. In addition to the stability against coalescence provided by the film of particles around dispersed droplets, the formation of a 3D-network of particles in the continuous phase surrounding the droplets may provide additional stabilisation. The network increases the viscosity of the continuous phase and prevents movement of the dispersed phase droplets (Binks, 2002).

Finally, the density of packing of particles at the oil-water interface is suggested to be a major determinant of the strength of the steric protection (Dickinson, 2012). Binks (2002) has reviewed the formation of particle monolayers and occurrence of hexagonal packing in monolayers. Interactions between particles may influence the packing that occurs.

2.10.6. Lignin as a Pickering stabiliser

Afanas'ev, Selyanina and Selivanova (2008) made an important note that kraft lignin is known to produce a stable lignin-tall oil emulsion in the pulp and paper industry, suggesting the effectiveness of lignin as a solid particle emulsifier. More recently, Wei, Yang, Yang and Wang (2012) developed a method for preparing pH-responsive emulsions stabilised by solid lignin. In this method, lignin powder was added to water to produce aqueous dispersions of lignin. Concentrated ammonia or sodium hydroxide was then added to the dispersions until the lignin completely dissolved, at which point the pH of the dispersions was around 11. The resulting solution was filtered to remove any impurities before the pH was reduced to about 3 by adding 1M HCl. Lignin particles formed and were dispersed in the solution, coagulating after 10 minutes of standing. Figure 14 shows a simplified diagram of the key steps in this process.

The droplet size of the styrene-in-water emulsions prepared by Wei et al. (2012) was quite polydisperse, ranging in size up to even ~140 μm in diameter for a lignin concentration in the aqueous phase of 0.05% (w/w). Furthermore, the emulsified oil

droplets creamed rapidly to give a cream layer on top and a continuous water phase on the bottom. However, the emulsion remained stable to coalescence for up to 6 months.

Although a lignin concentration of 0.05% was found to be sufficient to produce stable styrene-in-water emulsions, the effect of lignin concentration on droplet size was also investigated. Mean droplet diameter decreased with increasing lignin concentration in the aqueous phase. The extent of creaming decreased with decreasing droplet diameter.

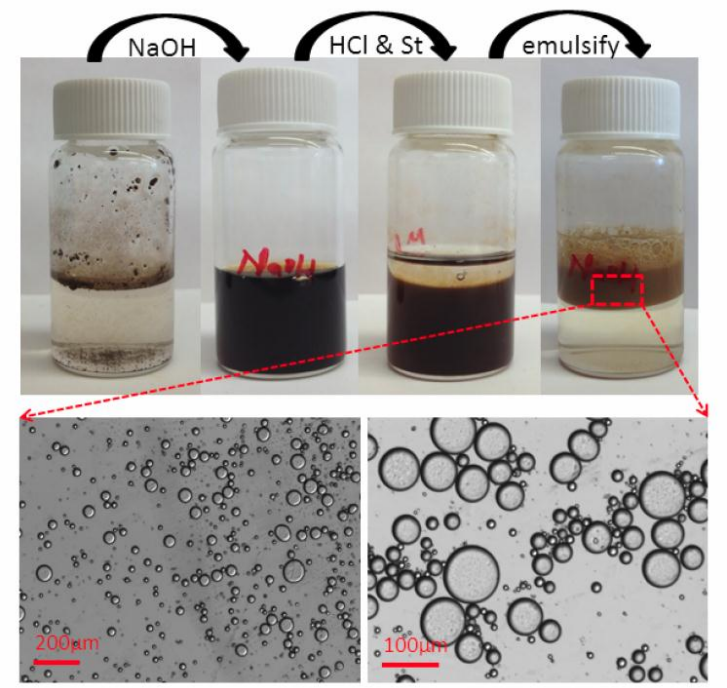


Figure 14. Major steps in the production of pH-responsive Pickering emulsions (styrene-in-water) prepared using lignin and an NaOH/HCl system (note: St = ten minutes of standing time) (Wei et al., 2012).

The ability of lignin to stabilise emulsions containing various oils, including soybean oil, was also investigated. Stable emulsions were formed regardless of the oil type and associated oil polarity and viscosity. These results indicate that food-grade lignin could have application as a Pickering stabiliser of food emulsions.

It may be important in the future to gain more understanding of the effectiveness of lignin as a Pickering stabiliser. Basic characterisation of the lignin may identify how lignin microparticles could better stabilise emulsions. For example, extractive resinous

compounds present in the lignin may influence the hydrophile-oleophile ratio of lignin and thus composition analysis may be important. Similarly, lignin with middle-range molecular weights have been found to produce more stable emulsions than lignins with very low or very high molecular weights (Afanas'ev et al., 2008; Selyanina & Selivanova, 2007).

Chapter Three. Lignin Extraction Vessel

The proposed "organosolv" process for this project involves heating wood chips in ethanol-water mixtures to 150-210°C for 40-120 minutes. Therefore, a vessel able to withstand these temperatures and the associated pressures of 2-3 MPa was required. The stages in the development of this vessel, along with the associated experimental trials, are outlined in this chapter.

3.1. Preliminary trials: Existing extraction vessel

Preliminary trials were carried out using a 370 mL vessel fabricated in our workshop. Once filled with wood chips and solvent, this vessel was sealed at both ends with O-rings and screw caps. The vessel was then submerged in silicone oil, which was temperature controlled at 200°C, to perform the lignin extraction. However, vessel contents leaked from the screw-capped ends of the vessel during heating. The leakage posed threats to safety since it caused bubbling of the silicone oil, and it also indicated that the conditions inside the vessel were changing during the heating time of the extraction. Although modifications and re-designs were considered, no further work was completed with this vessel, mostly due to safety reasons. Therefore, a new vessel able to withstand the conditions of the process was sourced.

3.2. Parr extraction vessel

A Parr 4767 reactor vessel (Parr Instrument Company, Moline, Illinois), locally supplied by John Morris Scientific (Auckland, New Zealand) was found to be a suitable alternative reactor for our small scale work. The vessel was a 450 mL stainless steel cylinder with bolted split ring closure and a four-port head unit containing ports for temperature measurement, pressure relief, burst release and a spare port. Appendix A contains the manufacturer's data report and technical drawing for this vessel. The vessel was also supplied with a heating mantle, a reactor controller and software for logging the measured temperatures. Although this was a large improvement compared to the existing vessel, two important features were missing: The first was a means of measuring pressure in the vessel. The second was a means of releasing extraction liquor

from the vessel while still under high temperature and pressure to avoid lignin re-precipitation on the surface of the wood fibres, which increases when liquor is slowly cooled before removal (Macfarlane, 2009). Therefore, modifications to the new vessel were required.

3.2.1. Modifications to the Parr vessel

To enable pressure to be measured, a pressure transducer was added to the vessel and a display module for the pressure readings was added to the reactor controller. A logging system compatible with the supplied Parr software was set up to enable pressure measurements to be logged throughout the extraction.

By rearrangement of the four ports, a 3 mm diameter stainless steel tube with a sintered metal filter element was added to the underside of the head unit to enable the removal of extraction liquor from the vessel. A relief valve and 3 mm stainless steel tube were also added to the top of the head unit. By opening the relief valve, the extraction liquor can flow from inside the vessel to outside due to the pressure gradient between the vessel and the outside environment. A gas inlet for applying an external gas such as Nitrogen gas was also added to the vessel. This was used to purge the vessel and ensure complete removal of the extraction liquor.

In addition to the modifications to the vessel itself, a safety casing was also built to surround the vessel during extraction runs. This casing was made out of aluminium, with a clear polycarbonate front cover. An inner wall was also fitted with a cooling water coil, to condense any vapour that might be produced if the vessel leaked. Finally, rigid stainless steel tubing was also added to the burst release disc port. This tubing was directed into a 20 L pail of cold water. This was to quench any vapours released in the case that the pressure inside the vessel exceeded the maximum pressure allowance and the burst release disc ruptured.

Figure 15 shows schematically the cross section of the modified reactor vessel. The split ring assembly, compression ring, drop band and gasket are not shown. A ¼” NPT safety head outlet leads to a 3000 psi rupture disc (1), which in turn is fitted with a 7 mm diameter (I.D) stainless steel tube that runs down into a 20 L pail of quench water.

Liquid can be removed from the reactor through a 1/8" outlet and tube, with flow controllable by needle valve (3), when there is a pressure gradient between the inside and outside of the reactor. A ½" diameter, 18 mm long sintered stainless steel filter element (4) allows liquid to be filtered from the wood chips and removed from the vessel. There is also a type-J thermocouple (2) and a pressure transducer (5). The reactor can be pressurised with nitrogen, with flow controllable by needle valve (6), through a tee (7) in the pressure transducer line. Refer to the technical drawing in Appendix A for vessel dimensions and further details.

Figure 16 shows the final set-up of the vessel and associated equipment (quench water not shown).

3.2.2. Testing the Parr vessel

3.2.2.1. Gasket material

The gaskets supplied with the original Parr vessel to seal the vessel were made of virgin PTFE. Although these gaskets were able to provide a good seal during extraction runs, with a one hour holding time at 200 °C, the gaskets deformed during only a single run and were unable to be used in subsequent runs unless trimmed back to shape. The deformation was likely to be caused by creep flow of the gasket under pressure, a problem enhanced by temperatures greater than 150 °C (Parr Instrument Company, 2012). Trimming the gaskets was not considered to be a safe option and thus alternative gasket materials were investigated.

Silicone was trialled in the form of two O-rings of different diameters. However, as with the PTFE gaskets, although an adequate seal was provided, the material degraded during the extraction and could not be used in subsequent extraction trials.

Finally, gaskets made from Polyether ether ketone (PEEK) (SustaPEEK, Dotmar Universal Plastics, Palmerston North, New Zealand) were trialled. These gaskets not only provided an adequate seal during the extraction, but did not degrade in any way. In fact, the same PEEK gasket was used in three eight-hour extraction runs and did not degrade over this time. Therefore, PEEK was chosen as the gasket material for all future work.

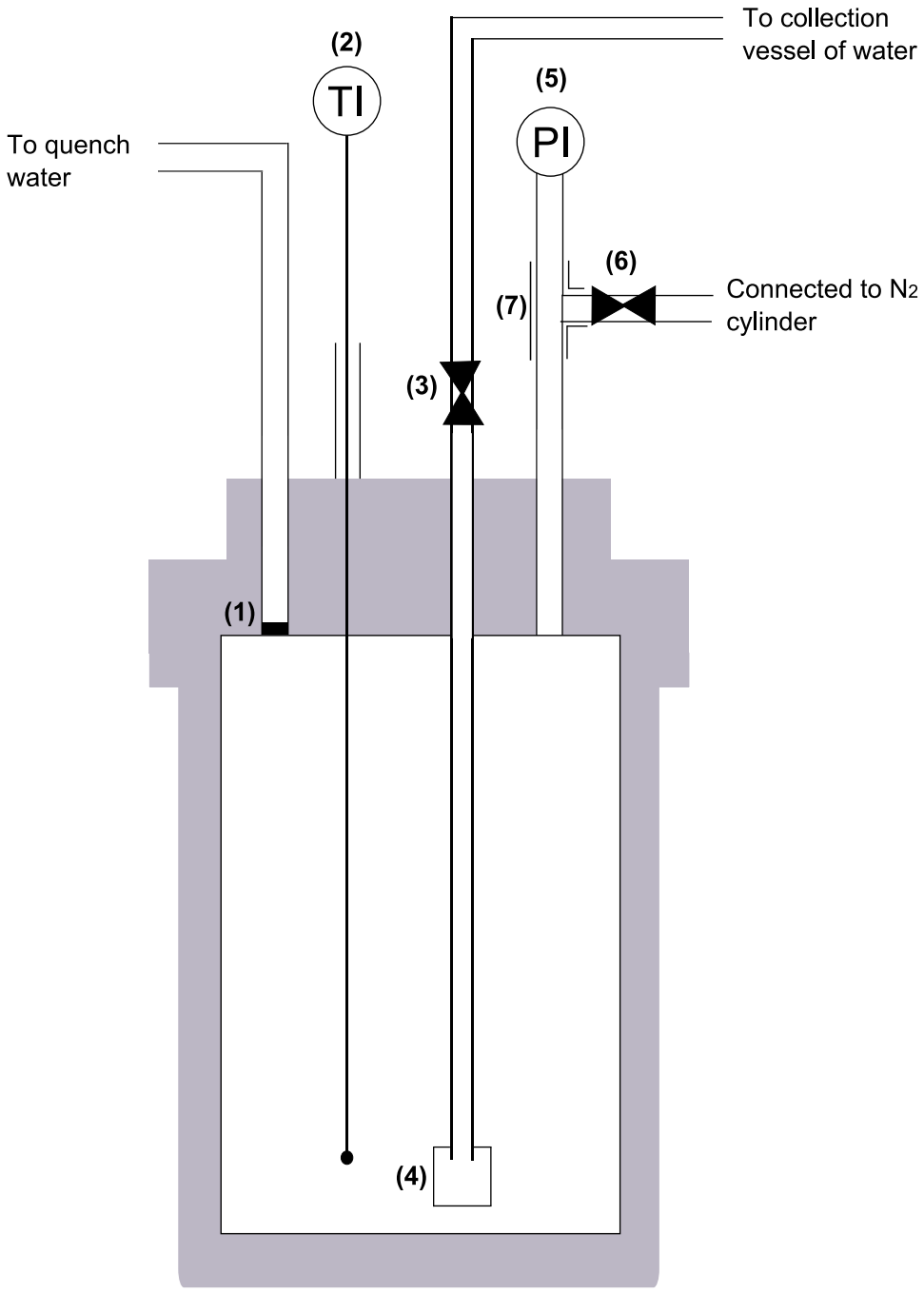


Figure 15. Schematic diagram (not to scale) of cross-section of modified reactor, with gasket, split ring assembly, compression ring and drop band excluded for clarity.

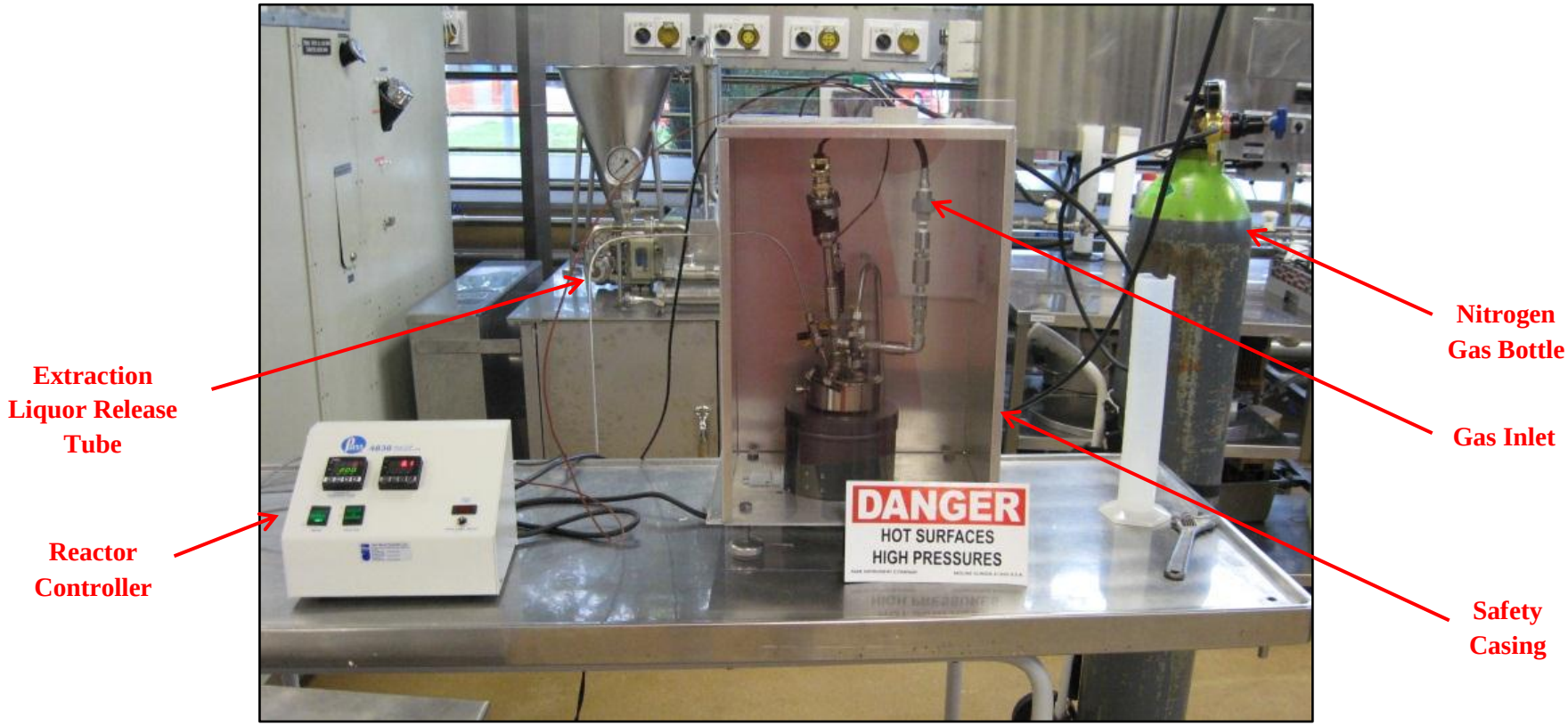


Figure 16. Final set-up of the Parr extraction vessel (quench water not shown).

3.2.2.2. Product discharge

A final factor to consider was the performance of the extraction liquor discharge mechanism. A 70% ethanol-water mixture was heated in the vessel and released using the relief valve at 200°C. The mixture turned to vapour as it exited the product release tube. However, the liquor was not lost as vapour if it was bubbled into water. Therefore, in subsequent trials the end of the release tube was placed under water to retain the extraction liquor.

In some experiments, the extraction liquor was not completely removed from the vessel (refer to section 4.1.2.2 in the next chapter). This was found to be caused by blockage of the sintered metal filter element, possibly with lignin. Therefore, a cleaning procedure utilising acetone and a sonicator (Soniclean 160HT, Soniclean Pty. Ltd, Thebarton, Australia) was devised to clean the filter element. In this procedure, the element was placed in a small beaker and acetone was added to cover the element. The beaker was placed in the sonicating water bath and covered with weights to hold the beaker in the water. The sonicating bath was degassed before applying various (low, medium and high) frequencies of sonication to remove material from the element by solubilisation in acetone. However, while this cleaning method worked the first few times, it soon failed to completely clean the filter, ultimately leading to failure of the release mechanism to remove all liquor from the vessel at the end of the extraction. Therefore, for all remaining trials, extraction liquor was manually removed from the vessel using the procedure outlined in section 4.1.2.2.

3.2.2.3. Commissioning inspection

The modified vessel underwent a commissioning inspection by SGS Ltd to certify its safety under the intended conditions of use. The vessel passed this inspection.

3.3. Safe operating procedure

A Safe Operating Procedure was written for the modified vessel and is shown in Appendix B. The document includes a hazard analysis, operating procedures and emergency procedures. High temperature and high pressure were the two main hazards identified.

Chapter Four. Lignin Extraction

This chapter outlines the optimisation of lignin extraction conditions for use in this project. Organosolv pulping using ethanol as the solvent has been selected as the pulping method for the extraction of lignin since it is a food-grade method and a reactor vessel capable of organosolv pulping is available. A variety of different factors is known to influence delignification when using organosolv pulping, with the balance between different factors also being of high importance. Along, with high yield, purity of the lignin is also important, since it influences the possible applications of the lignin. Therefore, this chapter focusses on the optimisation of lignin yield and purity through a central composite design in which both extraction time and ethanol concentration are varied.

4.1. Materials and methods

This section outlines the materials and methods used for the organosolv extraction of lignin from willow. Firstly, the preparation of *Salix purpurea* as the feedstock is described. The methods of analysis used to determine the yield and purity of extracted lignin are then given. These methods consist of measures of the mass of lignin recovered and both acid-insoluble and acid-soluble lignin analysis. Finally, the approaches followed to optimise the organosolv extraction of lignin from S. *purpurea* are described.

4.1.1. Feedstock preparation

Each lignin extraction experiment requires a representative sample of wood to be used each time. This limits the influence of variation in feedstock on the resulting yield and purity of extracted lignin (Macfarlane, 2009). To achieve this, wood must be de-barked, chipped, vacuum packed and frozen to preserve the wood and protect against microbial and oxidative degradation. The chipped wood should be separated into different size fractions to enable consistent sampling of wood chips for extraction experiments.

4.1.1.1. Selection of feedstock

S. *purpurea* was used as the feedstock for lignin extraction. It is well known that shrub willow is a high-yielding, fast growing perennial crop and has potential for use as a

feedstock in lignocellulosic biorefineries (Kuzovkina & Quigley, 2005; Lamlom & Savidge, 2003). Furthermore, the S. *purpurea* species was readily available.

4.1.1.2. Feedstock processing

Two-year-old stems (diameter ≈ 20-40mm, length ≈ 1m) of S. *purpurea* were obtained from Akura Nursery in Masterton, New Zealand approximately 24 hours after they were cut. They were de-barked via the application of steam to soften the bark, which was then peeled off by hand. The stems were then chipped in a Hansa C6 chipper (Hansa Products Ltd., Hamilton, New Zealand). The chipped wood was stored in plastic drums in the freezer. The frozen wood chips were then separated into five different size fractions using an air screen cleaner and indent cylinder separator. The fractionated wood chips were then vacuum-packed in aluminium foil pouches and stored in the freezer (-25°C) until use. Appendix C provides further details on the fractionation of the wood chips. Table 13 shows the size distribution of the wood chips prepared for this project.

Table 13. Names and proportions of the five wood chip fractions used for lignin extraction experiments

Fraction number	Fraction name	% of total (% w/w)
1	'Large'	23.5%
2	'> 10mm'	48.3%
3	'< 10mm'	23.9%
4	'Air-blown'	0.57%
5	'Sawdust'	3.8%

4.1.1.3. Feedstock composition

Table 14 shows the composition of the feedstock willow in terms of lignin, carbohydrate, extractives and ash. With the exception of the ethanol extractives, all analyses were carried out by Veritec (Scion, Rotorua, New Zealand). Appendix D contains the reports provided by Veritec and details of the analysis methods used. Table 14 reveals that the total lignin content of the willow is ~26%. This indicates that when

optimising the extraction conditions to be used for this project, the maximum lignin yield that can be achieved is ~26% of the oven-dry willow.

Table 14. Untreated *Salix purpurea* composition

	% (w/w)	% (w/w)
LIGNIN		26.0 ± 0.4
---Acid soluble lignin	3.38 ± 0.18	
---Acid insoluble lignin	22.6 ± 0.3	
CARBOHYDRATES		
Cellulose sugars		43.5 ± 1.8
---Glucosyl	43.5 ± 1.8	
Hemicellulose sugars		23.1 ± 0.9
---Xylosyl	20.7 ± 0.9	
---Arabinosyl	0.66 ± 0.014	
---Galactosyl	0.76 ± 0.04	
---Mannosyl	0.96 ± 0.06	
ETHANOL		4.32 ± 0.15
EXTRACTIVES		
ASH		0.25 ± 0.021
TOTAL		97.06

Notes: All values are based on oven-dried willow weight basis. Sugars are reported as anhydrosugar units. Uncertainties are standard deviations.

4.1.1.4. Extractives removal

Wood extractives are soluble in either water or ethanol during exhaustive extraction (National Renewable Energy Laboratory, 2005). It has been suggested that extractives may interfere with analyses for carbohydrates and lignin due to irreproducible

partitioning of the extractives in these methods (Milne, Chum, Agblevor, & Johnson, 1992). Therefore, extractives were removed from the willow prior to the lignin extraction optimisation trials according to the National Renewable Energy Laboratory (1994), involving a 24 hour ethanol extraction using standard soxhlet apparatus.

For many of the screening trials, it was not feasible to use extractives-free willow and thus untreated willow was used. The masses of the various willow chip fractions used in each run during these trials are given in Table 15.

Table 15. Oven-dry mass of each chip size fraction used in screening trial extraction experiments

Fraction	Oven-dry mass used in each run (g)
Large	3.99
>10 mm	8.21
<10 mm	4.06
Air-blown	0.097
Sawdust	0.64

More detail on the trials in which the extractives-free or untreated wood was used is given in the relevant sections of this chapter.

4.1.2. Extraction of lignin

4.1.2.1. Materials

The willow prepared in section 4.1.1 was used as the feedstock for lignin extraction. Absolute ethanol (analytical reagent grade, LabServ, Thermo Fisher Scientific New Zealand Ltd) was mixed with distilled water to prepare aqueous ethanol of various concentrations.

The extraction vessel was loaded with 136 mL of aqueous ethanol and 17 g of feedstock. This ratio of solvent to wood was chosen based on the literature reviewed in section 2.6.2.2.8. The vessel was then sealed according to the instructions given in the safe operating procedure for the vessel (Appendix B). The temperature controller was

set to the process temperature of 195°C. The extraction time was started when the internal temperature reached 195°C. Each extraction was allowed to proceed for a pre-determined time period, for which details are given in section 4.1.4.

At the end of the reaction time, power to the heating mantle was disconnected and the vessel was allowed to cool in the mantle. When the internal temperature reached 155°C, the solvent release valve was opened slightly to allow solvent to be discharged from the vessel. The outlet pipe from this valve was directed into a 2 L metal beaker containing 410 mL of distilled water at room temperature. The water was magnetically stirred at 500 rpm. The solvent was discharged into the water until the pressure inside the vessel dropped to approximately 10 bar. At this pressure, the solvent release valve was closed and the Nitrogen inlet valve was opened. Approximately 12 bar of nitrogen gas was applied to the vessel through the inlet valve. The solvent release valve was then opened, allowing nitrogen to flow from the cylinder into the headspace of the vessel for two minutes, forcing solvent liquor out through the filter element and solvent release valve where it was collected in the beaker of distilled water. The nitrogen inlet valve was then closed and the solvent release valve remained open until the pressure in the vessel dropped to a constant value. The stirring was stopped and the lignin suspension was stored in a 500 mL plastic container.

The lignin suspensions were stored in the 5°C chiller for at least 24 hours before the precipitated lignin was recovered by vacuum filtration. The lignin suspensions were vacuum filtered through Whatman #5 filter paper to obtain a brown solid product and yellow filtrate. The crude lignin was left to dry on the vacuum filter for two hours after the liquid had drained through. The lignin was then dried on watch glasses in a 30°C room.

4.1.2.2. Manual removal of solvent

During lignin extraction optimisation trials, the procedure outlined in section 4.1.2 began to fail to discharge all solvent from the vessel. It was assumed that either the filter was blocked or there was a leak at point where the discharge tube connected with the top of the vessel. An alternative method was then used. In this method, the extraction was carried out as outlined in section 4.1.2, however, instead of discharging the extraction liquor using the solvent release valve, the vessel was left to cool until the internal temperature reached 45°C. The cooling time was approximately 2 hours 50

minutes. The vessel was then opened and the solvent was obtained by filtering through glass wool. Ten millilitres of warm (~55°C) aqueous ethanol of the same concentration as that used in the extraction was used as a wash solution. The wood chips were left to drain on the glass wool for two hours.

To precipitate the lignin, the solvent and washings were added to 410 mL of distilled water under magnetic stirring at 250 rpm. One hundred millilitres of distilled water was used as the wash solution. The total mixing time, including addition of the solvent to the water, was 14 minutes. The lignin suspensions were then stored and lignin recovered, as outlined in section 4.1.2.

4.1.3. Lignin analysis

4.1.3.1. Lignin yield

Yield was measured initially as the mass of lignin collected on the watch glass after vacuum filtration after three days of drying at 30°C. The yield was then reported as a percentage of the total available lignin in extractives-free willow.

4.1.3.2. Lignin purity

Analyses for acid insoluble (AIL) and acid soluble (ASL) lignin were used to determine the total lignin (TL) content of the crude lignin precipitate obtained using the steps outlined in section 4.1.2. The National Renewable Energy Laboratory (NREL) analytical procedures for lignin determination were used (National Renewable Energy Laboratory, 2010). The dried lignin was ground with a mortar and pestle before 200-300 mg was hydrolysed with 72% H_2SO_4 in a 100 mL Duran bottle for 1 hour at 30°C. The sample was then diluted to 4% H_2SO_4 by the addition of 84 g of deionised water to the Duran bottle. The lids of the Duran bottles were loosely tightened, the samples heated in an autoclave at 121°C for one hour and then allowed to cool to room temperature. The samples were then filtered through pre-weighed glass filtering crucibles of porosity 4. The filtrate was analysed for acid soluble lignin content using UV-visible spectroscopy (Pharmacia Ultrospec II, Pharmacia LKB Biotechnology, Uppsala, Sweden). The absorbance at 320 nm was recorded and ASL content recorded using an absorptivity of 11.4 $Lg^{-1}cm^{-1}$ as suggested by Ramirez (2005) for measuring the ASL of Poplar and used by Macfarlane (2009) for measuring the ASL of *Salix schwerinii*. The retentate was dried in the filtering crucibles at 108°C for 12 hours, weighed, ashed in a muffle

furnace at 525°C for 16 hours and reweighed to determine the AIL fraction. The total lignin content was then calculated as the sum of ASL and AIL.

4.1.3.3. Verification of lignin composition

The lignin produced using the conditions found to give the highest yield and purity was also sent to Veritec for verification of the lignin purity. Along with AIL and ASL, cellulose sugars, hemicellulose sugars and ash present in the lignin were also determined.

4.1.4. Optimisation of lignin extraction conditions

The organosolv conditions used to extract lignin were optimised to ensure that lignin of both high yield and purity was used for lignin microparticle formation in later experiments. Ethanol concentration and extraction time were selected as the factors to optimise, while the extraction temperature was kept constant at 195°C. A temperature of 195°C was selected based on the literature. Notably, Pan et al. (2006) found that an increase in temperature beyond 195°C reduced the yield of organosolv lignin from poplar due to excessive degradation of lignin. The degradation produced fragments too small to be recovered by precipitation. A series of screening experiments was carried out initially to select suitable factor levels, followed by optimisation by central composite design.

4.1.4.1. Screening

The method of steepest ascent is recommended to find the "best known conditions" on which to base a central composite design. In this method, a contour plot of the first order model for the operating conditions is generated. The path of steepest ascent is then identified, which is the line along which the rate of increase of the response is maximal. Experiments are carried out stepwise up the path until an increase in the response is not seen. Fitting a new first order model and determining the lack-of-fit then shows whether or not the latest operating conditions are in the vicinity of the optimum (NIST/SEMATECH, 2013).

A more simple approach was used to find the best known conditions for the organosolv extraction of lignin from *S. purpurea*. To find the best known extraction time, the ethanol concentration was held constant at 65%, while the extraction time was increased from 60 to 110 minutes. The yield of lignin was measured to determine the extraction

time that produced the highest yield of lignin. Subsequently, the extraction time was held constant at 90 minutes while the ethanol concentration was varied between 50 and 75%. Again, the yield of lignin was measured to determine the ethanol concentration that produced the highest yield of lignin. It is important to note that although the conditions required for both high yield and purity were under investigation, only lignin yield was measured during the screening trials. This was because of the time-consuming nature of the lignin purity determination test and the limited time available for screening.

4.1.4.2. Optimisation

A 2^2 central composite design was applied to optimise the organosolv extraction. The design included ethanol concentration and extraction time as the two main effects and also a two-factor interaction between the two. The experimental matrix is shown in Table 16. The ethanol concentration and extraction time used for the centrepoint runs were selected based on the results of the screening trials. Thus the centrepoint runs represented the "best known" lignin extraction conditions in terms of high lignin yield.

The response variables measured were lignin yield and purity. Minitab was used to fit various models to the data. The statistical significance of the regression coefficients for each model, along with the R^2 value, was used to determine the adequacy of the model in predicting changes in yield and purity with ethanol concentration and extraction time.

A second smaller trial was required due to inconclusive results from the central composite design experiment. Table 17 gives details of the second trial.

Table 16. Experimental design matrix for optimisation of lignin extraction

Run	Coded Design Level		Ethanol Concentration (%)	Extraction Time (mins)
	X_1	X_2		
1	-1	-1	50	60
2	1	-1	70	60
3	-1	1	50	120
4	1	1	70	120
5	-1.68	0	46	90
6	1.68	0	74	90
7	0	-1.68	60	48
8	0	1.68	60	132
9	0	0	60	90
10	0	0	60	90
11	0	0	60	90
12	0	0	60	90
13	0	0	60	90

Table 17. Details of secondary trials in the optimisation of lignin extraction conditions

Run	Ethanol Concentration (%)	Extraction Time (mins)
1	60	132
2	60	150
3	60	150
4	60	180

4.2. Results

This section presents the results obtained for the optimisation of organosolv lignin extraction. This chapter is split into two sections, firstly to cover the results obtained from the screening trials and secondly to cover the results from the lignin extraction optimisation carried out via central composite design. Finally, a method for the extraction of lignin in future work is presented.

4.2.1. Screening

Eleven screening trials were carried out to determine the "best known conditions" as the starting point for the extraction optimisation trials. Details of these trials are presented in Table 18.

Screening trials 1 and 2 were carried out using extractives-free wood. Table 18 shows that for an ethanol concentration of 65%, increasing the extraction time from 60 to 110 minutes increased the yield of lignin from 49 to 55%. This indicates that increasing the extraction time during the organosolv pulping of *S. purpurea* causes an increase in the amount of lignin extracted.

Table 18. Influence of wood type, temperature, extraction time and ethanol concentration on lignin yield

Trial no.	Wood Type	Temperature (°C)	Time (minutes)	Ethanol Concentration (% v/v)	Lignin Yield (% of total lignin in willow)
1	Extractives-free	195	60	65	49.0
2	Extractives-free	195	110	65	55.0
3	Untreated	195	60	65	60.8
4	Untreated	195	60	65	56.9
5	Untreated	195	90	65	62.2
6	Untreated	195	90	65	58.0
7	Untreated	195	90	75	43.1
8	Untreated	195	90	50	58.9

Trials 3-8 were carried out using untreated (non-extractives-free) wood. It was not ideal to use untreated wood for the screening trials; however, preparation of enough extractives-free wood was impractical due to limitations of time and the size of soxhlet apparatus available. It was assumed that relative differences between the mass of crude lignin extracted under different pulping conditions would still provide a useful indication of the effect of different extraction times and temperatures on lignin yield.

Trials 3 and 4 were carried out using the same extraction conditions to provide an indication of the repeatability of the extraction procedure. The yields of lignin obtained

for trials 3 and 4 were 60.8 and 56.9%, respectively. A difference of only 3.9% is consistent with a reasonably repeatable extraction method.

Trials 5 and 6 were carried out using an extraction time of 90 minutes. For this longer extraction time, similar yields of lignin to those obtained for an extraction time of 60 minutes (at an ethanol concentration of 65%) were measured. Thus longer extraction time did not seem to increase the yield of extracted lignin. However, based on the review of extraction conditions in section 2.6.2.2 and the fact that there was limited time to carry out more screening trials at longer extraction times, 90 minutes was selected as the centrepoint extraction time for the central composite design.

Once the centrepoint extraction time of 90 minutes had been chosen, trials 7 and 8 were carried out to determine whether a suitable centrepoint for ethanol concentration had been chosen. Trial 7 showed that using an ethanol concentration of 75% reduced the lignin yield to 43.1%, while trial 8 showed that an ethanol concentration of 50% gave a similar lignin yield to using 65% ethanol. Based on these results, a centrepoint ethanol concentration of 60% was chosen for the subsequent central composite design.

It is important to note that three other screening trials were attempted. However, there was incomplete discharge of extraction liquor from the vessel in these trials and the yield of lignin could not be measured. Had these trials been completed, more early knowledge about the effect of ethanol concentration and extraction time on lignin yield may have been obtained.

4.2.2. Optimisation by central composite design

Table 19 shows the yield and purity of lignin produced using the various extraction conditions set out by the central composite design. Lignin was extracted from extractives-free willow in these trials.

Table 19. Influence of ethanol concentration and extraction time on lignin yield and purity

Ethanol Concentration (%)	Extraction Time (mins)	Lignin Yield (% of total lignin in willow)	Purity of Crude Lignin (%)
50	60	39.9	83.6
70	60	40.8	86.7
50	120	47.0	90.6
70	120	43.1	90.1
46	90	32.0	88.7
74	90	40.2	84.5
60	48	44.1	86.5
60	**132**	**47.7**	**91.6**
60	90	45.2	88.7
60	90	45.3	89.5
60	90	42.8	87.6

Table 19 shows that both the highest lignin yield and purity were obtained using 60% ethanol and an extraction time of 132 minutes. The lowest lignin yield was obtained using 46% ethanol and extraction time of 90 minutes. However, yield did not seem to consistently increase or decrease with an increase or decrease in either ethanol concentration or extraction time. Furthermore, the quadratic model fitted to the lignin yield data had an R^2 value of only 0.795 and only the coefficient for the square of ethanol concentration was significant. Other models were also fitted but had either similar or worse R^2 values. Thus neither ethanol concentration or extraction time seemed to significantly affect lignin yield within the ranges tested.

The purity of all samples fell within the range 83-92%, indicating quite a high purity of the samples overall. However, Figure 17 shows a surface plot of the effect of ethanol concentration and extraction time on lignin purity and reveals that lignin purity increases as extraction time increases. Furthermore, statistical analysis revealed that extraction time significantly affects lignin purity ($P = 0.010$).

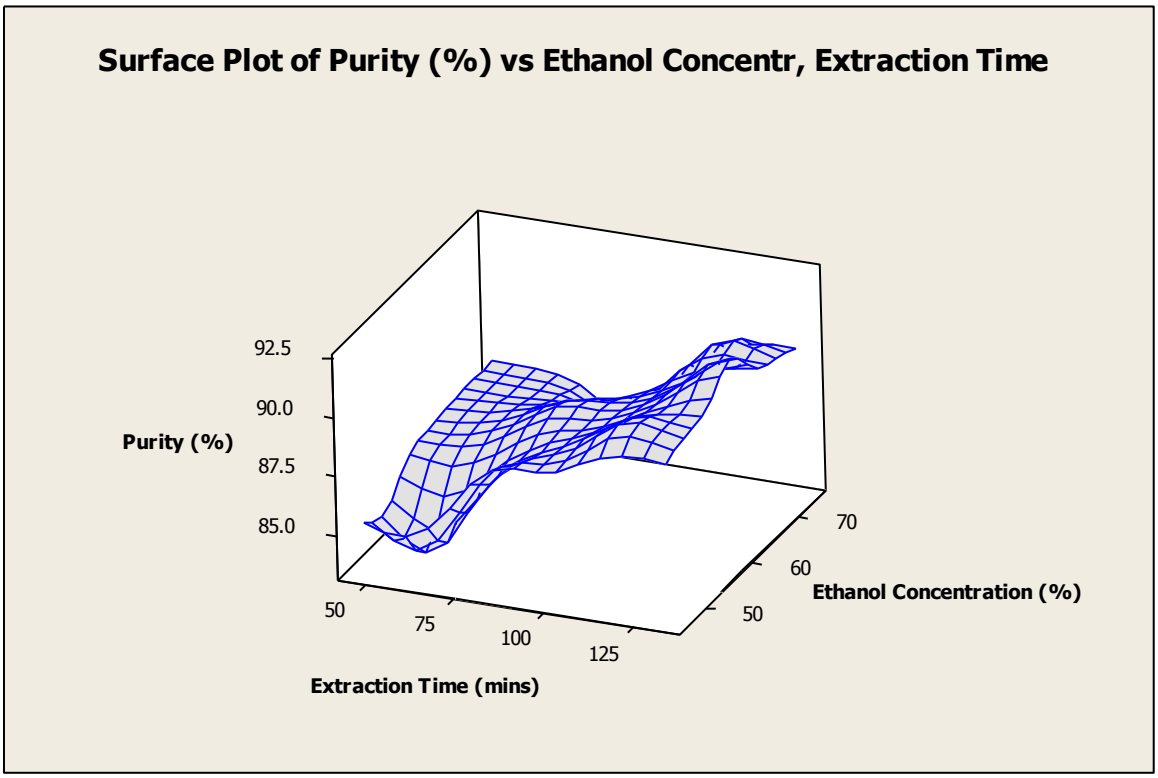


Figure 17. Surface plot for the effect of ethanol concentration and extraction time on lignin purity

Figure 17 may show a maximum in lignin purity in the region of 60-70% ethanol and 125-132 minutes. However, due to the continual upward slope of the response surface with extraction time, it cannot be concluded that maximum lignin purity is achieved using conditions in this region. This suggests that the optimum extraction conditions were not found and that higher lignin purity may be obtainable using longer extraction times.

Longer extraction times were quickly investigated by carrying out further trials in which the extraction time was increased to 150 and 180 minutes, keeping the ethanol concentration and temperature constant at 60% and 195°C, respectively. An extraction time of 180 minutes was selected based on a review of the literature, which indicated very little advantage in using extraction times longer than this. 150 minutes was then chosen as an intermediate time between 132 and 180 minutes. A replicate of the extraction running for 150 minutes was carried out. A 132 minute extraction was also

carried out to check consistency with the results of the optimisation trial. The results of these trials are shown in Table 20.

Table 20. Influence of extraction time on lignin yield and purity

Ethanol Concentration (%)	Extraction Time (mins)	Lignin Yield (% of wood)	Purity of Crude Lignin (%)
60	132	44.3	89.9
60	150	48.4	87.9
60	150	43.4	90.2
60	180	45.2	89.0

Table 20 shows that, as expected, there was no trend in yield with respect to extraction time. The yields were all close to 45%, regardless of the extraction time. Furthermore, the largest difference in measured yield was between the replicates of the 150 minute trial, suggesting that variation between the yields was experimental, rather than real variation due to the changes in extraction conditions.

There was also no increase or decrease in purity with increasing extraction time. Again, the largest difference in purity was between the replicates of the 150 minute trial. This indicates that using extraction times longer than 132 minutes does not produce lignin of higher yield or purity. Thus, extraction conditions of 195°C, 60% ethanol and 132 minutes, which had already been selected for use in other work in this project as a result of the optimisation trial, were confirmed as suitable extraction conditions to produce lignin for this project.

A limitation of these additional trials was that the combination of longer extraction times with different ethanol concentrations were not investigated and also that more extraction times, increasing at smaller intervals, were not tested. This may have better shown the conditions at which the maximum yield and purity are obtained. However, the major aim of this thesis was to prepare microparticles from lignin, rather than to perfectly optimise yield and purity.

4.2.3. Composition of lignin produced using optimised extraction conditions

4.2.3.1. Lignin composition

For the remainder of the project, lignin was extracted from untreated willow using the optimal extraction conditions. Therefore, it was important to know the purity and composition of this lignin to help understand its suitability for various applications. Table 21 shows the results of a composition analysis carried out by Veritec in terms of lignin, carbohydrates and ash. It can be seen that the lignin is of high purity and that the second major component present is sugar from xylose, the monomer comprising the hemicellulose component of willow. This indicates that sugar contamination is low and thus this lignin seemed suitable for use in all future work.

Table 21. Composition of lignin extracted from untreated willow

	% (w/w)	**% (w/w)**
LIGNIN		96.53 ± 0.10
---Acid soluble lignin	3.14 ± 0.03	
---Acid insoluble lignin	93.39 ± 0.10	
CARBOHYDRATES		
Cellulose sugars		0.33
---Glucosyl	0.33 ± 0.02	
Hemicellulose sugars		2.04 ± 0.007
---Xylosyl	1.86 ± 0.007	
---Arabinosyl	0.03 ± 0.0	
---Galactosyl	0.11 ± 0.0	
---Mannosyl	0.04 ± 0.0	
ASH		1.65 ± 0.16
TOTAL		100.54

Notes: All values are based on oven-dried lignin weight basis. Sugars are reported as anhydrosugar units.

4.3. Discussion

Lignin yields were all less than 50% of the total available lignin. It may be possible to achieve higher yields closer to the actual lignin content by catalysing the reaction using acid catalysts such as H_2S0_4. For example, Pan et al. (2006) obtained lignin yields up to 20.9% of the wood during the organosolv pulping of poplar with sulfuric acid as catalyst. Assuming poplar has a similar lignin content to the willow used in the current work, this equates to a yield of approximately 80% of the total available lignin. However, section 2.6.2.2 outlined that altering the extraction conditions can often achieve similar results to catalysis anyway and that the negative impacts of acid catalysis, such as excessive lignin degradation and corrosion of extraction vessels, often outweigh the benefits. Using acidified water rather than distilled water to precipitate lignin may also have allowed greater yields to be achieved.

Lignin re-precipitation on the wood during the cooling of the vessel may also have occurred (Macfarlane, 2009). Such re-precipitation would have reduced the yield of lignin recovered from the solvent liquor. Thus removing the solvent liquor sooner after extraction, for example by using the filter element set up that was initially trialled, may enable more lignin to be recovered after the extraction.

A purity of 91.6% is close to total lignin contents of 94.2% and 96.36% found for organosolv lignins by El Mansouri and Salvadó (2006) and Buranov, Ross and Mazza (2010), respectively. Furthermore, Veritec analysis of the lignin produced using the optimal conditions of 195°C, 60% ethanol and 132 minutes revealed a total lignin content of 98.5 ± 0.7%. Although there was a difference of 6.9% between the internal and external measurement of lignin purity, ultimately it is clear that high purity lignin was produced using the chosen extraction conditions, confirming the suitability of these conditions for use in the remainder of the project. These purity levels support the suggestion by Lora (2008) that organosolv lignins are high purity lignins.

Interestingly, it is suggested that very fine lignin can give spuriously high lignin contents (Kirk Torr, Scion, Rotorua, New Zealand, personal communication, 2013). However, considering that the high purity organosolv lignin (HP-L^{TM}) produced by Lignol Innovations in Canada has an ash content < 0.5% and carbohydrates < 1% (Lignol Innovations Ltd., 2009), and also that the lignin extracted in this case was from extractives-free willow, a lignin content of 98.5% does not seem unreasonable.

4.4. Conclusions and recommendations

Through a series of trials including screening trials and a central composite design, optimal conditions were found for the extraction of lignin from extractives-free willow (*Salix purpurea*) using ethanol organosolv pulping. It was found that neither ethanol concentration nor extraction time significantly affected lignin yield. Comparatively, extraction time was found to significantly affect lignin purity, with purity increasing as extraction time increased.

When extracting lignin from extractives-free willow, the highest lignin yield and purity were obtained using an extraction temperature of 195°C, extraction time of 132 minutes and ethanol concentration of 60% (v/v). These optimal conditions produced a yield of 47.7% (w/w) of total available lignin and purity of 91.6% (w/w). External analysis found the purity of this lignin to be higher at 98.5 ± 0.7%, supporting that the conditions produce high purity lignin.

Use of the optimal conditions to extract lignin from untreated willow also produced high purity lignin. External analysis revealed a lignin content of 96.53 ± 0.10%, cellulose content of 0.33%, hemicellulose content of 2.04% and ash content of 1.65% for this lignin. Lignin will be extracted from untreated willow chips for the remainder of the project and thus it is useful to know the composition of this lignin.

Chapter Five. Manufacture of Lignin Microparticles[1]

5.1. Introduction

For any particles that are added to foods, particle size, shape, hardness and taste are important to consider. Hinton (1970) has suggested that the minimum particle size that can be felt by the palate is around 25 μm. Tyle (1993) observed that particle shape and hardness heavily influence the perception of different particle sizes, finding that particles as large as 80 μm are not perceived as gritty if they are either soft and spherical or relatively hard and flat. Conversely, for hard and angular particles, grittiness can become evident as particles exceed a size range of about 11-22 μm. Thus there seems to be an important interplay between particle size, shape and hardness. Taste is also critical; a major commercial drawback for LITA®, an ingredient produced by microparticulating zein protein from corn gluten meal, was its inherent flavour problems (Jones, 1996).

For fat mimetic applications, in which microparticles are used to mimic the fat droplet, the microparticles must be insoluble in water. With diameters of only 0.1-3 μm, the particles are not detected individually, but rather as a smooth, creamy, fluid product that coats the mouth in a similar way to fat. In this way, fat droplets in emulsion-based foods can be replaced by equivalent phase volumes of microparticulate fat mimetics (Gómez, 2008; Setser & Racette, 1992; Singer, 1996). Although many fat replacing ingredients on the market have hydrophilic characteristics, hydrophobic microparticles may better mimic fat droplets (Jones, 1996). Hydrophobic microparticles exhibit less interaction with aqueous media and could potentially form films that provide a more fat-like mouth coating (Stark & Gross, 1994).

A technique based on anti-solvent precipitation may be an appropriate route for the preparation of lignin microparticles. In this technique, an organic solution containing the polymer of interest is added to an aqueous solution under shear. Through removal of the organic solvent by either evaporation or extraction, polymer precipitation occurs and

[1]Paper has been published based on this work:
Stewart, H., Golding, M., Matia-Merino, L., Archer, R., & Davies, C. (2014). Manufacture of lignin microparticles by anti-solvent precipitation: Effect of preparation temperature and presence of sodium dodecyl sulfate. *Food Research International, 66*(0), 93-99. doi: http://dx.doi.org/10.1016/j.foodres.2014.08.046

solid microparticles are produced. For example, Patel et al. (2010), Zhong and Jin (2009) and Parris et al. (2005) sheared ethanol solutions containing dissolved zein into water, and then removed the ethanol to produce zein microparticles or nanoparticles. Asrar and Ding (2006) patented a method of producing lignin microparticles for the controlled release of agricultural actives using a solvent evaporation technique. The method produced lignin particles with average size in the range 5.6-7.3 μm, depending on the mixing speed used. Finally, numerous parameters can be altered when using the anti-solvent precipitation method. These include mixing type and speed, polymer concentration, volume ratio of the dispersed and continuous phases, temperature, composition of the phases and the rate of solvent removal. Use of emulsifiers and stabilisers is also an option.

Temperature influences the rate of solvent evaporation during microparticle formation. Temperatures close to or above the boiling point of the dispersed phase solvent are suggested to increase the rate of solvent evaporation. Faster evaporation reduces the amount of shearing that the dispersion undergoes before precipitation of solid microparticles, leading to larger microparticles with larger size distributions (Yang, Chia, et al., 2000; Yang, Chung, et al., 2000). On the other hand, Stark and Gross (1994) have suggested that smaller microparticles are formed if higher temperatures are used during the precipitation process. However, the maximum temperature is limited by the boiling point of the system and also by the solution properties of the polymer. Asrar and Ding (2006) have recommended that the temperature should remain at least 20°C below the normal boiling point of the organic solvent.

Emulsifiers can influence particle size and stability by enabling the size of dispersed phase droplets to be better controlled (Asrar & Ding, 2006). Stabilisers can prevent aggregation of particles by providing, for example, electrostatic or steric repulsion (Patel et al., 2010). Asrar and Ding (2006) found cellulose derivatives, particularly methylcellulose, to be useful emulsifiers for the preparation of lignin microparticles. Stark and Gross (1994) suggest that carboxymethyl cellulose, gum Arabic, sodium caseinate, lecithin, DATEM-esters, polysorbates and sodium dodecyl sulfate (SDS) can all be used effectively to stabilise zein microparticles. Thus there is a variety of emulsifiers and stabilisers that may be useful for the production of lignin microparticles.

This chapter outlines the development of a method based on anti-solvent precipitation for the production of lignin microparticles from lignin extracted in the previous chapter. The effect of temperature on particle properties is discussed. An initial investigation into the effect of added surfactant on particle properties is also outlined.

5.2. Materials and methods

This section describes the materials and methods used in the development and understanding of a method for the preparation of lignin microparticles. The anti-solvent precipitation technique used to produce microparticles from the lignin extraction liquor is described, along with the method of drying the microparticles. Finally, the methods used to characterise the microparticles are described. These methods include static light scattering, scanning electron microscopy, Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC).

5.2.1. Materials

Lignin was extracted from chipped *Salix purpurea* and used in the form of extraction liquor containing 4.68 ± 0.04% solids. Lignin precipitated from this liquor was found to have a total lignin content of 96.5%, with the remainder mostly comprising ash (1.65%), xylan (1.78%) and glucan (0.33%).

Absolute ethanol (analytical reagent grade, LabServ, Thermo Fisher Scientific New Zealand Ltd) was mixed with distilled water to prepare 60% (v/v) ethanol.

Sodium dodecyl sulfate (SDS) (BDH, VWR International Ltd, New Zealand) was used as received.

5.2.2. Methods

5.2.2.1. Lignin extraction

Lignin was extracted from chipped *S. purpurea* by heating the feedstock with 60% aqueous ethanol to 195°C in a stainless steel vessel (Parr 4767, Parr Instrument Company, Illinois) and holding the mixture at this temperature for 132 minutes. The extraction conditions were chosen based on the results of the optimisation trials in Chapter Four. The extraction liquor containing the lignin was recovered by filtering the

mixture through glass wool to remove the wood chips. The extraction liquor was then stored at 5°C until use.

5.2.2.2. Microparticle preparation

Microparticles were prepared using a method based on anti-solvent precipitation (Patel et al., 2010). To study the influence of preparation conditions on morphology and size distribution, 30 mL samples of lignin extraction liquor (20°C) were added to 75 mL distilled water (4-80°C), under continuous stirring (875 rpm) using a magnetic stirrer. The resulting dispersions were stirred at 875 rpm for the first hour, followed by 21 hours of stirring at 500 rpm to enable slow evaporation of ethanol from the sample; a regime developed based on earlier trials. The lignin concentration of the resulting suspensions was calculated to be approximately 1.5% (w/v) based on the solids content of the extraction liquor and the volume of the resulting suspension after ethanol evaporation. The suspensions were analysed for particle size immediately after preparation. To obtain dry powder samples, the suspensions were concentrated by centrifugation at 30,000 rpm (65,043 x g) for 1 hour (Sorvall WX Ultra 100 Centrifuge, Thermo Scientific, Waltham, MA), air-dried at 30°C overnight and ground to powder using a mortar and pestle.

5.2.2.2.1. Effect of temperature

To determine the effect of temperature on microparticle size and shape, the distilled water was cooled or heated to temperatures between 4 and 80°C immediately prior to the addition of the lignin extraction liquor. The lignin extraction liquor was always at a temperature of 20°C when it was added to the water. Once the liquor had been added to the water, the temperature of the mixture was not controlled and thus naturally approached room temperature during the stirring period.

Additionally, after the 22 hour stirring period, suspensions prepared at 4°C were heated to 35.7, 51.4 or 60°C whilst still being magnetically stirred at 500 rpm. Heating was stopped once the temperature of the suspension reached the required temperature and the sample was then stirred until ambient temperature was reached.

5.2.2.2.2. Effect of surfactant

To determine the effect of surfactant on microparticle size and shape, SDS was added to the water in the range 0.0625-1.5% (w/v). The SDS was added to the water before

heating or cooling of the water and before the lignin extraction liquor was added. Where possible, the preparation of particles under different conditions of temperature and surfactant addition was replicated. However, only representative data is included in this chapter, unless stated otherwise.

5.2.2.3. Particle size analysis

Particle size analysis of the lignin suspensions was carried out using static light scattering (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). A refractive index of 1.6 (Donaldson, 1985) and absorption of 0.01 (based on analysis of fit of the light scattering data) for lignin were used by the instrument to calculate the particle size distributions. The mean particle size was reported in terms of both the D[4,3] and D[3,2] values, which are defined by Equation 5 and Equation 6, respectively.

$$D[4,3] = \sum \frac{n_i {d_i}^4}{n_i {d_i}^3}$$

Equation 5

$$D[3,2] = \sum \frac{n_i {d_i}^3}{n_i {d_i}^2}$$

Equation 6

where n_i is the number of particles of diameter d_i. The D[4,3] is the volume mean diameter and refers to the diameter of a sphere of equivalent volume to the particles in the sample. The D[4,3] is very sensitive to large particles or large aggregates of particles. The D[3,2] is the surface area mean diameter and refers to the diameter of a sphere of equivalent surface area to the particles in the sample. The measurement of the diameters was carried out in duplicate and the results were reported as averages of the readings.

