Abstract

CONTRERAS, IRMA. On the Propensity of Lignin to Associate; Static Light Scattering Measurements. (Under the direction of Dr. Lucian Lucia and Dr. Dimitris S. Argyropoulos).

Lignin, the glue that keeps fibers together, is a complex three dimensional network polymer which has shown association phenomena in solution. Following the molecular weight of the biopolymer in solution as a function of time allow us to explore their observable de-association phenomena. These measurements were carried out using multiple angle light scattering (MALS) photometry in the static mode. EMAL (Enzymatic Mild Acidolysis Lignin) from hardwood and softwood were isolated and an additional method for the complete dissolution of such biopolymers in THF was further developed. Once the challenge of the measurement procedures were worked out, some rather accurate *dn/dC* values for lignin solutions were obtained as a function of time. This effort when coupled to additional work using static light scattering measurements (Zimm plots) for the same solutions offered an insight into the self-assembly processes operating within the lignin biopolymer.

On the Propensity of Lignin to Associate Static Light Scattering Measurements

by
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Dedication

This work is dedicated to those who support me, even though some are not physically present in this world.

Biography

Irma Sofia Contreras was born in Merida, Venezuela in October 4th, 1979. She went to Univesidad de Los Andes in her country and she got her bachelor degree in Chemical Engineering in July 2003.

In spring 2006 she started the Master program in the Department of Wood and Paper Science having as advisor Dr. Lucian Lucia and Dr. Dimitris Argyropoulos.

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1. Introduction

Lignin, second only to cellulose as a source of fixed carbon in the biosphere, is a complex three dimensional polymer network; its structure in wood was visualized as branched with linear chains cross-linked by a variety of interchain covalent bonds.[1] Lignin content and composition are important traits in several tree breeding programs, but very little is known about their natural variation.[2] In general, between 15 and 35% of wood in trees consist of lignin. Lignin is required for waterproofing of the vascular system, for mechanical strength and for resistance to insects and pathogens.[3]

Recent interest in renewable natural resources has awakened interest in the field. The alkaline degradation of native lignin commonly referred to as the kraft pulping process produces over 16 million tons dissolved lignin per annum in U.S. alone.[4] Of this, only a very small fraction is recovered. The greater part is burned in internal recovery units, contributing to the heat balance in the operation.[5] From these applications and uses of lignin and those to come, emerge the effort of understanding lignin properties and behavior.

Lignin components in solution tend to associate with one another. There have been a lot of efforts trying to better understand lignin association phenomena. [5-10] Association forces are effective both in aqueous solution and in organic solvents.[11] Most of the previous studies with lignin were carried out using size exclusion chromatography (SEC) and gel permeation chromatography (GPC). It has been known there can be some problems as ion inclusion and ion exclusion or adsorption of the lignin components onto the gel matrix with the techniques

just mentioned. Even though, there have been several publications that worked out the possible artifacts, just mentioned above, in the measurements of lignin solutions with techniques that required the use of columns [6, 7, 11, 12]; it is a good idea to use a different technique to corroborate the results and to go further in this investigation. This research is focused in the study of two kinds of lignins in solution (Spruce lignin, Eucalyptus Globulus lignin), using organic solvent (THF) and alkaline solution (NaOH) with MALLS (Multi Angle Laser Light Scattering) in static mode to determine the change in the apparent molecular weight distributions of lignin solutions for different temperatures and different incubation times. MALLS is an absolute technique, based in the intensity of the light that is scattered when a laser passes through a sample. The intensity of the light scattered is directly proportional to the molar mass thus light scattering represents a powerful technique for monitoring the presence and formation of aggregates in solution. Therefore, light scattering is a good technique to study the interaction forces responsible for lignin association phenomena.

2. Literature review

2.1 Lignin

Crucial to the evolution of land plants, the lignin polymer is increasingly well understood while at the same time being increasingly misunderstood and misrepresented. [13]

The term lignin was introduced in 1819 by de Candolle and is derived from the Latin word *lignum* meaning wood. Lignin, second only to cellulose as a source of fixed carbon in the biosphere, is one of the major components of the plant cell wall (20% by *dry* weight), along with cellulose and hemicelluloses.[14] This biomaterial is generally considered to be highly resistant to rapid biological degradation[10] and it is a heterogeneous phenolic polymer that plays crucial roles in the development and physiology of vascular plants.[3] Their basic units are linked at least by 10 different linkages.[1]

Lignins are complex natural polymers resulting from oxidative coupling of, primarily, 4-hydroxyphenylpropanoids (coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol).[15] The actual structure of the lignin macromolecule is not absolutely defined or determined. The currently accepted theory is that the lignin polymer is formed by combinatorial-like phenolic coupling reactions, via radicals generated by peroxidase-H_2O_2, under simple chemical control where monolignols react endwise with the growing polymer.[13] Stereochemical studies on lignins have shown that the arylglycerol-β-aryl ether structure (1) is the predominant structural element in lignins. [1, 16] Hardwood lignins consist of guaiacyl (2) and syringyl (3) units in variable proportions, while spruce and other softwood lignins consist almost entirely of guaiacyl (2) units.[17] **Figure 1.**

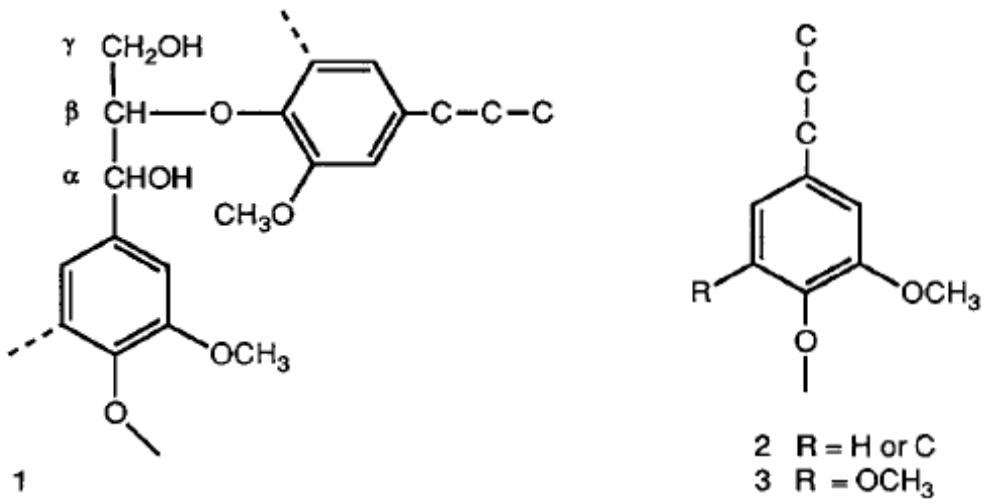
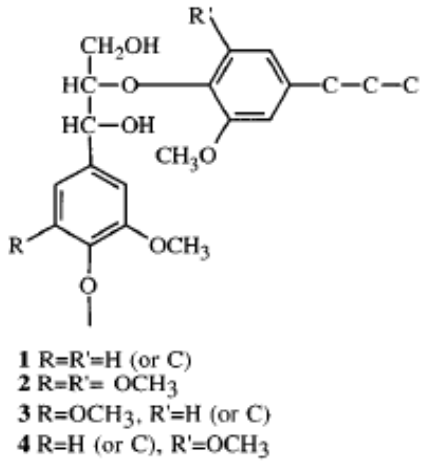


Figure 1. (1) arylglycerol-β-aryl ether structure and (2) guaiacyl and (3) syringyl units.

The "randomness" of linkage generation and the number of possible isomers of a simple polymer structure, lead to a wide polydisperse polymer. The interunit linkages found in lignin are primarily β-*O*-4, β-5, β-1, β-β, 4-*O*-5 and 5-5 couplings.[1] The β-*O*-4 linkage is the most frequent of these and is usually more abundant in hardwoods than in softwoods.

In hardwood lignins there are substantial amounts of guaiacylpropane units in addition to syringylpropane units. Therefore, four types of arylglycerol β-aryl ethers (1-4) have to be considered in studies of such lignins **Figure 2**.[18]



1 R=R'=H (or C)
2 R=R'= OCH_3
3 R=OCH_3, R'=H (or C)
4 R=H (or C), R'=OCH_3

Figure 2. The four types of arylglycerol β-aryl ethers found in lignin.

Furthermore, there are two forms, the *erythro* and *threo* stereoisomers of the β-*O*-4 linkage [17,18,19] **Figure 3**.

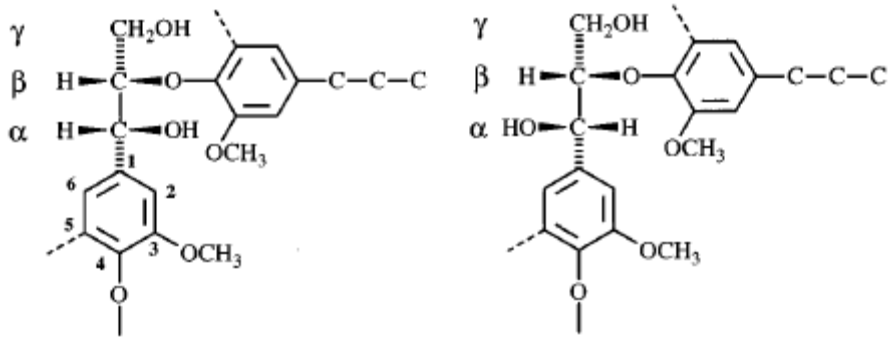


Figure 3. The *erythro* and *threo* forms of arylglycerol β-aryl ethers.

The structural characteristics of lignins vary according to biological parameters such as plant species or tissue type, thereby producing a wide range of chemical structures that influence the final properties of these complexes.[20] In **Figure 4** a model of lignin molecule is shown.

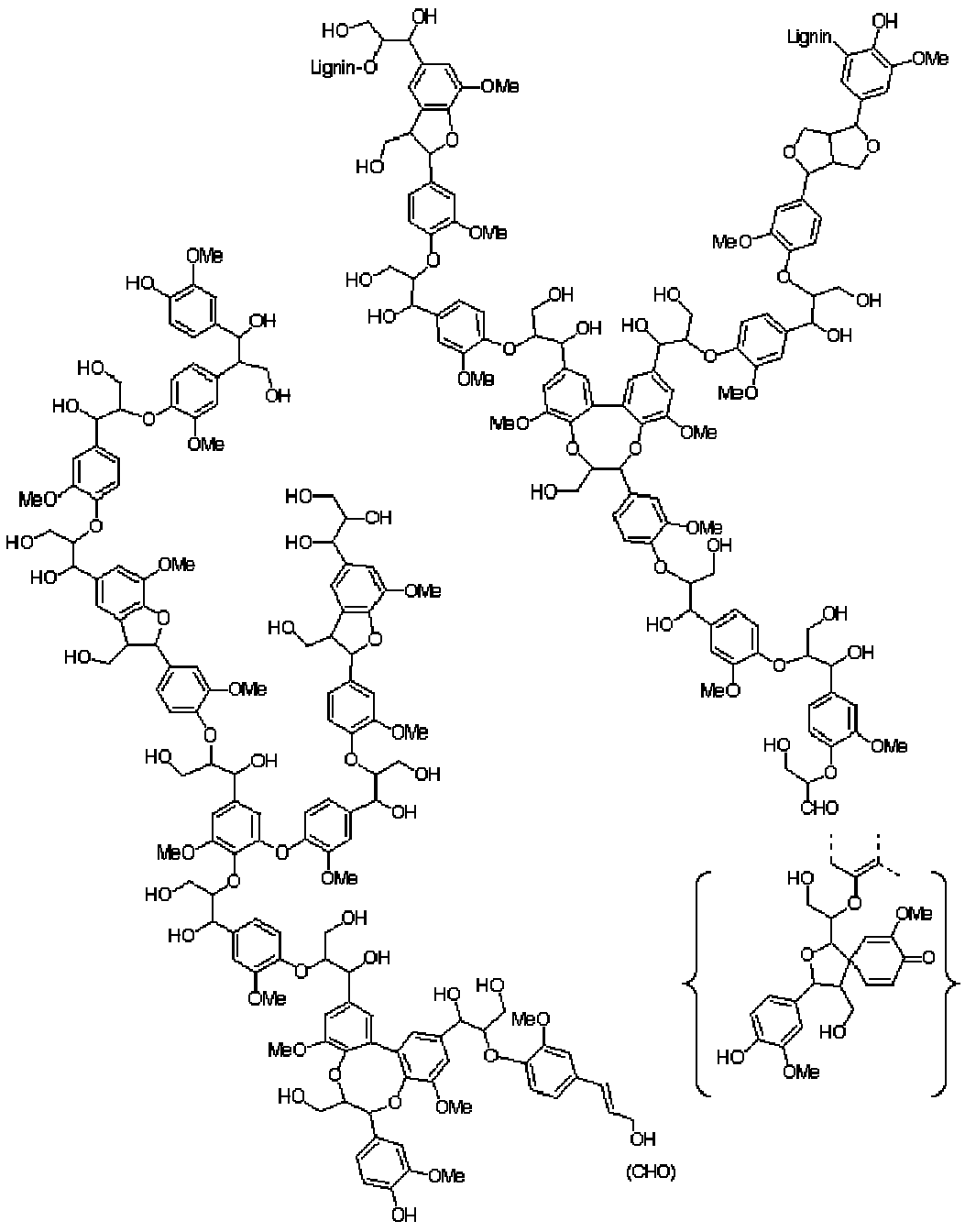


Figure 4. Model of lignin molecule.

Lignin fills the spaces in the cell wall between cellulose, hemicellulose and pectin components, especially in tracheids, sclereids and xylem. It is covalently linked to hemicellulose and thereby crosslinks different plant polysaccharides, conferring mechanical strength to the cell wall and by extension the plant as a whole.[21] It also plays a significant role in the carbon cycle, sequestering atmospheric carbon into the living tissues of woody perennial vegetation. Lignin is one of the most slowly decomposing components of dead vegetation, contributing a major fraction of the material that becomes humus as it decomposes. The resulting soil humus generally increases the photosynthetic productivity of plant communities growing on a site as the site transitions from disturbed mineral soil through the stages of ecological succession, by providing increased cation exchange capacity in the soil and expanding the capacity of moisture retention between flood and drought conditions.

2.1.1 Economical significance

Mechanical, or high yield pulp used to make newsprint contains most of the lignin originally present in the wood. This lignin is responsible for newsprint yellowing with age. Lignin must be removed from the pulp before high quality bleached paper can be manufactured from it.

Lignin removed via the kraft process is usually burned for its fuel value, since lignin yields more energy when burned than cellulose, providing more than enough energy to run the mill and its associated processes.

One of the processes used to remove lignin from wood pulp is called sulfite pulping, where lignin is removed as sulphonates. These lignosulfonates have several uses:[22]

- Binders: Lignosulfonates are a very effective and economical adhesive, acting as a binding agent in pellets or compressed materials. They reduce environmental concerns from airborne dust particles and stabilize the road surface when used on unpaved roads.[23]

- Dispersants: Lignosulfonate prevents the clumping and settling of undissolved particles in suspensions. By attaching to the particle surface, it keeps the particle from being attracted to other particles and reduces the amount of water needed to use the product effectively in high performance cement applications, water treatment formulations and textile dyes.[24, 25]
- Emulsifiers: Lignosulfonate stabilizes emulsions of immiscible liquids, such as oil and water, making them highly resistant to breaking. They are used in asphalt emulsions, pigments and dye, and wax emulsions. [26, 27]

- Additives in specialty oil field applications and agricultural chemicals.[28, 29]

- Raw materials for several chemicals, such as vanillin, DMSO, ethanol, torula yeast, xylitol sugar and humic acid.

- Environmentally sustainable dust suppression agent for roads.

2.2 Association

In order to study lignin behavior in solution it is important to understand how it interacts with solvents. Related studies started some years ago. Lindberg et al.[30] focused the study on hydrogen bonding in a thiolignin by infrared spectroscopy and measured the molecular

weight of the sample by cryoscopy, concluding that these lignins exist in solution as three-dimensional aggregates held together by hydrogen bonding and in turn kept in solution by hydrogen bonds to solvent molecules and that lignin molecule in solution consists of a strongly immobilized, tight network core and a looser surface region where random coil-like local motions of the macromolecule chains are possible.

Lignin has shown to be a polydisperse material with high apparent molecular weight. Therefore, back in 1967, there was an open question: whether the material of apparent molecular weight greater than that of approximately 1,000 consists of molecularly associated lignin or if it is made up of covalent macromolecules?[31] Later on, lignin studies generated bi- and multimodal distributions of species with high apparent molecular weight when analyzed by GPC using nonaqueous and aqueous mixtures. Later, work done by Sarkanen et al.[12] concluded that such disparities result from the association of lignin components to well-defined high apparent molecular weight complexes. They added that these effects should not be confused with aggregative interactions between associated complexes leading ultimately to gelation or precipitation.

In additional work, Sarkanen et al.[7] followed the variations in kraft lignin apparent molecular weight distributions arising from the method of isolating sample precipitated from black liquor at pH 2.5. Despite their common origin, these three kraft lignin samples differ markedly from one another in the contribution that the higher apparent molecular weight species make to their respective apparent molecular weight distributions as shown in **Figure 5**. Indeed an appropriately weighted sum of profiles 2 and 3 shows that the kraft lignin as a whole contained a larger proportion of high apparent molecular weight components after filtering and air-drying than after centrifuging the precipitate from acidified black liquor.

