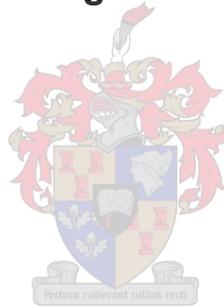


# **Lignin polysaccharide networks in biomass and corresponding processed materials**

**by**

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March 2015

## Declaration

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## Abstract

Lignocellulosic material is composed of three major macromolecule components i.e., cellulose, hemicelluloses and lignin. These components are chemically associated and directly linked to each other through covalent bonding which is scientifically denoted as lignin-carbohydrate complexes (LCCs) and their interaction is fundamentally in wood formation and reactivity during chemical and biological processing e.g. pulping and enzymatic hydrolysis.

These linkages exist in lignocellulosic materials from wood to herbaceous plants. In woody plants, they consist of ester and ether linkages through sugar hydroxyl to  $\alpha$ -carbonyl of phenylpropane unit on lignin. However, in herbaceous plants ferulic and *p*-coumaric acids are esterified to hemicelluloses and lignin respectively.

The study was aimed at isolating and fractionating LCCs from raw lignocellulosic materials (*E. grandis* and sugarcane bagasse) and corresponding processed materials (chemical pulps and water-insoluble residues (WIS)) in order to determine the chemical structure of the residual lignin associated with polysaccharides and how they affected industrial processing. Both feedstock were subjected to Kraft pulping and sugarcane bagasse was further processed for enzymatic hydrolysis. Hemicelluloses pre-extracted (mild alkali or dilute acid and autohydrolysis for sugarcane bagasse) pulps of Kraft or soda AQ from *E. grandis* and sugarcane bagasse were used to understand the effect of xylan pre-extraction prior to pulping on lignin-carbohydrate complexes has not been reported to the best knowledge of the primary author. Also prior to EH the material was subjected to two different treatment methods, i.e. steam explosion and ionic liquid fractionation in varying conditions. The study illustrated the types of extracted and fractionated LCCs from hemicelluloses pre-extracted pulps and WIS in comparison to the non-extracted pulps and reports from the literature. Lignin-carbohydrate complexes (LCCs) were isolated and fractionated by an inorganic method which yielded reasonable quantification quantities and no contamination and low yields for the hardwood compared to reports of using an enzymatic method. To the best knowledge of the authors, no work has been done on WIS material.

The lignocelluloses were subjected to ball milling which was followed by a sequence of inorganic solvents swelling and dissolution into 2 fractions i.e. glucan-lignin and xylan-lignin-glucan. Characterisation of the isolated LCCs was made using a variety of analytical tools such as FTIR-PCA, HPLC, GPC and GC-MS. LCCs were evident when FTIR and HPLC studies were conducted.

Residual lignin isolated from the lignocelluloses was assumed to be chemically bonded to carbohydrates and mostly to xylan. Approximately 60% and 30% of the lignin was linked to xylan while for the second and first LCC fractions respectively. Literature reports that lignin associated with xylan is more resistant to delignification than when linked to glucan which is easily hydrolysable.

With the FTIR and GPC analyses of LCC fractions, it was evident that the ester bonds of LCCs were destroyed through pre-extraction and pre-treatment, where this resulted to more cellulose being more accessible to alkaline pulping and enzymatic hydrolysis respectively. The linkages were either partially broken down or completely destroyed leading to significant changes of chemical structures. The polydispersity of the LCCs assisted in determining the structure of lignin, either existing as monolignols on the surfaces of fibres or as a complex two or three-dimensional structure that is linked to carbohydrates as the  $M_w$  increased or decreased. In general, these findings may have an important implication for the overall efficiency on bio-refinery.

The molecular weights ( $M_w$ ) of the extracted LCCs were measured by gel permeation chromatography. From the chromatograms, it was observed that the materials that were subjected to pre-processing prior to further processing, the  $M_w$  shifted to lower  $M_w$ s regions. It was found that LCCs isolated from mild alkali pre-extracted pulps had high lignin syringyl to guaiacyl lignin contents than LCCs isolated from dilute acid pre-extracted pulps.

High syringyl/guaiacyl ratio (S/G ratio) was an indication of low lignin content as a result of processing which will result to high product yields after downstream processing. The average S/G ratio for the pulps from *E. grandis* and sugarcane bagasse was ranging between 1.1 to 19.01 and 1.4 to 18.16 respectively, while for the WIS-material generated from ionic liquid fractionated and steam exploded materials ranged from 3.29 to 9.27 and 3.5 to 13.3 respectively. The S/G ratios of the LCCs extracted from *E. grandis* and sugarcane bagasse pulps ranged from 0.42 to 2.39 and 0.041 to 0.31 respectively while for the LCCs extracted from water-insoluble-solids (WIS) material generated from steam exploded material was from 4.87 to 10.40. The determination of S/G ratio is recommended for the LCC extraction and characterisation study as an evaluation of residual lignin in processed materials such as pulps and WIS.

The obtained degree of saccharification was low, possibly due to the severity of the steam explosion pre-treatment and ionic liquid fractionation conditions which resulted on high accumulation of acetic acid and increased in cellulose crystallinity respectively. From

quantitative analysis of the LCCs perspective it could be concluded that free lignin was present in mild alkali pre-extracted pulps than for the dilute acid pre-extracted pulps.

**Keywords:** Lignin-carbohydrate complexes (LCCs), pre-treatment, pulping, enzymatic hydrolysis,

## Opsomming

Cellulose materiaal is saamgestel uit drie groot makromolekule komponente naamlik, sellulose, hemisellulose en lignien. Hierdie komponente is chemies verwante en direk met mekaar verbind deur kovalente binding wat wetenskaplik aangedui as lignien - koolhidraat komplekse (LCCs) en hul interaksie is fundamenteel in hout vorming en reaktiwiteit tydens chemiese en biologiese verwerking bv verpulping en ensiematiese hidrolise. Hierdie skakeling bestaan in cellulose materiaal uit hout te kruidagtige plante. In houtagtige plante, hulle bestaan uit ester en eter bindings deur suiker hidroksiel te  $\alpha$  - karboniel van feniel - propaan eenheid op lignien. Maar in kruidagtige plante ferulic en p- coumaric sure veresterd te hemisellulose en lignien onderskeidelik.

Die studie is daarop gemik om te isoleer en fraksionering LCCs van rou cellulose materiaal ( E. grandis en suikerriet bagasse ) en die ooreenstemmende verwerkte materiaal ( chemiese pulp en water - oplosbare residue ( WIS) ) ten einde die chemiese struktuur van die oorblywende lignien wat verband hou met te bepaal polisakkariede en hoe hulle geraak industriële verwerking. Beide roumateriaal is onderwerp aan Kraft verpulping en suikerriet bagasse is verder verwerk vir ensiematiese hidrolise. Hemisellulose pre -onttrek ( ligte alkali of verdunde suur en autohydrolysis vir suikerriet bagasse ) pulp van Kraft of soda AQ van E. grandis en suikerriet bagasse is gebruik om die effek van Xylan pre- onttrekking te voor verstaan verpulping op lignien - koolhidraat komplekse het nie is na die beste kennis van die primêre skrywer berig . Ook voor EH die materiaal is onderworpe aan twee verskillende behandeling metodes, naamlik stoom ontploffing en ioniese vloeistof fraksionering in wisselende toestande. Die studie geïllustreer die tipes onttrek en gefractioneerd LCCs van hemisellulose pre -onttrek pulp en WIS in vergelyking met die nie -onttrek pulp en verslae van die literatuur. Lignien - koolhidraat komplekse (LCCs) is geïsoleer en gefraksioneer deur 'n anorganiese metode wat redelike kwantifisering hoeveelhede en geen besoedeling en lae opbrengste opgelewer vir die hardhout vergelyking met verslae van die gebruik van 'n ensiematiese metode. Na die beste kennis van die skrywers, het geen werk op WIS materiaal gedoen.

Die lignocelluloses is onderworpe aan die bal maal wat gevolg is deur 'n reeks van anorganiese oplosmiddels swelling en ontbinding in 2 breuke dws glucan - lignien en Xylan - lignien - glucan . Karakterisering van die geïsoleerde LCCs is gemaak met behulp van 'n verskeidenheid van analitiese gereedskap soos FTIR – PCA, HPLC, GPC en GC- MS. LCCs was duidelik wanneer FTIR en HPLC studies is uitgevoer . Residuele lignien geïsoleerd van die lignocelluloses is aanvaar moet word chemies gebind aan koolhidrate en meestal te Xylan. Ongeveer 60% en 30 % van die lignien is gekoppel aan Xylan terwyl dit vir die tweede en eerste LCC breuke onderskeidelik . Literatuur berig dat lignien wat verband hou met Xylan is meer bestand teen delignification as wanneer gekoppel aan glucane wat maklik hidroliseerbare .

Met die FTIR en GPC ontledings van LCC breuke, was dit duidelik dat die ester bande van LCCs is deur pre- ontginning en pre- behandeling, waar dit gelei tot meer sellulose

om meer toeganklik te alkaliese verpulping en ensiematiese hidrolise onderskeidelik vernietig . Die skakeling is óf gedeeltelik afgebreek of heeltemal vernietig lei tot beduidende veranderinge van chemiese strukture . Die polydispersity van die LCCs bygestaan in die bepaling van die struktuur van lignien , hetsy bestaande as monolignols op die oppervlak van die vesel of 'n as komplekse twee of drie - dimensionele struktuur wat gekoppel is aan koolhidrate as die Mw vermeerder of verminder . In die algemeen, kan hierdie bevindinge het 'n belangrike implikasie vir die algehele doeltreffendheid op bio - raffinadery.

Die molekulêre gewigte (MW) die onttrek LCCs gemeet deur gelpermeasie-chromatografie. Van die chromatograms, was dit opgemerk dat die materiaal wat blootgestel is aan die pre- verwerking voor verdere verwerking , die Mw verskuif MWS streke te verlaag. Daar is gevind dat LCCs geïsoleerd van ligte alkali pre -onttrek pulp het hoë lignien syringyl lignien inhoud as LCCs geïsoleerd van verdunde suur vooraf onttrek pulp te guaiacyl.

Hoë syringyl / guaiacyl verhouding (S / G -verhouding ) was 'n aanduiding van 'n lae lignien inhoud as 'n resultaat van verwerking wat sal lei tot 'n hoë produk opbrengste ná stroomaf verwerking. Die gemiddelde S / G -verhouding vir die pulp van E. grandis en suikerriet bagasse was wat wissel tussen 1,1-19,01 en 1,4-18,16 onderskeidelik, terwyl dit vir die WIS - materiaal gegeneer uit ioniese vloeistof gefraksioneer en stoom ontplof materiaal het gewissel 3,29-9,27 en 3.5 13,3 onderskeidelik . Die S / G verhoudings van die LCCs onttrek uit E. grandis en suikerriet bagasse pulp gewissel 0,42-2,39 en ,041-,31 was onderskeidelik terwyl dit vir die LCCs onttrek uit water - oplosbare - vastestowwe ( WIS ) materiaal gegeneer uit stoom ontplof materiaal was van 4,87-10,40 . Die bepaling van S / G -verhouding word aanbeveel vir die LCC ontginning en karakterisering studie as 'n evaluering van die oorblywende lignien in verwerkte materiaal soos pulp en WIS .

Die verkry saccharifications was laag , moontlik as gevolg van die erns van die stoom ontploffing pre- behandeling en ioniese vloeistof fraksionering voorwaardes wat gelei op 'n hoë opeenhoping van asynsuur en vermeerder in sellulose kristalliniteit onderskeidelik . Van kwantitatiewe ontleding van die LCCs perspektief kan dit afgelei word dat 'n vrye lignien teenwoordig is in ligte alkali pre -onttrek pulp as vir die verdunde suur vooraf onttrek pulp was.

## Acknowledgements

My appreciation and thanks for the accomplishment of this study are directed to Dr. Luvuyo Tyhoda and Prof. Johann Görgens for their years of patience and guidance of this thesis. Without them this would not have been possible.

I am forever grateful to PAMSA and Sappi Ltd. for funding this study.

I am much in debt to Dr. Phumla Vena for answering what seemed to be unanswerable questions and supplying desperately needed assistance and suggestions. I will forever miss you Miss Vena.

I also thank Dr. Michel Brienzo for keeping me on track and giving valuable advice.

Dr. Maria Garcia for her invaluable help.

My deepest gratitude goes to Sappi Personnel; Mr. Stephen Brent, Dr. Nelson Safari my mentor, Mrs Helga Easom and the entire Cape Kraft Mill staff for their unconditional support they showed towards me.

I am thankful to the following people, Wood Science staff; Mrs. Manda Rossouw for HPLC analysis, Mr. Lucky Mokoena for GC-MS analysis, Danie Diedericks for proving ionic liquid fractionated material, Paul McIntosh for supplying steam exploded sugarcane bagasse.

I thank the Forestry and Wood Science Department and Process Engineering for providing the facilities and laboratories and Polymer Science and Organic Chemistry Departments for allowing me to use their GPC and FTIR instruments.

This thesis is dedicated to all the people who never stop believing in me; Loyiso and Thabisa, my beloved siblings; my entire family and my zillion nieces and nephews, much love to my dear friends at Sappi Saaicor for pushing me so hard to finish, thank you!.

But most importantly God, who been my 'footprints in the sand' blessing me with the brains.

*This thesis is dedicated to my beloved Mom No-africa and my precious Boy, Aziwa-Kungawo who taught me that even the largest task can be accomplished even if it is done one step at a time*

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**Abbreviations**

<b>Abbreviation</b>	<b>Abbreviated word</b>
<b>LCC</b>	Lignin-carbohydrate complex
<b>SCB</b>	Sugarcane bagasse
<b><i>E. grandis</i></b>	<i>Eucalyptus grandis</i>
<b>HLPC</b>	High Performance Liquid Chromatography
<b>FTIR</b>	Fourier Transform Infrared
<b>SEC</b>	Size Exclusion Chromatography
<b>GC-MS</b>	Gas Chromatography with Mass Spectrometry

## **CHAPTER 1: BACKGROUND**

### **1.1 Introduction**

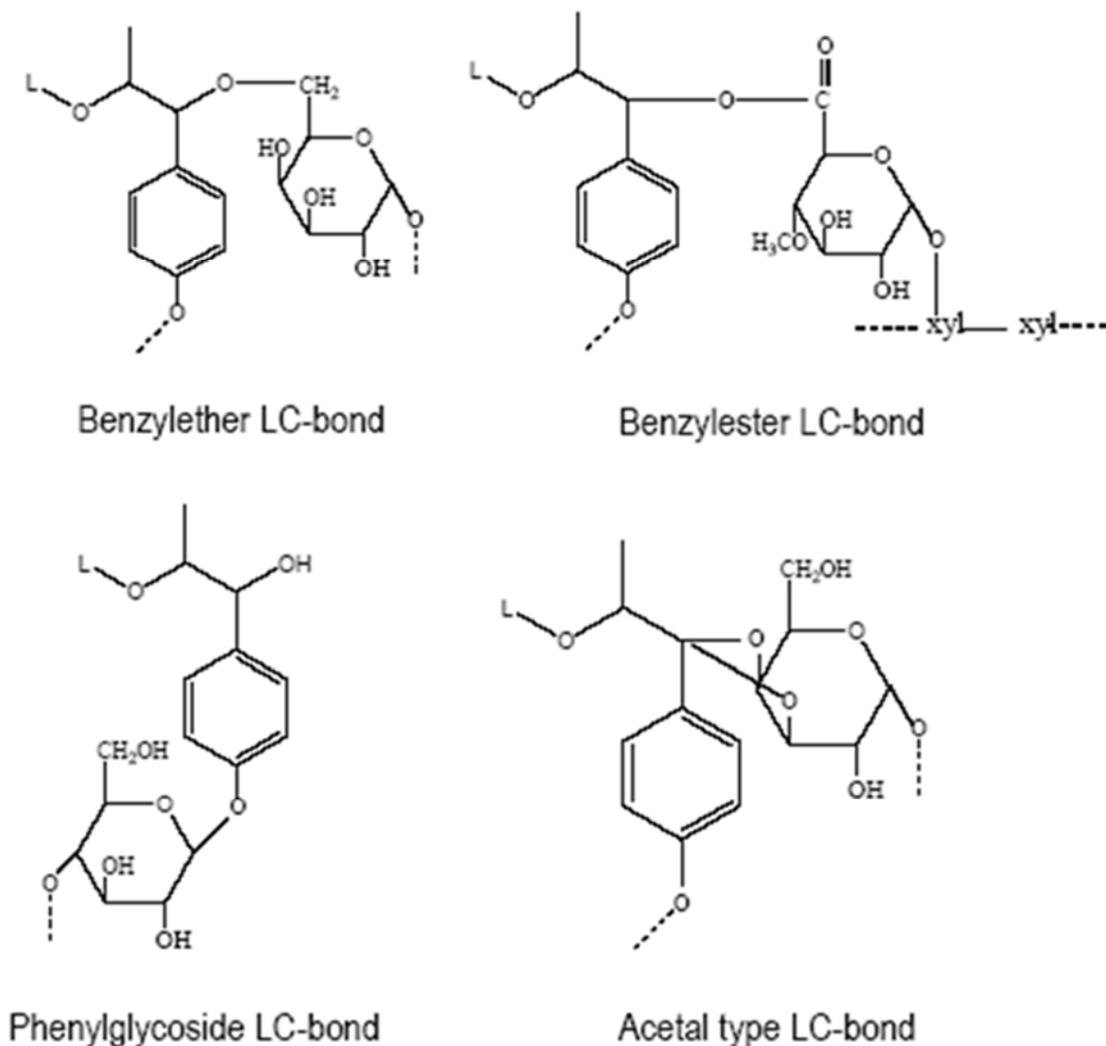
The existence of covalent bonds between lignin and carbohydrates is of considerable interest in connection with a number of issues in wood chemistry. These include the reactions taking place during the formation of wood, the natural molecular weight distribution of lignin and carbohydrates, swelling and accessibility properties and the reactivity of wood during its processing, e.g. chemical pulping. Such linkages may be responsible for the retardation of delignification in the final phases of chemical pulping (Lawoko et al. 2003; Lawoko, 2005 and Li et al. 2011). The stable nature of lignin-carbohydrate complexes is one of the main reasons preventing selective separation of the wood components during processes. Understanding the mechanism of various processes for the chemical utilisation of lignocellulosic requires good knowledge of the lignin and carbohydrate structures and their interaction in the starting raw material and their transformation during processing.

Lignin is an aromatic biopolymer with heterogeneous composed of phenyl-propane units of the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) types. These phenyl-propane units are linked together by various ether linkages (C-O-C) and carbon-carbon (C-C) bonds (Adler, 1977). Lignin in lignocellulosic materials is linked to polysaccharides, mainly through the hemicelluloses fraction of the biomass. Figure 1.1 below illustrates how the two lignocellulosic fractions are linked together. The different linkages are further described below i.e.:

(a) *Benzyl ether*: This type of bond is formed when an  $\alpha$ -hydroxyl group of the lignin is etherified with the primary hydroxyl group of a carbohydrate. This usually takes place through carbon 6 of the carbohydrate due to its high reactivity.

(b) *Benzyl ester*: this is formed by the  $\alpha$ -hydroxyl which esterified with the carboxyl group of the glucuronic acid residue.

(c) *glycoside linkage* – This is formed when a hydroxyl group of the phenol structure is glycosylated by the reducing end groups of the carbohydrate (Iiyama et al. 1994 and Watanabe 2002). The acetal type occurs when two hydroxyl groups of a carbohydrate are linked to lignin by an acetal (Xie et al. 2000). The benzyl ester and benzyl ether types are regarded as the most alkaline stable linkages, while the glycoside and acetal types are rare.



**Figure 1.1:** Proposed lignin-carbohydrate linkages (Lawoko, 2005).

Linkages between lignin and carbohydrates also create significant problems in the selective isolation of lignin preparation from lignocellulosic materials. A significant amount of work has been done to elucidate the structure of lignin carbohydrate complexes (LCCs) and how it influences biomass processing (Du et al., 2013). However, the results obtained still remain controversial for a number of reasons. These include the fact that the LCC yield from wood and pulps has been very low, making it impossible to quantify them. The reason for this is their inaccessibility both in the wood and pulp, making hydrolytic techniques necessary to access them (Lawoko, 2005). However, the use of such techniques results in the degradations some of the lignin carbohydrate linkages hydrolytic through bond cleavage, or alternatively, artificial linkages may also be formed. If such degradations are controlled by selectively protecting the lignin-carbohydrate linkages or selectively cleaving them in order to analyse the new functional groups formed upon their cleavage, then the data obtained can be valid. There is already

significant progress in this regard although the results are not free from criticism (Kim et al. 2003 and Choi et al. 2007).