5.2.2.4. Scanning electron microscopy

Dried, crushed lignin samples were further dried under vacuum at room temperature for 24 hours. The dried samples were then sputter coated with a thin layer of gold and their morphology was characterised using scanning electron microscopy (SEM) (FEI Quanta 200, Hillsboro, Oregon). Images were taken at magnifications of 400, 2500, 5000, 10000 and 20000X, in two different areas for each sample.

5.2.2.5. Fourier transform infrared (FTIR) spectroscopy

FTIR analysis was used to determine and compare the functional groups present in lignin microparticles prepared at 4, 20, 40 and 60°C. Crushed, vacuum-dried lignin was analysed using a Nicolet 5700 infrared spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a diamond anvil ATR probe. 256 scans were recorded over the range 4000-400 cm^{-1}.

5.2.2.6. Differential scanning calorimetry

Samples were vacuum dried at room temperature for 24 hours and analysed by differential scanning calorimetry (DSC) (Q200, TA Instruments Inc., New Castle, DE). Lignin samples (1.5-2 mg) were accurately weighed and hermetically sealed in DSC aluminium pans. The samples were heated from 10 to 200°C at a rate of 10°C/min, cooled to 10°C at a rate of 10°C/min and heated a second time from 10 to 200°C at a rate of 10°C/min. To eliminate any thermal history effects of the sample, the second heating scan was used for determination of glass transition temperature. An empty pan was subjected to a temperature ramp covering the same temperature range as the samples (10-200°C) to collect a baseline for the data collected. The data collected during each run was the heat flow (W/g) to the sample at each temperature and time. Moisture content of the samples was also measured and found to be $\leq$ 1% for each sample.

Data were analysed using TA Instruments Universal Analysis 2000 software. To determine the glass transition temperature, the baseline data were subtracted from the data from the second heating scan of each sample. The glass transition temperature was then determined using a function of the Universal Analysis software that calculates the glass transition temperatures after pivot markers have been placed either side of the transition by the user. An onset, mid and end glass transition temperature are calculated

by the software, but only the onset temperature of the glass transition is referred to in this chapter. Figure E1 in Appendix E shows an example of the calculation of glass transition temperature using the software.

5.3. Results

5.3.1. Effect of temperature

Figure 18 shows the size distribution of precipitated microparticles as a function of water temperature. The temperatures shown in Figure 18 are the temperatures of the water immediately before addition of the lignin extraction liquor. However, since the extraction liquor was at a temperature of 20°C when it was added to the water, the final mixing temperature was intermediate between those of the two starting liquids. Assuming that there was no heat or volume of mixing, using energy balances the temperature at mixing where the water temperatures were 4, 20, 40, 60 and 80°C were calculated to be 7.4, 20, 35.7, 51.4 and 67.2°C, respectively.

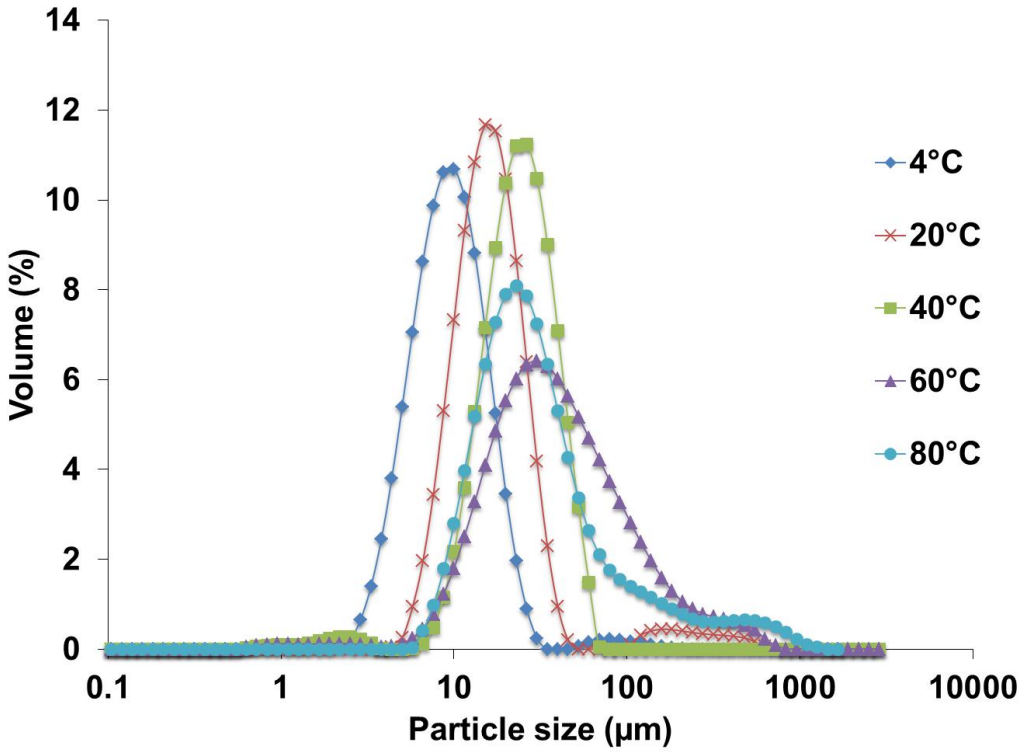


Figure 18. Particle size distributions of microparticle suspensions prepared by shearing 30 mL of lignin extraction liquor into 75 mL water at temperatures of 4, 20, 40, 60 and 80°C.

Microparticles prepared at all temperatures exhibit a major peak in particle size between approximately 10-100 µm. For water temperatures of 4-60°C, as the temperature increases, the peaks shift to the right, indicating an increase in particle size with temperature. At 80°C, the particle size peak occurs in a similar position to that for 40°C, seemingly going against the trend of increasing particle size with increasing temperature. However, the peak for 80°C is skewed, with more particles in the 100-1000 µm size range than the particles prepared at aqueous phase temperatures of 4-40°C. Consideration of the D[4,3] and D[3,2] particle size means (Table 22) shows that the size of particles prepared using a water temperature of 80°C is higher than the particle size of those prepared at 4-40°C. Therefore, although particles prepared at 60°C and 80°C were similar in size, increasing temperature generally leads to increasing particle size.

Table 22. D[4,3] and D[3,2] values of lignin particles in suspensions prepared using initial water temperatures of 4-80°C

Water temperature (°C)	D[4,3] (µm)	D[3,2] (µm)
4	10.6	7.7
20	25.5	13.8
40	24.0	16.2
60	59.8	21.1
80	61.5	22.2

Increasing particle size with increasing water temperature was also reflected in the colour of the microparticulate suspensions (Figure 19). Although all suspensions were brown, the suspension of particles prepared at 4°C had a lighter brown and more milky appearance than the other suspensions, which became increasingly darker and more watery with increasing temperature. Visible particulate material in the suspensions was more easily discerned at higher temperatures. Lighter colour and milkiness indicate the existence of smaller particles (Swaisgood, 2008). Irrespective of appearance of the suspensions, in all samples, visible sedimentation of the particles had commenced within five minutes of being at rest.

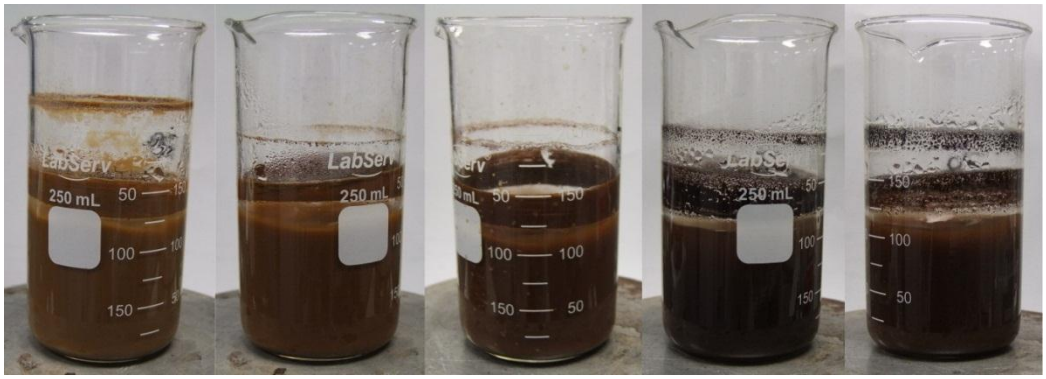


Figure 19. Photographs of microparticle suspensions prepared by shearing 30 mL of lignin extraction liquor into 75 mL water at temperatures of (from left to right) 4, 20, 40, 60 and 80°C.

Figure 20 shows scanning electron microscopy (SEM) images of the microparticles prepared at 4, 20, 40, 60 and 80°C. The images show that microparticles prepared at 4°C are quite distinct clusters that appear to comprise high densities of submicron particles. With increasing temperature, the clusters become decreasingly well-defined, larger and more amorphous. At 60 and 80°C, the images show large continuous masses of material.

Figure 21 shows an SEM image of microparticles initially prepared using a water temperature of 4°C, where the particle suspension was subsequently heated to 60°C. It can be seen that the particles have formed an amorphous mass similar to that produced when the water was initially 60°C (Figure 20D). The change in particle size with this temperature-cycling treatment is also consistent with the particle size distribution shown in Figure 18 for particles prepared using a water temperature of 60°C (data not shown). The size and appearance of the particles did not return to their original state when the suspension was cooled, indicating that irreversible changes occur with increasing temperature.

Figure 22 shows FTIR spectra of lignin microparticles prepared using aqueous water temperatures of 4, 20, 40 and 60°C. The similarity of the FTIR spectra suggests that microparticles prepared at different temperatures have similar chemical structures, indicating that chemical changes are not responsible for the microstructural changes observed at different microparticle preparation temperatures.

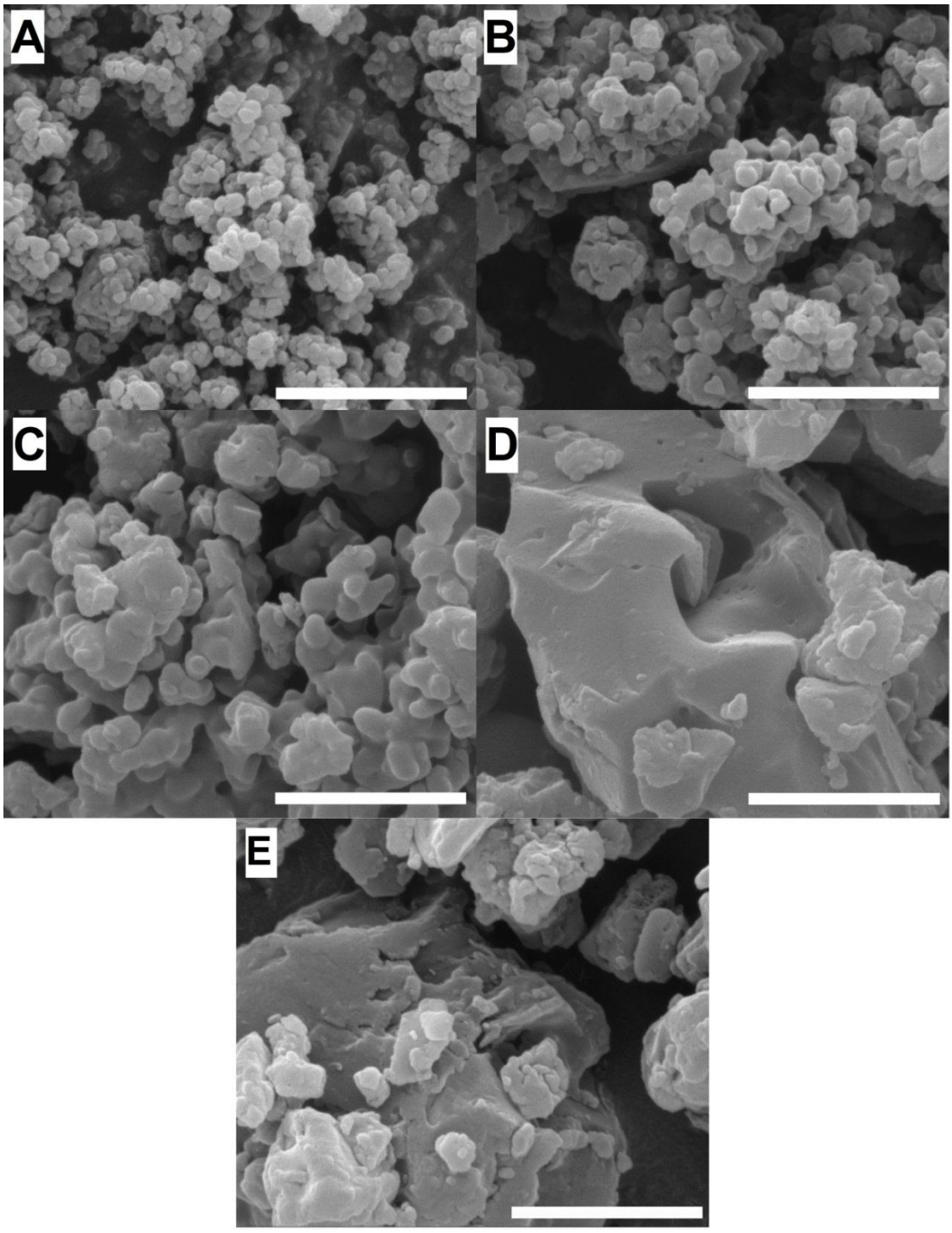


Figure 20. SEM images of the dried, crushed lignin microparticles prepared by shearing 30 mL of lignin extraction liquor into 75 mL of water at temperatures of (A) 4°C, (B) 20°C, (C) 40°C, (D) 60°C and (E) 80°C. All images were taken at a magnification of 20,000X (bar scale = 3µm).

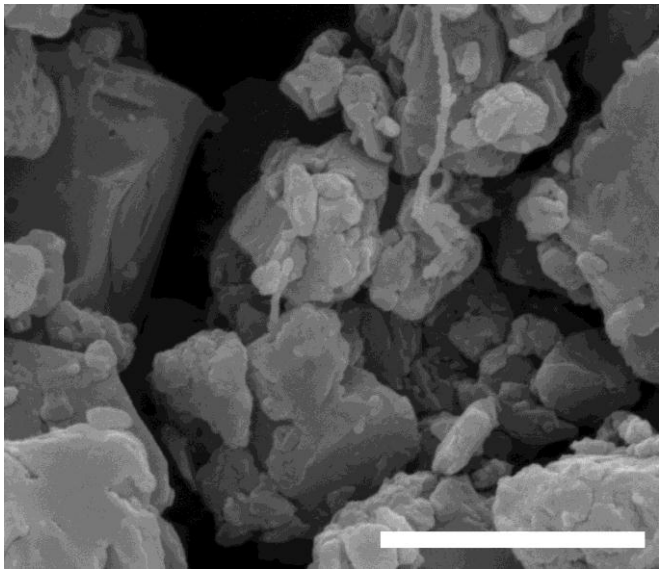


Figure 21. SEM image of the dried, crushed microparticles prepared by shearing 30 mL of lignin extraction liquor into 75 mL of water at 4°C, stirring for 22 hours to remove ethanol and subsequently heating the particle suspension to 60°C. The image was taken at a magnification of 20,000X (bar scale = 3 μm).

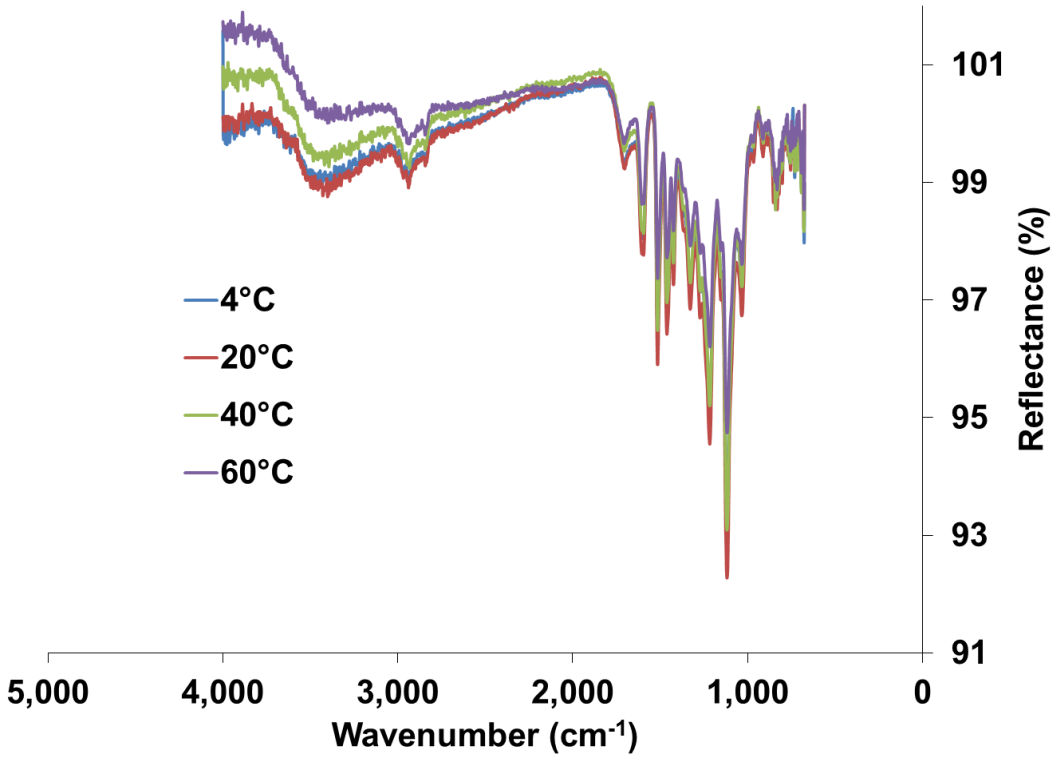


Figure 22. FTIR spectra for dried, crushed lignin particles prepared by shearing 30 mL of lignin extraction liquor into 75 mL water at temperatures of 4, 20, 40 and 60°C

The peaks shown in the spectra in Figure 22 are consistent with those expected for organosolv, hardwood lignin. Hardwood lignins generally contain mainly guaiacyl and syringyl units and traces of *p*-hydroxyphenylpropane groups. Guaiacyl units contain one methoxy group on the aromatic ring, while syringyl units contain two. Thus hardwoods would be expected to show a strong band in FTIR spectra at 1300-1000 cm^{-1} corresponding to C-O stretch in the ether groups. This is consistent with the high intensity peak at 1100 cm^{-1} in Figure 22. The next highest intensity peak is at about 1200 cm^{-1}, which is also likely to be due to C-O stretch. The peak observed at 1450-1600 cm-1 can be assigned to C=C-H asymmetric stretch and C-C=C symmetric stretch of the aromatic ring, again a dominant functional group in lignin. Finally, the broad peak at 3600-3100 can be assigned to the hydrogen-bonded O-H stretch of both phenols and other alcohols, which are expected in lignin based on proposed structure. (Bhat et al., 2009; Liu et al., 2011; Wellesley College, n.d.).

Figure 23 shows the traces obtained when dried lignin samples originally prepared using water temperatures of 4, 20 and 60°C were analysed using differential scanning calorimetry (DSC). Only a representative trace for each preparation temperature is shown, however, Table E1 in Appendix E gives details of the glass transition temperature for all replicates of each preparation temperature. The glass transition temperatures are shown by the bold arrows in Figure 23. All fall within the range 75-87°C. These temperatures are lower than glass transition temperatures measured for other organosolv lignins: 91 and 97°C for organosolv lignins from pine and aspen, respectively (Fox, 2006). Organosolv lignin from Sigma Aldrich has a glass transition temperature of 106°C (Buranov et al., 2010). The lower measured glass transition temperature may reflect the different feedstock and conditions used to extract lignin in our work.

Further experiments were carried out in which the effect of water temperatures between 40 and 60°C on particle morphology was investigated. Figure 24 shows SEM images of microparticles prepared using water temperatures of 45 and 50°C. At both temperatures, the lignin microparticles appear monolithic, indicating that the transition from individual yet increasingly agglomerated structures to completely amorphous structures occurs at lower temperatures than 60°C.

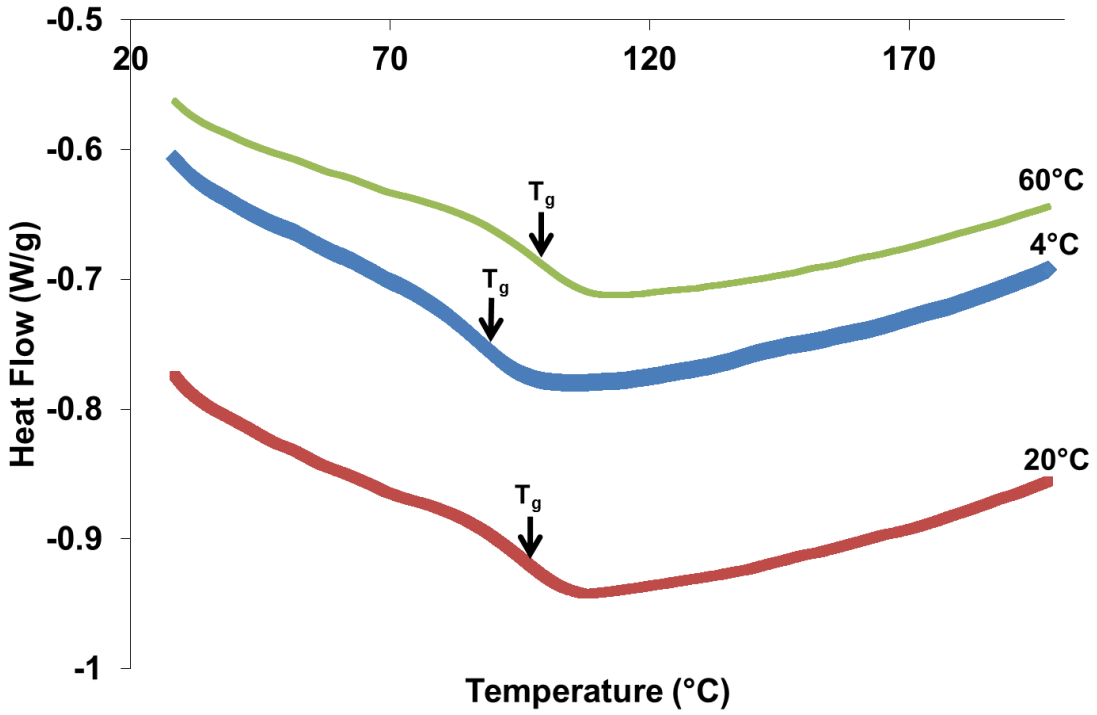


Figure 23. DSC curve for dried, crushed lignin particles prepared by shearing 30 mL of lignin extraction liquor into 75 mL water at temperatures of 4, 20 and 60°C.

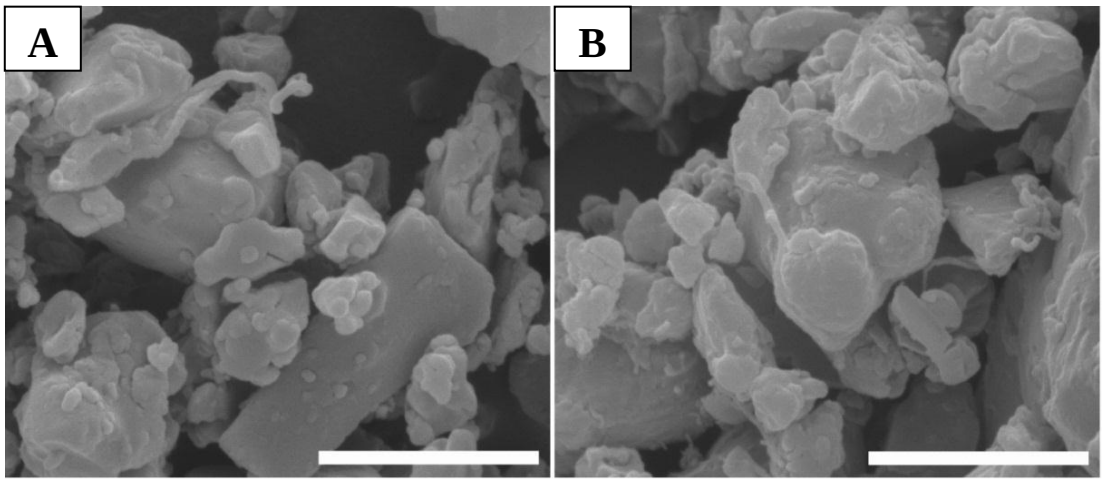


Figure 24. SEM images of dried, crushed lignin microparticles prepared by shearing 30 mL of lignin extraction liquor into 75 mL of water at temperatures of (A) 45°C and (B) 50°C. The images were taken at a magnification of 20,000X (bar scale = 3 µm).

Figure 25 shows SEM images of microparticles initially prepared using a water temperature of 4°C, for which the suspension was subsequently heated up to 35.7 and 51.4°C, which were the temperatures of mixing for suspensions prepared using water temperatures of 40 and 60°C, respectively. The particles heated to 51.4°C appear monolithic, while individual particles are evident in the image of particles heated to only 35.7°C.

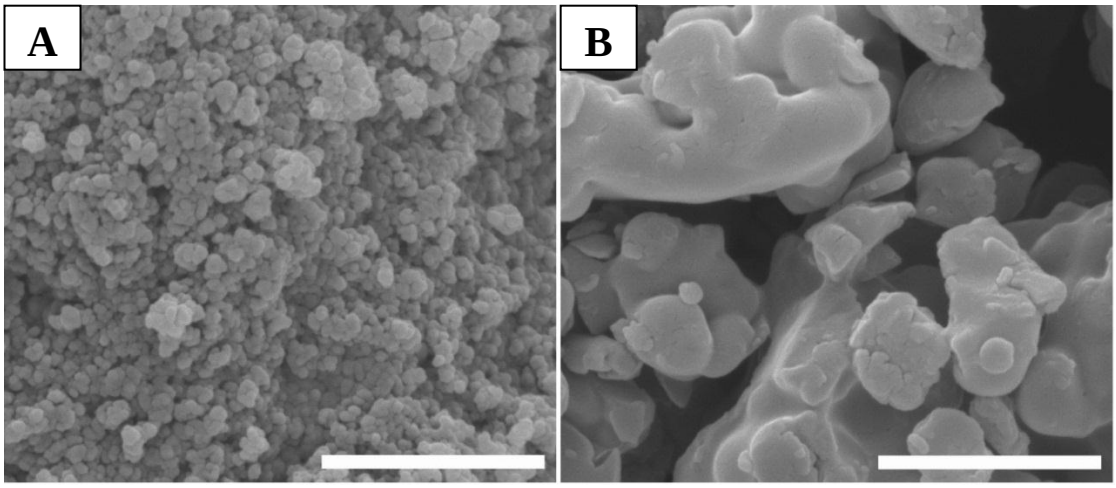


Figure 25. SEM images of dried, crushed lignin microparticles prepared by shearing 30 mL of lignin extraction liquor into 75 mL of water at 4°C, stirring for 22 hours to remove ethanol and subsequently heating the particle suspension to (A) 35.7°C and (B) 51.4°C. The images were taken at a magnification of 20,000X (bar scale = 3 μm).

5.3.2. Effect of sodium dodecyl sulfate

Figure 26 shows the particle size distributions of microparticle suspensions prepared using water containing 0.5% (w/v) SDS at temperatures of 4, 20, 40 and 60°C. For initial water temperatures between 20 and 60°C, the particle size distributions are narrow and centre around 0.1-0.2 μm. There is also a smaller population of agglomerated structures. Consistent with these size distributions, SEM of dried particles prepared using an SDS concentration of 0.5% (w/v) and temperature of 60°C reveals spherical, smooth and monodisperse particles (Figure 27). For an initial water temperature of 4°C the distribution is bimodal (Figure 26); some particles are in the 0.1-0.2 μm size range and others centre around 10 μm. This indicates that stabilisation of

sub-micron particles by SDS is less effective at lower temperatures. Nonetheless, addition of SDS has reduced particle size to some degree at all temperatures.

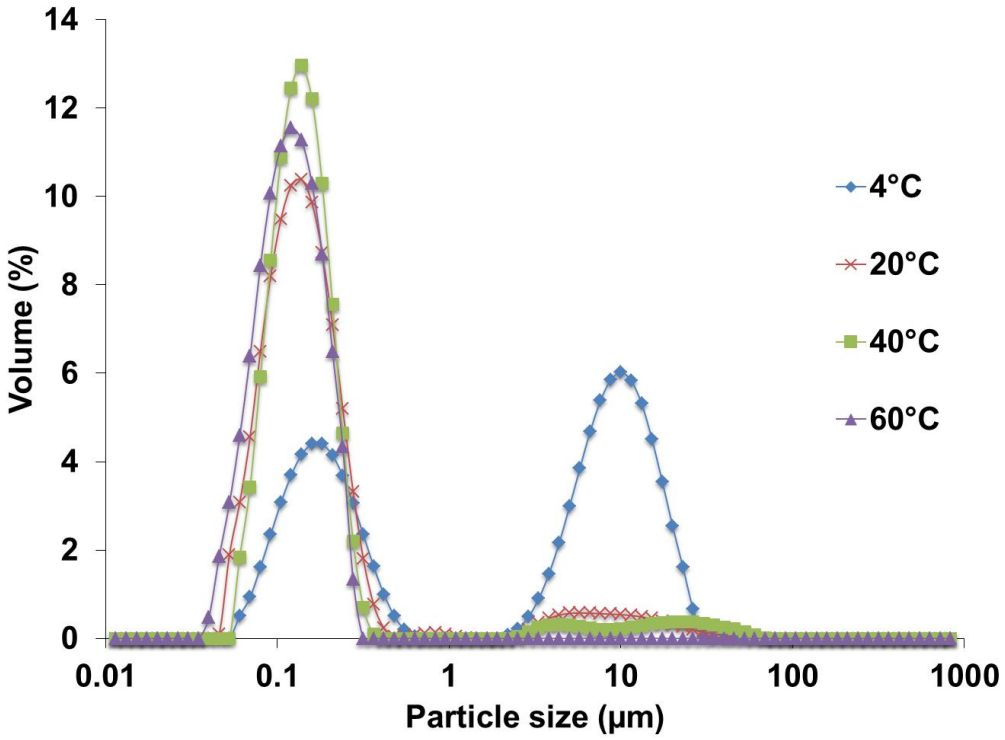


Figure 26. Particle size distributions of microparticle suspensions prepared by shearing 30 mL of lignin extraction liquor into 75 mL water containing 0.5% (w/v) SDS at temperatures of 4, 20, 40 and 60°C.

Figure 27 shows an SEM image of dried particles prepared using an SDS concentration of 0.5% (w/v) and temperature of 60°C. The particles are spherical, smooth and monodisperse, consistent with the narrow size distribution shown in Figure 26.

Lignin extraction liquor, which had been stored at 5°C for ≥ 12 hours, was centrifuged at 98,922 x g for one hour at 15°C to determine whether any particles or nuclei in the sub-micron size range were already present in the cooled extraction liquor before it was mixed with water to form microparticles. Figure 28 shows photographs of the liquor before and after centrifugation, revealing that the appearance of the liquor did not change after centrifugation. There was no sedimentation of solids. Since centrifugation at only 65,043 x g for one hour at 20°C was required during lignin microparticle

recovery steps to sediment 0.1-0.2 μm lignin particles earlier in this work, the lack of sedimentation in the extraction liquor at a higher speed indicates that particles of this size are not pre-existing in the liquor. Therefore, the mixing regime, evaporation of solvent and/or presence of SDS seem to be important if sub-micron particles are to be produced.

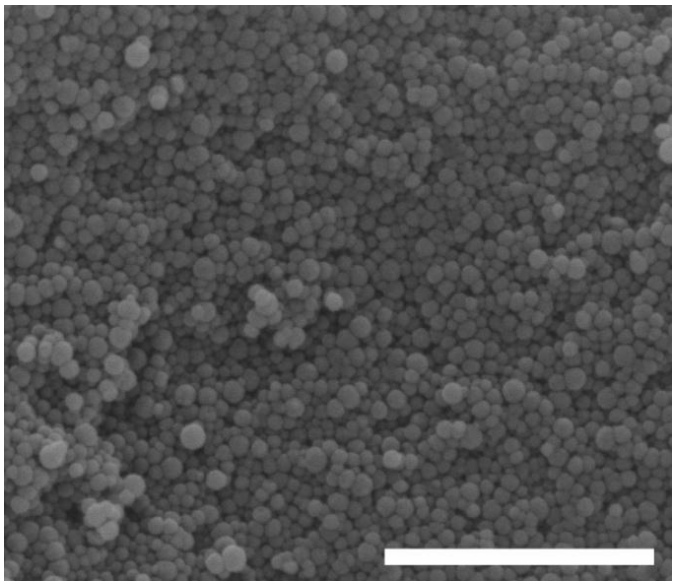


Figure 27. SEM image of the dried, crushed lignin microparticles prepared by shearing 30 mL of lignin extraction liquor into 75 mL water containing 0.5% (w/v) SDS at 60°C (bar scale = 3 μm).

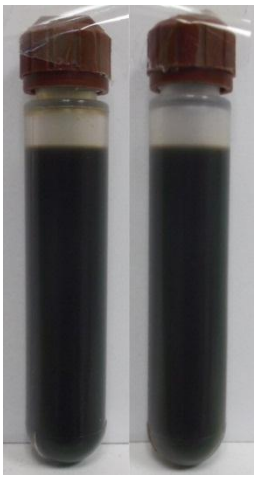


Figure 28. Appearance of lignin extraction liquor before (left) and after (right) centrifugation at 98,922 x g.

5.4. Discussion

5.4.1. Effect of temperature

Increases in particle size and agglomeration with increasing temperature of precipitation in the absence of surfactant may simply be the result of an increase in the rate of solvent evaporation at higher temperatures. Faster removal of solvent may cause more rapid precipitation of lignin and reduced time for the dispersion of liquor into smaller droplets. The increase in agglomeration could also be a result of increased hydrophobic interactions at higher temperatures. The hydrophobic interactions may arise between the phenyl moieties and possibly the methoxy groups in lignin, although methoxy groups are suggested to have little impact on hydrophobicity (Yanagita et al., 2013). "Diffusion limited coalescence" (DLC) may also be responsible for the changes with temperature. DLC takes place when the mixing of the two phases during anti-solvent precipitation occurs very quickly and when the polymers have a low molecular mass and thus behave as liquids. The rapid mixing and liquid-like state cause the polymer particles to diffuse, collide and stick (Stepanyan, Lebouille, Slot, Tuinier, & Stuart, 2012). According to collision theory, as the temperature of mixing increases, polymer molecules may be moving faster and collide more frequently. This may support the increased fusion at higher temperatures. The fact that sub-micron lignin particles were formed at a high precipitation temperature of 60°C when SDS was present indicates that small particles are still formed at higher temperatures, but they agglomerate in the absence of surfactant.

The increase in particle size and agglomeration of a particle suspension initially prepared at a water temperature of 4°C and subsequently heated to 60°C suggests that the temperature at which lignin precipitates to form solid "primary" particles is not necessarily the critical factor influencing ultimate particle size. It may be that particles precipitate in the same size range regardless of temperature due to the near constant conditions of shear. Temperature may then influence only the interaction of particles with one another. It could even be possible that a process such as sintering occurs, where solid particles fuse at temperatures below their melting point. Heating a suspension originally prepared using a water temperature of 20°C and SDS concentration of 0.5% (w/v) to 60°C did not cause any change in particle size (data not shown). This indicates that the presence of surfactant prevents particle interaction.

Apart from adjustment of the initial water temperature, the temperature during the mixing of the lignin extraction liquor and water was uncontrolled. Using a water bath to control the temperature of the mixture during the evaporation stage may provide more conclusive information about the effect of temperature on particle formation as the temperature would be controlled throughout the time of mixing and solvent evaporation, rather than just at the onset of mixing. This temperature control may enable particles of different sizes to those obtained in the current work to be produced. For example, perhaps at 4°C, the ethanol may evaporate much slower and produce smaller microparticles. At the higher temperatures, particularly 60 and 80°C, a mixing time of 22 hours may not be required since the ethanol will evaporate quickly and the long mixing time at higher temperature may cause evaporation of water. Additionally, for mixing temperatures ≥ 20°C, particles with the same size or larger than those produced using the uncontrolled temperature method in this work are likely to be produced.

FTIR analysis was carried out for dried particles produced using water temperatures of 4-60°C. The peaks related to expected functional groups in hardwood lignin. The peaks were also consistent across the particles produced at the four different temperatures, suggesting that changes in the chemical structure were not responsible for the morphological changes observed with increasing temperature.

Glass transition temperatures for lignin particles prepared at water temperatures of 4-60°C were in the range 75-87°C, seemingly above the temperature range over which changes in particle morphology were observed. However, it is known that water plasticises lignin and shifts the glass transition temperature to lower values (Irvine, 1985). This is thought to be due to replacement of intermolecular hydrogen bonds within lignin by lignin-water linkages (Sakata & Senju, 1975; Atack & Heitner, 1978, as cited by Irvine, 1985). Perhaps during the anti-solvent precipitation process, the presence of a large volume of water depressed the glass transition temperature, leading to softening at temperatures lower than 75-87°C. Glasser, Kelley, Ryials and Ciemniecky (1986, as cited by Lora & Glasser, 2002) reported that the T_g of lignin decreased by over 20°C when 5% water was present, indicating that the glass transition temperature of lignin during microparticle formation in the current thesis project could have been low enough to contribute to the morphological changes observed. It may be useful to determine the glass transition temperature at the water content present during particle formation.

SEM images reveal that when microparticle suspensions initially prepared using a water temperature of 4°C were heated to 51.4°C, the particles appeared monolithic, while individual particles were evident in the image of particles heated to only 35.7°C. The monolithic appearance of the particles heated to 51.4°C was expected, since it seems that once lignin is heated through temperatures of 45-50°C, a thermal change occurs that causes its agglomeration. The non-monolithic appearance of particles heated to only 35.7°C was also expected since the apparent critical temperature of 45-50°C had not yet been reached. However, the individual particles in this sample appear less agglomerated than particles prepared using an initial water temperature of 20°C, which was unexpected since the sample was heated through 20°C to reach 35.7°C. This may indicate that although a temperature of 45-50°C is a critical temperature for the lignin in this work, temperatures below this may only cause some agglomeration if the lignin particles are still being precipitated and not if the particles have previously been formed at a lower temperature.

5.4.2. Effect of SDS

The production of 0.1-0.2 μm spherical, homogeneous lignin particles in the presence of 0.5% (w/v) SDS at 20-60°C indicates that anionic small molecule surfactants are effective in stabilising the "primary" lignin microparticles. It is likely that during particle formation, the hydrophobic tails of SDS molecules bind to hydrophobic surfaces of the lignin particles, while the anionic head groups remain in the aqueous phase and provide stabilisation by electrostatic repulsion. At 60°C, SDS concentrations as low as 0.0625% (w/v) were found to stabilise sub-micron particles, which is also likely to be the case at 20-40°C, revealing that low concentrations of SDS can effectively stabilise lignin particles.

The larger particle size observed for a water temperature of 4°C in the presence of SDS could be a result of lignin becoming less hydrophobic as temperature decreases, reducing the affinity of the hydrophobic tails of SDS for lignin. The Krafft point of SDS may also be an important factor. The Krafft point is the critical temperature above which surfactants become soluble enough to function properly (McClements, 2005). Values reported for the Krafft point of SDS are in the range 12± 4°C (Beheshti, Kjøniksen, Zhu, Knudsen, & Nyström, 2010). When the extraction liquor was added to the water to bring the temperature of mixing to 7.4°C, the temperature may not have

been high enough for the SDS to function as a surfactant, leading to some larger diameter particles. When the microparticle suspension temperature increased to room temperature over the 22 hour shearing stage of microparticle formation, it is likely that the particles had already precipitated at the larger size; the now more functional SDS unable to influence particle size.

Changing the concentration of SDS may enable lignin microparticles of different size to be formed and stabilised. Other types of surfactants or emulsifiers may also enable particles of different size to form. Therefore, it is recommended that the effect of SDS concentration and also of other types of emulsifier on lignin particle properties is investigated, and this forms the basis of the following chapter.

5.5. Conclusions and recommendations

In this chapter, lignin microparticles were successfully manufactured using a method based on anti-solvent precipitation. An organic solution of lignin, obtained by aqueous ethanol extraction from shrub willow, was added to an aqueous phase of distilled water under simple stirring. Slow removal of ethanol by evaporation allowed the lignin to precipitate into hardened microparticles.

Water temperature markedly influences particle morphology. Increasing initial water temperature between 4 and 80°C led to increasing agglomeration and fusion of sub-micron “primary” particles.

DSC analysis of dried recovered particles showed glass transition temperatures of 75-87°C, above the range of interest; however, it is possible that the glass transition temperature of lignin in the presence of a large volume of water during particle formation was reduced due to plasticisation. The transition of lignin from the brittle to rubbery state may influence the observed increase in particle fusion with increasing preparation temperature.

Incorporating the surfactant SDS in the water at a level of 0.5% (w/v) seemed to inhibit agglomeration of primary particles at most temperatures, but least effectively at 4°C. Investigation into the effect of SDS concentration on particle morphology is recommended. The ability of other emulsifiers to produce stable microparticles of various sizes is also recommended.

Chapter Six. Control of Lignin Microparticle Size

6.1. Introduction

In the previous chapters, lignin was extracted and mixed with water in an anti-solvent precipitation process to obtain lignin microparticles. For lignin microparticles to be used in different applications, it is important that particles of varied and controlled size can be produced. For example, particles to be used as fat mimetics must be spherical and fall in the 0.1-0.3 μm size range (Jones, 1996). Therefore, there is a need for methods that can be used to manipulate lignin particle size.

Zhong and Jin (2009) have pointed out the three competing mechanisms occurring during particle formation by liquid-liquid dispersion processes such as solvent evaporation: the break-up of dispersed phase droplets due to the shear force, the precipitation of the dispersed polymer during solvent attrition and the coalescence or partial coalescence of the droplets. It is noted that the relative time scale of each process controls the resulting particle size and shape. For example, irregular particle structures may be produced if the polymer precipitates before the dispersed phase has been adequately broken up into droplets or if droplets are allowed to coalesce before the polymer precipitates. Thus particle size can be manipulated through control of processing parameters such as temperature, relative volumes of the dispersed and continuous phase, speed of mixing of the two phases and the addition of surfactants.

In the preparation of lignin microparticles, temperature has already been shown to influence particle size, possibly due to a faster rate of lignin precipitation at higher temperatures. It was also shown that incorporation of the surfactant sodium dodecyl sulfate (SDS) enabled stable sub-micron lignin particles to form. Since the dispersed and continuous phases are very miscible in this system, surfactants that lower the interfacial tension between the two phases to allow smaller droplets to form and thus smaller particles to precipitate are likely to be non-critical. This miscibility, in addition to rapid evaporation of the ethanol at high water phase temperatures such as 60°C, leads to fast precipitation of lignin. Hence it is likely most important to incorporate surfactants or stabilisers that can prevent coalescence or aggregation of precipitated lignin particles in suspension. Stabilisers are also often necessary to prevent aggregation during drying of the particles (Patel et al., 2010).

Stabilisers are generally added to the continuous phase and function either by adsorbing at the surface of newly formed droplets or particles, or by enhancing the viscosity of the continuous phase to limit droplet interaction (Freitas et al., 2005). Surfactants adsorb from the aqueous continuous phase at the hydrophobic droplet or particle surface; the hydrophobic group of the surfactant orients towards the hydrophobic surface and the polar head group orients towards the water (Holmberg, Jönsson, Kronberg, & Lindman, 2002). This reduces the surface tension between the surface and the surrounding water, but depending on the type of surfactant, can also prevent coalescence or aggregation by electrostatic and/or steric stabilisation. Thus surfactants that are water soluble and can provide electrostatic or steric stabilisation are required for the current system. Highly water soluble surfactants can be selected based on their hydrophile-lipophile balance or HLB number; values of the HLB greater than 7 indicate greater solubility in water than in oil (Walstra & van Vliet, 2008). In this chapter, small molecule, high-HLB surfactants containing polar head groups and hydrocarbon chain tails are evaluated on their ability to stabilise lignin microparticles. The surfactants of interest are SDS, cetyltrimethylammonium bromide (CTAB), Tween 20 and a sucrose ester.

Both SDS and CTAB are ionic surfactants. SDS has a negatively charged sulfonate head group and HLB number of 40 (Walstra & van Vliet, 2008). CTAB has a positively charged trimethylammonium head group and HLB number of 10 (Barut, Ari, & Öner, 2005). Hsieh, Korkut, Punckt and Aksay (2013) reported that adsorption of SDS onto functionalised graphene sheets introduces electrostatic repulsion between the sheets and prevents aggregation. Based on this information, we might expect SDS and CTAB to be effective in stabilising lignin particles by the electrostatic mechanism. On the other hand, Tween 20 and sucrose ester are non-ionic surfactants. The polar head group of Tween 20 consists of polymerised oxyethylene units (Holmberg et al., 2002), which are suggested to make the head group bulky and thus able to provide a steric barrier to flocculation or aggregation (Ariyaprakai, Limpachoti, & Pradipasena, 2013; Moore et al., 2003; Rastogi et al., 2008). With an HLB number of 16 (Sigma-Aldrich, 2014b), Tween 20 is a suitable surfactant to evaluate in this work. Finally, sucrose esters comprise sucrose as the hydrophilic head group and up to eight fatty acid tails per molecule. The variation in the number of fatty acid groups means that the HLB number can vary depending on the particular ester (Szűts & Szabó-Révész, 2012). High HLB value sucrose esters can be used to stabilise oil-in-water emulsions (Nelen & Cooper,

2004) and various HLB emulsifiers have been used as surfactants at 0.5% (w/v) to prepare discrete and spherical microparticles using a solvent evaporation method (Youan, Hussain, & Nguyen, 2003). It is suggested that sucrose esters decrease the surface tension of water (Szűts & Szabó-Révész, 2012). Thus sucrose ester P-1670, with HLB number of 16 (Mitsubishi-Kagaku Foods, 2002) may be useful for the preparation of lignin microparticles by precipitation in water. Furthermore, sucrose esters are non-toxic and widely used as additives in the food industry (Ariyaprakai et al., 2013; Szűts & Szabó-Révész, 2012).

Once stable particles have been produced, it may be possible to further manipulate particle size through controlled destabilisation of the particles in suspension. For example, electrostatically-stabilised particles may flocculate upon the addition of salt or adjustment of suspension pH via screening of repulsions between surfactant head groups (Holmberg et al., 2002). The aggregation or flocculation of aqueous dispersions in this way can be explained by DLVO theory. This theory recognises that repulsive electrostatic double-layer forces stabilise dispersions, whereas attractive van der Waals forces promote aggregation. When little or no salt is present, particles are prevented from aggregating by the repulsive electrostatic energy barrier. However, as salt concentration increases, the repulsive energy barrier decreases, until the van der Waals attraction dominates, leading to aggregation (Butt, Graf, & Kappl, 2003).

pH is known to influence the electrostatic repulsion provided by SDS (Hirano, Tanaka, Urabe, & Kataura, 2013; Umlong & Ismail, 2007). It is also known that pH affects the solubility of lignin; lignin is soluble in water at high pH but insoluble at low pH (Wei et al., 2012). In this chapter, the effect of addition of the monovalent salt potassium chloride and divalent salt calcium chloride on the size of SDS-stabilised lignin microparticles is examined. It is known that salts containing divalent counterions weaken electrostatic repulsions more effectively than monovalent counterions (Butt et al., 2003). The divalent calcium chloride may also form bridges between lignin particles through interaction with two sulfonate head groups, which may allow particles of different size to be obtained. Changes in pH of the system both before and after the precipitation of lignin particles are then studied.

Finally, adjustment of some of the other parameters during the anti-solvent precipitation process is also investigated. Pace (as cited in Bennion & Daggett, 2003) states that the

organic compound urea is commonly used to denature proteins to evaluate protein stability and unfolding. Although the exact mechanism by which urea is able to achieve this is unknown, it is recognised that urea somehow disrupts the hydrophobic attractions that keep proteins in the folded conformation (Bennion & Daggett, 2003; Shpiruk & Khajehpour, 2013; Zangi, Zhou, & Berne, 2009). Hence the presence of urea during microparticle formation is investigated to determine whether hydrophobic interactions play any role in particle agglomeration and whether urea could be a useful agent for controlling particle size. Addressing the possibility that mixing speed during anti-solvent precipitation could influence particle size, ultrasonication as a substitute for simple magnetic stirring is then examined.

6.2. Materials and methods

6.2.1. Materials

Potassium chloride (KCl) (LabServ Pronalys, Biolab (Aust) Ltd, Victoria, Australia), Calcium chloride ($CaCl_{2)}$ (LabServ Pronalys, Biolab (Aust) Ltd, Victoria, Australia), urea (LabServ Pronalys, Thermo Fisher Scientific New Zealand Ltd, North Shore City, New Zealand), Sucrose ester (P-1670, Ryoto, Mitsubishi Kagaku Foods Corporation, Tokyo, Japan), Tween 20 (BDH, VWR International Ltd.) and cetyltrimethylammonium bromide (CTAB) (Ajax Chemicals Ltd, Sydney, Australia) were used as received.

6.2.2. Methods

6.2.2.1. Lignin extraction

Lignin was extracted from shrub willow using the method outlined in Chapter Five.

6.2.2.2. Microparticle preparation

Microparticles were prepared using the solvent evaporation method outlined in Chapter Five. The temperature of the water that the lignin extraction liquor was added to was always 60°C unless otherwise stated. Where possible, the preparation of particles under different conditions was replicated. However, only representative data is included in this chapter, unless stated otherwise.

6.2.2.3. Effect of various emulsifiers

To determine the effect of emulsifiers other than SDS on lignin microparticle size, Cetyltrimethylammonium bromide (CTAB), Tween 20 and Sucrose Ester were added to the water prior to the addition of lignin extraction liquor. The SDS, CTAB, Tween 20 and Sucrose Ester concentrations evaluated were 0.03-1.5% (w/v), 0.03-1.5% (w/v), 0.5-25% (w/v or v/v) and 0.05-2.5% (w/v), respectively. Addition of Tween 20 to the extraction liquor rather than to the water phase was also investigated. In this case the concentration of Tween 20 was still calculated in terms of concentration of the aqueous phase, but the Tween 20 was briefly pre-mixed with the extraction liquor before microparticles were formed using the usual method.

6.2.2.4. Destabilisation of particles as a method of controlling particle size

6.2.2.4.1. Effect of potassium chloride

Initially, solutions of potassium chloride (KCl) at concentrations of 0.01, 0.05 and 0.1 M were prepared using deionised water. These solutions were then used as the water phase during microparticle formation. SDS was added to the solutions to give an SDS concentration of either 0.25 or 0.5% (w/v). The aqueous phase was then heated to 60°C before the addition of lignin extraction liquor to prepare microparticles.

In later experiments, the effect of the addition of KCl after lignin microparticle formation was evaluated. KCl was added to suspensions after the 22 hour stirring period involved in microparticle formation. KCl was added in amounts that lead to concentrations of KCl in the final suspension of 0.01, 0.05 and 0.1 M.

6.2.2.4.2. Effect of $CaCl_2$

$CaCl_2$ at concentrations of 5.1, 10 and 20 mM was added to microparticle suspensions after the 22 hour stirring period involved in microparticle formation.

6.2.2.4.3. Effect of pH

Changes in pH were made both before and after microparticle formation. Aqueous phases with unbuffered pH of 3 and 10 were used to form microparticles. The pH of water was adjusted to pH 3 and 10 by the addition of HCl and NaOH. SDS was added to the aqueous phase at a concentration of 0.5% (w/v) before the addition of the lignin extraction liquor.

For changes made after microparticle formation, the pH of the microparticle suspension was adjusted to 1.5, 2, 7 or 10 after the 22 hour stirring period, via the addition of HCl and NaOH. The suspensions were mixed by magnetic stirring (500 rpm) for 30 minutes after the adjustment of pH.

6.2.2.5. Effect of urea on particle size

Aqueous phases containing urea were used to prepare microparticles. Urea concentrations of 6.6 and 8 M were used, along with aqueous phase temperatures of 4, 20 and 60°C.

6.2.2.6. Effect of ultrasonic mixing on particle size

Microparticles were also prepared using an ultrasonic mixing set-up in place of magnetic stirring. A system for ultrasonic mixing using a flow-cell was specially designed for this work. The design featured an ultrasonic transducer (UIP1000hd, Hielscher Ultrasonics GmbH, Teltow, Germany), 22 mm diameter sonotrode (BS2d22, Hielscher Ultrasonics), mono-pump, peristaltic pump and receiver tank. Extraction liquor was pumped through a 2 mm inside diameter tube directly into the flow-cell (Hielscher Ultrasonics). Water was pumped through the flow-cell simultaneously. This set-up enabled extraction liquor to be mixed with water by the sonotrode immediately upon entry into the flow-cell. The mixed liquids continuously recirculated between the flow-cell and receiver tank until three minutes after all liquor had been pumped in. The suspension was then transferred to a metal beaker and mixed under magnetic stirring (500 rpm) for 21 hours. Further details of the ultrasonic mixing set-up and method are given in Appendix F.

6.2.2.7. Particle characterisation

6.2.2.7.1. Static light scattering

Particle size analysis was carried out on the lignin suspensions using static light scattering (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). A refractive index of 1.6 (Donaldson, 1985) and absorption of 0.01 (based on analysis of fit of the light scattering data) were used by the instrument to calculate the particle size distributions.

The ultrasonic probe attachment of the Mastersizer 2000 was used to apply sonication to some samples prior to light scattering measurement. The maximum ultrasonic probe tip displacement of 20 μm was used, for periods of up to two minutes.

6.2.2.7.2. Laser Doppler electrophoresis

Determination of zeta potential was carried out for lignin particle suspensions using Laser Doppler Electrophoresis (Malvern Zetasizer Nano ZS, Malvern Instruments Ltd., UK). Dispersants used to dilute the suspensions were prepared using deionised water with salt added to give the same salt concentration as the suspension. Dispersant pH was adjusted to be the same as suspension pH in each case using dropwise addition of ≤ 1 M HCl and NaOH as required. Suspensions containing only SDS or CTAB-stabilised lignin particles were diluted to 0.1. Samples containing 0.1 M KCl did not require dilution since the larger lignin particles present in the sample naturally sedimented, leaving a more dilute phase that could be sampled from. This was also the case for suspension samples in which 0.05 M KCl had been added after particle formation. For all other suspension samples with added KCl, dilutions of 0.2 were prepared. All measurements were carried out at 20°C, and the results reported are the mean of two readings.

6.2.2.7.3. Scanning electron microscopy

Dried, crushed lignin samples were further dried under vacuum at room temperature for 24 hours. The dried samples were then sputter coated with a thin layer of gold and their morphology was characterised using scanning electron microscopy (SEM) (FEI Quanta 200, Hillsboro, Oregon). Images were taken at magnifications of 400, 2500, 5000, 10000 and 20000X, in two different areas for each sample.

6.2.2.7.4. Cryo-SEM

Liquid samples of the lignin extraction liquor, the microparticle suspension containing 0.5% (w/v) SDS and the microparticle suspension containing 1% (w/v) SDS were analysed by cryo-SEM. This technique allows liquid samples to be fractured cryogenically to expose internal microstructure and the dispersion of components in the liquid (Gatan, 2007).