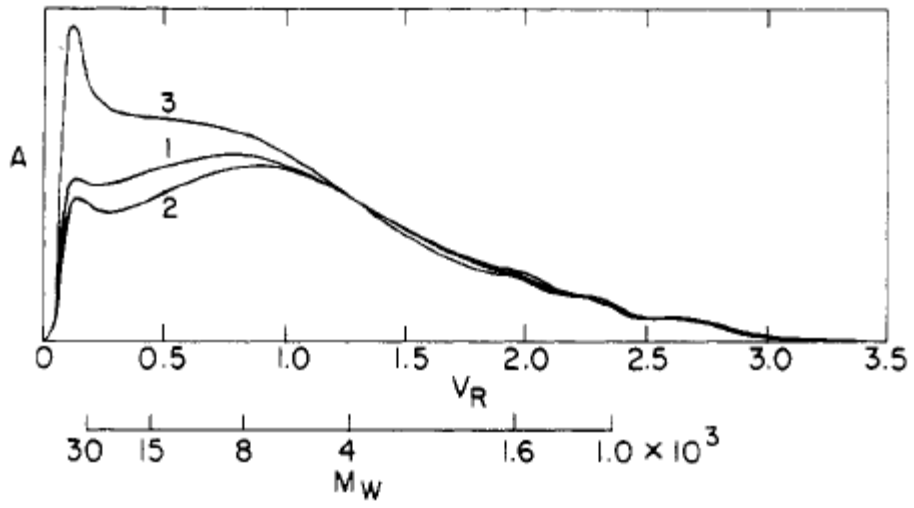


Figure 5. Apparent molecular weight distributions of kraft lignin samples from different isolating method. (Sephadex G75/0.10 M aqueous NaOH, monitored at 254 nm): after (1) centrifugation and subsequent freeze-drying from aqueous solution at pH 8.5; following filtration and air-drying, portions (2) soluble in (and freeze-dried from) and (3) insoluble in aqueous solution at pH 8.5.[7]

Clearly, then, air-drying of the kraft lignin precipitate significantly affect the degree of association between the components in the sample; these observations could not have arisen from covalent chemical changes such as oxidative coupling between phenolic moieties. It is worth emphasizing that the behavior of Organosolv and kraft lignin components at varying concentrations in alkaline aqueous solution (pH 13-14) is appropriately interpreted in terms of intermolecular association and de-association and is not due to covalent chemical changes such as oxidative coupling of phenols or reactions of the retroaldol type.[12]Additional work showed an increase in apparent molecular weight of kraft lignin solutions over time.[5-9, 32]

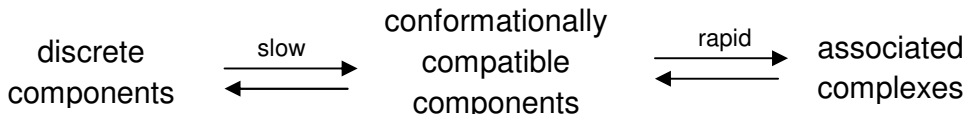
These authors suggested the presence of "noncovalent" interactions between individual lignin molecular components, meaning that lignin molecules are not sharing electrons, but rather electromagnetic interactions are involved. Lindström reported the presence of stable lignin sols in aqueous solution, whose extent of aggregation was followed by gel permeation chromatography and viscometric measurements, however, no light scattering measurements were made.[5] He explained the sol or gel formation by association due to hydrogen bonding between the carboxylic groups and various ether oxygens and hydroxylic groups. It was concluded that, the extent of association of kraft lignin sols in the hydrogen form increases with increasing storage time and temperature**.** They also concluded that van der Waals forces contribute strongly to the interactions between the particles and that irreversible nature of the association process is thus essentially a coagulation process where the particle size is increased and the shape of the aggregates is changed although the sol is still stable.[33]

On the other hand, Sarkanen et al.[12] made the study with organosolv lignin and kraft lignin concluding that the intermolecular associative effects are apparently governed by nonbonded orbital interaction presumably between the aromatic moieties in the components since exhaustive methylation and acetylation of lignin samples have not appreciably affected the proportions of high apparent molecular weight species studied by GPC. Therefore, hydrogen bonding does not appear to play a direct role in the association phenomenon. They explain the presence of kraft lignin association complexes in alkaline solutions by means of HOMO-LUMO interactions (interactions of the highest occupied molecular orbital with the lowest unoccupied molecular orbital). Later, an idea was developed, if the interactions just mentioned are present in the association phenomenon, then the presence of iodine would

reduce the association of lignin dislocating the aromatic rings from each other.[34] This assumption is based on the ability of iodine to form iodine-aromatic hydrocarbon complexes with different compounds characterized by intense absorption peaks in the 280–400 nm region.[35] It was found that the addition of I_2 changed the SEC chromatograms and furthermore, the amount of I_2 added was also affecting the apparent molecular weight changes. De-association was faster when I_2 was present in the lignin solutions. This finding may indicate that non-bonded intermolecular orbital interactions prevail over entanglements as far as the operating association forces are concerned. After disrupting such intermolecular interactions, chain entanglement can be easily dislocated and the de-association process is completed easier.[34]

In an effort to go deeper in the understanding of lignin association/de-association phenomenon, Sarkanen et al.[7] developed a mechanism to explain the kinetics of this process. He worked with kraft lignins, adding zwitterion (betaine) which are chemical compunds that are electrically neutral but carry positive and negative charge in different atoms. Then he studied the zwitteroin effect in lignin solutions. A particular manner in which betaine influences the apparent molecular weight distribution of kraft lignin has revealed that the associative process involves at least two kinetically distinguishable steps. Preincubation of the kraft lignin sample in aqueous NaOH containing betaine, was followed and the elution profiles were identical with those observed when prior incubation was carried out under the same conditions but without the zwitterion. Thus, a sudden reduction in the concentration of betaine allows a rate of reassociation which is at least 2 orders of magnitude faster in 0.10 M aqueous NaOH than that observed when the zwitterion had not been initially present. These

findings can be rationalized in terms of a slow (rate-controlling) process leading to component conformations which are compatible with subsequently rapid associated complex formation.[7]



Discrete components that can not associate undergo a slow conformational change to components that can then interact with the complexes. After deriving the equations for the kinects of the association/de-association process and assuming steady state concentration of the associated complexes for gymnosperm kraft lignin in aqueous alkaline solution,[7] it was concluded that association is not simply a random process: each kraft lignin complex possesses a locus or position, which is complementary to only one type of component. Of course the chemical structures of the components that are compatible with a particular locus need not be identical in every detail, but they presumably have in common a dominant molecular feature which serves to distinguish between component types.

Despite the various studies that point to a prevailing consensus that lignin associates in both aqueous and organic media, the magnitude, and the underlying driving forces behind these processes are still a matter of discussion.[34] There is an important issue to point out; association between lignin components can be further complicated by aggregation between the resulting associated complexes leading to gelation or precipitation.[12]

Additional work based on lignin association followed by GPC measurements of lignin of

several sources and species, showed the apparent molecular weight distributions in lignin solutions change according to several factors, the method to isolate the lignin from wood, the type of lignin (softwood lignin or hardwood lignin), the solvent used to prepare the solutions, and the age of the solution shows.[6, 7, 11, 12, 34]

Some other lignin studies have been carried out with light scattering [32, 36-40] concluding that MALLS method that can be used for determination of the apparent molecular weight and the apparent molecular weight distribution of lignins.

Following the literature, these are the possible driving forces responsible for the lignin association behavior:

2.3 Possible driving forces

2.3.1 van der Waals forces

Molecules can attract each other at moderate distances and repel each other at close range. The attractive forces are collectively called "van der Waals forces". van der Waals forces are much weaker than chemical bonds, and random thermal motion around room temperature can usually overcome or disrupt them. The attractive component is due to the induction of dipoles in the electron cloud of neighboring atoms. The dipoles will be coupled, leading to attractive forces. The repulsive component is due to steric hindrance when neighboring atoms start to have overlap of the electron clouds. The more electrons a molecule has, the more distance over which they can move, the bigger the possible temporary dipoles and therefore the bigger the dispersion forces. The shapes of the molecules also matter. Long thin molecules can develop bigger temporary dipoles due to electron movement than short fat

ones containing the same numbers of electrons.

As shown in **Figure 6**, the magnitude of the attractive and repulsive forces decreases when the distance between the molecules increases (dotted lines). The result force (continuous line) illustrates, there is an attractive force between the molecules when they are far from each other, usually low magnitude. As the molecules get closer the resultant attractive force increases until it reaches a maximum and then the molecules repel each other. As the molecules get even closer the magnitude of the repelling force become higher.

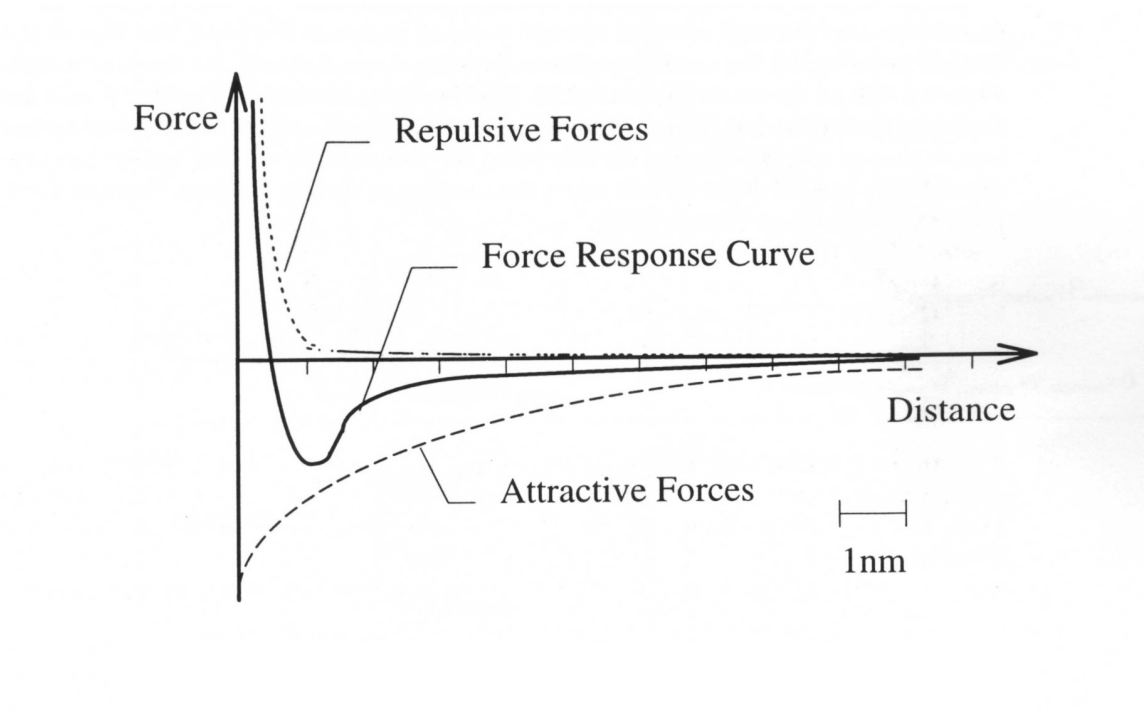


Figure 6. van der Waals forces versus distance.

2.3.1.1. Hydrogen bonding

It is a very strong dipole-dipole van der Waals interaction but weaker than covalent, ionic or metallic bonds. The strength of a hydrogen bond can vary from very weak (1-2 KJ/mol)

to very strong (155KJ/mol). In a hydrogen bond the H atom is noncovalently attracted to an electronegative atom. The H must have a large positive delta and the other atom must have a source of electrons to be attracted to H, [41] shown in **Figure 7**.

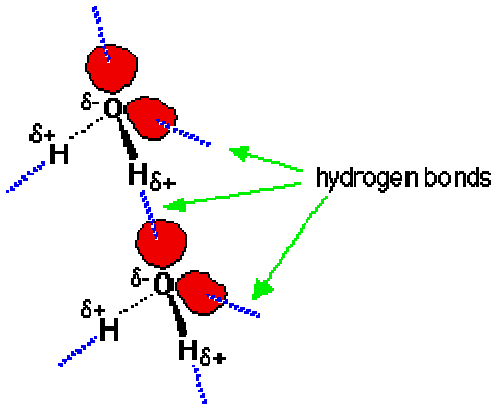


Figure 7. Hydrogen bonding between water molecules.

The hydrogen is attached directly to one of the most electronegative elements, causing the hydrogen to acquire a significant amount of positive charge. Each of the elements to which the hydrogen is attached is not only significantly negative, but also has at least one "active" lone pair. The δ^+ hydrogen is so strongly attracted to the lone pair that it is almost as if they were beginning to form a co-ordinate (dative covalent) bond. It doesn't go that far, but the attraction is significantly stronger than an ordinary dipole-dipole interaction. Hydrogen bonds have about a tenth of the strength of an average covalent bond, and are being constantly broken and reformed in liquid water.[42]

Several authors attribute lignin association to hydrogen bonding. The hydroxyl and/or the carbonyl groups present in lignin molecules tend to form hydrogen bonds and thus, form high

apparent molecular weight associate molecules.[5, 30, 31]

2.3.1.2. π – stacking

It is one of the van der Waals forces of the dipersion type, it exists between non polar molecules and it is a weak attraction of the order of 0.5-0.75 kcal/mol for parallel-displaced structure and 1 kcal/mol for T-shaped structure. It results from the distortion of two different π-electron clouds. When the π-electron cloud of one benzene ring becomes attracted to that of another as illustrated in (**Figure 8**), the negative dipoles repel each other so the electrons shift away from the other molecule. A positive dipole is created on the other side of the π-electron cloud; they become stronger as the number of π electrons increases. The negative dipole movement of a second benzene ring is attracted to this induced positive dipole.[41] The exact nature of such interactions (electrostatic or nonelectrostatic) is a matter of debates.

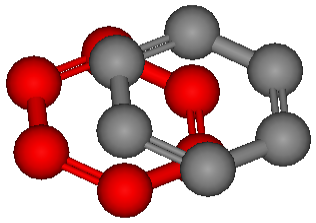


Figure 8. Face to face pi stacking interaction.

Aromatic rings can also adapt different arrangement like the one illustrated in **Figure 10** called edge- to-face interaction.[43]

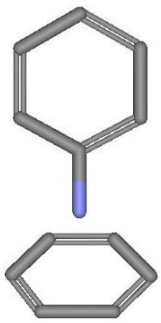


Figure 9. Edge-to-face pi stacking interaction.

The aromatic interactions can be attraction or repulsion depending on the angle and the offset between the aromatic rings, as shown in **Figure 10.**

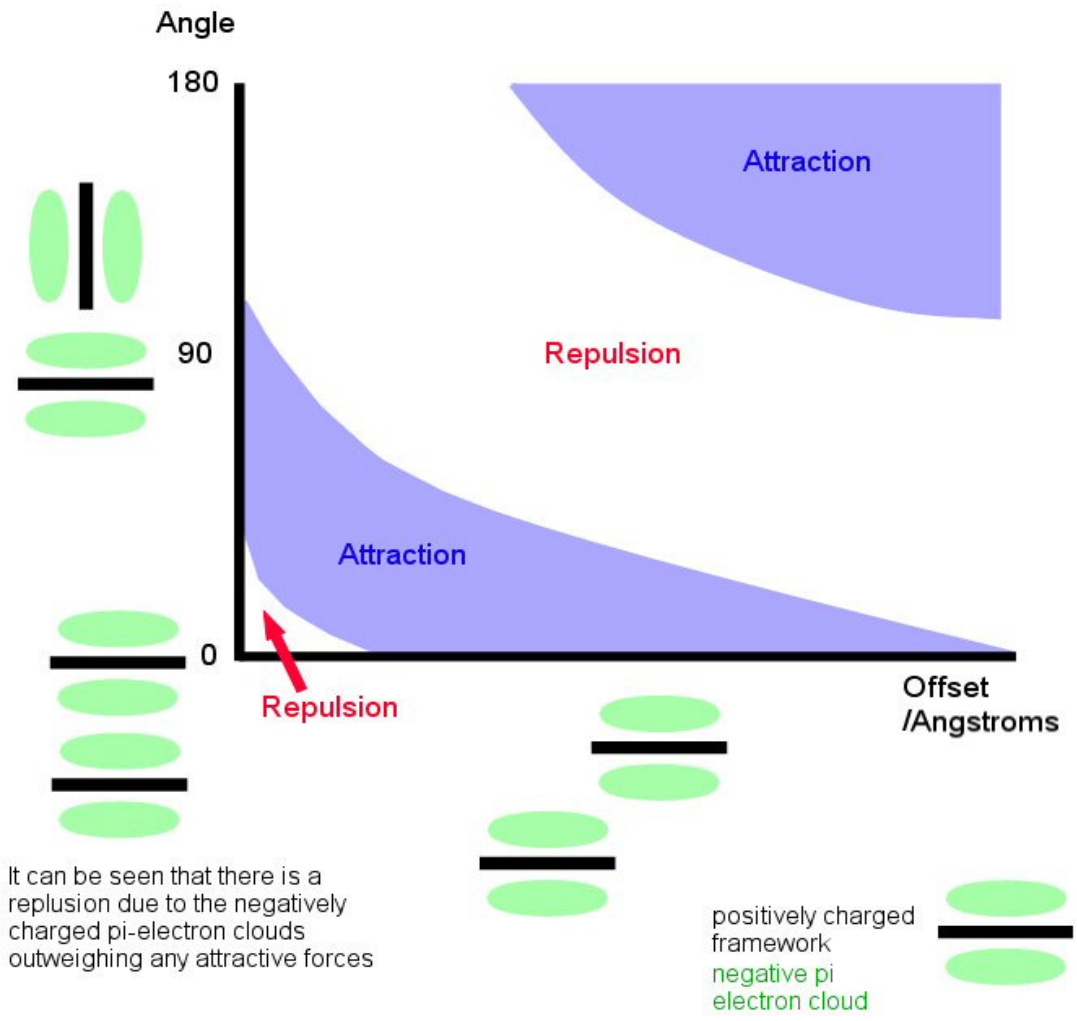


Figure 10. Relation between the angle and the offset between the aromatic rings in pi stacking interactions.

2.3.2 Entanglements

Molecular theories for polymer dynamics are based on the topological constraint known as chain entanglement. Physically, this means that the random walk polymer chains cannot move through one another. However, there is no precise definition available for exactly what

an entanglement is. As such, several models have been proposed to account for how entanglement spacing changes with polymer structure and concentration. The concept of molecular entanglement is simple (**Figure 11**). Although their existence for assemblies for long chains seems to be reasonable enough, the exact nature of entanglements and its mechanical properties are not obvious. This is true particularly in a uncross-linked system where, due to thermal motion, all the entanglements are temporary.[44]

The manner in which entanglement spacing depends on concentration is important in many aspects of polymer melt dynamics. For example, effects of long-chain branching and linear polymer polydispersity depend strongly on how entanglement spacing changes when part of the system relaxes.

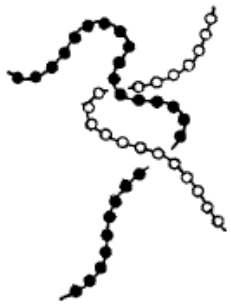


Figure 11. Typical entanglement junction.

2.4 Light Scattering

This technique was selected for this research because light scattering permits the measurement of the solution properties of macromolecules in solution. Guerra et al.[34]

followed the apparent molecular weight changes for different kinds of lignin in THF using gel permeation chromatography (GPC). GPC gives a relative value because the measurements are based in a standard, usually polystyrene and as mentioned before there can be ion inclusion or ion exclusion and adsorption of the lignin components in the column gel matrix. The next step was to confirm the behavior found by Guerra et al.[34] with a different technique that gives absolute values and without dealing with columns. Therefore, the idea for this research, where the apparent molecular weight of lignin in solution is determined using multi angle laser light scattering (MALLS). Light scattering permits measurement of the solution properties of macromolecules and it gives absolute values of the weight-average apparent molecular weight.

Light scattering is a non-invasive technique for characterizing macromolecules and a wide range of particles in solution. In contrast to most methods of characterization, it does not require external calibration standards. In this sense it is an absolute technique. There are two different types of light scattering measurements for absolute molecular characterization:

- Classical light scattering or Static light scattering: the intensity of the scattered light is measured as a function of angle. For the case of macromolecules, this is often called Rayleigh scattering and can yield the molar mass, rms radius, and second virial coefficient (A_2).

- Quasi-elastic light scattering (QELS) or dynamic light scattering (DLS): time-dependent fluctuations in the scattered light signal are measured using a fast photon counter. QELS measurements can determine the hydrodynamic radius of

macromolecules or particles.

Light scattering can be either applied in batch or chromatography mode. For this study the batch mode was used providing weight average apparent molecular weight for the different kinds of lignin in solution studied.

In the 19th century Lord Rayleigh offered the explanation of why the sky is blue. It was a simplification of the Maxwell's electromagnetic theory for small particles compared to the wavelength of the incident light. This theory was extended to macromolecules in solution.

In a light scattering experiment, a single frequency of a polarized light beam (laser) is used to illuminate a solution containing a suspension of the macromolecules. The electric field of the polarized light beam is generally produced perpendicular to the plane in which the intensity and angular dependence of the subsequently scattered light is to be measured. The intensity carries information about the molar mass, while the angular dependence carries information about the size of the macromolecule.[45]

2.4.1 Rayleigh theory

The intensity of the scattered light is related to different factors:

- Polarizability of the sample: when laser light impinges on a macromolecule, the oscillating electric field of the light induces an oscillating dipole within it. This oscillating dipole will re-radiate light. The intensity of the radiated light depends on the magnitude of the dipole induced within the macromolecule. The more polarizable the macromolecule, the larger the induced dipole, and hence, the greater the intensity of the scattered light. Therefore, in order to characterize the scattering

from a solution of such macromolecules, it is first necessary to know their polarizability. This may be determined from a measurement of the change (Δn) of the solution's refractive index (n) with the molecular concentration change (ΔC) by measuring the dn/dC ($=\Delta n/\Delta C$).[45]

- Concentration: each molecule in solution scatters light thus the intensity of the light scattered is proportional to the concentration of the solution.

- The intensity of light scattered by a molecule is directly proportional to the molar mass. The net result is that the scattered light from the dimer is twice as intense as that from the individual monomers. Simply by doubling the mass, even while keeping the concentration the same, the intensity of the scattered light doubles.

- Angular Dependence of Scattered Light: For macromolecules smaller than the wavelength of the incident light, there is no dependence with the scattering angle but for larger macromolecules there are variations in the phase of the scattered light from different parts in the macromolecule. Each macromolecule is assumed to be made up of very small elements, each of which scatters independently of each other. However, during the time of passage, each element scatters in phase with the scattering of adjacent elements. Thus the scattered waves will add destructively or constructively producing constructive or destructive interference in certain directions.[45]

This theory offers a good support to study the behavior of macromolecules in solution, which is our case. Gidh et al.[10] also states that light scattering technique could offer great insight into lignin aggregate characterization.