## 1.2 Study aims

The main objective of the study was to determine the occurrence of LCCs in raw and processed biomass materials as well as to determine the changes that they go through as a result of processing. The specific objectives of the study are as follows:

- (i) isolate LCCs in order to determine the type and extent of occurrence in the hardwood (*E. grandis*) and herbaceous (sugarcane bagasse) raw materials
- (ii) isolate LCCs in order to determine the extent of occurrence in hemicelluloses pre-extracted, pre-treated and fractionated lignocellulosic materials and their pulps and enzymatically hydrolysed materials, respectively
- (iii) Identify pulping processes with reduced LCC components, thus perhaps facilitating easier bleaching.

The results of this study could be used as a model to study other biomass materials in order to predict the best processing routes as determined by the LCCs structure and occurrence.

## 1.3 Scientific approach

A method for complete isolation of lignin-carbohydrate complexes from softwood and its pulps has been well established and developed by (Lawoko et al. 2003 and Lawoko, 2005). The isolation process involves enzymatic degradation of the cellulosic component of the biomass through the use of  $\beta$ -endoglucanase. This is followed by treatment of the residue with a series of inorganic solvents including urea solution, boric acid and others with the aim of swelling the structure so that there will be easy penetration of chemicals that will facilitate the separation of cellulose from lignin and hemicelluloses, as cellulose is reported to interfere with the structure of LCCs. However, the application of this method to hardwood and its pulp resulted in high protein contamination that interfered with quantification (Capanema, 2004 and Lapierre et al. 1995).

New methods applicable to hardwoods (Li et al. 2011 and Lawoko et al. 2011) and hardwood pulps based. In both methods the material is firstly subjected to ball milling to destroy the crystalline structure of cellulose without affecting lignin structure. The ball milling stage is followed by the use of inorganic solvents such as urea, boric acid and others with the aim of

opening up and entangling the complex structure of the lignocellulosic material preserving the bonding structure between lignin and the carbohydrates. The isolated fractions are analysed through wet chemical methods to quantify the lignin and carbohydrate contents. Further, the fractions are degraded using a thioacidolysis as described by Lapierre et al. 1995 and the degradation products are analysed using size exclusion and gas chromatography respectively. These provide the information needed on the effect of hemicelluloses processing techniques such as alkaline hemicelluloses pre-extraction from biomass prior to pulping or biomass pre-treatment prior hydrolysis and enzymatic fermentation to produce bioethanol on the structure of LCCs.

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## **CHAPTER 2: LITERATURE REVIEW**

### **2.1 Introduction**

Many theories have been proposed to explain the existence of lignin-carbohydrate complexes (LCCs) in lignocellulosic material. Although the literature covers a wide variety of studies, this review will focus on the effect of biomass pre-treatment for bio-ethanol production, that is, physico-chemically (i.e. steam explosion) processed material, hemicelluloses pre-extraction (value prior to pulping (VPP)) using the dilute acid and mild alkali method and the fractionation of sugarcane bagasse using ionic liquids and determine the structural changes on LCCs as a result of such biomass processing procedures. Although the literature presents these studies in a variety of contexts, this study focuses on their application to LCC isolation and characterisation. The study pays particular attention to hemicelluloses pre-extraction on biomass prior to pulping for paper production. VPP is the concept that is used where the hemicelluloses are pre-extracted for biofuel or biopolymer production Liu et al. (2011). The extracted hemicellulose biopolymers have high potential of application in various industries e.g. pharmaceutical, pulp and paper, etc. (Parajo et al. 1998).

Current studies on hemicelluloses pre-extraction have shown that lignin remains in the extracted xylan (Postma et al. 2012). It is hoped that the LCC structure and degree of occurrence could explain how lignin is associated with the hemicelluloses.

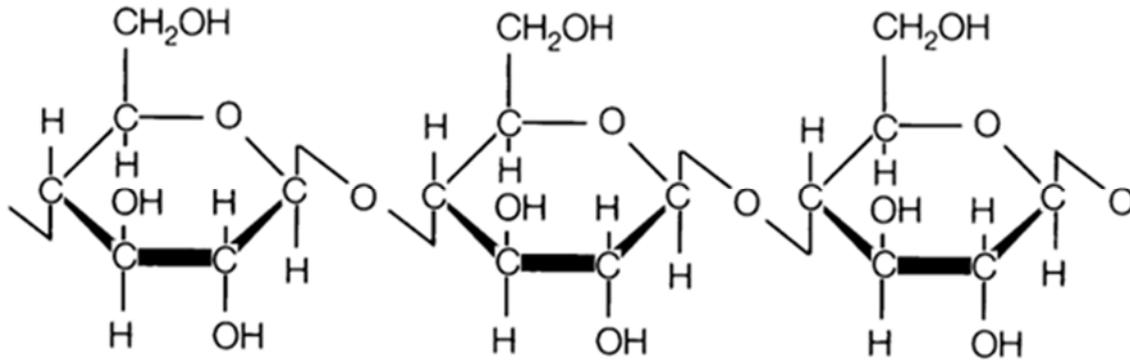
### **2.2 Cell wall components of lignocellulosic material**

Lignocellulose is the most abundant renewable carbon source on Earth. It is a heterogeneous biological material consisting of different types of cells. The cell walls are composed mainly of cellulose, hemicelluloses, lignin and small amounts of extractives, proteins and inorganic components, all in different proportions which vary from species to species.

#### **2.2.1 The structure and composition of lignocellulosic materials**

##### **2.2.1.1 Cellulose**

Cellulose is a polymer of glucose. Cellulose consists of anhydro-glucopyranose units which are linked to form a molecular chain as Figure 2.1 shows.



**Figure 2.1:** Schematic cellulose chain structure (re-drawn from Fengel and Wegener, 2003).

Therefore cellulose is described as a linear polymer with a uniform chain structure. The repeating unit of the cellulose chain is referred to as cellobiose with a length of 1.03nm. The units are bound together by  $\beta$ -(1→4)-glycosidic linkages. These are linked through the elimination of a water molecule between their hydroxyl groups of two glucose molecules at C<sub>1</sub> and C-4 (Freudenberg, 1968) of the respective glucose units.

The fibrils represent the association of cellulose and contain ordered and less ordered regions. The smallest units are called microfibrils where they have a diameter of 10 to 25nm (Vogel, 1953); the even smaller units are called elementary fibrils (Muhlethaler, 1965). It makes approximately 40-50% of dry wood biomass with a degree of polymerisation of 5000-10 000.

### 2.2.1.2 Hemicelluloses

Hemicelluloses or polyoses represent branched polysaccharides in wood having shorter chains and are formed from limited number of different five- and six-carbon saccharides namely: xylose, mannose, glucose and others (Staudinger and Reinecke, 1939) and have a lower molecular weight than cellulose. They make up to 15-25% of the total dry mass of wood. They can be hydrolysed with acid and heat, and they can also be extracted with dilute alkali. They are found between cellulose and lignin in the cell wall. The saccharide units are linked through alpha- or beta-(1→4)-linkage. They are classified in terms of the main sugar components in the main chain or backbone. Examples include xylan, mannan, galactan and arabinan. Hemicelluloses are highly branched while cellulose is a straight unbranched polymer consisting of only  $\beta$ -glucose units. Hardwoods contain more polyoses than softwoods and the

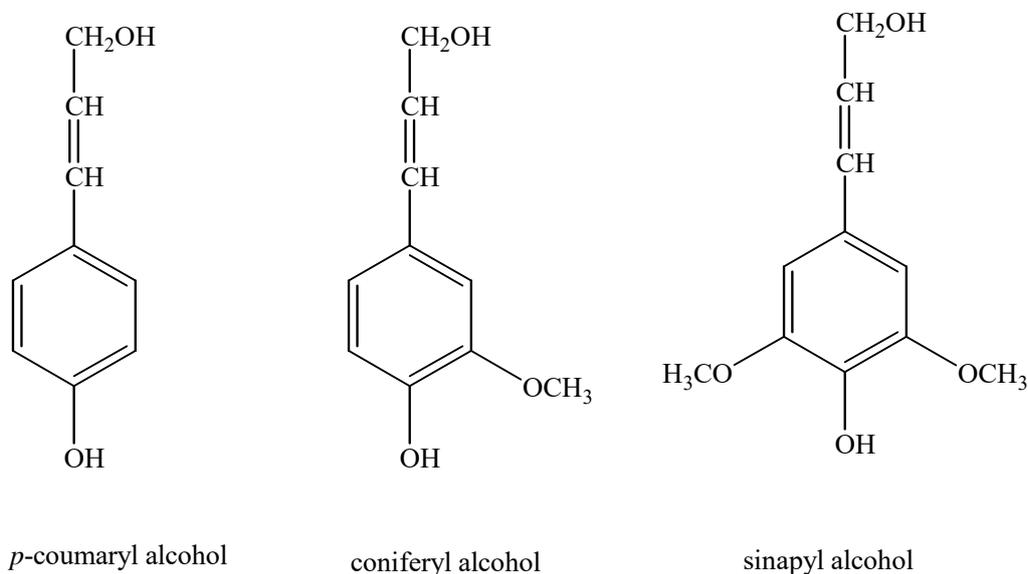
sugar composition is different (Schuerch, 1963). Hemicelluloses are also acetylated. The degree of branching in hemicelluloses depends on the species and type of wood.

Glucuronoxylan is the main hemicelluloses in hardwood species and its content varies between 15-30%. The backbone consists of (1→4)-linked  $\beta$ -D-xylopyranose units, with about seven out of ten of the xylose residues containing O-acetyl groups at the C-2 and C-3 positions. One of ten xylose units carries a (1→2)-linked 4-O-methyl-  $\alpha$ -D-glucuronic acid group (Sjöström 1993).

*Glucomannan*: Around 2-5% of the dry wood is glucomannan material. These are composed of  $\beta$ -D-glucopyranose and  $\beta$ -D-mannopyranose units linked by (1→4)-bonds. The glucose:mannose ratio varies between 1:2 and 1:1 depending on the wood species.

### 2.2.1.3 Lignin

Lignin is a polymeric product that is formed by an enzyme initiated dehydrogenative polymerisation of the three primary precursors which are coniferyl-, sinapyl- and coumaryl-alcohols (Figure 2.2) (Adler, 1977). Polymerisation of lignin results in a highly branched three-dimensional cross linked polymer of unknown molecular mass. It makes about 27-30% of the dry wood.

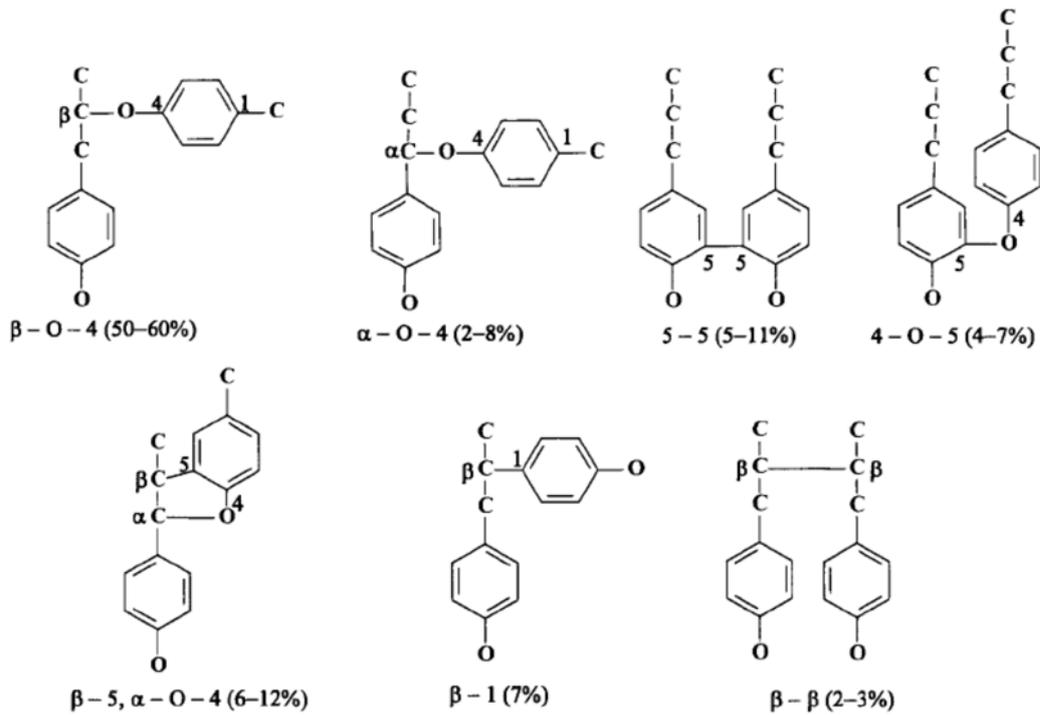


**Figure 2.2:** Schematic representation of lignin precursors (Re-drawn from Fengel and Wegener, 2003).

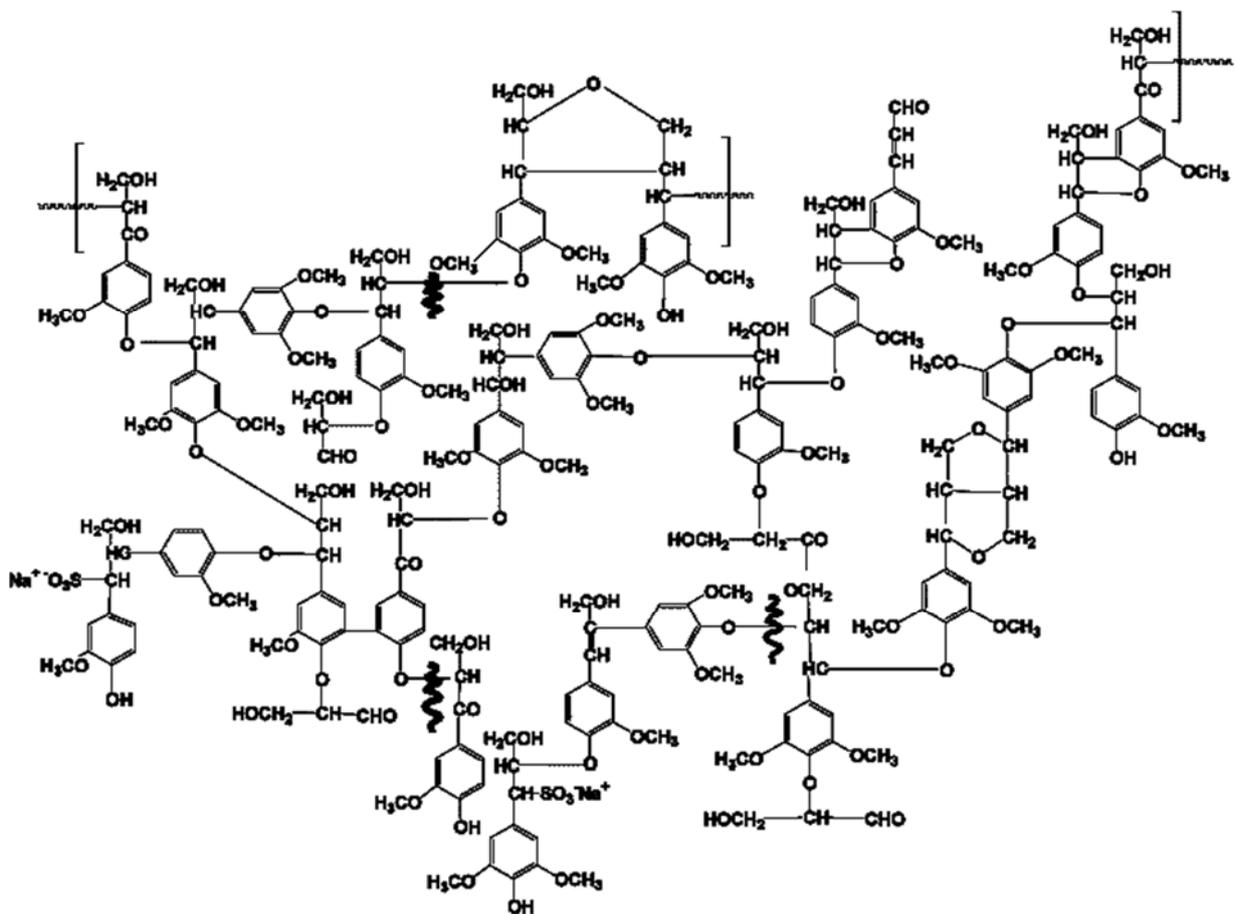
There are various types of bonds that join the building units together which are  $\beta$ -O-4,  $\beta$ - $\beta$ , 5-5,  $\beta$ -5, 4-O-5 (see Table 2.1 and Figures 2.3 and 2.4). The lignin polymer works as a binder within the cell wall and is found in various parts of the cell wall.

**Table 2.1:** Inter-unit linkages of lignin in lignocelluloses (Fengel and Wegener, 2003)

Linkage type	Dimer structure	Percent of total linkages	
		Softwood	Hardwood
$\beta$ -O-4	Arylglycerol – $\beta$ -aryl ether	50	60
$\alpha$ -O-4	Noncyclic benzyl aryl ether	2-8	7
$\beta$ -5	Phenylcoumaran	9-12	6
5-5	Biphenyl	10-11	5
4-O-5	Diaryl ether	4	7
$\beta$ -1	1,2-Diaryl propane	7	7
$\beta$ – $\beta$	Pinoresinol/lignin type	2	3



**Figure 2.3:** Schematic representation of linkages between monolignols (Fengel and Wegener, 2003).

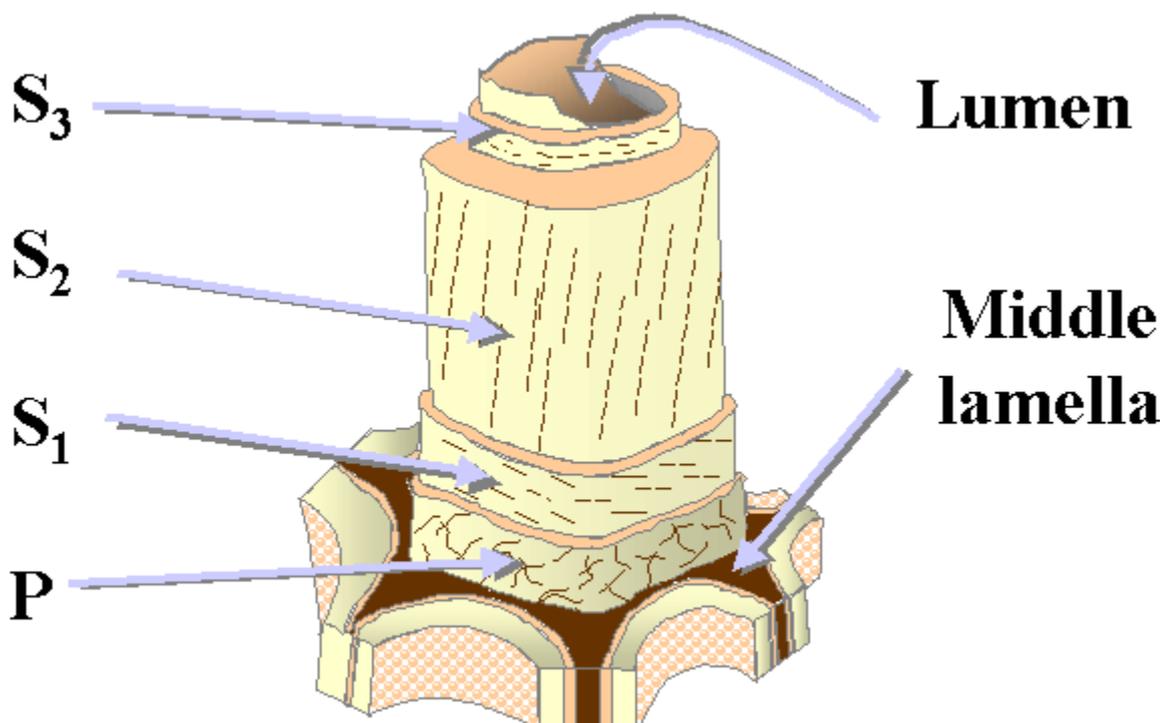


**Figure 2.4:** A hypothesized hardwood structure (Re-drawn from Fengel and Wagener, 2003).

Lignin has few functions in plants where it provides rigidity to the plant cell walls confers water impermeability and is also a physico-chemical barrier against microbial attacks (Fengel and Wagener, 2003). Lignin is structurally linked to polysaccharides in wood and other biomass materials. As a result of this, any process aimed at extracting polysaccharides from wood always requires the removal of lignin first, which always affects the yield of polysaccharides in the end, e.g. in pulping for isolation of cellulose fibres.