The samples were frozen in a nitrogen slush. The frozen samples were then fractured under liquid nitrogen in the cryo unit (Gatan Alto 2500, Gatan, Inc., CA). Once the

temperature in the unit dropped below -120°C, the samples were sputter coated with gold under vacuum. Then once the temperature dropped below -140°C, samples were transferred to the gas-cooled module of the SEM stage where they were examined microscopically at magnifications of 71-20,000X. Three series of images (i.e. three images of different areas of the sample at each magnification) were obtained for the lignin extraction liquor and 1% (w/v) SDS suspension samples. Only one series of images was obtained for the 0.5% (w/v) SDS suspension sample. The SEM unit used was a Philips XL30S FEG (Netherlands).

6.3. Results

6.3.1. Effect of SDS concentration on particle size

Based on earlier indications that SDS is an effective stabiliser of sub-micron lignin microparticles, the effect of SDS concentration on lignin particle size was determined. Figure 29 shows lignin particle size as a function of SDS concentration for suspensions prepared at 60°C. SDS concentration in the aqueous phase ranged from 0.03 to 1.5% (w/v), corresponding to SDS-to-lignin mass ratios in the final suspensions of 0.04 to 1.03 (Table 23).

Figure 29 shows that in the absence of surfactant, lignin particles are in the 10-100 μm size range. For an SDS concentration of 0.03% (w/v) a bimodal particle size distribution exists, in which the majority of particles centre around 10 μm with a smaller modality centring around 0.1-0.2 μm. Narrow particle size distributions in the range 0.1-0.2 μm are exhibited at SDS concentrations of 0.0625-0.75% (w/v). Finally, for SDS concentrations of 1 and 1.5% (w/v), although there is a narrow distribution of particles in the range 0.1-0.2 μm, there is also a small yet broad shoulder of particles in the 1-100 μm size range.

Figure 30 shows SEM images of particles prepared using 0.0625 and 1% (w/v) SDS, revealing that although 0.0625% SDS was sufficient to produce stable microparticles in suspension, some fusion of the particles appears to have occurred during the microparticle recovery or drying process. This may indicate insufficient coverage of particulates at this low concentration, and that a higher concentration of SDS is required to produce particles that are stable both in suspension and during recovery.

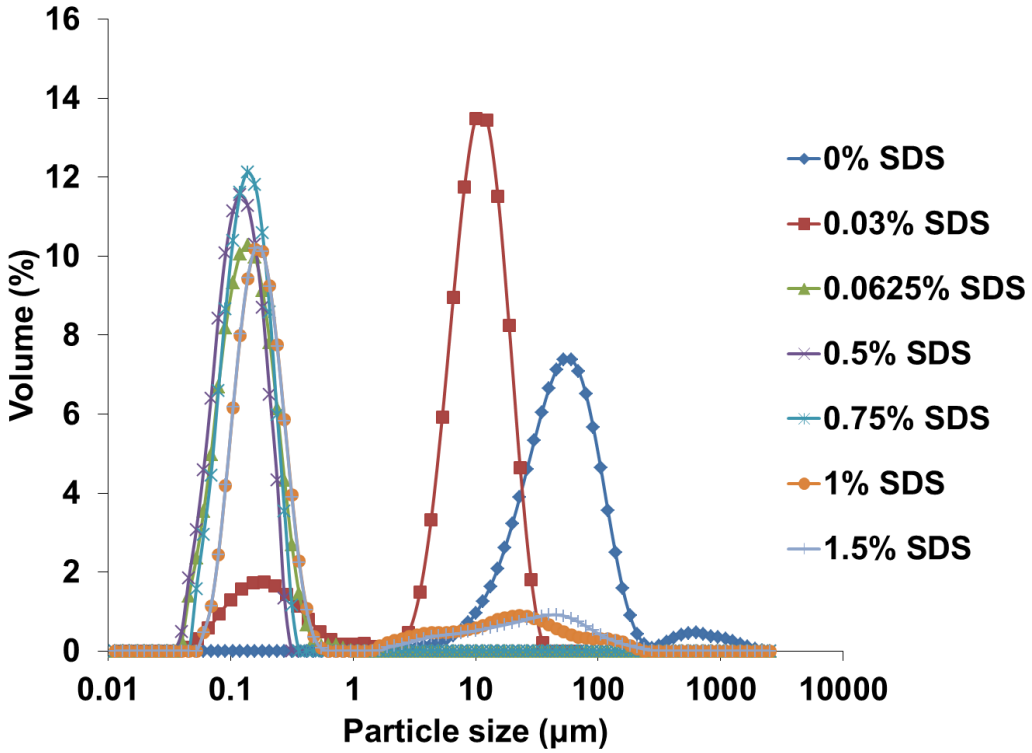


Figure 29. Particle size distributions of microparticle suspensions prepared by shearing 30 mL of lignin extraction liquor into 75 mL of water (60°C) containing SDS concentrations of 0-1.5% (w/v).

Table 23. Mass ratio of SDS to precipitated lignin at various SDS concentrations

SDS concentration (% w/v)	SDS-to-lignin ratio
0.03	0.02
0.0625	0.04
0.125	0.09
0.5	0.34
0.75	0.52
1.0	0.69
1.5	1.03

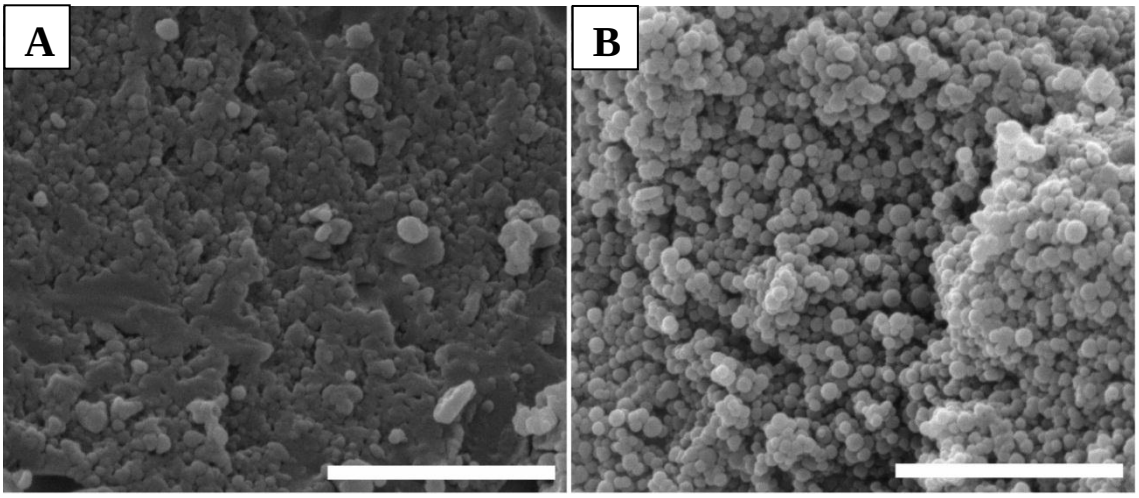


Figure 30. SEM images of microparticles prepared using an aqueous phase temperature of 60°C with (A) 0.0625% (w/v) SDS and (B) 1% (w/v) SDS (bar scale = 3 μm).

Figure 30 also shows that for an SDS concentration of 1% (w/v), spherical, sub-micron particles were still produced. This indicates that the broad peak of particles in the 1-100 μm size range must be due to aggregation of primary (0.1-0.2 μm) particles, rather than the formation of larger particles.

To further investigate the strength of the interactions responsible for particle-particle aggregation during precipitation in the presence of 1% (w/v) SDS, sonication was applied. Figure 31 shows particle size distributions for the suspensions prepared with the application of increasing periods of sonication prior to particle size measurement.

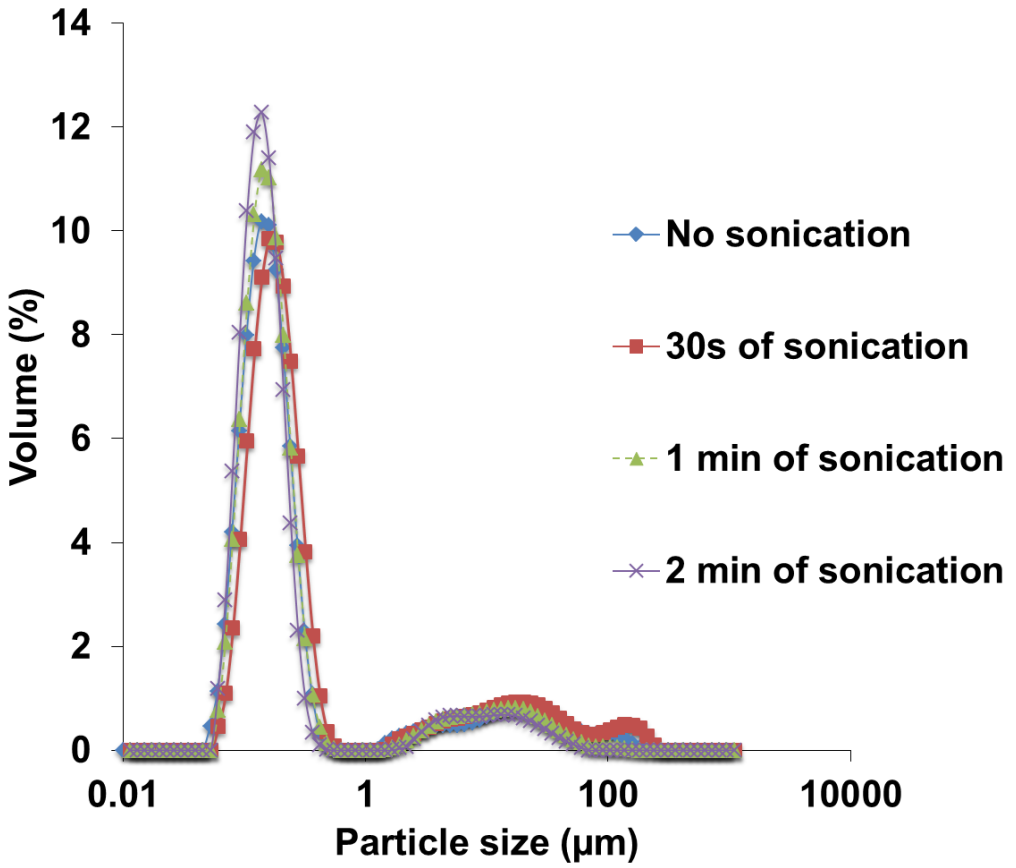


Figure 31. Particle size distributions of microparticle suspensions prepared by shearing 30 mL of extraction liquor into 75 mL of water (60°C) containing an SDS concentration of 1% (w/v) that were subsequently treated with different periods of sonication time before laser light scattering measurements were made.

Figure 31 reveals that an increase in sonication time did not remove the upper modal distribution of particles between 1-100 µm. This observation suggests that the larger size of the particles in this peak is due to strong aggregation of primary particles. In support of this, Figure 32 shows a cryo-SEM image of a suspension of microparticles prepared using 1% SDS. The particles appear to be agglomerated or aggregated together and perhaps even as though they are glued together by a film-like structure. Comparatively, Figure 33 shows lignin particles in a suspension prepared using 0.5% SDS (w/v), revealing that at this lower SDS concentration, the particle size is similar yet the particles are less agglomerated.

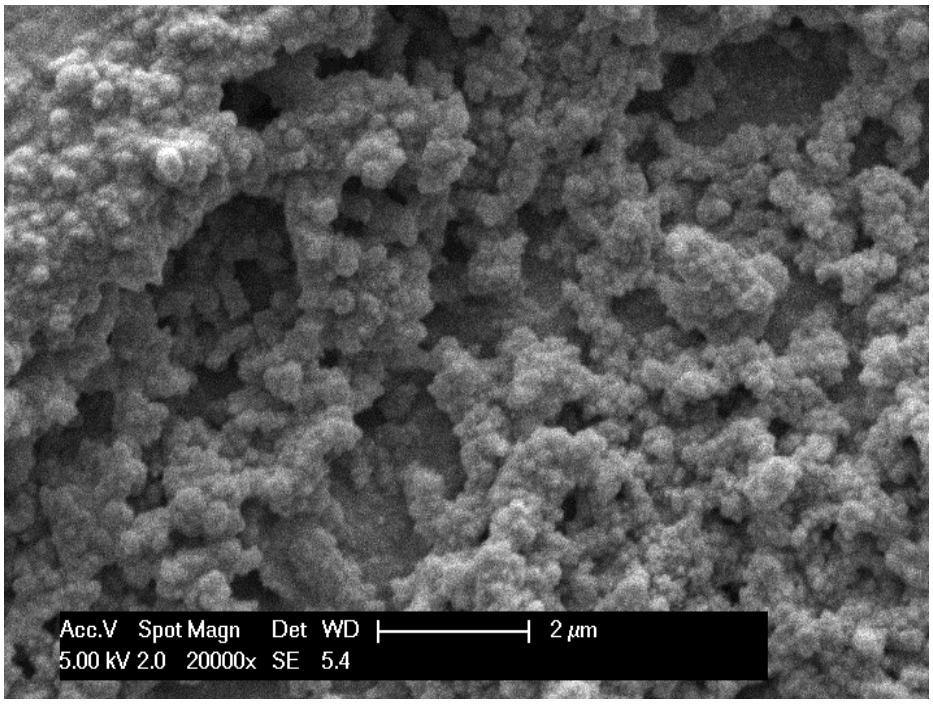


Figure 32. Cryo-SEM image of liquid suspension of lignin microparticles prepared using 1% (w/v) SDS and water temperature of 60°C.

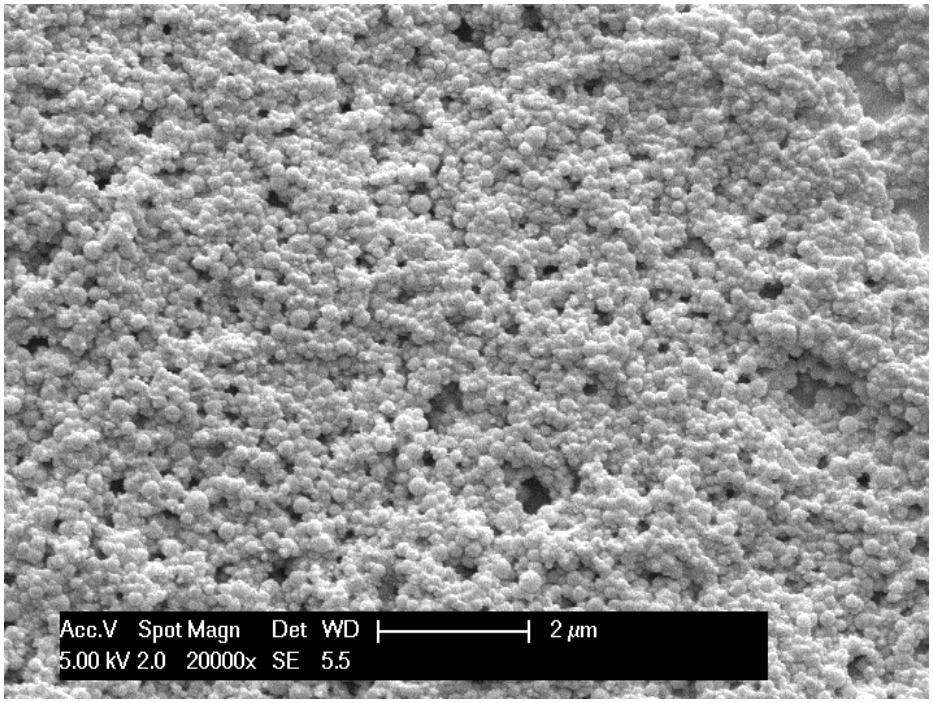


Figure 33. Cryo-SEM of liquid suspension of lignin microparticles prepared using 0.5% (w/v) SDS and water temperature of 60°C.

In the absence of added surfactants, particle size was shown to be strongly influenced by temperature. To determine whether this observation was consistent in the presence of surfactants, Figure 34 provides the size distributions of particles prepared using 0.125, 0.25 and 0.5% SDS with a water phase temperature of 4°C.

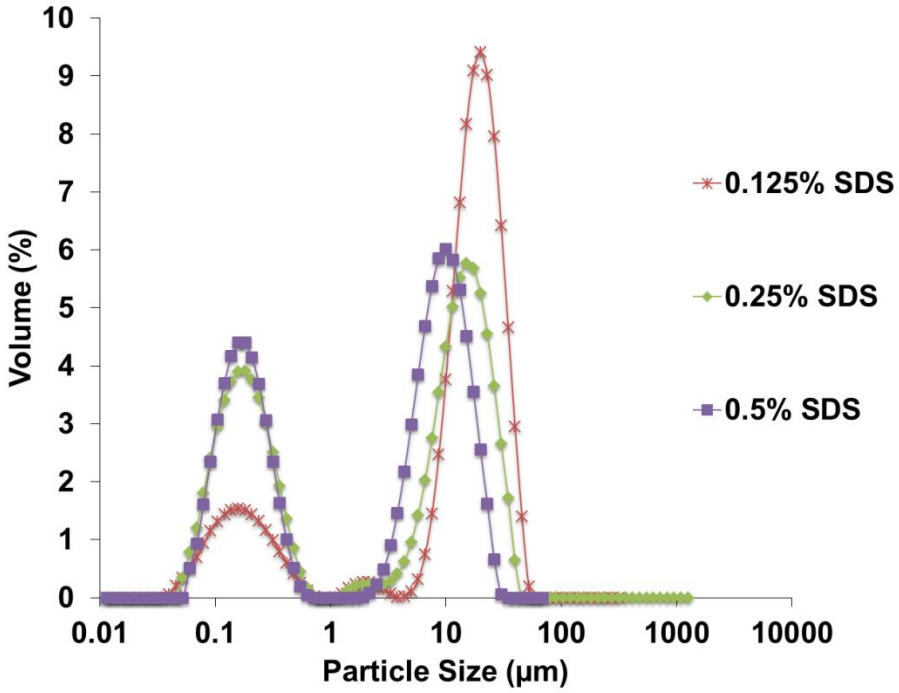


Figure 34. Particle size distributions of particle suspensions prepared by shearing 30 mL of extraction liquor into 75 mL of water (4°C) containing SDS concentrations of 0.125, 0.25 and 0.5% SDS (w/v).

Figure 34 shows that for the lower SDS concentration of 0.125%, there is a small volume of particles centring around 0.1-0.2 µm, a smaller volume of particles centring around 1-2 µm and the majority of particles falling within approximately 10-20 µm. For the particles prepared using 0.25 and 0.5% SDS, there is a larger volume of particles in the sub-micron size range and a smaller volume in the 10-20 µm range. It seems that the size of particles or particle aggregates changes between the sub-micron and ≥ 10 µm size ranges rather than existing at sizes in between. This may indicate that generation of particles of sizes in the 1-10 µm size range may be difficult. Findings are also in marked contrast to samples prepared at 60°C (Figure 29) using equivalent concentrations of

SDS, where the upper modal distribution was seen to be absent across that concentration range.

6.3.2. Effect of CTAB, Tween 20 and sucrose ester on lignin particle size

Considering the success of SDS in stabilising sub-micron lignin particles, the ability of other emulsifiers was of interest. Hence CTAB, Tween 20 and sucrose ester were trialled. Figure 35 shows particle size distributions for microparticle suspensions prepared using CTAB concentrations in the water of 0-1.5% (w/v) and water temperature of 60°C.

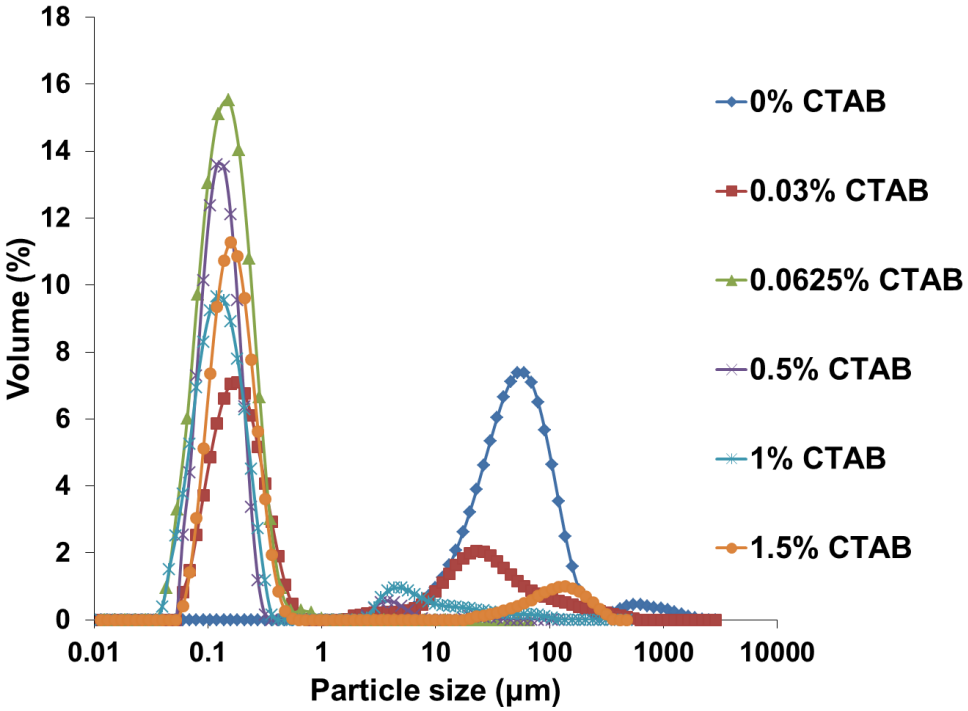


Figure 35. Particle size distributions of microparticle suspensions prepared by shearing 30 mL of lignin extraction liquor into 75 mL water (60°C) containing CTAB concentrations of 0-1.5% (w/v).

Figure 35 shows that for all concentrations of CTAB in the range 0.03-1.5% (w/v) a narrow peak of particles near 0.1 µm was observed. However, a smaller peak of particles in the 10-1000 µm size range is evident for a CTAB concentration of 0.03% (w/v). This may indicate that 0.03% CTAB is insufficient to stabilise all surfaces of small lignin particles, leading to some larger particles. Broad peaks of particles between

1-1000 μm are also evident for particles prepared using higher CTAB concentrations of 1 and 1.5% (w/v), similar to observations for SDS concentrations greater than 1% (w/v).

To compare the sizes of particles produced using SDS or CTAB as surfactant, Table 24 presents the D[4,3] and D[3,2] values for particles prepared using various concentrations of these surfactants.

Table 24. D[4,3] and D[3,2] values of lignin particles in suspensions stabilised by various concentrations of SDS and CTAB

Particles prepared with SDS			Particles prepared with CTAB		
Concentration (% w/v)	**D[4,3] (μm)**	**D[3,2] (μm)**	**Concentration (% w/v)**	**D[4,3] (μm)**	**D[3,2] (μm)**
0.03	9.1	0.9	0.03	15.4	0.2
0.0625	0.1	0.1	0.0625	0.1	0.1
0.5	0.1	0.1	0.10	0.6	0.1
0.75	0.1	0.1	0.5	0.4	0.1
1	5.2	0.2	1	1.7	0.1
1.5	6.6	0.2	1.5	12.7	0.2

From Table 24, it can be seen that apart from 0.03% (w/v) SDS, particles stabilised by all concentrations of SDS and CTAB have D[3,2] values in the range 0.1-0.2 μm. Meanwhile, D[4,3] values are between 0.1-0.2 μm for particles stabilised by 0.0625-0.75% (w/v) SDS, and between 0.2-0.4 μm for particles stabilised by 0.0625-0.5% (w/v) CTAB. The slightly lower D[4,3] values for the particles stabilised by SDS indicate that although both surfactants can stabilise sub-micron lignin particles, SDS is slightly more effective than CTAB.

To determine whether the effectiveness of the surface charge at various surfactant concentrations influenced particle size, the zeta potential of SDS and CTAB-stabilised particle suspensions was measured. Figure 36 shows that the zeta potential of lignin suspensions becomes more negative with increasing SDS concentration. Figure 37 shows that the zeta potential of lignin suspensions becomes more positive with

increasing CTAB concentration, but the zeta potential seems to plateau once a concentration of 0.1% (w/v) CTAB is reached.

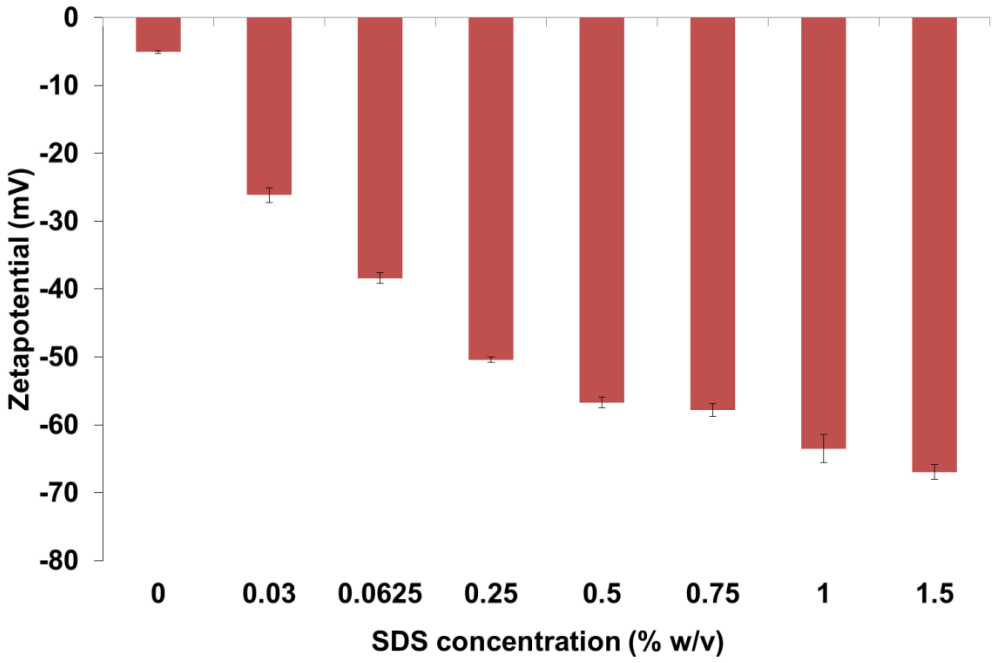


Figure 36. Zeta potential of particles prepared using SDS concentrations of 0-1.5% (w/v) (error bars based on standard deviation).

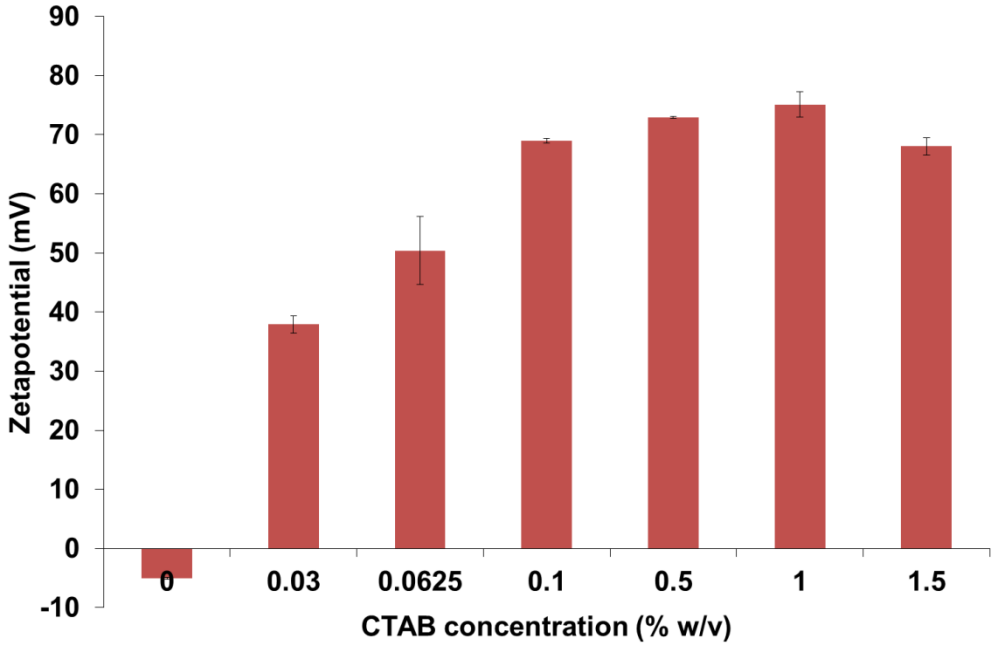


Figure 37. Zeta potential of particles prepared using CTAB concentrations of 0-1.5% (w/v) (error bars based on standard deviation).

Figure 38 shows particle size distributions for microparticle suspensions prepared using Tween 20 concentrations in the water of 10-25% (w/v) and water temperature of 60°C.

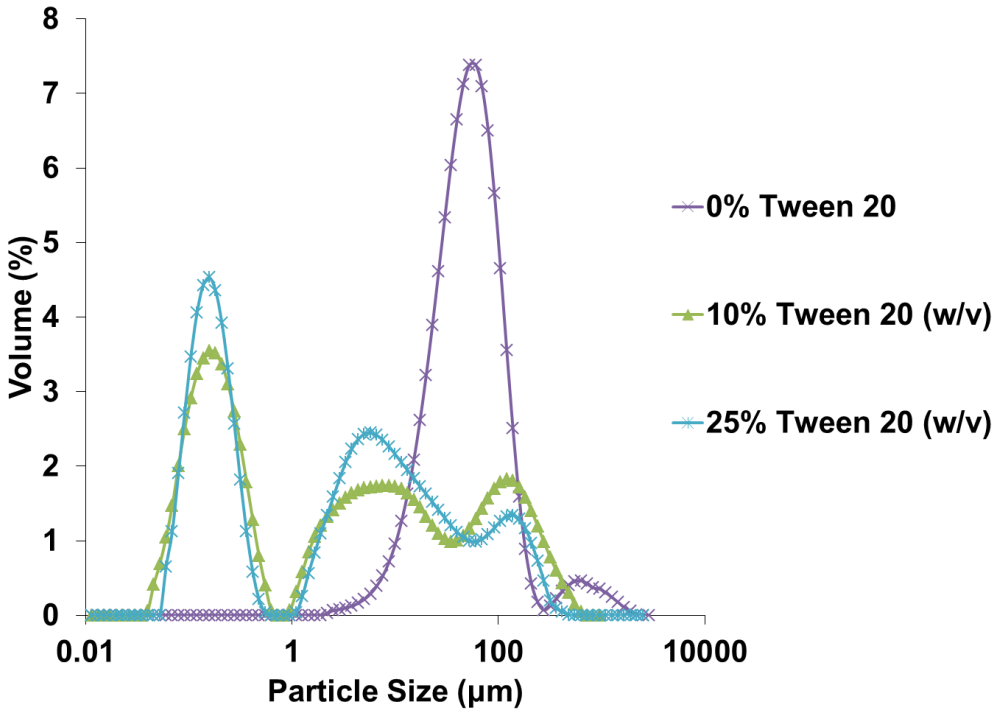


Figure 38. Particle size distributions of microparticle suspensions prepared by shearing 30 mL of lignin extraction liquor into 75 mL of water (60°C) containing 10-25% Tween 20.

In Figure 38, it can be seen that over the range of Tween 20 concentrations, the resulting lignin particles have a wide size distribution. Particles are present in the sub-micron range, but also in the 1-100 µm range. As the concentration of Tween 20 increases, the volume of particles in the sub-micron range increases. Concentrations as low as 0.5% (w/v) Tween 20 were also evaluated. However, at lower concentrations, large clumps of lignin were formed, leaving very little lignin suspended in the water for size measurement. The addition of Tween 20 to the extraction liquor rather than to the water before microparticle formation was also investigated yet did not provide a reduction in particle size.

Figure 39 shows particle size distributions for microparticle suspensions prepared using sucrose ester at concentrations in the water of 0.05-2.5% (w/v) and water temperature of 60°C.

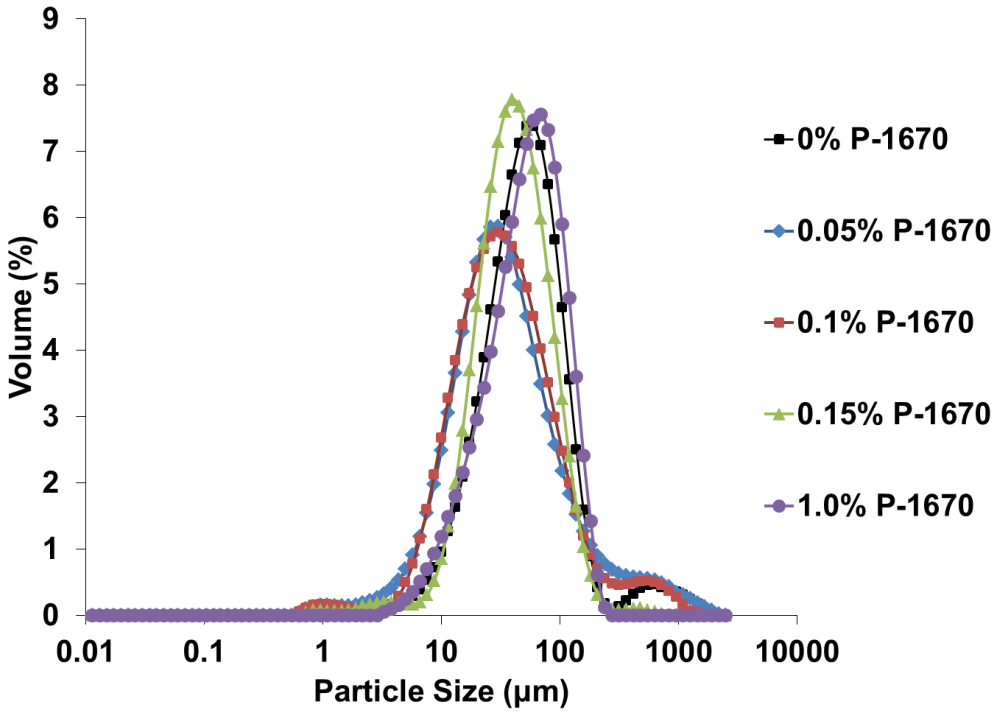


Figure 39. Particle size distributions of microparticle suspensions prepared by shearing 30 mL of lignin extraction liquor into 75 mL water (60°C) containing sucrose ester at concentrations of 0-2.5% (w/v).

Figure 39 reveals that the addition of sucrose ester at levels of 0.05-2.5% made very little change to lignin particle size compared to if no surfactant was used. This observation indicates that sucrose ester is not an effective stabiliser of lignin particles.

Figure 40 compares size distributions of lignin microparticles prepared using SDS, CTAB, Tween 20 or sucrose ester. Although a range of concentrations for each emulsifier was investigated, only the concentration that produced the smallest lignin particle size is shown.

Figure 40 highlights that the charged emulsifiers are able to stabilise smaller lignin particles than non-ionic emulsifiers. SDS and CTAB, at concentrations of 0.5% (w/v) resulted in particles with narrow size distributions and size < 1 µm. Non-ionic Tween 20 produced particles with a bimodal size distribution; a narrow distribution of particles <

1 μm and a broad peak of particles centring around 10 μm. However, this was only achieved using a high concentration of 19% (v/v) Tween 20. Incorporation of sucrose ester produced lignin particles of similar size to those produced using no emulsifier at all, indicating that sucrose esters do not appear to contribute to the stabilisation of small lignin particles.

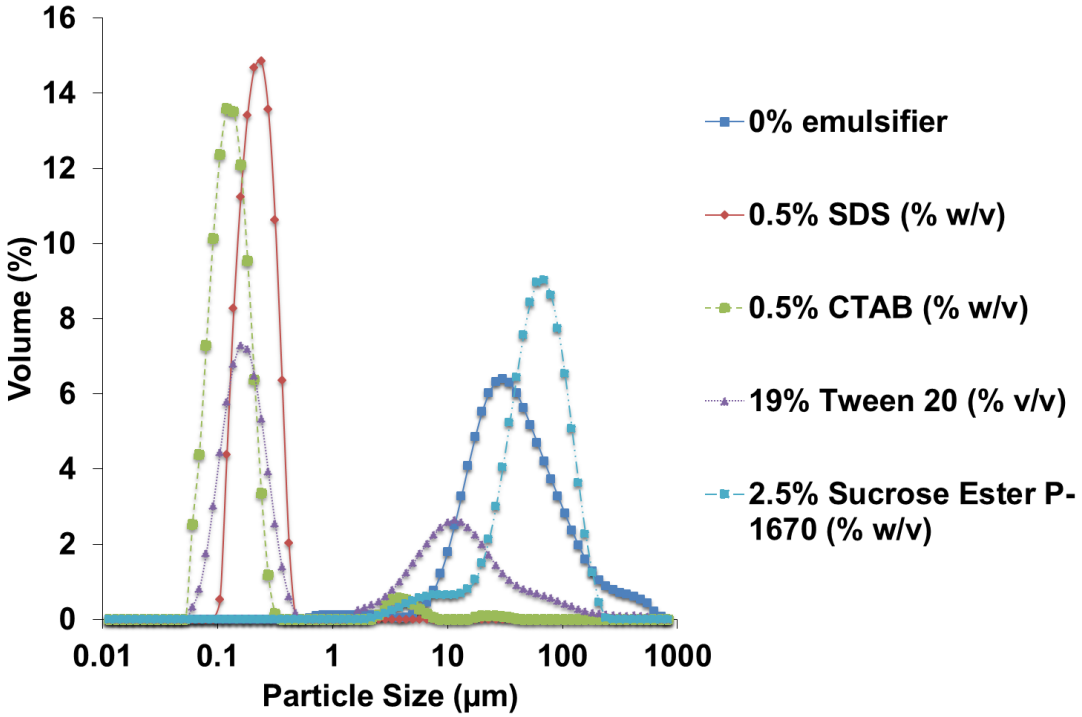


Figure 40. Size distributions of lignin microparticles prepared using various emulsifiers.

To further support the difference in particles stabilised by the four surfactants, Figure 41 compares SEM images of microparticles prepared using SDS, CTAB, Tween 20 and sucrose ester. Figure 41 shows that spherical, monodisperse, sub-micron-sized particles were produced when either SDS or CTAB were used as the emulsifier. The more clumped appearance of the particles stabilised by CTAB (Figure 41B) may indicate that these particles are more aggregated than those stabilised by SDS. This may help to explain the small distribution of CTAB-stabilised particles present at ~3-6 μm in Figure 40. On the other hand, when Tween 20 or sucrose ester were used as the emulsifying agent, monolithic masses of particles similar to those produced with no emulsifier were obtained.

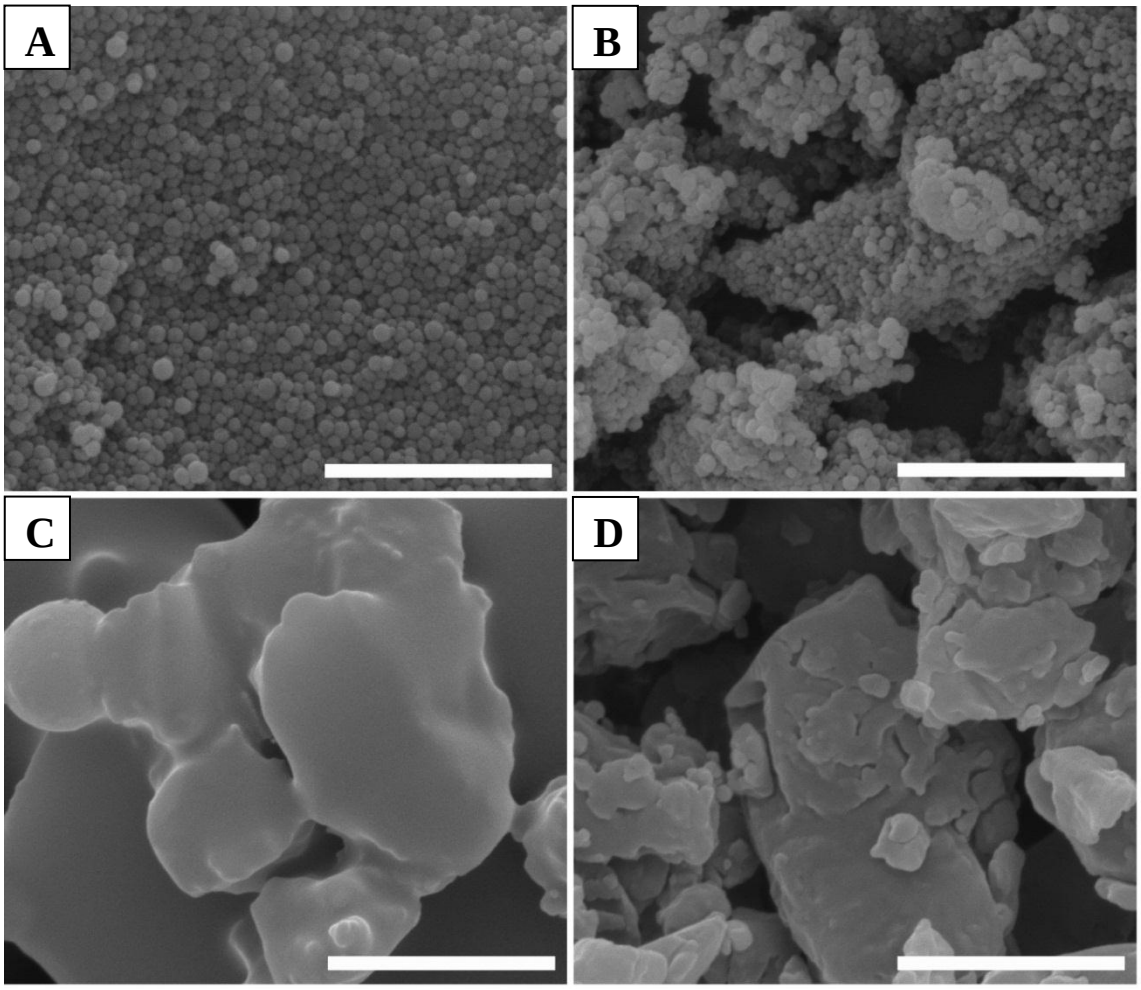


Figure 41. SEM images of dried, crushed lignin microparticles prepared by shearing 30 mL of lignin extraction liquor into 75 mL water containing (A) 0.5% (w/v) SDS, (B) 0.5% (w/v) CTAB, (C) 10% (w/v) Tween 20 and (D) 2.5% (w/v) sucrose ester at 60°C (bar scale = 3 µm).

It is important to note that the microstructure shown in Figure 41C is for particles prepared using only 10% Tween 20 rather than the 19% shown in Figure 40. Smaller particles may have been observed for the 19% Tween 20 sample. However, the key point is while only 0.5% SDS and CTAB are required for sub-micron lignin particles to be produced, far larger concentrations of Tween 20 were required to produce particles of partially the same size or greater. Therefore, only SDS and CTAB were further investigated in this work.

6.3.3. Controlled destabilisation of lignin microparticles as a tool for particle size manipulation

6.3.3.1. Destabilisation with KCl

The ability of added KCl to screen electrostatic repulsions between SDS-stabilised particles and induce particle aggregation was evaluated. Of particular interest was the production of particles with narrow size distributions in the 1-10 μm range since particles of this size had not been obtained using other methods.

The addition of salt to either the aqueous phase before SDS and extraction liquor addition or to particle suspensions after the 22 hour stirring period of particle formation was investigated. Figure 42 shows the size distributions of particles produced with both KCl and SDS present in the aqueous phase before microparticle formation.

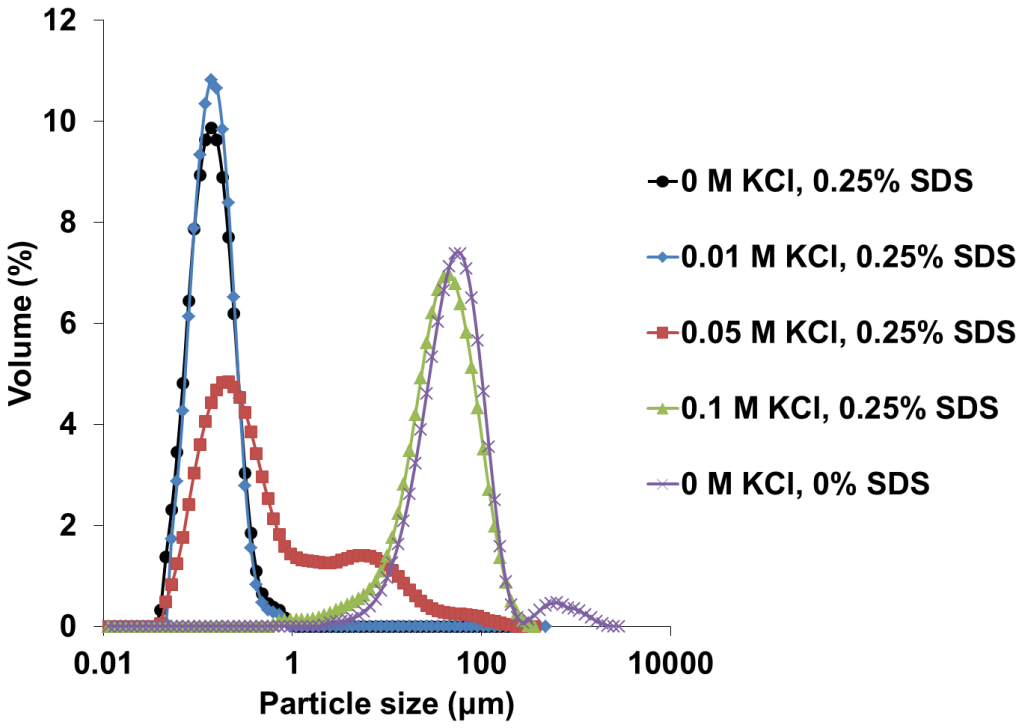


Figure 42. Size distributions of lignin microparticles prepared using a water phase containing both 0.25% (w/v) SDS and 0-0.1 M KCl at 60°C.

Figure 42 shows that the concentration of added salt influences lignin particle size. Particles produced in the presence of 0.1 M KCl exhibit a size distribution very similar

to that of particles produced with no SDS present as stabiliser. At the other extreme, particles produced in the presence of only 0.01 M KCl exhibit a size distribution very similar to that of particles produced with no added KCl, indicating that 0.01 M KCl is insufficient to cause aggregation of SDS-stabilised lignin particles.

The presence of 0.05 M KCl in the aqueous phase prior to microparticle formation produced particles with a wide size distribution spanning ~0.1-100 μm. The majority of particles fall in the 0.1-1 μm range but there is also a significant shoulder of particles in the 1-100 μm range. This indicates that the addition of KCl at concentrations between 0.01 and 0.1 M can produce lignin particles or aggregates with sizes in the 1-10 μm range. However, Figure 42 shows that these particles are not narrowly distributed and thus that destabilisation with 0.05 M KCl is not an efficient method for the production of 1-10 μm particles. Earlier trials involving a higher SDS concentration of 0.5% (w/v) and another intermediate KCl concentration of 0.06 M KCl generated particles with size between those produced using 0.05 and 0.1 M KCl (Figure 43). However, these particles still centred around ~10 μm, rather than around 1 μm or between 1-10 μm. Therefore, it seems that a transition from predominantly sub-micron to ~10 μm sized particles occurs quite suddenly between 0.05 and 0.06 M KCl and thus it may be difficult to gain further control of lignin particle size at an intermediate KCl concentration.

Figure 44 shows the microstructure of lignin particles formed in the presence of 0.5% (w/v) SDS and 0.05, 0.06 or 0.1 M KCl. As the concentration of KCl increases the particles become larger, until at 0.1 M they are monolithic, as observed for the particles prepared using 0% SDS at an aqueous phase temperature of 60°C.

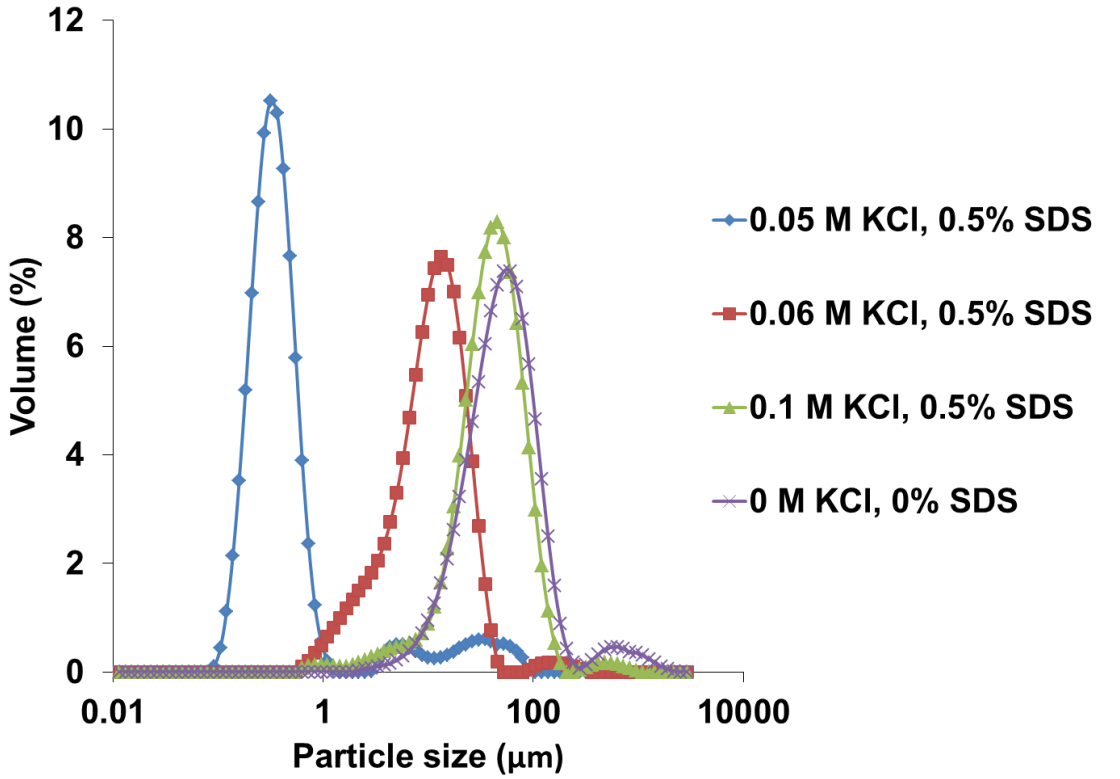


Figure 43. Size distributions of lignin microparticles prepared using a water phase containing both 0.5% (w/v) SDS and 0-0.1M KCl at 60°C.

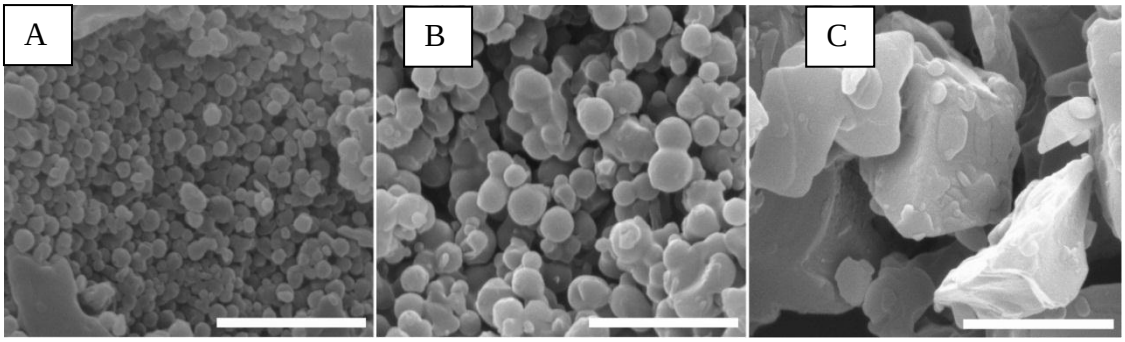


Figure 44. SEM images of lignin microparticles formed in the presence of 0.5% (w/v) SDS and (A) 0.05 M KCl, (B) 0.06 M KCl and (C) 0.1 M KCl, all at 60°C (bar scale = 3 µm).

Figure 45 shows the size distributions of particles produced in the presence of SDS, stirred for 22 hours and subsequently destabilised by the addition of 0.01-0.1 M KCl.

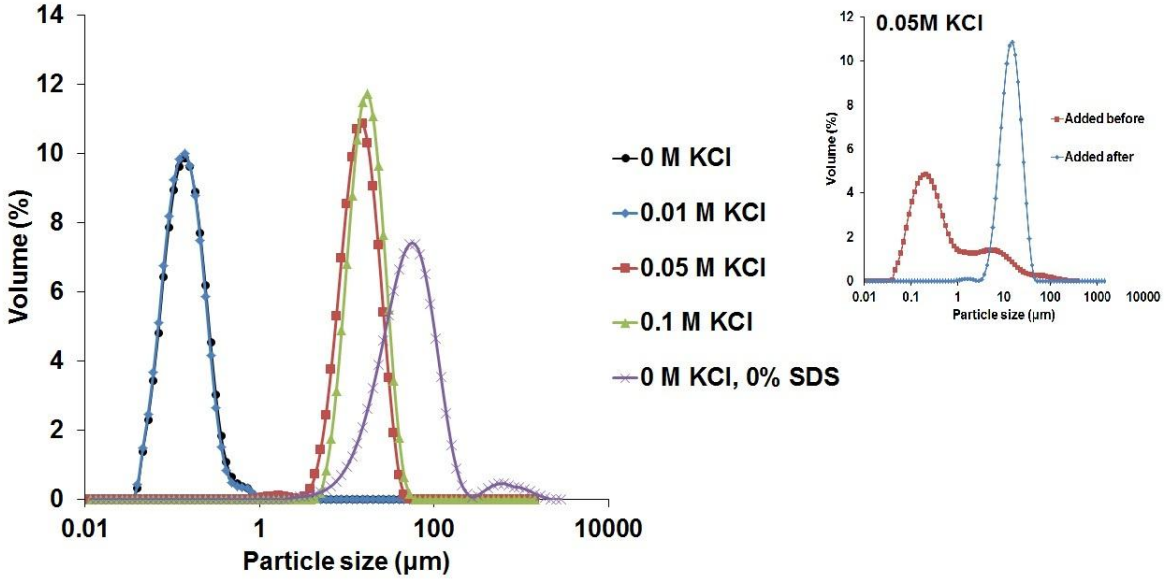


Figure 45. Size distributions of lignin microparticles prepared using a water phase at 60°C containing 0.25% (w/v) SDS, with 0-0.1 M KCl added after the 22-hour stirring period of the lignin particle formation process.

Similar to when KCl was present before microparticle formation, Figure 45 shows that particles with only 0.01 M KCl added have a similar size distribution to particles with no KCl added. Particles with 0.05 and 0.1 M added KCl exhibit very similar particle size and size distribution. This indicates that when added after particle formation, 0.05 M KCl is able to cause the same extent of charge screening as 0.1 M KCl. Comparatively, when KCl was present at 0.05 M before microparticle formation, particles more similar to those produced in the presence of 0.01 M KCl (i.e. a greater volume of sub-micron particles) were produced (Figure 45 inset). Furthermore, the addition of 0.1 M KCl after microparticle formation did not cause particles as large as those produced using no SDS to form. Therefore, the addition of 0.1 M KCl after particle formation did not cause as large an increase in particle size as the presence of 0.1 M KCl before particle formation. This suggests that the effect of KCl concentration on lignin particle size depends on whether the KCl is added before or after microparticle formation.

It is also interesting to note the existence of particles in two different size ranges. Particles fall within either the 0.1-1 μm size range or the >10 μm size range. Apart from

the particles prepared in the presence of 0.05 M KCl, this was also observed for particles prepared in the presence of KCl.