2.4.2 What values do we get from the light scattering?

- Radius of gyration: It is a measure of the size of a molecule weighted by the mass distribution about its center of mass as shown in the **Figure 12**. It is calculated as the root mean square (rms) distance of the parts from its center of mass, **Equation 1**. It is determined with the measurement of the angular dependence of the scattered light. Since the chain conformations of a polymer sample are infinite and constantly change over time, the radius of gyration in polymer physics is understood as a mean over all polymer molecules of the sample and over time.

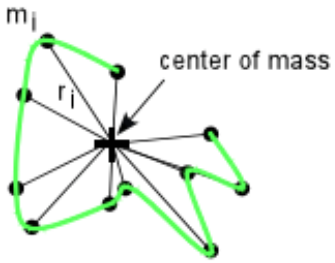


Figure 12. Radius of gyration of a polymer.

$$R_g^{\,2} = \frac{\sum m_i \times r_i^{\,2}}{m_i} \qquad \textbf{Equation 1}$$

- Molecular weight: Since the intensity of the scattered light is proportional to the molar mass, it is possible to determine the molecular weight of the molecules in solution with the light scattering technique. This relationship can be written as in

Equation 2.

$$\frac{K^* c}{R(\theta, c)} = \frac{1}{M_w P(\theta)} + 2A_2 c \qquad \textbf{Equation 2}$$

Where:

- $R(\theta,c)$ is the excess Rayleigh ratio of the solution as a function of scattering angle θ and concentration c. It is directly proportional to the intensity of the scattered light in excess of the light scattered by the pure solvent.
- c is the solute concentration.
- M_w is the weight-averaged solute molar mass.
- A_2 is the second virial coefficient in the virial expansion of the osmotic pressure.
- K^* is the constant $4\pi^2(dn/dc)^2 n_0^2/N_a\lambda_0^4$.
- N_a is Avogadro's number. This number always appears when concentration is measured in g/ml and molar mass in g/mol.
- n_0 is the refractive index of the solvent.
- λ_0 is the vacuum wavelength of the laser.
- $P(\theta)$ describes the angular dependence of the scattered light, and can be related to the rms radius.

- Second virial coefficient A_2: The second virial coefficient is a property describing

the strength of the interaction between the molecule and the solvent. For samples where $A_2>0$, the molecules tend to stay in solution. When $A_2 = 0$, the molecule-solvent interaction strength is equivalent to the molecule-molecule interaction strength and the solvent is described as being a theta solvent. When $A_2<0$, the molecules will tend to crystallize or aggregate. In a study done by Brown with kraft lignin in different solvents, the second virial coeficient increased as the apparent molecular weight decreased in the three solvents he was using, DMSO, DMF and dioxane. As the second virial coefficient is a measure of the thermodynamic interaction between solute and solvent, this shows that the variation in apparent molecular weight is a result of lignin association, diminishes as the solvent becomes "better".[31]

3. Experimental Part

3.1 Lignin Isolation by enzymatic mild acidolysis

One of the problems when studying lignin is whether the sample is representative of the overall material within the wood from which it was isolated and that is because there is no ideal isolation method that gives unaltered native lignin.[46]

Wu and Argyropoulos [46] have proposed a novel lignin isolation procedure composed of an initial mild enzymatic hydrolysis of milled wood, followed by a mild acid hydrolysis stage. In this procedure, the initial cellulolytic action removes most of the carbohydrates while the mild acidolysis is designed to cleave the remaining lignin-carbohydrate bonds, liberating lignin in high yield and purity.

3.1.1 Procedure for Isolation of EMAL from wood

1. **Wiley Mill**

    a. Wood chips were air-dried at room temperature and stored in a desiccator under vacuum before use;

    b. About 200g of air-dried wood chips were grounded to pass a 20-mesh screen in a Wiley mill;

2. **Acetone Extraction**

    c. The wood powder was then dried in vacuum oven set at 40°C overnight and stored in a desiccator under vacuum before use.

    d. About 150 g of OD wood powder were extracted in a Soxhlet apparatus for 16 hours. Some inert bumping control granules were necessary in round bottom flask to obtain

stable acetone vapor;

e. The extracted wood powder was air dried and stored in a desiccator under vacuum.

3. Ball Milling

f. About 100 g of OD extracted wood powder were placed into a 5.5-liter porcelain

g. 1,660 kg of balls were added (474 glass balls with 9.4mm in diameter). This amount of balls should occupy 18% of the active jar volume, allowing for a glass ball/wood weight ratio of 16.6.

h. The milling process was conducted in dry conditions at room temperature for up to 28 days with a rotation speed of 60 rpm;

i. The milled-wood from the jar was removed, dried in a vacuum oven set at 40°C and stored in a desiccator under vacuum before use.

4. Enzymatic Treatment with cellulase

j. A solution of 0.1M acetate buffer was prepared as follow: dissolve 9.84 g of NaOAc ($82 \times 0.1 \times 1.2 = 9.84$ g) in 500 ml of deionized H_2O. About 15ml of AcOH were added to adjust the NaOAc solution to pH 4.5. The resulting solution was diluted with deionized water to 1.2L;

k. 10 g of OD milled-wood were placed in an Erlenmeyer flask and add 200mL of acetate buffer solution in order to reach 5 % consistency;

l. Cellulase enzyme was added into the flask (40 filter paper units (FPU) per gram of milled-wood; cellulase activity ~130 FPU), then it was covered and placed in a shaker set at 40°C for 48h;

m. The insoluble material was collected after enzymatic hydrolysis by centrifugation (3500 rpm; 30 min.) and washed twice with acidified deionized water (pH 2) and freeze-dried.

n. The freeze-dried material was dried in a vacuum oven set at 40°C and stored in a desiccator under vacuum before using.

5. Acidolysis

o. The oil bath was preheated to 80°C;

p. 2 g of OD crude lignin recovered from the enzymatic treatment were suspended in 60mL of aqueous dioxane (dioxane/water 85:15, v/v, containing 0.01 mol l^{-1} HCl). The resulting suspension was refluxed (azeotrope boiling point 88°C) under Argon for 2 hrs. Reaction time starts when the temperature of the suspension reaches 85°C;

q. The suspension was cooled down to room temperature under Ar; and centrifugeted for 2 hours (3,500 rpm);

r. The supernatant was withdrawn carefully and neutralized with sodium bicarbonate to pH around 7 and finally 1L of acidified deionized water (pH 2) was added, dropwise, under vigorous stirring

s. The precipitated lignin was allowed to be equilibrated with the aqueous phase overnight;

t. The precipitated lignin was recovered by centrifugation (3,500 rpm; 1 h), wash (2x) with deionized water and freeze-dried and oven dried at 40°C, the lignin was ready for analysis.

3.2 Acetobromination derivatization procedure

The acetobromination procedure was carried out in order to make the lignin samples soluble in THF to be analyzed. By dissolving a lignin sample in acetyl bromide diluted with glacial acetic acid (acetobromination), the primary alcoholic and the phenolic hydroxyl groups are acetylated, while the benzylic α-hydroxyls are displaced by bromide.[47]

Acetobromination was carried out following the procedure described elsewhere.[47-49] Specifically, a lignin sample (10 mg) was added into a solution of AcBr:AcOH (2.5 mL, 8:52 v/v); followed by stirring for 2 h at 50^{o}C, the solvent was evaporated in vacuum (using a high vacuum pump and a cold trap) and the resulting residue was immediately dissolved in THF (5 mL) [34] and subjected to light scattering analyses.

3.3 Procedure for selective blocking of lignin hydroxyl groups

3.3.1 Selective Blocking of Phenolic and COOH groups (Methylation of Lignin with Diazomethane)

For this reaction the Diazomethane is needed. It was generated by slowly adding a solution of diazald (2.64g) in diethyl ether (38 mL) into an alkaline (0.75g KOH) solution of carbitol (23 mL) at 65°C (thermostatted water bath); Carbitol:[2-(2-Ethoxyethoxy)Ethanol]; Diazald: [N-Methyl N-Nitroso p-Toluensulfonamid], this reaction is shown in **Figure 13**. (The procedure requires the use of specially constructed round bottom flask and distillation equipment with no ground glass joints to avoid the risks of explosion)

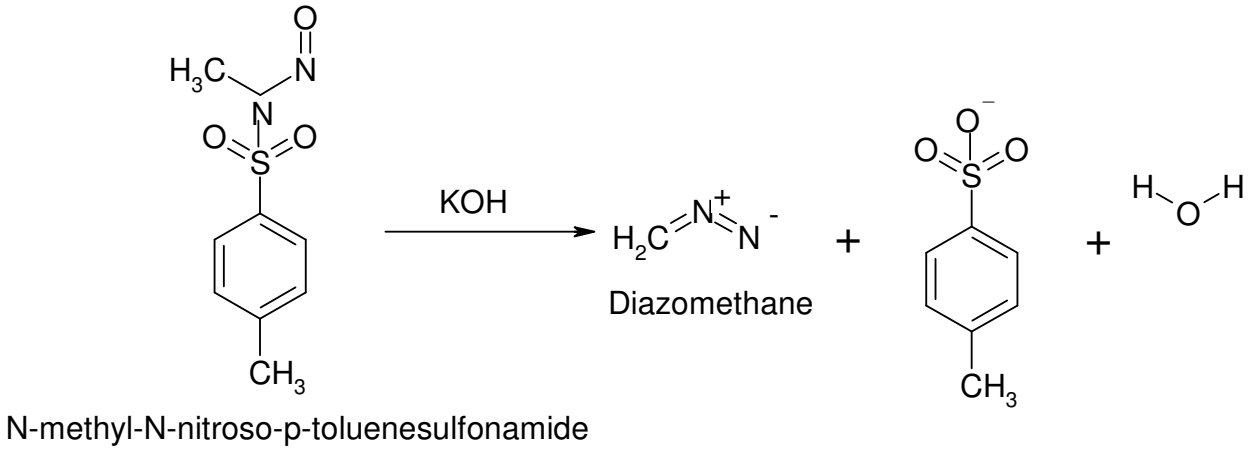


Figure 13. Diazomethane generation.

Once diazomethane was produced, 1g of lignin was suspended in 38 mL of diethyl ether and treated with an excess of diazomethane for 24 hours at room temperature. [50] The treatment was repeated twice. The solid was isolated by centrifation and the methylated lignin was washed with diethyl ether and centrifuged again. The lignin was finally dried under reduced pressure at room temperature. **Figure 14** illustrates the general reaction.

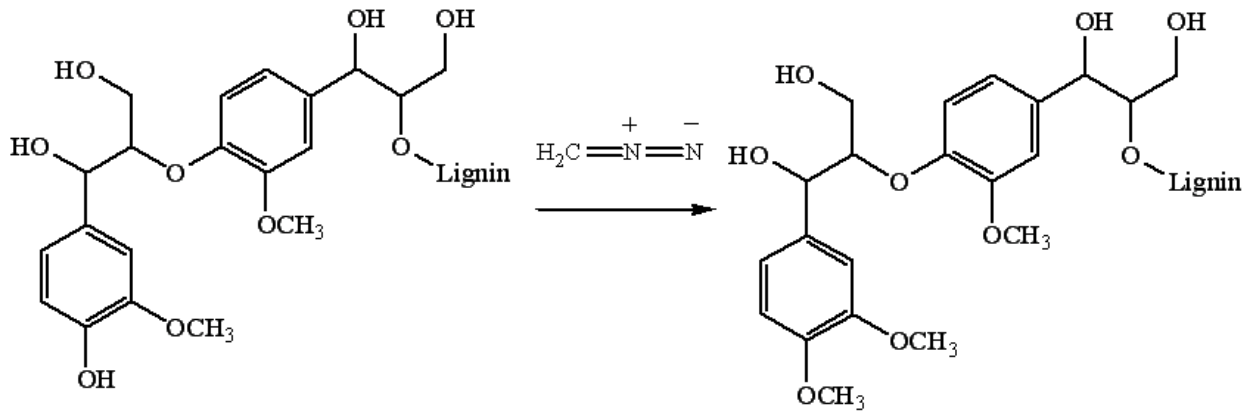


Figure 14. Selective methylation of phenolic hydroxyl groups in Spruce lignin general reaction.

3.3.2 Acid Catalyzed Methylation of Lignin

Selective Blocking of alpha aliphatic OH groups

Lignin (1g) was dissolved in a mixture of 67 mL of dry dioxane (distilled from sodium) and 134 mL of dry methanol in a 250 mL round-bottomed flask. p-Toluenesulfonic acid monohydrate (5.73g) were added and the flask was sealed using a septum and kept at 30°C (thermostatted water bath) for 5 days in accordance to Alder et al.[51] The solution was 0.15 M with respect to p-toluenesulfonic acid. The reaction was quenched by the addition of 2.798 g of solid $NaHCO_3$. The resultant mixture was stirred for 2 hours and then the amount of solvent was reduced to 30 mL by film evaporation. The concentrated mixture was then precipitated by dropwise slow addition into 500 mL of 1% Na_2SO_4 (pH adjusted to 2.8 by the addition of 0.1 M HCl). The precipitate was centrifuged, washed in several portions with 200 mL of 1% Na_2SO_4 and dried in a vacuum oven at room temperature. The reaction is presented in **Figure 15**.

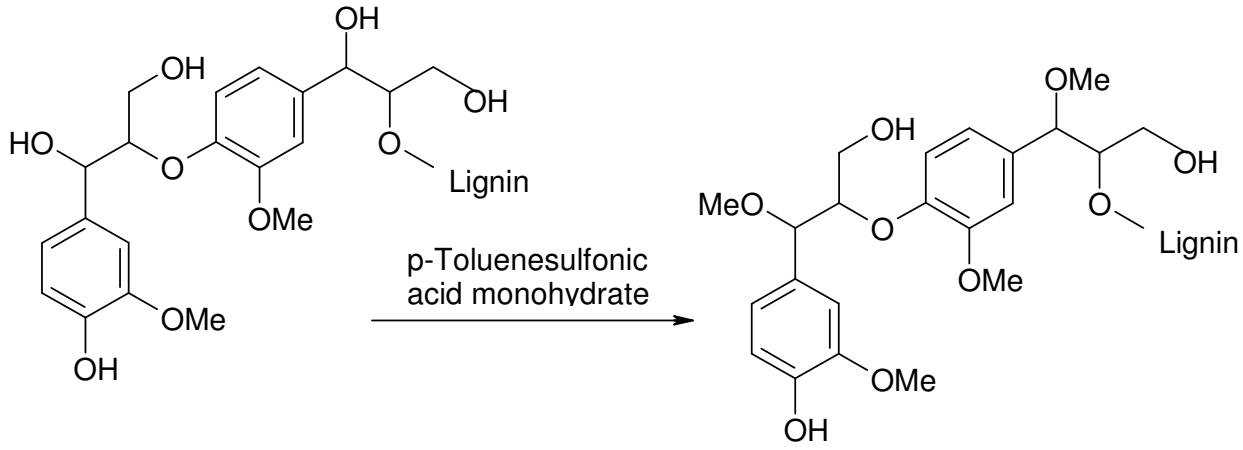


Figure 15. Selective methylation of alpha aliphatic hydroxyl groups in Spruce lignin.

4. Results and discussion

4.1 Light scattering

Lignins can be characterized by light scattering (LS) photometry,[52] a very useful method, although not as widely used as it could be, especially under the **static mode**. This study is focused on the usage of this method, exclusively under the static mode, applied to lignin biopolymers. Mainly, measurements of the weight-averaged molecular mass ($\overline{M}_W$) of lignins in solution were carried out. In some cases, the parameter average root mean squared (rms) radius of gyration was also measured.

Multiple angle laser light scattering (MALLS) photometry makes use of multiple (in this case eighteen) different angles to detect the light scattered from the samples. The intensities of the light scattered correlated with the different concentrations let acquire the weight-averaged apparent molecular weight of the lignins in solution, among other useful parameters. By measuring the light scattering signal as a function of angle and concentration, light scattering (Wyatt Technology) instruments create a global fit that evaluates the data as a whole. The quality of the fit can be assessed via a Zimm plot. This type of plot is a two-dimensional slice of a three-dimensional data set. The global fit results are presented as a grid, and the data as points. In **Figure 16** a typical Zimm plot of a Spruce lignin in THF solution is presented. It is shown that the data points fit nicely in the final global fit grid. The final results are further presented in the sub-sections below.

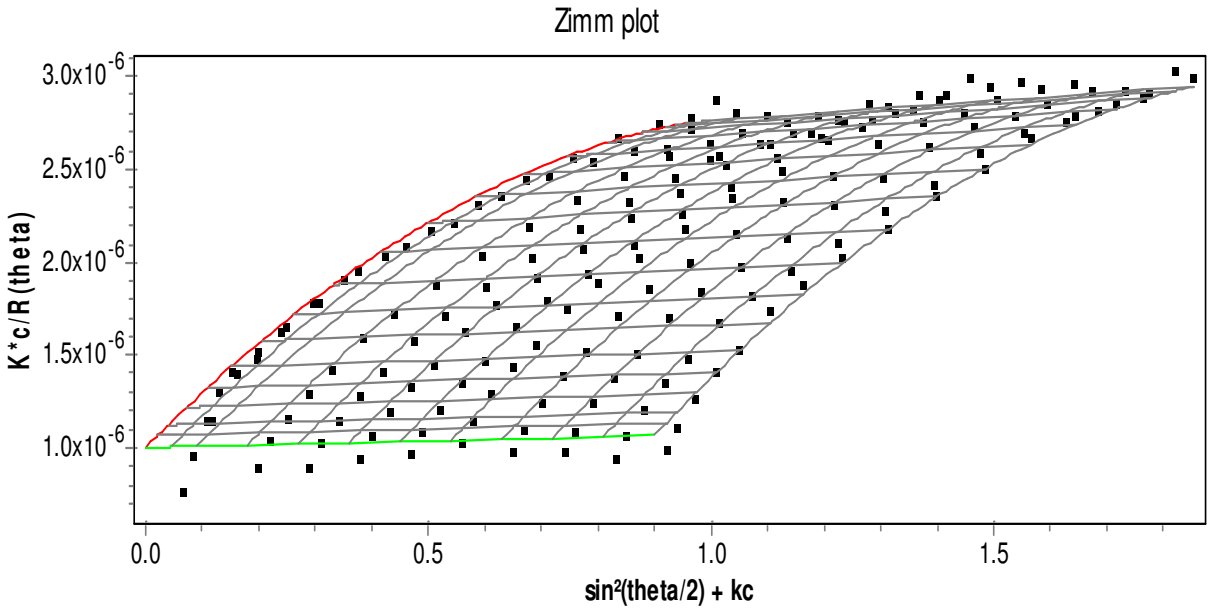


Figure 16. Typical Zimm plot by light scattering photometry from a Spruce lignin solution in THF.

To better understand light scattering measurements of lignins, several optical phenomena should be overcome to obtain reproducible and accurate results.[52, 38] There are attempts in the literature to correct or minimize the specific optical properties of lignins, i.e., their anisotropy, absorbance, and fluorescence.[52, 38]

The optical anisotropy, that is the dependence of optical properties on a preferred direction, caused by the depolarization of the incident beam gives rise to an enhancement of the light scattered, overestimating the final apparent molecular weight measured. One possible adjustment makes use of an analyzing polarizer inserted between the sample and the detector, which allows the corrections from the vertical and horizontal excess Rayleigh factors.[53] Another method involved the use of the Cabannes factor, in the case the incident light is vertically polarized.[53] More recently, it has been shown that the anisotropy effect exhibited is

mainly due to the solvent itself rather than by the lignin.[38] As far as the anisotropy effect correction is concerned, the necessary mathematical procedures taking also into account the anisotropy effect from the solvent were followed by the ASTRA software.