#### 2.2.1.4 Cell wall organisation

The cell wall has different layers, mainly primary- and secondary walls in which wood components are distributed with different proportions of chemical composition in different layers (Figure 2.5).



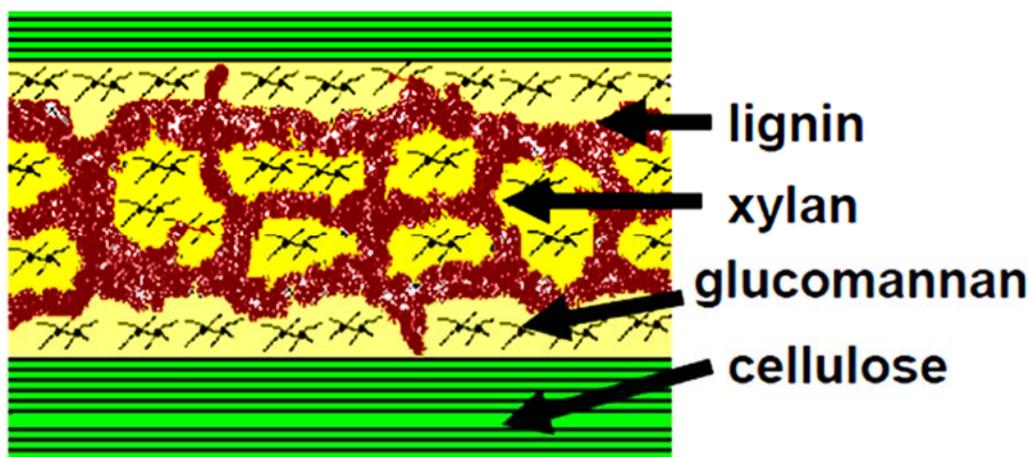
**Figure 2.5:** A diagrammatic sketch of a multi-layered wood cell wall showing the middle lamella (ML) the primary wall (P), the three layers of the secondary wall i.e. S1, S2 and S3 (Fengel and Wegener, 2003).

The growth of the cell wall results in the formation of a multi-layered secondary wall. Dimensionally, the primary cell wall is very thin (0.1 – 0.2 $\mu\text{m}$ ) and consists of cellulose, hemicelluloses, lignin and pectins. Some of the hemicelluloses found in this region are distinct in structure from the hemicelluloses found in the secondary wall such as xyloglucan (Adler, 1977).

The outer layer of the secondary layer (S1) is about 0.1 to 0.3 $\mu\text{m}$  thick with a micro-fibril angle between 50 – 90°. The S1 layer closely resembles the primary wall to which it is closely attached (Adler, 1977). Thus it is also known as the transition layer. The central secondary wall (S2) is less firmly attached to the S1 layer. A continuous envelope of hemicelluloses between the layers causes less cohesion between the layers. The S2 layer makes the bulk of the cell wall and it is about 2 – 8 $\mu\text{m}$  thick.

Individual fibres in the structure are held together by the middle lamella (ML) which is mainly composed of lignin and it is about 1 – 2 $\mu\text{m}$  thick. The removal of this cementing layer between the individual fibres is the key to pre-treatment, pre-extraction and chemical processing, as individual cell are separated by removing the layer to produce single fibres (Fengel and Wegener, 2003).

Several studies that have been done based on the softening behaviour of glucomannan and xylan suggests that xylan is more linked to lignin, while glucomannan is more associated with cellulose in the secondary cell wall (Figure 2.6) (Salmen and Olsson 1998). The findings were further supported by Åkerholm and Salmen in 2001 using FT-IR spectroscopy.



**Figure 2.6:** Ultra-structural arrangement of wood polymers in the secondary cell wall (Salmen and Olsson 1998).

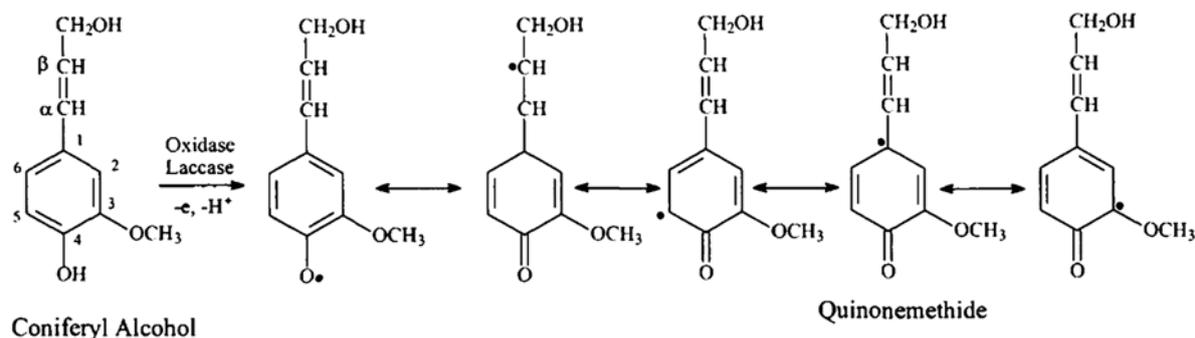
## 2.3 Lignin–carbohydrate complexes: Structure and properties

Ester linkages (CO-O-C) occur between free carboxyl groups of uronic acid in hemicelluloses. Others are found between uronic acid and lignin, while others are found between hemicelluloses. Monomeric side chains in wood xylan consist of 4-*O*-methylglucuronic acid units. The glucuronic acids present in LCCs are involved in an ester linkage between lignin and glucuronoxylan (Takahishi and Koshijima, 1988b). However, many glucuronic acid units may be esterified within the xylan polymer (Wang et al. 1967).

Direct evidence for the chemical nature of ester linkages between lignin and carbohydrates have been obtained through the selective oxidation of carbonyl groups in lignin. It has been proposed that the 4-*O*-methylglucuronic acid residue in arabino-glucuronoxylan binds to lignin through an ester linkage (Watanabe and Koshijima, 1988). The linkage position is probably  $\alpha$ - or conjugated  $\gamma$ - position of guaiacyl-alkane units. Glucose is *O*-6 ether linked and xylose *O*-2 or *O*-3 ether linked to the benzyl hydroxyl in a neutral fraction of LCC.

### 2.3.1 Biosynthesis of ester and ether lignin-carbohydrate complexes

Koshijima and Watanabe in 2003 described the biosynthesis of ester and ether lignin-carbohydrate complex linkages that has been proposed for many decades. The biosynthesis mechanism of these linkages is related to the biosynthesis of lignin. Coniferyl, sinapyl and *p*-coumaryl alcohols are three main monomers for lignin. The relative abundance for the monomers depends on the lignocellulosic material. The biosynthesis of lignin is initiated by plant peroxidases and phenol oxidases by dehydrogenation and polymerization. Peroxidases or laccases in the wood tissue initiate the dehydrogenation step of the lignin biosynthesis, which generates the phenoxy radical and several resonance structures. The biosynthesis starts from coniferyl alcohol, which is initiated by dehydrogenation of the phenolic hydrogen by peroxidase or laccase. Figure 2.7 below shows the schematic mechanism of biosynthesis starting from coniferyl alcohol.



**Figure 2.7:** Schematic mechanism reaction of biosynthesis (redrawn from Fengel and Wegner, 2003).

### 2.3.2 Isolation of lignin-carbohydrate complexes

The question of whether lignin is chemically bound to polysaccharides in the plant cell or whether it is present in free-state has been one of the most frequently debated issues in the history of wood chemistry. Earlier results on the existence of LCC in wood were reviewed by Merewether (1957).

In 1866 Erdmann hypothesized that covalent bonds occurred between lignin and carbohydrates in wood; basing his hypothesis on the observation that it was difficult to separate the two components. He called this material “glycolignose”. Many decades later, several works in support of Erdmann’s hypothesis emerged. Traynard et al (1953) observed that when poplar was hydrolysed with water buffered at four different pH levels (range 2.2 to 4.2), the ratio of the lignin dissolved to the pentosans dissolved was constant. The interpretation of this was that there existed covalent linkages between pentosans and lignin. . Earlier on, Sarkar and co-workers (1952) had observed that the treatment of jute with weak alkali doubled its acid value. The additional free acid was interpreted to be a result of the cleavage of ester linkages between lignin and polyuronides. Tachi and Yamamori (1951) made the observation that the carboxyl content of holocellulose was higher than that of the original wood, and concluded that the cleavage of an ester linkage between lignin and polyuronides had occurred.

The concept of a lignin-polyuronide linkage was thus further substantiated. From finely divided wood, Brauns (1952) was able to extract 2 – 3% of the total wood lignin with cold ethanol. The soluble fraction was named native lignin, also commonly referred to as Brauns native lignin. This fraction was free from carbohydrates. The low yield of soluble lignin was interpreted to

mean that the remaining lignin in wood was highly polymerised or that at least a part of it was bound to carbohydrate that restricted its extraction.

It was reported that the degree of milling did not lead to an increase in the yield of Braun's native lignin. In support of Brauns, Björkman (1956) observed no increase in the alcohol solubility when wood was ground in a vibratory mill for 48 hours. Björkman developed a method for isolating lignin from wood after ball milling. The lignin preparation, globally referred to as milled wood lignin (MWL), was obtained by extracting the ball milled wood with dioxane: water mixture. Furthermore, Björkman found an "inseparable mixture" of lignin and carbohydrates and introduced the term "lignin-carbohydrate complexes" (LCC), a term which has claimed global recognition. From his works on spruce wood, Björkman concluded that ~25% of the lignin was extracted as MWL, ~20% as lignin carbohydrate complexes (LCC) with other organic solvents (such as dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), and acetic acid: water mixtures), ~9% as "intermediate" fractions on purification of the crude LCC, and ~42% remained in the residue. The LCC obtained in the Björkman preparation have been referred to as Björkman LCCs.

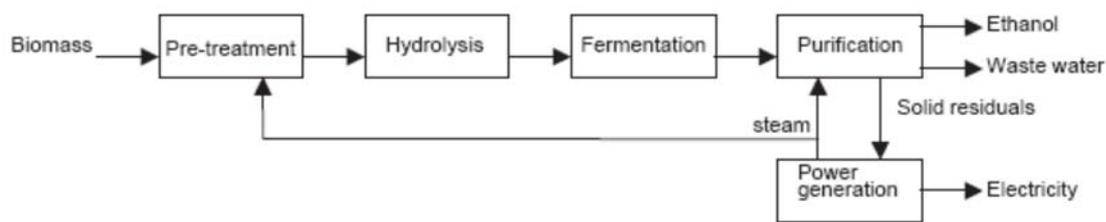
Four types of native lignin carbohydrate bonds (LC-bonds) have been proposed in the literature (Figure 1.1) viz., benzylethers (Freudenberg and Grion, 1959; Eriksson and Lindgren 1977; Kosikova et al 1979; Yaku et al. 1981; Koshijima et al. 1984; Watanabe 1989), benzylesters (Freudenberg and Harkin 1960; Yaku et al. 1976; Eriksson et al. 1980; Obst et al 1982; Lundquist et al. 1983; Watanabe and Koshijima 1988), phenylglycosides (Smeltorious 1974; Kosikova et al. 1972; Yaku et al. 1976; Joseleau and Kesraoui 1986; Kondo et al. 1990) and acetal bonds (Xie et al 2000).

#### **2.4 An overview of key lignocelluloses conversion technologies where LCCs play a role**

Lignocellulosic biomass can be utilized to produce ethanol; a promising alternative energy source for the limited crude oil as well as biopolymers and chemicals. However, the structural linkage between cellulose, hemicelluloses and lignin makes efficient conversion of biomass to either ethanol or value added chemicals difficult. Therefore, efficient pre-treatment to remove or modify the lignin/hemicelluloses protective sheath around cellulose or efficient fractionation of the biomass into individual components in high purity and yield is necessary before efficient conversion of each of the individual components can occur. Pre-treatment is especially necessary to reduce the crystalline structure of cellulose which makes it highly insoluble and resistant to enzymatic conversion. To achieve this, advanced pre-treatment

technologies for biomass are required than in the sugar or starch crops processing (Hamelinck et al. 2005).

There are mainly four processes involved in the conversion: pre-treatment, hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars, fermentation of the sugars to ethanol and purification (Figure 2.8) (Sun et al. 2002). The key process steps will be discussed hereafter, following the here presented order.



**Figure 2.8:** Generalised biomass to ethanol process redrawn from Hamelinck et al. (2005).

Biomass fractionation (mentioned above) can be viewed as an alternative route by which the individual biomass components can be efficiently separated and converted to products. With regards to bio-ethanol, the majority of feedstocks are agricultural residues, e.g. sugarcane bagasse, sorghum, straw, corn stovers and other types of materials. The main components of interest in these residues are cellulose and hemicelluloses regarding bioethanol. However, cellulose is also used in pulp and paper production. In this conversion process, hemicelluloses are underutilised and are lost in the black liquor together with lignin which is then used to generate heat energy in pulp mills. Hemicelluloses can be pre-extracted from biomass prior to pulping using a fractionation method widely known as the Value Prior Pulping (VPP) which can also be viewed as a pre-treatment for the biomass prior to pulping. This process can be viewed as a new or improved biomass processing route by which value can be obtained from these polysaccharides e.g. by enzymatically converting them to ethanol or conversion to high value biopolymers and chemicals (Vena et al. 2013).

Other fractionation methods include the use of ionic liquids, which are mainly organic salts with large organic cations and inorganic anions which can augment the (decrystallise) the lignocellulose structure in three ways, i.e. selective lignin dissolution, selective hemicellulose) dissolution or complete dissolution of the biomass.

This study is therefore aimed at examining all the biomass processed mentioned above with a specific aim of elucidating the effect of the presence of LCCs and its structures on the fractionation of the wood components with ionic liquids. A more detailed discussion of each of the biomass processing techniques is given in the subsections below prior to the discussion on the isolation and characterisation of the LCCs.

## **2.4.1 Biomass pre-treatment**

Pre-treatment is an important stage in biomass processing prior to enzymatic hydrolysis. The stage is essential since it makes the lignocelluloses structure to be more porous and accessible to further processing of the material for enzymatic hydrolysis. This stage is regarded as one of the most expensive stages since it requires the use of additional chemicals and energy. Adaptation of pre-treatment has resulted on the improvement of yield quantities and qualities, the structure being more accessible to biological catalysts and chemicals since it is more porous.

### **2.4.1.1 Physical pre-treatment**

Physical pre-treatment which is also referred to as mechanical pre-treatment includes: (a) milling, which is the initial stage of processing. Milling reduces the biomass material into a particle sizes that are easily amenable to processing or conversion.

### **2.4.1.2 Physico-chemical pre-treatment**

This process combines both physical pre-treatment and chemical pre-treatment. Examples include:

(a) Steam explosion; a process whereby chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials undergo an explosive decompression. By subjecting wood or other biomass to high temperature steam treatment, followed by pressure release, the fibrous mass is exploded and liberated together with fibre bundles are formed. By adjusting the time and temperature, different degrees of wood polymer modification and degradation can be achieved. The method has gained much attention as a possible means for a simple and cheap separation of wood polymers (e.g. for the production of micro-crystalline cellulose and bio-based ethanol) (DeLong, 1981). In particular, hardwood species are suitable raw materials since lignin portions can be extracted

to a large extent by either aqueous alkali or by organic solvents leaving residue highly enriched in cellulose.

In a pure steam explosion process without any added chemicals, the reaction conditions are weakly acidic due to the release of acetic acid from hemicelluloses. Thus, the major reaction types are similar to those present in acidic sulphite pulping, i.e. hydrolysis of polysaccharides and hydrolysis and condensation of lignin (Mörck et al. 1986). In addition, due to the high temperature usually employed ( $\sim 200^\circ \text{C}$ ); homolytic cleavage reactions of for example,  $\beta$ -O-4 linkages in lignin can be assumed to take place. Altogether, these reactions result in a highly heterogeneous lignin structure containing both degraded lignin fragments and recombined fragments through condensation reaction (Josefsson, 2001). Consequently, the number of  $\beta$ -O-4 structures is much lower, compared with the product of the starting material and the content of phenolic end-groups higher. In addition; the number of carbonyl groups is considerably increased due to hydrolytic or homolytic cleavage reactions (Robert et al. 1984). The LCC isolation and characterisation is therefore aimed at quantifying the changes on the lignin and lignin-carbohydrate structures due to this process.

(b) Ammonia fiber explosion (AFEX) whereby lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is swiftly reduced. (Similar to steam explosion)

(c)  $\text{CO}_2$  explosion is similar to steam and ammonia fibre explosion, high pressure  $\text{CO}_2$  is injected into the batch reactor and liberated by an explosive decompression. It is believed that  $\text{CO}_2$  reacts to the carbonic acid, thereby improving the hydrolysis rate.

#### **2.4.2 Biological processing of biomass**

Biological conversion of pre-treated lignocellulose into fermentable sugars can be achieved by enzymatic hydrolysis, followed by fermentation by yeast or bacteria to alcohols. Since the early 1970s, extensive studies on the hydrolysis of lignocellulosic materials using enzymes have been done and the objective behind was to develop cost effective methods for the production of ethanol. According to the survey done by Duff and Marray (1996), biological hydrolysis holds more advantages over chemical hydrolysis e.g. acid hydrolysis due to less of inhibitory bi-products which are generated during enzymatic hydrolysis (Qin, 2010). It has been concluded that this is a method of choice for the future of lignocelluloses-to-ethanol

process because of its mild processing conditions and there are no corrosion problems that are reported compared to acid hydrolysis.

The hydrolytic enzymes degrade cellulose and hemicelluloses into their basic sugar components. There are various types of enzymes that are commercially available which include cellulases and hemicellulases that act on cellulose and hemicelluloses respectively. The conversion of lignocellulosic material rates by specific enzyme are dependent on the type of enzyme cocktails used and the substrate source. The lignin content and its distribution in the material has a major influence in enzymatic hydrolysis in two ways (a) lignin prevents enzymes from effective binding to cellulose (Ucar and Fengel, 1988) and (b) lignin irreversibly adsorbs the cellulases, thus preventing their reaction with the substrate. The removal of lignin by fractionation or pre-treatment makes cellulose to be more accessible and swollen for cellulases interaction. Further, the protective sheath that the lignin and hemicelluloses form around the cellulose fibrils, and the types of bonds (LCCs) that link the biomass components together, make the components of the native fibre less susceptible to enzyme action. The question to answer for this project is whether LCCs have a key role in determining the rate and extent of lignocellulose hydrolysis by enzymes, subsequent to pre-treatment.

### 2.4.3 Chemical pre-treatment

Chemical pre-treatment is defined as a process by which chemical substances are used. These include the following:

- a) **Auto-hydrolysis:** In this process, hot water is used to pre-treat the material at an elevated temperature (maximum 300° C) and pressure value in a short time frame (max. 1 hour). Most of the woody components are dissolved during processing, ~20% cellulose and ~60% lignin are removed and all the remaining components can be recovered. Most polyoses are recovered as monomeric sugars because the high acetic acid content accumulated during processing hydrolyses the sugars resulting, which could also result in formation of fermentation inhibitors like furfural.
- b) **Acid pre-treatment:** This process is designated according to the strength of the acid viz, dilute acid; the most widely used method because it is an effective biomass hydrolysis method. This pre-treatment can be performed either using a high temperature and continuous flow process for low-solid loadings ( $T \sim 150^{\circ}\text{C}$ , 5 – 10wt% biomass) or low temperature and batch process for high-solid loadings ( $T \leq 150^{\circ}\text{C}$ , 10 – 40wt% biomass). The hemicelluloses are hydrolysed into monomeric sugars and

soluble oligomers. The removal of hemicelluloses results on the structure being more porous. The method has major drawbacks that include the generation of high quantities of microbial fermentation inhibitors e.g. furfural and hydroxymethyl-furfural (HMF) and high lignin condensation. Further, the acids are very corrosive to the equipment.