Figure 46 shows the microstructure of lignin particles that had KCl at concentrations of 0.05 and 0.1 M added to the microparticle suspensions after the 22 hour stirring period. The particles destabilised by KCl in this way generally exhibit smaller primary particles that are increasingly fused together with increasing KCl concentration, rather than larger individual particles.

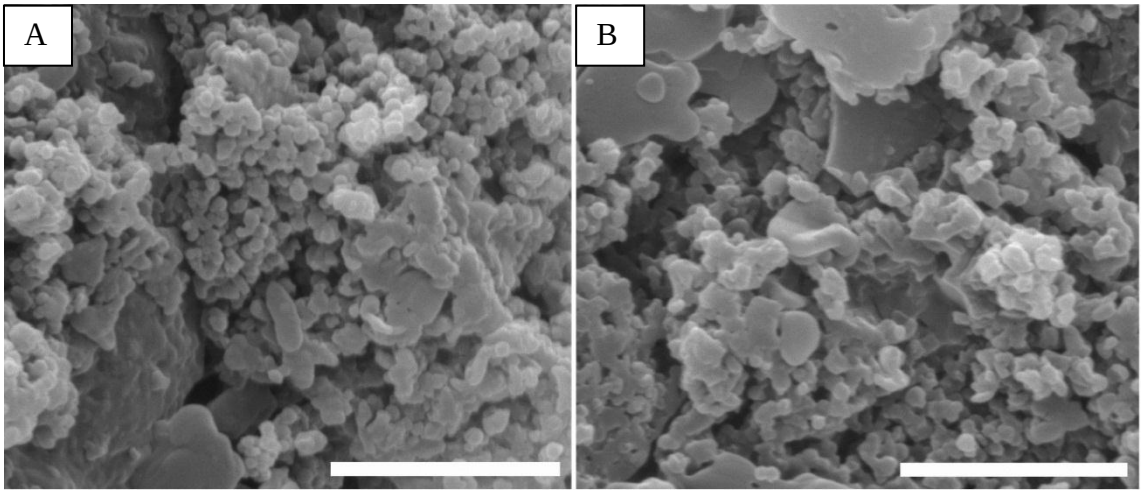


Figure 46. SEM images of lignin microparticles prepared using a water phase containing 0.5% (w/v) SDS at 60°C where KCl has been added after the 22 hour stirring process of microparticle formation at concentrations of (A) 0.05 M and (B) 0.1 M KCl (bar scale = 3 μm).

Finally, Figure 47 compares the zeta potential of particles prepared using different concentrations of KCl added either before or after microparticle formation. Figure 47 shows that irrespective of whether added before or after particle formation, particles prepared with 0.01 M KCl have the same zeta potential as particles with no KCl added. This was expected based on the lack of change in particle size at this salt concentration, as shown in Figure 42 and Figure 45.

Figure 47 also shows that the zeta potentials of particles prepared with 0.05 M KCl were less negative than those of the particles prepared with 0.01 M KCl. This is again consistent with the changes in particle size seen with increasing KCl concentration. For

a KCl concentration of 0.05 M, the zeta potentials of particles for which the salt had been added before particle formation were slightly less negative than those for which salt had been added after particle formation.

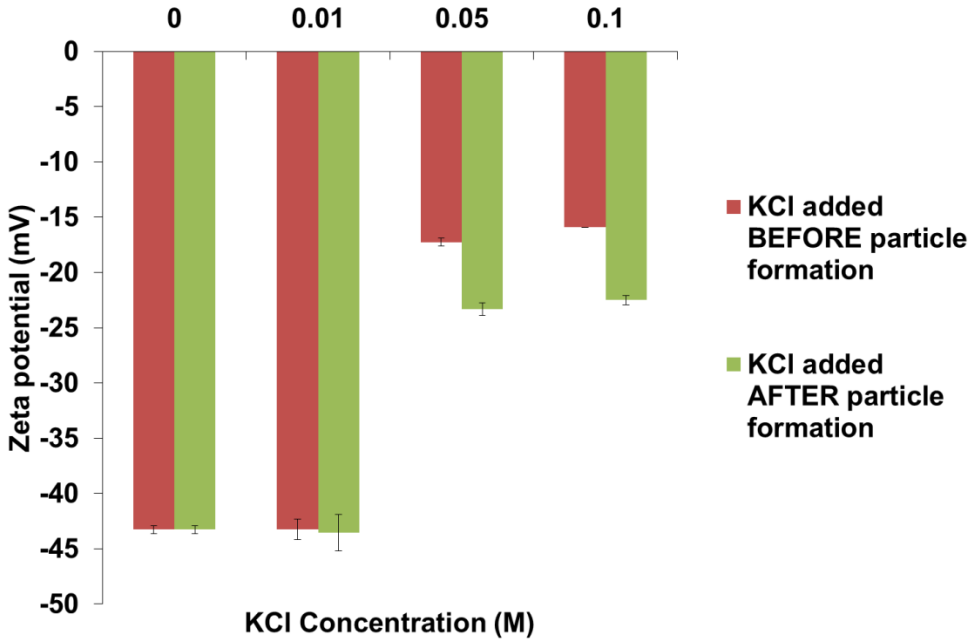


Figure 47. Bar graph of zeta potential values measured for particle suspensions containing 0.25% (w/v) SDS and 0-0.1M KCl, which was added either before or after microparticle formation.

There was little or no difference in zeta potential between particles prepared with 0.05 and 0.1 M KCl. The slightly less negative zeta potential for the particles prepared in the presence of 0.1 M KCl rather than with 0.1 M KCl added later was expected since they were clearly larger and more monolithic.

6.3.3.2. Destabilisation with $CaCl_2$

Figure 48 shows the particle size distributions of particle suspensions initially prepared using 0.5% (w/v) SDS at a temperature of 60°C where 5.1, 10 or 20 mM $CaCl_2$ was added after the stirring period of microparticle formation.

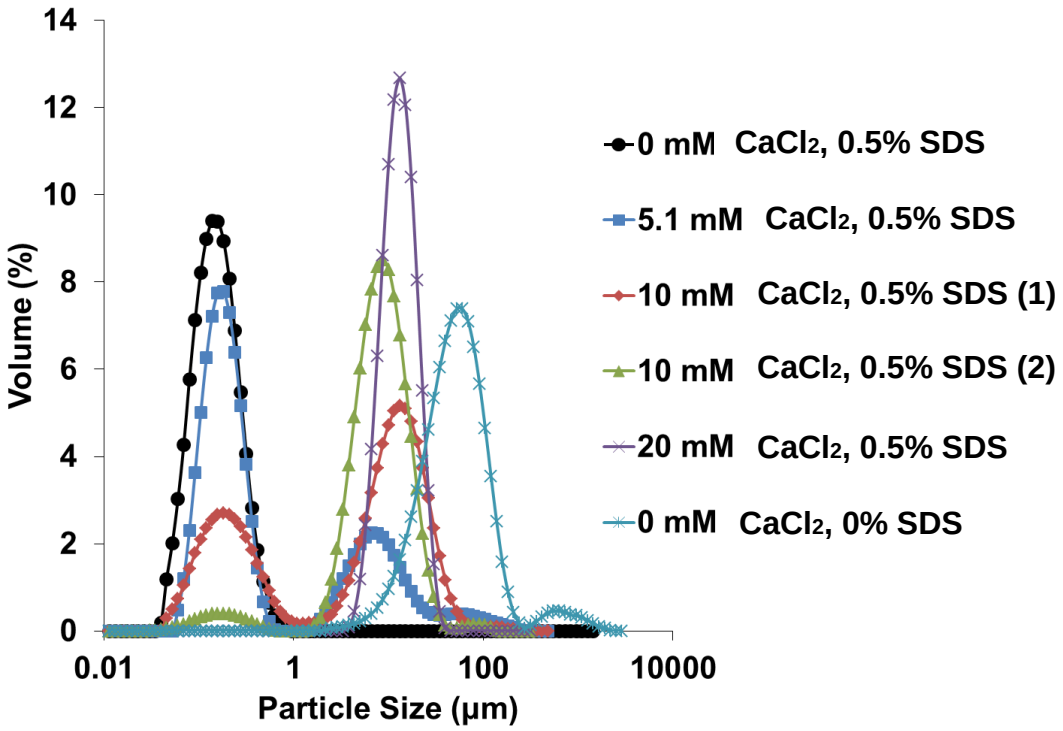


Figure 48. Size distributions of lignin microparticles prepared using a water phase containing 0.5% (w/v) SDS where $CaCl_2$ has been added to the suspension after microparticle formation at concentrations of 5.1-20 mM.

Figure 48 shows that particle size increased with increasing $CaCl_2$ concentration. The incorporation of 5.1 mM or 10 mM $CaCl_2$ produced bimodal particle size distributions, in which particles were present in both the 0.1-1 µm size range and the 1-10 µm size range. As the $CaCl_2$ concentration increased from 5.1 to 10 mM, the volume of particles in the lower size range decreased and the volume of particles in the higher size range increased. There was some variation in the relative volumes of particles in the 0.1-1 µm and 1-10 µm size ranges for replicate trials of 10 mM $CaCl_2$ addition. However, in both cases bimodal size distributions were evident, with a greater volume of particles present in the 1-10 µm size range. When 20 mM $CaCl_2$ was added, all particles were centred around approximately 10 µm, with no particles in the lower size range.

6.3.3.3. Destabilisation using changes in pH

Figure 49 shows the size distributions of particles prepared using water phases with unbuffered pH of 3 and 10, revealing that apart from very small peaks of particles in the

1-100 µm size range, the initial pH of the aqueous phase did not influence lignin particle size.

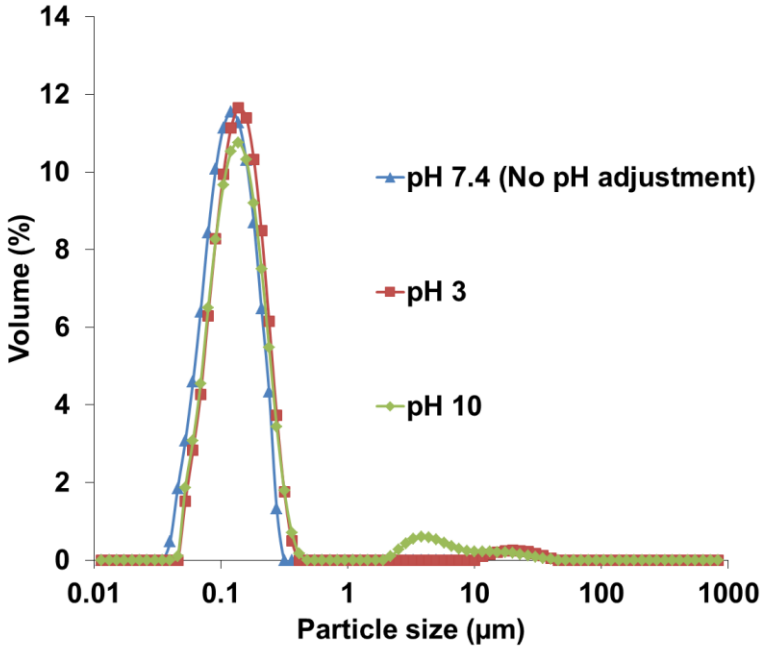


Figure 49. Size distributions of lignin microparticles prepared using a water phase containing 0.5% (w/v) SDS, with pH adjusted to 3, 10 or 7.4 (unadjusted pH) and temperature adjusted to 60°C before mixing with the extraction liquor.

Figure 50 shows size distributions of particles prepared using a water phase that contained SDS but had no pH adjustment; the pH of the suspension was instead adjusted to pH 1.5, 2, 7 or 10 after the 22 hour stirring period of microparticle formation and the suspension then stirred for another 30 minutes. Figure 50 shows that the size distribution of lignin particles differed from the control only when the pH was adjusted to 1.5. At pH 1.5, the size distribution became bimodal, with some particles evident in the 1-10 µm size range. It is interesting to note that bimodal distributions were also observed when KCl or CaCl2 were added during or after microparticle formation. Figure 51 reveals that the primary particles in the suspension at pH 1.5 are still in the sub-micron size range, indicating that the upper modality of particles in Figure 50 are the result of primary particle aggregation.

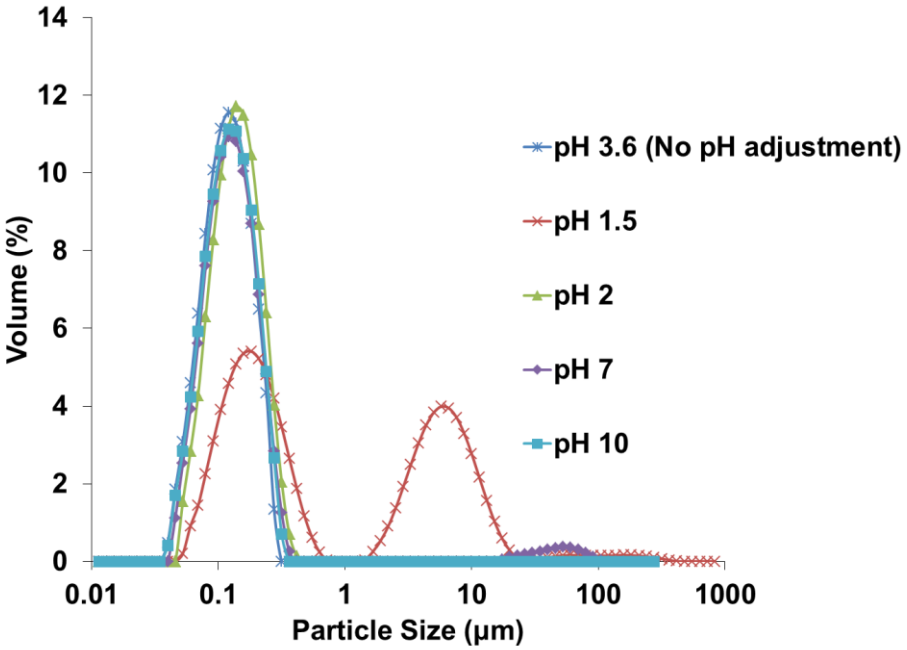


Figure 50. Size distributions of lignin microparticles prepared using a water phase containing 0.5% (w/v) SDS at 60°C, where the pH of the suspension has been adjusted after the 22 hour stirring process of microparticle formation to pH 1.5, 2, 7 or 10.

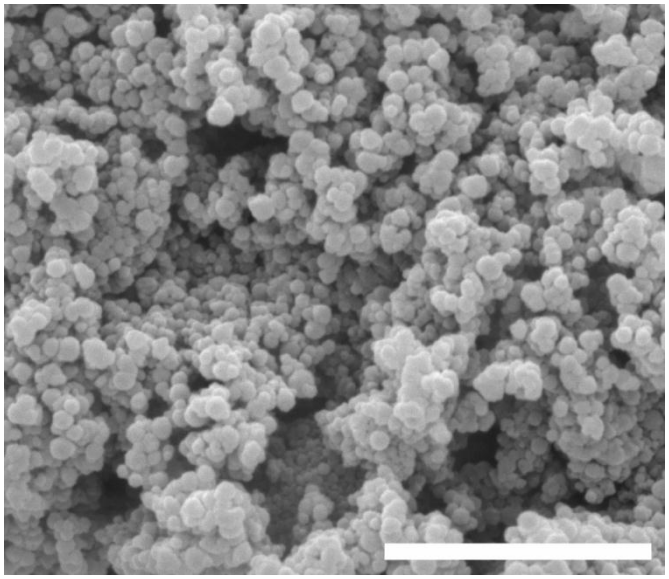


Figure 51. SEM image of particles prepared using a water phase containing 0.5% SDS (w/v) at 60°C where the pH of the suspension has been adjusted to pH 1.5 after the 22 hour stirring process of microparticle formation (bar scale = 3 µm).

Perhaps if the pH of the water phase is reduced below 1.5 before the extraction liquor is added, larger (1-10 μm) yet narrowly distributed particles may be formed. However, it is likely to be important to buffer the system to ensure that the addition of liquor does not increase the pH above 1.5. Initial experiments using buffered aqueous phases were carried out, as described in Appendix G. However, more work is required in this area to prepare buffers with sufficient capacity to prevent the liquor changing the pH of the system but also a low ionic strength, since it is known that the addition of salt also destabilises particles.

6.3.4. Control of hydrophobic interactions during particle formation

Solutions of 6.6 and 8 M urea were prepared and used as the aqueous phase in microparticle formation. The particle size distributions in Figure 52 show that for an aqueous phase temperature of 20°C, the presence of 6.6 M urea caused very little change in particle size compared to those prepared without urea. For an aqueous phase temperature of 60°C, the presence of either 6.6 or 8 M urea caused a slight reduction in particle size compared to those prepared with no urea at the same temperature.

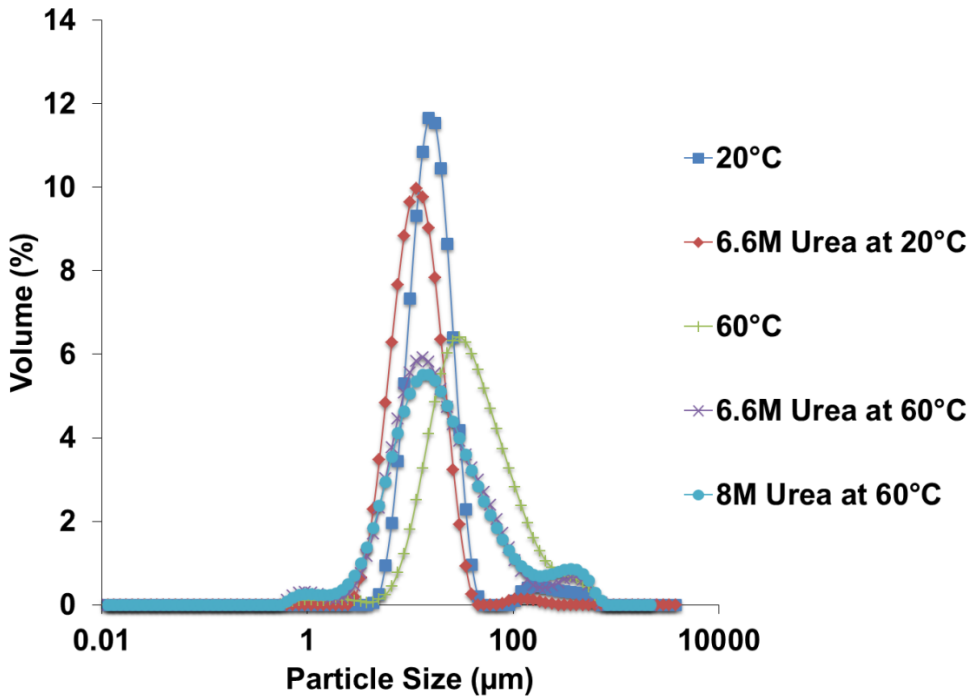


Figure 52. Size distributions of lignin microparticles prepared using water phases containing either water alone or varying concentrations of urea, at either 20 or 60°C.

However, scanning electron microscopy revealed that although a slight reduction in particle size was observed with incorporation of urea for an aqueous phase temperature of 60°C, the monolithic appearance is still evident (Figure 53).

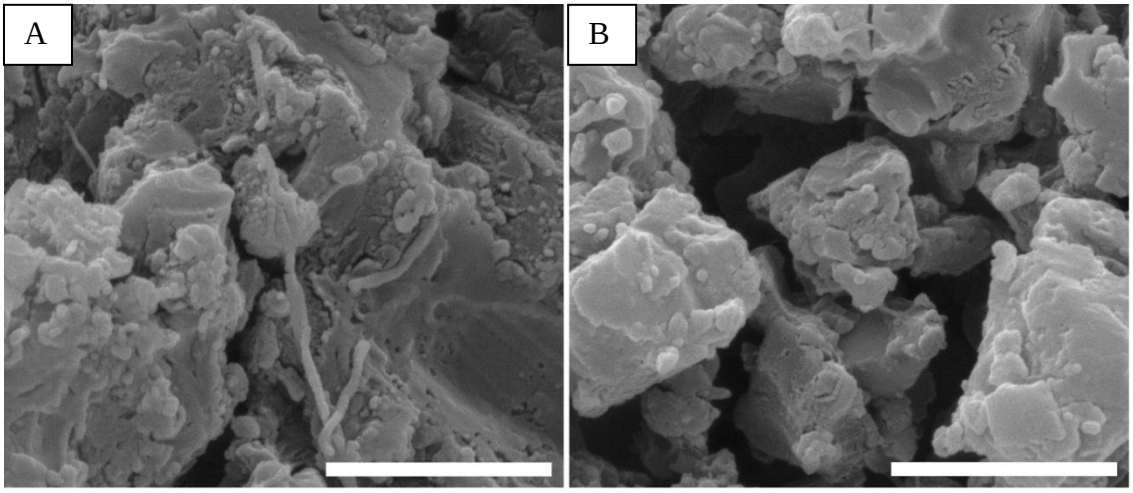


Figure 53. SEM images of particles prepared using water phases containing (A) 6.6 M urea and (B) 8 M urea, at 60°C with no SDS present (bar scale = 3 μm).

Interestingly, when both 6.6 M urea and 0.5% (w/v) SDS were present in the aqueous phase, for an initial aqueous phase temperature of 4°C, the presence of urea caused a large reduction in particle size (Figure 54).

6.3.5. Effect of ultrasonic mixing on particle size

The ability of ultrasonic mixing to produce particles in different size ranges was investigated. A system for ultrasonic mixing using a flow cell was used to enable small volumes of lignin liquor and water to be mixed at any one time. Significant work was involved in setting up the system and carrying out each run. Experimental details and results of this work are detailed in Appendix F. Initial results showed a slight decrease in particle size when using ultrasonic mixing compared to magnetic stirring under the same conditions. However, no further trials were carried out due to the time input required for a single run. More time would be required to optimise the process and determine whether any significant advantage can be gained from the use of ultrasonic mixing.

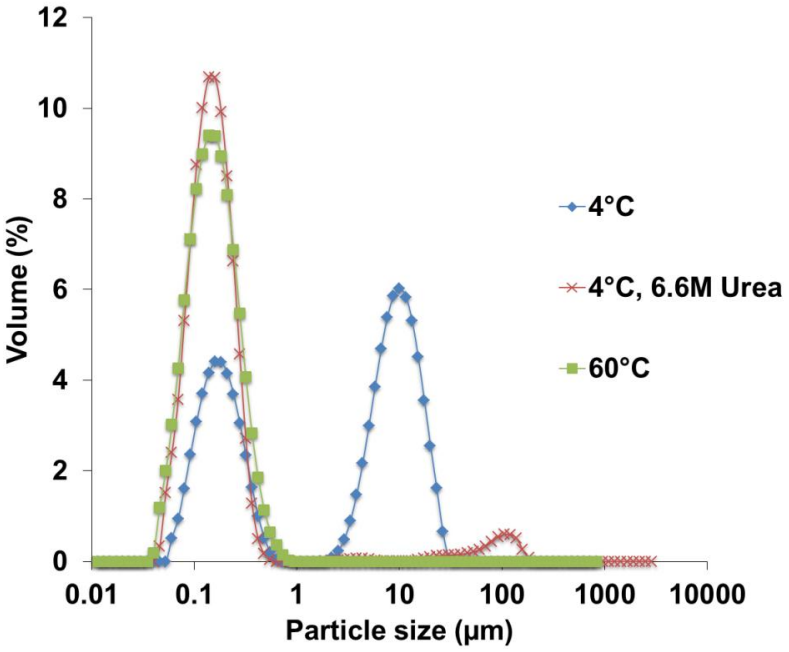


Figure 54. Size distributions of lignin microparticles prepared using water phases containing either 0.5% SDS (w/v) alone or both 0.5% SDS (w/v) and 6.6 M urea, at temperatures of either 4 or 60°C.

6.3.6. Re-dispersibility of lignin particles

A preliminary investigation into the ability of dried lignin particles to be re-dispersed in water to the same size as the original microparticle suspension was carried out. This work is outlined in Appendix G. It was found that sonication was able to return non-SDS-stabilised particles to their original size in suspension. On the other hand, sub-micron SDS-stabilised particles were not re-dispersed by sonication. Further investigation into particle drying and re-dispersion methods, including re-dispersion into different solvents is recommended before food applications of the particles can be considered.

6.4. Discussion

As noted in the introduction to this chapter in section 6.1, Zhong and Jin (2009) have outlined the dependence of particle size and shape on the relative timescales of droplet break-up, polymer precipitation and droplet coalescence. Further to this, Lannibois, Hasmy, Botet, Chariol and Cabane (1997) suggest that where surfactants are involved, final particle size is determined by the interplay between coalescence of the

hydrophobic polymer material and surfactant adsorption at the interface. Surfactant adsorption hinders coalescence of the polymer particles, thereby kinetically stabilising the particles. Thus factors such as the speed of adsorption of surfactants, the long-term stability able to be provided and the presence of any destabilising agents have an important influence on particle size.

6.4.1. Effect of emulsifier type

The presence of ionic surfactants SDS and CTAB at the point of precipitation of extracted lignin resulted in smaller lignin particles than the presence of non-ionic surfactants sucrose ester and Tween 20, even over the wide range of concentrations of the non-ionics tested. Moreover, structurally, the particles stabilised by sucrose ester and Tween 20 existed in the form of monolithic masses of lignin rather than smaller particles or agglomerates of smaller particles as observed for stabilisation with SDS and CTAB. The fused structure indicates that there was limited interfacial stabilisation of the colloidal particles when sucrose ester or Tween 20 were used. This is contrary to the results obtained by Rastogi et al. (2008), who found that Tween 20 produced a more stable dispersion of carbon nanotubes than SDS when used at the same concentration. It was suggested that although SDS has a longer hydrophobic tail than Tween 20 by one carbon and thus would be expected to bind more strongly to the surface of carbon nanotubes, Tween 20 has a bulkier hydrophilic head group, which produces greater steric stabilisation. In the work presented here, it seems that the charge on the head groups of SDS and CTAB provides more effective particle stabilisation by electrostatic repulsion.

Temperature is known to affect non-ionic oxyethylene-based surfactants such as Tween 20. It is suggested that conformational changes in the polyoxyethylene chains occur with increasing temperature that make them progressively less polar. The reduction in polarity weakens the interaction of the chains with water, thus reducing hydration of the head groups and favouring interactions between them. Ultimately, this leads to closer packing of head groups and phase inversion of the micelles (Holmberg et al., 2002). Phase changes and reduction in head group hydration may have been responsible for the poorer performance of Tween 20 in stabilising sub-micron particles, since the water temperature used for lignin precipitation was 60°C. It is recommended that Tween 20 be

trialled again with a lower water temperature, such as 20°C, to determine whether this more food-grade surfactant can better stabilise lignin microparticles.

On the contrary, the solubility of sugar-based non-ionic surfactants in water increases with temperature (Holmberg et al., 2002). Based on this knowledge, sucrose ester would have been expected to be an effective stabiliser of lignin particles under the preparation conditions used. However, perhaps the hydration of the polyhydroxyl head group is insufficient at any temperature to keep lignin particles dispersed and stabilised in water. In support of this, Ariyaprakai et al. (2013) found that although sucrose ester was more effective than Tween 60 in reducing the interfacial tension between coconut oil and water in the formation of coconut milk emulsions, the emulsion droplets produced were larger. Furthermore, although Youan et al (2003) produced discrete, spherical PLGA microparticles using HLB 16 sucrose ester, the particles had an average diameter of 50 μm, which is close to the size of the lignin particles produced with a similar sucrose ester concentration in the current work (D[4,3] = 76.9 μm, D[3,2] = 16.7 μm). Lignin microparticles stabilised by lignin were fairly narrowly distributed and may have appeared spherical and discrete if viewed under light microscopy rather than by SEM after drying. This may indicate that sucrose esters do not provide adequate stability during drying and also that the emulsifier is unable to produce particles of novel size in this case. It is unfortunate that sucrose ester was not found to be a more successful particle stabiliser in this work. As a low-toxicity, biodegradable and food-grade material, it would fit well with the food-grade requirement of this project.

It is recommended that to gain more understanding of the mechanism of stabilisation of lignin particles, the study should be extended in future work to include more emulsifiers. In particular, effective food-grade emulsifiers must be found to enable stabilised lignin particles to be used in foods. Sodium stearoyl lactylate may provide a good starting point, since it is a charged, food-grade material.

6.4.2. Effect of emulsifier concentration

SDS concentrations as low as 0.0625% (w/v) were found to stabilise lignin particles effectively in the 0.1-0.2 μm size range. However, particles stabilised by 0.0625% (w/v) SDS exhibited some fusion during drying, unlike those stabilised by higher concentrations. Furthermore, SDS concentrations greater than 1% (w/v) led to agglomeration of some of the sub-micron particles. Instability at very low concentration

is attributed to insufficient coverage of particulates by surfactant, as indicated by zeta potential measurements. The zeta potentials of particles containing 0 or 0.03% (w/v) SDS were -5.1 and -26.1 mV, respectively and only "particles with zeta potentials more positive than +30 mV or more negative than -30 mV are normally considered stable" by charges (Malvern, 2013).

It is less immediately apparent why a small shoulder of larger (1-100 μm) particles were produced when SDS concentrations greater than 1% (w/v) were used. Ultrasonication of the microparticle suspensions containing these higher concentrations of SDS, as well as the dilution that occurs in the light scattering equipment when measuring particle size, did not eliminate the broad shoulder of larger particles. This indicates that weak flocculation or agglomeration was not the cause of the larger particle size, and that more permanent bonds were being formed between particles. It was also clear from the microstructure of particles prepared using an SDS concentration of 1% that the increase in size was not due to the formation of a continuous mass of material as with increasing temperature, but instead strong aggregation or agglomeration of "primary" particles.

Rastogi et al. (2008) observed aggregation of carbon nanotubes dispersed by various surfactants, including SDS. It was suggested that just as surfactants form micelles at high concentration in solution, surfactants at high concentration can form multilayers on surfaces. The multilayers may cause parts of the surfactant molecule to extend into the liquid phase and interact with surfactant molecules on neighbouring surfaces. Interaction between hydrophobic tails in this way can cause flocculation by bridging. Figure 55 shows the proposed mechanism of flocculation of carbon nanotubes (shown in grey) via bridging of surfactant molecules at high concentration.

It is highly possible that a mechanism such as the one shown in Figure 55 is responsible for the aggregation of lignin primary particles at high SDS concentration in the current work. It is important to note that in this case the hydrophobic interactions between the hydrocarbon tails must have been strong enough or high enough in number to prevent break-up during static light scattering measurement, including sonication in the Mastersizer.

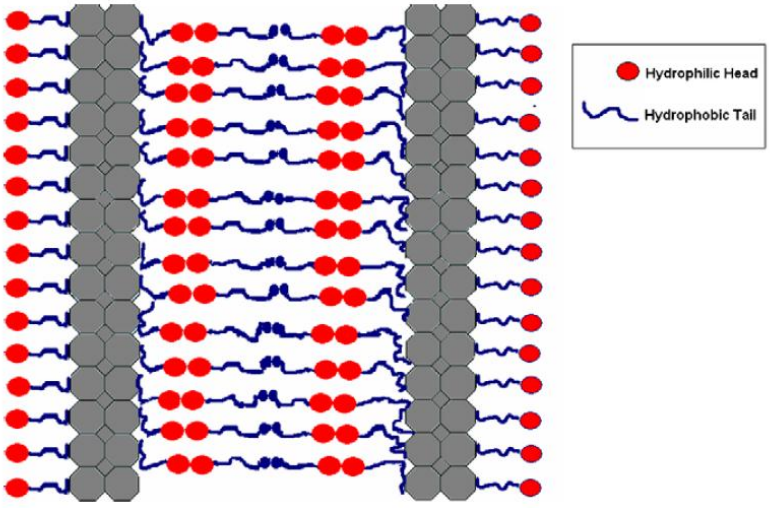


Figure 55. Mechanism of flocculation of carbon nanotubes via surfactant molecules (Rastogi et al., 2008)

Given the wide concentration range over which SDS stabilises sub-micron lignin particles, it was hypothesised that the zeta potentials measured with increasing SDS concentration would begin to level off, indicating that particle surfaces had been completely coated with surfactant. However, the increasing negativity of zeta potential values with increasing SDS concentration indicates that SDS continues to be adsorbed at the particle surface but does not cause further reduction in particle size at higher concentrations. This may indicate that only a relatively low level of surface charge is actually required to provide effective stabilisation.

CTAB behaved similarly to SDS; concentrations of 0.0625-0.5% (w/v) produced narrowly distributed sub-micron particles, while concentrations of 1 and 1.5% (w/v) produced particles with a bimodal size distribution. As with SDS, it is likely that particles observed in the larger size range were agglomerates or aggregates of primary particles. It is recommended that particles prepared using 1 or 1.5% CTAB are examined using scanning electron microscopy to confirm particle aggregation at high surfactant concentration. Zeta potential increased as CTAB concentration increased from 0 to 0.1%, but from 0.1 to 1.5% was very similar. This may indicate that 0.1% CTAB fully coats the surface of the particles, which is why no further increases in zeta potential with increasing CTAB concentration were observed.

6.4.3. Effect of salt concentration

Addition of KCl to SDS-stabilised lignin microparticle suspensions caused increases in particle size. The increase in size and decrease in zeta potential with increasing salt concentration indicate that the added salt effectively screens electrostatic repulsions between the charged head groups, leading to aggregation of particles or coalescence of liquor droplets or newly precipitated particles. This supports the notion that that the stability provided by the ionic surfactants SDS and CTAB is primarily via electrostatic repulsion. In fact, Butt et al. (2003) suggest that although lateral repulsion between head groups also arises due to hydration and steric effects, electrostatic effects provide additional repulsion but can be screened by added salt.

The addition of 0.1 M KCl before particle formation was sufficient to completely destabilise the lignin particles to the same size as those produced in the absence of SDS. On the other hand, addition of 0.1 M KCl after particle formation destabilised the particles to a large extent, but not to the degree of initially non-SDS-stabilised particles. Furthermore, a greater degree of fusion and increase in primary particle size was also observed for particles prepared in the presence of KCl than for those for which KCl was added after particle formation. The increase in primary particle size with increase in salt concentration may be due to the inability of SDS to stabilise particles as soon as they precipitate. With effectively no SDS available to stabilise the particles due to charge screening at high salt concentration, the particles may easily agglomerate during mixing, particularly at the onset of mixing when the temperature is higher. This is consistent with the theory that particle size is determined by the competition between the adsorption of surfactants and coalescence of hydrophobic material (Lannibois et al., 1997; Lebouille, Stepanyan, Slot, Cohen Stuart, & Tuinier, 2014) Comparatively, when particles are formed first in the presence of SDS only, small particles are able to be stabilised. Then when KCl is added after the long stirring period, the electrostatic charges are screened and the primary particles aggregate into clusters, leading to larger measured particle size.

It has been found that monovalent counterion concentrations of 25-150 mM cause precipitation in most dispersions (Schulze (1882) and Hardy (1990), as cited by Butt et al., 2003). Thus the coalescence or aggregation of lignin particles with 0.05-0.1 M KCl was expected. Some discrepancies were seen in the influence of intermediate

concentrations of KCl, in that the addition of 0.05 M KCl after particle formation led to larger particles than the addition of 0.05 M KCl before particle formation. However, additional work carried out with a higher aqueous phase SDS concentration of 0.5% (w/v) indicates that a sudden change in size from the 0.1-1 μm to the 1-100 μm range occurs for salt concentrations in the 0.05-0.06 M range. Thus the unexpected larger size observed for 0.05 M KCl added after particle formation may merely reflect the nature of the effect of KCl in this concentration range.

Addition of $CaCl_2$ to SDS-stabilised lignin microparticle suspensions also caused increases in particle size. For addition of both KCl and $CaCl_2$, rather than gradual increases in particle size with increases in salt concentration, particles seem to exist in two particle size ranges, with only the relative volume of particles in each size range increasing or decreasing with changes in salt concentration. Similar switches in particle size were observed with changes in pH and with addition of urea. Lebouille et al. (2014) suggest that when solid particles collide and stick to form larger particles, fractal aggregates are formed. The existence of aggregates of "primary" particles in the SEM images for particles prepared in the presence of SDS and subsequently destabilised by salt or reduction in pH may support this idea. Lebovka (2014) states that the morphology of aggregates or clusters depends on the type of interactions that exist between particles, the mechanism of attachment of particles to the growing cluster, and the available space in the system. Four models for cluster morphology simulation – diffusion limited aggregation, reaction limited aggregation, ballistic aggregation and Eden-like aggregation – are explained. Gaining an understanding of aggregate growth for lignin particles may help to explain the existence of two modal distributions of either aggregated or non-aggregated particles in the current system.

The change in the relative volume of particles in two discrete size ranges may suggest that the addition of salt can only produce particles in certain size ranges, which could be a limitation of this method.

6.4.4. Effect of pH

Reduction of suspension pH to 1.5 after microparticle formation caused an increase in measured particle size, shown to be a result of primary particle aggregation rather than an increase in primary particle size. Ko, Schlautman and Carraway (1998) suggest that the pKa of SDS is 2.3. Thus the aggregation of primary particles at pH 1.5 could be a

result of protonation of the SDS below the pKa and subsequent inability to provide stability via electrostatic repulsion. However, reduction of suspension pH to 2 did not cause particle aggregation, which may indicate that protonation of SDS below the pH of dissociation was not the only cause of particle aggregation at low pH. Perhaps at pH 2, although electrostatic repulsion is no longer possible, the hydration and steric effects of the SDS head groups are still sufficient to keep lignin particles stable and dispersed. However, on this basis we might have expected Tween 20 to be an effective stabiliser of lignin particles too. Therefore, further work is necessary to understand the influence of pH on particle aggregation.

Similar to the addition of KCl before particle formation, perhaps if the pH of the water phase is reduced below 1.5 before the extraction liquor is added, larger primary particles may form. Further experiments using buffered aqueous phases are recommended. However, it may be that particles will fall within two size ranges as observed for manipulation with KCl and $CaCl_2$ rather than give discrete particles in the intermediate size range. Changes in pH may also influence particle size due to the effect of pH on lignin itself. Podaralla and Perumal (2012) found that smaller and less polydisperse zein nanoparticles were produced when the pH during precipitation was close to the isoelectric point of zein (pI = 6.8). Larger particles were produced when the precipitation pH was greater than 8 because the higher solubility of zein at higher pH affects the rate of precipitation (Cabra, Arreguin, Vazquez-Duhalt, & Farres, 2006). Thus the effect of pH on lignin solubility may enable particles of novel size and shape to be produced.

6.4.5. Effect of urea

Urea did not reduce the size of lignin particles prepared in the absence of surfactants. Although the measured size of particles prepared at 60°C was slightly smaller in the presence of urea, SEM analysis of particles prepared at both 20 and 60°C revealed that the particles still had a monolithic appearance when urea was used. This indicates that coalescence of lignin during particle precipitation and subsequent stirring still occurred, further indicating that hydrophobic interactions are not responsible or are only partially responsible for this coalescence. Lignin contains many ether groups, as indicated by FTIR analysis in Chapter Five, which are often polar. Perhaps dipole-dipole interactions (a type of van der Waals interaction) between lignin particles play a major role in their

coalescence. Further characterisation of the lignin using a technique such as thin layer chromatography may help to identify what the common lignin fragments are and thus whether dipole-dipole interactions could be contributory to the coalescence. It could also be possible that the phenolic groups in lignin, which are also polar, arrange in a way that limits hydrophobic interactions.

The ability of 6.6 M urea to produce particles almost exclusively in the sub-micron range in the presence of 0.5% (w/v) SDS for a water temperature of 4°C was unexpected based on the distribution of particles produced under these conditions without urea. The result may indicate that hydrophobic interactions do play some role in the structure of lignin. In this case, perhaps the urea has helped to reduce residual hydrophobic interactions that still exist due to poorer functionality of SDS at low temperature. It would be interesting to determine the effect of urea on particle size for an aqueous phase temperature of 4°C with no SDS present.

Ultimately, the addition of urea did not enable particles of sizes different to those previously fabricated with or without SDS to be produced. Therefore, other than providing an indication that hydrophobic attractions do not play a major role in the agglomeration of lignin particles, the addition of urea does not appear to be a useful tool for the manipulation of particle size.

Although more work is necessary to make strong conclusions about the effects of pH, urea and ultrasonication on lignin particle size, ultimately these results show that the preparation of particles with a narrow size distribution in the range of ~0.5-10 μm is difficult. Therefore, the size of lignin particles that can be produced efficiently may be limited in terms of achievable particle size distributions.

6.5. Conclusions and recommendations

SDS and CTAB were found to be effective emulsifiers for the formation of sub-micron lignin microparticles. Concentrations as low as 0.0625% (w/v) of both surfactants were sufficient to stabilise lignin particles in the sub-micron size range. Concentrations greater than 1% (w/v) were not only unnecessary for stability, but also caused particle aggregation. Therefore, it is recommended that the lowest concentration that can provide stability is used, which may require further investigation into stability during particle drying.

The non-ionic surfactants sucrose ester and Tween 20 were unable to stabilise sub-micron lignin particles. It is recommended that the performance of other emulsifiers is evaluated to determine whether charged surfactants are a necessity.

The addition of KCl or $CaCl_2$, along with the adjustment of pH, were found to destabilise primary particles of lignin, causing either larger primary particles or aggregation of primary particles depending on whether alterations were made before or after particle formation. However, in any case, particles fell within two size ranges: 0.1-1 μm or 1-100 μm. A narrow distribution of particles in the 1-10 μm size range was not produced due to the aggregation kinetics of the system. Control of pH throughout particle formation is yet to be studied; however, it may provide further indication of the scope of lignin particle size control.

Initial determination of the effect of urea and ultrasonic mixing during particle formation failed to produce lignin particles in size ranges different from those previously produced. Further work at different aqueous phase temperatures is recommended.

Although more work is necessary to make strong conclusions about the effects of pH, urea and ultrasonic mixing on lignin particle size, the current work has shown that size can be manipulated. However, it is not yet clear whether particles can be produced in well-controlled size ranges, or only in two modal distributions. There may be limitations in the variation in particle size that can be achieved, which may limit applications of the particles.

Chapter Seven. Lignin Microparticles as Pickering Stabilisers

7.1. Introduction

Some of the lignin particles produced in this work are 100-200 nm in diameter, while others seem to have primary particles this size that are clustered together to form larger aggregates. Organosolv lignin literature also suggests that organosolv lignins are relatively hydrophobic (Lora & Glasser, 2002; Nakagame, 2010). Nakagame (2010) found that the contact angle of organosolv lignin extracted from poplar, a plant in the same family as willow, was 77.5°. It was suggested in section 2.10 that particles with contact angles close to 90° are adsorbed strongly at oil-water interfaces. It was also noted that particles with contact angles less than 90° are hydrophilic and will generally form oil-in-water emulsions. Furthermore, the requirement for particles to be at least a magnitude smaller than the emulsion droplet size to provide effective stabilisation suggests that smaller particles lead to smaller emulsion droplets. Therefore, it seems that sub-micron organosolv lignin particles may have the ability to stabilise oil-in-water emulsions by the Pickering mechanism.

Section 2.10 covered much of the necessary background to Pickering emulsions, including a previous investigation by Wei et al. (2012) into the ability of lignin particles to stabilise emulsions. This literature method will be evaluated in this chapter to determine whether the organosolv lignin extracted and processed into microparticles in the previous chapters can produce stable emulsions. However, an alternative method in which fresh lignin microparticle suspensions are used as the aqueous phase in emulsion formation will also be evaluated. In this method, the pH modification steps critical to the literature method can be avoided because the lignin in the suspensions is already solid, homogenously dispersed throughout the liquid and also fairly monodisperse. In both methods, mostly non-SDS stabilised lignin particles will be used. These particles range in diameter from approximately 10-100 μm and thus are much larger than the SDS-stabilised sub-micron particles. Although the sub-micron particles would be expected to allow stabilisation of smaller emulsion droplets, it was unclear whether the SDS present on the surface of the particles would influence the wettability of the particles or contribute itself to emulsion droplet size and stability. Hence only

preliminary trial results concerning the performance of sub-micron lignin particles as Pickering stabilisers are included here.

Emulsion droplet size and stability will be used to compare the efficacy of the two methods. The ratio of stabilising particle to oil is also known to be important for droplet size (Frelichowska et al., 2010; Wei et al., 2012). Therefore, the effect of lignin-to-oil ratio will be investigated in this chapter by varying either the lignin or oil concentration used in emulsion formation.

7.2. Materials and methods

7.2.1. Materials

Soybean oil (Gilmours, Auckland, New Zealand) and sodium dodecyl sulfate (SDS) (BDH, VWR International Ltd) were used as received. Lignin was extracted from chipped *Salix purpurea* as described in Chapter Five. Lignin extraction liquor at 20°C, containing 4.68 ± 0.04% solids, was mixed with water (4-60°C) to prepare suspensions of microparticles. Lignin was either dried from these suspensions by the centrifuging and crushing steps outlined in Chapter Five or used in the form of the microparticle suspension either directly or diluted with distilled water. Lignin used in this work had a total lignin content of 96.5%, with the remainder mostly comprising ash (1.65%), xylan (1.78%) and glucan (0.33%).

7.2.2. Methods

7.2.2.1. Preparation of Pickering emulsions using the pH-modification method

Lignin-stabilised emulsions were prepared based on the method developed by Wei et al. (2012). Dried, crushed organosolv lignin prepared according to Chapter Five with an initial water phase temperature of 60°C, was added to water. NaOH (0.1-8 M) was then added to the lignin dispersion until all the lignin dissolved, at which point the pH was around 12.1. HCl (0.1-3 M) was then added to the dispersion until the pH dropped to 3.0. The dispersion was then left to stand for at least 10 minutes over which time the precipitated lignin particles coagulated. Soybean oil was then added to the dispersions prior to mechanical mixing.

7.2.2.2. Preparation of Pickering emulsions using fresh lignin microparticle suspensions

Lignin-stabilised emulsions were also prepared using fresh lignin microparticle suspensions. Soybean oil was added either directly to the suspensions or to suspensions that had been diluted with distilled water or concentrated by natural evaporation in air to achieve certain lignin concentrations. Lignin concentration was measured in terms of total solids content of the suspensions, which was measured using the standard air oven method. The concentration of the original suspension was initially assumed similar to previous trials and dilutions were made based on this assumed concentration. The total solids content of the original suspension was later measured to enable the actual lignin concentration to be calculated and reported. In some experiments, however, the lignin concentration of the original suspension was measured before dilutions were carried out. Where required, it will be made clear throughout the chapter whether a specified concentration is a 'target' or 'actual' value. The microparticle suspensions used were always those that had been prepared using an initial water temperature of 4°C, since particles in these suspensions were known to be smaller than those in suspensions prepared at higher temperatures (Chapter Five).

For both the pH-modification and fresh suspension (FS) methods, when discussing the emulsions, the concentration of lignin is always expressed as the concentration of lignin in the aqueous phase.

7.2.2.3. Effect of lignin concentration

The effect of lignin concentration on emulsion droplet size was investigated in emulsions containing 10% (w/w) soybean oil. Target concentrations of 0.1, 0.5, 1, 1.5 and 3% (w/v) lignin were trialled when using the FS method. The total solids content of the original fresh suspension used to prepare the aqueous phase of these emulsions was found to be 1.4% and therefore the actual lignin concentrations studied were 0.1, 0.5, 1.0, 1.4 and 2.9% (w/v). Lignin concentrations of 0.05, 0.1, 0.5 and 2% (w/v) were trialled when using the pH-modification method. The lignin concentrations used in the pH-modification method were chosen based on the work of Wei et al. (2012) and also on the results of preliminary trials. The lignin concentrations used in the FS method were chosen based on preliminary trials (Appendix H). All emulsions were prepared

using a bench-top rotor-stator homogeniser (Heidolph DIAX 600, Heidolph Instruments, Germany) at a speed of ~17,000 rpm for one minute.

7.2.2.4. Effect of oil concentration

The effect of oil concentration on emulsion droplet size and stability was investigated using both methods of emulsion formation. The oil concentrations tested were 3.8, 13.8, 21.9 and 28.6% (w/w). In the FS method, the lignin concentration of the aqueous phase was kept constant at 1.39% (w/v), which was the total solids content of the original suspension. In the pH-modification method, the lignin concentration of the aqueous phase was kept constant at 0.5% (w/v). All emulsions were prepared using a rotor-stator homogeniser (model L4RT, Silverson Machines Ltd., Chesham, England) fitted with a fine emulsor screen (mesh 800 μm) at its maximum speed of ~10,300 rpm. Aluminium foil was used to cover the sample beakers during mixing to try to prevent loss of sample over the sides during mixing. In the FS method, a replicate of the emulsion containing 3.8% oil was prepared. In the pH-responsive emulsion, a replicate of the emulsion containing 21.9% oil was prepared. The emulsions were stored in the chiller at 5°C for five months after manufacture.

7.2.2.5. Emulsion characterisation

7.2.2.5.1. Droplet size measurement

The droplet size distributions of the emulsions were determined using static light scattering (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). Distilled water was used as the dispersant. The refractive index and absorption index used were those of soybean oil; 1.414 and 0.001, respectively. The size of the emulsion droplets was measured on the day of preparation and also after subsequent time periods (1 week, 2 weeks, 4 weeks, 6 weeks, 8 weeks, 12 weeks and 20 weeks) under storage at 5°C.

7.2.2.5.2. Light microscopy

Emulsion droplets were analysed using light microscopy (Olympus BX53, Olympus Corporation, Tokyo, Japan) with 10X and 40X objectives. Images were captured using an Olympus XC50 camera attachment. Light microscopy was generally only carried out on the day of sample preparation. However, any light microscopy carried out on subsequent days will be made clear in the relevant section.

7.2.2.5.3. Confocal microscopy

Emulsion droplets were analysed using confocal microscopy (Leica TCS SP5, Leica Microsystems, Germany) with a 40x N.A. 1.25 lens. Images were excited and acquired sequentially at 1024x1024 resolution with a line average of 4. A 405 nm laser line was used to excite lignin and its autofluorescence was captured with a PMT over the range of 490-620 nm. The samples were also stained with Nile Red (N3013 Technical Grade, Sigma-Aldrich, St. Louis, MO) to visualise the oil. A 561 nm laser line was used to excite the Nile Red dye and its emission was captured with a PMT over the range 570-676 nm. A brightfield image was simultaneously acquired with the brightfield detector. All acquisitions were controlled by Leica LAS AF software v 2.6.3.8173.

Lignin is known to autofluoresce (Chapman, Oparka, & Roberts, 2005; Donaldson & Radotic, 2013; Donaldson, Radotić, Kalauzi, Djikanović, & Jeremić, 2010). The excitation and emission wavelengths for lignin were chosen based on the work of Chapman et al. (2005), who found that excitation of lignin with 405 nm light gave strong emission between 490 and 620 nm.

Nile Red is a fluorescent probe of intracellular lipids and hydrophobic domains of proteins (Sigma-Aldrich, 2014a). Nile Red was used to stain the oil present in the emulsions to confirm that oil droplets had been produced. The difference in emission wavelength range of the Nile Red to lignin was a key factor in its selection. Staining with Nile Red was carried out immediately before confocal examination unless otherwise stated.

7.2.2.5.4. Laser Doppler electrophoresis

The zeta potential of emulsions was determined using Laser Doppler Electrophoresis (Malvern Zetasizer Nano ZS, Malvern Instruments Ltd., UK). Dispersants used to dilute the emulsions prepared using the pH-modification method were made by taking deionised water through the same pH-modification steps that were used in emulsion formation (i.e. 1-8 M NaOH used to increase pH and 1-3 M HCl used to decrease pH) until the pH was the same as the final emulsion pH. Dispersants used to dilute emulsions prepared using the FS method were made by adjusting the pH of deionised water to be the same as the pH of the emulsion using drop wise addition of ≤ 1 M HCl. All measurements were carried out at 20°C, and the results reported are the average of two readings.

7.3. Results

This section presents the results of the evaluation of the effect of lignin and oil concentration on emulsion droplet size and stability for emulsions prepared using both the FS and pH-modification methods. The effect of lignin concentration is outlined first, covering the results for the two emulsion preparation methods separately. The effect of oil concentration is then examined, again covering the results for the two emulsion preparation methods separately. Finally, the results of experiments concerning differences between the two methods are presented.

7.3.1. Effect of lignin concentration

In trials concerning the effect of lignin concentration on emulsion droplet size and stability, the concentration of oil and water was kept constant and only the concentration of lignin was varied.

7.3.1.1. Fresh suspension method

Figure 56 shows photographs of emulsions prepared using lignin microparticle suspensions with lignin concentrations of 0.1-2.9%, revealing an increase in cream height layer with increase in lignin concentration.

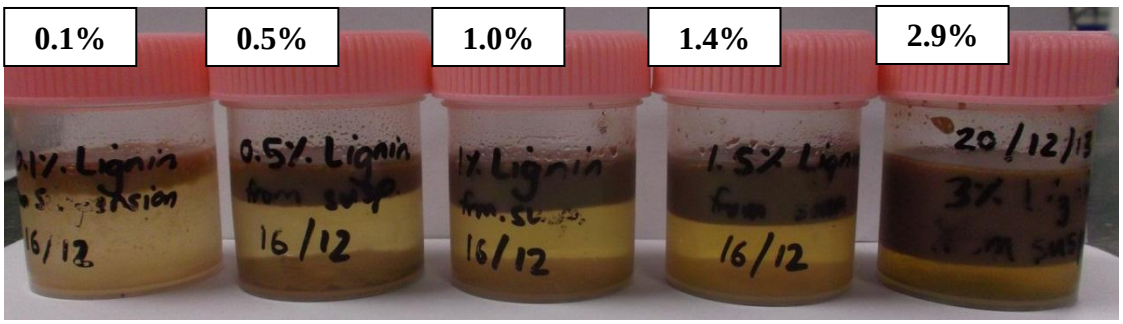


Figure 56. 10% (w/v) soybean oil emulsions stabilised with 0.1, 0.5, 1.0, 1.4 and 2.9% (w/v) lignin using the FS method.

Figure 57 shows size distributions for emulsion droplets in these emulsions. Figure 57 shows that emulsion droplet size decreases as lignin concentration increases. This is also shown by the D[4,3] and D[3,2] values in Table 25.

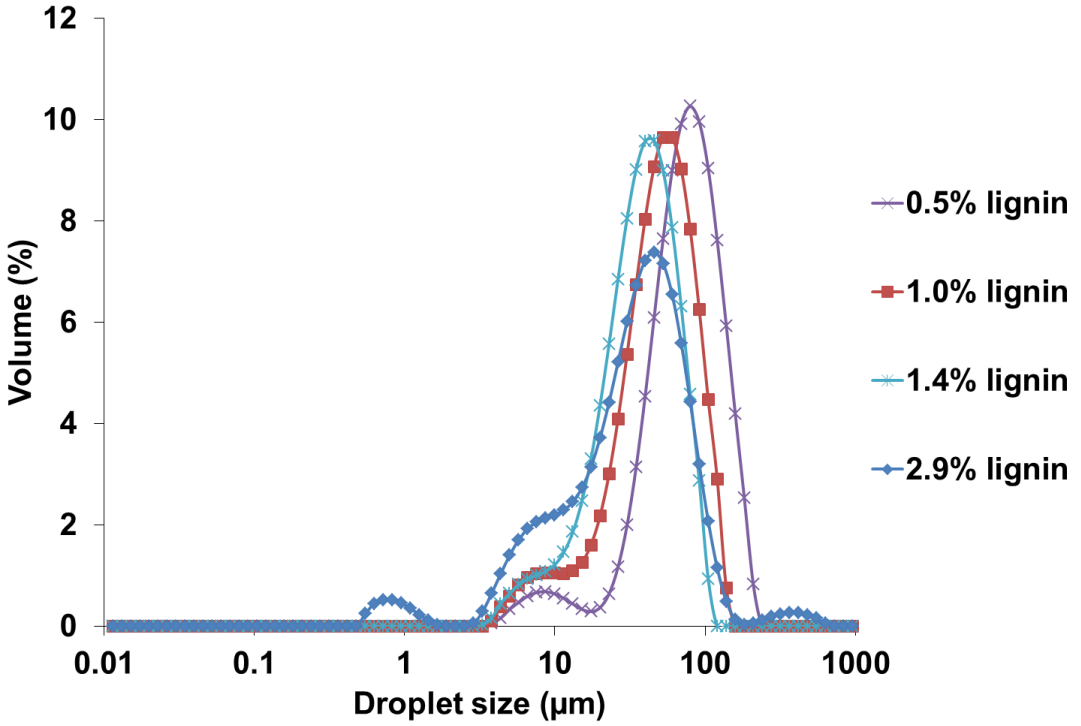


Figure 57. Droplet size distributions in 10% (w/v) soybean oil emulsions containing 0.5-2.9% (w/v) lignin, prepared using the FS method.