The absorption of near ultraviolet light tailing to the visible light by lignin may attenuate the incident and scattered light, leading to an underestimated apparent molecular weight. When the measuring cell is cylindrical and the optical density of the solution is sufficiently weak, the correction becomes partially independent of the scattering angle. In this case, the absorption effect can be corrected for simply by dividing the apparent Rayleigh factor by the transmittance of the solution.[54, 55] For these experiments, the absorption by lignin solution is very low, because at the wavelength used (633 nm) the absorption is minor. On the other hand, the concentration of the lignin solutions analyzed were also low; therefore, even for the most concentrate solutions the transmittance was negligible, keeping the Rayleigh factor constant for all the concentrations of the lignin solutions.

The optical fluorescence from lignin solutions results from excited electrons returning to a lower energy (ground) state after being excited to a higher, thermally unstable state by the absorption of light. Typically, the higher energy state thermally equilibrates to the lowest, non-degenerate excited state during the excitation event. Therefore, fluorescent light has lower energy and hence, greater wavelength than that of the absorbed incident light. Nevertheless, this fluorescence is more pronounced at lower wavelengths.[55] Without correction, the photomultiplier in the LS photometer will detect both the scattered and fluorescent light. Even at 633 nm, under the acquisition conditions of the LS, lignin fluorescence effect leads to an overestimation of the apparent molecular weight. These findings are clearly demonstrated with the measurements carried out with and without

interferometric filters (**Figure 17**). It is clearly observed that the lignin samples analyzed without the filters show much higher apparent molecular weights than the ones analyzed with the 1 nm filters (samples A and B in **Figure 17** top graph). The overestimation was around 62-111%. On the other hand, the error associated to the measurements without the filters is much more pronounced than when the 1 nm filters are used. The use of interferometric filters with a bandwidth of 10 nm was also investigated. Even with the 10 nm filters, the final measured apparent molecular weights were overestimated by around 62-65% when compared to the data using of the 1 nm filters (samples A and B in **Figure 17** top graph). For each sample, its specific determined dn/dC was used for the apparent molecular weight calculations in the cases with and without the use of the filters. Similar findings are observed with respect to the rms radius measurements (samples A and B in **Figure 17** bottom graph). Surprisingly, even with the use of the a 10 nm bandwidth filters, the optical fluorescence effect takes place and creates an overestimation of the (rms) by light scattering. In a previous report,[54] it was indicated that dramatic change occurred for kraft lignins at the low angle laser light scattering (LALLS) signal when a 632.8 nm filter was placed between the sample solution and the photomultiplier, besides nothing significant was observed on the fluorescence spectrum at an excitation wavelength of 633 nm.

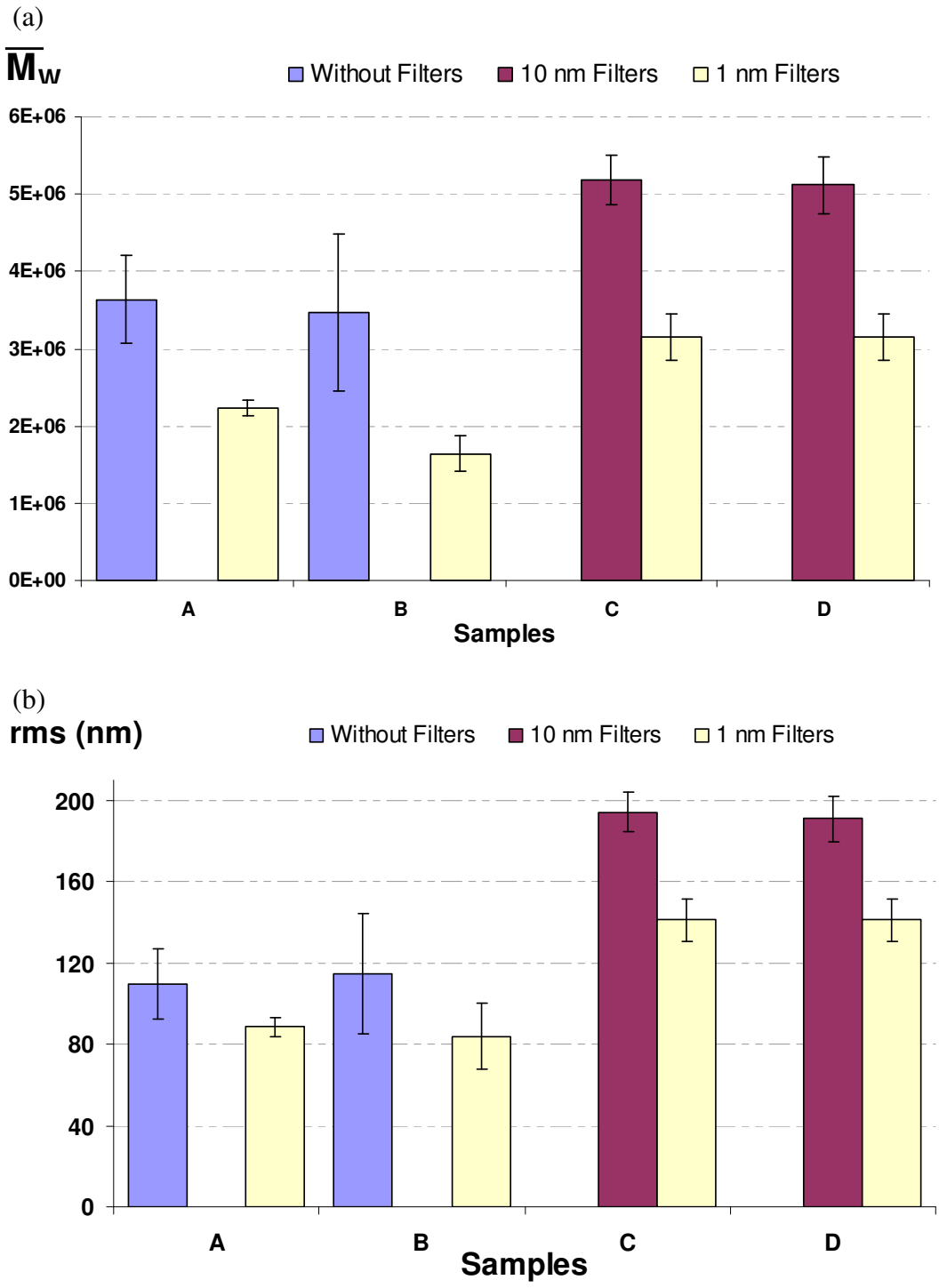
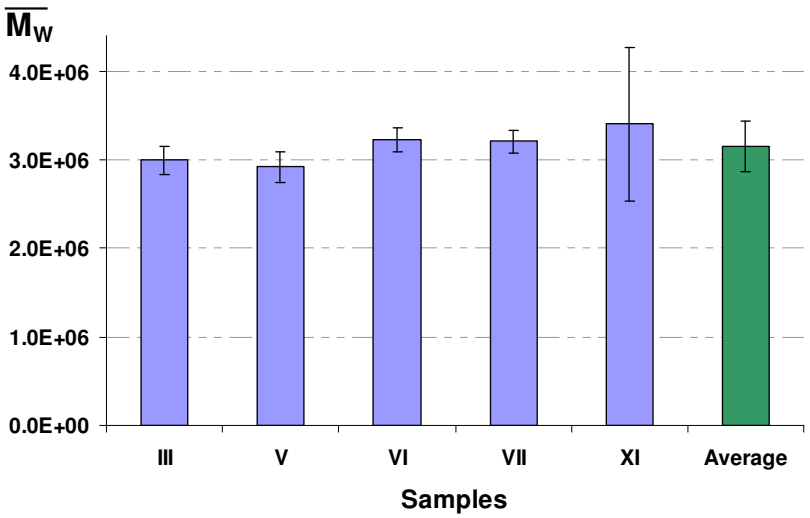


Figure 17. Effect of light scattering filters on the weight average apparent molecular weight (a) and the root mean square radius (b) of fresh EMAL Spruce solutions in THF.

Taking into account the above mentioned optical effects and their detailed adjustments for the light scattering measurements, the reproducibility observed for the final results was excellent. When the weight-averaged apparent molecular weight was measured for several samples of the same lignin, i.e. for each sample the lignin was acetobrominated, dried, dissolved in THF and diluted to the proper concentrations for analysis, all the final values were within the experimental error (**Figure 18**). The apparent molecular weight calculations took into account the specific dn/dC values for the fresh Spruce lignin solutions in THF, analyzed by light scattering photometry. Similar results of good reproducibility were observed for the rms radius of the lignin (**Figure 18**). These achievements allowed us then to confidently continue further investigations shown in the sub-sections presented below.

(a)



(b)

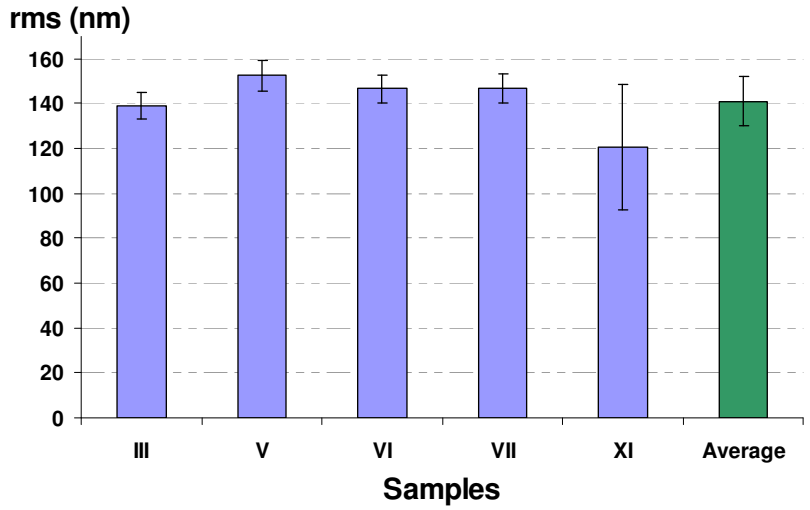


Figure 18. Reproducibility of the weight-averaged apparent molecular weight (a) and of the root mean square radius (b) by light scattering photometry from fresh EMAL Spruce solutions in THF.

4.2 Specific refractive index increment

In order to calculate the weight-averaged apparent molecular weight by light scattering photometry, the specific refractive index increment (dn/dC) of the material in solution needs to be known. Therefore, the dn/dC values for each lignin being characterized were

determined and the reproducibility was validated. For both fresh Spruce lignin and fresh Eucalyptus Globulus lignin solutions in THF, it was observed that the reproducibility of the dn/dC values was within the experimental error (**Figure 19**). Thus, the consistency of the measured dn/dC values was reliable. The dn/dC values were 0.140 ± 0.011 cm^3g^{-1} and 0.160 ± 0.008 cm^3g^{-1} for the Spruce and the Eucalyptus Globulus lignin, respectively, both freshly dissolved in THF.

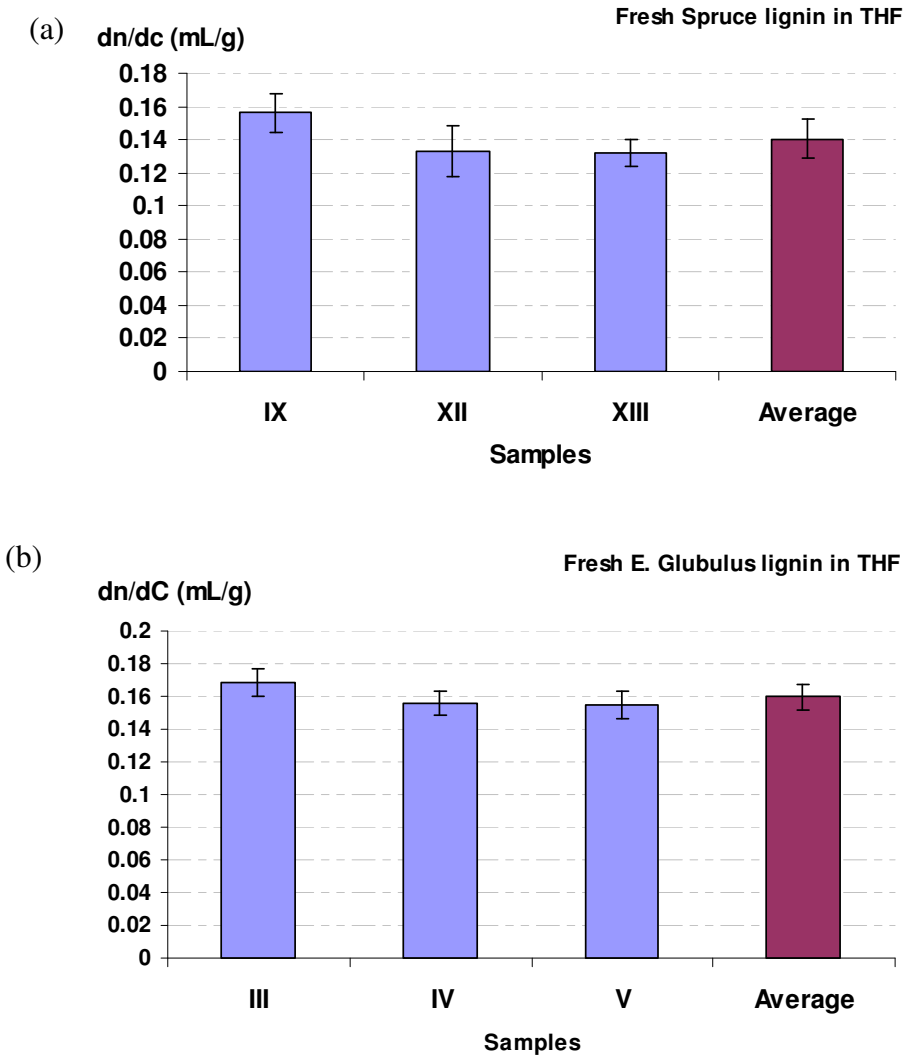


Figure 19. Reproducibility of the specific refractive index increment (dn/dC) of fresh EMAL Spruce solutions (a) and fresh EMAL Eucalyptus solutions (b), both in THF.

As the idea was to follow the behavior of lignins solutions with time, the dependability of the dn/dC values with the aging of the lignin solutions was investigated. The results are presented in **Figure 20**. In the case of Spruce lignin in THF, it was observed that from a freshly prepared solution to an older solution, there were changes in the dn/dC values. There was an increase of the dn/dC from 0.140 ± 0.011 cm^3g^{-1} (fresh solution) to 0.28 ± 0.011 cm^3g^{-1} after two days of aging of Spruce lignin solution in THF. For longer times, the dn/dC values have been found steady. The dn/dC values used for calculations of the weight-averaged apparent molecular weight have been 0.140, 0.149, 0.250 and 0.284 for fresh, 0.5, 1 and older than two days inclusive aged solutions, respectively. In the case of Eucalyptus Globulus lignin in THF, it resulted in dn/dC values with insignificant changes along the aging of the solution. The dn/dC value was considered 0.165 ± 0.008 cm^3g^{-1} for any incubation time. The dn/dC values of the Spruce lignin dissolved in 0.01 N NaOH were also investigated in time (**Figure 20**). It was observed that the dn/dC values are very similar along the solution incubation time. The dn/dC of the lignin in 0.01 N NaOH was 0.182 ± 0.002 cm^3g^{-1}.

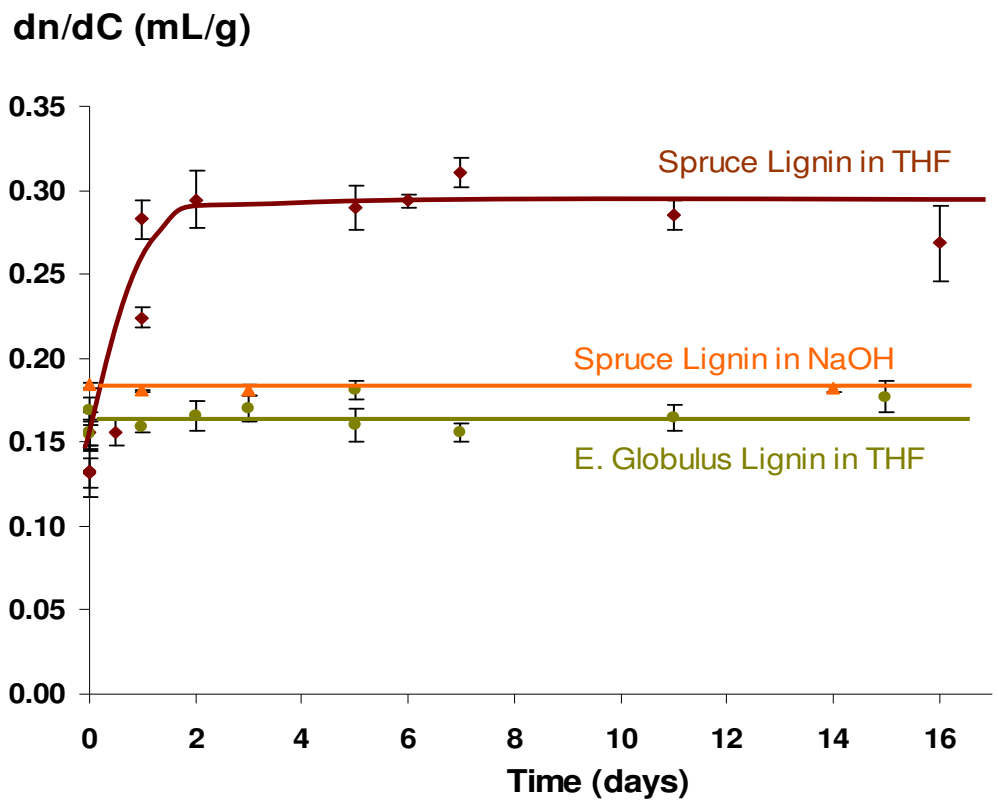


Figure 20. Incubation effect on the specific refractive index increment (dn/dc) of the spruce and eucalypt lignin in THF and spruce lignin in 0.01 N NaOH.

One remarkable observation regarding the associated errors of the dn/dC measurements is that accuracy of the lignin in NaOH solution determinations was much higher than in the case of the lignins in THF. The error associated to the dn/dC of the lignin in NaOH solution was about 1%, while the dn/dC of the lignins in THF was in the range of 4-8%. This corroborates the idea of the difficulties associated with the use of THF as a solvent for refractive index measurements, since THF is more compressible than water. Compressibility of THF is higher than for water, there are difficulties when pumping THF throughout the system and this fact generates error in the experiment.

The dn/dC of polymers has been shown to change with the charge density of the polymer, degree of substitution, degree of de-association, chain scission, conformational changes, chemical changes and molar mass, where only some previous studies are cited to recognize these features.[56-59] But, there is no systematic study on the changes of dn/dC with incubation or aggregation of polymers in solution. As shown in **Figure 20**, an unexpected result was obtained regarding the change of the dn/dC with the incubation for the case of the Spruce lignin in THF solution. Nothing in the literature was found to show changes of the dn/dC with the aging of a polymer in solution. It is generally accepted that the dn/dC value for a homopolymer is almost entirely dependent on the monomer and weakly dependent on (or independent of) apparent molecular weight.[60] Copolymers may be very different, however, because apparent molecular weight and composition can be closely linked.[61] In almost all the cases typically the dn/dC that is once determined for a polymer is assumed for the rest of the developed investigation. However, changes of the dn/dC due to chemical modifications were reported once.[56] It was shown that the dn/dC of chitosans in 0.1 mol/L $KClO_4$ is dependent upon the degree of acetylation and the degree of de-association. These changes induce adjustments in the ionic/hydrophobic environment of the polymers. In the case of the Spruce lignin in THF, the lignin chemical environment may display a similar role as demonstrated by the chitosan ionic/hydrophobic environment. In the incubation time of the Spruce lignin in THF, showing de-association phenomena as it is described in the next sub-chapter and by Guerra et al.[34] there may be a strong dependence between d*n*/d*C* and the charge density of the polymer, in relation to the modification of the dielectric properties of the polymer chains in dilute solution. Since the behavior of lignin is that of a typical polyelectrolyte controlled by the charge density and the interactions are mainly of electrostatic nature. Then, when the

degree of association increases, the weakening of the charge density induces the progressive replacement of the electrostatic interactions by both hydrophobic interactions and H bonds. This is the consequence of the increase of hydrophobicity in the polymer structure, which modifies the dielectric environment of the residual charges that can explain the change in the dn/dC observed for the EMAL Spruce in THF varying the age of the solution. This is not the case for EMAL E. Globulus solutions in THF, neither for EMAL Spruce in NaOH, since their dn/dC was found to be constant upon incubation time of the solution. Maybe the behavior of the refractive index increments is affected by the extent of dissociation; at high extent of de-association there is an important change in the hydrophobicity of the polymer in solution due to de-association and thus, an increase in the refractive index increments. It seems that for EMAL E. Globulus solutions in THF and EMAL Spruce in NaOH the extent of de-association was not high enough to cause a change in the hydrophobicity of the lignin in the solution. A better knowledge of the dependency of the dn/dC of polymers is still required and further investigations should be carried out.