- c) **Alkaline pre-treatment:** This pre-treatment process includes the use of strong bases like sodium hydroxide (NaOH) and ammonia at elevated temperatures. Improved hemicelluloses removal has been reported in many studies (Sun et al. 2000). The removal of these components makes the substrate be more accessible to biological catalysts or chemicals. Harsem et al. (2010) reported that alkaline hydrolysis mechanism is based on saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and other components such as lignin.
- d) **Organosolv**, it is a process that uses organic solvents or mixtures with water for removal of lignin before enzymatic hydrolysis of the cellulose reactions. In addition to lignin removal, hemicelluloses hydrolysis occurs leading to improved enzymatic digestibility of cellulose fractions. Common solvents used for this process include ethanol, acetone, methanol and others
- e) **Oxidative delignification**, delignification of lignocellulose can also be achieved by treatment with an oxidising agent such as hydrogen peroxide, ozone and air or oxygen. The effectiveness in delignification can be attributed to the high reactivity of oxidising chemicals with an aromatic ring. Thus, the high lignin polymer is converted into carboxylic acid. The formed acids as inhibitors in the fermentation process hence they need to be neutralised or removed.
- f) **Ionic liquids**, they are salts that are in a liquid state at room temperature. There are different types of ionic liquids, however, they share a common characteristic, which is they are comprised of an inorganic anion and organic cation of a very heterogeneous molecular structure. The difference in the molecular structure renders the bonding of the ions weak enough for the salt to be liquid at room temperature (van Rantwijk, 2003).

#### 2.4.4 Chemical pulping

Wood is converted into pulp in one of two ways i.e. mechanical defibrillation whereby the wood is disintegrated into fibres by grinding or refining. The resulting pulp is obtained in high yield.

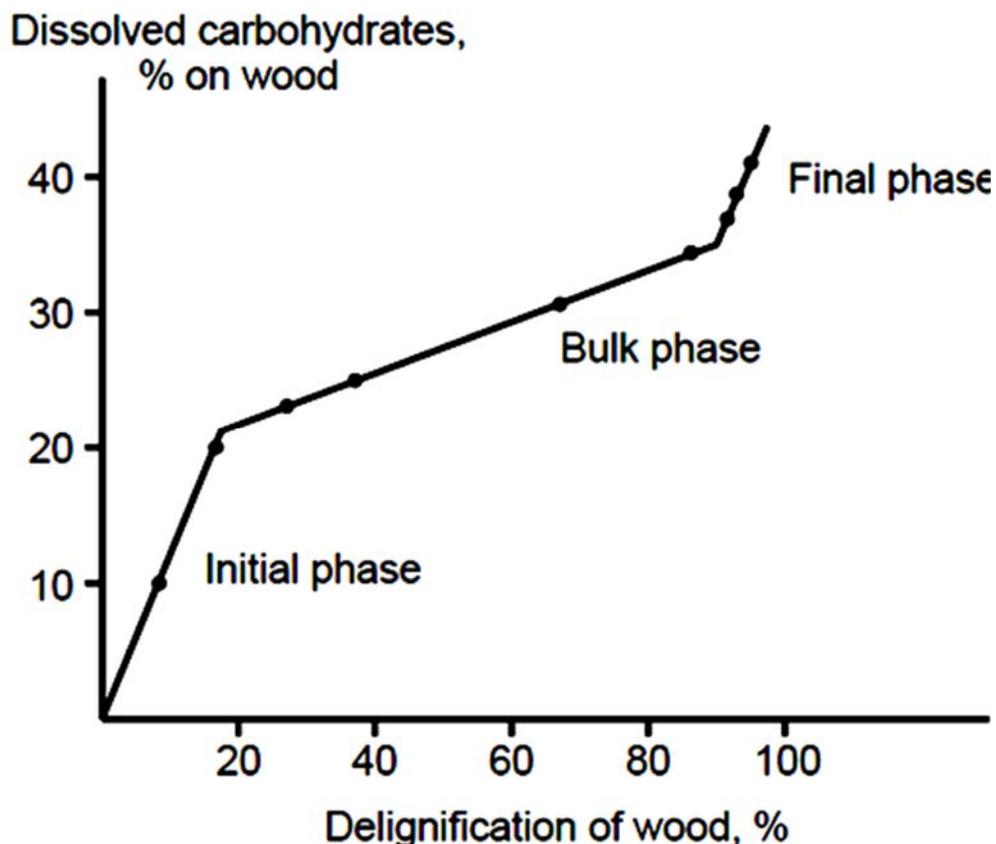
The process is however demanding in terms of the mechanical energy needed to separate the fibres. Alternatively, pulp is produced by chemical processing of wood, whereby the lignin is degraded and dissolved to release the fibres. For this purpose, two methods are commonly used namely; Kraft or sulphate pulping, and sulphite pulping.

#### **2.4.4.1 Kraft pulping general aspects**

The Kraft pulping process is also referred to as sodium sulphide process and was first invented by Dahl in 1879 (Grace and Malcolm 1989). Since its initial discovery it has been developed tremendously and has become the leading pulping process in the pulping industry in the world to produce unbleached pulp. Advantages of this method are as follows: (a) better pulp physical properties (b) shorter cooking time (c) insensitivity to wood species and (d) efficient energy and chemical recovery capabilities.

The wood chips are pulped at 150 - 170°C using an alkaline solution with hydroxide- and hydrosulphide ions as the active delignifying agents.

The selectivity of delignification is however observed to change during the Kraft pulping (Gellerstedt and Lindfors 1984a, Lindgren and Lindström 1996). Delignification is thus divided into 3 phases namely; initial, bulk and residual delignification (Figure 2.9). The yield losses during the Kraft pulping have been found to be substantial, especially in the case of the hemicelluloses due to degradation. The chemical reactions occurring during the different phases have been studied. The main lignin reactions occur during the first two phases. The residual delignification phase is unique in that very little lignin is dissolved and the carbohydrate losses become substantial. Therefore, the Kraft cook has to be terminated at this stage. The degree of delignification at termination stage is often around 90%, but may depend on the pulping conditions. The remaining lignin is removed in the more selective oxygen delignification process (i.e. bleaching). However, even in this case a slow delignification rate is observed in the residual phase, which occurs when ~50% delignification has been achieved (Olm and Teder 1979).



**Figure 2.9:** The selectivity of delignification in the three phases of Kraft pulping (Lawoko 2003).

#### 2.4.4.2 Chemistry of Kraft pulping

A mixture of sodium sulphide ( $\text{Na}_2\text{S}$ ) and sodium hydroxide ( $\text{NaOH}$ ) are used in this pulping process for wood to liberate the individual cellular elements. Fibre separation is achieved by dissolving the lignin and hemicelluloses that hold the fibres together in the middle lamella or the region between adjacent cells. The chemicals in the cooking liquor penetrate the fibre walls and dissolve the lignin that is found in between. The cooking chemicals not only react with lignin but also react with the carbohydrate polymers that exist in the cell wall. The latter reactions are not desirable because they degrade the carbohydrates to small components that are soluble which results in low pulp yields and reduced fibre quality.

The  $\beta$ -O-4 structures in lignin are hydrolysed (~95%) and the resulting lignin fragments dissolve in the alkaline solution. Several other degradative lignin reactions also take place under the harsh conditions prevailing in the digester and most of the phenyl-propane side-chains are in part eliminated, in part modified. The process results in the dissolution of around 90 – 95% of all lignin present in the starting material (Gellerstedt and Zhang, 2001). By

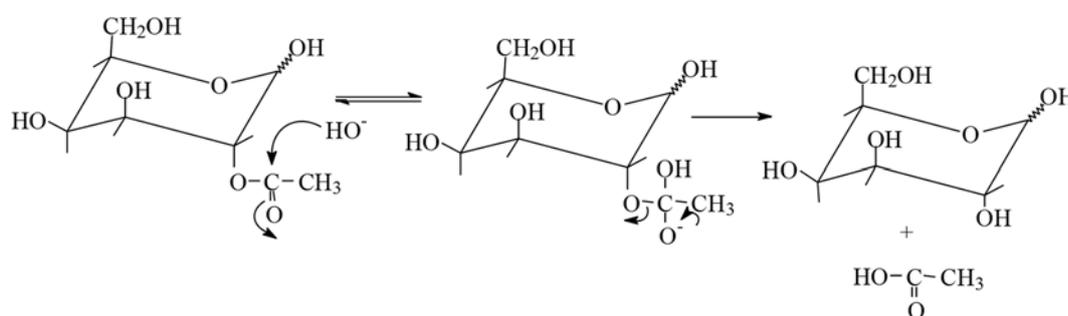
acidification of the pulping liquor, the dissolved lignin can be recovered to a great extent as a complex mixture of phenolic structures with molecular mass in the range of ~150 – 200 000.

The manner in which the Kraft pulping process is executed has a significant effect on pulp bleaching and oxygen delignification. The amount of reduced lignin remaining in the pulp depends upon the conditions used in the pulping process. Also, the chemical demand required to bleach the pulp to a given brightness value can be different even when the lignin content in the pulp is the same. In addition, variations in the hemicelluloses content and structure resulting from different pulping conditions would be expected to have a significant effect on oxygen delignification and bleaching.

#### 2.4.4.2.1 Carbohydrate reactions

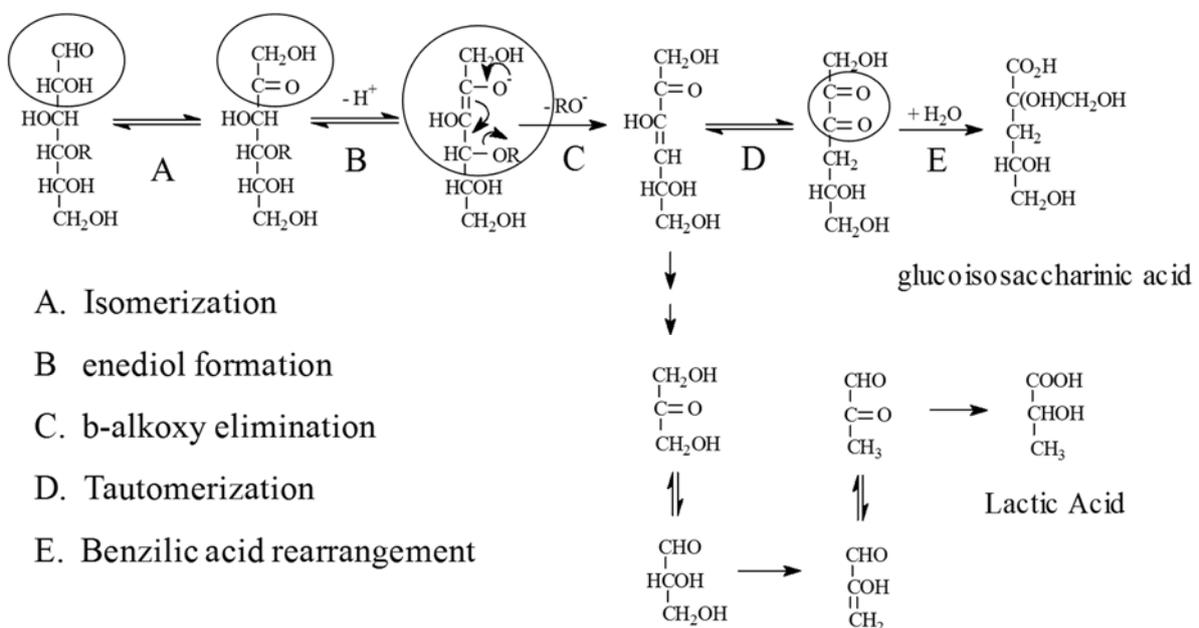
The amount and composition of the carbohydrate polymers are changed continuously during pulping. The extent of these transformations affects the yield and the properties of the final pulp. The main carbohydrate reactions include deacetylation, end-wise degradation (peeling reaction) and alkaline hydrolysis (Sjöström, 1993).

*Deacetylation* – reactions that involve the saponification of acetyl groups in acetylated hemicelluloses, i.e., the galactoglucomannan in softwoods and 4-O-methyl-glucuronoxylan in hardwoods. Deacetylation reactions are rapid. The hemicelluloses are deacetylated and the amount of the uronic acid groups is reduced during the initial delignification phase see Figure 2.10. The yield loss during the initial delignification is ascribed to the peeling reaction and direct dissolution of hemicelluloses.



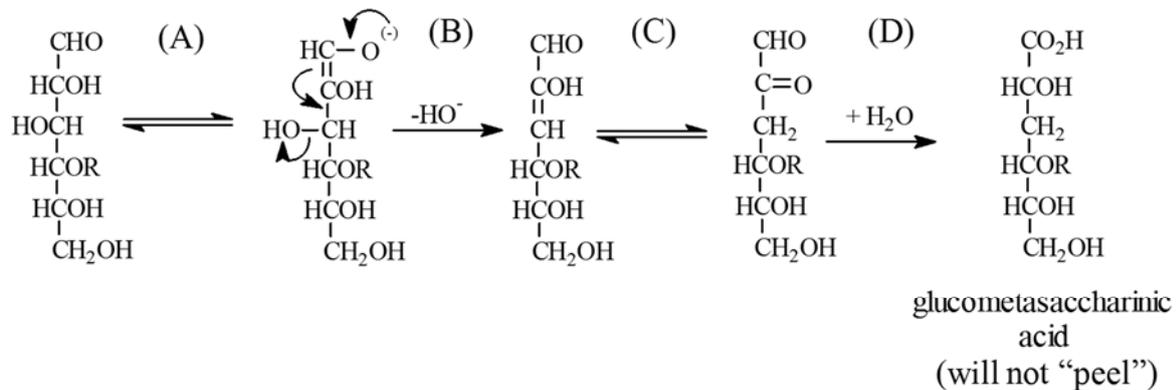
**Figure 2.10:** Schematic mechanism of deacetylation reaction under alkaline hydrolysis using acetyl groups as an example (Patil et al., 2012).

*Peeling reaction and stopping reaction* - Peeling is the process in which sugars are peeled of carbohydrates from the reducing end. This can cause yield losses during pulping. The first step in the process is the alkaline induced isomerisation of the aldose to a ketose. The second step is formation of an enediol through keto-enol isomerisation. This enediol is in equilibrium with the ketone but can also go through  $\beta$ -alkoxy elimination (follow the arrows) thus cleaving the glycosidic linkage. Through another keto enol tautomerization a ketone is formed. The final step in this pathway is the benzylic acid rearrangement forming an isosaccharinic acid. This shows that as the monosaccharides are released they are converted to acids (Fengel and Wegener, 2003). The peeling reaction occurs during the heat-up period of cooking and this result in yield loss (starts at about 100°C) (Gierer, 1980). During the peeling reaction as shown in Figure 2.11 (Fengel and Wegener, 2003), the terminal units of the polysaccharide chain are removed in a stepwise manner until a stopping reaction occurs that stabilizes the polysaccharide against further peeling. The carbohydrate material lost during peeling is converted to soluble acids, which reduce the effective alkali concentration of the cooking liquor. The cessation of peeling may be due to a rearrangement of the reducing end group which makes it stable to peeling ("chemical stopping"), or because the reducing end group has become physically inaccessible to the alkaline reagent ("physical stopping") (Fengel and Wegener, 2003). The polysaccharides which have undergone a stopping reaction are relatively stable until the high temperature portion of the cook is reached.



**Figure 2.11:** Represents the reaction mechanism for the peeling reaction (Fengel and Wegener, 2003).

The stopping reaction does what its name implies; it stops the peeling reaction. The end group is converted to a metasaccharinic acid. This group does not undergo the peeling reaction. It is also not cleaved from the carbohydrate polymer.

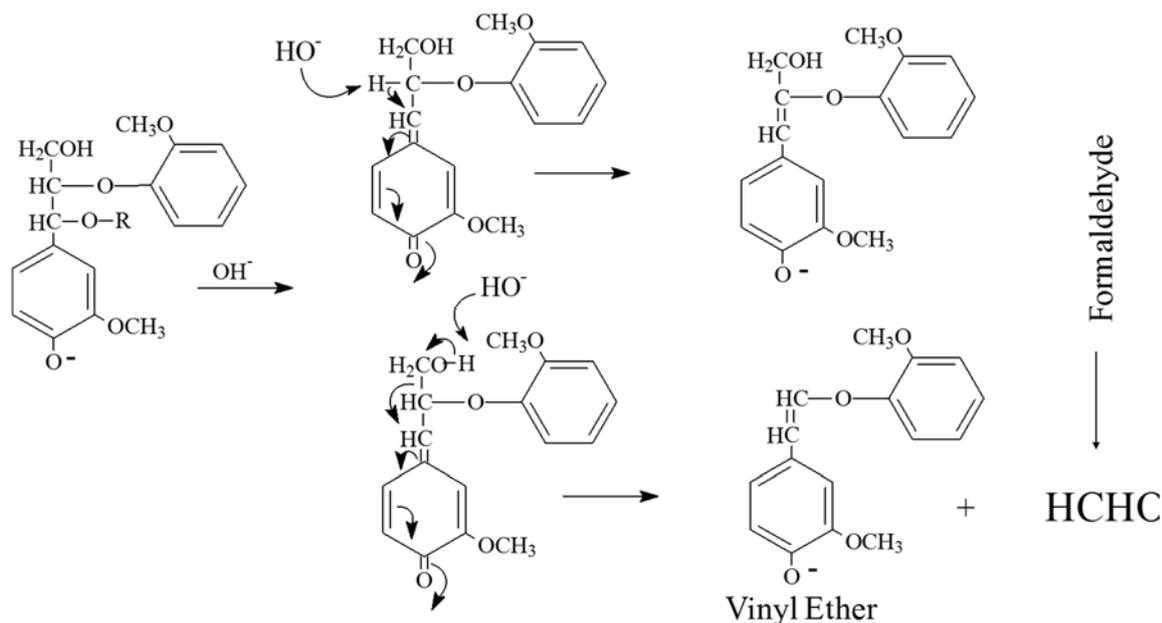


- A. 1,2 Enediol formation
- B.  $\beta$ -hydroxy elimination
- C. Tautomerisation
- D. Benzilic acid rearrangement

**Figure 2.12:** Represents the reaction mechanism for the stopping reaction (Fengel and Wegener, 2003).

#### 2.4.4.2.2 Lignin reaction

The main reactions involve the cleavage of  $\beta$ -O-4 linkages resulting in the formation of new phenolic hydroxyl groups. Generally, Kraft pulping involves mercaptation, alkaline hydrolysis and alkaline condensation of lignin. The reaction of sulphur with lignin is still not understood. But it appears to result in the stabilisation of the phenolic groups thus preventing lignin condensation and assisting in the cleavage of ether bonds between phenyl-propane units that have free phenolic hydroxyl groups. Under alkaline conditions, the phenolate anions formed upon protolysis of phenolic  $\beta$ -O-4 structures are converted into a quinone methide intermediate as Figure 2.13 shows. A nucleophilic attack by a hydrosulphide ion at the  $\alpha$ -carbon of the quinone methide leads to the formation of a benzyl-thiol structure. The cleavage of  $\beta$ -O-4 bonds leading to the fragmentation of lignin then occurs through another nucleophilic attack by the thiol group at the  $\alpha$ -carbon, or by another hydrosulphide ion. However, the desirable lignin fragmentation can be restricted by the low availability of hydrosulphide ions (Fengel and Wegener, 2003).



**Figure 2.13:** The  $\beta$ -O-4 reactions during Kraft pulping (Gierer, 1980).

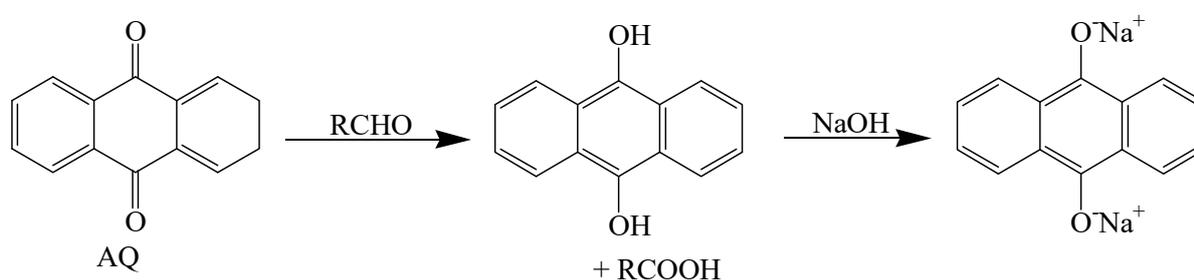
The quinone methide also undergoes lignin condensation reactions (Gierer 1980), side chain reduction reactions (Gellerstedt and Robert 1987) and conversion into enol ether structures (Gellerstedt and Lindfors 1987a). The condensation of the quinone methide with polysaccharide peeling intermediate has also been proposed (Gierer and Wännström, 1984) forming lignin carbohydrate bonds (LCC) and could lead to restriction of dissolution of lignin as a result of an increased molecular weight. Recently, a new mode of radical-initiated lignin condensation reaction has been proposed to restrict delignification (Gellerstedt et al., 2004).