Table 25. D[4,3], D[3,2] and specific surface area values of droplets in 10% (w/v) soybean oil emulsions containing 0.5-2.9% (w/v) lignin, prepared using the FS method

Lignin concentration (% w/v)	Lignin-to-oil ratio	D[4,3] (µm)	D[3,2] (µm)	Specific Surface Area (m^2/g oil)
0.5	0.05	75.2	45.6	0.143
1.0	0.09	49.4	30.0	0.218
1.4	0.13	37.9	24.8	0.263
2.9	0.26	41.5	11.4	0.573

Table 25 also shows the specific surface area (SSA) values of droplets in emulsions containing 0.5-2.9% lignin. The SSA values were calculated using the Mastersizer 2000 software, but are based on Equation 4 in Chapter Two and indicate the interfacial area

of the droplets. The SSA of emulsions increases as lignin concentration, and thus lignin-to-oil ratio, increases. This was expected based on the decrease in droplet size with increase in lignin concentration.

The emulsions were also analysed by light microscopy. However, it was difficult to see any differences between the droplets in the images as the droplet analysed under the microscope was very concentrated. Samples were thereafter gently mixed by inversion and swirling before analysis to obtain a less concentrated specimen for analysis.

7.3.1.2. pH-modification method

Figure 58 shows photographs of 10% (w/v) soybean oil emulsions prepared using the pH-modification method with lignin concentrations of 0.05-2%. Figure 58 shows that similar to the results for the FS method, the height of the cream layer in emulsions prepared using the pH-modification method increases as lignin concentration increases.

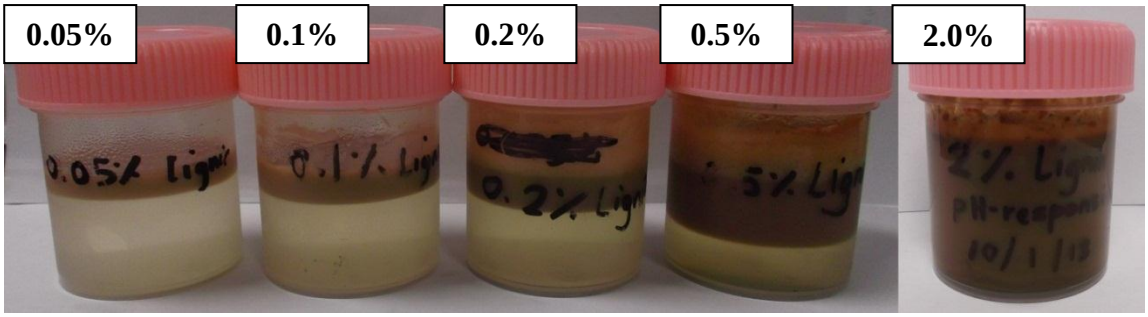


Figure 58. 10% (w/v) soybean oil emulsions stabilised with 0.05, 0.1, 0.2, 0.5 and 2% (w/v) lignin using the pH-modification method.

Figure 59 shows droplet size distributions for these emulsions, with D[4,3], D[3,2] and SSA values shown in Table 26. Figure 59 and Table 26 reveal that although the height of the cream layer increases with increasing lignin concentration, the oil droplet size remains consistent. It can also be seen that the oil droplets produced using the pH-modification method are smaller than those produced using the FS method, at all lignin concentrations. This was mainly a result of the small peak of droplets present in the 1 μm size range. Additionally, the calculated SSA at all lignin concentrations was ~0.7 m^2/g oil; larger than the SSA for emulsions prepared using the FS method, even those

containing a higher lignin-to-oil ratio. This may suggest that the pH-modification method provides lignin in a form that can better stabilise small oil droplets.

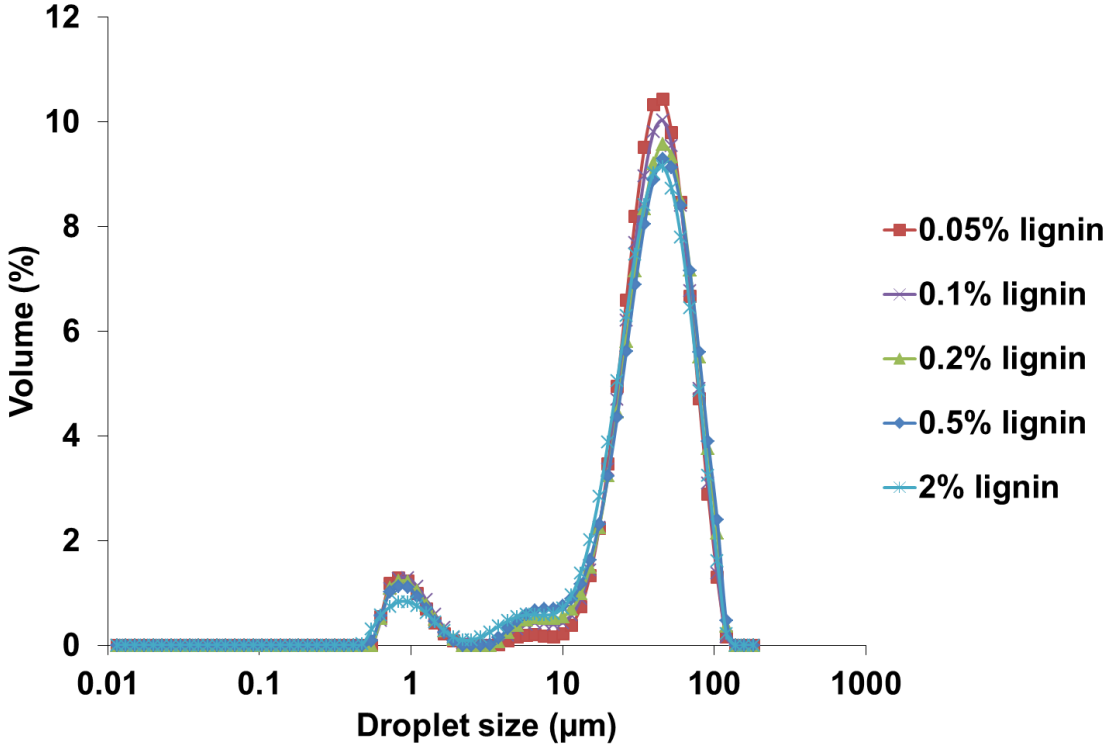


Figure 59. Droplet size distributions in 10% (w/v) soybean oil emulsions containing 0.05-2% (w/v) lignin, prepared using the pH-modification method.

Table 26. D[4,3], D[3,2] and specific surface area values of droplets in 10% (w/v) soybean oil emulsions containing 0.05-2% (w/v) lignin, prepared using the pH-modification method

Lignin concentration (% w/v)	Lignin-to-oil ratio	D[4,3] (µm)	D[3,2] (µm)	Specific Surface Area (m^2/g oil)
0.05	0.005	39.1	9.4	0.698
0.1	0.009	38.7	9.0	0.729
0.2	0.018	40.0	9.2	0.708
0.5	0.045	40.1	9.4	0.696
2.0	0.180	37.8	9.7	0.676

7.3.2. Effect of oil concentration

With the knowledge that the particle-to-oil ratio is an important parameter determining droplet size (Frelichowska et al., 2010), experiments in which the soybean oil concentration was varied while the lignin concentration was held constant were carried out. Since we were limited in the amount of lignin available, varying the oil concentration provided an alternative method of evaluating the effect of lignin-to-oil ratio on droplet size and stability.

7.3.2.1. Fresh suspension method

Figure 60 shows droplet size distributions for 3.8-28.6% soybean oil emulsions prepared using a fresh lignin microparticle suspension containing 1.39% (w/v) lignin. Figure 60 shows that oil droplet size increases with increasing oil concentration, which is supported by the D[4,3] and D[3,2] values shown in Table 27.

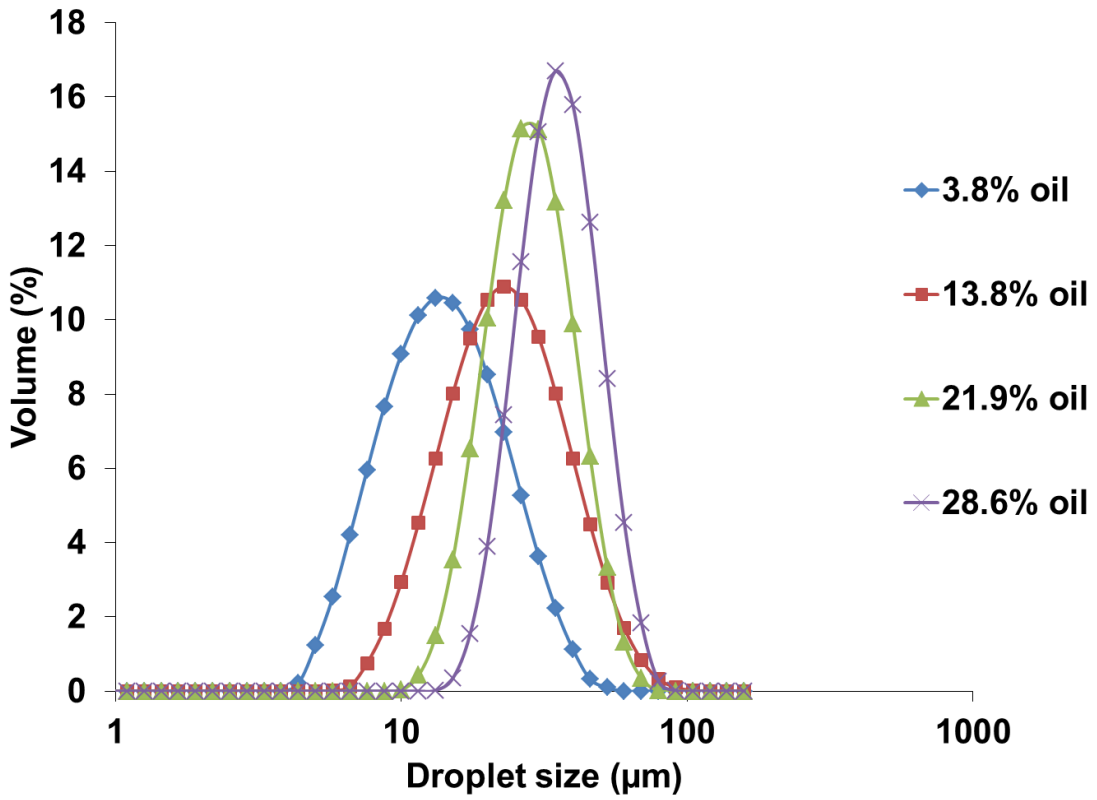


Figure 60. Droplet size distributions in emulsions prepared using the FS method with 1.39% (w/v) lignin and 3.8-28.6% oil (w/w).

Table 27. D[4,3] and D[3,2] values of droplets in emulsions prepared using the FS method with 1.39% (w/v) lignin and 3.8-28.6% (w/w) soybean oil

Oil concentration (% w/w)	Lignin-to-oil ratio	D[4,3] (µm)	D[3,2] (µm)
3.8	0.35	14.5	11.6
3.8 (replicate)	0.35	17.0	13.3
13.8	0.09	24.1	19.2
21.9	0.05	27.7	24.7
28.6	0.04	34.4	31.2

Table 27 also indicates that, considering the lignin-to-oil ratio, smaller oil droplets have been produced using the FS method in this set of experiments in which oil concentration has been varied compared to the previous set in which lignin concentration was varied. For example, the emulsion containing 13.8% oil had a D[4,3] droplet size of 24.1 µm and D[3,2] of 19.2 µm. The emulsion in section 7.3.1.1 containing 1% lignin had the same lignin-to-oil ratio of 0.09, however, the D[4,3] and D[3,2] values were higher, at 49.4 µm and 30.0 µm, respectively. It is likely that the difference in droplet size is due to the use of a Silverson rotor-stator homogeniser rather than Heidolph rotor-stator mixer to mix the oil and water phases during emulsion formation in the later work.

Figure 61 shows light microscopy images of these emulsion droplets. The images show a clear increase in droplet size with increase in oil concentration. The droplets also seem to become less spherical and more oval shaped with increasing oil concentration.

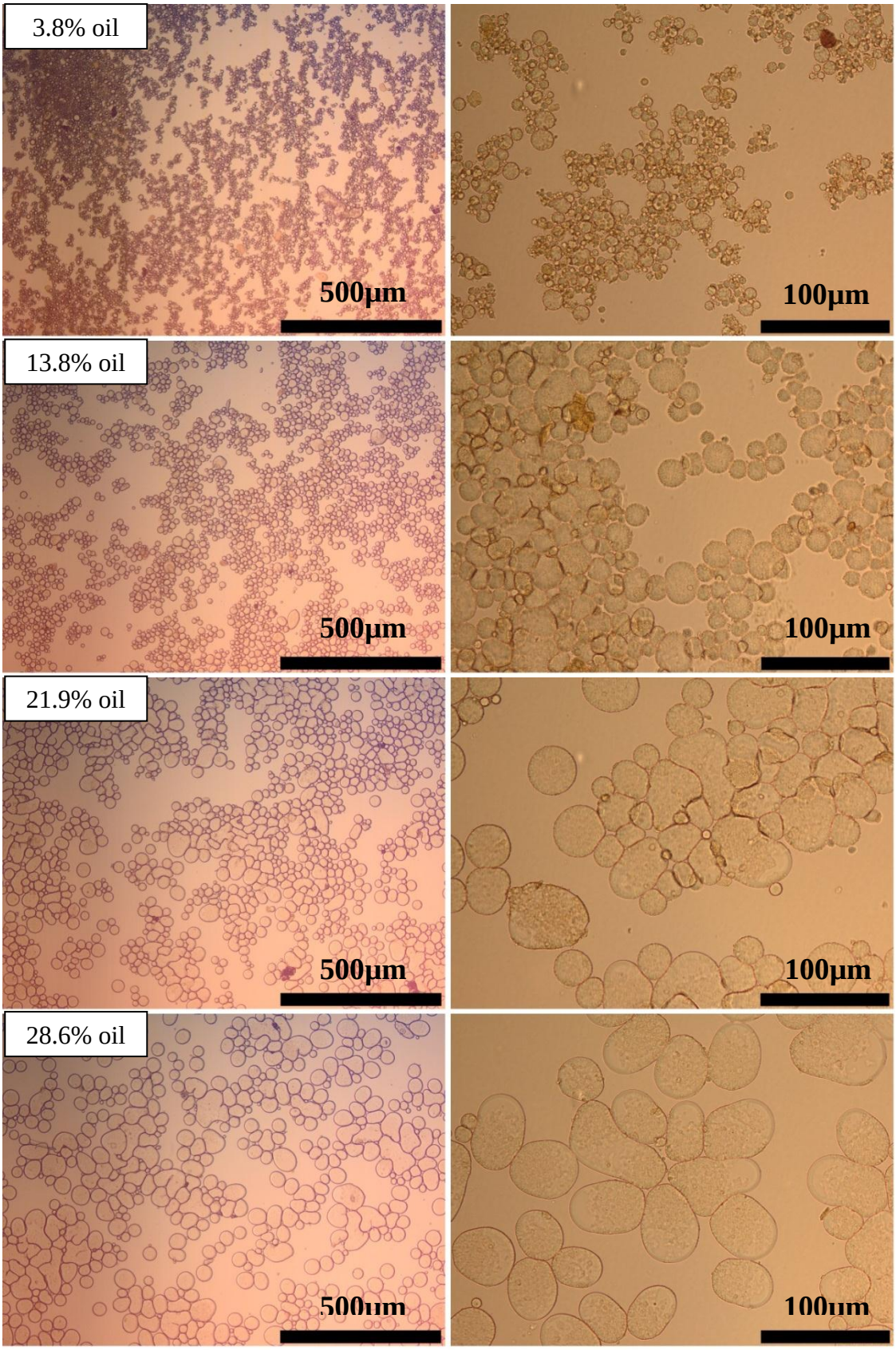


Figure 61. Light microscopy images of droplets in emulsions prepared using the FS method with 1.39% (w/v) lignin and 3.8-28.6% (w/w) oil.

Figure 62 shows confocal microscopy images of droplets in the emulsions containing 13.8 and 28.6% oil. The images again reveal an increase in droplet size with increase in oil concentration. However, the autofluorescence of lignin was unable to be used to show the location and structure of lignin particles at the emulsion droplet surface. Appendix I outlines the limitations of the technique used. It is recommended that the excitation and emission wavelengths of lignin autofluorescence for the lignin prepared and used in this work is determined before future confocal analysis.

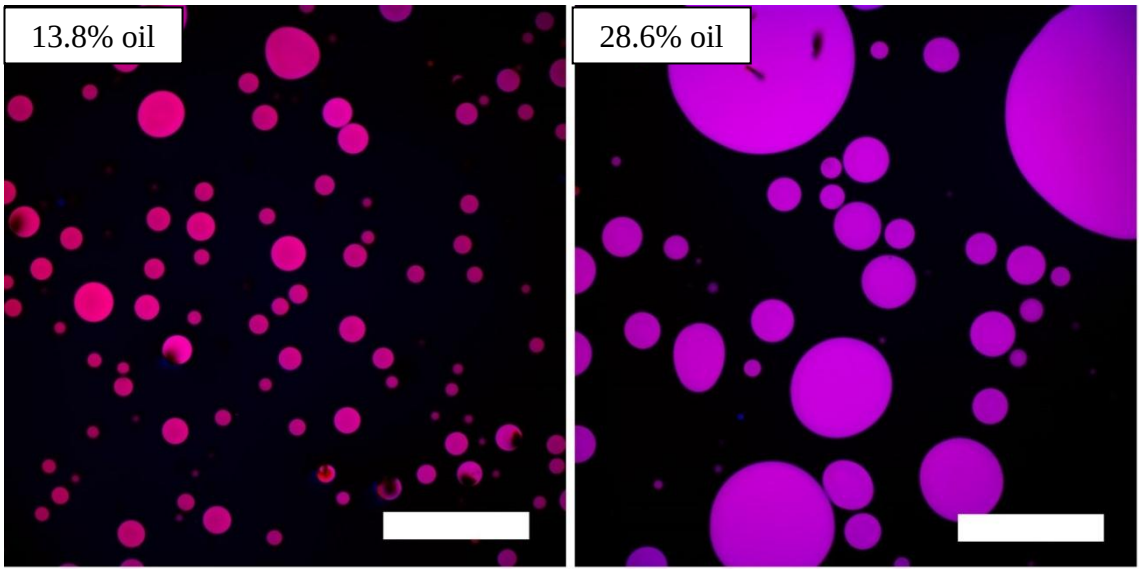


Figure 62. Confocal images of droplets in emulsions prepared using the FS method with 1.39% (w/v) lignin and either 13.8% or 28.6% (w/w) oil. Samples were stained with Nile Red before analysis (bar scale = 100 µm).

Figure 63 shows photographs of the emulsions containing various oil concentrations two minutes after emulsion manufacture. Figure 64 shows the appearance of the emulsions approximately 45 minutes after manufacture.

Figure 63 shows that within two minutes after manufacture, all emulsions exhibit creaming destabilisation. The height of the cream layer decreases with decreasing oil content, probably due to the reduced amount of oil available for emulsion formation at lower oil concentrations. Lignin particles are also visible in the cream layer and are most discernible at lower oil concentrations, which may be a result of lignin being present in excess at lower oil concentrations.

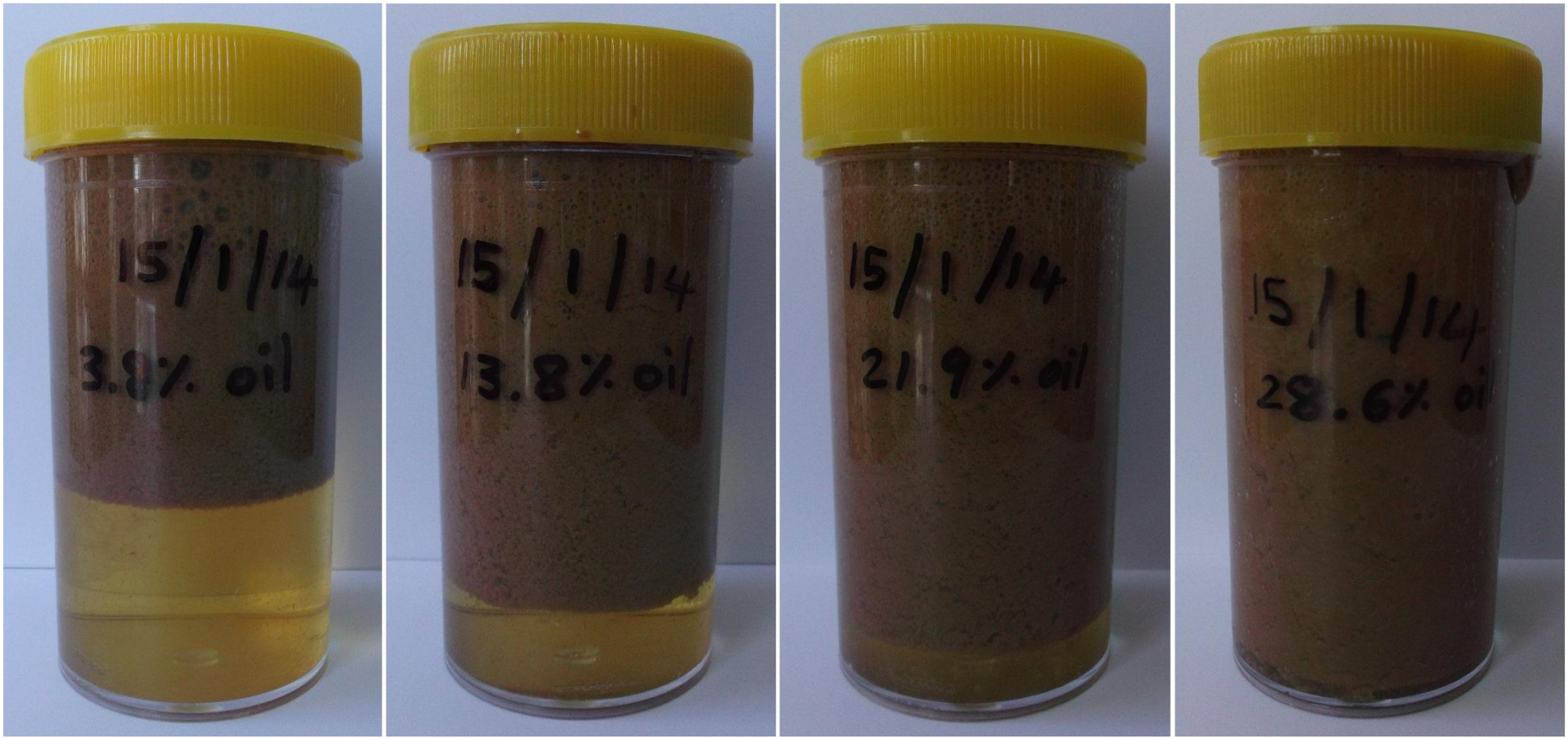


Figure 63. Photographs of emulsions prepared using the FS method with 1.39% (w/v) lignin and 3.8-28.6% (w/w) soybean oil. Photographs were taken two minutes after emulsion manufacture.

Figure 64 shows that 45 minutes after manufacture, the differences in volume of the cream layers are still evident. The volume of the cream layer increases as oil concentration increases. Lignin is not visible in the excess water phase below the cream layer, indicating that lignin remains associated with the oil phase.

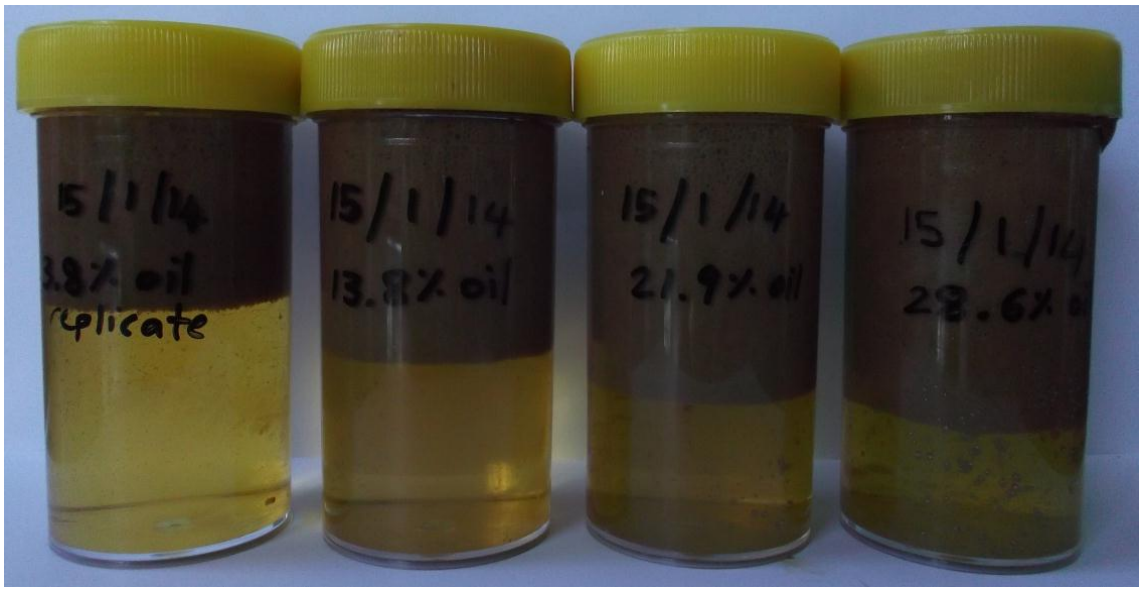


Figure 64. Photographs of emulsions prepared using the FS method with 1.39% (w/v) lignin and 3.8-28.6% (w/w) soybean oil. Photographs were taken ~45 minutes after emulsion manufacture.

Finally, the size of emulsion droplets was monitored over a period of five months using static light scattering. Figure 65 and Figure 66 show the size distributions of oil droplets in the emulsions containing 3.8 and 28.6% oil immediately after preparation, and two, four, eight, twelve and twenty weeks after preparation, revealing a slight increase in droplet size in both samples over the five months of chilled storage. However, in both samples the increase in droplet size over time was quite small, corresponding to increases in D[4,3] of approximately 5 μm and 4 μm for the emulsions containing 3.8 and 28.6% oil, respectively. The droplets in the emulsions containing 13.8 and 21.9% oil showed similar small increases in size over the five month storage period (data not shown). The appearance of the emulsions remained the same over the five month period.

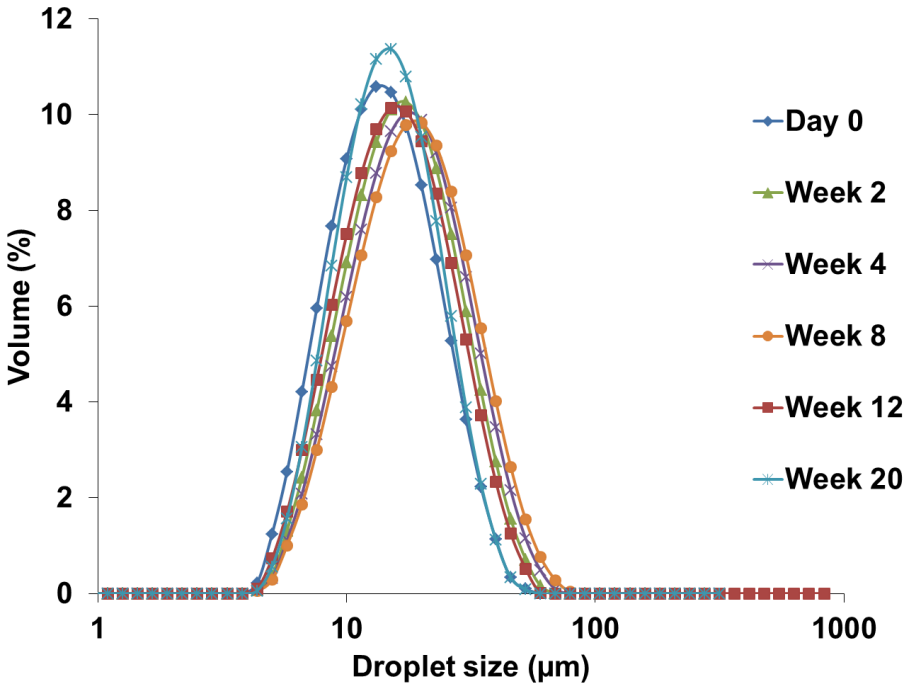


Figure 65. Droplet size distributions in FS method emulsion containing 1.39% (w/v) lignin and 3.8% (w/w) oil on the day of manufacture and 2, 4, 8, 12 and 20 weeks after manufacture.

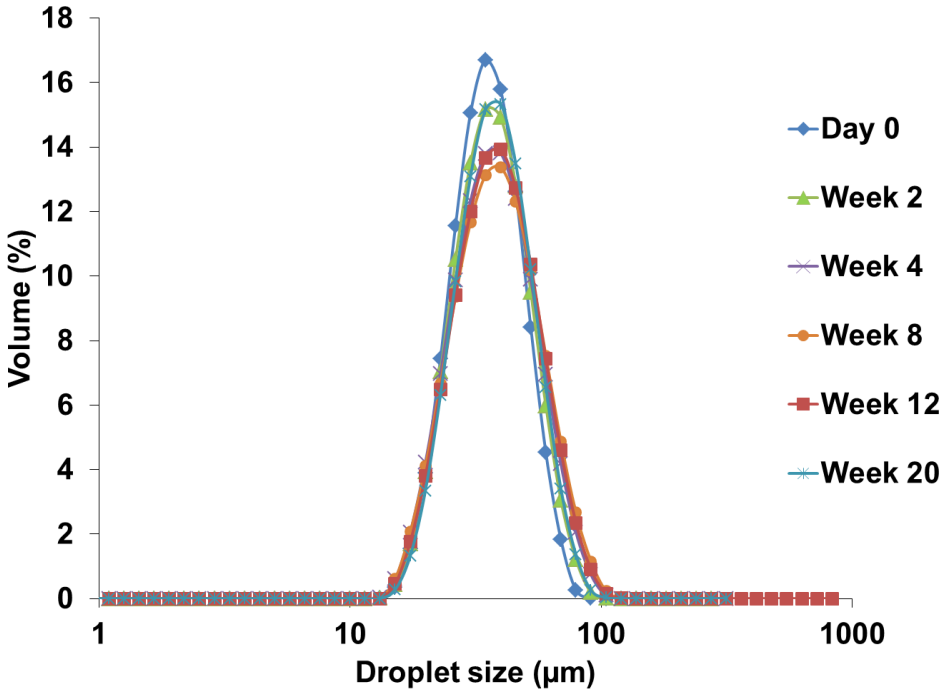


Figure 66. Droplet size distributions in FS method emulsion containing 1.39% (w/v) lignin and 28.6% (w/w) oil on the day of manufacture and 2, 4, 8, 12 and 20 weeks after manufacture.

7.3.2.2. pH-modification method

Figure 67 shows droplet size distributions for emulsions containing 3.8-28.6% oil prepared using the pH-modification method with 0.5% (w/v) lignin. Figure 67 shows that for oil concentrations of 3.8-21.9%, the distribution of droplet sizes is largely bimodal, with a small peak of droplets centring around 1 µm and a larger peak of droplets centring around 10-20 µm. As the oil concentration increases, the height of the peak at 10-20 µm increases while the height of the 1 µm peak decreases slightly and the peak becomes narrower. In the emulsion containing 28.6% oil, no peak exists at 1 µm; all the droplets are in the 10-20 µm size range. Emulsions containing 21.9 and 28.6% oil also show a small volume of droplets centring around 100 µm. These small peaks could represent larger droplets; however, the peaks could also be due to clusters of oil droplets, air bubbles during static light scattering or to larger lignin particles present in the emulsion. Overall, the differences in oil droplet size distributions suggest that, as observed for the FS method, emulsion droplet size increases with increasing oil concentration.

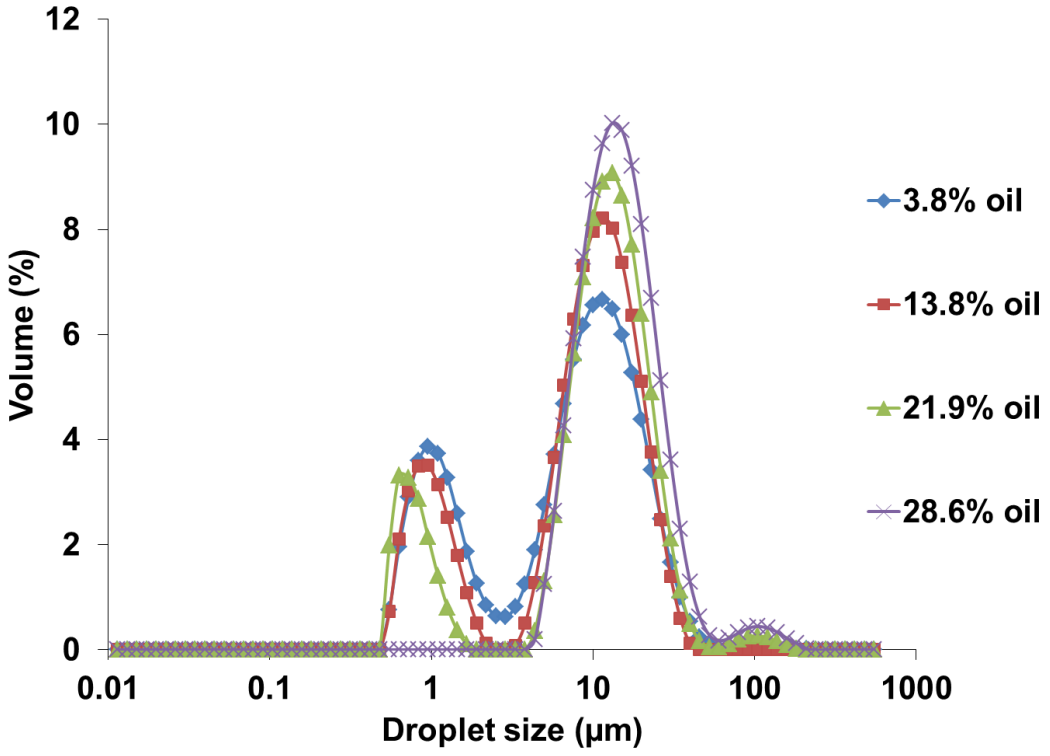


Figure 67. Droplet size distributions in emulsions prepared using the pH-modification method with 0.5% (w/v) lignin and 3.8-28.6% (w/w) oil.

Table 28 shows the D[4,3] and D[3,2] values of the emulsion droplets. It can be seen that the increases in these values with increasing oil content are quite small for oil concentrations in the range 3.8-21.9%. The increase in droplet size is larger when the oil concentration increases from 21.9% to 28.6%, reflecting the loss of the smaller ~1 μm droplets as shown in the droplet size distribution in Figure 67.

Table 28. D[4,3] and D[3,2] values of droplets in emulsions prepared using the pH-modification method with 0.5% (w/v) lignin and 3.8-28.6% (w/w) oil

Oil concentration (% w/w)	Lignin-to-oil ratio	D[4,3] (μm)	D[3,2] (μm)
3.8	0.13	9.5	2.7
13.8	0.03	9.6	3.0
21.9	0.02	12.6	3.3
21.9 (2)	0.02	11.1	3.4
28.6	0.01	17.1	11.9

Table 28 again indicates that, considering the lignin-to-oil ratio, smaller oil droplets have been produced in experiments in which oil concentration has been varied compared to the previous set in which lignin concentration was varied.

Figure 68 shows light microscopy images of the emulsion droplets. Unlike the emulsions prepared using the FS method, using the pH-modification method, there is no clear increase in droplet size with increasing oil concentration. This was expected based on the similarities in size distributions of the oil droplets shown in Figure 67. However, we might have expected to see a greater number of larger droplets under light microscopy for the emulsion containing 28.6% oil.

Figure 69 shows confocal microscopy images of the emulsion droplets. The images support that a major increase in droplet size did not occur with increasing oil concentration.

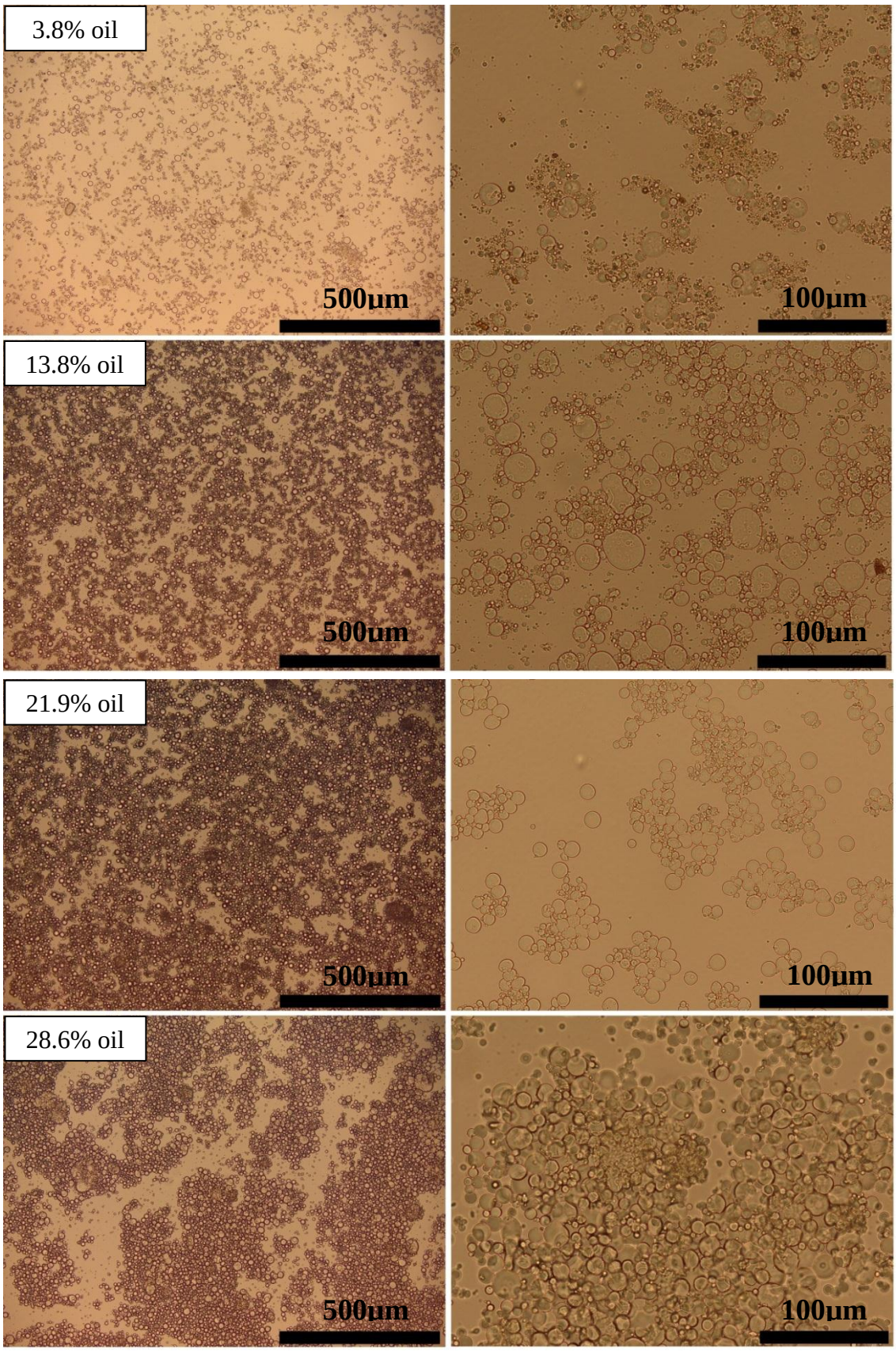


Figure 68. Light microscopy images of droplets in emulsions prepared using the pH-modification method with 0.5% (w/v) lignin and 3.8-28.6% (w/w) oil.

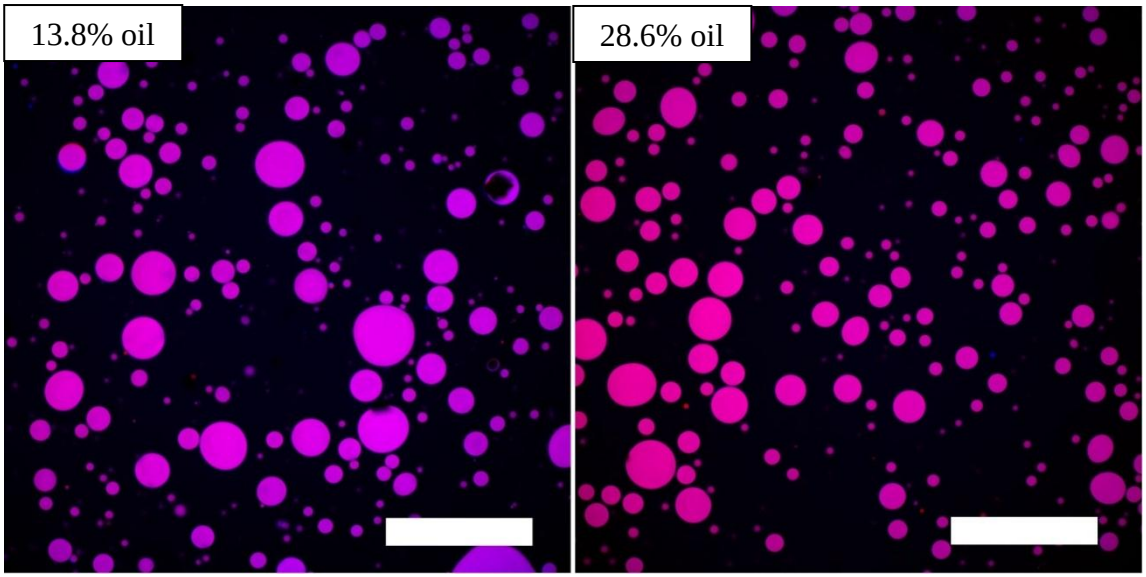


Figure 69. Confocal microscopy images of droplets in emulsions prepared using the pH-modification method with 0.5% (w/v) lignin and either 13.8% or 28.6% (w/w) oil. Samples were stained with Nile Red before analysis (bar scale = 100 µm).

Figure 70 shows photographs of the emulsions two minutes after manufacture. Figure 70 reveals that the extent of creaming destabilisation after two minutes for these emulsions is less than that observed for emulsions prepared using the FS method, indicating that the pH-modification method produces smaller emulsion droplets. Furthermore, although lignin particles are visible in the 3.8% (w/w) soybean oil emulsion, they are less easy to see in the 13.8% (w/w) oil emulsion and not evident in the 21.9-28.6% (w/w) oil emulsions.

Figure 71 shows photographs of the emulsions approximately 45 minutes after manufacture, revealing the occurrence of creaming destabilisation in all emulsions. Again, the height of the cream layer decreases with decreasing oil content.

Figure 70. Photographs of emulsions prepared using the pH-modification method with 0.5% (w/v) lignin and 3.8-28.6% (w/w) soybean oil. Photographs were taken two minutes after emulsion manufacture.

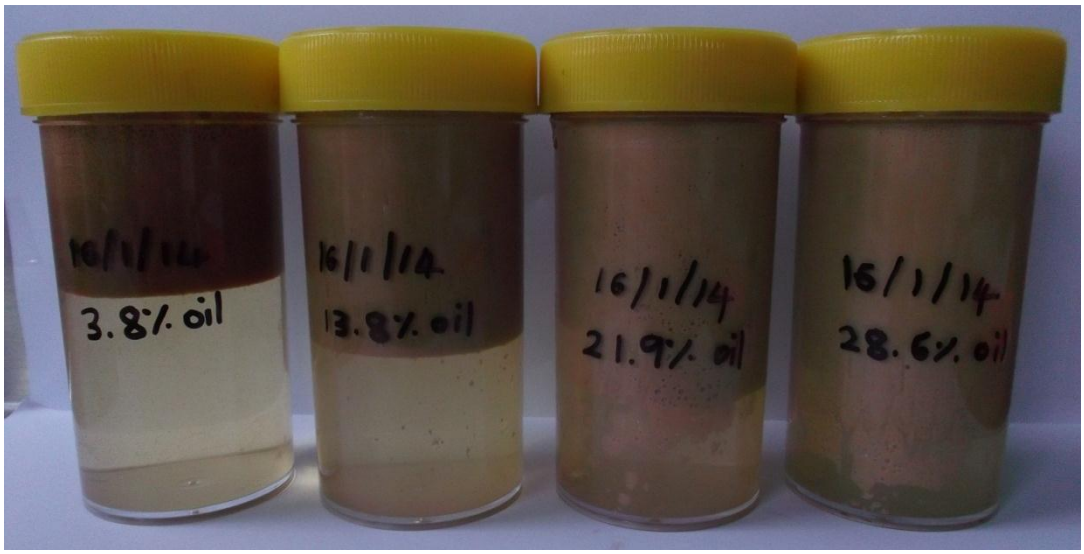


Figure 71. Photographs of emulsions prepared using the pH-modification method with 0.5% (w/v) lignin and 3.8-28.6% (w/w) oil. Photographs were taken ~45 minutes after emulsion manufacture.

Figure 72 and Figure 73 show the size distributions of oil droplets in emulsions containing 3.8 and 28.6% oil immediately after preparation, and two, four, eight, twelve and twenty weeks after preparation. Almost no change in the size distribution of either emulsion occurred over the five month storage period. At both oil concentrations there was a very slight decrease in the volume of the droplets present in the lower size range and increase in the volume present at the higher size range as the storage time increased, indicating a small shift to larger droplet size. However, increases in the D[4,3] and D[3,2] values of emulsion droplets in these emulsions were only 2 μm or less over the five months of storage. Therefore, emulsions prepared using the pH-modification can be regarded to be stable against coalescence over a five month period of chilled storage. Emulsions prepared using this method may also be slightly more stable than those prepared using fresh lignin suspensions, which exhibited larger increases in droplet size over time.

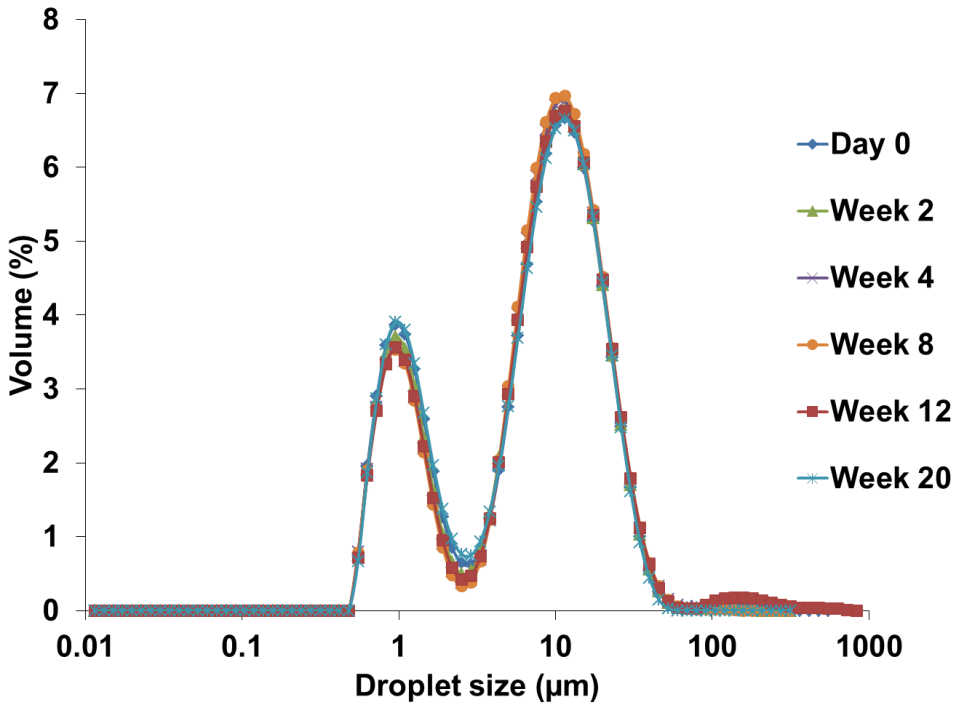


Figure 72. Droplet size distributions in pH-method emulsion prepared using 0.5% (w/v) lignin and 3.8% (w/w) oil on the day of manufacture and 2, 4, 8, 12 and 20 weeks after manufacture.

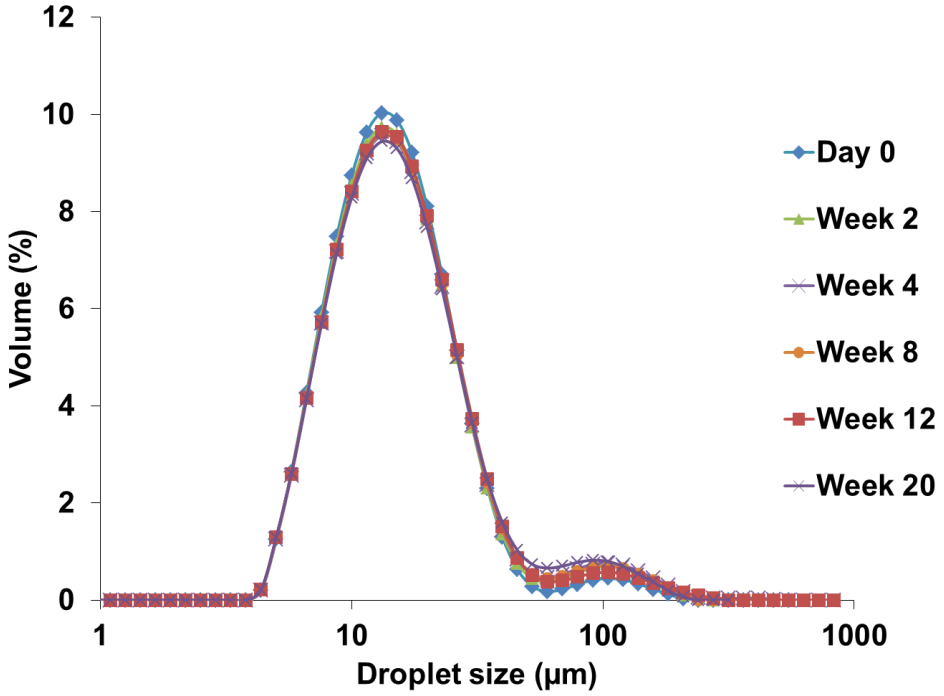


Figure 73. Droplet size distributions in pH-method emulsion prepared using 0.5% (w/v) lignin and 26.8% (w/w) oil on the day of manufacture and 2, 4 8, 12 and 20 weeks after manufacture.

Ultimately, the pH-modifcation method produced smaller emulsion droplets than the FS method, even though a lower concentration of lignin was used, as confirmed by the D[4,3] and D[3,2] values in Table 29. The values in Table 29 also support that the increase in droplet size with increasing oil concentration was smaller for emulsions prepared using the pH-modification method. Therefore, it was recommended that an investigation be carried out into why the pH-modification was able to produce smaller emulsion droplets.

Table 29. D[4,3] and D[3,2] values of droplets in emulsions containing oil concentrations of 3.8-28.6% (w/w) and prepared using either the pH-modification (0.5% lignin) or FS (1.39% lignin) method

	D[4,3] (μm)		D[3,2] (μm)	
Oil concentration (%)	**pH-modification method**	**Fresh suspension method**	**pH-modification method**	**Fresh suspension method**
3.8	9.51	14.53	2.69	11.61
13.8	9.63	24.09	2.98	19.20
21.9	12.59	27.73	3.28	24.69
28.6	17.08	34.40	11.89	31.20

7.3.3. Comparison of the fresh suspension and pH-modification methods

This section outlines the results of experiments carried out to determine why the pH-modification method was able to produce smaller oil droplets. The size and structure of the lignin particles present in each method, along with properties of the final emulsions, were investigated as potential sources of difference between the methods.

7.3.3.1. Size and structure of lignin immediately prior to emulsion formation

The size and microstructure of lignin when dispersed in water immediately before the addition of oil and emulsification in both methods was analysed to determine whether they could have influenced oil droplet size and stability. Figure 74 shows size distributions of the lignin in the dispersions for the two methods.

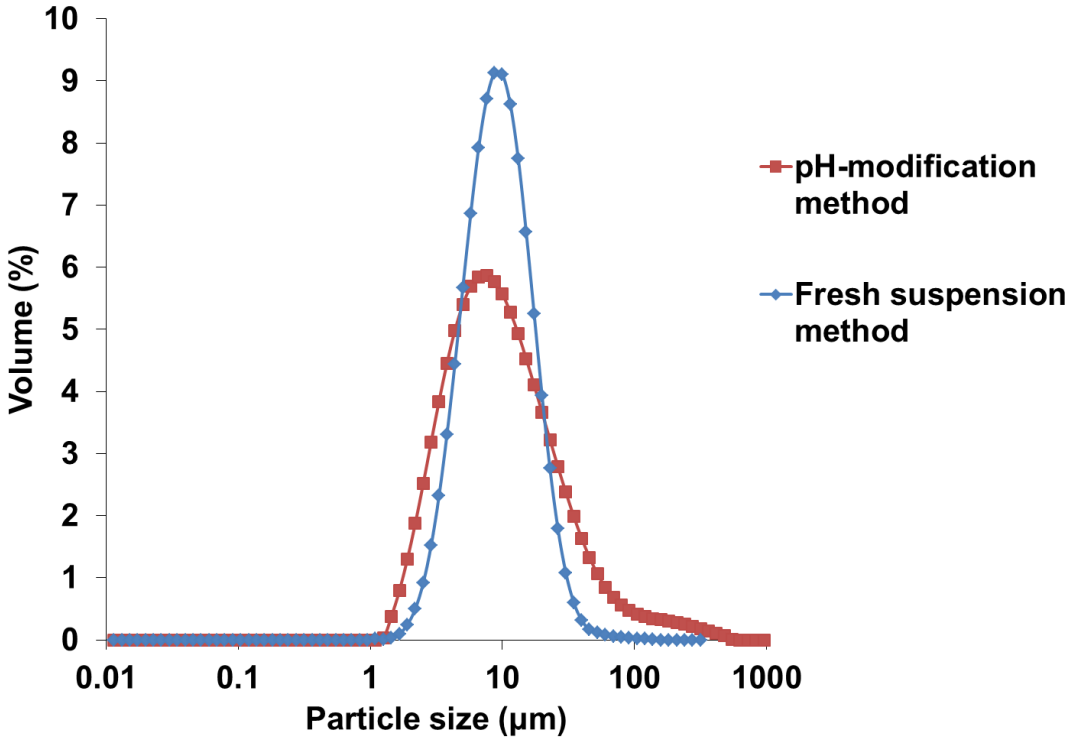


Figure 74. Size distributions of lignin particles in the water phase used for the emulsification process in the pH-modification and FS methods.