4.3 De-association of the lignins in solution

In order to explore the propensity of lignin to associate, the weight-averaged apparent molecular weight of lignins in solution along the incubation time was investigated by light scattering photometry. The lignins studied were Spruce and Eucalyptus Globulus EMALs, acetobrominated and dissolved in THF, and the Spruce EMAL dissolved in an aqueous 0.1 N NaOH solution and the study for E. Globulus in alkaline solutions is is not included because of time issues. The incubation of lignin samples was carried out at 25 ± 3℃ (room temperature) or at 4 ± 1℃ without stirring and at a concentration of 2.0 gL^{-1}. The weight-

averaged apparent molecular weights were calculated taking into account the above indicated dn/dC measured values.

In an effort to ensure that the mentioned effects on the apparent molecular weight distribution of acetobrominated EMALs were not due to degradation of covalent linkages within lignin, the incubation of such EMALs derivatives was also monitored by quantitative ^{31}P NMR[34] in a parallel work in our laboratory because it has been reported ^{31}P NMR is an accurate technique to quantify the amount of hydroxyl groups in lignin molecule.[62, 63] The ^{31}P NMR analyses were done for fresh solutions and right after the measurement with GPC. After the hydroxyl groups in lignin were acetylated, they were not detectable by ^{31}P NMR spectroscopic analysis because they can no longer be phosphitylated. As a result, no signals due to phenolic or aliphatic hydroxyl groups were detected in the ^{31}P NMR spectra of the freshly prepared lignin solutions. If aryl ether linkage cleavage or oxidative reactions are taking place within these lignin preparations, they would be promptly recognized by the appearance of the corresponding signal in the ^{31}P NMR of such samples after incubation. When the aryl ether linkages are cleaved, the corresponding phenolic hydroxyls released can be quantified by ^{31}P NMR spectroscopy, while oxidation reactions may result in oxidative fragmentation of the lignin macromolecule with simultaneous creation of carboxylic acid groups, which are also detectable by ^{31}P NMR[62]. Nevertheless, there was no evidence of an increase in the hydroxyl groups signal of the samples analyzed after incubation.[34] Thus, there are no cleavages in covalent bonds between lignin molecules, and the decrease in the weight-average apparent molecular weight of lignin samples must be due to disruption of physical association and the nature of that physical association is the aim of this research.

4.3.1 Effect of temperature in the de-association of lignin

The incubated acetobrominated Spruce EMAL in THF showed a decrease of the apparent $\overline{M}_W$ with time, with a different profile as their incubation at room temperature or at 4℃ is concerned (**Figure 21**). It is observed that the apparent molecular weight rapidly decreases from the freshly prepared solutions until 24 hours of incubation, in both cases at room temperature and 4℃. It is also observed that the initial rates of the apparent $\overline{M}_W$ decrease are very similar. From 24 until 48 hours, only a slight reduction is seen. After 48 hours of incubation, steady final values are achieved. In the case of the acetobrominated Spruce EMAL in THF solution incubated at 4℃, the apparent $\overline{M}_W$ has dropped from 3.15×10^6 to an averaged steady value of 5.0×10^5 gmol^{-1}, equivalent to an 84% reduction in apparent $\overline{M}_W$. When the acetobrominated Spruce EMAL in THF solution was incubated at room temperature, the apparent $\overline{M}_W$ fell from 3.15×10^6 to the averaged final value 1.5×10^5 gmol^{-1}, corresponding to a 95% decrease. The steady apparent molecular weights achieved by the lignin in THF kept at 4℃ was more than 3 times compared to the lignin solution kept at room temperature. This singular behavior of the apparent molecular weight dropping is an indication of the de-association phenomena taking place in lignin that is thermally controlled. The relationship between of energy levels of molecules with temperature is expressed in **equation 3** (Boltzmann equation), where we can determine the fraction of particles that are in high energy level when changing the temperature. As temperature increases the ratio of particles in a higher energy level increases, which means that at high temperature there is more energy for molecules motion.

$$\frac{n_i}{n_j} = e^{-\frac{(Ei-Ej)}{KT}}$$

equation 3

Where $\mathbf{n_i}$ and $\mathbf{n_j}$ are the number of particles in energy level **i** and **j** respectively. Ei and Ej are the energy levels **i** and **j** respectively, being **Ei** higher than **Ej**. **K** is Boltzmann constant, which value is $1.380\ 6504 \times 10^{-23}$ J/K and **T** is the temperature in K. The relationship between the number of molecules in high energy levels and the temperature is shown in **Figure 21**. Where the number of molecules in the ground energy level (blue ΔE=0 Kcal/mol) is decreasing as the temperature increases. Meanwhile the number of molecules in higher energy levels (pink (ΔE=1 Kcal/mol) and yellow (ΔE=2.5 Kcal/mol)) increases with the temperature because there is more energy for molecular motion. When comparing the idea shown in **Figure 21** with the de-association phenomena it can be concluded that for associated complexes the energy level is higher than when those associated complexes de-associate and tend to form discrete components, since all systems tend to go to lower energy levels that are thermodynamically better. In lignin as associated complexes form the high energy level is related to the possible driving forces for the association phenomena energy, which are: hydrogen bonding, entanglement and π-π stacking, which specific strength value in lignin association is not known. When de-association phenomenon takes place, lignin associated complexes molecules in solution fall to lower energy levels as they get apart becoming discrete components in which the interaction forces responsible for association are not longer present or present in a lower extent.

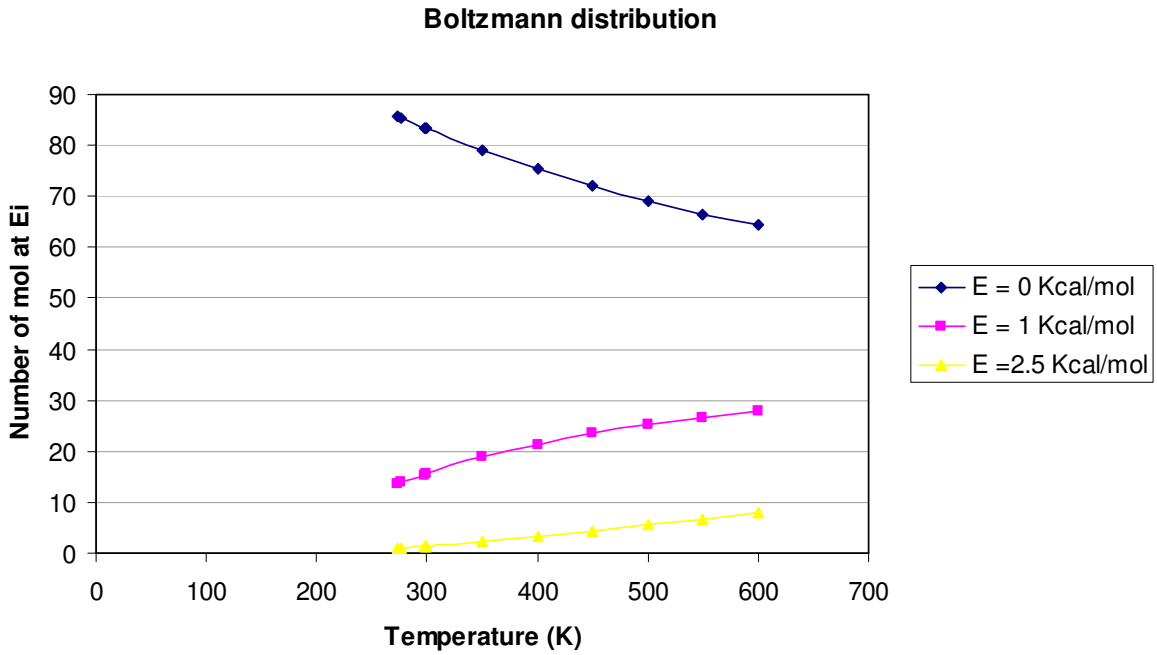


Figure 21. Relationship between the number of molecules in different energy levels with temperature.

The prevention of the further de-association at 4°C than to room temperature may be understandable taking into account that is related to the average internal energy of the molecular motions in the system (temperature). At higher temperature than 4°C the further lignin de-association may be only promoted by slightly upper level of the average internal energy of the motions of the lignin molecules. Raman et al [64] made some ultrasonic and computational study of intermolecular association through hydrogen bonding in aqueous solutions of D-mannitol at different temperatures and they found a weakening of intermolecular forces due to thermal agitation of the molecules at higher temperatures. They calculated the internal pressure of the liquid system that can be used to study the molecular interactions in liquids. When temperature increases, the hydrogen bonds break up due to

thermal vibrations in solvent and also the thermal vibrations between solute and solvent molecules. As a result, the weakened intermolecular forces lead to a decrease in internal pressure.

Likely, the strength of the interactions that promote the association of lignin molecules is reduced due to the increase in molecular motion in solution caused by higher temperatures in the system. This idea was confirmed in our study with the drop of the weight-averaged apparent molecular weight of the lignin, once the solution kept at 4°C was then left at room temperature. After 14 days of the solution being kept at 4°C, it was then allowed to warm to room temperature and at the 16 days incubation (14 days at 4°C plus 2 days at room temperature) the apparent $\overline{M}_W$ measured was 1.4×10^5 $gmol^{-1}$ (point at 16 days in **Figure 22**); this being the same value the same as the one reached for the lignin solution incubated continiusly at room temperature. Brown et al. [31] reported an increase in the solvent-solute interactions with the increase in the temperature meaning that higher temperatures promote lignin molecules movement favoring de-association phenomenon.

Similar results were obtained by Guerra et al. [34], they followed the apparent molecular weight of EMAL from different species (Spruce, Eucalyptus Globlulus, White Fir, Straw and Redwood) at the two temperatures that are used in this investigation, 4 and 25°C, using GPC. It was found that incubation in THF at 4°C induces some association between components of lignins from all evaluated woody species with the exception of lignins from Spruce and Corn. This may indicate that for the lignin from this species the initial sample was already fully associated under the conditions evaluated in their work[34]. However, here the effect of temperature was studied just with Spruce lignin but by comparing these results it can be concluded that Spruce lignin does not show a reversible association/de-association behavior

as observed in the other species Guerra studied[34] and the kraft and organosolv lignins Sarkanen analyzed[12]. Further work should be done in this area in order to determine the behavior of different kinds of lignin in solution at different temperatures.

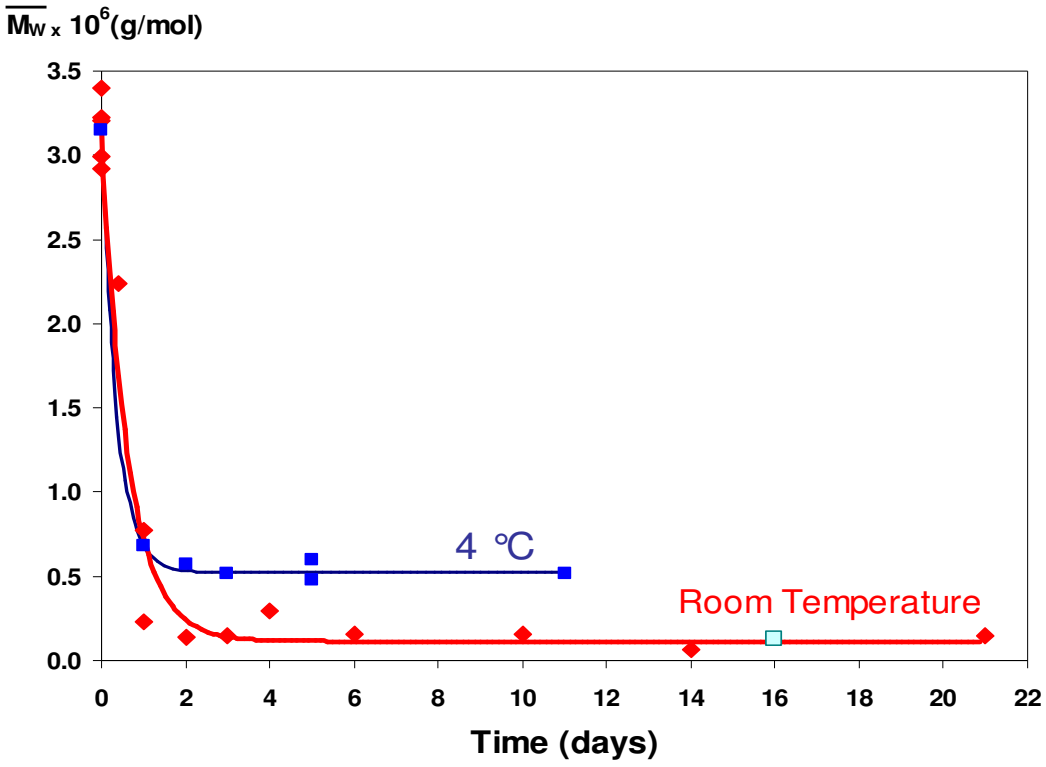


Figure 22. Incubation effect on the weight-averaged apparent molecular weight of the EMAL Spruce solution in THF kept at room temperature and at 4°C.

The data was fitted in a math function using a kinetics program. The function that fitted nicely the experimental points was one phase exponential decay, which is applicable when the decrease of a variable is at a rate proportional to its value, the general equation is as shown in **equation 4**.

$$\overline{M}_W = A \times \exp^{-K \times t} + B \qquad \textbf{equation 4}$$

Where $\overline{M}_W$ is the weight-average apparent molecular weight of lignin, A is related to the extend of de-association and the A units are g/mol, K (day^{-1}) is related with the velocity of the de-association process and B is the final weight-average apparent molecular weight of the sample in g/mol or when it is completely de-associated.

After regression of the points for Spruce lignin at 4°C and at room temperature the **equations are 5 and 6** respectively.

$$\overline{M}_W = 2.625\times10^6 \times \exp(-2.784\times t) + 522903 \qquad \textbf{equation 5}$$

The corresponding halflife, referring to half of the time needed to complete de-association, for Spruce lignin at 4°C was 0.2490 and the R^2=0.9980, showing good fit between the experimental data and the equation.

$$\overline{M}_W = 3.033\times10^6 \times \exp(-1.623\times t) + 147897 \qquad \textbf{equation 6}$$

The halflife for Spruce lignin incubated at room temperature was 0.4271, almost twice as for incubation at 25°C, meaning that as the extent of the de-association phenomenon is lower for lower temperature it takes shorter time to complete half de-association, this can be also seen when comparing the coefficient A (extent of de-association) for the **equations 5 and 6**, since they are 2.625x10^6 and 3.033x10^6 respectively. The R^2=0.9750 for fitted points at 25°C was also good correlation of the points with exponential decay. It is also important to notice that the rate of de-association is higher at 4°C than at 25°C since the K values are 2.784 and 1.623, respectively. This was not expected, but maybe the temperature influences the extent of de-association but not the rate of de-association.

4.3.2 De-association behavior of lignins from different species

Softwood Spruce lignin and a hardwood EMAL Eucalyptus Globulus were also investigated to study their de-association phenomena. The weight-averaged apparent molecular weight of acetobrominated Eucalyptus Globulus lignin in THF was followed along the incubation time at room temperature by light scattering photometry and compared to what observed with the Spruce lignin (**Figure 23**). Interestingly, the apparent $\overline{M}_W$ incubation profile of the Eucalyptus Globulus lignin is very similar with the one observed by Guerra et al.[34] measured by gel permeation chromatography (GPC). While for the case of the Spruce lignin compared to the results measured by GPC,[34] the values obtained by LS are much higher. The apparent $\overline{M}_W$ of the fresh Spruce lignin solutions are around 30 times higher measured by LS than by GPC. An explanation for this difference may be attributed to the fact that the light scattering photometry has a much higher sensitivity to the high apparent molecular weight fractions than gel permeation chromatography and because the values obtained from light scattering are absolute, this technique does not use any reference as GPC where usually polystyrene is used as reference for this kind of measurements as mentioned before.

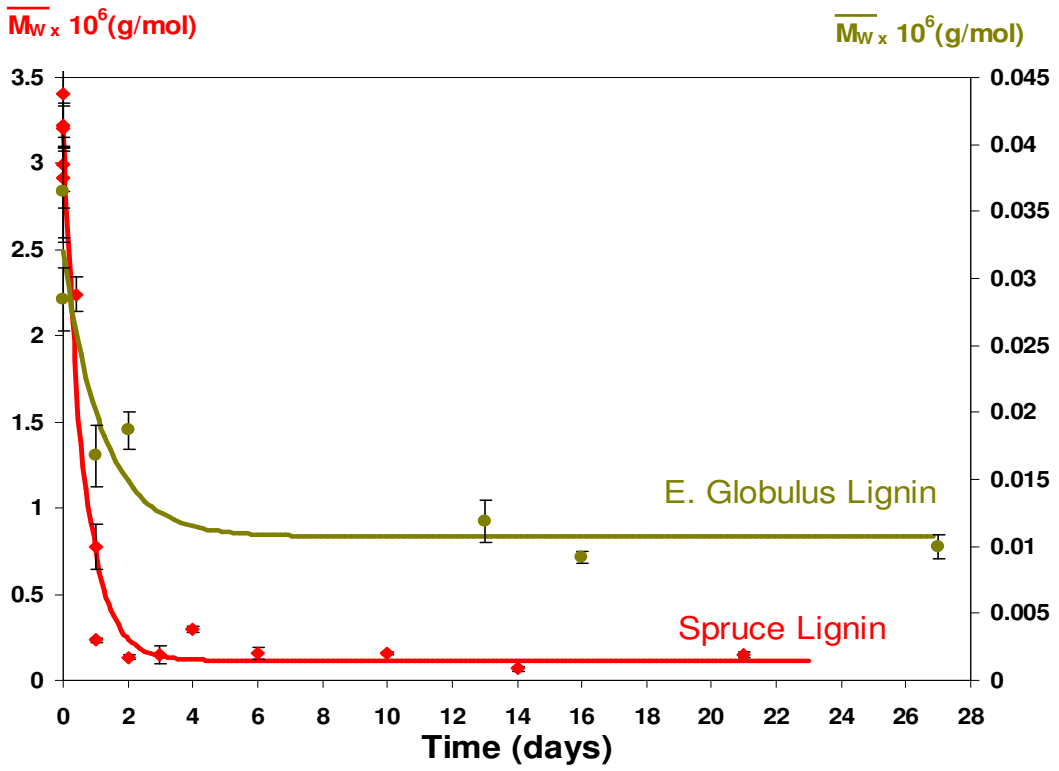


Figure 23. Incubation effect on the weight-averaged apparent molecular weight of the Spruce and Eucalyptus Globulus lignin in THF solution kept at room temperature.

The weight-averaged apparent molecular weight of the fresh acetobrominated Eucalyptus Globulus lignin in THF was 3.2×10^4 gmol^{-1}. There is a decrease in the weight-average apparent molecular weight of E. Globulus and after 4 days a plateau was achieved at 1.0×10^4 gmol^{-1} with a reduction of 68% compared to the fresh solution. Previously, it was shown that the apparent $\overline{M}_W$ reduction for the Spruce lignin was 95%. The de-association phenomena of the hardwood lignin took place with a contrasting profile and different degree to the softwood Spruce lignin. These profiles can be seen in **equations 6 and 7** which are the kinetic equation for Spruce and E. Globulus lignin respectively at room temperature.

$$\overline{M}_W = 21314 \times \exp(-0.8247 \times t) + 10735$$ **equation 7**

Halflife for de-association of hardwood E. Globulus was 0.8405 and the R^2 was 0.9003. It can be deduced that de-association of E. Globulus takes longer time than de-association of Spruce in THF when comparing the respective halflifes, 0.8405 and 0.4271 respectively. De-association for E. Globulus is slower than for Spruce lignin as concluded when observing the K value for equations 5 and 6 the K value for Spruce is higher than the K value for E. Globulus, which are 1.623 and 0.8247 respectively. Therefore, we can conclude that softwood lignin de-associates faster than hardwood lignin.