#### 2.4.4.3 Anthraquinone

It has been reported that the use of AQ as an additive for Kraft and soda pulping resulted in significant increase in pulp yields and reduced amounts of pulp rejects (Dommissie, 1998). In some studies, AQ salts have been used as a pre-treatment before alkaline pulping. The chemistry of AQ is mostly centred on the reactions of carbohydrate fraction of wood. The carbohydrate degradation is reported to be reduced, which is attributed to the oxidation of aldehyde end-groups, which leads to the stabilisation against the peeling reaction. However, few reviews have been done on lignin reaction chemistry.

Anthraquinone is reported to be insoluble in water and partially soluble in organic solvents (Holton, 1977). AQ is reduced to hydroquinone (AHQ) only in the presence of reducing sugars and alkali (Fleming et al. 1978).

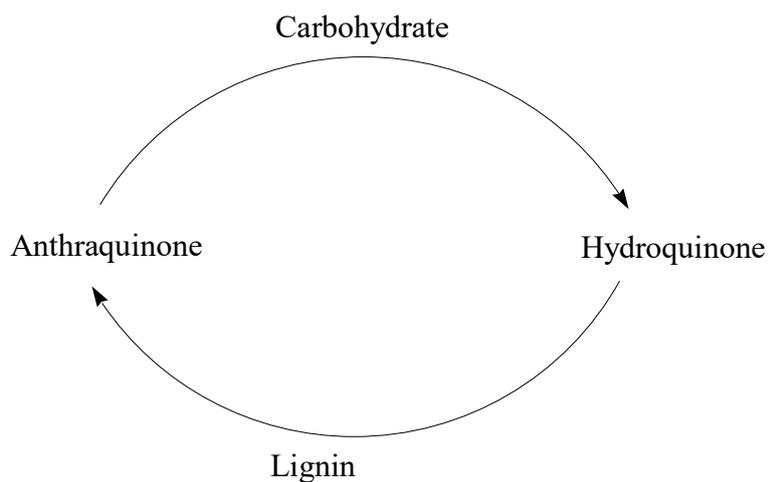
Hydroquinone is acidic and forms water-soluble disodium, salts when reacted with an alkali (Figure 2.14). The reaction occurs rapidly with soluble carbohydrates, and a deep red colour of disodium salts can be easily observed. The reduced form of AQ is kept in a solution and is readily available if a catalytic cycle is involved. Anthraquinone is an electron-donating effective alkali.



**Figure 2.14:** The anthraquinone reduction reaction to form hydroquinone reaction at room temperature (Wilsconsin, 1978)

#### 2.4.4.3.1 The mechanism of AQ pulping

The mechanism of AQ pulping is not well understood. However, AQ is considered to be functioning as a reduction-oxidation (redox) catalyst. It is assumed that the carbohydrate end-groups are oxidised by AQ and lignin completes the catalytic cycle by oxidising AHQ to AQ again summarised in Figure 2.15.



**Figure 2.15:** The mechanism of AQ pulping, where the cycle is applicable to both soda and Kraft pulping.

The reaction involves pulp hydrolysis, an increase in aldonic acids which correspond to the end-group oxidation and lastly, reduced amount of alkaline stopping acids. The reduction in alkaline stopping acids is due to the low peeling and is consistent with high carbohydrate content during pulping (Bach and Fiehn, 1972).

A correlation between the delignification rate and the redox potential of spent liquor for a series of amines has been reported. This was discovered by Sjöström and Ahlstrom, 1976 who showed the importance of redox potential on delignification rates of non-sulphur pulping. They developed a method for monitoring the electron potential of the cook relative to the saturated calomel electrode. At the start of the reaction, the potentials are positive and become more negative as the reaction proceeds. The soda-amine pulps are found to have high tear strength.

The proposed AQ mechanism involving a catalyst reduces and prevents lignin condensation and thus, enhancing the solubilisation reactions of delignification.

## 2.5 Analysis of lignin and lignin-carbohydrate complexes

Lignin is a polyphenolic biopolymer with an irregular structure. Due to this heterogeneous structure, it is not possible to degrade lignin into repetitive regular monomeric fragments like most other biopolymers. In addition to the difficulty of preparing representative lignin samples that have not undergone any chemical change, all known methods of chemical degradation yield identifiable products of small molecular weight in modest yield only. Under such

circumstances the task of devising a satisfactory structural picture of lignin macromolecules have been likened to an attempt to compose a picture-puzzle with an incomplete number of pieces (Sarkanen and Ludwig, 1971). The degradative methods described below are all aimed at some particular aspect of the structure of lignin and an intensive structural analysis implies the use of more than one method and a combination with a spectroscopic method.

### **2.5.1 Acidolysis and thioacidolysis**

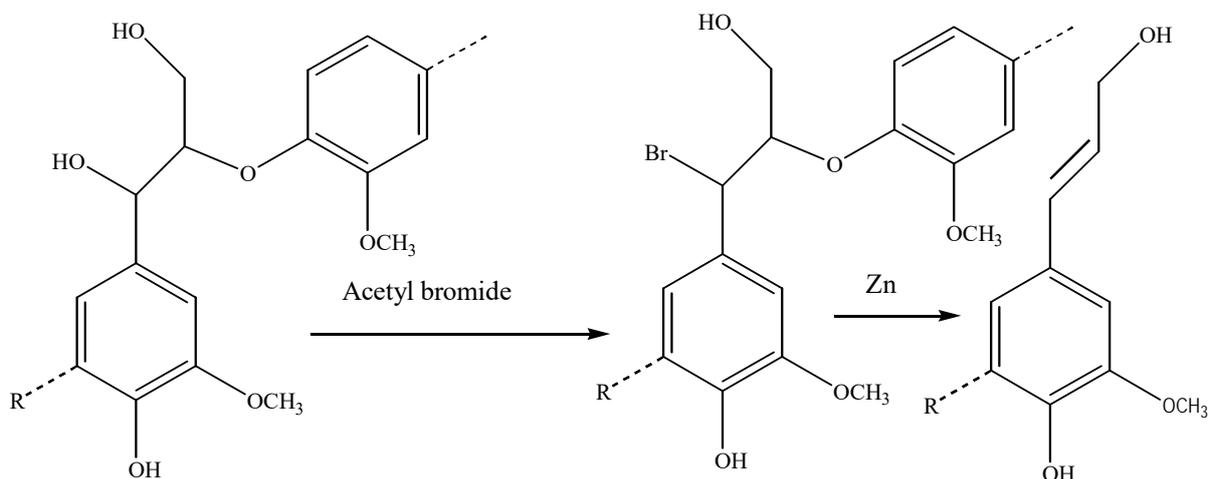
Acid-catalysed degradation aims at cleaving the most important ether bonds in lignin i.e. the aryl-glycerol- $\beta$ -aryl ethers. Acidolysis involves heating the sample at 100° C in 0.2M HCl dioxane-water (9:1, v/v) mixture (Lundquist, 1973). This causes selective cleavage of aryl-glycerol- $\beta$ -ethers and other types of labile ether linkages.

Monomeric and dimeric acidolysis products that contain a phenyl-propane skeleton can be analysed by gas chromatography after silylation which will be discussed later. The results can be interpreted with an aid of low molecular weight model compounds as standards that have undergone the same treatment. The structural elements detected with the aid of the acidolysis studies include  $\beta$ -O-4,  $\beta$ -5,  $\beta$ - $\beta$ ,  $\beta$ -1, glyceraldehydes-2-aryl-ether, 2-aryl-oxypropiophenone, cinnamaldehyde, cinnamic acid, benzaldehyde, benzoic acid and quinoid types. Some of the components have been estimated quantitatively. The same structural units are detected by thioacidolysis, where the sample is treated with boron-trifluoride in dioxane-ethanethiol solution (Lapierre et al. 1986). Monomeric products substituted with the thiol-ethyl are formed and they are analysed by GC after silylation.

### **2.5.2 Reductive cleavage after derivatisation (DFRC)**

Acidolysis and thioacidolysis are specific reactions that cleave  $\beta$ -aryl ether bonds in lignin. The strong acidic treatments involved causes extensive acid-catalysed reactions that complicate the analysis of degradation products. In an effort to develop a simpler method with cleaner cleavage reactions of  $\beta$ -aryl ether bonds, a procedure has been developed that involves derivatisation of lignin with acetyl bromide and reductive cleavage of the resulting benzyl bromides with zinc dust. The reaction sequence cleaves the  $\beta$ -aryl ether bonds and liberates phenyl-propane units that can be analysed as cinnamyl alcohol derivatives. The product mixtures in the derivatisation followed by reductive cleavage (Lu and Ralph, 1997) (Figure 2.16) tend to be simpler than those analysed by acidolysis and thioacidolysis. Apart from monomeric products, a number of dimmers and trimmers have been identified. These include

representatives of all the common inter-unit linkages in softwood lignin except the  $\beta$ -O-4 which is efficiently cleaved in the degradation reaction (Peng et al. 1999). Among the trimmers, a novel isochroman structure has been found and it is suggested that such structures may exist in unchanged lignin (Ralph et al. 1998).

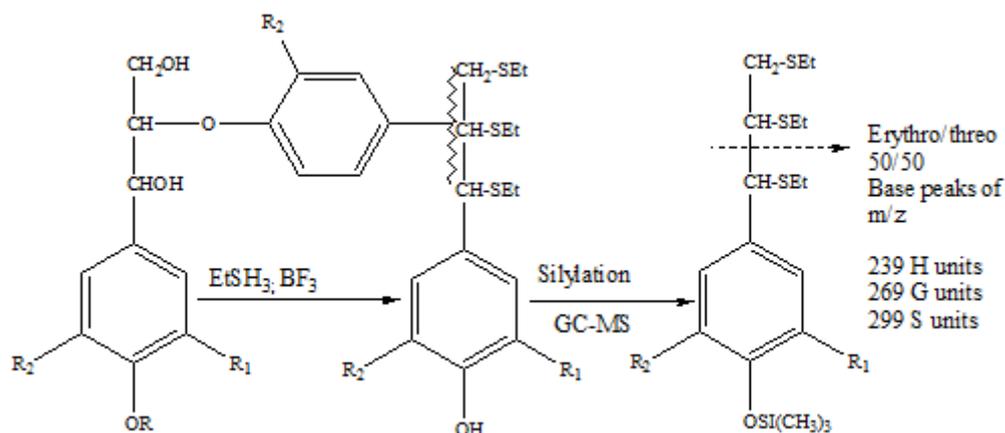


**Figure 2.16:** Reaction mechanism of reductive cleavage after derivatisation redrawn from Lu and Ralph, (1997).

### 2.5.3 Thioacidolysis

Thioacidolysis; a solvolytic procedure which specifically provides monomers and dimers from lignin was developed about two decades ago and still continues to develop. This method is one of the most widely used routine methods in the study of lignin structure. With this method an in depth evaluation of lignin structure is possible as it provides information on the biosynthetic pathways and mechanisms involved in lignification.

Thioacidolysis provides lignin-derived monomers from *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units only involved in  $\beta$ -O-4 linkages (Lapierre et al. 1995). It proceeds through the cleavage of  $\beta$ -O-4 bonds, the most frequent linkage in native wood lignin. Lignin units only involved in arylglycerol- $\beta$ -aryl ether structures mainly afford  $C_3C_6$  thioethylated monomers which can be analysed as their trimethylsilylated (TMS) derivatives by GC-MS as shown in Figure 2.17.



**Figure 2.17:** Schematic reaction principle for thioacidolysis (Lapierre 2008,). The H ( $R_1=R_2=H$ ), G ( $R_1=OMe$ ;  $R_2=H$ ) and S ( $R_1=R_2=OMe$ ) units are only involved in  $\beta$ -O-4 bonds and yield H, G and S monomers analysed as their TMS derivatives.

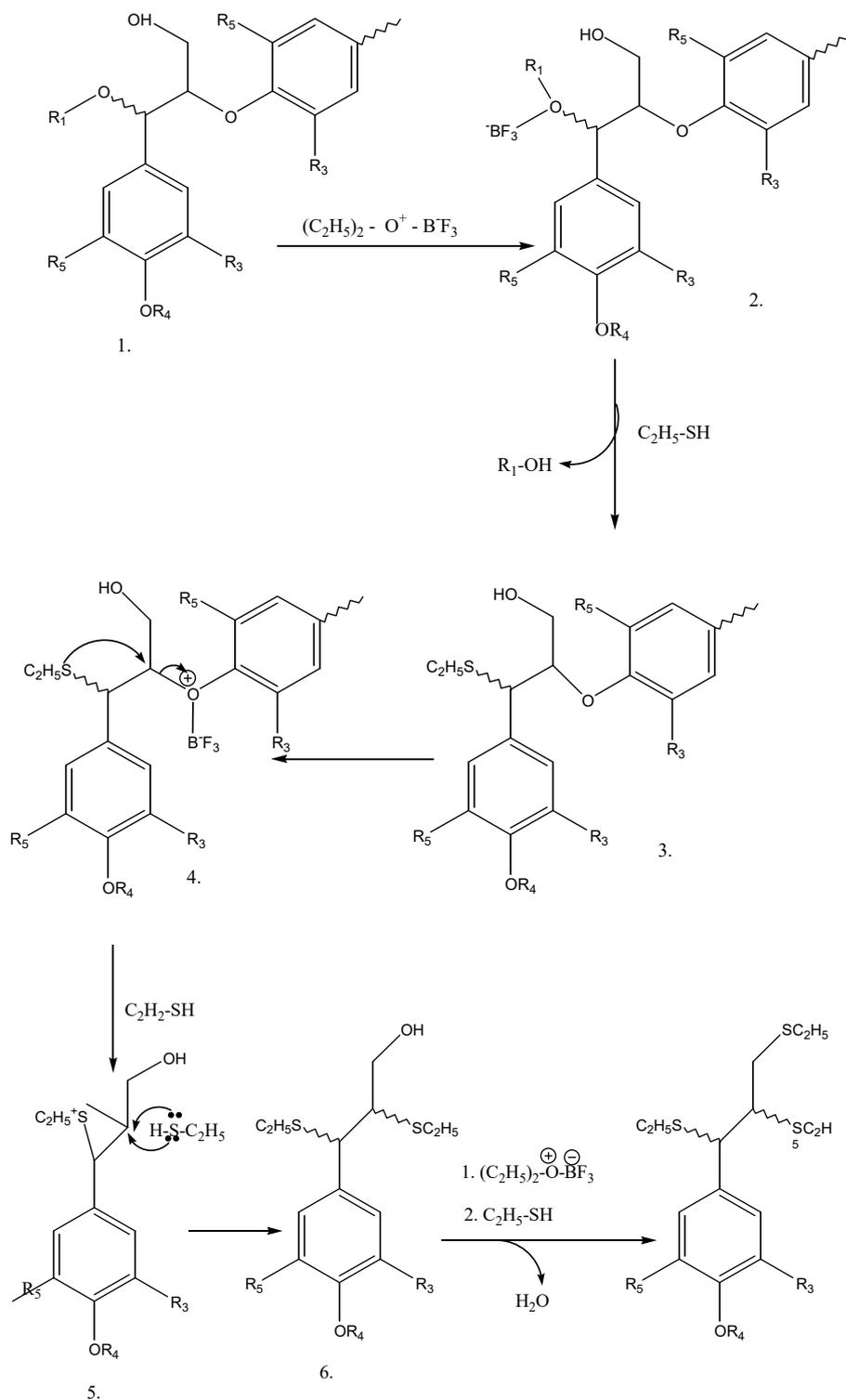
Determination of the TMS monomers is done from ion chromatogram respectively reconstructed at 239, 269 and 299 m/z for H, G and S monomers. By doing so, precise characterisation of the monomers is obtained even when present in trace amounts.

The main outcomes of thioacidolysis have been discussed intensively in literature. The thioacidolysis yield of the main monomers which mirror the frequency of units only involved in  $\beta$ -O-4 bonds; which in some cases the frequency exceeds 50% of  $C_3C_6$  units in constitutive lignin, which suggests the occurrence of linear and  $\beta$ -O-4 rich domains in lignin (Lapierre et al. 1995 and Lawoko et al. 2005). There is also the relative frequency of minor monomers issued from coniferyl alcohol end-groups, which provides insight to the oxidative coupling mechanism (end-wise type reactions between a monolignol and a growing polymer versus bulk-type coupling between two monolignols) (Lapierre and Rolando, 1998). Also the significance of thioacidolysis performed after an exhaustive permethylation of the sample is important. This method provides the first evidence that H units are terminal units with free phenolic groups, in contrast to S units that are internal units etherified at  $C_4$  (Lapierre and Rolando, 1998). The heterogeneous distribution has been rationalised on the basis of different redox potentials of monolignols (Brunow et al. 1998). The relative frequency of the lignin-derived dimmers that are representatives of the resistant inter-unit bonding modes which with its variations reflect the heterogeneous distribution of these bonding modes (Lapierre et al. 1991), such as the  $\beta$ -1 inter-unit linkage which has been found to be the matter of conflict in results of Lapierre et al., in 1991. The last outcome is the occurrence of unusual units and bonding patterns in lignin mutants and transgenic which further establish the biochemical

plasticity of lignification and provides novel insight into casual relationships of lignin structure and cell wall properties.

### **2.5.3.1 Reactions mechanism of the thioacidolysis process**

Thioacidolysis relies on the use of boron-trifluoride etherate  $(C_2H_5)_2O-BF_3$  and ethanethiol  $C_2H_5-SH$  to cleave  $\beta-O-4$  bonds in lignin. The mechanism is shown in Figure 2.18. At first the  $\alpha$ -carbon is substituted by  $BF_3$ , resulting in compound 2. The activated  $\alpha$ -carbon makes the compound reactive to ethanethiol, resulting in formation of compound 3.



**Figure 2.18:** Reaction mechanism for the formation of 1,2,3-trithioethane phenyl-propanoid monomers from lignin. R<sub>1</sub> is either an aryl group or hydrogen atom. In H-residue, both R<sub>3</sub> and R<sub>5</sub> are hydrogen atoms; in G-residue; R<sub>3</sub> is a methoxyl group and R<sub>5</sub> is a hydrogen atom and in S-residue; both R<sub>3</sub> and R<sub>5</sub> are methoxyl groups. R<sub>4</sub> is either a hydrogen atom or an alkyl group. The wavy bonds indicate that both the *S*- and *R*- stereoisomers are present.

The thioethyl group attacks the activated  $\beta$ -carbon of compound 3, resulting in an intermediate compound 4. The attack of ethanethiol results in the formation of compound 5 and through a similar mechanism, the  $\gamma$ -carbon is substituted resulting in the formation of 1,2,3-trithioethane phenyl-propanoid monomer 7 (Lapierre et al. 1986). Thioacidolysis allows the distinction between products derived from lignin and products derived from *p*-coumaric and ferulic acid, and the distinction between products derived from cinnamaldehyde and cinnamyl alcohols. Recent improvements have made it possible to estimate the fraction of free phenolic groups in uncondensed lignin and to depolymerise the dimmers so that they can be included in the analysis of the lignin composition (Vermiss and Nicholson, 2008).

### 2.5.3.2 Silylation

Silylation is the introduction of a substituted silyl group ( $R_3Si-$ ) to a molecule. Nearly all functional groups which present problems in gas chromatographic separation (hydroxyl, carbonic acid, amine, thiol, phosphate) can be derivatised by silylation reagents. This involves the replacement of an acidic hydrogen on the compound with an alkyl silyl group, for example, trimethylsilyl ( $-SiMe_3$ ). The derivatives are generally less polar, more volatile and more thermally stable. The introduction of a silyl group(s) can also serve to enhance mass spectrometric properties of derivatives by producing either more favourable diagnostic fragmentation patterns of use in structure investigation, or characteristic ions of use in trace analyses employing selected ion monitoring and related techniques.