Figure 74 shows that the size distributions of the lignin particles in both methods are centred around 10 μm, indicating that the lignin particles available to go to the oil-water interface in both methods are approximately the same size. However, the pH-modification method generates a more polydisperse lignin suspension. This indicates that both slightly smaller and larger lignin particles are available for droplet stabilisation in the pH-modification method.

Figure 75 shows SEM images of particles dried from the dispersions immediately before the addition of oil for emulsion formation. Figure 75 shows that in both methods, sub-micron lignin microparticles are present. Considering the size distributions in Figure 74, these images suggest that in both methods, lignin is in the form of 1-100 μm aggregates of sub-micron particles. It is interesting to note that particles of this size are similar in size to the oil droplets in the emulsions.

Figure 76 shows the structure of lignin particles in dispersion before mixing with oil to prepare emulsions, confirming that the lignin available for emulsifying in both methods

is in the form of 1-100 μm aggregates of primary particles, with the aggregate size centring around ~10 μm.

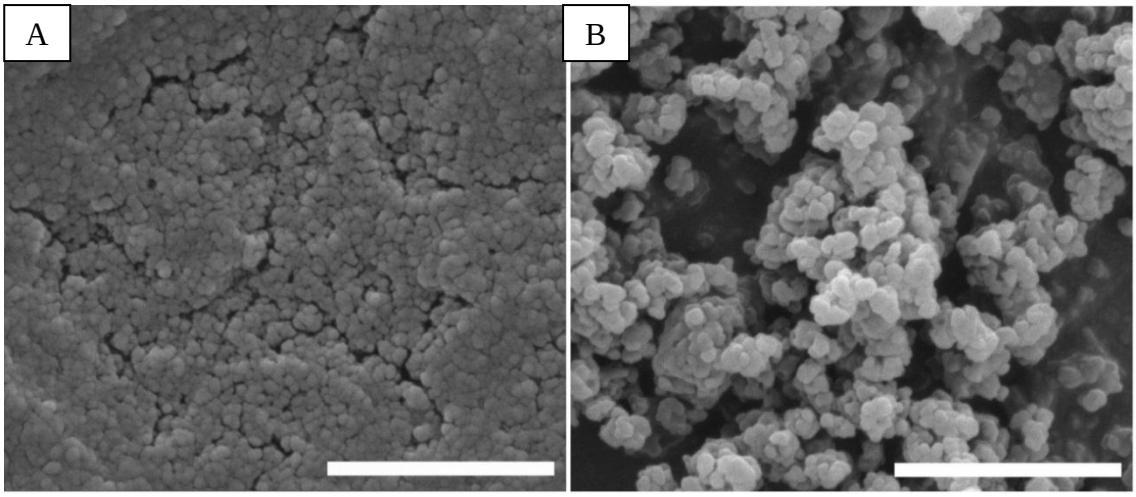


Figure 75. SEM images showing lignin in water immediately prior to the addition of oil to form emulsions. The samples were (A) dried from the lignin dispersion at pH 3 in the pH-modification method (dispersion dried on glass slide for SEM analysis) and (B) dried from the lignin microparticle suspension (suspension centrifuged and the pellet dried and crushed for SEM analysis) (bar scale = 3 μm).

7.3.3.2. Differences between the final emulsions

Along with differences in the size and structure of lignin particles available for droplet stabilisation in the two methods, differences in the properties of the final emulsions may also influence droplet size and stability. Therefore, the final pH and zeta potential of the emulsions were measured to give an indication of the environment that the droplets were in in each method.

Table 30 shows the final pH of emulsions prepared using the pH-modification and FS methods, revealing that the final pH of emulsions prepared using both methods falls between 3 and 4. However, the pHs of the emulsions prepared using the pH-modification method are lower than the pHs of the emulsions prepared using the FS method. The emulsion pH also increases with increasing oil concentration in the pH-modification method. The greater acidity of the emulsions prepared using the pH-modification method is likely to be a result of the greater acidity of the initial lignin dispersion, for which the pH was adjusted to 3, compared to the natural pH of the fresh

lignin suspension of 3.59. The higher acidity of the emulsions prepared using the pH-modification method may play a role in the smaller size and slightly better stability of the oil droplets in these emulsions.

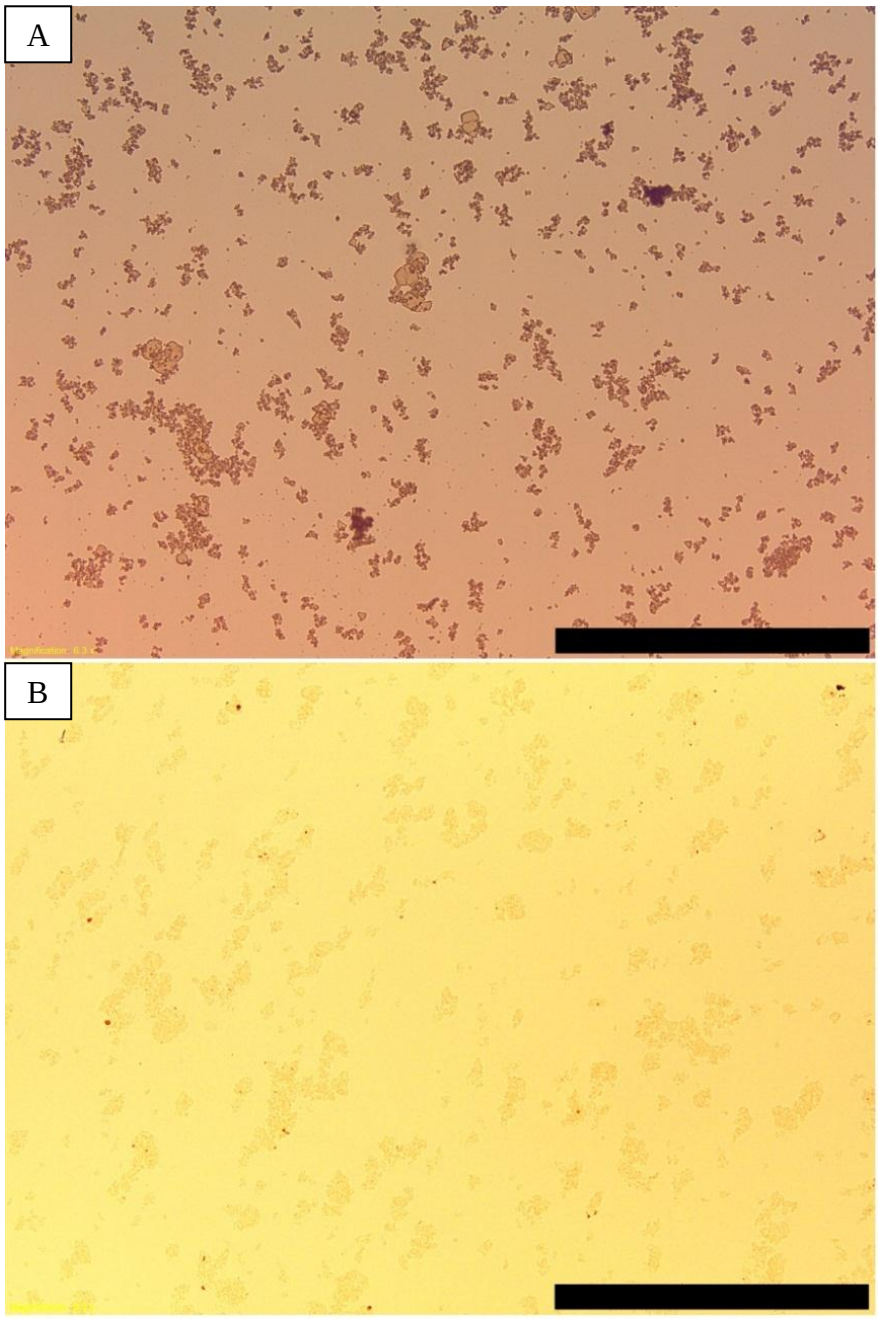


Figure 76. Light microscopy images of lignin microparticle dispersions before mixing with oil in (A) the FS method and (B) the pH-modification method (bar scale = 500 µm).

Table 30. Final pH of emulsions prepared using the pH-modification method and FS method with oil concentrations of 3.8-28.6% (w/w)

Oil concentration (% w/v)	pH	
	pH-modification method	Fresh suspension method
3.8	3.11	3.60
13.8	3.20	3.62
21.9	3.34	3.65
28.6	3.46	3.68

Figure 77 shows the zeta potentials of emulsions containing 3.8 and 28.6% oil prepared using both emulsion preparation methods. The zeta potentials of the four emulsions tested generally fell between -1 and -5 mV. These values are consistent with the zeta potential of the fresh lignin microparticle suspension, which was found to be -5 mV (Chapter Six). Since the lignin particles are only lightly charged, we expect the same for the emulsion droplets coated with this lignin. The lack of charge may be due to the large amount of phenol and hydroxyl groups present in lignin. The lignin may become charged at extreme pH but not at pH 3-4.

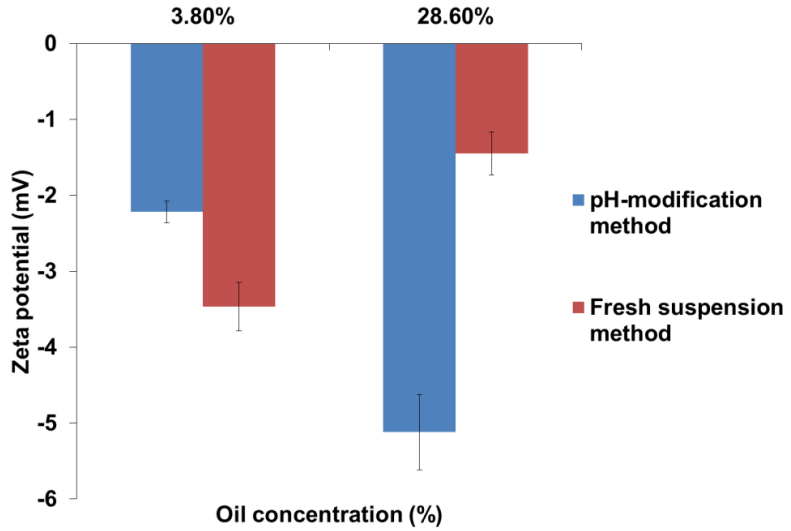


Figure 77. Zeta potential of 3.8 and 28.6% oil emulsions prepared using the pH-modification method (with 0.5% lignin) and the FS method (with 1.39% lignin).

The similarity of the zeta potential values of all emulsions indicates that the state of the droplet surfaces in emulsions produced using different methods was not responsible for the differences in emulsion droplet size and stability.

7.4. Discussion

7.4.1. Emulsion appearance

The FS and pH-modification methods both produced o/w emulsions. The emulsions rapidly separated into a cream or emulsion phase on the top, and a water phase on the bottom. Li et al. (2013) suggest that creaming is typical of Pickering emulsions due to the large droplet size. For both methods of emulsion preparation, as the concentration of lignin increased, the height of the cream layer increased. This is consistent with the work of Gould et al. (2013), Li et al. (2013) and Wei et al. (2012). It is suggested that the increase in creaming stability with particle concentration is a result of increasing surface coverage of oil droplets (Li et al., 2013). Creaming may also be inhibited by the formation of a particle assisted network between oil droplets. The network may incorporate either free particles or particles adsorbed at droplet surfaces (Gould et al., 2013; Li et al., 2013). The height of the cream layer also increased with increasing oil content, however, this was probably due to the increased amount of oil available for emulsification.

Immediately after emulsion manufacture, lignin particles were clearly visible in the emulsions, particularly in those containing a high lignin-to-oil ratio. The presence of these particles may support that part of the stabilisation provided by lignin particles is the formation of a 3D particle network that effectively traps oil droplets and prevents creaming through an increase in the viscosity of the continuous phase. It could be that larger particles form this 3D network, while fine lignin particles go the interface and provide steric stabilisation. Alternatively, the visible particles may be excess particles that do not contribute to any stabilisation of oil droplets. This is supported by the observation of more visible particles in emulsions containing less oil, for which more excess lignin would be expected. Particles were also less discernible in the emulsions prepared using the pH-modification method, in which a lower lignin concentration was present. Either way, it is interesting that the lignin always remains associated with the oil phase, rather than sedimenting to the bottom of the excess water layer.

7.4.2. Effect of lignin and oil concentration

When using the FS method, an increase in lignin concentration or decrease in oil concentration both resulted in a decrease in emulsion droplet size. This was accompanied by an increase in the specific surface area, suggesting that the lignin-to-oil ratio is an important parameter for controlling droplet size. The higher the lignin-to-oil ratio, the higher the interfacial area that can be stabilised by lignin particles and thus the smaller the resulting emulsion droplet size.

When using the pH-modification method, although an increase in lignin concentration caused a decrease in creaming instability, the droplet size was unaffected. This may support the suggestion that part of the ability of lignin to stabilise emulsions is due to the formation of a particle-assisted network, since the reduction in creaming stability was not associated with a reduction in oil droplet size. Similarly, only small increases in droplet size were observed with increases in oil concentration, with the largest increase in droplet size occurring when oil concentration increased from 21.9 to 28.6% (w/w) oil. The lack of reduction in droplet size with increase in lignin-to-oil ratio may indicate that the high-shear rotor-stator mixing was insufficient to reduce droplet size any further. In fact, Gould et al. (2013) and Midmore (1999) found that reductions in emulsion droplet size were limited by the maximum energy supplied by a Silverson mixer. Therefore, perhaps only the stability of emulsion droplets to coalescence can be concluded from this work, rather than the ability of higher lignin concentrations to reduce droplet size and produce emulsions stable to both coalescence and creaming. It is recommended that higher energy emulsification methods such as microfluidisation or high pressure homogenisation are investigated to better understand the efficacy of lignin to stabilise emulsions.

The lack of change in droplet appearance and size with change in lignin-to-oil ratio when analysed by light and confocal microscopy may also indicate that the measured increases in droplet size were due to droplet flocculation. The flocculation may be caused by depletion effects. However, depletion effects would be expected to be greater at lower oil concentration. Flocculation may also be caused by attractive forces between the lignin particles surrounding different droplets. Although the physical lignin barrier prevents droplet coalescence, polymer-polymer interactions, such as hydrophobic

interactions, may cause the droplets to flocculate. Such flocculation may have contributed to the high degree of creaming observed.

7.4.3. Differences between the FS and pH-modification methods

Smaller droplets were produced using the pH-modification than the FS method. For example, the D[3,2] for the emulsion containing 3.8% (w/w) oil was 2.7 μm, compared to 11.6 μm for the same emulsion prepared using the FS method. Moreover, in the pH-modification method, a lignin concentration of only 0.5% (w/v) was used, compared to 1.39% (w/w) in the FS method. It appears that irregularly shaped aggregates of sub-micron lignin particles were present in both methods. However, lignin particles present in the pH-modification method were slightly more polydisperse, with some particles smaller than those present in the fresh lignin microparticle suspension. These smaller particles (~1-2 μm) may enable more interfacial area to be stabilised, resulting in smaller oil droplets. Smaller particles may also pack more efficiently at the oil droplet surface or adsorb more rapidly to the interface, again allowing stabilisation of smaller droplets (Ashby & Binks, 2000).

Differences in the final emulsions may also have contributed to differences in droplet size. In the pH-modification method, flocs of precipitated lignin were formed by reducing the pH of the lignin dispersion to around 3. However, the pH of suspensions used to prepare emulsions in the FS method was ~3.59. Lignin may already have been in a flocculated form at this pH. In fact, Wei et al. (2012) found that both flocs of lignin formed at pH < 3 and lignin particles formed at around pH 4 were able to stabilise o/w emulsions. Gould et al. (2013) found that cocoa particles worked better as single particles than flocs because the acid that induced the flocculation also caused the particle hydrophobicity to change. This provides support for the preparation of stable emulsions using both methods in the current work. However, perhaps the definite formation of flocs at pH 3 in the pH-modification method enabled lower concentrations of lignin to be used and smaller emulsion droplets to be formed compared to the FS method. It could also be possible that the lignin flocs/aggregates in the pH-modification method are more loosely bound and thus individual lignin particles can break off more easily and go to the oil-water interface. The absence of irregularly shaped lignin flocs in the confocal images (Appendix I) may support this idea. It may be argued then that there is little or no point in forming the flocs, however, perhaps the pH-modification

process provides control of particle size, with the initial increase in pH being important for dissolving the lignin so that it can then be re-precipitated in a controlled size range.

Zeta potential measurements revealed that the state of the surface of emulsion droplets prepared by both methods was very similar; the effectiveness of the surface charge to repel other droplets was low in both cases. This was expected since the charge density of lignin surfaces is known to decrease with decreasing pH, due to screening of negative charges by H^+ (Dong, Fricke, Moudgil, & Johnson, 1996).

Although the droplets in the emulsions produced using the FS method had D[3,2] values ranging from ~10-30 μm depending on the oil concentration, these droplets remained stable in terms of droplet size over five months of chilled storage. Some small increases in droplet size were observed; however, these increases were only up to 5 μm and were likely to have been due to measurement variation. Droplets in the emulsions produced using the pH-modification method were also stable to increases in size over the five month storage period. These observations confirm suggestions that particle-stabilised emulsions are extremely stable to droplet coalescence, regardless of large droplet size and creaming instability (Binks, 2002).

7.4.4. Size of the lignin particles used for stabilisation

It is important to note the close agreement in size between the lignin particles present and the resulting oil droplet size in both methods. The D[4,3] value for the lignin particles was in the range ~10-18 μm depending on the method used, and the D[4,3] values of the emulsion droplets produced were in the range ~9.5-34.4 μm. However, it is known that particles must be at least an order of magnitude smaller than the droplets they are stabilising (Dickinson, 2012). It could be possible that a smaller lignin size fraction not detected by static light scattering was responsible for the stabilisation of oil droplets in the emulsions. Gould et al. (2013) found that in the stabilisation of emulsions with cocoa particles, the size distributions of the cocoa particles in aqueous suspension and the emulsion droplets stabilised by the cocoa particles were very similar. However, it was shown by SEM that smaller particles were responsible for droplet stabilisation. In fact, when cocoa particles larger than 2 μm were removed before emulsion formation, stable emulsion droplets were still formed. This indicated that that the larger particles that were of similar size to the oil droplets had little influence on the emulsion droplet size and stability. It was suggested that the larger particles may

influence network formation between the droplets and thus have an impact on the rheological properties of the emulsions. Given the creaming observations, small and large lignin particles may be operating in a similar way to these cocoa particles. Although all lignin particles were ≥ 1 μm, it is unlikely that the 3D network mechanism is the only mechanism by which the particles provide stabilisation. This is because no oiling off was observed in the emulsions, which would have been expected due to more coalescence occurring with this type of stabilisation. There was also little lignin present in the excess water layer, indicating a high level of interaction between the lignin and the oil.

It is recommended that further work be carried out looking at the position and importance of variously-sized lignin particles in emulsions. This could include removing certain size fractions of lignin particles before forming the emulsions and evaluating the effect on droplet size and stability. It could also include developing better microscopy techniques for visualising the position of lignin in the emulsions. Improving the confocal microscopy technique is recommended as a first step before possibly moving on to a technique such as atomic force microscopy to see more detail of the arrangement of particles at the interface. Perhaps even cryo-SEM could help to conclude whether lignin particles are adsorbed at the oil droplet surface or exist in a 3D network between the oil droplets.

It is also recommended that the performance of sub-micron lignin particles as Pickering stabilisers is evaluated. Smaller particles can pack more efficiently around droplets and it is also known that the average size of the stabilising particles should be at least an order of magnitude smaller than the size of the emulsion droplets (Dickinson, 2012; Gould et al., 2013). Thus the sub-micron "primary" lignin particles may enable droplets in the ~1 μm size range to be stabilised. Initial trials in this area were carried out as detailed in Appendix H. Furthermore, it was uncertain whether or not the SDS present in the sub-micron lignin particles used to prepare the emulsions would influence the wettability of the lignin and hence the size and stability of the oil droplets produced. Now that more is understood about emulsion characterisation, the ability of SDS-stabilised sub-micron lignin particles can be determined. The lowest concentration of SDS that still enables sub-micron lignin particles to form in suspension, found to be 0.0625% (w/v), should be used to limit the influence of SDS itself on droplet size and stability.

7.5. Conclusions and recommendations

Both a method based on the pH-responsiveness of lignin and a method utilising fresh lignin microparticle suspensions were able to produce oil-in-water emulsions that were stable to coalescence for at least five months of chilled storage.

Although the pH-modification method used a lower lignin concentration of 0.5% compared to 1.39% for the FS method, it produced smaller emulsion droplets than the FS method. Depending on the oil concentration, D[3,2] values for emulsion droplets prepared by the pH-modification method ranged from 2.7-11.9 μm. For the FS method, this range was 11.6-31.2 μm. It is not clear why this difference occurs, since the lignin in both methods was found to be of similar size and structure immediately before emulsion formation in both methods. It may be that the aggregates of lignin are held together more strongly in one method, influencing whether lignin aggregates or single particles that adsorb at the interface.

The oil droplets in the emulsions were similar in size to the lignin particles or flocs. The similarity in size may suggest that part of the stabilisation of oil droplets by lignin is provided by lignin particles forming a 3D network that traps particles and prevents creaming. Smaller lignin particles may still adsorb at the interface and provide a steric barrier against coalescence. It is recommended that more knowledge is gained on the fate of the lignin particles or flocs after emulsification. For example, improvement to the confocal microscopy method may better show the position of lignin in the emulsions. Additionally, the evaluation of sub-micron lignin particles as Pickering stabilisers is recommended.

When using the pH-modification method, oil droplet size did not decrease with increasing lignin-to-oil ratio. The lack of decrease in size may indicate that the Silverson high shear mixer used for emulsification was not sufficient to reduce droplet size any further. To determine whether an increase in the lignin-to-oil ratio can produce smaller oil droplets, it is recommended that alternative mixing types are used, such as high pressure homogenisation.

Overall, many opportunities exist for more work concerning the use of lignin particles as Pickering stabilisers. Although the initial work detailed in this chapter is positive, it

is recommended that the emulsions are examined and characterised more thoroughly to better gauge the effectiveness of lignin as a Pickering stabiliser.

Chapter Eight. Overall Discussion and Conclusions

Interesting results in a range of areas have been obtained in this work, illustrated by the key steps and major outcomes in the summary diagram in Figure 78. It is now important to assess whether the original research questions were answered, highlight key successes, discuss interesting observations and outline recommendations for future work.

8.1. Can food-grade lignin be extracted from wood?

A vessel capable of small-scale organosolv pulping of willow chips was commissioned in this work. A Parr 4767 reactor vessel with a maximum working pressure at 350°C of 207 bar and maximum working temperature when fitted with PTFE confined flat gasket of 350°C was obtained. The reactor vessel was modified to enable pressure inside the vessel to be measured and solvent liquor to be discharged from the vessel while still at high temperature and pressure to minimise re-precipitation of lignin on the wood chip surfaces. A filter element enabled liquor to be removed from the vessel separately to the residual wood chips. The vessel was successfully used for many lignin extractions in this work. However, ultimately, solvent liquor was unable to be removed from the vessel before cooling due to continual and accumulated blockage of the filter element. Therefore, solvent liquor was only removed from the vessel after cooling. This approach was also used by Quesada-Medina et al. (2010), Pan et al. (2006) and Sánchez et al. (2010) and thus was considered appropriate. However, this method may have reduced lignin yield since lignin may have re-precipitated on the wood chips during cooling. Cooling the vessel naturally before opening also introduced approximately an extra three hours to the extraction each time, along with more intermediate steps of manually filtering the solvent from the residual wood chips. Therefore, benefits in terms of yield, time and labour could be gained by improving the solvent release mechanism.

Conditions for extracting lignin of high yield and purity from *S. purpurea* were established using a central composite design. An ethanol concentration of 60% (v/v), an extraction time of 132 minutes and extraction temperature of 195°C were found to be the optimal conditions in terms of high lignin yield and purity. The yield obtained using these optimal conditions was 47.7% (w/w) of total available lignin and the purity, when extracted from untreated willow chips, was found to be 96.53 ± 0.10 % (w/w).

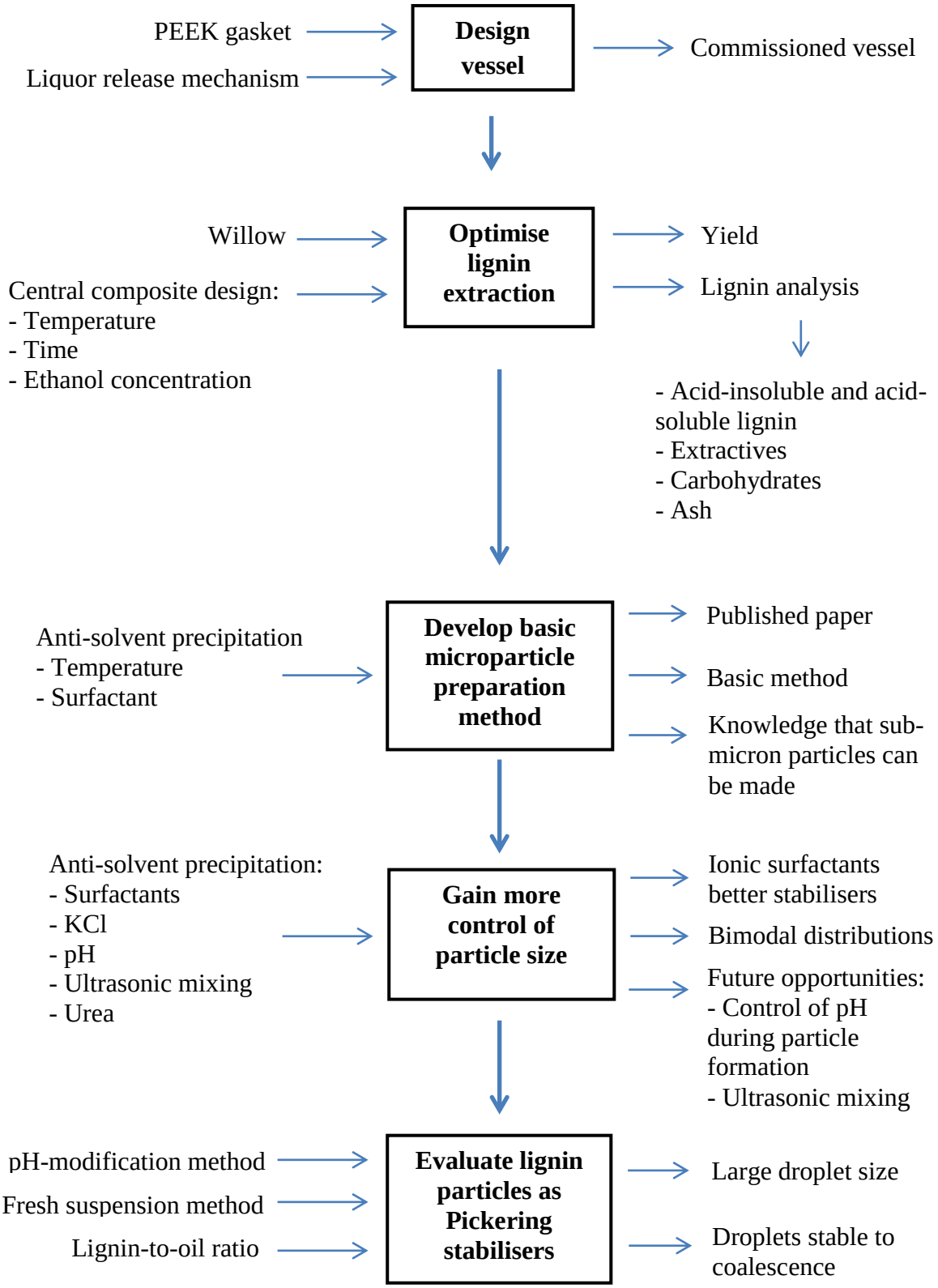


Figure 78.Major steps and outcomes of project

8.2. Can the structure and functional properties of lignin be controlled?

Answering this research question formed the major focus of the thesis. A basic lignin microparticle preparation method was developed based on the anti-solvent precipitation method used by Patel et al. (2010) for the preparation of zein colloidal particles. The method involves the addition of an aqueous ethanolic solution of lignin to a much larger volume of distilled water under simple stirring, leading to the precipitation of lignin particles.

The water temperature used to precipitate lignin during the anti-solvent precipitation method influenced particle size and morphology. Particles became increasingly fused and thus had larger measured particle size as the temperature of precipitation increased over the temperature range 4-80°C. For water temperatures ≥ 60°C, particles were completely fused into monolithic masses, which rapidly sedimented. The increased agglomeration may have been due to the faster rate of solvent evaporation at higher temperatures, which reduced the time available for the initial lignin dispersion to be sheared into smaller droplets. Increased hydrophobicity at higher temperatures may also have contributed to the agglomeration. However, although it is known that organosolv lignins are relatively hydrophobic (Lora & Glasser, 2002; Nakagame, 2010), the hydrophobicity of the lignin extracted from willow in this work needs to be determined. The agglomeration may also have been caused by softening of the lignin due to lowering of its glass transition temperature in the presence of a large volume of water during the anti-solvent precipitation process. Finally, "diffusion limited coalescence" (DLC), which can occur during rapid precipitation of low molecular mass polymers that behave like liquids, may also be responsible for the trend observed. However, the diminishing boundaries between primary particles with increasing temperature may align better with the idea of increased hydrophobic interactions between particles at higher temperature.

Although FTIR analysis revealed that changes in functional groups did not occur with increasing temperature, it would be interesting to determine whether the conformation of the lignin changes with temperature. Techniques that allow spectra to be obtained over a range of temperatures, such as solid state NMR or an infra-red microscope with a temperature stage are recommended for this purpose. It could also be useful to analyse

liquid samples such as mixtures of lignin extraction liquor and water with DSC to determine whether the presence of water and/or ethanol caused a depression of glass transition temperature and thus the agglomeration of lignin particles.

It is possible to manipulate particle size using ionic surfactants. SDS and CTAB were able to stabilise spherical, monodisperse lignin particles in the sub-micron size range (~0.1-0.2 μm). However, Tween 20 and sucrose ester were not effective, indicating that ionic surfactants provide the most effective stabilisation. It may be that the electrostatic repulsion provided by the charged surfactants provides the most effective stabilisation, or that the preparation temperature of 60°C reduced the hydration of the polyoxyetheylene chains in Tween 20. Sucrose ester may simply be a better agent for reducing interfacial tension during particle formation rather than for providing stability against aggregation.

SDS, also known as sodium lauryl sulfate (SLS), is only permitted in certain foods in very restricted concentrations and for specific purposes. For example, among other applications, the FDA Code of Federal Regulations lists SLS as a permitted surfactant in fumaric acid-acidulated dry beverage bases in an amount not exceeding 25 ppm of the finished drink. CTAB is not permitted for use in foods. Thus although good model anionic surfactants for this thesis project, SDS and CTAB may not be suitable to stabilise lignin for all foods – a more generally permitted alternative may be required. It is recommended that food-grade ionic surfactants, such as sodium stearoyl lactylate, are evaluated for the stabilisation of lignin microparticles in future work.

The electrostatic mechanism of stabilisation by SDS was confirmed by the ability of added KCl and $CaCl_2$ to destabilise lignin particles. However, control of particle size through modification of salt concentration, pH and even surfactant concentration was limited. Rather than the production of particles in a range of sizes as a result of these modifications, generally only the relative volume of particles in two modal distributions (~0.1-0.2 μm and ~1-100 μm) appeared to change.

The addition of KCl before particle formation produced either unaltered primary particles or increasingly fused particles, falling in either of the modal distributions, depending on the KCl concentration. On the other hand, addition of KCl after particle formation caused aggregation of primary particles, with particles still falling in either of the two modal distributions, depending on the KCl concentration. This is controlled by

the competition between the adsorption of surfactants and the coalescence of the lignin material (Schulze (1882) and Hardy (1990), as cited by Butt et al., 2003).

Reduction of suspension pH to 1.5 after microparticle formation caused aggregation of SDS-stabilised lignin particles. Again, two modal distributions of either aggregated or non-aggregated particles were evident, suggesting that aggregation kinetics prevent the production of aggregates in an intermediate size range. It is recommended that further work be carried out in which pH is controlled throughout the particle formation process. This may influence the ability of SDS or other ionic surfactants to stabilise particles or even influence the rate or extent of lignin precipitation, possibly enabling narrowly distributed particles in the 1-10 μm size range to form. Use of different mixing types may also enable particles in the intermediate size range to be produced. Further work with the ultrasonic mixer is recommended. Other processes such as jet-break up, microfluidisation or supercritical emulsions extraction, as described in the literature review, may provide better control of particle size.

Lignin particles in the sub-micron size range produced in this work could have application as fat mimetics since they are just within the suggested 0.1-0.3 μm size range for microparticulate fat mimetics. For example, Simplesse® (CPKelco, Atlanta, Georgia), a fat mimetic made from spherical 0.1-0.2 μm microparticulates of gelled whey protein, mimics the creaminess of fat in foods such as ice cream, frozen desserts, cheese, dressings, sauces, soups and baked goods (Gómez, 2008; Setser & Racette, 1992; Singer, 1996).

However, the stability of lignin particles of this size to drying and when mixed with other food ingredients would need to be established. It is recommended that spray-drying and freeze-drying are evaluated for the ability to dry lignin microparticles or particle aggregates to a constant size. Unless applied as the wet suspension, the re-dispersibility of the particles will also be important to improve. Finally, safety and consumer acceptance of lignin microparticles would be important to evaluate before these particles can be added to foods. In particular, at 0.1-0.2 μm, the particles are almost in the nano-scale and thus there may be additional safety concerns if the particles remain in this size range in foods. Currently, there are limited commercialised applications of nanotechnology in foods (World Health Organisation (WHO), 2014) and scientific advice surrounding the use of the technology is still under development.

Another aspect that may be important to consider is that organosolv lignin is known to consist of low molecular weight fragments. Baurhoo, Ruiz-Feria and Zhao (2008) suggest that these fragments are digestible by both ruminant and monogastric animals. This may indicate that unlike native lignin, which is considered to be part of dietary fibre, organosolv lignin may be absorbed into the blood. However, this does not necessarily mean that the lignin will contribute any energy. In fact, it may even have a more positive effect such as increasing the antioxidant level of the blood. Thin layer chromatography may help to firstly confirm that the lignin has been hydrolysed to mono-phenolic fragments and secondly to identify what fragments are present. The likelihood of absorption into the blood and any resulting effects can then be determined before microparticles prepared from this lignin are approved for use in foods.

8.3. Can the lignin microparticles act as Pickering stabilisers of emulsions?

The suggested contact angle for organosolv lignin of between 70-80° (Nakagame, 2010), indicated that lignin microparticles may have use as Pickering stabilisers of emulsions. Congruously, lignin-stabilised emulsions prepared using both the pH-modification method of Wei et al. (2012) and the method using lignin microparticles in the form of freshly prepared suspensions were stable to coalescence for at least five months of chilled storage. This stability supports the notion that Pickering emulsions are extremely stable to coalescence (Binks, 2002).

Creaming instability was observed in emulsions prepared using both methods. Creaming stability increased as the lignin-to-oil ratio increased, possibly due to an increase in particles available to form a 3D-network between oil droplets to reduce creaming. It is interesting to note that although all emulsion samples exhibited creaming instability, lignin particles always remained in the oil phase and did not sediment. The dispersed particles or aggregates before emulsion formation were approximately 10 μm and sedimented upon standing. The absence of sediment lignin particles in the emulsions may indicate that the aggregates were broken up during emulsion formation and therefore were too small to sediment. However, larger lignin particles were visible in some of the cream layers, indicating that the lignin remains associated with the oil phase.

The pH-modification method produced emulsions with D[3,2] size ranging from 2.7 to 11.9 μm for oil concentrations ranging between 3.8 and 28.6%. In comparison, the FS method resulted in D[3,2] values ranging from 11.6 to 31.2 μm. Thus the pH-modification method produced smaller emulsion droplets. The presence of a fraction of smaller lignin particles or the easier break-up of flocs may contribute to the better performance of the pH-modification method; however, it is not yet clear why the differences occur.

Changes in droplet size with increase in lignin-to-oil ratio were generally small for the pH-modification method, perhaps due to limitation in the energy provided by the Silverson high shear mixer to form small oil droplets in the first place. It is recommended that higher emulsification speeds are studied to determine whether smaller oil droplets can be produced and stabilised by lignin particles. For example, higher speeds may be obtained using a high pressure homogeniser. It would also be interesting to determine the shelf-stability of lignin-stabilised emulsions at ambient temperature, since this would provide further indication of possible food applications. Analysis of whether the lignin exists as a monolayer, in multiple layers, or in clumps on the surface of oil droplets may also provide more information on the effect of lignin-to-oil ratio. Determination of the hydrophobicity of lignin using measurement of contact angle may also help to explain how lignin stabilises oil droplets. Finally, there may be better methods of forming the emulsion or getting the lignin to the surface of the oil droplets. For example, emulsions could first be prepared with traditional surfactants to give small and monodisperse oil droplets. Particle-stabilised emulsions could then be formed by sequentially replacing surfactants at droplet surfaces with particles using dialysis. This method may improve the monodispersity of emulsions (Giermanska-Kahn, Schmitt, Binks, & Leal-Calderon, 2002).

Determination of the hydrophobicity or wettability of the lignin is a critical next step. Some work was carried out in this area; however, a reliable value was not obtained. It is recommended that the measurement method is improved. Nakagame (2010) prepared a thin film of isolated lignin using a spin coater, while Heiss-Blanquet, Zheng, Ferreira, Lapierre, and Baumberger (2011) prepared sample pellets using an infrared press, which applied a pressure of 10 tonnes to the ground sample for five minutes. Methods similar to these should be looked at for preparing samples of lignin in this work. Contact angle

can then be measured by analysing changes in shape of a drop of water applied to the sample surface (Heiss-Blanquet et al., 2011; Nakagame, 2010).

Finally, the size of lignin particles used to prepare the emulsions will be important to better control in future work. Since it is known that particles should be at least an order of magnitude smaller than the oil droplets that they stabilise, it is not yet clear how the lignin particles and flocs in both methods were able to stabilise some of the smaller oil droplets. However, the requirement for particles at least a magnitude smaller than the emulsion droplets presents an opportunity for preparation of smaller lignin-stabilised oil droplets in the future. The use of fresh suspensions of sub-micron (i.e. stabilised) lignin particles may enable smaller oil droplets to be stabilised due to more efficient packing of the smaller particles at the interface. Initial work in this area was not progressed due to difficulties separating the effect of the SDS coating on the particles from the effect of the lignin particles. The SDS may also increase the wettability of the particles, which may reduce the strength with which the particles are held at the interface. However, even if any surfactant present on the particle surface contributes to droplet stabilisation, this may still enable small droplets that are extremely stable to coalescence to be produced, which may be beneficial in the food industry.

8.4. Can lignin microparticles be used in other food applications?

Investigating other applications of lignin in foods will be critical for the further development of lignin microparticles as food ingredients. Along with the evaluation of lignin as an emulsion stabiliser, preliminary trials on the incorporation of lignin particles into processed cheese spread and chocolate muffins were also carried out. The trials are not covered in this thesis because the lignin was of unknown particle size. Replacing up to 17% of the oil in processed cheese, or 5% of the total cheese formulation, with lignin did not affect the meltability of the cheese. In chocolate muffins, increasing replacement of oil with lignin increased hardness and decreased springiness and cohesiveness. Therefore, further work is necessary to determine the best route for incorporation of lignin into baked goods. Furthermore, the processed cheese became increasingly darker brown in colour as the concentration of added lignin increased. This indicates that the colour of lignin needs to be improved. A better understanding of the chemistry of the extracted lignin may enable colour, and also taste and odour, to be controlled better.

Finally, lignin particles may also have application as foam stabilisers. It is known that hydrophilic yet close to intermediate hydrophobicity particles can stabilise water-in-oil emulsions or aqueous foams (Binks, 2002). Thus lignin may be useful as a stabiliser of food foams.

8.5. Summary of recommendations

Numerous recommendations have been made throughout the thesis. Practical, obvious improvements have been identified, along with recommendations regarding new areas to explore. Recommendations specific to each research question can be taken from the experimental chapters and from sections 8.1 to 8.4 above, however, the main recommendations are summarised here:

Lignin extraction

- Modifications to the vessel to allow liquor to be removed from the vessel before cooling may increase lignin yield.
- Modifications to the extraction conditions, such as the use of acid catalysts, may enable higher lignin yield to be obtained.

Microparticle preparation

- DSC analysis of liquid lignin samples may determine whether plasticisation of lignin reduces glass transition temperature and influences agglomeration during particle formation.
- Adjustment and control of pH throughout anti-solvent precipitation may enable the production of particles in different size ranges.
- Other methods of mixing during anti-solvent precipitation, such as ultrasonic mixing, may enable the production of particles in different size ranges.
- The stability of particles during drying, along with re-dispersibility in different solvents must be determined.
- More food-grade surfactants for lignin microparticles must be explored.
- Understanding the aggregation kinetics of lignin particles may enable more control of particle size.

Pickering stabilisation

- More extensive characterisation of emulsions, including more thorough determination of the location of lignin in the emulsions, is highly recommended.
- Higher energy emulsification techniques may enable the production of smaller emulsion droplets.
- The use of sub-micron lignin particles for stabilisation of emulsions may enable the stabilisation of smaller droplets. The impact of any surfactant coating on particle wettability is critical to determine.

Food applications

- Lignin may also be effective as a Pickering stabiliser of foams. The production of lignin microparticles in a variety of shapes may be important for this and other applications.
- Determination of the molecular weight and specific fragments present in the extracted lignin may indicate whether it is non-digestible and what other effects it may have in the body. Knowledge in this area would be critical for the development of lignin microparticles as a non-caloric fat mimetic.
- Sensory attributes such as the colour, taste and odour of the lignin will need to be characterised before food applications can be considered.

Given that yield of extracted lignin is likely to be controlled by biorefining processes, the recommendations concerning lignin yield are purely practical and not critical to the main focus of this thesis. This leaves three main areas that are considered to be important for exploration in the future:

- Lignin characterisation – including molecular weight, determination of lignin fragment types and contact angle.
- Control of particle size – including the investigation of more food-grade surfactants, ultrasonic mixing and controlled pH changes during anti-solvent precipitation.
- Food applications – including further investigation into Pickering emulsions, foam stabilisation and fat mimetic applications.

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Appendix A

Figure A1 and A2 below show the Manufacturer's Data Report for the Parr 4767 vessel supplied. Figure A3 shows the technical drawing supplied with this vessel.

334865 Order #
3508 PO #

FORM U-1A MANUFACTURER'S DATA REPORT FOR PRESSURE VESSELS
(Alternative Form for Single Chamber, Completely Shop or Field Fabricated Vessels Only)
As Required by the Provisions of the ASME Boiler and Pressure Vessel Code Rules, Section VIII, Division 1

1. Manufactured and certified by **Parr Instrument Company, 211 - 53rd Street, Moline, Illinois, 61265, USA** (Name and address of manufacturer)
2. Manufactured for **John Morris Sci Ltd, Unit 2, 74-80 Wellesley Street, Auckland, NEW ZEALAND** (Name and address of purchaser)
3. Location of Installation **John Morris Sci Ltd, Unit 2, 74-80 Wellesley Street, Auckland, NEW ZEALAND** (Name and address)
4. Type **Vertical** (Horizontal or vertical, tank) **4760-1012-21892** (Manufacturer's serial number) - (CRN) **4766 Sheet 10** (Drawing number) **2550** (National Board number) **2011** (Year built)
5. The chemical and physical properties of all parts meet the requirements of material specifications of the ASME BOILER AND PRESSURE VESSEL CODE. The design, construction, and workmanship conform to ASME Rules, Section VIII, Division 1 **2010** (year) to **N/A** (Addenda, if applicable (Date))
2155, 2156-1 (Code Case numbers) - (Special Service per UG-120(d))
6. Shell: **SA479 T316SS** (Material spec. number, grade) **0.249 in** (Nominal thickness) **0.001 in** (Corr. allow.) **2.50" (ID)** (Inner diameter) **8.00"** (Length (overall))
7. Seams: **None** [Long. (welded, dbl., sngl., lap, butt)] - R.T.(Spot or Full) **100** Eff.(%) - (H.T. temp) - Time (hr) **None** [Girth. (welded, dbl., sngl., lap, butt)] - [R.T. (spot or full)] **100** Eff(%) - No. of Courses
8. Heads: (a) Material **SA479 T316SS** (Spec. no., grade) (b) Material **Integral with shell** (Spec. no., grade)

	Location (Top, Bottom, Ends)	Minimum Thickness	Corrosion Allowance	Crown Radius	Knuckle Radius	Elliptical Ratio	Conical Apex Angle	Hemispherical Radius	Flat Diameter	Side to Pressure (Convex or Concave)
(a)	Top	0.250"	0.021"	N/A	N/A	N/A	N/A	N/A	2.49"	N/A
(b)	Bottom	0.500"	0.025"	N/A	N/A	N/A	N/A	N/A	2.50"	N/A

If removable, bolts used (describe other fastenings) **Split ring SA193-B7 with six 3/8-24 thd bolts** (Material spec. number, grade, size, number)

9. MAWP **3000 psi** (Internal) **N/A** (External) at max. temp. **662 °F** (Internal) **N/A** (External)
Min. design metal temp. **-20 °F** at **3000 psi** Hydro, pneu., or comb. test pressure **HYDRO at 4800 psi**
Proof test -

10. Nozzles, inspection and safety valve openings:

Purpose (Inlet, Outlet, Drain, etc.)	No.	Diameter or Size	Type	Material		Nozzle Thickness		Reinforcement Material	Attachment Details		Location (Insp. Open.)
				Nozzle	Flange	Nom.	Corr.		Nozzle	Flange	
RD	1	5/8-18	THD		SA479 T316SS				Screwed		Head a
T/C	1	1/8"	NPT		SA479 T316SS				Screwed		Head a
I/O	2	1/8"	NPT		SA479 T316SS				Screwed		Head a

11. Supports: Skirt **No** (Yes or no) Lugs - (Number) Legs - (Number) Other - (Describe) Attached - (Where and how)
12. Remarks: Manufacturer's Partial Data Reports properly identified and signed by Commissioned Inspectors, have been furnished for the following items of the report:
N/A (Name of part, item number, Manufacturer's name and identifying stamp)
Laboratory Pressure Vessel:
Head marked: 816HC45-334865-T316-120410-4760-1012-21892
Cylinder marked: 452HC2-334865-T316-120410-4760-1012-21892 Closure marked: 113010
Additional Remarks - See Attached U-4...

CERTIFICATE OF SHOP/FIELD COMPLIANCE
We certify that the statements made in this report are correct and that all details of design, material, construction, and workmanship of this vessel conform to the ASME BOILER AND PRESSURE VESSEL CODE, Section VIII, Division 1. "U" Certificate of Authorization No. **10187** expires **08/19/2012**
Date **01/24/2011** Co name **Parr Instrument Company** (Manufacturer) Signed [signature] (Representative)

CERTIFICATE OF SHOP/FIELD INSPECTION
Vessel constructed by Parr Instrument Company at 211 - 53rd Street, Moline, Illinois, 61265, USA I, the undersigned, holding a valid commission issued by The National Board of Boiler and Pressure Vessel Inspectors and/or the State or Province IA, IL, WI and employed by HSB CT of Hartford, CT have inspected the component described in this Manufacturer's Data Report on January 24, 2011 and state that, to the best of my knowledge and belief, the Manufacturer has constructed this pressure vessel in accordance with ASME BOILER AND PRESSURE VESSEL CODE, Section VIII, Division 1. By signing this certificate neither the Inspector nor his/her employer makes any warranty, expressed or implied, concerning the pressure vessel described in this Manufacturer's Data Report. Furthermore, neither the Inspector nor his/her employer shall be liable in any manner for any personal injury or property damage or a loss of any kind arising from or connected with this inspection.
Date 01/25/2011 Signed [signature] (Authorized Inspector) Commissions 10744A, IA 251, IL1496, WI100133 (National Board (incl. endorsements), State, Province and number)

1283281 exe: v4.8.43 Form and version: U1A-17

Figure A1. Manufacturer's Data Report for the Parr 4767 reactor vessel as supplied (page 1 of 2).

FORM U-4 MANUFACTURER'S DATA REPORT SUPPLEMENTARY SHEET

As Required by the Provisions of the ASME Boiler and Pressure Vessel Code Rules, Section VIII, Division 1

1. Manufactured and certified by Parr Instrument Company, 211 - 53rd Street, Moline, Illinois, 61265, USA
(Name and address of Manufacturer)

2. Manufactured for John Morris Sci Ltd, Unit 2, 74-80 Wellesley Street, Auckland, NEW ZEALAND
(Name and address of Purchaser)

3. Location of installation John Morris Sci Ltd, Unit 2, 74-80 Wellesley Street, Auckland, NEW ZEALAND
(Name and address)

4. Type

Vertical	N/A	4760-1012-21892
(Horizontal, vertical, or sphere)	(Tank, separator, heat exch., etc.)	(Manufacturer's serial number)

-	4766 Sheet 10	2550	2011
(CRN)	(Drawing number)	(National Board number)	(Year built)

Additional Remarks:

Vessel tested vertically. No impact test per UHA 51(d). Safety relief device installed by others.

Certificate of Authorization: Type "U" No. 10187 Expires 08/19/2012

Date 01/24/2011 Name Parr Instrument Company (Manufacturer) Signed (Representative)

Date 01/25/2011 Name (Authorized Inspector) Commissions: 10744A, IA1251, IL1496, WI100133 (National Board (incl. endorsements), State, Province and number)

1283281 exe: v4.8.43 NB67 Rev. 5

Figure A2. Manufacturer's Data Report for the Parr 4767 reactor vessel as supplied (page 2 of 2).

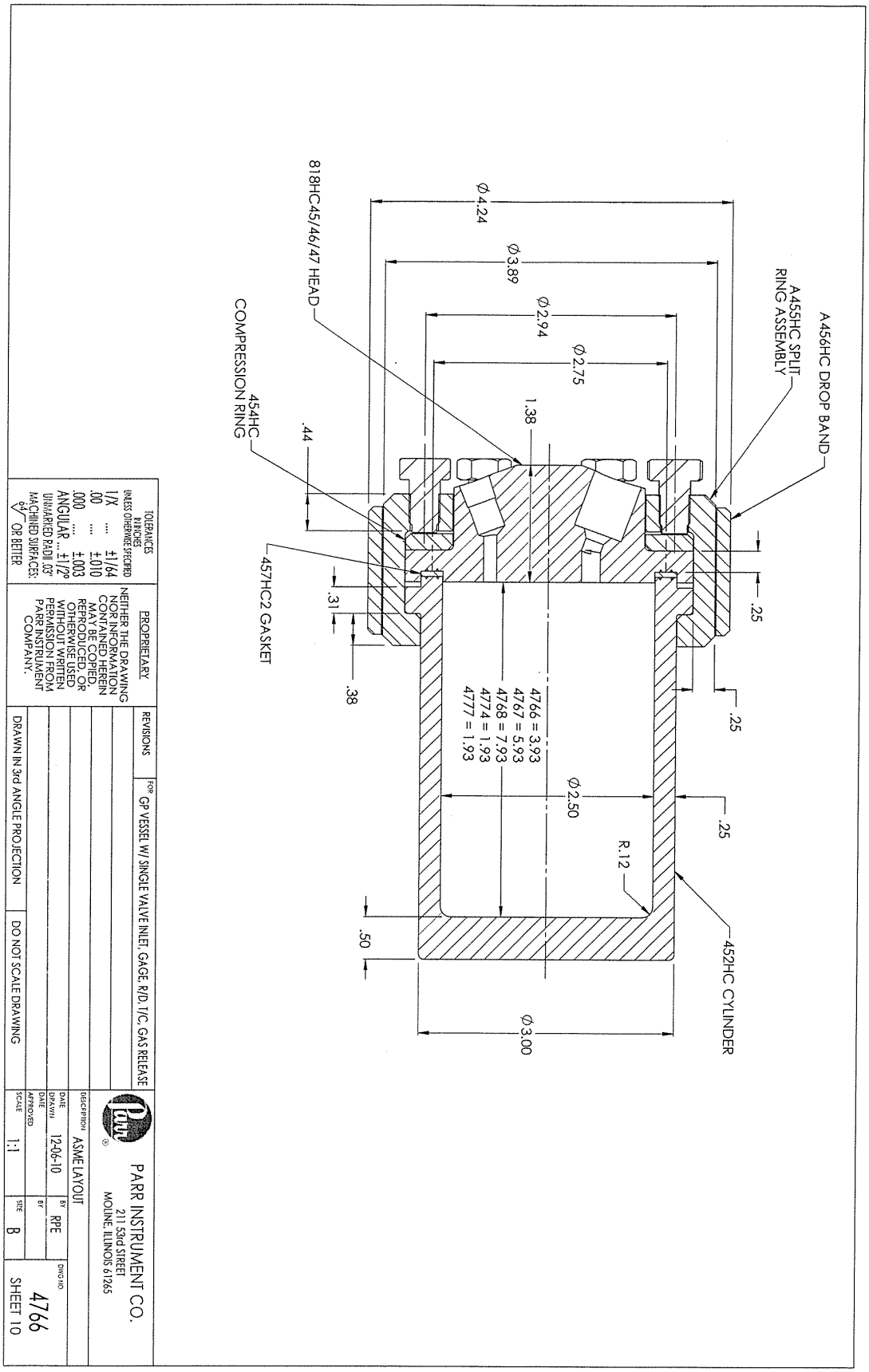


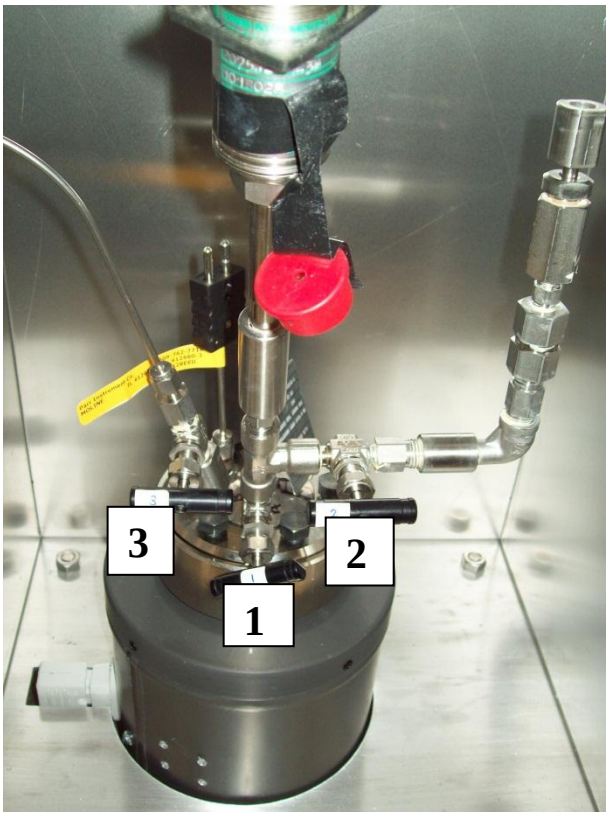
Figure A3. Technical drawing for Parr 4767 reactor vessel

Appendix B

This Appendix contains the Safe Operating Procedures document prepared for the modified vessel.