Hardwood lignins consist of guaiacyl and syringyl units in variable proportions, while Spruce and other softwood lignins consist almost entirely of guaiacyl units.[17] Since hardwood lignin has syringyl units which have one methoxy group more than guaiacyl units, there are more electron donating groups in the aromatic ring what can make the π-π stacking stronger; however, there is another factor to take into account, the steric hindrance. If an aromatic ring has more substituted groups there is less capability to get together and form a π-π interaction because of the steric hindrance. Therefore, there would be easier for softwood lignin, which guaiacyl units are less substituted than syringyl units, to form π-π stacking since there are more possible conformations favoring the π-π stacking. The difference in the behavior in the de-association phenomena for hardwood and softwood lignin can be due to their different structure and the different linkages. The relative proportion of each linkage is related to the ratio of the mesomeric forms of the radicals which are influenced by the physico-chemical environment and kinetics parameters of the reaction.[16]

Such differences in the behavior between Spruce and Eucalyptus Globulus lignin upon incubation time were observed in a previous study in our laboratory,[34] the measurements were carried out using GPC and a bimodal elution curve was observed for Spruce lignin, meanwhile Eucalyptus Globulus lignin displayed an almost unimodal elution profile for freshly prepared samples. Meaning that E. Globulus did not have the fraction of higher apparent molecular weight but it did show de-association behavior with aging of the solution. Thus, they concluded that the extent and pattern of the associative process appears to depend upon the woody species; furthermore, there are different propensities to associate even among lignins from different species of softwood.

In an additional study, hydrogen bonding in lignin with Fourier transform infrared, it was found that aliphatic hydroxyl groups appear to form stronger and more extensive multiple-hydrogen-bonding complexes as compared to the phenolic groups. In fact, the relative intensity of these bands in softwood kraft lignin is greater than that of the bands in the hardwood kraft lignin, consistent with the higher aliphatic hydroxyl group content of the softwood lignin.[65] This fact can explain the higher weight average apparent apparent molecular weight values observed in our experiments for softwood lignin than for hardwood lignin, since softwood lignins with higher content of hydroxyl groups can form more and stronger hydrogen bonding complexes. On the other hand, Guerra et al.[34] tried to correlate the presence of different functional groups in lignin with the change in the apparent apparent molecular weight; the data indicates that there is no clear correlation between the extent of de-association and total amount of hydroxyl groups, carboxylic acids and condensed phenolic groups within lignin. This means that ionization and conjugation of various functional groups cannot explain the large differences observed in de-association behavior of lignins from

different woody species in THF. But there was a correlation found, E. globulus contains much more uncondensed β-aryl-ether structures than any of the softwoods evaluated. These corroborates that hardwood lignin is more linear than softwood lignin. As a result, there is a correlation between the amounts of uncondensed β-aryl-ether in lignin molecules with their extent of association. This finding is indicative that the observed effects may have their origin, at least in part, in chain entanglements operating within different macromolecules.[34] In order to develop this entanglement idea even further, the apparent molecular weight of Redwood and Eucalyptus Globulus sample was followed with stirring and without stirring. The extent of de-association was found not to be dependent on the stirring but the rate of the de-association showed to be higher when the mechanical energy was applied to the system. With all this in mind Guerra et al.[34] concluded, that chain entanglements, may also operate in the underlying mechanisms of lignin association and that another possibility may also operate during the stirring experiments; stirring can perturb any possible equilibrium that may occur between various lignin components enhancing their effective rate of diffusion away from one another and/or reducing the probability of re-association. Further studies need to be completed to clarify these effects are currently in progress.

4.3.3 De-association behavior of lignins in different solvents

The apparent molecular weight of EMAL Spruce was followed in alkaline solution of NaOH 0.1N. It is important to mention that EMALs in NaOH were not acetobrominated; therefore we have native lignin in an alkaline solution, pH 13, where we monitored the changes in the apparent molecular weight with the static light scattering technique. There was a decrease in the apparent molecular weight value, from $3.77x10^6$ to an average final value of

$6.22x10^5$ that represents 84% of reduction. The trend of lignin de-association phenomenon in NaOH is similar to THF as is shown in **Figure 24**.

The initial molecular weights for the Spruce lignin in the two different solvents are different and also the values when the plateaus are reached. Furthermore, there is a difference between the lignins that are dissolved in the two solvents. The lignin in THF was acetobrominated and the lignin in NaOH was not. This means that lignin in the THF solutions do not have hydrogen bond interactions because all the hydroxyl groups are acetylated. Meanwhile, in the NaOH solutions lignin was not pretreated and therefore it has hydroxyl groups. The fact that the two lignins (the one that was acetobrominated and the one that was not) show the same behavior in solution demonstrating that the acetobromination procedure is a good method to use in the study of lignin in organic solutions.

For the measurements, lignin solutions in NaOH 0,1N with a corresponding pH of 13 were prepared, and the apparent molecular weight was measured right after preparation. Those were called fresh solutions. Then, part of the solution was kept at room temperature in order to follow the change in the apparent molecular weight with the age of the solution (the same procedure used in THF solutions). There was a decrease in the apparent molecular weight values of lignin with time. This shows a de-association behavior of lignin in aqueous solvent. It is important to point out that there is no oxidation of lignin when incubated in alkaline solutions with air contact; since, comparative studies indicated that exposure to air did not have a detectable effect upon the form of the kraft lignin apparent molecular weight distributions which were developed during incubation under identical solution conditions.[7]

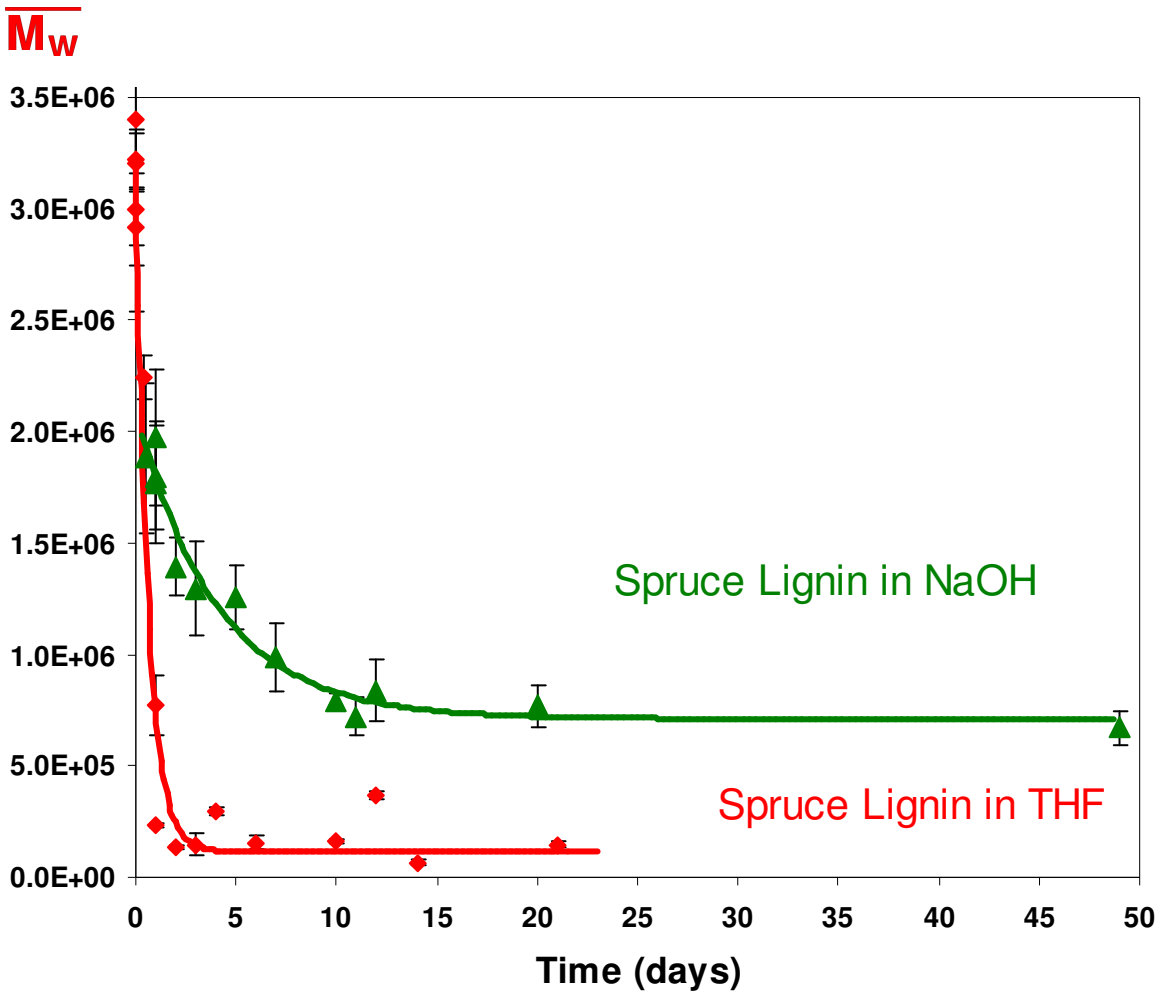


Figure 24. Incubation effect on the weight-averaged apparent molecular weight of the Spruce lignin in THF and in 0.01 N NaOH solutions kept at room temperature.

The curves from **Figure 23** can be fitted in an exponential decay equation as it was done for the de-association data at different temperatures and different lignins (hardwood and softwood); in **equation 8**, where the plateau or the final molecular weight reached is 538082g/mol and the time required to achieve the plateau value is twice its halflife, 4.132. The R^2 for this fit was 0.9319, thus the correlation of the experimental data with the equation is quite acceptable.

$$\overline{M}_W = 1.466\times10^6 \times \exp(-0.167\times t) + 538082 \qquad \textbf{equation 8}$$

When comparing halflifes for Spruce in THF and NaOH, which were 0.4271 and 4.132, it can be concluded that de-association in THF is much faster than in NaOH, around ten times faster and this can be seen in the K values for in **equations 6 and 8** corresponding to de-association kinetics of Spruce in THF and NaOH respectively. The K value for Spruce in THF is 1.623, higher than the K value for Spruce in NaOH, 0.1677. The extent of de-association is higher when lignin is in THF solutions, A values are $3.033x10^6$g/mol for Spruce lignin in THF, higher than $1.466x10^6$g/mol for Spruce lignin in alkaline solution.

Similar work was done by Lindström et al. with kraft lignins gels concluding that at high additions of sodium hydroxide, the increased ionic strength shields the charged groups in the network, thus decreasing the electrostatic repulsion between the charged groups and the degree of swelling is lowered.[33] Although the ion-exchange capacity of the network increases at higher pH , the effect of the higher ionic strength is more important.[33] They said that the increased ion-exchange capacity at higher pH-values (higher than 12) is presumably due to de-association of catecholic, phenolic and aliphatic hydroxyl groups.[33] In our EMALs samples, analyzed in aqueous alkaline solutions, there are no cathecolic groups but we do have phenolic and aliphatic hydroxyl groups; thus, the de-association of these groups maybe present in our solutions. In additional work with kraft lignin in alkaline media,[9] it was observed that the ionization of various lignin functional groups is followed by the formation of the corresponding intermediates and its oxidation; leading to the conclusion that ionization and conjugation govern both elution behaviour and the apparent molecular weight

distribution patterns of kraft lignins in aqueous media is in contrast with the findings of Guerra et al.[34] that, as mentioned before, concluded that the ionization of hydroxyl and carboxylic groups can not explain the de-association phenomena as there was no correlation between their amount and the change in the weight-average apparent molecular weight when working with EMAL.

Benko[66] measured, by a diffusion technique, the relative apparent molecular weights of kraft lignins and lignosulfonates. He found very large differences in the apparent molecular weights measured in aqueous and organic solvents. For example, a polymerized hardwood lignosulfonate gave an apparent molecular weight of 50,000g/mol in 0.1N aqueous KC1 which decreased to about 2000g/mol when measured in DMSO. We can observe a similar tend in our results; the final molecular weight, when the plateau is reached, of Spruce lignin is higher in NaOH than in THF indicating that, the molecular weight value may depend on the solvent used to prepare the solutions because of the solvent-solute interactions. This is in contrast with another study,[67] in which the molecular weight of lignosulfonates in two different solvents, 0.1M aqueous NaCl and DMSO, was measured. There was not significant difference in the Mw values for the lignin prepared in the two solvents and there was no further association with storage time or with temperature. The authors state that the small change was within the experimental error.

In previous studies it was concluded that a prime cause of the lignin apparent molecular weight variation lies in molecular association which, is to a large extent responsible for the actual molecular weight measured under any given set of conditions, for example, in different solvents.[66, 68] Consequently, in order to form a more soundly based picture of lignin

structure, the nature of the interactions of lignin with its solvents needs to be clarified.[31]

In **table 1** we can see the rate of de-association of Spruce EMAL in the two different solvents being used in our experiments. We can observe these values are different, which means lignin de-associates faster in THF than in NaOH 0.1N. Probably the interactions present between lignin and THF promote de-association process to occur faster or maybe, since lignin in THF was acetobrominated before preparing the solutions, one of the interactions among associated lignin molecules (hydrogen bonding) is not present anymore, allowing de-association phenomena to take shorter time. It is experimentally evident that kraft lignin have a pronounced tendency to associate and form more complex structures to a degree dependent on the extent of interaction with the solvent.[31] The actual disparity in the de-association of lignin between the different solvents may also indicate that the association forces that operate in aqueous and organic media are not essentially the same.[34]

The de-association phenomenon takes 10 days until the apparent molecular weight reaches a constant value.

Table 1. Kinetic factors for EMALs incubated in different solvents and different temperatures. **(See equation 4)**

EMAL	Temperature (°C)	Solvent	Rate ($days^{-1}$)	A ($g\ mol^{-1}$)	B ($g\ mol^{-1}$)
Spruce	25	THF	1.623	$3.033x10^6$	$1.48x10^5$
Spruce	4		2.784	$2.625x10^6$	$5.23x10^5$
E. Globulus	25		0.8247	$2.13x10^4$	$1.07x10^4$
Spruce	25	NaOH (0.01 N)	0.1677	$1.466x10^6$	$5.38x10^5$

In trying to compare these results, we came across a study carried out many years ago where kraft lignin association was studied in three different solvents; dimethyl sulfoxide (DMSO), dimethyl formamide and dioxane.[31] One point taken to make a comparison is the electronegativity of the solvents. DMSO is the most electronegative one having a dielectric constant of 45.0 and dioxane is the least electronegative having a dielectric constant of 2.2. In our study the solvents used are water with a dielectric constant of 78.4[69] and THF with a dielectric constant of 7.58.[70] Brown et al.[31] found the best solvent for kraft lignins was DMSO because the corresponding mixing free energy was the lowest one compared to the other two solvents. The better the solvent the more negative the mixing free energy is[31].

In order to determine the mixing free energy it was required to estimate the enthalpy and the entropy of the solutions, following the **equation 9**

$$\Delta F = \Delta H + T\Delta S \qquad \textbf{equation 9}$$

Where Δ is the value of the solution minus the values for the pure components. ΔF correspond to the mixing free energy, ΔH is the enthalpy, T is temperature and ΔS is the entropy of the solution. If solvent-solute interactions are greater than solute-solute and solvent-solvent interactions, heat is evolved on the solution and the value of ΔH will be negative. This occurs when there is solvation and orientation of solvent molecules in the vicinity of the solute molecules. Brown [31] found that all the values of ΔH for his solutions were large and positive and increase with the polar nature of the solvent. He said that those values are probably due to lignin forming hydrogen-bonded complexes with these highly polar solvents. This is because very strong solute-solvent interactions are necessary to overcome the attractive forces between lignin molecules which are the root cause of the

observed association. One would consequently expect that the ΔH values would be negative or at least show small positive values. This is not the case here, however, because the above reasoning ignores an important feature of polar organic solvents which is their considerable degree of internal structure. This means solvent molecules can be associated and consequently the solvent-solvent interactions are quite strong and there is less capability to form lignin-solvent bonds. The internal structure of solvents limits the hydrogen bonds between lignin and solvent. These effects dominate the picture and overshadow the thermodynamic effects arising from the conventional interaction of the lignin with the solvent. In the present study the solvents used were polar (H_2O) and the other moderately-polar (THF). THF has a molecular shape of an envelope. Water is a tiny V-shaped molecule with the molecular formula H_2O. Its molecular diameter is about 2.75 Å. In the liquid state, in spite of 80% of the electrons being concerned with bonding, the three atoms do not stay together as the hydrogen atoms are constantly exchanging between water molecules due to protonation/deprotonation processes. Following Brown's conclusions,[31] perhaps the change in the shape of the solvents molecules plays a role in the solvation and de-association behavior of lignins in solution. Further work in this area needs to be carried out in order to elucidate this idea.

Brown conclusions were that good solvents for kraft lignin (referring to those in which lignin is little associated) are highly polar and thus possess varying degrees of internal structure. Consequently, it is the change in solvent structure, rather than the nature of the lignin-solvent interactions, which govern the magnitudes of the estimated thermodynamic parameters.[31]

4.4 Selective methylation of hydroxyl groups in EMAL Spruce.

Having the behavior of lignins in solution, we observed a de-association trend. In THF we could study the possible π-π stacking interaction responsible for the de-association phenomena, since lignin was acetobrominated; but in NaOH we may have the hydrogen bonding effects as well. Then, next step was to determine how each interaction (hydrogen bonding), if it is present, contributes to the observed phenomenon. Therefore the new approach was the selective blocking of the hydroxyl groups of lignin (Spruce) as described in the experimental part. After methylation the sample was analyzed using ^{31}P NMR.

In **Figure 25** the ^{31}P NMR spectrum for EMAL Spruce is shown. The experiment was done using phosphytilation Reagent II (2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane). The reaction of Reagent II with hydroxyl groups in lignin is shown in **Figure 25** (a). In (b) the spectrum before methylation is presented. (c) shows the ^{31}P NMR after two treatments of methylation. It can be deduced from the Figures b and c that almost all the phenolic hydroxyl groups were methylated because their corresponding signal almost disappear in c.