Similarly, silylation is used to introduce silyl groups as protecting groups. A general technique is to deprotonate the substrate with a suitable strong base (e.g. butyl lithium), and allow it to react with a silyl chloride (e.g. trimethylsilyl chloride). An auxiliary base is usually used to remove hydrogen chloride formed:



## 2.5.4 Instrumental techniques

### 2.5.4.1 Size exclusion chromatography

The molecular mass of a polymer is an important parameter which determines its properties such as viscosity, tensile strength and etc. Most polymers are poly-disperse, meaning that the polymer contains molecules with different molecular mass-distributions. Methods such as Size Exclusion Chromatography (SEC) have led to the exact determination of molecular mass and molecular mass-distributions, provided that the methods are calibrated properly. SEC has also been called Gel Permeation Chromatography (GPC) and Gel Filtration Chromatography (GFC). The molecules are separated according to their hydrodynamic size in column packed with porous particles which are the stationary phase. To determine the molecular mass, calibrations with polymers with known molecular masses are used. The calibration polymers have properties similar to those of the samples to be analysed (Josefsson, 2001). According to Sjöholm (1999) size exclusion chromatography requires that the following criteria be met:

- (a) Dissolution of samples should be possible without any degradation
- (b) Adsorption of samples should not occur onto the stationary phase of the column
- (c) Samples should be compatible with the stationary phase and
- (d) The dissolved samples should be stable.

Lignin samples are often derivatised by methylation or acetylation before analysis.

### 2.5.4.2 Gas chromatography-mass spectroscopy (GC-MS)

Gas chromatography-mass spectroscopy is one of the hyphenated analytical techniques. It is composed of two techniques that are combined to form a single method of analysing mixtures of chemical compounds. Gas chromatography separates the compounds and mass spectroscopy characterises each component individually.

Chromatography separation occurs when the sample mixture is introduced into a mobile phase. In the liquid chromatography, the mobile phase is a solvent. In gas chromatography, the mobile phase is an inert gas such as helium. The mixture in the mobile phase interacts with the stationary phase. Each compound in the mixture interacts at a different rate. Those that interact the fastest are eluted first and those that interact the slowest are eluted from the column lastly. Different mixtures can be separated by changing the characteristics of the

mobile and stationary phases. Further refinements to separation process can be made by changing the temperature of the stationary phase or the pressure of the mobile phase.

## 2.6 Summary

A lot of research in the past and on-going, focuses on trying to understand the reasons for the retarded delignification observed during the final phases of the Kraft pulping and oxygen delignification. Three major conclusions have been derived; 1) The presence of alkaline stable native lignin structures, 2) condensation reactions occur in lignin and 3) The presence of alkaline stable covalent linkages between lignin and carbohydrates (LCCs). It has not been possible to determine the relative importance of the three reasons given above; however, experimental support for all three has been presented (Lawoko, 2003). The current study is aimed at elucidating the presence of the LCC structures on our selected biomass sources i.e. *E. grandis* and sugarcane bagasse as well as our selected processes i.e. value prior pulping, steam explosion and ionic liquid treatment of biomass which have been extensively discussed in this review. The stable LCCs that survive during processing may contribute significantly to the difficulty in removing lignin at the end of processing the biomass material.

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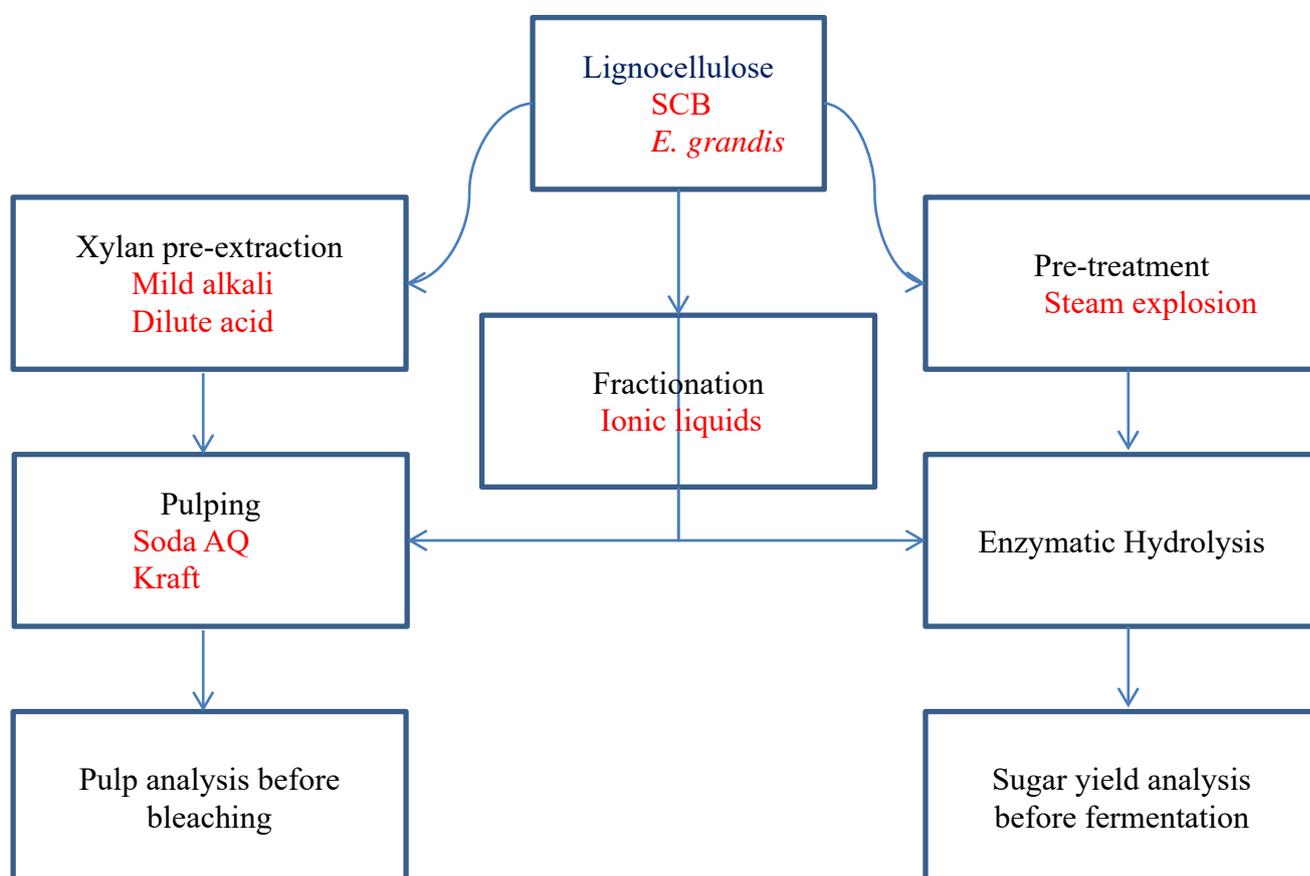
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**CHAPTER 3: METHODOLOGIES****3.1 Introduction**

Figure 3.1 presents a flow sheet of the process steps that the raw materials selected for this study went through and the chemical analysis carried out in each of the steps. As described in the figure, the biomass was subjected to steam explosion, ionic liquid fractionation, xylan extraction prior to Kraft pulping. Samples were collected from each of the processes in order to investigate the structural changes of lignin-carbohydrate complexes (LCCs) for each process in comparison to the raw feed stocks (control), that is, sugarcane bagasse (SCB) and *Eucalyptus grandis* (*E. grandis*).



**Figure 3.1:** Summary of the methodology chapter

### 3.2 Materials

Two raw feed stocks i.e. *Eucalyptus grandis* and sugarcane bagasse were selected for this study. The *E. grandis* chips were supplied by Sappi Manufacturing, Pty (Ltd), South Africa. The chips were screened and a 4-8mm chip size fraction was selected for experiments.

Air dried and depithed sugarcane bagasse (*Saccharum officinarum*) was provided by TSB Sugar, located in the Mpumalanga Province, South Africa. Both biomass materials were conditioned at 23°C and 55% relative humidity before use. The chemicals used were obtained from Merck chemical supplies and Sigma and Aldrich. 1-ethyl-3-methylimidazolium acetate (96%) and 1-butyl-3-methylimidazolium methyl sulphate (96%) were obtained from Sigma and Aldrich (St. Louis, MO).

### 3.3 Chemical characterisation of the raw materials

The 4-8mm fractions of *E. grandis* chips were sub-sampled and ground in a Retsch mill to 40 mesh size. The depithed SCB was also ground to 40 mesh size using a vibratory mill from Retsch GmbH. The composition of the raw biomass materials was determined using methods standard methods for the Technical Association of the Pulp and Paper Industry (TAPPI) (T264 om-88; T211 om-85; T222 om-88 and T223 om-84) and the standard Laboratory Analytical Procedures for biomass analysis provided by the National Renewable Energy Laboratory (NREL) as summarised in Table 3.1 below.

**Table 3.1:** The analytical methods used to determine the chemical composition of the feed stocks

	<b>Method</b>	<b>Reference</b>
<b>Moisture content</b>	T264 om-85	Technical Association of the Pulp and Paper Industry (TAPPI), 1992-1993
<b>Ash content</b>	T211 om-88	Technical Association of the Pulp and Paper Industry, 1992-1993
<b>Ethanol/cyclohexane+ water soluble extractives</b>	T264 om-84	Technical Association of the Pulp and Paper Industry, 1992-1993
<b>Klason lignin</b> <b>Carbohydrate content</b>	T222 om-88 and NREL/TP – 510 -42618	Technical Association of the Pulp and Paper Industry, 1992-1993, National Renewable Energy Laboratory, 2010
<b>Syringyl/guaiacyl analysis</b>	Acidolysis	Govender et al. 2009

### 3.3.1 Moisture content

A biomass sample with a particle size of 250µm and known mass (5.0g) was placed in an oven at 105°C for 12 hours after which it was re-weighed and the mass loss recorded. The moisture content of the material was calculated using equation 3.1.

$$\% MC = \frac{Mass_B - Mass_A}{Mass_A} \times 100 \times 1/\% MC \quad \text{Equation 3.1}$$

Where, MC is moisture content of the material, Mass<sub>B</sub> is the mass of the sample before it was placed in an oven and Mass<sub>A</sub>, is the mass after the sample was removed from the oven after 12 hours.

### 3.3.2 Ash content

The ash represents the inorganic elements that are present in the biomass material. A sample of known (3.0g with 250µm particle size) mass was placed in a Gallenkamp Muffle furnace at 575±25°C for 4 hours. The sample was then transferred into a desiccator with silica gel and allowed to cool to room temperature after which it was re-weighed. The ash content of the samples was expressed using equation 3.2.

$$\% AC = \frac{\text{mass of ash}}{\text{mass of sample}} \times 100 \times 1/\% MC \quad \text{Equation} \quad \mathbf{3.2}$$

Where AC is the percentage ash content of the material

### 3.3.3 Extractive content in biomass

Five grams of material were placed in a cellulose thimble. The extractives in the biomass material were determined in a two-way step. The first step was to determine the organic solvent soluble extractives using a mixture of an alcohol and cyclohexane in a 1:2 ratio (73ml ethanol + 147ml cyclohexane). The sample in the cellulose thimble was Soxhlet extracted overnight (~12 hours). The quantity of soluble extractives was determined by evaporating the solvent in a rotary evaporator at 60°C. The same cellulose thimble was used to determine the water soluble extractives in the second step. In this step 250mL of distilled water was placed in a round bottom flask. The sample was refluxed using Soxhlet set-up apparatus for 24 hours, after which the water was evaporated to determine the water-soluble extractives. The extractives were reported according to equation 3.3.

$$\% EC = \frac{M_{OE} + M_{WE}}{\text{Mass sample}} \times 100 \times 1/\% MC \quad \text{Equation} \quad \mathbf{3.3}$$

Where,  $M_{OE}$  is the mass of the extractives soluble in solvent mixture and  $M_{WE}$  is the mass of extractives soluble in water.

### 3.3.4 Klason lignin and carbohydrate content

The Klason lignin content of the samples was determined as described in the TAPPI and NREL standard methods for biomass characterisation (Table 3.1).

As described in the standard method, the analysis involved two consecutive acid hydrolysis steps during which 0.3g the raw biomass was first hydrolysed at high acid concentration (3ml of 72% (w/w) H<sub>2</sub>SO<sub>4</sub>) and low temperature (30°C) for an hour. In the second step, the acid content was reduced to 4% (w/w) H<sub>2</sub>SO<sub>4</sub> by diluting with water. The biomass was then further hydrolysed at high temperature in an autoclave for 1 hour at 121kPa after which the lignin residue was separated from the hydrolysate through vacuum filtration, dried at 105°C for 24 hours and quantified through weighing. The ash content of lignin residue was determined so that the quantity of the lignin can be reported on an ash free basis. This was achieved through heating the samples for 4 hours in a Gallenkamp furnace (Loughborough, UK) at to 575±25°C. The difference in the mass before and after combustion was used as an indication of the acid insoluble lignin. A small amount of lignin is usually solubilised by the acid hydrolysis and this is referred to as acid soluble lignin. This was done using a UV-Visible spectrophotometer at wavelengths of 240 and 205nm for *E. grandis* and SCB respectively.

The carbohydrate content of the biomass was quantified through the analysis of the individual sugar components of the hydrolysate i.e. glucose, xylose and arabinose via high performance liquid chromatography (HPLC) equipped with an Aminex HPX-87H Ion Exclusion Column with an IG Cation-H cartridge guard column. Detection was conducted using a Shodex RI-101 which is a refractive index detector. The column was operated at 65°C with a mobile phase of 5mM sulphuric acid at a flow rate 0.6 ml/min. The sugar content of the samples was quantified by using a standard curve of the combined sugar components. The data obtained was processed using a Chromeleon® Version 6.80 software package.

### **3.4 Biomass processing**

#### **3.4.1 Hemicelluloses pre-extraction from raw biomasses and pulping of the biomass**

The hemicelluloses pre-extraction conditions of raw feed stocks which were subjected to pulping are summarised in Table 3.2 and below are further discussed in subsequent subsections. The experiments were carried as described by Vena (2013) and Vena et al. (2013a and 2013b). A range of conditions were tested (Table 3.2). The best condition indicated in the table was selected and the samples from this condition were analysed for LCCs.

**Table 3.2:** Pre-extraction conditions for *E. grandis* and sugarcane bagasse

Hemicelluloses Extraction process	Extraction conditions	Extracted xylan (%)	Pulping process	Kappa number	Reference
<b><i>Eucalyptus grandis</i></b>					
<b>Control</b>	-	-	Soda AQ	22.80	Vena, 2013 and Vena et al. (2013b)
<b>H<sub>2</sub>SO<sub>4</sub></b>	Acid concentration (0.3% v/v); Temperature (140°C); Reaction time (20 minutes)	27.70		20.90	
<b>Control</b>	-	-	Kraft	20.00	Vena, 2013
<b>H<sub>2</sub>SO<sub>4</sub></b>	0.1 - 0.5% v/v; 120-140°C; 0.5-1.5 hr	27.70		26.80	
<b>Sugarcane bagasse</b>					
<b>Control</b>	-	-	Soda AQ	22.80	Vena, 2013
<b>Hot water</b>	Hot water; 120°C; 0.33 hr	12.00		20.90	
<b>Alkaline</b>	Base conc.(1-2M); Temp.(40-90°C);	69.10		15.50	
<b>H<sub>2</sub>SO<sub>4</sub></b>	Time (0.33 hr) 0.1 - 0.5% v/v; 120-140°C; 0.5-1.5 hr	57.60		33.2	

#### **3.4.1.1 Dilute acid pre-extraction**

According to the reaction conditions described in Table 3.2, 50g oven dry (ODM) raw biomass (SCB and *E. grandis*) was treated with sulphuric acid solution in a ratio of 1g ODM raw material: 4ml diluted acid; (g/v)] at micro reactors (bombs). The reactors were placed in a 15dm<sup>3</sup> capacity batch digester which uses an electric heating jackets for heating and equipped with a thermocouple for monitoring the temperature. At the end of the desired extraction time, the reactors were cooled in water to room temperature. The solid residues were recovered by fractionation on a 100 mesh screen, washed with water and then air dried. The solid residues were stored in a conditioning room in which the temperature and the relative humidity are regulated prior to pulping.

#### **3.4.1.2 Mild alkali pre-extraction**

According to the reaction conditions described in Table 3.2, 50g ODM sample material was treated with a NaOH solution in autoclave (or autoclavable bottles) bottles and placed in a shaking water bath and kept at desired temperature and time. The solid to liquid ratio was fixed at 1g ODM biomass: 4ml sodium hydroxide (NaOH) solution at different concentrations see Table 3.2. At the end of the extraction time, the bottles were cooled in cold water to room temperature. The solid fractions were collected on a 100 mesh screen, rinsed with water and then air dried. The solid residues were stored in a conditioning room prior to pulping.

#### **3.4.1.3 Hot water pre-extraction**

Forty grams of OD materials were placed into micro bomb reactors and treated with hot water at 120°C for 40 minutes. The solid to liquid ratio was fixed at 1g ODM: 6ml hot water. The reactors were placed in a batch digester 15dm<sup>3</sup> capacity batch digester which uses electric heating jackets for heating, and equipped with a thermocouple for monitoring the temperature. At the end of the extraction period, the fibres were separated from the liquid fraction and treated the same way as described for dilute acid and alkali extraction sections (Sections 3.4.1.1 and 3.4.1.2 respectively).

#### **3.4.1.4 Pilot scale pulping of the control and hemicelluloses pre-extracted biomass samples**

Control samples and solid residues from dilute acid, alkaline and hot water pre-extraction described in Table 3.2 and sections 3.4.1.1, 3.4.1.2 and 3.4.1.3 respectively were subjected to Kraft or soda AQ micro pulping. Pulping was carried out in a 15dm<sup>3</sup> batch type digester. Kraft pulps were prepared by treating 1000g of biomass to 18.7% NaOH and 25% sulphidity for 30 minutes at 170°C. Soda AQ, pulping was carried out by cooking the biomass with 18.7% NaOH and 0.15% anthraquinone (AQ) for 60 minutes at 170°C. At the end of cooking cycle, the fibres were separated from the black liquor and washed through a 10 mesh screen. For alkali pre-extracted biomass, no additional sodium hydroxide was added. Only 35.7% sodium sulphide was added. The hot water pre-extracted biomass was subjected to soda AQ pulping where it was treated with 14% NaOH and 0.1% AQ for 30 minutes at 170°C. The pulps were then screened in a Packer slotted laboratory screen. The Kappa numbers were determined using TAPPI standard method T236 cm-85.

#### **3.4.2 Ionic liquid fractionation of the biomass**

This experiment was only carried out with sugarcane bagasse. This study was done to fractionate SCB through the application of processes utilising the ionic liquids ([EMiM]CH<sub>3</sub>COO or [BMiM]MeSO<sub>4</sub>) (Diedericks et al. 2011). In preparation for ionic liquid treatment, a 1kg of sugarcane bagasse sample was ground to a particle size range between 0.425 and 0.850mm using a centrifugal mill (Retsch ZM 200, Haan, Germany) at 6 000rpm. The sample fraction, which represented 70% (w/w) of the original sample, was oven-dried at 45°C until a moisture content of less than 1% (w/w) was reached. The chemical composition of the biomass was determined according to the NREL standard procedures described in Table 3.1 and subsequent sections 3.3.1 for MC, 3.3.2 for ash, 3.3.3 for extractives and 3.3.4 for Klason lignin and carbohydrate content respectively. The ionic liquid fraction was carried out as described by Diedericks et al. (2011) (Table 3.3).

**Table 3.3:** Ionic liquid fractionation conditions

Condition	Temperature (°C)	Time (minutes)	Description	Reference
1.	100	60	+	Diedericks et al. 2011
2.	100	120	++	Diedericks et al. 2011
3.	125	240	+++	Diedericks et al. 2011

+ The condition was poor; ++ the condition was good and +++ the condition was severe.