Safe Operating Procedures

Name / Description: Parr high temperature-high pressure extraction vessel



Hazard	Significant Hazard	Effect of Hazard	Safety Measures Required
High temperature	Yes	Burns	Heat proof gloves, face shield and "hot surface" sign in front of vessel
High pressure	Yes	Explosion Leak	Heat proof gloves, face shield, stainless steel discharge tube for burst release disc, cooling coil to condense leaking vapours, monitor pressure, ensure pressure is released before opening vessel and "high pressure" sign in front of vessel
<u>If ultrasonic mixer used:</u>			
Noise	Yes	Ear damage	Hearing protection for operator and others in pilot plant
Transducer tipping over	No	Crushing, spillage	Ensure stand is on flat surface, clear surrounding area
High temperature of the probe	Yes	Burns	Leave probe to cool before touching
<u>If Silverson used:</u>			
Moving rotor	Yes	Tangling of hair or fingers	Hair net, keep hands away from rotor when operating

Operating Procedures / Safety Procedure

1. Fill vessel with contents. Never fill vessel to more than 2/3 of its available free space.
2. Before closing the vessel, examine the head seal (flat PEEK gasket) carefully to be sure that it is in good condition. The seal should not have any nicks or be hardened discoloured or deformed. Examine the mating surfaces on the cylinder and head to be sure they are clean and free from burrs; then set the head on the cylinder.
3. To close the vessel, put the two split ring halves around the head and cylinder flanges, fasten the latches or tighten the bolts*. Tighten the compression bolts using a criss-cross pattern, applying a firm but hard pull to each screw. Use a torque wrench to apply 25-ft lbs to each compression bolt. Let the vessel stand for about five minutes after the initial tightening, then tighten again to

25-ft lbs.
4. Mount the vessel in the heating mantle within the safety cage. Connect the burst release disc outlet to the stainless steel pipe end in the safety cage using a wrench. Ensure that the bucket that the stainless steel pipe flows into contains sufficient water to cover the end of the pipe.
5. Ensure product release valve (valve 2) and gas inlet valve (valve 3) are closed and valve to pressure transducer (valve 1) open.
6. If gas is to be applied to the vessel, connect gas inlet hose and tighten using wrenches.
7. Connect temperature and pressure communication cables (these must always be connected to the transducer before the power is switched on).
8. Slide the clear front safety shield into place in the safety cage.
9. Turn on reactor controller, set set-point temperature and start heating (use heater II switch if heating to temperature > 175°C).
10. Ensure vessel reaches set-point temperature.
11. Monitor vessel over time by monitoring the pressure every hour.
Emergency Procedures
If pressure in vessel exceeds 170 bar, shut off power to the vessel, clear people from area and wait for the vessel to cool.
If a leak occurs, shut off power to the vessel.

* Routinely inspect the bolts on split ring closures for lubrication and cleanliness. These screws should not be allowed to dry because the threads will seize. Regularly apply Parr high temperature anti-seize lubricant before this happens.

NPT (National Pipe Taper) threads should not be disassembled any more than necessary. It will become increasingly difficult to maintain a tight seal with these tapered threads if the joint is made and repeatedly broken.

Do not use oil or anti-seize lubricant on threads or fittings if the vessel is to be used with oxygen

Prepared by: Hayley Stewart

Date Prepared: 26/11/2012

Reviewed by:

Date reviewed:

Version Number:

Appendix C

This Appendix provides details of the preparation of willow for lignin extraction.

Willow receipt

560 stakes (1 m tall) of *Salix purpurea* 'Pohangina' were received from Akura Nursery in Masterton. The stakes were stored in the Pilot Plant Loading Bay overnight.

Willow steaming and debarking

Each stake was cut in half using a band saw. The stakes were loaded into a steam jacketed vessel in batches of approximately 20 stake pieces, stacked as shown in Figure C1(A) below. Each batch was covered and steamed for 10 minutes. The stakes were removed from the pan immediately after steaming and the bark stripped by hand. The resulting stems were smooth and seemed to be consistently debarked. The stems were then stacked on the shelves in the Pilot Plant Loading Bay and left for 2-3 days (Figure C1(B)). This was not ideal timing as the stems suffered some drying. However, the band-sawing and debarking took longer than expected.

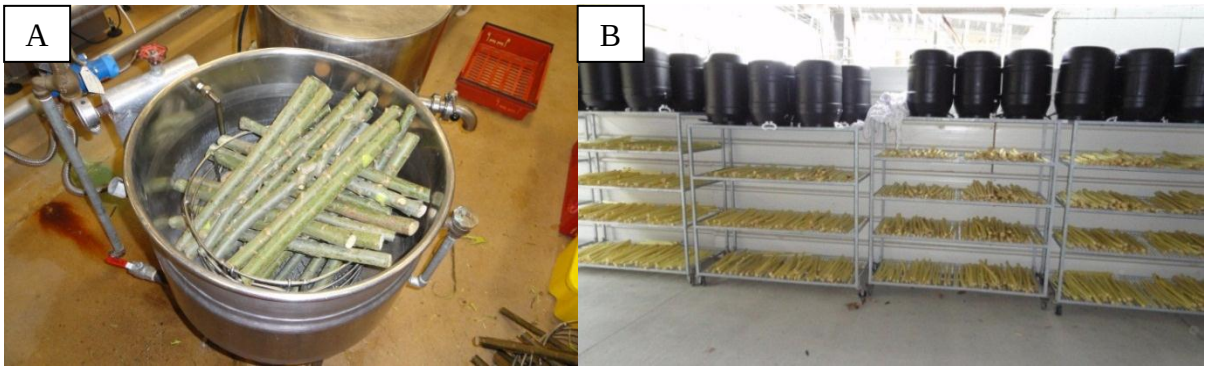


Figure C1. (A) Willow stakes loaded in steam jacketed vessel and (B) Storage of debarked stems on shelves (outside but undercover) for 2-3 days.

Willow Chipping

The debarked stakes were chipped using a Hansa Model C6 chipper (Figure C2). A square metal chute was placed down the centre of the inlet chute of the chipper to ensure that the stems hit the blades straight on. This was to the avoid shard-like chips that were produced when the stakes were simply let go down the chute. Any stems that

were too large or bent to be fed through the inner metal chute were fed between the inner metal chute and the main chute. The chips were collected into a large cardboard box. To avoid loss of chipped material between the outlet chute of the chipper and the cardboard box, a large sack was tied around the outlet chute and hung into the cardboard box. Chipping was completed over a period of three days. Once chipped, the material was stored in 100 L storage drums in a freezer at -25°C.

Figure C2. Use of chipper to obtain chipped willow, showing metal chute placed down the centre of the inlet chute.

Willow Chip Fractionating

The chips were fractionated into different size fractions to ensure that when chips were subsampled for extraction runs during the project, consistently sized chips were used to increase the consistency of the extracted lignin. Size fractionation was achieved using an Air Screen Cleaner and Indent Cylinder Separator.

Using two screens and an air flow, the air screen cleaner separated the wood chips into five different size fractions. Figure C3 below shows the air screen cleaner. Figure C4 then shows the inner set-up of the machine and the fractions produced.

Figure C3. Air screen cleaner.

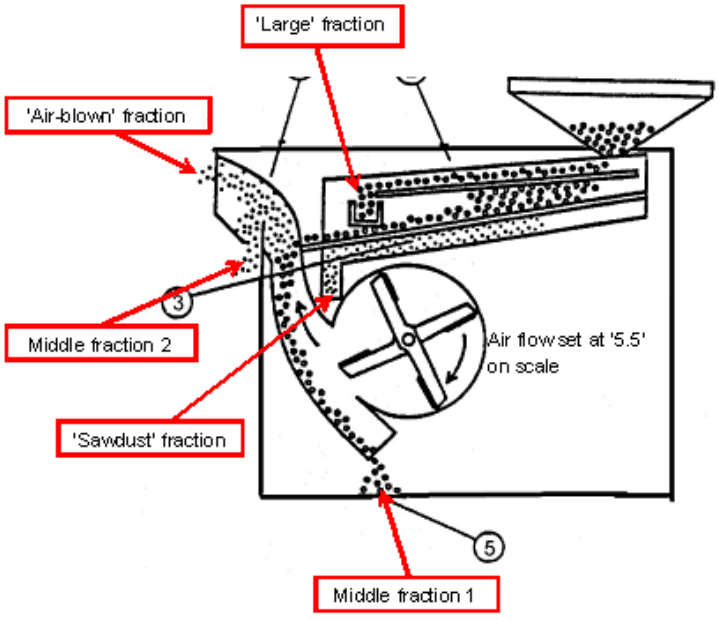


Figure C4. Schematic diagram of the air screen cleaner showing the five size fractions produced.

The two ‘middle’ fractions were then mixed before passing through the Indent Cylinder Separator shown in Figure C5. The indent cylinder worked by collecting any wood chips that were small enough to fit in 10 mm long indents in a rotating drum. These

chips were then rotated around until reaching the top of the rotation cycle, where they fell into a collection chamber. All chips that were too large to be held in the 10 mm indent until the top of the cycle passed through the drum and into a different collection chamber. Therefore, two different size fractions were obtained in this process – denoted as > 10 mm and < 10 mm. Although it is understood that the shape of the wood chips had an impact on this sorting procedure, it was considered to provide adequate fractionation of the 'middle fraction' of wood chips.

Figure C5. Indent cylinder separator.

Ultimately, five different size fractions were produced by the overall fractionation process. These fractions were labelled as:

1. Large
2. > 10mm
3. < 10mm
4. Air-blown
5. Sawdust

Appendix D

This Appendix contains the analysis reports provided by Veritec for the composition of the willow, extractives-free lignin and lignin from untreated willow.

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Facsimile: +64 7 348 0852

ANALYSIS REPORT

27 November 2013

Institute of Food, Nutrition and Human Health
Massey University
PO Box 11222
Palmerston North 4442

Attention: Hayley Stewart

Report No: AL13233
Date Received: 22/10/2013

Project Code: T02082
Order No: N/A

Analysis Requested: Ash, Extractives, Carbohydrate and Lignin Analysis
Sample Description: Wood

Client ID	Lab ID	Ash %w/w oven dried sample	Extractives %w/w oven dried sample	%w/w extracted oven dried sample							
				Lignin		Neutral carbohydrates					
				Acid-insoluble (Klason)	Acid-Soluble	Arabinan	Galactan	Glucan	Xylan	Mannan	Total
Willow (Salix Purpurea)	2013/233/1	0.23	0.86	22.40	3.50	0.67	0.78	44.73	21.34	1.01	95.52
	2013/233/1	0.26		22.83	3.25	0.65	0.73	42.17	20.02	0.92	91.69
	average	0.25	0.86	22.62	3.37	0.66	0.75	43.45	20.68	0.96	93.36

Notes about table: Sugars are reported as anhydrosugar units
Total % w/w is the sum of the sugars and lignin values

Method: TAPPI Standard Methods T 211 om-93 - ash in wood,pulp, paper and paperboard: combustion at 525°C
Samples are extracted using dichloromethane (DCM) solvent on a Soxtec apparatus, boiling time 1 hour, rinse time 1 hour
Acid-insoluble lignin in wood and pulp, TAPPI Standard Methods T 222 om-88
Acid-soluble lignin in wood and pulp, TAPPI Useful Method UM 250
Wood Sugar Analysis by Anion Chromatography, R.C. Pettersen, V.H. Schwandt, Journal Of Wood Chemistry And Technology, 11(4), 495-501 (1991)

General note: Descriptions of methods and repeatability analysis of a standard Pinus radiata sample run with all samples available on request

Analysis: Sunita Jeram / Claire Armstrong **Validated:** Sunita Jeram

Disclaimer: All results reported herein are legally privileged and confidential. All results apply to the samples as labelled and received on the date stated. Veritec will not be responsible for any claim or proceedings arising from errors or omissions in or arising from samples, documents or data which have not been prepared by Veritec staff or for any incidental, indirect, special or consequential loss or damage.

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Figure D1. Ash, extractives, lignin and neutral carbohydrates content of feedstock willow.

VERITEC

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ANALYSIS REPORT

25 November 2013

Institute of Food, Nutrition and Human Health
Massey University
PO Box 11222
Palmerston North 4442

Attention: Hayley Stewart

Report No: AL13231
Date Received: 22/10/2013

Project Code: T02082
Order No: N/A

Analysis Requested: Lignin Analysis
Sample Description: Lignin

Client ID	Lab ID	Lignin %w/w OD basis	
		Acid-insoluble (Klason)	Acid-Soluble
Lignin - from extractive free willow	2013/231/1a	97.13	1.86
	2013/231/1b	96.21	1.78
	average	96.67	1.82

Method: Acid-insoluble lignin in wood and pulp, modified method based on TAPPI Standard Methods, T 222 om-88
Acid-soluble lignin in wood and pulp, TAPPI Useful Method UM 250

General note: Descriptions of methods and repepatability analysis of a standard *Pinus radiata* sample run with all samples available on request

Analyst/s: Sunita Jeram / Claire Armstrong **Validated:** Sunita Jeram

Disclaimer: All results reported herein are legally privileged and confidential. All results apply to the samples as labelled and received on the date stated. Veritec will not be responsible for any claim or proceedings arising from errors or omissions in or arising from samples, documents or data which have not been prepared by Veritec staff or for any incidental, indirect, special or consequential loss or damage.

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Figure D2. Lignin content of lignin extracted from extractives-free willow using optimised extraction conditions.

Figure D3. Ash, lignin and neutral carbohydrates content of lignin extracted from untreated willow using the optimised extraction conditions.

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ANALYSIS REPORT

19 December 2013

Institute of Food, Nutrition and Human Health
Massey University
PO Box 11222
Palmerston North 4442

Attention: Hayley Stewart

Report No: AL13232
Date Received: 26/11/2013

Project Code: T02082
Order No: N/A

Analysis Requested: Ash, Lignin & Carbohydrate Analysis
Sample Description: Lignin

Client ID	Lab ID	%w/w oven dried sample								
			Lignin		Neutral carbohydrates anhydrosugars					
		Ash %w/w oven dried sample	Acid-Insoluble (Klason)	Acid-Soluble	Arabinosyl units	Galactosyl units	Glucosyl units	Xylosyl units	Mannosyl units	Total
Lignin (extracted from willow using 60% ethanol	2013/232/1a	1.54	93.32	3.16	0.03	0.11	0.34	1.86	0.04	100.39
	2013/232/1b	1.77	93.46	3.12	0.03	0.11	0.31	1.85	0.04	100.69
	average	1.66	93.39	3.14	0.03	0.11	0.33	1.86	0.04	100.54

Comments: Sugars are reported as anhydrosugar units
Total % w/w is the sum of Ash, Lignin & Carbohydrate values

Method: TAPPI Standard Methods T 211 om=93 - ash in wood,pulp, paper and paperboard: combustion at 525°C
Acid-insoluble lignin in wood and pulp, TAPPI Standard Methods T 222 om-88
Acid-soluble lignin in wood and pulp, TAPPI Useful Method UM 250
Wood Sugar Analysis by Anion Chromatography, R.C. Pettersen, V.H. Schwandt, Journal Of Wood Chemistry And Technology, 11(4), 495-501 (1991)

Analyst/s: Sunita Jeram / Claire Armstrong **Validated:** Sunita Jeram

Disclaimer: The contents of this report, including all results therein, are confidential to the client. All results apply to the samples as labelled and received on the dated stated. Veritec will not be responsible for any loss or damages arising from errors or omissions in, or arising from samples, documents or data which have not been prepared by Veritec staff. Veritec makes every effort to ensure that the requested tests are performed correctly, but will not be liable for any loss or damage arising from the use or application of the results by the Client or any third party.

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Appendix E

This appendix details the analysis of glass transition temperature of lignin samples prepared using different initial water temperatures. Figure E1 below shows how glass transition temperatures were determined from the DSC traces.

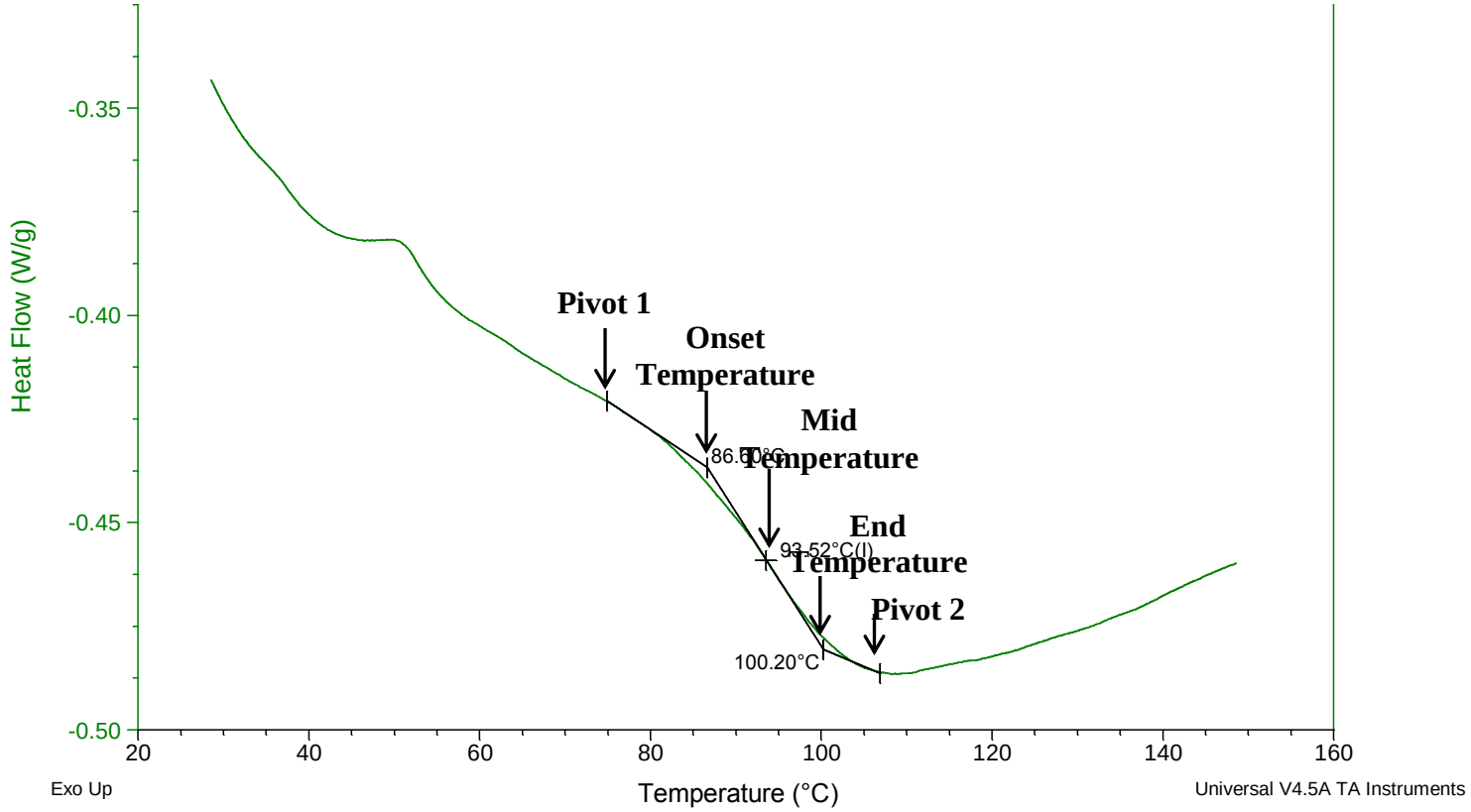

Figure E1. Annotated DSC trace showing how the Universal Analysis 2000 software uses tangents to calculate the onset, mid and end glass transition temperatures based on pivot points identified by the user (note that the small peak at ~50°C shown in this DSC trace is due to a baseline error).

Table E1. Values of the onset, mid and end glass transition temperatures obtained by analysis of DSC traces for dried lignin samples initially prepared using water temperatures of 4, 20 and 60°C

		Glass Transition		
Sample	**Replicate number**	**Onset (°C)**	**Mid (°C)**	**End (°C)**
4°C	~~1~~	~~85.49~~	~~93.72~~	~~100.46~~
	2	77.19	86.63	94.36
	3	74.38	84.63	90.54
20°C	~~1~~	~~99.35~~	~~109.17~~	~~114.79~~
	~~2~~	~~85.56~~	~~97.29~~	~~103.34~~
	~~3~~	~~86.21~~	~~94.55~~	~~102.64~~
	4	86.07	95.95	101.87
60°C	~~1~~	~~103.12~~	~~113.82~~	~~119.09~~
	~~2~~	~~101.54~~	~~114.01~~	~~119.31~~
	~~3~~	~~97.55~~	~~109.10~~	~~114.62~~
	4	87.31	98.07	106.42

The ruled out measurement results show data that was not included in the analysis because it was later found to have a baseline error.

Appendix F

This Appendix outlines the brief experimental work carried out concerning the use of ultrasonic mixing for the preparation of lignin microparticles.

Background

Ultrasonic mixing was trialled as an alternative method of mixing the dispersed and continuous phases during the preparation of lignin microparticles. Sonication has been used to prepare microspheres (Tabata, Gutta & Langer, 1993, as cited in O' Donnell & McGinity, 1997) and it was hypothesised that it may allow further manipulation of lignin microparticle size, perhaps producing smaller particles.

Materials and Methods

Equipment

A system for ultrasonic mixing using a flow cell was specially designed for this work. Figure F1 shows the initial design provided by DKSH (Palmerston North, New Zealand). The design features an ultrasonic transducer (UIP1000hd, Hielscher Ultrasonics GmbH, Teltow, Germany) and sonotrode, flow cell, receiver tank and mono-pump. This design enables liquid to be pumped from the receiver tank to the flow cell where ultrasonic mixing occurs and back to the receiver tank. The receiver tank can be jacketed to aid in temperature control of the sample during continuous ultrasonication.

The final experimental set-up used in this work is shown in the photograph in Figure F2. Although based on the initial design, an important addition was an inlet tube to the flow cell (Hielscher Ultrasonics GmbH) through which to feed the extraction liquor during operation. Extraction liquor was released from the tube and mixed with the recirculating water phase directly at the tip of the sonotrode (Figure F2B). The extraction liquor was pumped into the system using a peristaltic pump (MasterFlex L/S^{TM} Economy Drive Model 7554-85, Cole Parmer Instrument Company, Vernon Hills, Illinois).

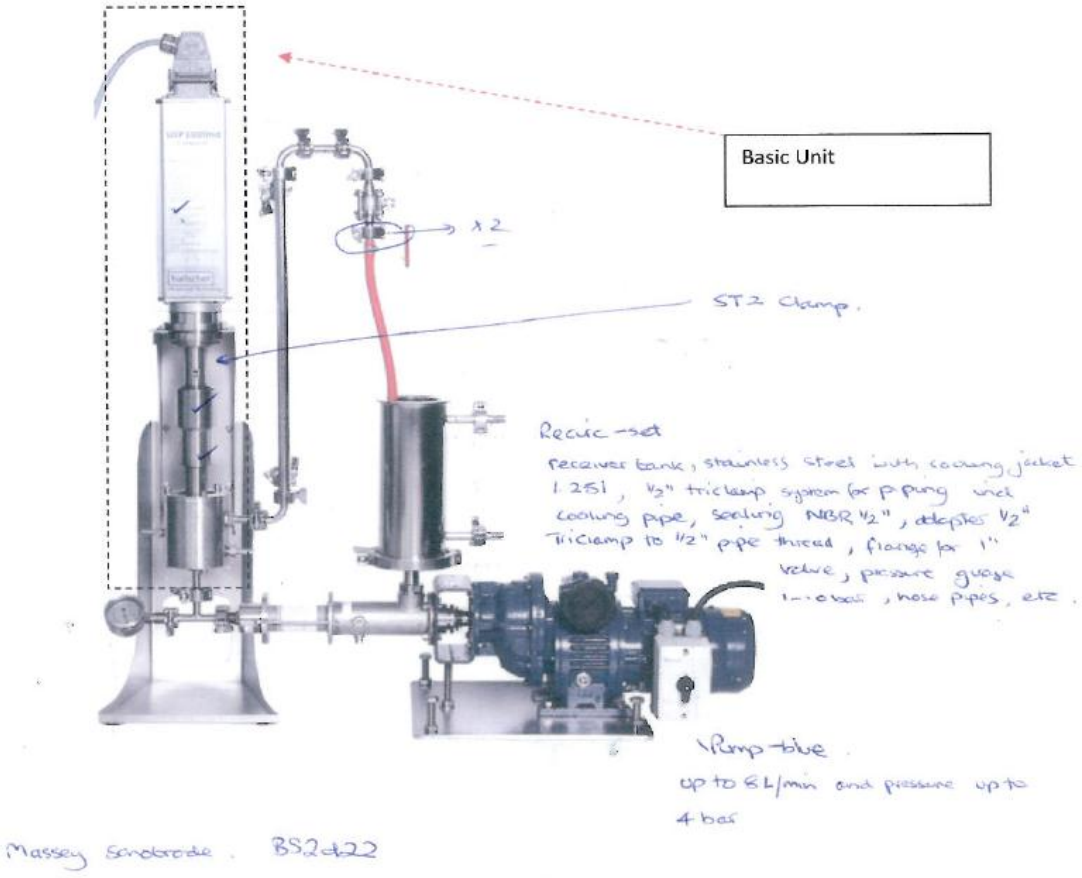

Figure F1. Proposed equipment set-up for preparation of lignin microparticles using ultrasonic mixing (DKSH, Palmerston North).

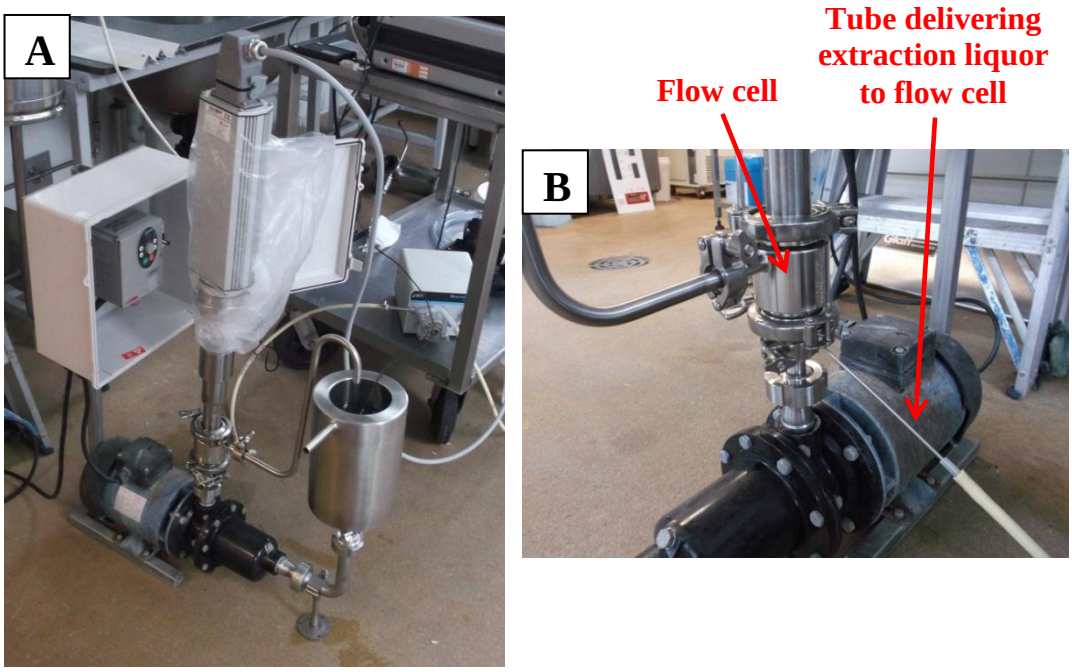

Figure F2. (A) Actual rig used for preparation of lignin microparticles by ultrasonic mixing. (B) Point where extraction liquor is pumped into flow cell.

Method

Eight hundred millilitres of distilled water with or without SDS was added to the jacketed vessel (1.5 L) and circulated around the system using a mono-pump (Brook Crompton UK Ltd., Doncaster, England) operating at 8 rpm. Cold water at approximately 20°C was used as the coolant in the vessel jacket. Once the temperature of the distilled water circulating around the system equilibrated to 20 ± 1°C, ultrasonication at 20 kHz and 100% amplitude was commenced. The peristaltic pump was immediately started and set to a speed of 0.5 rpm. The end of the feed tube to the peristaltic pump was then placed in a measuring cylinder containing 340 mL of extraction liquor. The liquor was pumped into the flow-cell, mixed with water at the tip of the sonotrode (22 mm diameter, BS2d22, Hielscher Ultrasonics GmbH) and the mixture pumped to the receiver tank for recirculation back through the flow-cell. Ultrasonication continued until three minutes after all extraction liquor had been fed into the system. The microparticle suspension was then collected from the receiver tank and stirred at 500 rpm under magnetic stirring for 21 h. The resulting suspensions were immediately analysed for particle size. To obtain dry powder samples, the dispersions were concentrated by centrifugation at 30,000 rpm for 1 hour (Sorvall WX Ultra 100 Centrifuge, Thermo Scientific, NZ), air-dried at 30°C overnight and ground to powder using a mortar and pestle.

After initial trials to finalise the methodology, two ultrasonic mixing experiments were carried out. Details of these trials are given in Table F1 below.

Table F1. Aqueous phase temperature and SDS concentration used in each trial

Trial	Aqueous Phase Temperature (°C)	SDS Concentration (% w/v)
1	20	0
2	20	0.5

Results and discussion

Figure F3 shows particle size distributions for lignin microparticles prepared using both ultrasonic mixing and magnetic stirring for the initial mixing stage with a water phase temperature of 20°C and no surfactant present. Microparticles prepared using both mixing types exhibit similar particle size distributions, with a major peak in particle size at around 10 µm. Both also exhibit a smaller volume of particles in approximately the 100-1000 µm size range. However, the D[3,2] for particles prepared using magnetic stirring is 13.8 µm compared to only 7.0 µm for particles prepared using ultrasonic mixing, reflecting the differences in peak position in Figure F3. Therefore, ultrasonic mixing may present an opportunity to produce smaller particles than magnetic stirring at the same temperature without the use of surfactant. Due to the time involved in carrying out each trial, the effect of ultrasonication on particle size at different preparation temperatures was not investigated. However, this may be useful to investigate in the future to determine what other particle sizes can be produced and whether other advantages, such as reduced energy input or narrower particle size distribution, exist.

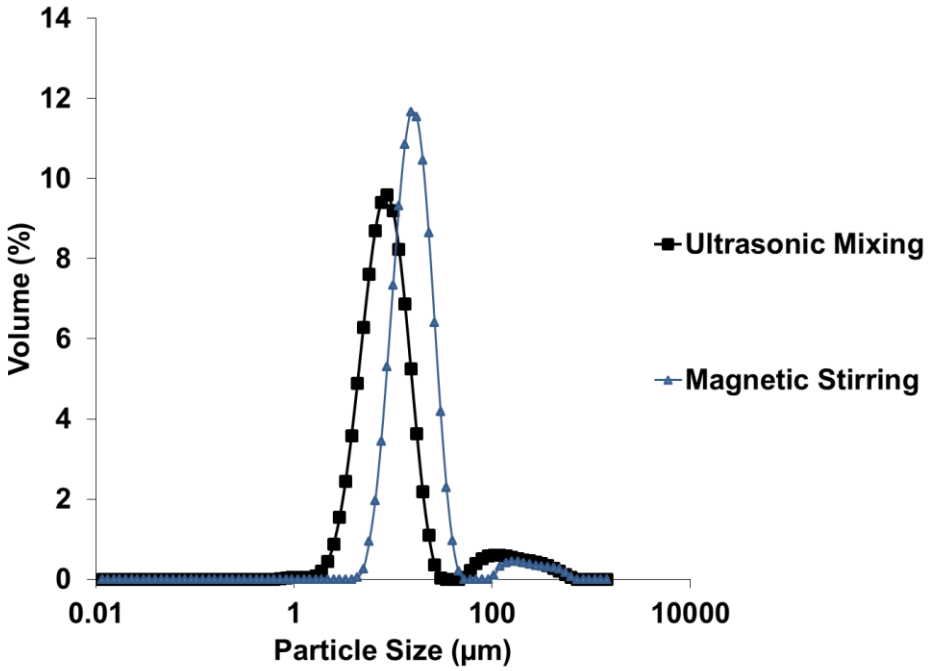

Figure F3. Particle size distributions of particle suspensions prepared by mixing extraction liquor (20°C) with water (20°C) in the absence of surfactant using either ultrasonic mixing or magnetic stirring.

The effect of ultrasonic mixing on particle size in the presence of SDS was also investigated. Figure F4 below shows microparticles prepared using both ultrasonic mixing and magnetic stirring using water phases containing 0.5% (w/v) at 20°C.

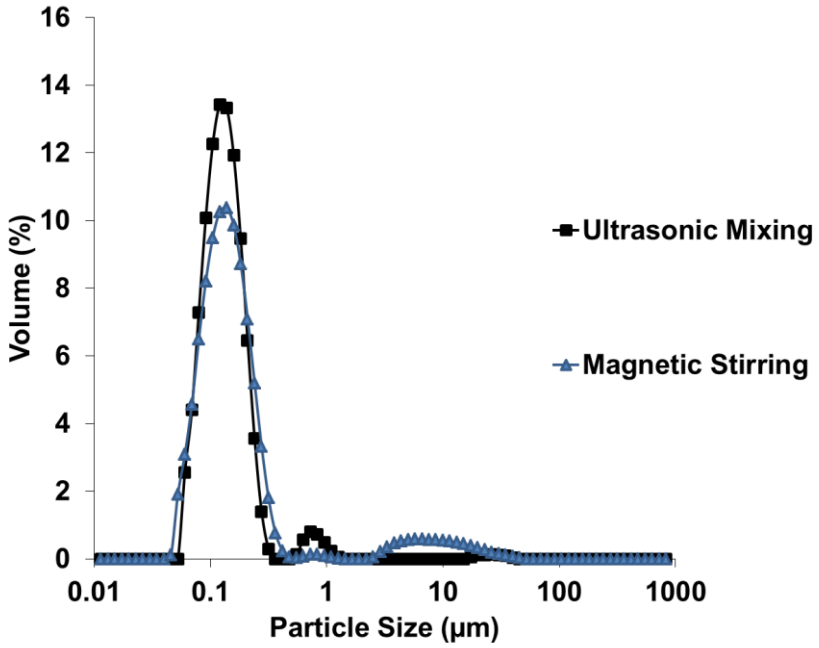

Figure F4. Particle size distributions of particle suspensions prepared by mixing extraction liquor (20°C) with water (20°C) containing 0.5% (w/v) SDS using either ultrasonic mixing or magnetic stirring.

The particle size distributions for particles prepared in the presence of SDS using either ultrasonication or magnetic stirring are similar. Both sets of microparticles exhibit a major particle size peak at approximately 0.1 µm, with small modalities of particles in a larger size range. There are fewer particles in the higher size range in the sample prepared using ultrasonic mixing than in the suspension prepared using magnetic stirring, and the larger particles are only in approximately the 1 µm range rather than centring around approximately 10 µm. This indicates that ultrasonic mixing produces slightly smaller lignin particles. However, since the majority of particles are in the ~0.1 µm size range for both methods, it seems that although ultrasonication is able to produce the same small particle size as magnetic stirring, it does not enable significant further reduction in particle size when SDS is present. This result may indicate that the presence of SDS and subsequent stabilisation of the high surface area of small lignin

particles is more important than the type and speed of mixing used. However, again it may be important to examine different water or liquor temperatures. For example, ultrasonication of an SDS-containing water phase at 4°C may produce a monomodal distribution of sub-micron particles, rather than the bimodal distribution produced by magnetic stirring at this temperature.

Conclusions and recommendations

In the absence of surfactant, ultrasonication appears to have allowed smaller lignin particles to form. However, a trial at only one water temperature was carried out. Evaluating the size of particles produced at other water temperatures will be important to gain more indication of the effectiveness of ultrasonic mixing to produce smaller particles than magnetic stirring.

In the presence of SDS, ultrasonication did not produce particles of different size to those produced using magnetic stirring. It may be that small particles are formed easily using a range of mixing types and speeds, but are only stable if surfactant is present.

Overall, it appears that ultrasonic mixing has not provided an advantage over magnetic stirring in terms of producing particles of smaller size with less energy input. However, it may be useful to carry out further trials in the future with more careful selection of ultrasonication conditions, such as amplitude, time of mixing and the use of cycles of sonication. Evaluating the effect of water and/or liquor temperature may also provide more indication of the effectiveness of ultrasonication for lignin particle preparation.

Appendix G

This Appendix outlines the preliminary trials carried out to study the effect of controlling pH during microparticle preparation and the re-dispersal of lignin particles in water.

Effect of pH

To gain more knowledge on the effect of pH on microparticle size, initial trials using buffered aqueous phases were carried out. Buffering was thought to be important due to the acidity of the lignin extraction liquor, which otherwise shifts the final pH of the suspensions to ~3.4-3.6. In these initial trials, buffer solutions at pH 4, 7 and 10 were used as the aqueous phase for microparticle formation. SDS was added to the buffer solutions at a concentration of 0.5% (w/v) before the addition of the lignin extraction liquor. The pH 4 and 7 buffers were commercial buffers of unknown concentration. The pH 4 buffer was a citric acid buffer and the pH 7 buffer was a phosphate buffer (Certipur®, Merck KGaA, Darmstadt, Germany). The pH 10 buffer was a 1 M carbonate buffer. Figure G1 shows the size distribution of particles prepared using pH 4, 7 and 10 buffers containing SDS as the aqueous phase.

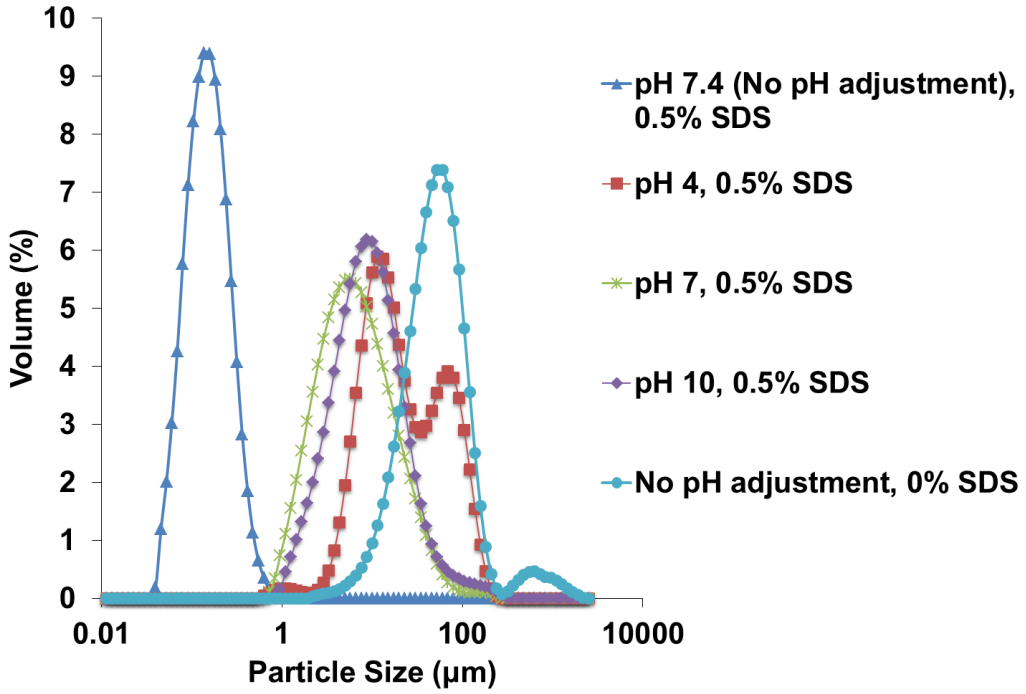

Figure G1. Size distributions of lignin microparticles prepared using aqueous phases containing 0.5% (w/v) SDS in pH 4, 7 and 10 buffers at 60°C.

Figure G1 shows that the pH 4 buffer produced a bimodal distribution with particle size ranging between about 1-200 µm. The pH 7 and 10 buffers produced particles with similar size distributions, with particle size ranging between 1-100 µm. The larger particles produced at pH 4 may be the result of decreased negative charge of SDS at the lower pH. However, in trials where the pH of the aqueous phase has not been adjusted, addition of the extraction liquor reduced the pH of the suspension to 3.6. Therefore, this change was not expected.

Differences in the ionic strength of the three buffers were not controlled in this experiment. The ionic strength of the carbonate buffer was 2.2 M and its concentration was 1 M. Since we know that 0.1 M KCl destabilised SDS-stabilised lignin particles to an extent similar to if no SDS was present, it would be expected that 1 M NaHCO3 would also destabilise the particles due to screening of electrostatic repulsions by the counter-ions. The ionic strengths of the pH 4 and pH 7 buffers were not known. Furthermore, the pH 7 buffer could not maintain the pH at 7, since the pH of the final suspension was 6.4. Therefore, it was necessary to prepare buffers for this work and to more carefully control the concentration and ionic strength.

However, it was difficult to prepare buffers of low concentration that could buffer the addition of the extraction liquor, which made up approximately one third of the final volume of the suspension. Therefore, only the effect of change in unbuffered pH was studied for the purpose of the thesis.

Changes in pH of microparticle suspensions revealed that reduction of the suspension pH to 1.5 caused aggregation of primary particles. The aggregation suggested that pH 1.5 was sufficiently low to cause screening of the electrostatic repulsions provided by SDS. Screening of the electrostatic repulsions before particle formation may cause larger primary particles to form, as indicated by the addition of KCl to the water phase before particle formation. Therefore, it is recommended that a pH 1.5 buffer is prepared and used to prepare microparticles.

Re-dispersibility of lignin particles

The ability of dried lignin particles to be re-dispersed to the same size as in the original microparticle suspension was determined. Figure G2 shows particle size as a function of increasing periods of sonication for non-SDS-stabilised particles re-dispersed in water.

The particles were initially re-dispersed by gentle magnetic stirring for five minutes at room temperature. Sonication decreased particle size compared to particles mixed with water by magnetic stirring. Particles the same size and even smaller than the particles present in the original microparticle suspension before drying and re-dispersion were obtained after 10 minutes of sonication. The smaller particles may support the conjecture that sub-micron primary particles are always formed under the same conditions of shear, but that these particles are increasingly fused at higher temperatures. Under sonication the agglomerated particles can be somewhat re-dispersed back to primary particles; however, the particles are more widely distributed.

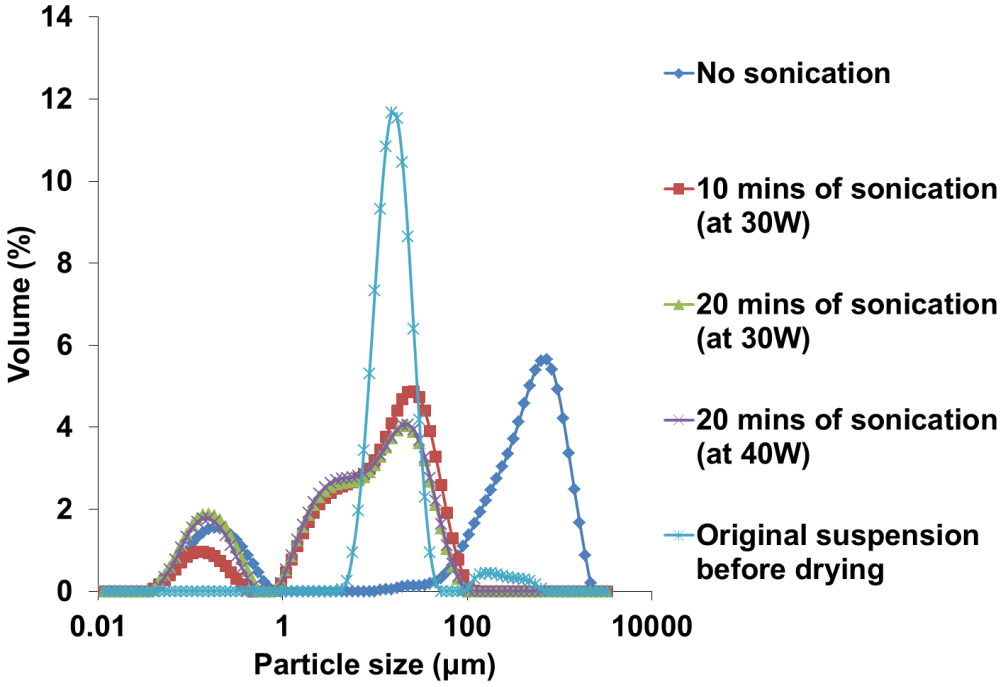

Figure G2. Size distributions of re-dispersed lignin microparticles. Samples were initially prepared at 20°C with no SDS present and dried using the centrifugation and air-drying procedure. Ultrasonication by the light scattering equipment for increasing periods of time at either 30 or 40W was evaluated for its ability to reduce particle size.

Figure G3 shows particle size as a function of increasing periods of sonication for SDS-stabilised particles re-dispersed in water. Sonication decreased particle size compared to particles re-dispersed by magnetic stirring. However, as also observed for the non-

stabilised particles, increasing length of sonication time and power did not cause additional decreases in particle size. Furthermore, the particle size distributions are wide and, apart from a small volume of particles, sonication has not reduced particles to the sub-micron size present in the original suspension. Only a small volume of particles in the sub-micron size range was obtained, with the majority of particles being widely distributed between 1-100 μm. This may indicate that little SDS remains on the surface of the lignin particles when they are dried, making it difficult to achieve stable sub-micron particles on re-dispersion. It is recommended that the particle drying process is studied more closely and improved to enable better control of particle size and re-dispersion behaviour. It is also recommended that other re-dispersal methods are studied, such as mixing with a rotor-stator mixer or propeller blade stirrer. Efficient re-dispersal with common food industry mixers is a goal.

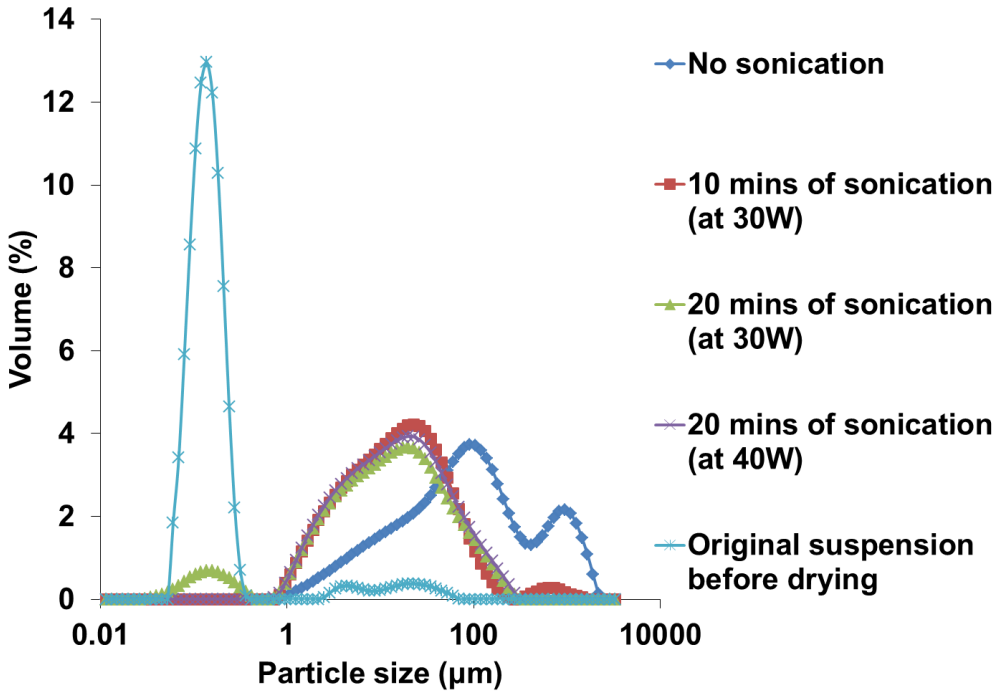

Figure G3. Size distributions of re-dispersed lignin microparticles. Samples were initially prepared at 40°C with 0.5% (w/v) SDS and dried using the centrifugation and air-drying procedure. Ultrasonication by the light scattering equipment for increasing periods of time at either 30 or 40W was evaluated for its ability to reduce particle size.

Appendix H

Introduction

This Appendix outlines preliminary trials carried out for the evaluation of the ability of lignin microparticles to stabilise emulsions. The initial trials to determine the potential of lignin particles to stabilise emulsions using both the pH-modification and fresh suspension method are described first. A short investigation of the effect of ultrasonic mixing on emulsion formation is then outlined. Finally, the ability of SDS-stabilised, sub-micron lignin particles to stabilise emulsions is covered.

Materials and Methods

Potential of lignin as a Pickering stabiliser

Emulsions were prepared using both the pH-modification and fresh suspension methods. The emulsions contained 20% (v/v) soybean oil and were homogenised using a rotor-stator bench-top homogeniser (Heidolph DIAX 600, Heidolph Instruments, Germany) at a speed of ~12500 rpm for 45 seconds. In the pH-modification method the concentration of lignin in the aqueous phase was 0.1% lignin (w/v). In the fresh suspension method, the concentration of lignin was 1.9% (w/v) since this was the final concentration of the suspension.

Ultrasonication for emulsion formation

The use of an ultrasonic mixer (UIP1000hd, Hielscher Ultrasonics GmbH, Teltow, Germany) to prepare emulsions was investigated. A 22 mm diameter probe was used and the tip was always placed 10 mm deep into the samples. The frequency of ultrasonication was 20 kHz and amplitudes of 30 and 40% were trialled. The time of ultrasonication varied between 45 seconds and 4 minutes. During mixing, the sample was either sitting in ice, an ice slurry or at room temperature.

Sub-micron lignin particles as Pickering stabilisers

Microparticle suspensions containing sub-micron particles stabilised by SDS were used to form emulsions in order to determine whether smaller lignin microparticles were able to produce smaller emulsion droplets. Suspensions containing initial SDS concentrations of 0.0625-0.5% (w/v) were used. To gauge the effect of SDS alone on droplet formation and stability, emulsions containing no lignin but equivalent concentrations of SDS were also prepared for some SDS concentrations.

Unless otherwise stated, a soybean oil concentration of 10% (w/w) was used for all emulsions, which were made using Silverson rotor-stator homogeniser, running at 5000-8000 rpm for 1-1.5 minutes.

Results and Discussion

Potential of lignin as a Pickering stabiliser

Figure H1 shows a photograph and light microscopy (LM) image of a 20% (v/v) soybean oil-in-water emulsion prepared using the pH-modification method and stabilised by 0.1% lignin. Figure H1 reveals that emulsions similar to those produced by Wei et al. (2012) are produced using the pH-modification method. This result indicates that further investigation into the ability of lignin microparticles to stabilise emulsions is a valid research path.

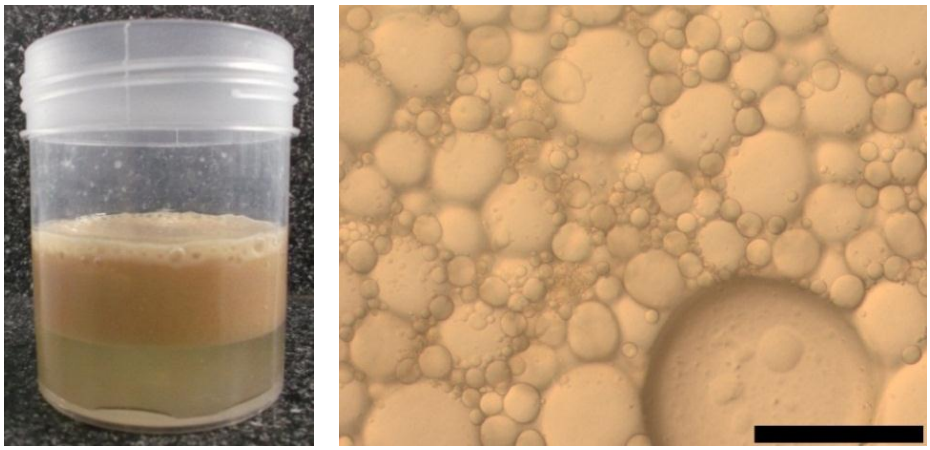

Figure H1. Photograph and LM image of a 20% (v/v) oil emulsion prepared using the pH-modification method with 0.1% (w/v) lignin (bar scale = 100 µm).

Figure H2 shows a photograph and LM image of a 20% (v/v) soybean oil-in-water emulsion stabilised by 1.9% (w/v) lignin, prepared using the fresh microparticle suspension method. Figure H2 shows that emulsions are also able to be produced using lignin directly in the form of fresh microparticle suspensions initially prepared using an aqueous phase temperature of 4°C. The emulsion droplets are dissimilar to those produced using the pH-modification method in that they are more irregularly shaped, but similar in that they are polydisperse and exhibit creaming instability. This result,

particularly the appearance of the emulsion layer indicated that this method of producing lignin-stabilised emulsions should also be further investigated.

Figure H2. Photograph and LM image of a 20% (v/v) oil emulsion prepared using the fresh suspension method with 1.9% (w/v) lignin (bar scale = 500 µm).

Ultrasonication for emulsion formation

Table H1 shows the observations made when emulsions of various compositions were prepared using an ultrasonic probe rather than the Heidolph mixer. The fresh suspension and pH-modification methods were used in emulsion preparation, as specified in Table H1.

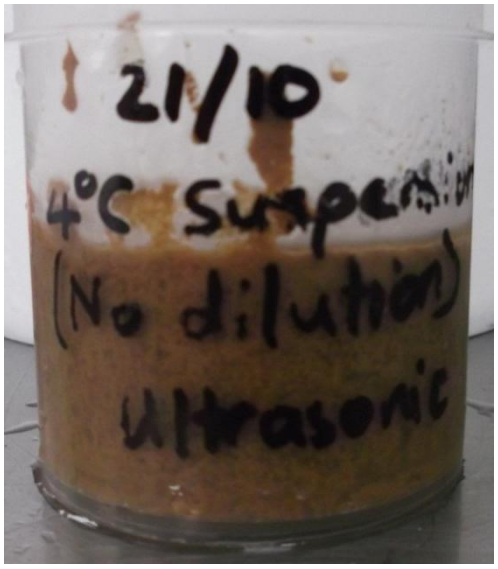

Figure H3. Emulsion prepared using an undiluted lignin microparticle suspension with homogenisation by ultrasonic probe.