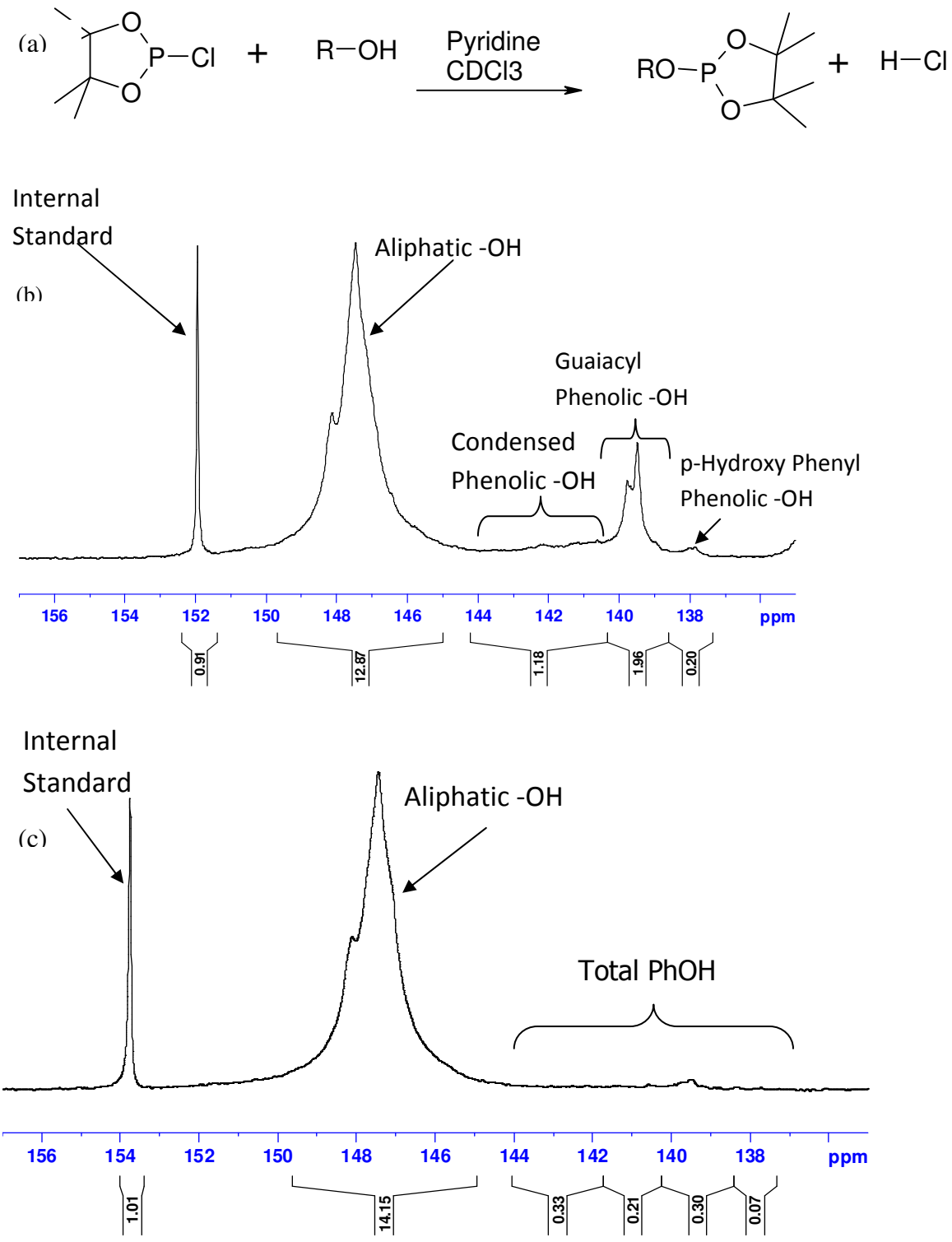


Figure 25. Selective methylation of phenolic hydroxyl groups (a) general reaction (b) ^{31}P NMR spectra for EMAL Spruce before methylation. (c) ^{31}P NMR spectra for EMAL Spruce after methylation.

After methylation of the phenolic units of EMAL Spruce, the solubility in the solvents used in this study was verified. Phenolic methylated Spruce lignin was not soluble in THF, neither in NaOH 0.1N alkaline solution. Therefore, the measurements of the corresponding weight-average apparent molecular weight in light scattering for those samples could not be determined.

Next step was to try selective methylation of alpha aliphatic hydroxyl groups of EMAL Spruce lignin. **Figure 26** the ^{31}P NMR spectrum for EMAL Spruce is shown. The experiment was done using phosphytilation Reagent I (1,3,2-dioxaphopholanyl chloride) .[71] The reaction of Reagent I with hydroxyl groups in lignin is shown in **Figure 26** (a). In (b) the spectrum before methylation is presented. (c) shows the ^{31}P NMR after methylation treatment. It can be observed in the Figures b and c that almost all the alpha aliphatic hydroxyl groups were methylated because their corresponding signal almost disappear in c.

FTIR analysis in lignin model compounds revealed aliphatic hydroxyl groups form stronger hydrogen bonds than phenolic hydroxyl groups.[65] Hence, it was interesting to test the EMAL Spruce with the alpha aliphatic hydroxyl groups blocked in order to determine if they play a role in the association phenomenon of lignin in solution. These samples showed good solubility in THF and in NaOH 0.1N.

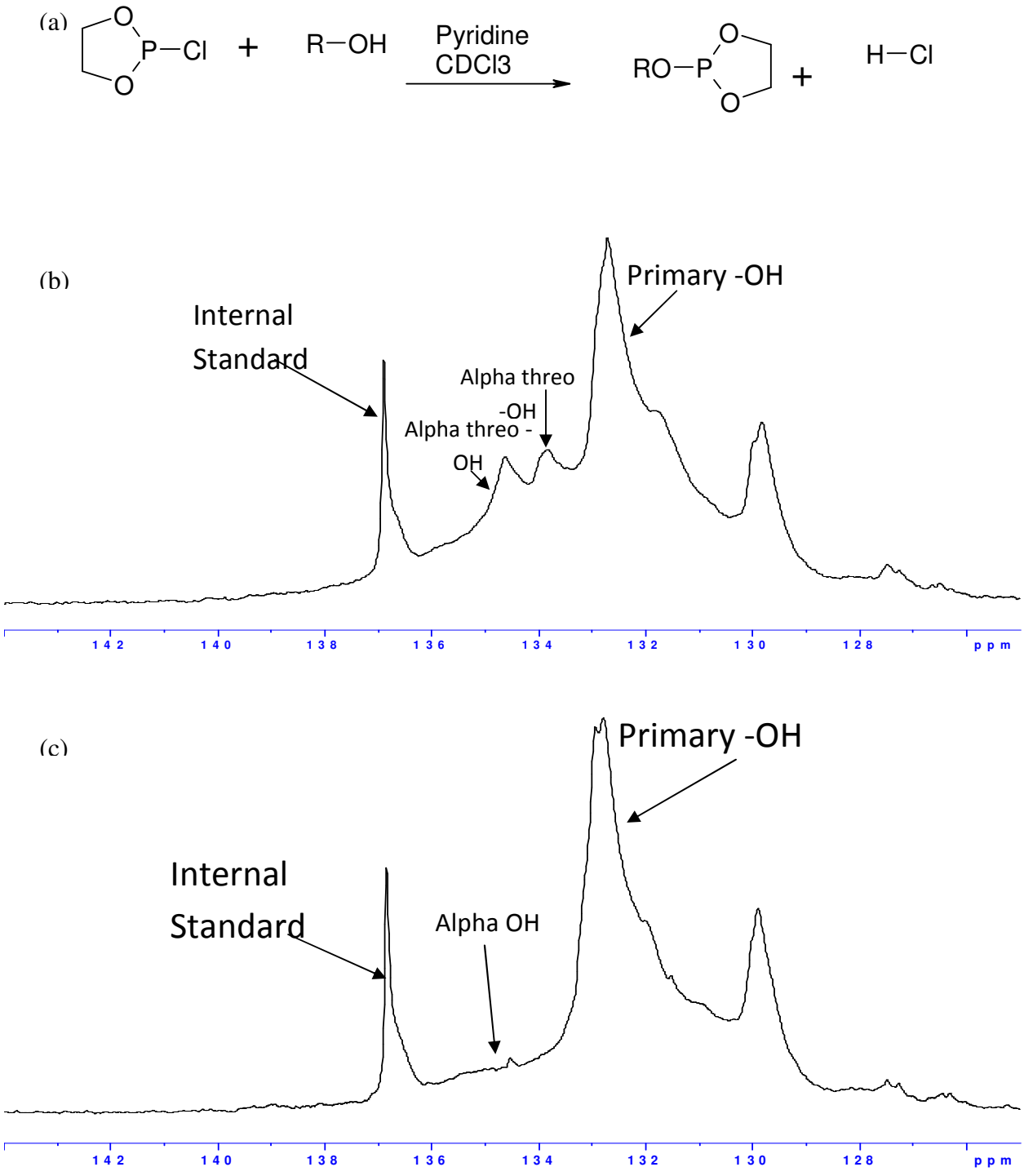


Figure 26. Selective methylation of alpha aliphatic hydroxyl groups (a) general reaction (b) ^{31}P NMR spectra for EMAL Spruce before methylation. (c) ^{31}P NMR spectra for EMAL Spruce after methylation.

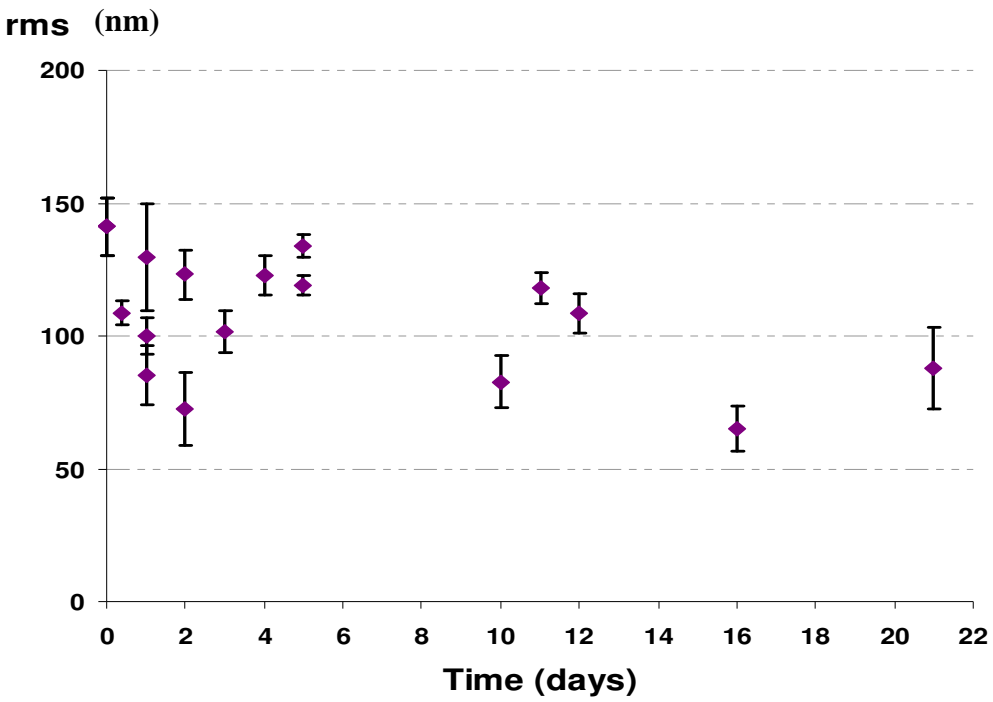
4.5 Radius of gyration (rms)

Another parameter obtained with static light scattering measurements is the root mean square (rms) or radius of gyration of the lignin molecules in solution. Radius of gyration is referred to the root mean square distance of the subunits of a molecule from its center of mass. As observed in **Figure 27** there is not as extensive dependence variation trend between the radius of gyration of lignin samples and the age of the solution. The behavior is not the expected because as lignin weight-average apparent molecular weight decreases the radius of gyration is not decreasing in the same way, showing an exponential decay with time. Even though, there is not a define relationship, a reduction in the rms of the molecules with time can be observed.

The change in the radius of gyration of lignin samples in THF with aging of the solution is random. It has been determined that discrete kraft lignin components in alkaline solutions behave as expanded random coil molecules.[6] A random coil is a polymer conformation where the monomer subunits are oriented randomly while still being bonded to adjacent units. It is not one specific shape, but a statistical distribution of shapes for all the chains in a population of macromolecules. Discrete lignin components exhibit a range of conformations in solution that will be distinct from the configuration of the complexes formed through association between them and the associated complexes showed a flexible lamellar configuration.[6] The lamellar configuration of lignin was described elsewhere[72] after preparing films of lignosulfonates and taking photomicrographs of the samples. It was shown that lignin tends to adopt flat configuration similar to the lamellae structure in the wood. Moreover, such molecules in solution would be expected to be flexible and disk-like having various

shapes and sizes and their hydrodynamic behavior will lie between that of a sphere and a random coil.[72] Gidh et al.[73] studied kraft lignin in aqueous solutions with multi angle laser light scattering technique in the static and dynamic mode and found that the distribution in the radius of gyration (rg) became narrower with time. At the same time the hydrodynamic radius (rh) and apparent molecular weight distribution did not vary, indicating that molecular conformation (which can be deduced from the log (rg)/log(MW) and (rg/rh ratio) changed to a more uniform molecular shape. Since the cumulative radius of gyration is decreasing the molecules become more compact. Our results can support the just mentioned conclusion, perhaps the high polydispersity of the fresh samples make the reproducibility of the measurements poor and therefore, there is no a clear behavior for 5 days incubation solution, but after 5 days the rms of the molecule has decreased due to de-association of lignin complexes that become more compact and possess a more define shape.

(a)



(b)

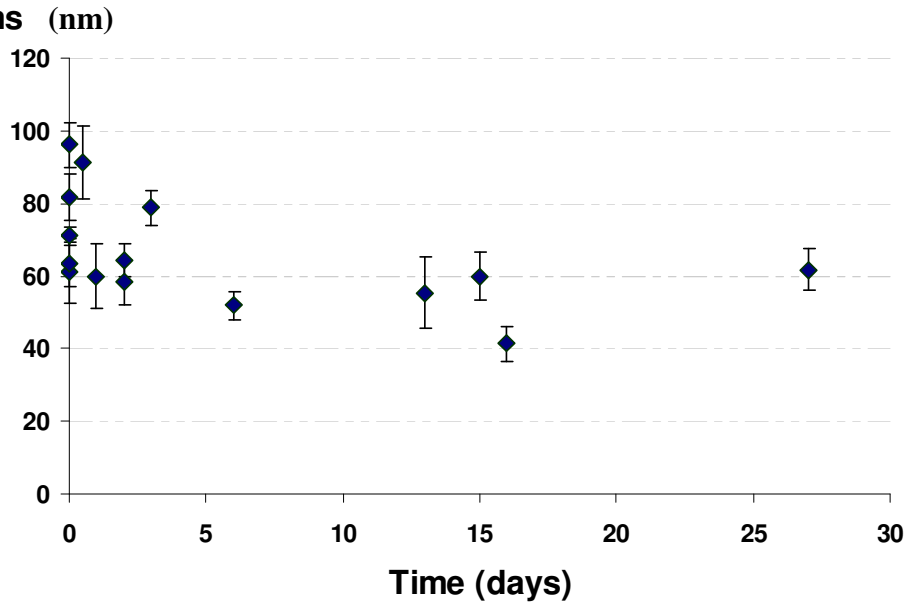


Figure 27. Root mean square of EMAL Spruce in THF (a) and EMAL E. Globulus in THF (b).

4.6 Second virial coeficcient (A_2)

Light scattering can be used to measure the second virial coefficient (A_2) of a macromolecule. A_2 is a measure of macromolecular self association, and is one of the few parameters that can be used to predict the crystallization properties of a sample. Moreover, light scattering techniques used to measure A_2 can be extended to characterize hetero-association between two different proteins.[45] Quantitatively, the theta state is defined through the second virial coefficient (A_2), which measures the excess chemical potential (excess Gibbs free energy of dilution) between polymer and solvent molecules in solution. Theta conditions are defined as those at which $A_2 = 0$. Conversely, $A_2 > 0$ signifies that the polymer is dissolved in a thermodynamically 'good' solvent at the given temperature, while $A_2 < 0$ signifies that the solvent/temperature conditions are thermodynamically 'poor.' At 'good' conditions, the chemical potential between the analyte and solvent is minimized, the molecule is more extensively solvated, the chain assumes a more extended configuration due to the strengthening effect of the solvent, and the excluded volume is positive. The opposite of this is true at 'poor' conditions.[74]

As shown in **Figure 28**, the second virial coefficient for EMAL Spruce solutions, in THF and in NaOH (0.01N), decreases as the weight-average apparent molecular weight increases. Indicating that, for associated complexes having high apparent molecular weight, the second virial coefficient is low and for discrete components with low apparent molecular weight, after de-association takes place, the second virial coefficient increases, representing better solvation. This is in agreement to what Brown[31] observed. This autor found that the second virial coeficient A_2 increases as the apparent molecular weight decreases. Since, A_2 is a

measure of the thermodynamic interaction between solute and solvent, this indicates that the variation in apparent molecular weight is a result of lignin association.[31] In the case of EMAL Spruce dissolved in THF there are no negative values, as shown in Figure 27 a, in contrast to what is exposed in Figure 27 b, where EMAL Spruce is dissolved in NaOH 0.01N having several negative values of second virial coefficient. This indicates there is better interaction between lignin molecules and THF than between lignin and aqueous alkaline solution.

The errors associated to the second virial coefficient measurements were much higher for lower apparent molecular weight samples, when de-association was taking place. This effect can be due to the fact that light scattering technique is more sensitive to high apparent molecular weight samples. Consequently it gives high errors when determining the parameters for low apparent molecular weight samples.

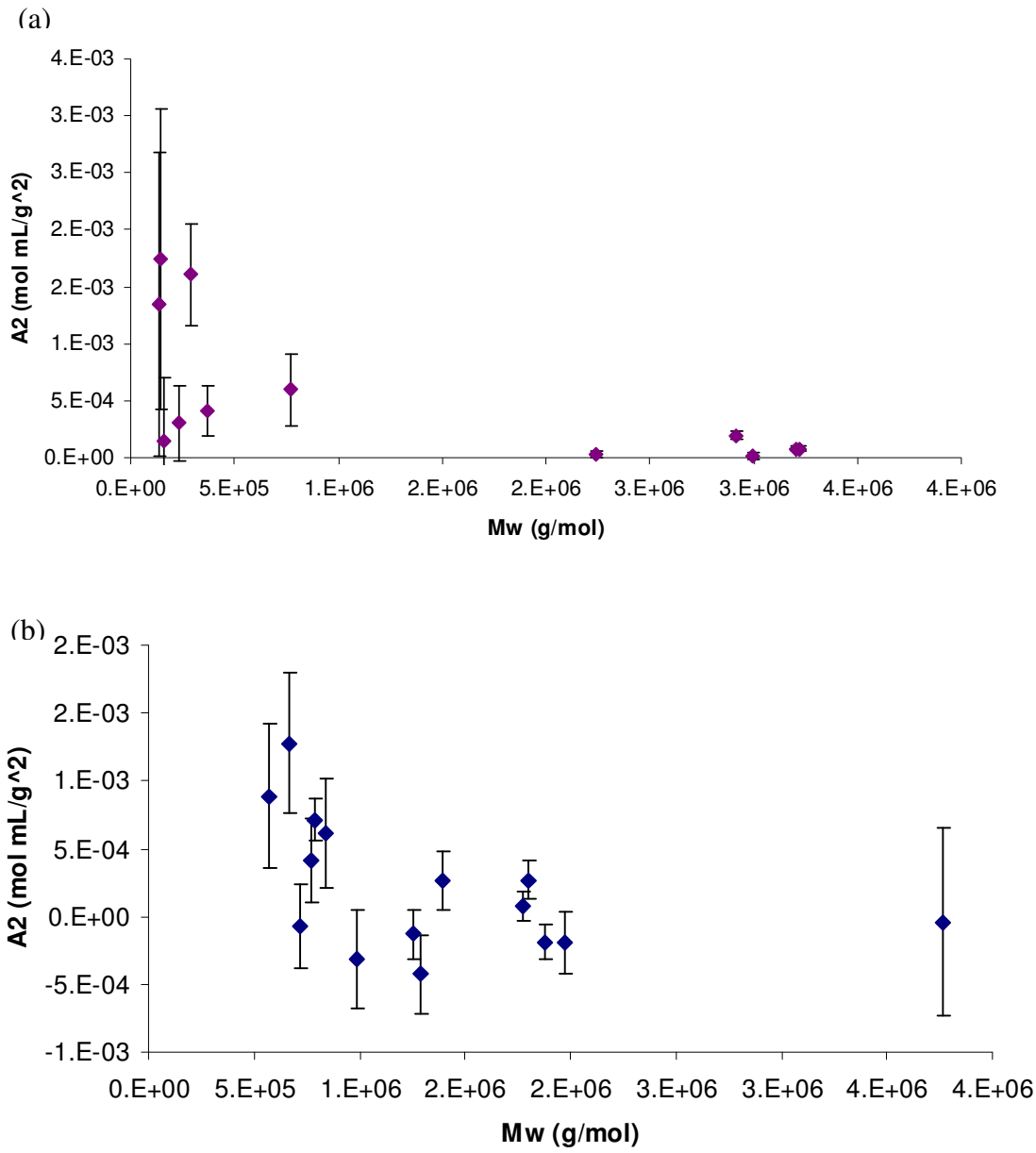


Figure 28. Second virial coefficient (A_2) of EMAL Spruce in THF (a) and EMAL Spruce in NaOH (b).