A 0.6 g sample was transferred into a tubular reactor, where-after a predetermined mass of either [EMiM]CH<sub>3</sub>COO or [BMiM]MeSO<sub>4</sub> was added in order to ensure a solid/ionic liquid ratio of 10% (w/w) was achieved (Diedericks et al. 2011). Ionic liquid was added under a nitrogen blanket to prevent the uptake of atmospheric moisture. Mixing occurred gravitationally by leaving the loaded reactor overnight in an upright position. The reactor was heated in a sand-baths. Treatment was conducted at three different treatment conditions of which the severity increased from 60 minutes at 100°C (TS 1) to 120 minutes at 100°C (TS 2) and finally at 125°C for 120 minutes (TS 3). Treatment was terminated by placing the loaded reactor in a water bath at room temperature.

The content of the reactor (i.e. product mixture) was removed and recovered in 40 ml centrifuge tube. The product mixture was washed at room temperature using two different anti-solvents for cellulose i.e. Neat acetone ( $\bar{A}$ ), an acetone-water mixture (50% (v/v)) (AW) or a 0.1M sodium hydroxide solution (NaOH) served as the first anti-solvent. The second anti-solvent was composed of a 0.05M sodium citrate buffer (Diedericks et al. 2011). During the first anti-solvent wash, the product mixture was suspended by adding 30ml anti-solvent where after it was vigorously mixed under vortex followed by centrifugation at 1500xg for 20 minutes to separate and recover the residual solid from the supernatant. This process was repeated three times throughout which the supernatant was collected and stored at -20°C. The remaining solid after the first anti-solvent wash was re-suspended in the second anti-solvent by adding 30mL of the second anti-solvent where it was vigorously mixed under vortex followed by centrifugation at 1500xg for 20 minutes to separate and recover the residual solid from the supernatant. The process was repeated three times throughout which the supernatant was collected and stored at -20°C.

### 3.4.3 Biomass pre-treatment with steam explosion

This experiment was carried out with sugarcane bagasse as a potential lignocellulosic feedstock for the production of secondary generation bioethanol. Steam explosion of sugarcane bagasse was carried out in a steam gun explosion set-up, designed and built by IAP, GmbH, Austria. The specific conditions of steam explosion treatment were carried out as described by Wallace (2013) is shown in Table 3.4.

600g of sample material was weighed and fed into a 19L pressure reactor. Saturated steam at 30 Bar was then injected into the pressure reactor and the temperature of the vessel was controlled through manipulation of the vessel pressure by means of a steam injection control valve. Following the steam injection, the pressure reactor took approximately 2 minutes to heat up, upon which the timing of the pre-treatment commenced. For each experiment, time and pressure were kept constant, while temperature changed in relation to the chosen severity. At the end of the residence time, an automatic ball valve capable of opening within less than 0.5 seconds was automatically opened. Subsequently, an explosive expansion of the steam occurred and exploded samples were collected in a cyclone type vessel with the excess steam escaping to the atmosphere. The pre-treated sugarcane bagasse samples were weighed, packed into a plastic bag and stored at 4°C until further analysis.

**Table 3.4:** Specific conditions of steam explosion pre-treatment

Run	Temperature (°C)	Time (minutes)	Description	Reference
1.	185	5	+	Wallace (2013)
2.	200	5	+ +	Wallace (2013)
3.	215	5	+ + +	Wallace (2013)

+ The condition was poor; + + the condition was good and + + + the condition was severe.

The terms poor, good and severe refer to the amount of lignin, hemicelluloses and other biomass components removed as affected by steam gun explosion pre-treatment.

### 3.5 Enzymatic hydrolysis of steam exploded pre-treated and ionic liquid fractionated sugarcane bagasse material

The enzymatic hydrolysis of the biomass samples was carried out as described by Diedericks et al. (2012) and García-Aparicio et al. (2006). The method is detailed in sections 3.5.1, 3.5.2 and 3.5.3. Accelerase 1500; a cellulases enzyme produced by Genencor and Novozyme 188, a  $\beta$ -glucosidases produced by Novozyme, both were used to convert cellulose to glucose. The glucose concentration was measured after complete hydrolysis while conditions such as temperature, rotation, time and pH were kept constant.

#### 3.5.1 Enzyme preparation and loading

The enzymes used had a 15 FPU (filter paper unit) enzyme activity. The amount of enzyme added was determined as follows:

$$15 \text{ FPU} \times 40 = 600 \text{ FPU}$$

$$1 \text{ ml} - 60 \text{ FPU}$$

Therefore, 10ml of Accelerase + 1ml Novozyme 188

Therefore, 11ml was the total volume of the enzymes used for a 2% solid loading.

All experiments were carried out with a 2% solid load i.e. 2g of solid dry mass sample was added to every 100ml buffer/enzyme mixture. Enzymatic hydrolysis has its rule of thumb that 80% of the starting material is completely hydrolysed to simple sugars while 20% remains as a solid residue which is always referred to as water-insoluble-solid (WIS). Therefore, it was important that the WIS was approximately 4g as the LCC isolation and analysis method required 3g as a starting material. The calculations were carried out as shown in Equation 3.4:

For example WIS of 0.5g then the starting material was 2.5g

$$2.5\text{g} \rightarrow 0.5\text{g}$$

$$X \text{ g} \rightarrow 3\text{g}$$

$$\text{Therefore, } x \text{ g} = 15\text{g} \qquad \text{Equation} \qquad \mathbf{3.4}$$

Where, **x** is an unknown mass of the sample

Therefore, the starting material was 15g but the moisture content (% MC) of the material was also considered, and the total mass loading was based on the wetness of the material. The actual mass load of the material was based on Equation 3.5:

$$\text{Sample mass load (g)} = \frac{\text{starting mass sample (g)}}{1 - \% \text{ MC}} \quad \text{Equation 3.5}$$

The amount volume of the buffer/enzyme mixture used was based on Equation 3.6 of 2% solid load calculation:

**2g → 100ml**

$$\text{SML g} \rightarrow \text{xml} \quad \text{Equation 3.6}$$

Where; SML is the sample mass load obtained from Equation 3.5.

### 3.5.2 Sample feeds and the buffer volumes

**Table 3.5:** The pre-treated mass samples and buffer volumes used

Sample	Moisture content (%)	Sample load (g)	Buffer/enzyme (ml)
<u>Raw sugarcane bagasse</u>			
Control	7.61	5.42	270.59
<u>Steam exploded material</u>			
1.	4.85	5.25	262.75
2.	5.72	5.30	265.17
3.	6.21	5.33	266.56
<u>Ionic liquid fractionated material</u>			
1.	7.62	1.07	53.60
2.	7.67	1.08	54.15
3.	7.23	1.08	53.88

The sample codes used in Table 3.5 refer to the conditions that sugarcane bagasse was subjected to during ionic liquid fractionation and steam explosion pre-treatment processes.

### 3.5.3 Preparation of buffer and enzyme activation

The citrate buffer with pH 4.8 was used for the experimental runs. A solid dry mass of 9.6646g citric acid was added to 15.8814g sodium acetate and both solids were added into a 2L volumetric flask where 0.02% sodium azide (0.4g) was added to reduce contamination. The mixture was stirred with a magnetic stirrer at 350rpm for 5 minutes at room temperature. The pH of the solution was adjusted to 4.8 with 7N KOH solution. The activation of enzymes was done by extracting 11ml of the buffer solution which was then compensated by adding 11ml

of enzyme mixture. The solid sample and buffer/enzyme mixture were incubated in an agitator set-up at 50°C, shaking at 150rpm for 72 hours.

After hydrolysis, the samples were removed from the agitator and cooled at room temperature for an hour. After the cooling period had elapsed and sedimentation at the bottom of the undigested solid residue; 5ml of the hydrolysate was extracted into centrifugation tubes for sugar analysis at 10 000rpm for 5 minutes. Then 1.8ml of the supernatant was extracted into a test tube and was treated with 109.8µl of 35% perchloric acid (PCA) to denature the enzymes to avoid HPLC column contamination. It was then placed in an ice bath for an hour for complete denaturing of the enzymes. The hydrolysates were then treated with 99µl of 7N KOH and were left standing in an ice bath overnight. The clear supernatant was carefully extracted through filtered pipette tips and was directly filtered into the HPLC vials through 0.22µm Nylon filters.

The undigested water-insoluble-residue was washed several times with running tap water through Packer slotted laboratory screens. The wet solid residue was then air dried at room temperature for 3 days. It was weighed, packed into a plastic bag and stored in a conditioned room until further analysis.

#### **3.5.4 Spectroscopic analysis of biomass**

Fourier Transform Infrared Spectroscopy (FTIR) was used to identify and determine the presence of lignin, hemicelluloses and cellulose in terms of their intensity as the samples were subjected to different processing methods (hemicelluloses pre-extraction, ionic liquid fractionation, steam explosion and pulping). The processed samples were compared against control samples (biomass material that has not been subjected to any kind of processing). About 1g of each sample that was to be analysed with FTIR was placed in a Petri-dish in which it was placed in a desiccator under phosphorus pentoxide for drying.

The IR spectra of the samples were recorded in reflectance mode using a Smart Performer detector from Thermo equipped with ZnSe lenses. Small portion of sample was placed on the ZnSe horizontal ATR and 32 scans with a resolution of 4cm<sup>-1</sup> were accumulated. FTIR spectra were obtained directly from untreated, pre-treated and pulp samples utilising diffuse reflectance infrared with Fourier transform technique (Perkin Elmer - Spectrum GX). The spectra were normalised by the absorption at 900 – 2000cm<sup>-1</sup> after baseline correction. The data of the samples was converted and reordered into a spread sheet on MS-Excel using OMNIC software. The normalised absorbances in the range of 900 – 2000cm<sup>-1</sup> were submitted to principal component analysis (PCA) using STATISTICA 10.

### 3.5.5 Guaiacyl-syringyl analysis

The syringyl-to-guaiacyl ratio (S/G) was done to determine the acidolysis products. It is a significant identifying parameter for in the delignification processes of biomass materials. The acidolysis products were separated HPLC to obtain S/G ratio. The lignin composition in relation to its basic phenyl-propane units is an important parameter. Determination of guaiacyl to syringyl ratio in lignin wood was done according to a method developed by Govender et al. (2009) with slight modifications. The biomass samples were ground to 40 mesh sizes. They were then hydrolysed by adding 2ml of reagent A<sup>a</sup> into 0.05 g of sample in a screw top vial with a Teflon lined cap. The vial was placed in an oven at 102°C for 4 hours with constant stirring at 30 minute intervals. The mixture was cooled to room temperature for approximately 30 minutes. It was then neutralised with 0.9ml of 0.4M NaHCO<sub>3</sub>. The neutralised mixture was filtered through 0.22µm Nylon syringe filters into HPLC vials without any dilution. The extracts were analysed via HPLC.

## 3.6 Isolation, fractionation and characterisation of lignin-carbohydrate complexes obtained from raw biomass samples in comparison with processed samples

### 3.6.1 Biomass preparation

The isolation of LCC from raw biomass and chemically processed samples was done according to a method developed by Lawoko et al. (2011), which is schematically presented in Figure 3.2. Slight modifications were done on the method in order to be applicable to pre-treated or hemicelluloses extorted samples and pulp samples for both *E. grandis* and sugarcane bagasse biomass materials.

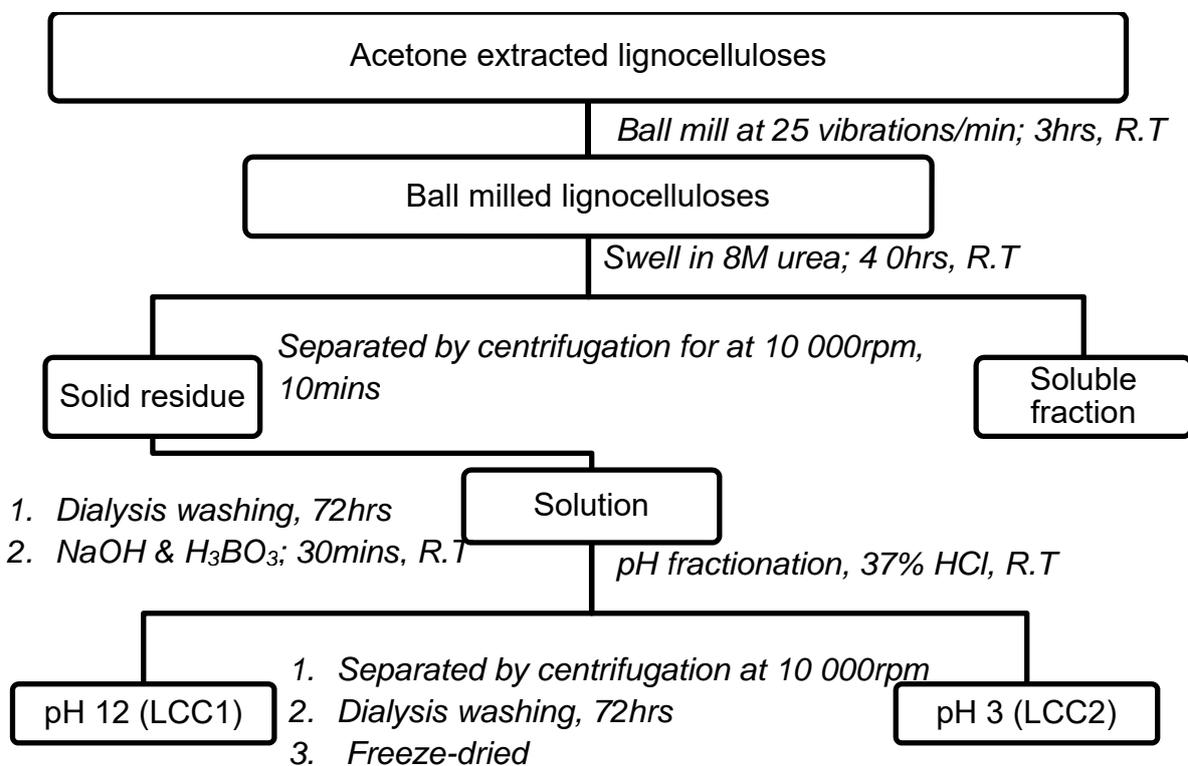
The biomass materials were milled in a Wiley mill through a 60 mesh size. The milled materials were then purified by refluxing in a Soxhlet set up using acetone to obtain an extractive free material. Extractives and neutral sugars interfere with the composition of isolated LCCs by influencing the molecular weight of the fraction (Filisetti-Cozzi et al. 1991). The materials were then subjected to ball milling in order to destroy the crystallinity of cellulose. Cellulose also influences the molecular weight of LCCs. Cellulose has a high molecular mass, this causes difficulties when detecting and assigning signals because of severe decrease in signal sensitivity (Du et al. 2013).

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<sup>a</sup> Reagent A - 0.1g butylated hydroxytoluene + 90ml dioxane + 10ml of 2M HCl into a 100ml volumetric flask

The desirable “powder-like” material was obtained by using 50ml steel jar with 0.5g of sample and 40g steel balls at a frequency of 25 vibrations per minute for 3 hours. After a 30 minute run, the jars were suspended in liquid nitrogen to reduce the heat generated by friction between balls and the jar that may alter the structure of biomass.

### 3.6.1.1 Isolation and fractionation of LCCs



**Figure 3.2:** Summary of the lignin-carbohydrate complex isolation method (Lawoko et al. 2011).

Three grams of each bio-material was treated with 300ml of 8M (for control sample), 4M (for pre-treated samples) and 2M (for pulp samples) urea solutions with continuous stirring at room temperature for 40 hours. The supernatant and the solid fraction were separated by centrifugation at 10 000rpm for 10 minutes. The solid residue was purified through dialysis several times (72 hours) with distilled water. The molecular weight cut-off of the dialysis tubes was 10 000Da. After several washings (dialysis process), the solid pellet was treated with

50ml of 18% NaOH and 4% boric acid with continuous stirring at room temperature for 25 minutes. The alkaline-borate mixture was diluted with 50ml of distilled water. The pH of the mixture was carefully adjusted to pH 12 and 3 by addition of 37% HCl. The fraction obtained through precipitation at pH 12 was separated by centrifugation. All the fractions obtained at pH12 and pH 3 was dialysed for several days (3 days/ 72 hours) as they were sensitive to water washing. The fractions were freeze-dried after which they were weighed to determine total recovery of each the fraction.

### **3.7 Characterisation of LCC fractions**

#### **3.7.1 Reducing sugars**

The reducing sugars were determined in untreated and LCC materials using a method adopted from Iversen (1985). The analysis was done to predict the structure of lignin-carbohydrate complexes that can be obtained by varying the sources of biomass materials (SCB and *E. grandis*), method of processing (hemicelluloses pre-extraction, pulping and other methods). According to this method, 0.002g of sample material was dissolved in 2.5ml of 90% aqueous tri-fluoro-acetic acid (TFA) and hydrolysed in a water bath for an hour at 95°C in a screw top vial with a Teflon lined cap. The hydrolysed samples were diluted with 12ml of water and further hydrolysed for 18 hours at 95°C. After complete hydrolysis, the reaction mixtures were allowed to cool to room temperature. The samples were derivatised to be volatile prior to GC-MS analysis. Derivatisation was achieved by treating each sample with 0.1 and 0.02ml TMS and pyridine respectively. The reaction was enhanced by heating at 65°C in a water bath for 20 minutes. The hydrolysates were directly filtered to vials through 0.22µm Nylon syringe filters without any dilutions. The liberated monosaccharides were converted into alditol acetates and were analysed by GC-MS. The sugars were quantified by Agilent 6890N GC Coupled with an Agilent 5975 MSD detector: column Rtx®-5MSsil MS w/Integra Guard (30m, 0.25mm ID, 0.5µm film thickness); He carrier gas 1ml/min; injection temperature 280°C, initial column temperature 150°C, ramped at 8°C/min to 325°C, hold 10 minutes; scanning mass range 35 to 750 m/z. Chemstation program (NIST05) was used for data analysis and for library matches.

#### **3.7.2 Structural characterisation of the LCC fractions**

The isolated lignin-carbohydrate fractions were analysed through thioacidolysis according to a method developed by Hoon et al. (2002). The objective of doing thioacidolysis prior to

chromatographic analysis is that it gives high yields of monomeric products through hydrolyses to yield less complex mixtures that can be quantified and assigned. Analysis by thioacidolysis reveals that approximately 10% of the  $\beta$ -O-4- structure in lignin is cleaved due to processing (Önnerud et al. 2003). The cleavage of  $\beta$ -O-4- structures is an indication of better processing resulting to high yield either cellulose during pulping of simple sugars for enzymatic hydrolysis (Wu et al. 2014).

The isolated lignin-carbohydrate fractions were dried in a desiccator under phosphorous pentoxide overnight. 0.03g of each sample of was dissolved in 6ml of dioxane with continuous stirring for 20 minutes. The mixture was then transferred to a 9ml screw top vial with a Teflon cap and 0.9ml of ethanethiol and 0.225ml boron-triflouride were added. The pH of the mixture was adjusted by adding dioxane to the mark (to 9mL of the vial). The vials were placed in an oil bath at 100°C for 4 hours to facilitate complete hydrolysis. The vials were then cooled in an ice bath for 20 minutes. The mixture was then transferred into a separating funnel separated through washing 3 times with 50ml of a 1:1 ratio dichloro-methane water mixture. The aqueous phase was adjusted to pH 3 – 4 by adding 2.5ml of a 0.4M NaHCO<sub>3</sub> (sodium hydrogen carbonate) solution. Three extractions were done with 10ml dichloro-methane and dried over magnesium sulphate. The solutions were concentrated on a rotator vapour at 40°C. The concentrated samples (~ 0.002ml) were dissolved in 2ml THF and stabilised with butylated hydroxytoluene. For GC-MS analysis, the samples were dissolved in 200 $\mu$ l pyridine and derivatised with 100 $\mu$ l N,O-Bis-(trimethylsilyl)-acetamide. The reaction was carried out in a water bath for 20 minutes at 65°C. The supernatant was filtered through 0.22 $\mu$ m Nylon syringe filters prior to injection directly into the gas chromatography coupled with mass spectroscopy (GCMS).