Table H1. Sample details, ultrasonication conditions and visual observations for emulsions prepared by homogenisation with an ultrasonic probe

Sample details	Ultrasonication conditions	Results/observations
▪ 20% (v/v) soybean oil ▪ 31.25 mL of total emulsion ▪ 0% lignin	▪ 40% amplitude ▪ 4 minutes ▪ Sample was sitting in ice throughout processing	A white, creamy emulsion formed. However, there was rapid separation to give a white, creamy bulk fluid with a layer of oil on the top.
▪ 20% (v/v) soybean oil ▪ 31.25 mL of total emulsion ▪ 0.1% lignin (diluted fresh suspension)	▪ 40% amplitude ▪ 4 minutes ▪ Sample was sitting in ice throughout processing	Did not work. Lumps of lignin. Oil layer on top. Container very warm to hold.
▪ 20% (w/v) soybean oil ▪ 31.25 mL of total emulsion ▪ 0.1% lignin (diluted fresh suspension)	▪ 30% amplitude ▪ 2 minutes ▪ Sample was sitting in ice bath (i.e slush) throughout processing	No big lumps like those seen in the previous trial. Sample container not quite as hot, but still warm. Two layers formed. Bottom layer was creamy/white. Top layer was brown with some lumps. Perhaps the ultrasonic was too powerful?
▪ 20% (w/v) soybean oil ▪ 31.25 mL of total emulsion ▪ 2.1% lignin (fresh suspension)	▪ Pre-mixed oil and suspension together by shaking ▪ 30% amplitude ▪ 1 minute ▪ Sample was sitting in ice bath (i.e slush) throughout processing	Did not produce the same result as when this emulsion was prepared using the Heidolph mixer. Lignin seemed to precipitate out. Formed a homogeneous but chunky mixture (Figure H3).
▪ 20% (w/v) soybean oil ▪ 31.25 mL of total emulsion ▪ 0.1% lignin (dried lignin, using pH-modification method)	▪ Pre-mixed oil and suspension together by shaking ▪ 30% amplitude ▪ 45 seconds ▪ Sample was not sitting in ice bath	Better than previous attempts with ultrasonic probe. Two layers formed. Top layer was caramel brown and much smoother than in previous samples. However, there were still some quite big particles. Bottom layer was pale/dirty white. Perhaps the ultrasonication was so powerful that it emulsified the bottom layer without needing any lignin

		(Figure H4).
Sample details	**Ultrasonication conditions**	**Results/observations**
▪ 20% (w/v) soybean oil ▪ 31.25 mL of total emulsion ▪ 0.1% lignin (dried lignin, using pH-modification method)	▪ Pre-mixed oil and suspension together by shaking ▪ 30% amplitude ▪ 45 seconds ▪ Sample was sitting in ice bath (i.e slush) for two minutes before processing and also throughout processing	Very similar to the trial with no ice bath above. The brown top layer was perhaps a bit chunkier (more precipitated-looking material). (Figure H4).

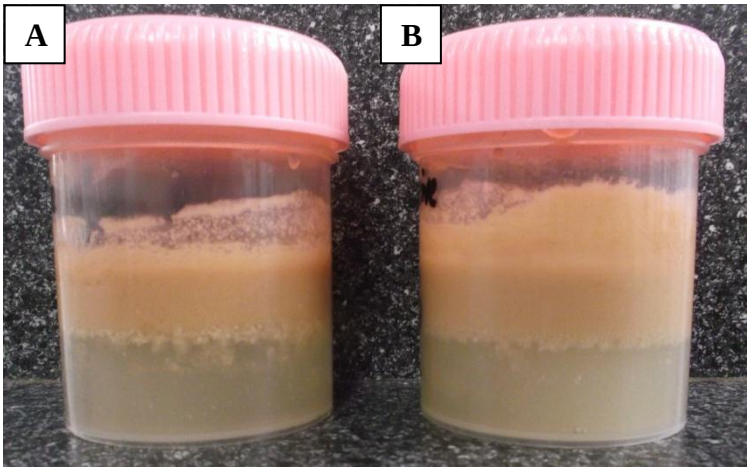

Figure H4. Emulsions prepared using the pH-modification method with emulsification by ultrasonic probe where the sample was (A) chilled in an ice bath during emulsification or (B) not chilled in an ice bath during emulsification.

Although no particle sizing or LM was carried out for the emulsion samples prepared by ultrasonication, the observations given in Table H1, along with the photos shown in Figure H3 and Figure H4 suggest that using ultrasonication for emulsion preparation did not produce emulsions with smaller droplets or more stability to creaming than those prepared using the Heidolph mixer. In fact, in many cases ultrasonication seemed to produce emulsions that contained clumps of lignin. However, it is likely that the method

just requires more development since ultrasonication is known to provide many benefits over conventional emulsification methods, including smaller droplet size, greater stability and lower surfactant requirement (Abbas, Hayat, Karangwa, Bashari, & Zhang, 2013). It is recommended that ultrasonication be trialled again later in the project once more is understood about sample composition, suitable lignin concentrations and how performance of lignin as an emulsifier will be evaluated.

Sub-micron lignin particles as Pickering stabilisers

Figure H5 shows photographs of emulsions prepared using fresh lignin suspensions containing SDS-stabilised, sub-micron lignin microparticles. The suspensions contained 0.5% (w/v) SDS in the water phase. The suspensions were diluted to give concentrations of lignin of 0.1 and 0.5%, which also diluted the SDS concentration in these samples to 0.028 and 0.14% (w/v), respectively. The concentration of soybean oil in the emulsion was 9.5% (w/v).

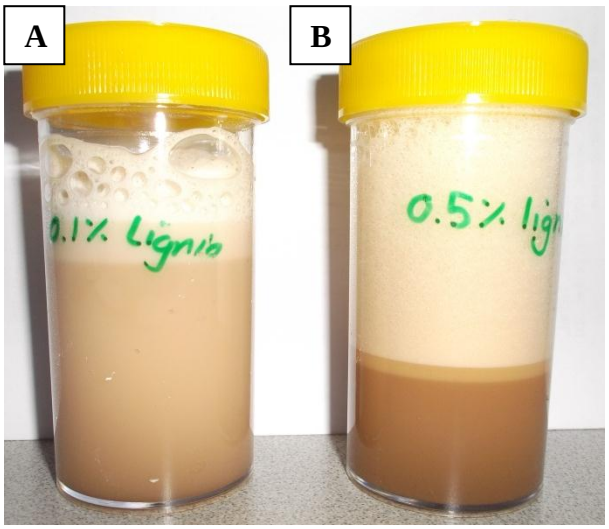

Figure H5. 9.5% (w/v) oil emulsions prepared using fresh lignin microparticle suspensions that were diluted to contain (A) 0.1% lignin and 0.028% SDS and (B) 0.5% lignin and 0.14% SDS.

Figure H5 shows that emulsions formed using SDS-containing suspensions had three different layers. The top layer consisted of foam, which could have been produced due to the presence of SDS during homogenisation. There was also a middle and lower layer of unknown composition. It was unclear whether the middle layer was the emulsion, similar to the emulsion layer seen in previous samples, whether the lower layer was the

emulsion, or whether both layers were emulsions. Therefore, the layers were analysed by LM to try to distinguish between them. Figure H6 below shows the LM images.

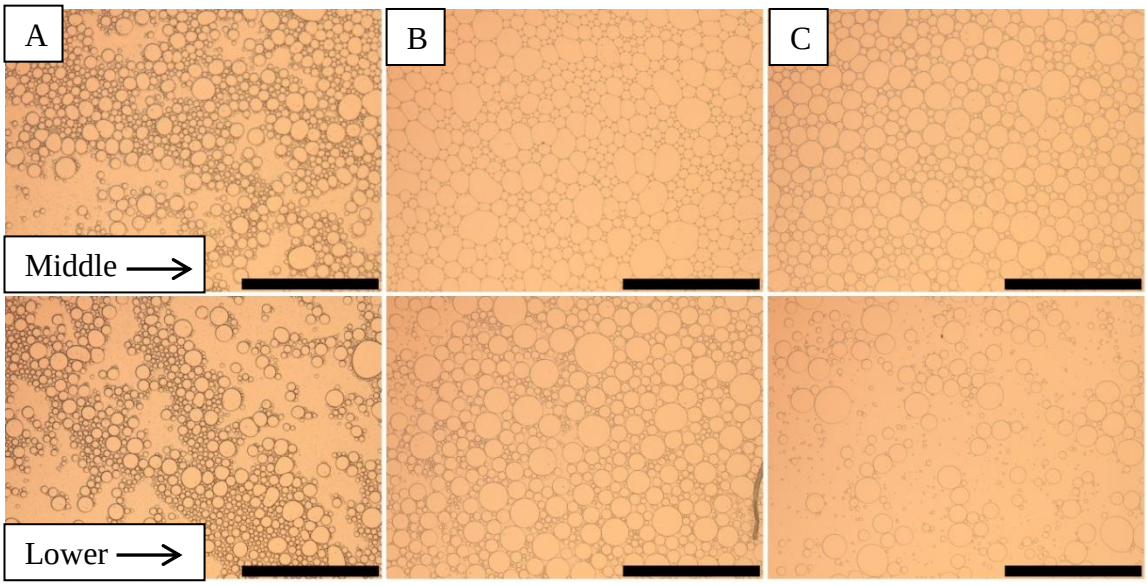

Figure H6. LM images of 9.5% (w/v) oil emulsions prepared using fresh microparticle suspensions diluted to contain (A) 0.1% lignin and 0.028% SDS, (B) 0.5% lignin and 0.028% SDS and (C) 0.14% SDS alone. Images of the middle layer are on the top, while images of the lower layer are along the bottom (bar scale = 500µm).

Figure H6 shows that mostly spherical oil droplets were produced for all lignin and SDS concentrations. Oil droplets were also present in both the middle and lower layers, with no major differences between the droplets in different layers. In some images that are not shown here, droplets in the middle layer looked more like foam bubbles. This could have been a result of the droplets being very tightly packed in this layer or to some foam being sampled from the top layer due to difficulties in sampling from the thin middle layer. For the emulsion containing 0.5% lignin, the droplets in the lower layer seem to be more polydisperse than those in the middle layer. This may suggest that although both layers contain emulsion droplets, the bottom layer contains droplets of a range of sizes including very small droplets, while many of the larger droplets have gathered in the middle layer.

Figure H6 also suggests that the presence of sub-micron lignin microparticles in the emulsion does not provide any advantage in terms of reducing droplet size over the

presence of SDS alone, since droplet size appears to be relatively similar across the three emulsions. Furthermore, an increase in lignin (and SDS) concentration does not appear to have caused a decrease in droplet size. There even appears to be a greater number of smaller oil droplets in the emulsion containing 0.1% lignin than in the emulsion containing 0.5% lignin. However, this may just be the case in the particular images shown. The lack of influence of SDS and lignin concentration on particle size may be due to the low emulsification speed used in the preparation of the samples. Speeds of only 5000-8000 rpm were used to emulsify the oil and water in these samples. Perhaps if higher speeds had been used, smaller droplets could have been produced at higher lignin concentration. Therefore, it is recommended that the top speed of the Silverson (~10, 300 rpm) be used in future experiments.

The samples were monitored over a three day period to gain an indication of the effect of SDS and lignin concentration on emulsion stability. The emulsion containing 0.1% lignin had destabilised by the third day, with a layer of oil sitting on the top, indicating that when using fresh suspensions to prepare emulsions, either a lignin concentration of 0.1% or SDS concentration of 0.028% is not sufficient to produce a stable emulsion. However, the emulsion containing 0.5% lignin had not destabilised by the third day. Figure H7 shows an LM image of oil droplets in this sample after three days; the oil droplets look very spherical, even more so than when the emulsion was first prepared. Figure H7 also shows that oil droplets in the emulsion containing SDS alone looked similar to their appearance on the day of preparation. This indicates that a certain level of either SDS or lignin is required for stability.

These results indicate that to determine better whether sub-micron lignin particles can

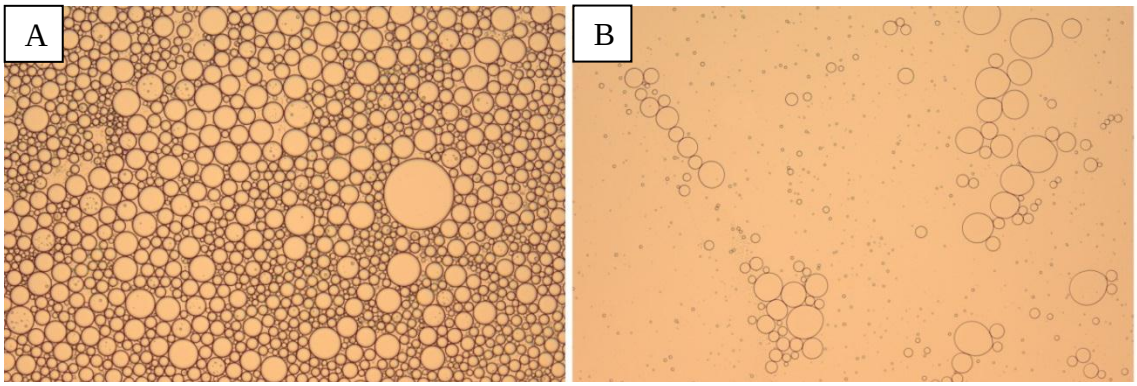

Figure H7. LM images of 3-day old 9.5% (w/v) oil emulsions prepared using (A) fresh lignin microparticle suspension diluted to contain 0.5% lignin and 0.14% SDS and (B) 0.14% SDS alone (bar scale = 500 µm).

produce smaller emulsion droplets, sub-micron particles stabilised by a very low concentration of SDS should be trialled. This should limit the effect of SDS on oil droplet formation and stability. Figure H8 below shows lignin particle size distribution as a function of SDS concentration, revealing that 0.0625% SDS is sufficient to produce sub-micron lignin microparticles in suspension.

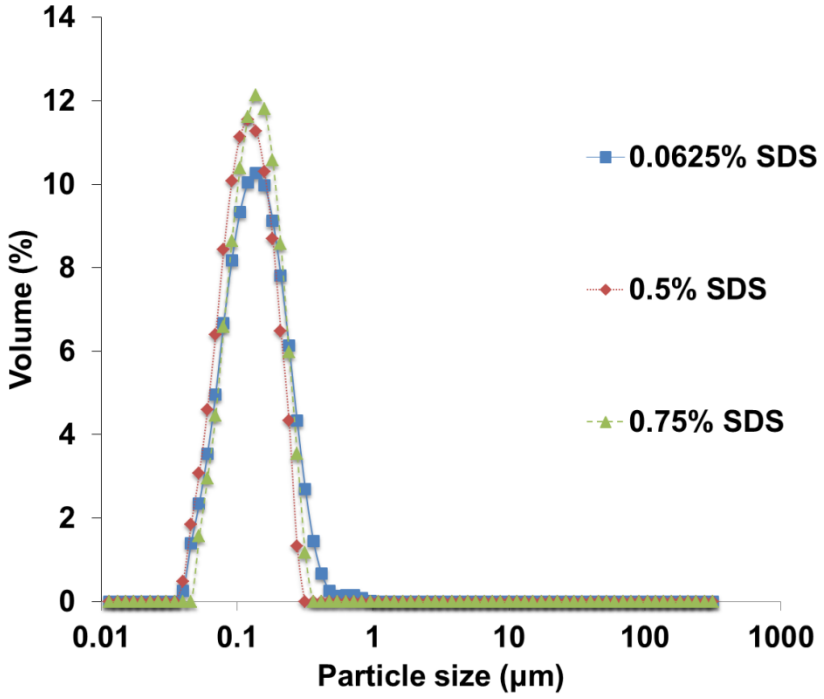

Figure H8. Size distributions of lignin particles prepared in the presence of 0.0625-0.75% (w/v) SDS at 60°C.

Hence emulsions were prepared using lignin microparticle suspensions that were diluted to give various lignin concentrations from a stock suspension containing 0.1% (w/v) SDS. Although the initial SDS concentration of the aqueous phase used for particle preparation was 0.0625% (w/v), a concentration of 0.1% (w/v) was estimated based on the concentration of SDS in the water phase prior to microparticle formation and the final volume of the suspension. Figure H9 shows photographs of 10% (w/w) soybean oil emulsions prepared using fresh lignin microparticle diluted from this stock suspension to contain 0.1 and 0.5% lignin and associated diluted SDS concentrations. An emulsion prepared using an equivalent concentration of SDS to the 0.5% lignin emulsion is also shown.

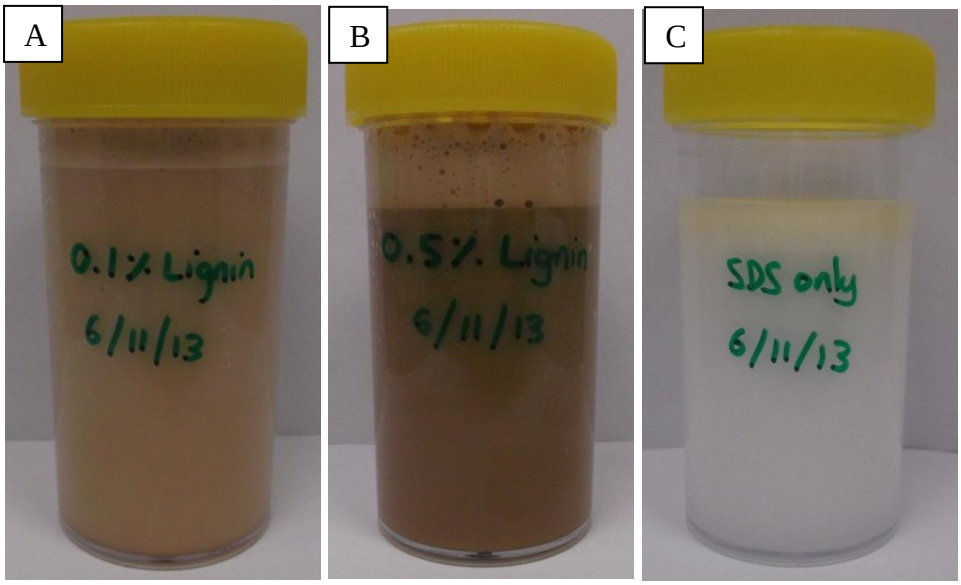

Figure H9. 10% (w/w) oil emulsions prepared using fresh lignin microparticle suspensions diluted to contain (A) 0.1% lignin and 0.006% SDS (B) 0.5% lignin and 0.032% SDS, and (C) 0.032% SDS alone. Photographs were taken 30 minutes after emulsion preparation.

Figure H9 shows that similar emulsions to those shown in Figure H5 were formed using the sub-micron lignin particle suspension. An opaque, creamy lower layer of the emulsion is evident, with no bottom layer of excess water. This may support earlier indications that sub-micron lignin particles can produce some smaller emulsion droplets that do not cream. Interestingly, there does not appear to be distinct "middle" and "lower" layers present in these emulsions. This may indicate that the droplets are less polydisperse, since there does not seem to be a cream layer present.

Figure H9 also shows that for oil and water emulsified under the same conditions in the presence of 0.032% SDS only, an oil layer had formed on top of the emulsion within approximately 30 minutes of standing. Since the emulsions containing lignin contained either the same or a lower concentration of SDS as the emulsion containing SDS alone, we can infer that the stability of these emulsions to major destabilisation has been provided by the lignin content rather than the SDS itself.

Figure H10 shows LM images of the lower layers of these emulsions, revealing an apparent decrease in droplet size with increase in lignin concentration. When examined the day after preparation, the emulsions containing 0.1% lignin (and 0.006% SDS) and SDS alone exhibited oil coalescence, with a layer of oil sitting on top of the emulsion.

The emulsion containing 0.5% lignin did not have a layer of oil on the top. These observations, along with the droplet size differences shown in Figure H10, suggest that lignin concentration influences emulsion droplet size. Therefore, it is recommended that higher lignin concentrations be trialled to determine whether even smaller oil droplets can be produced.

Compared to the emulsion droplets shown in the LM image in Figure H2, the emulsion droplets shown in Figure H10 are smaller and more spherical, indicating that the use of sub-micron lignin microparticles has enabled smaller emulsion droplets to be produced and stabilised. However, the emulsions shown in Figure H10 were produced using the Silverson high shear mixer and only 10% (w/w) oil, whereas in the emulsions in the previous trial contained 20% (v/v) (~18.6% (w/w)) oil and were prepared using the Heidolph. Therefore, comparisons between these emulsions prepared using lignin of different particle size cannot be made. Therefore, it is also recommended that further trials be carried out where sub-micron and ~10 μm lignin particles in fresh suspension are able to be directly compared.

Figure H11 shows the size distributions of oil droplets in emulsions prepared using both SDS-stabilised and non-stabilised fresh microparticle suspensions containing a similar lignin content. The non-stabilised suspensions contained 1.54% (w/v) lignin in the form of ~10 μm particles. The SDS-stabilised suspension contained 1.63% (w/v) lignin in the form of 0.1-0.2 μm particles. Figure H11 shows that the emulsion containing non-stabilised lignin particles has a high volume of droplets in the 10-100 μm size range. The droplets in the emulsion containing sub-micron lignin also show a small peak of droplets in this size range, but a higher volume of droplets in the 0.1-1 μm size range. This indicates that sub-micron lignin particles enable smaller droplets to be produced. However, since the peak in the 0.1-1 μm size range is in approximately the same range as the peak exhibited for sub-micron lignin in suspension (Figure H8), it is possible that sub-micron lignin particles have been detected as oil during the light scattering measurement. Therefore, analysis of the samples by LM was carried out to determine whether or not this had occurred.

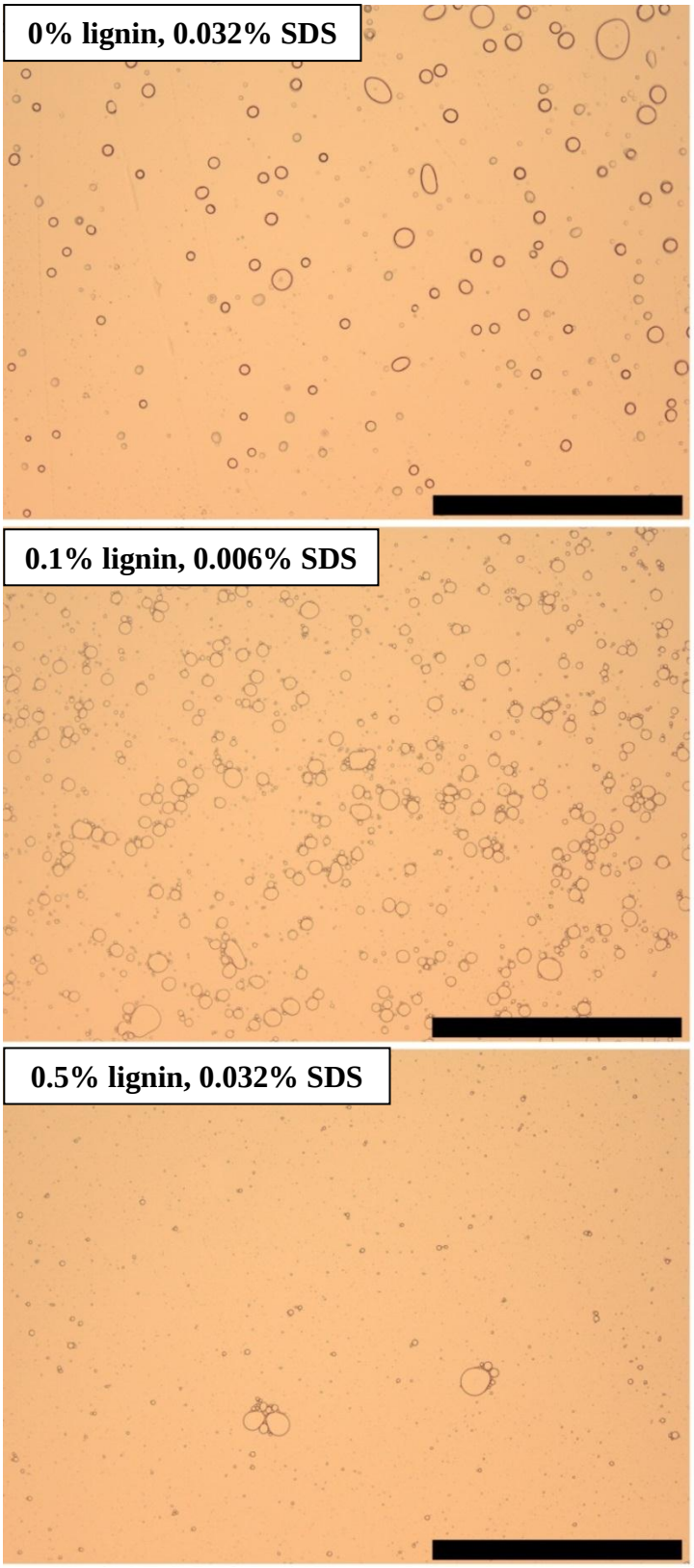

Figure H10. LM images of 10% (w/w) oil emulsions prepared using (A) 0.032% SDS alone, and fresh lignin microparticle suspensions diluted to contain (B) 0.1% lignin and 0.006% SDS and (C) 0.5% lignin and 0.032% SDS (bar scale = 500 µm).

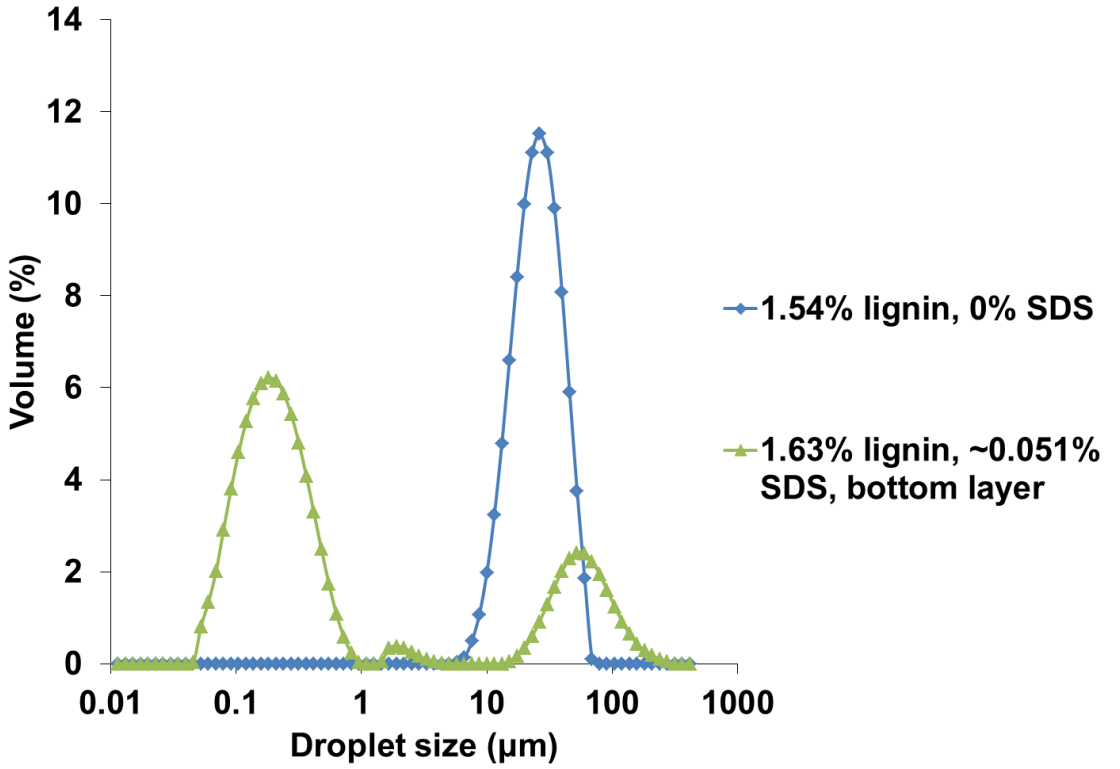

Figure H11. Droplet size distributions for 10% (w/w) oil emulsions prepared using fresh lignin microparticle suspensions containing (A) 1.54% (w/v) lignin and 0% SDS and (B) 1.63% (w/v) lignin and ~0.051% (w/v) SDS.

Figure H12 shows LM images of oil droplets in emulsions prepared using the stabilised and non-stabilised suspensions.

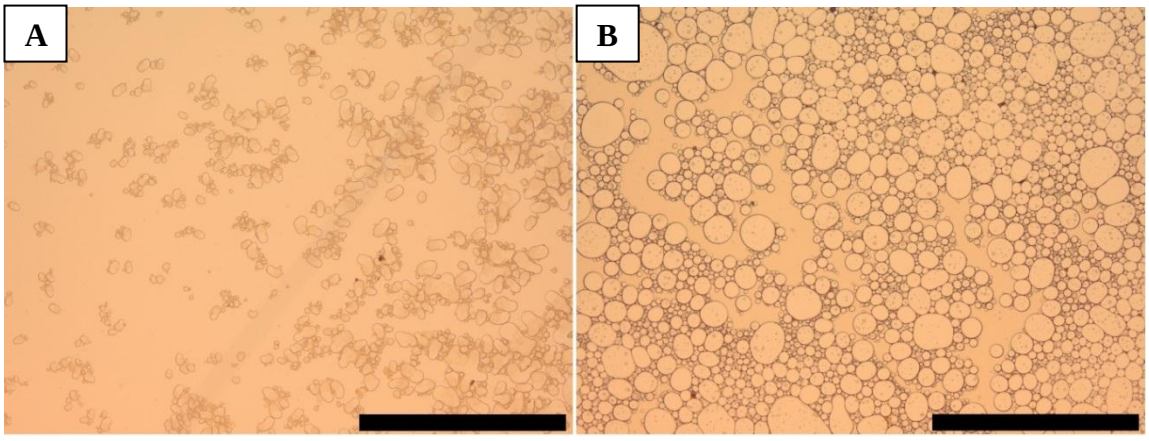

Figure H12. LM images of oil droplets in emulsions prepared using microparticle suspensions containing (A) 1.54% (w/v) lignin and 0% SDS, and (B) 1.63% (w/v) lignin and ~0.051% (w/v) SDS (bar scale = 500 µm).

Figure H12 reveals that more spherical droplets are produced when sub-micron lignin particles are used to prepare emulsions. However, these particles are also more polydisperse; some droplets are much larger than those produced using the lignin suspension containing non-SDS-stabilised particles. This may be due to the higher concentration of lignin required to stabilise the higher interfacial area of smaller droplets, which leaves insufficient lignin available for stabilising all droplets and thus some much larger droplets result. The resolution of the light microscope is not high enough to show the droplets in the 0.1-0.2 μm size range. Therefore, no conclusions can be made about whether the peak in this size range in Figure H11 represents oil droplets or lignin particles.

Figure H13 and Figure H14 show confocal microscopy images of oil droplets in the emulsions stabilised by the sub-micron and ~10 μm lignin. Lignin is shown as red fluorescence in these images. The images again support that droplets produced using sub-micron lignin particles are not smaller than those produced using non-stabilised lignin and that in fact some larger droplets are produced.

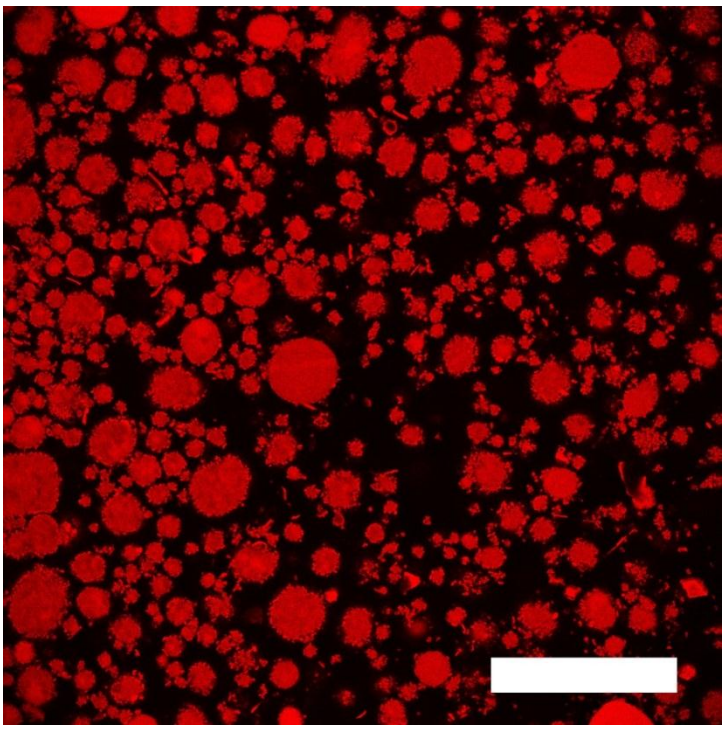

Figure H13. Confocal microscopy image of droplets in 10% (w/w) oil emulsion prepared using microparticle suspension containing 1.54% (w/v) lignin (bar scale = 100 μm).

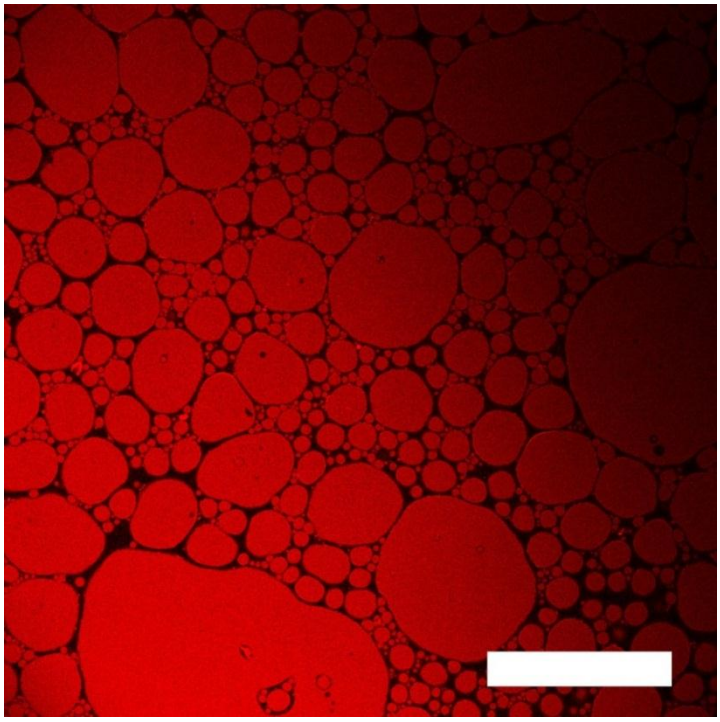

Figure H14. Confocal microscopy image of droplets in 10% (w/w) oil emulsion prepared using microparticle suspension containing 1.63% (w/v) lignin and ~0.051% (w/v) SDS (bar scale = 100 μm).

It is expected that when we examine a cross-section of a droplet, the centre of the droplet should not fluorescence since it is only oil, whereas the outside edge of the droplet should fluoresce because of the particle coating. However, images of the emulsions prepared using the sub-micron or ~10 μm lignin suspensions show that the whole surface of the droplet fluoresces. This may have been due to the high concentration of lignin in these emulsions. Interestingly, the droplets in the emulsion made from ~10 μm lignin suspensions exhibit rougher edges, giving the impression that the droplets are coated by small lignin particles.

Figure H15 shows a confocal image of droplets prepared using the pH-modification method and looks as we would expect for particle-stabilised droplets. The lower lignin content of only 0.1% (w/v) may be responsible for the better image.

It was suggested that the oil be stained with a fluorescent dye to better visualise the position of oil and lignin in the prepared emulsions. Therefore, in subsequent experiments, emulsions were stained with Nile red dye. Appendix I gives further details

of the development of the confocal method, including some critical limitations of the method and recommendations for future work.

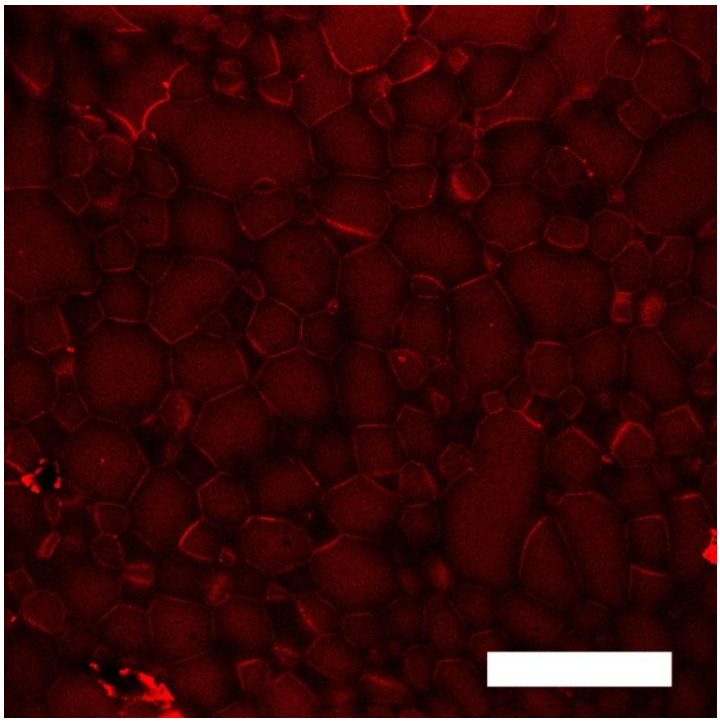

Figure H15. Confocal microscopy image of oil droplets in 20% (v/v) oil emulsion prepared using pH-modification method with 0.1% (w/v) lignin (bar scale = 100 µm).

Ultimately, it is unclear whether or not the use of sub-micron lignin particles enables smaller oil droplets to be formed. The microscopy images may provide the best indication, since the images allow oil droplets to be directly visualised. The microscopy images indicate that the use of sub-micron lignin particles does not provide an advantage over the use of ~10 µm sized particles in terms of resultant oil droplet size. However, this may have been due to lack of certainty in the method of Pickering emulsion preparation and characterisation at this stage of the project. For example, only Silverson (rotor-stator) mixing, running at less than top speed, was used, which may have limited oil droplets to a certain size regardless of the size of lignin particles used. Good static light scattering and LM technique was also still being developed. Therefore, it is recommended that lignin suspensions containing non-SDS-stabilised particles were

used all future experiments until the method and system are more understood. The effect of lignin particle size can then be investigated at a later time.

Appendix I

This Appendix outlines the development of a confocal microscopy method for the analysis of lignin-stabilised emulsions.

It was suggested that emulsion samples should be stained with Nile Red to enable both the oil and lignin in the emulsions to be visualised. Initially, samples of the emulsions were stained with Nile Red about an hour before examination under the microscope. Figure I1 below shows the images obtained for samples prepared using the fresh suspension method with both SDS-stabilised lignin particles and non-stabilised lignin particles. The fluorescence of the Nile Red is shown as blue in these images. It can be seen that the lignin and oil are in the same position in the emulsions, since the fluorescence for lignin and oil – coloured red and blue in this system – overlap to give a purple colour. This observation confirms that emulsion droplets were observed with the confocal fluorescence and supports that lignin goes to the surface of oil droplets to stabilise the emulsion.

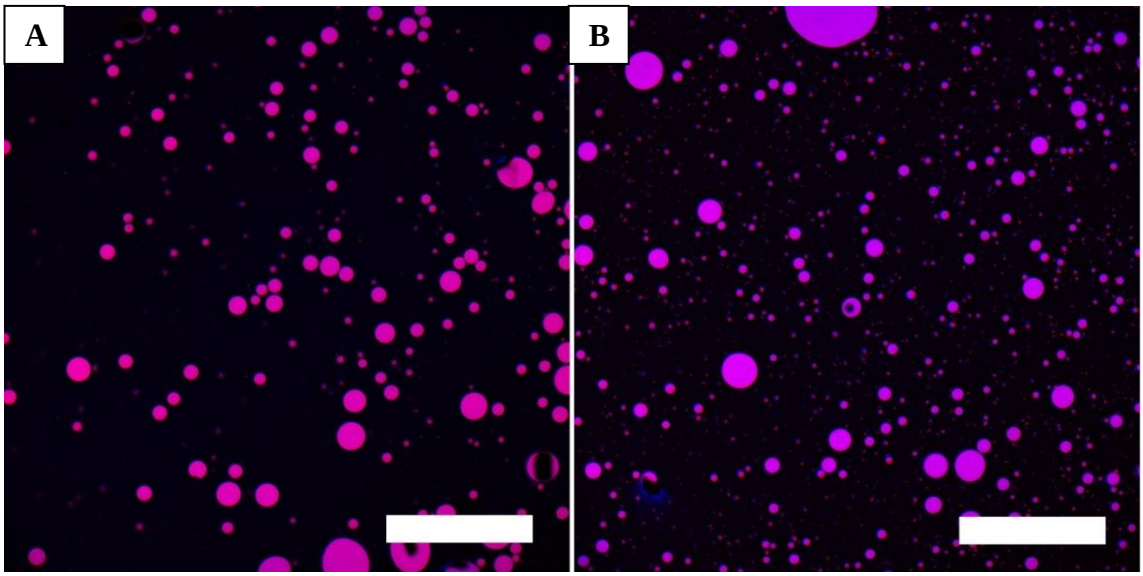

Figure I1. Droplets in 10% (w/w) oil emulsion containing (A) 1.48% (w/v) lignin, 0% SDS and (B) 1.55% (w/v) lignin, 0.064% (w/v) SDS (bar scale = 100 µm).

To check the expected appearance of oil droplets alone, a 'control' emulsion containing only 13.8% soybean oil and water was stained with Nile Red and examined. The purple colour of the droplets shown in Figure I2 indicates that fluorescence in both the lignin

and Nile Red wavelength ranges was detected. Since no lignin was present, this suggests that Nile Red gives a false positive for the presence of lignin. To test this, the control sample was analysed again but with no staining. The image obtained is shown in Figure I3.

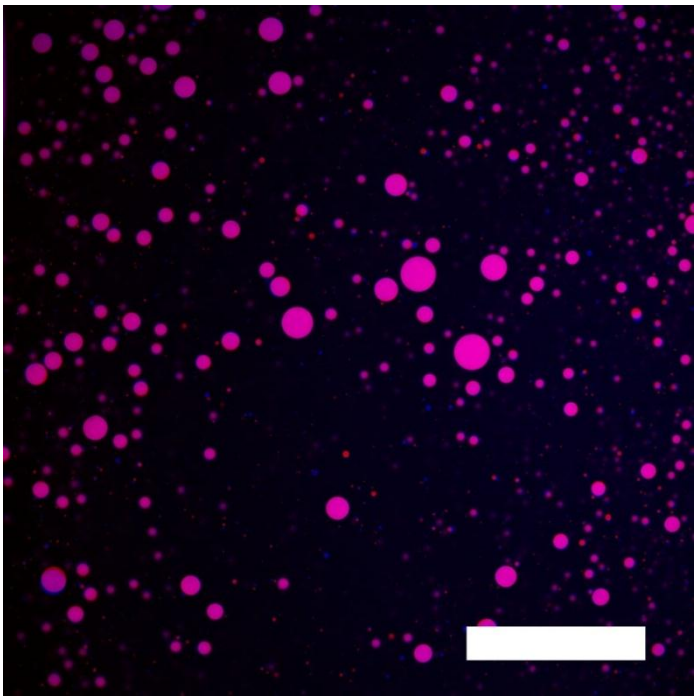

Figure I2. Droplets in 10% (w/w) oil emulsion containing no lignin or SDS. Sample was stained with Nile Red (bar scale = 100 µm).

Figure I3 shows dark droplets in a red background. The fact that only the background shows some redness rather than the droplets themselves supports that it was the use of Nile Red in the previous image of the control that gave a false positive for lignin. Unfortunately, the controls were only examined after the examination of most of the emulsion samples in this work. Therefore, different lipid dyes were not investigated. However, the confocal still provided another useful technique to visualise the droplets produced using various concentrations of lignin and oil.

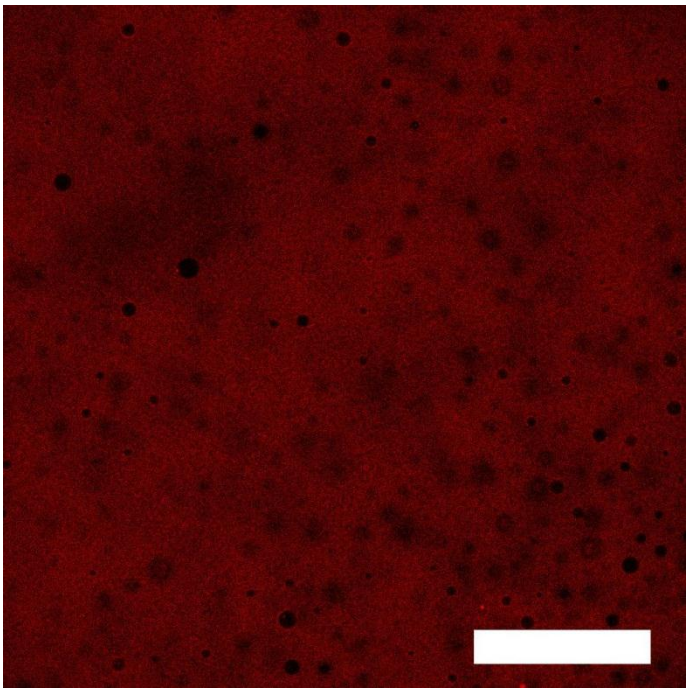

Figure I3. Droplets in 10% (w/w) oil emulsion containing no lignin or SDS. Sample was not stained for analysis (bar scale = 100 µm).

Figures I4-I7 below show images obtained for emulsion samples prepared using the pH-modification method with oil levels of 13.8 and 28.6% lignin where no staining of the sample was carried out. As for the examination of the unstained control, fluorescence in the emission range of the dye (570-676 nm) was still captured.

Figures I4-I7 show that without staining with Nile Red, red coloured droplets are observed, indicating lignin coated oil droplets. However, there are also some blue or purple coloured areas, indicating that fluorescence was detected in the emission range of Nile Red even though no Nile Red was present. The blue/purple colour is evident around the edges and between some droplets. The images indicate that lignin fluoresces in two separate emission regions.

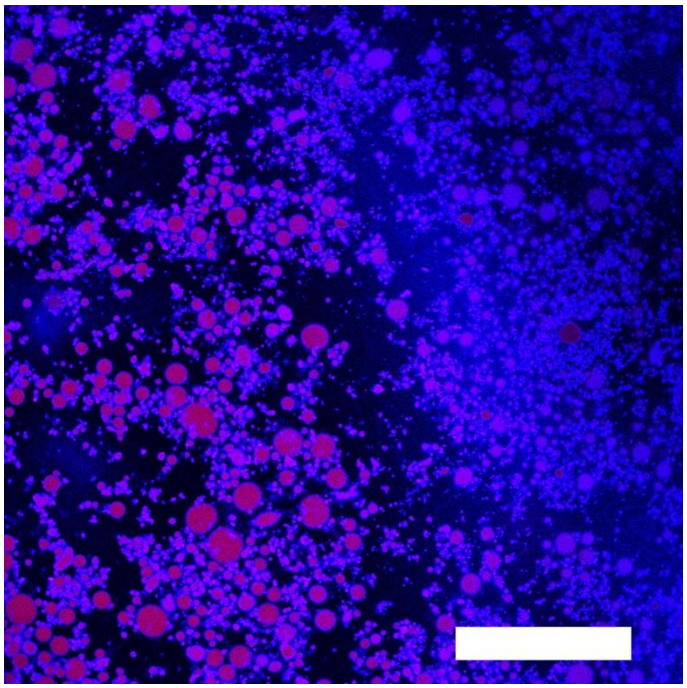

Figure I4. Droplets in 13.8% (w/w) oil emulsion prepared using the pH-modification method with 0.5% (w/v) lignin. The sample was not stained (bar scale = 100 µm).

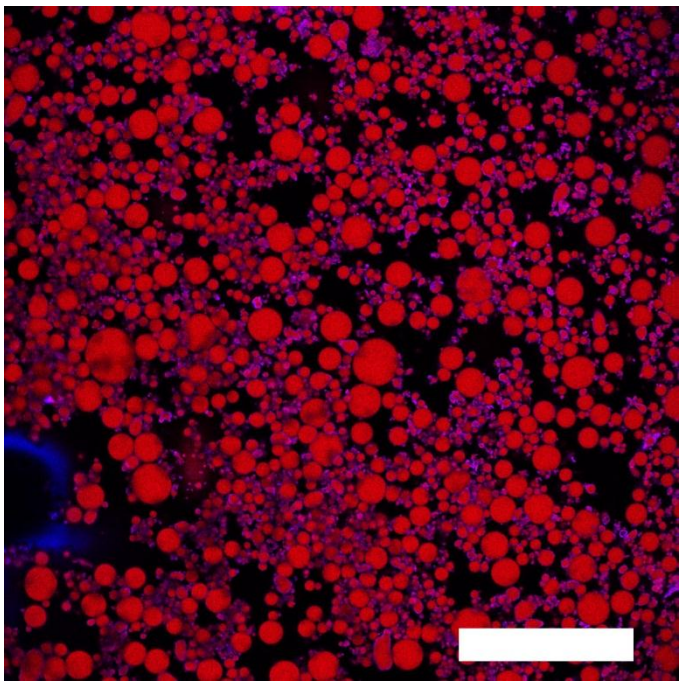

Figure I5. Droplets in 13.8% (w/w) oil emulsion prepared using the pH-modification method with 0.5% (w/v) lignin. The sample was not stained (bar scale = 100 µm).

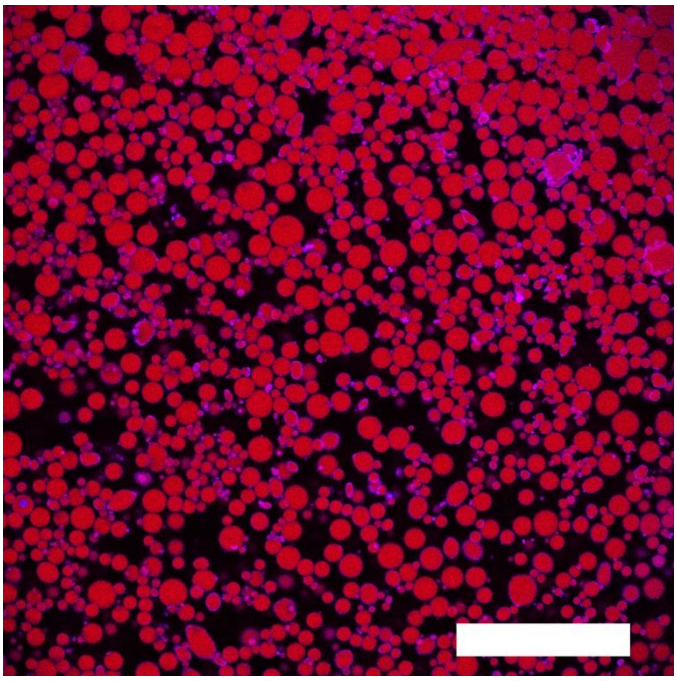

Figure I6. Droplets in 28.6% (w/w) oil emulsion prepared using the pH-modification method with 0.5% (w/v) lignin. The sample was not stained (bar scale = 100 μm).

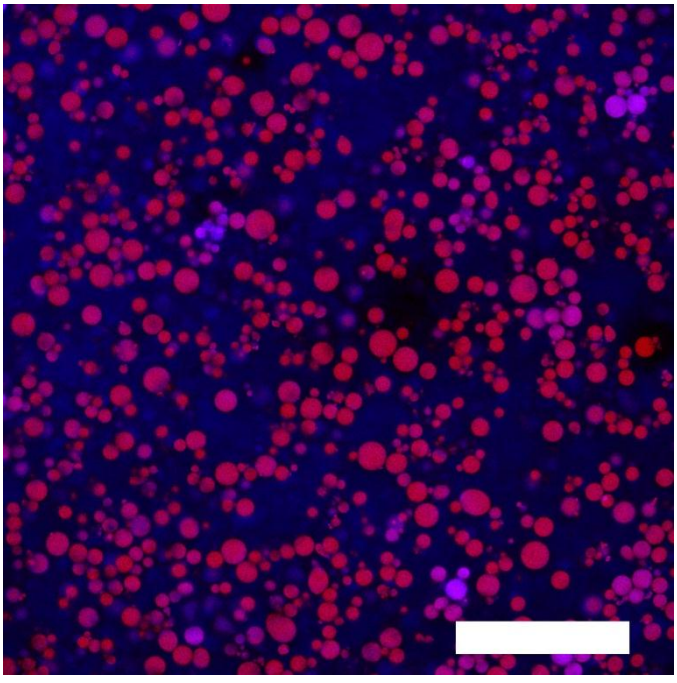

Figure I7. Droplets in 28.6% (w/w) oil emulsion prepared using the pH-modification method with 0.5% (w/v) lignin. The sample was not stained (bar scale = 100 μm).

Consistent with the idea that lignin emits in two different wavelength ranges, Donaldson and Radotić (2013) suggest that lignin has a broad fluorescence emission range and can be excited with both UV and visible light. Furthermore, "its autofluorescence is characteristically multimodal, indicating the presence of multiple fluorescent structures within the lignin molecule." In fact, Donaldson, Radotić, Kalauzi, Djikanović, and Jeremic (2010) suggest that there are at least five discrete fluorophores present in cell walls, including coniferyl alcohol, biphenyl, phenylcoumarane and stilbene. Therefore, Donaldson and Radotić (2013) used UV excitation (355 nm) and acquired emission spectra over the range 400-700 nm (at intervals of 5 nm) when imaging lignin fluorescence in normal and compression wood.

It has been shown that normal and compression wood lignins fluoresce at different wavelengths, likely to be due to differences in the amounts of *p*-hydroxy type lignin. Although in this work we are not interested in distinguishing between normal and compression wood lignins, this information indicates that excitation and emission wavelengths need to be specifically chosen when imaging certain types of lignin. In this work, an excitation wavelength of 405 nm and emission range of 490-620 nm were used for lignin. The chosen wavelengths were based on the work of Chapman, Oparka, and Roberts (2005). However, Chapman et al. (2005) were interested in distinguishing lignin from chlorophyll in roots and leaves of plants, rather than looking at refined lignin. The exploitation of the differences in lignin fluorescence depending on the specific type of lignin present by Donaldson et al. (2010) and Donaldson and Radotić (2013) suggests that conditions should have been found for the detection of fluorescence of refined lignin, particularly lignins from hardwoods, since we know these have a different structure to native lignin. Recent literature searches have not shown up any information for refined lignin. Therefore, it is recommended that if better images of the emulsions are to be obtained, in which the lignin and oil are clearly distinguished, preliminary experiments may need to be carried out to determine the best excitation and emission wavelengths to use. However, it is important to again note that the confocal analysis carried out in this work has still been helpful in confirming the presence, size and shape of emulsion droplets.

As a final step in the evaluation of the confocal method, images of lignin suspensions containing only lignin and water with no staining were obtained. Figure I8 shows one of the images obtained; revealing that the lignin in the suspensions used to prepare

emulsions fluoresces in the expected emission ranges for both lignin autofluorescence and Nile Red fluorescence. This observation further supports that lignin emits in a broad or multiple wavelength range.

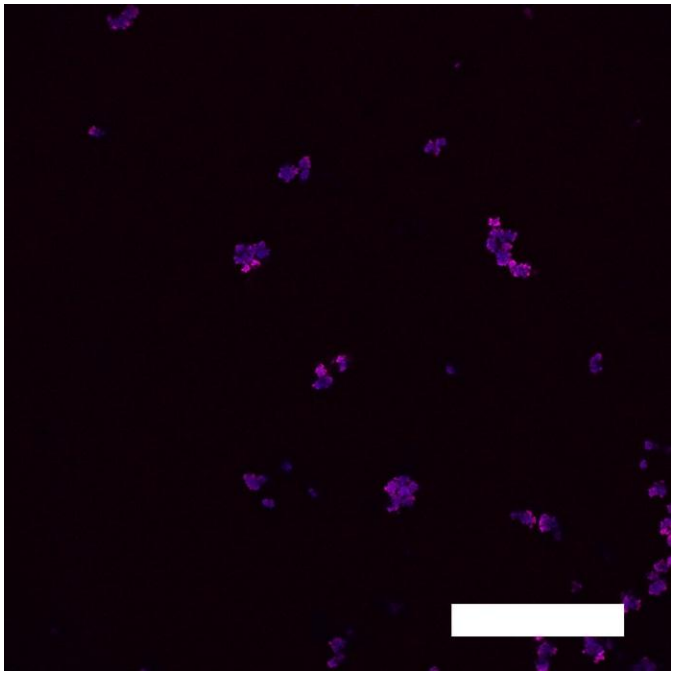

Figure I8. Sample of fresh microparticle suspension used in this work. No oil. No staining (bar scale = 100 µm).

It is interesting to note that irregular structures such as those shown in Figure I8 were not observed in the majority of confocal images of emulsion samples, indicating that lignin is not present in this form after emulsion formation. This may suggest that the lignin flocs or aggregates break up upon emulsification and adsorb at the oil droplet surface as single particles.