5. Conclusions

After this study it can be concluded that:

- Static light scattering measurements are revealing as far as the propensity of lignin to associate is concerned.
- Static light scattering studies may reveal more details as to the hydrophobic and hydrophilic forces operating in defining lignin association in the cell wall.
- The change in the dn/dC of Spruce lignin in THF can be due to a change in the hydrophobicity of the molecules when changing from associated complexes to discrete components.
- Lignin de-association can be described as an exponential decay of the apparent molecular weight with the age of the solution.
- Lignin de-association phenomena are temperature dependent.
- However, the rate of lignin de-association phenomena may not be temperature dependent
- Hardwoods and softwoods have different propensities to associate with different extents and rate of de-association.
- The rate and extent of de-association of Spruce lignin vary with the solvent used to prepare the solutions.
- Lignin association forces may be governed amongst others by hydrophobic

interactions non-bonded orbital (π-π stacking)

- The radius of gyration of lignin solutions decreases with time but does not show the same trend as the weight-average apparent molecular weight.

- Second virial coefficient (A_2) values increase when de-association takes place.

6. Future work

As far as the tendency of lignin molecules in solution to associate/de-associate is concerned, there are more experiments that could help in the understanding of this complex and still unknown phenomenon:

- Study of the change in weigh-average apparent molecular weight of E. Globulus lignin in NaOH in order to compare the behavior of softwood and hardwood lignin in aqueous alkaline solutions.

- Follow the apparent molecular weight with time of the previously described selectively blocked lignin to determine the role, if present, of the phenolic hydroxyl group and aliphatic alpha hydroxyl groups interactions in the association/de-association phenomenon.

- Viscosity or dynamic light scattering measurements can help to determine the hydrodynamic radius with the aim of studying the change in conformation of lignin molecules during the association/de-association phenomenon.

- Track the change in the apparent molecular weight of both softwood and hardwood lignin at different temperatures of incubation and try to establish relationships with the energy of activation needed for this phenomenon to occur.

- Molecular dynamics simulation of softwood and hardwood lignin model compounds in order to determine the energy associated to each interaction force present in associated complexes.

- Create lignin films and study their interaction with lignin or other polymers using quartz crystal microbalance (QCM-D) and also get images of those films using scanning electron microscopy (SEM) in order to study the shape of lignin aggregates.

- As mentioned previously, lignin can be used as emulsifier. The study of lignin behavior in emulsions is an interesting field. How the apparent molecular weight, the different functional groups of lignin and the different sizes of lignin molecules influence emulsion when using lignin as a surfactant.

7. References

1. Adler, E. "Lignin chemistry-past, present and future" *Wood science and technology, Vol*. 11, *(3),* pp. 169, 1977.

2. Wadenback, J.; Clapham, D.; Gellerstedt, G.; von Arnold, S. "Variation in content and composition of lignin in young wood of Norway spruce" *Holzforschung,* Vol. 58, *(2),* pp. 107, 2004.

3. Chen, C.; Baucher, M.; Christensen, J. H. and Boerjan, W. "Biotechnology in trees: Towards improved paper pulping by lignin engineering" *Euphytica, Vol*. 118, *(2),* pp. 185, 2001.

4. Falkehag, S. I.; Braddon, D. V.; Dougherty, W. K. "Lignin polymer applications" *Abstracts of papers of the american chemical society, (169),* pp. 6, 1975.

5. Lindström, T. "The colloidal behaviour of kraft lignin Part I.: Association and gelation of kraft lignin in aqueous solutions" *Colloid polymer science, Vol*. 257, *(3),* pp. 277, 1979.

6. Sarkanen, S.; Teller, D. C.; Abramowski, Edward and McCarthy, Joseph L. "Lignin. 19. Kraft lignin component conformation and associated complex configuration in aqueous alkaline solution" *Macromolecules,* Vol. 15, *(4),* pp. 1098, 1982.

7. Sarkanen, S.; Teller, D. C.; Stevens, Clyde R. and McCarthy, Joseph L. "Lignin. 20. Associative interactions between kraft lignin components" *Macromolecules,* Vol. 17, *(12),* pp. 2588, 1984.

8. Norgren, M.; Edlund, Håkan and Wågberg, Lars "Aggregation of lignin derivatives under alkaline conditions. Kinetics and aggregate structure" *Langmuir,* Vol. 18, *(7),* pp. 2859, 2002.

9. Bikova, T.; Treimanis, A.; Rossinska, G. and Telysheva, G. "On-line study of lignin behaviour in dilute alkaline solution by the SEC-UV method" *Holzforschung,* Vol. 58, *(5),* pp. 489, 2004.

10. Gidh, A. V.; Decker, S. R.; See, C. H.; Himmel, Michael E. and Williford, Clint W. "Characterization of lignin using multi-angle laser light scattering and atomic force microscopy" *Analytica chimica acta,* Vol. 555, *(2),* pp. 250, 2006.

11. Cathala, B.; Saake, B.; Faix, O. and Monties, B. "Association behaviour of lignins and lignin model compounds studied by multidetector size-exclusion chromatography" *Journal of chromatography,* Vol. 1020, *(2),* pp. 229, 2003.

12. Sarkanen, S.; Teller, D. C.; Hall, John and McCarthy, Joseph L. "Lignin. 18. Associative effects among organosolv lignin components" *Macromolecules,* Vol. 14, *(2),* pp. 426, 1981.

13. Ralph, J.; Lundquist, K.; Brunow, G.; Lu, F.; Kim, H.; Schatz, P. F.; Marita, J. M.; Hatfield, R. D.; Ralph, S. A.; Christensen, Jørgen Holst and Boerjan, Wout "Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenyl-propanoids" *Phytochemistry reviews,* Vol. 3, *(1/2),* pp. 29, 2004.

14. Gilardi, Gianfranco and Cass, Anthony E. G. "Associative and colloidal behavior of

lignin and implications for its biodegradation in vitro" *Langmuir,* Vol. 9, *(7),* pp. 1721, 1993.

15. Feldman, D. "Wood - chemistry, ultrastructure, reactions, by D. Fengel and G. Wegener, Walter de Gruyter, Berlin and New York, 1984." *Nature,* Vol. 310, *(5977),* pp. 521, 1984.

16. Sarkanen, K.V. and Ludwig, C.H. *Lignins—Occurrence, Formation, Structure and Reactions.* Wiley: New York, 1971.

17. Brunow, G.; Karlsson, O.; Lundquist, K. and Sipilä, J. "On the distribution of the diastereomers of the structural elements in lignins: the steric course of reactions mimicking lignin biosynthesis" *Wood science and technology,* Vol. 27, *(4),* pp. 281, 1993.

18. Helm, RF. and Li, KC. "Complete threo-stereoselectivity for the preparation of beta-o-4 lignin model dimers" *Holzforschung,* Vol. 49, *(6),* pp. 533, 1995.

19. Bardet, M.; Robert, D.; Lundquist, K. and von Unge, S. "Distribution of erythro and threo forms of different types of beta-0-4 structures in aspen lignin by C-13 NMR using the 2D INADEQUATE experiment" *Magnetic resonance in chemistry,* Vol. 36, *(8),* pp. 597, 1998.

20. Barakat, A.; Gaillard, C.; Lairez, D.; Saulnier, L.; Chabbert, B. and Cathala, B. "Supramolecular organization of heteroxylan-dehydrogenation polymers (synthetic lignin) nanoparticles" *Biomacromolecules,* Vol. 9, *(2),* pp.487-493, 2008.

21. Chabannes, M.; Ruel, K.; Yoshinaga, A.; Chabbert, B.; Jauneau, A.; Joseleau, Jean-Paul and Boudet, Alain-Michel "In situ analysis of lignins in transgenic tobacco reveals a

differential impact of individual transformations on the spatial patterns of lignin deposition at the cellular and subcellular levels" *Plant Journal,* Vol. 28, *(3),* pp.271-282, 2001.

22. http://www.lignin.org

23. Van Dam, J. E. G.; Van Den Oever, M.J.A.; Teunissen, W.; Keijsers, E. R. P.; Peralta, A. G. "Process for production of high density/high performance binderless boards from whole coconut husk - Part 1: Lignin as intrinsic thermosetting binder resin" *Industrial crops and products,* Vol. 19, *(3),* pp. 207, 2004.

24. Zhou, M.; Qiu, X.; Yang, D.; Lou, H.; Ouyang, X. "High-performance dispersant of coal-water slurry synthesized from wheat straw alkali lignin" *Fuel processing technology,* Vol. 88, *(4),* pp. 375, 2007.

25. Matsushita, Y.; Imai, M.; Tamura, Takashi and Fukushima, Kazuhiko "Preparation and evaluation of a dispersant for gypsum paste from acid hydrolysis lignin" *Journal of applied polymer science,* Vol. 98, *(6),* pp. 2508, 2005.

26. Tolosa, L. I.; Rodríguez-Malaver, A. J.; González, Aura M. and Rojas, Orlando J. "Effect of Fenton's reagent on O/W emulsions stabilized by black liquor" *Journal of colloid and interface science,* Vol. 294, *(1),* pp. 182, 2006.

27. Gundersen, S. A.; Sæther, Øystein and Sjöblom, Johan "Salt effects on lignosulfonate and kraft lignin stabilized O/W-emulsions studied by means of electrical conductivity and video-enhanced microscopy" *Colloids and surfaces. A, Physicochemical and engineering aspects,* Vol. 186, *(3),* pp. 141, 2001.

28. Davis, J. N.; Baier, J. E.; Fulton, J. P.; Brown, D. A. and McDonald, T. P. "Use of chemical soil additives to stabilize off-road vehicle trails" *Applied engineering in agriculture,* Vol. 23, *(5),* pp. 597, 2007.

29. Kramárová, Z.; Alexy, P.; Chodák, I.; Špirk, E.; Hudec, I.; Košíková, B.; Gregorová, A.; Šúri, P.; Feranc, J.; Bugaj, P. and Ďuračka, M. "Biopolymers as fillers for rubber blends" *Polymers for advanced technologies,* Vol. 18, *(2),* pp. 135, 2007.

30. Lindberg, J. J.; Bulla, I. and Tormala, P. "Spin labeling studies on constituents of wood .2. Correlation times of spin probes and rigidity of network structure in isolated lignin" *Journal of polymer science. Polymer symposia, (53),* pp. 167, 1975.

31. Brown, W. "Solution properties of lignin. Thermodynamic properties and molecular weight determinations" *Journal of applied polymer science,* Vol. 11, *(11),* pp. 2381, 1967.

32. Gidh, A. V.; Decker, S. R.; Vinzant, T. B.; Himmel, Michael E. and Williford, Clint "Determination of lignin by size exclusion chromatography using multi angle laser light scattering" *Journal of chromatography,* Vol. 1114, *(1),* pp. 102, 2006.

33. Lindström, T. and Westman, L. "The colloidal behaviour of kraft lignin III. Swelling behaviour and mechanical properties of kraft lignin gels" *Colloid polymer science,* Vol. 258, *(4),* pp. 390, 1980.

34. Guerra, A.; Gaspar, A.; Contreras, I. S.; Lucia, L.; Crestini, C.; Argyropoulos, D. S. "On the Propensity of Lignin to Associate, Part I , A Size Exclusion Chromatographic Study with Native Lignins Isolated from Different Wood Species" Vol.68, *(19),* 2007.

35. Benesi, H.A. and Hildebrand, J.H. "A spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons" *J. Am. Chem. Soc.,* Vol. 71, *(8),* pp.2703-2707, 1949.

36. Merkle, G.; Auerbach, S.; Burchard, W.; Lindner, Albert and Wegener, Gerd "Light scattering of acetylated lignin" *Journal of applied polymer science,* Vol. 45, *(3),* pp. 407, 1992.

37. Moacanin, J.; Felicetta, V. F.; Haller, Wolfgang and McCarthy, Joseph L. "Lignin. VI. Molecular weights of lgnin sulfonates by light scattering" *Journal of the American Chemical Society,* Vol. 77, *(13),* pp. 3470, 1955.

38. Dong, D. and Fricke, A. L. "Investigation of optical effect of lignin solution and determination of w of kraft lignin by LALLS" *Journal of applied polymer science,* Vol. 50, *(7),* pp. 1131, 1993.

39. Beignon, M.; Bambab, S. E. H.; Evaina, K.; Le Goffa, D. and Proutiere, A. "Molecular weights by light scattering in macromolecules solutions. Large deviations observed between a new method and the classical method" *Journal of molecular structure,* Vol. 477, *(1-3),* pp. 255, 1999.

40. Fredheim, G. E.; Braatenb, S. M. and Christensen, B. E. "Molecular weight determination of lignosulfonates by size-exclusion chromatography and multi-angle laser light scattering" *Journal of chromatography,* Vol. 942, *(1-2),* pp. 191, 2002.

41. ucla web.chem.ucla.edu/~harding/notes.

42. http://www.chemguide.co.uk/atoms/bonding/hbond.html.

43. www.chemsoc.org/ExemplarChem/entries/2004/warwick_robinson/Pi.htm.

44. Graessley, W. W. "Molecular entanglement theory of flow behavior in amorphous polymers" *The Journal of chemical physics,* Vol. 43, *(8),* pp. 2696, 1965.

45. Wyatt Technology Corporation.

46. Wu, S.; Argyropoulos, D. S. "An improved method for isolating lignin in high yield and purity" *Journal of pulp and paper science,* Vol. 29, *(7),* pp. 235, 2003.

47. Lu, Fachuang and Ralph, John "The DFRC method for lignin analysis. 2. Monomers from isolated lignins" *Journal of agricultural and food chemistry,* Vol. 46, *(2),* pp. 547, 1998.

48. Guerra, A.; Filpponen, I.; Lucia, Lucian A. and Argyropoulos, Dimitris S. "Comparative evaluation of three lignin isolation protocols for various wood species" *Journal of agricultural and food chemistry,* Vol. 54, *(26),* pp. 9696, 2006.

49. Guerra, A.; Filpponen, I.; Lucia, L. A.; Saquing, C.; Baumberger, Stephanie and Argyropoulos, Dimitris S. "Toward a better understanding of the lignin isolation process from wood" *Journal of agricultural and food chemistry,* Vol. 54, *(16),* pp. 5939, 2006.

50. Crestini, C.; Argyropoulos, D. S. "Structural Analysis of Wheat Straw Lignin by Quantitative 31P and 2D NMR Spectroscopy. The Occurrence of Ester Bonds and α-O-4 Substructures" *J. Agric. Food Chem.,* Vol. 45, *(4),* pp.1212-1219, 1997.

51. Adler, E.; Brunow, G. and Lundquist, K. "Investigation of the acid-catalyzed alkylation of lignins by means of nmr spectroscopic methods" *Holzforschung,* Vol. 41, *(4),* pp. 199, 1987.

52. Pla, F.; Lin, S. Y.; Dence, C. In *Handbook of size exclution chromatography and related techniques.* 1992; Vol. 91, pp. 689.

53. Stacey, K. A. "Light-Scattering in Physical Chemistry" 1956.

54. Kolpak, F. J.; Cietek, D. J.; Fookes, W.; and Cael, J. J. "Analysis of lignins from spent alkaline pulping liquors by gel permeation chromatography/low-angle laser light scattering (GPC-LALLS)" Vol.37, pp. 431, 1983.

55. Forss, K. and Stenlund, B. "Light absorption and fluorescence of lignosulfonates dissolved in water and dimethyl sulfoxide" *Paper ja Puu-Papper och tra,* Vol. 49, *(8),* pp. 525, 1967.

56. Sorlier, P.; Rochas, C.; Morfin, I.; Viton, Christophe and Domard, Alain "Light scattering studies of the solution properties of chitosans of varying degrees of acetylation" *Biomacromolecules,* Vol. 4, *(4),* pp. 1034, 2003.

57. Kulicke, W. M.; Horl, H. H. "Characterization of aqueous polystyrene sulfonate solutions and their unusual viscosity-time-behavior" *Angewandte Makromolekulare Chemie,* Vol.116, pp. 149, 1983.

58. Stejskal, Jaroslav and Horská, Jitka "Refractive index increments of polyacrylamide and comments on the light scattering from its solutions" *Die Makromolekulare Chemie,* Vol.

183, *(10),* pp. 2527, 1982.

59. Dupont, Anne-Laurence and Harrison, Gabrielle "Conformation and dn/dc determination of cellulose in N,N-dimethylacetamide containing lithium chloride" *Carbohydrate polymers,* Vol. 58, *(3),* pp. 233, 2004.

60. Brandrup, J. and Immergut, E. H. In *Polymer Handbook.* Wiley InterScience: New York, 1989.

61. Leamen, M. J.; McManus, N. T. and Penlidis, A. "Refractive index increment (dn/dc) using GPC for the alpha-methyl styrene/methyl methacrylate copolymer at 670 nm in tetrahydrofuran" *Journal of applied polymer science,* Vol. 94, *(6),* pp. 2545, 2004.

62. Argyropoulos, D. S. "Quantitative Phosphorus-31 NMR analysis of lignins, a new tool for the lignin chemist" *Journal of wood chemistry and technology,* Vol. 14, *(1),* pp. 45, 1994.

63. Granata, Alessandro and Argyropoulos, Dimitris S. "2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, a reagent for the accurate determination of the uncondensed and condensed phenolic moieties in lignins" *Journal of agricultural and food chemistry,* Vol. 43, *(6),* pp. 1538, 1995.

64. Raman, M. S.; Ponnuswamy, V.; Kolandaivel, P. and Perumal, K. "Ultrasonic and computational study of intermolecular association through hydrogen bonding in aqueous solutions of D-mannitol" *Journal of molecular liquids,* Vol. 135, *(1-3),* pp. 46, 2007.

65. Kubo, S.; Kadla, J. F. "Hydrogen bonding in lignin: A Fourier transform infrared model

compound study" *Biomacromolecules,* Vol. 6, *(5),* pp.2815-2821, 2005.

66. Benko, J. "Empirische zusammenhange zwischen der atomstruktur und einigen physikalischen konstanten der elemente" *Acta chimica,* Vol. 41, *(4),* pp. 401, 1964.

67. Yean, W. Q.; Goring, D. A. I. "The molecular weight of a sodium lignosulfonate in dimethylsulfoxide and in aqueous 0.1*M* sodium chlorine" *Journal of applied polymer science,* Vol. 14, *(4),* pp. 1115, 1970.

68. Gross, S. K.; Sarkanen, Kyosti and Schuerch, Conrad "Determinations of molecular weight of lignin degradation products by three methods" *Anal. Chem.,* Vol.30, *(4),* pp.518-521, 1958.

69. http://www.lsbu.ac.uk/water/data.html.

70. http://macro.lsu.edu/howto/solvents/THF.htm.

71. Saake, B.; Argyropoulos, D. S.; Beinhoff, Ottokar and Faix, Oskar "A comparison of lignin polymer models (DHPs) and lignins by P-31 NMR spectroscopy" *Phytochemistry,* Vol. 43, *(2),* pp. 499, 1996.

72. Goring, D. A. I.; Vuong, R.; Gancet, C. and Chanzy, H. "The flatness of lignosulfonate macromolecules as demonstrated by electron microscopy" *Journal of applied polymer science,* Vol. 24, *(4),* pp. 931, 1979.

73. Gidh, A. V.; Decker, S. R.; Vinzant, T. B.; Himmel, M. E.; Williford, C. W. "Fungal-Induced Redistribution of Kraft Lignin Molecular Weight by Multi-Angle Laser Light

Scattering" *Chem. Eng. Commun.,* Vol.193, *(12),* pp.1546-1561, 2006.

74. Smith, M. J.; Haidar, Imad A. and Striegel, André M. "Measuring the size of polymers with negative radii using MALS/QELS: an exploration of the thermodynamic radius" *The Analyst,* Vol. 132, *(5),* pp. 455, 2007.