The thioacidolysed LCCs were also analysed via GC-MS analysis and were identified by the mass spectrometer. The degraded thioacidolysis monomers (30 $\mu$ l) were silylated with 100 $\mu$ l N,O-bis-(trimethyl)-trifluoroacetamide (BSTFA) and 50 $\mu$ l pyridine in a 200 $\mu$ l GC vial. The derived monomers were quantified by Agilent 6890N GC Coupled with an Agilent 5975 MSD detector: column **Rtx®-5MSsil** MS w/Integra Guard (30m, 0.25mm ID, 0.5 $\mu$ m film thickness); He carrier gas 1ml/min; injector temperature 280°C, initial column temperature 150°C, ramped at 8°C/min to 325°C, hold 10 minutes; scanning mass range 35 to 750 m/z. Chemstation program was used for data analysis and for library matches, NIST05 was used.

### 3.8 Size exclusion chromatography (SEC) analysis method

Prior to size exclusion chromatography the samples were acetylated so that they can be less polar and more volatile to be suitable for analysis. Acetylation is a process of introducing an acetyl group into a compound, substituting an active hydrogen atom. This is usually done by using acetic anhydride as an acetylating agent and the procedure was done as follows for this study: The thioacidolysed samples of 0.1ml were acetylated in 1.5ml dichloro-methane containing 0.2ml acetic anhydride and 0.2ml pyridine for 40 minutes in a water bath at 50°C. All volatile components were evaporated completely by co-evaporation with ethanol under reduced pressure in a vacuum pump. The residues were used for GC-MS characterisation. The amounts of the individual lignin monomers were determined using response factors derived from pure monomer standards using tetracosane was used as an internal standard.

The SEC setup runs using tetrahydrofuran (THF) as a mobile phase at a flow rate of 1ml/min. Butylated hydroxytoluene (BHT) was added to the THF as a stabiliser at a concentration of 0.0125% (w/v). The columns installed at the time were 2 x PL-Gel Mixed-C, 5µm particle size, columns (300 x 7.5mm) from Polymer Laboratories, with a 50 x 7.5mm, 3µm (particle size) PL-Gel guard columns (from Polymer Laboratories).

The instrument was a Waters with various components: Waters 717 plus auto-sampler, Waters isocratic 1515 pump with inline degasser, Waters 2487 dual wavelength UV detector, Waters 2414 RI detector. The use of SEC system would assist in identifying the type of linkages between lignin monomers depending on the quantity of monomeric structures, dimers and oligomers.

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## **CHAPTER 4: RESULTS AND DISCUSSIONS**

### **4.1 Chemical composition of the biomass materials**

The aim of this chapter is to elaborate on the finding gathered of this study on lignin-carbohydrate complexes (LCCs). Based on the results obtained, effect of LCCs on the processing of biomass materials will be discussed.

Table 4.1 presents the chemical composition of the raw feedstocks i.e. *Eucalyptus grandis* (*E. grandis*) and sugarcane bagasse (SCB). The 0.45% ash content observed with for *E. grandis* was slightly higher than the reported in literature where it ranges between 0.06 and 0.37% (da Silva et al. 2010) whereas it was a bit lower compared to other *E. grandis* used at Stellenbosch University in previous years where the range was between 1.1 and 1.5% (Vena, 2013). The 1 – 4% ash content obtained with sugarcane bagasse was within the range reported in the literature (Hugo, 2010). The high ash content is due to high silica and soil contamination of sugarcane bagasse when it is harvested (Sun et al. 2002).

**Table 4.1:** Chemical composition of *Eucalyptus grandis* and sugarcane bagasse.

Feedstock	Moisture [%]	Ash [%]	Extractives [%]	*Cellulose		*Hemicelluloses			Lignin				S/G ratio
				Total [%]	Glucose [%]	Total [%]	Xylose [%]	Arabinose [%]	Total [%]	Guaiacyl [%]	Syringyl [%]	Hydroxyphenyl [%]	
<i>E. grandis</i>	2.64	0.45	2.37	46.01	42.10	19.59	17.61	1.98	#26.57	0.20	3.80	n.d	1.1
SCB	3.15	1.58	2.62	46.57	47.04	25.6	24.92	1.49	#19.52	0.17	0.81	0.80	2.35

#Klason lignin

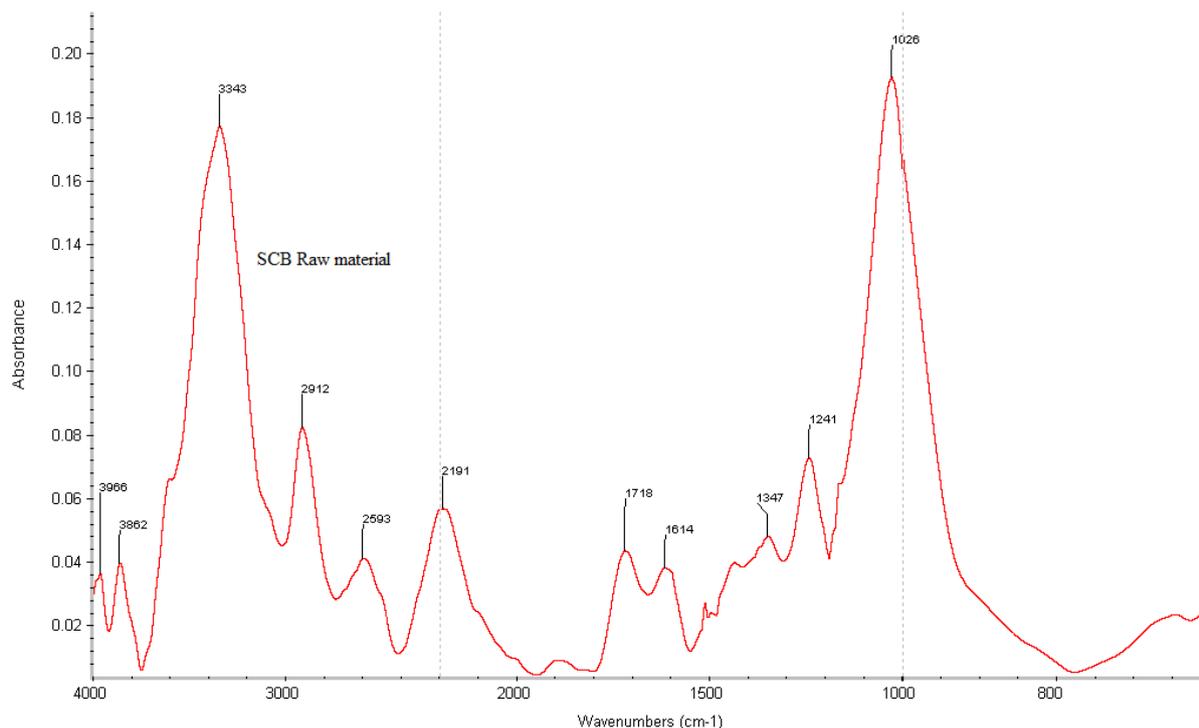
\*Based on oven dry mass

n.d. = not determined

Table 4.1 showed that the lignin content of the sugarcane bagasse (19.52%) was less than that of *E. grandis* (26.57%) which is typical of grasses (Fengel and Wegener, 2003). This is considered a positive raw material characteristic as the material then needs less harsh conditions for processing e.g. pulping and steam explosion than enzymatic hydrolysis. The easier processing of sugarcane bagasse compared to *E. grandis* is due to its S/G ratio aspect. In addition, the lignin structure of sugarcane bagasse the values were also similar to those reported in the literature (McKendry, 2002). The syringyl/guaiacyl (S/G) ratio was 1.1 (Table 4.1) for *E. grandis* which is the close value obtained by Rencoret et al. (2008), where they compared different methods to determine the S/G ratio of *Eucalyptus grandis*. The S/G ratio for SCB was slightly higher at 2.35 compared to the 1.1 for *E. grandis*. The ratio of S/G provides a pivotal parameter for gauging the expected chemical reactivity of delignifying plant cell walls and for determining the energy requirements for pulping and bleaching feedstocks (Tsutsumi et al. 1995). This means that a material with high S/G ratio requires less severe conditions of pre-treatment/processing to the same degree of delignification. The exact effect of lignin S/G ratio on processing has not been elucidated, this metric has proven to be integral to understanding the role lignin structure plays in deconstructing biomass (Lupoi et al. 2014).

The average hemicelluloses content of *Eucalyptus grandis* is ~22.10% based on oven dry weight (Postma, 2012). The hemicelluloses content which is 19.59% for the *Eucalyptus* samples used in this study falls within the range. The glucose and hemicelluloses contents of *E. grandis* and sugarcane bagasse are similar to the values reported in literature as they range from 42.10 and 19.59% for *E. grandis* and 47.04 and 26.41% for sugarcane bagasse respectively.

Further characterisation of sugarcane bagasse raw material with FTIR spectroscopy revealed some similarities and differences in support of the wet chemical data presented in Table 4.1. Figure 4.1 shows the spectrum of sugarcane bagasse. Table 4.2 presents a summary of the higher and main bands observed in the feedstock.



**Figure 4.1:** FTIR spectrum of raw sugarcane bagasse lignocellulosic material

**Table 4.2:** Infrared main transition for lignocellulosic material

Wavenumber (cm <sup>-1</sup> )	Vibration	Source
3343	O-H linked shearing	Polysaccharides
2912	C-H symmetrical stretching	Polysaccharides
1718	C=O unconjugated stretching	Xylans
1614	C-O aromatic ring	Lignin
1347	C-O-C asymmetrical stretching	Cellulose

The cellulose, hemicelluloses and lignin peaks were recorded at 1000cm<sup>-1</sup>, 1718cm<sup>-1</sup> and 1614cm<sup>-1</sup> region respectively. The hemicelluloses content was intensely high while the lignin content was slightly low. The FTIR analysis clearly supports the data obtained through wet chemistry analysis as it is indicated in Table 4.1 for sugarcane bagasse analysis where the lignin content was 19.52% while hemicelluloses content was 25.6%.

The sugarcane bagasse material showed to have intense guaiacyl content while the 1347cm<sup>-1</sup> indicated less pronounced syringyl breathing with CO stretching (Fengel and Wegener, 2003). High guaiacyl content has been reported to negatively affect processing of lignocellulosic material in more ways than one, either being in pulping and bleaching or enzymatic hydrolysis and bio-fuel production and etc. (Ramos et al. 1992 and Muguet et al. 2012). This is due to the fact that there is an absence of methoxyl groups and therefore, there will be high cross-linking that might occur when the material is subjected to processing. The structure of lignin is important for processing not only the content. Low presence of methoxyl group content is a good property for processing. So therefore, low lignin content is a good property however, the structure of lignin is also important.

#### **4.1.1 Compositional analysis of the pre-processed materials**

The data tabulated in Table 4.5 are the values for the solid residue after hemicelluloses pre-extraction and not for the extracted polysaccharides. It is observed from Table 4.5 that the glucan content of the alkaline-pre-extracted xylan from *Eucalyptus grandis* (*E. grandis*) double that of the raw material. This is due to the high lignin content that is removed during the lignin reactions with an alkali as has been explained in the reactions in Section 2.2.3. This has been clearly explained by Vena et al. (2013a) that this increase might be due to the disruption of the bonds between lignin and carbohydrates (Peng et al. 2012). The amount of the lignin that is removed is compensated by the cellulose (Fengel and Wegener, 2003). This is due to the high solubility of hemicelluloses in alkaline solutions than in acidic medium with less degradation of furfural substances (Cheng et al. 2010). Considering the above statement around the calculation, it is assumed that the glucan content is 95% that of the original material meaning that 5% has been removed. Table 4.5 shows that the alkaline extraction methods selectively removed the hemicelluloses and the lignin and preserved most of the cellulose. For application purposes where cellulose is the key component e.g. in pulping this means that the method can be integrated into the process provided that the strengths properties meet product specifications.

**Table 4.5:** Compositional analysis of *E. grandis* chips after xylan pre-extraction.

Feedstock	Moisture [%]	Ash [%]	Extractives [%]	Cellulose		Hemicelluloses			Lignin				S/G ratio
				Total [%]	Glucose [%]	Total [%]	Xylose [%]	Arabinose [%]	Total [%]	Guaiacyl [%]	Syringyl [%]	Hydroxyphenyl [%]	
<i>E. grandis</i> (control)	2.64	0.45	2.37	46.01	42.10	19.59	17.61	1.98	26.57	0.20	3.80	n.d	1.10
<i>E. grandis</i> Alkaline pre- extracted	7.87	0.17	n.d	50.59	51.10	32.22	32.01	0.21	10.12	0.93	6.23	n.d	7.70
<i>E. grandis</i> Dilute acid pre- extracted	15.13	2.34	n.d	42.70	43.13	10.52	10.52	n.d	14.39	2.54	0.31	n.d	1.33

The similar phenomenon was observed for the dilute acid-pre-extracted *Eucalyptus grandis*, but lignin was detected as this is due to the formation of pseudo-lignin that result because of condensation reactions that occur when lignocellulosic material is subjected to acidic conditions. The mechanisms of condensation reactions are detailed in Section 2.2.3. An increase in the solubilisation of xylan content (Vena et al. 2013b and Vena et al. 2013c) and low lignin content in the material will result in low quantities of chemicals being used when the material is being subjected to pulping or enzymatic hydrolysis (Li et al. 2010). The material will be easily delignified as the structure has been physical loosened and non-cellulosic components have been partially or completely removed (Vena, 2013).

Dilute acid-pre-extraction resulted in an elevated ash content of the material. An increase in ash content is not a satisfactory indication of further processing the material, as lots of chemicals will be required for downstream processing (Mustajoki et al. 2010). It was also observed that the S/G ratio was inversely proportional to the lignin content of the material. For alkaline pre-extracted material, the S/G ratio of the material was higher than the control material. This was due to high degradation of the lignin as a result of the process. This was not the case for the dilute acid pre-extracted material. The guaiacyl content was higher than syringyl which shows that the material will required high quantities of delignifying chemicals as the material is less reactive. According to literature (Muguet et al. 2012), a material that has high guaiacyl content than syringyl is difficult to delignify as guaiacyl is less reactive than syringyl. This is due to the availability of the methoxyl groups attached in positions 3 and 5 of the *p*-hydroxyphenyl as seen in Figure 2.5.

The present section summarizes steam explosion process parameters and product properties. Steam explosion of biomass is a pre-treatment process that opens up the fibres, and makes the biomass polymers more accessible for subsequent processes, i.e. fermentation, hydrolysis or densification processes. Steam explosion has been shown to be a valuable and important technology to open up the biomass fibres, to improve the recovery of sugars and other useful compounds from biomass.

The component concentrations are based on oven dry weight of the original material. It can be observed in Table 4.6 that the steam exploded sugarcane bagasse material at conditions 2 and 3 (abbreviated as SE 2 and SE 3 on the Table) had the highest glucan content than condition 1 (SE 1). However, low hemicelluloses content (xylan) was detected for SE 3 whereas SE 1 had the highest content than the other two steam explosion conditions (SE 2

and SE 3). Studer et al. (2011) reported that xylose does not have a negative effect on the sugar yield during enzymatic hydrolysis as the furfural and other by-products affect enzyme digestibility. The steam exploded material has a proportionally higher amount of ash content compared to the untreated (control) material. This is due to that ash is preserved during steam explosion; the change in the ash content can be used to estimate the loss of organic materials as volatiles. The similar phenomenon of ash content change was observed by Horn et al. (2011). The high S/G ratio in the material indicates highly decreased lignin content in the materials. The lignin content was decreased for the steam exploded materials compared to the control material that was around 19%. The similar reduced lignin content in the range obtained in Table 4.6 for the steam exploded sugarcane bagasse has been observed reported in various studies for wheat straw and other grass lignocellulosic materials carried by Carvalheiro et al. (2008); Zhang et al. (2008); Sun et al. (2002) and Fang et al. (2011).

**Table 4.6:** Compositional analysis of steam exploded pre-treated sugarcane bagasse in various processing methods compared to the raw material (control).

Feedstock	Moisture [%]	Ash [%]	Extractives [%]	*Cellulose		*Hemicelluloses			Lignin				S/G ratio
				Total [%]	Glucose [%]	Total [%]	Xylose [%]	Arabinose [%]	Total [%]	G [%]	S [%]	H [%]	
SCB (control)	3.15	1.58	2.62	46.57	47.04	25.6	24.92	1.49	19.52	0.17	0.81	0.80	2.35
SCB SE 1	4.85	5.00	0.74	79.20	80.00	58.00	56.00	2.00	n.d	6.35	8.60	0.99	10.00
SCB SE 2	5.72	6.00	1.96	112.86	114.00	52.30	51.00	1.30	0.10	0.18	16.06	2.82	10.00
SCB SE 3	6.21	5.00	2.15	112.86	114.00	13.00	13.00	n.d	0.2	0.13	19.86	1.22	10.00

\*The components are based on Oven Dry weight of the original material. AE = alkaline extracted; DE = dilute acid extracted; SE = steam explosion based on conditions on Section 3.5.3; IL = ionic liquid based on conditions in Section 3.5.2.

**Table 4.7:** Compositional analysis of ionic liquid fractionated sugarcane bagasse in various processing methods compared to the raw material (control).

Feedstock	Moisture [%]	Ash [%]	Extractives [%]	*Cellulose		*Hemicelluloses			Lignin				S/G ratio
				Total [%]	Glucose [%]	Total [%]	Xylose [%]	Arabinose [%]	Total [%]	Guaiacyl [%]	Syringyl [%]	Hydroxyphenyl [%]	
SCB IL 1	7.62	0.0	n.d	21.54	21.76	16.40	15.13	1.27	0.1	16.58	0.68	7.83	9.27
SCB IL 2	7.77	0.0	n.d	38.41	38.80	13.88	12.91	0.97	0.1	17.13	27.77	5.34	3.29
SCB IL 3	7.23	0.0	n.d	49.98	50.48	15.24	14.22	1.02	0.01	5.02	31.16	7.06	3.54

The ionic liquid fractionated sugarcane bagasse was analysed according to the data presented in Table 4.7. The material that was processed under severe conditions (i.e. Ionic Liquid fractionation process 1 denoted as (IL3)) had high glucan content than any other condition (i.e. the milder condition of ionic liquid fractionation denoted as IL1 and IL2). All the ionic liquid fractionated materials had a high xylan content compared to the untreated (control SCB material). The S/G ratio of the material was high in all treatments however compared to the SCB control material, and there was no significant difference from each ionic liquid fractionation as they all resulted in high lignin content removal and same S/G content which was 10. The ionic liquid method did not affect the sugars content (there were no sugar losses) according to Table 4.7 as it targeted lignin removal. The high S/G ratio in the material indicates high glucan and xylan release (Studer et al. 2011), in the case of this study there will be easy digestibility when the material has been subjected to enzymatic hydrolysis and the less to minimal substrate (lignin) will influence enzyme conversion (Tahezardeh et al. 2008).

## Summary

In general, alkaline xylan pre-extraction does not only remove the xylan, but lignin as well due to the amenability of lignin under alkaline conditions. In essence, alkaline pre-extraction is a pre-pulping step; and as such subsequent pulping of xylan pre-extracted biomass feedstock should require less harsh conditions compared to when un-extracted material is pulped as also observed by Cheng et al. (2010). Dilute acid pre-extraction does not enhance the cleavage of hemicelluloses-lignin complexes compared to alkaline pre-extraction (Lawoko, 2005). It is therefore important to understand how the pseudo-lignins that are formed during condensation are associated with hemicelluloses, as it has been reported in study by Hu et al. (2012) on how they affect the rate of pulping and enzymatic processing.

Table 4.6 in general shows that there will be less substrate interference with digestibility as the material has high cellulose for the ionic liquid fractionated (Karatzos, 2011). The high crystallinity cellulose structure is disrupted by the ionic liquid in addition to lignin dissolution and results in complete digestion.

But for steam exploded material, this is one method that is highly recommended for biomass pre-treatment prior to enzymatic hydrolysis because there is high recovery of sugars for this pre-treatment process.

### *Steam explosion*

Figure 4.2 shows FTIR spectra of steam explosion treated sugarcane bagasse in comparison to the control. The graphic illustrates the compositional and structural changes that the material went through as a result of the process. Further, the data presented here support the data obtained with wet chemical methods. Differences were observed between the raw and steam exploded sugarcane bagasse and the in the region from 3500 to 1000 $\text{cm}^{-1}$ . The absorption bands at around 1700, 1600 and 1500 $\text{cm}^{-1}$  were attributed to the functional groups present in the lignin that is associated with cellulose and hemicelluloses before and after the process of delignification (pre-treatment and enzymatic hydrolysis). Two major bands were of importance were also observed i.e., the bands in the 1500 and 1200 $\text{cm}^{-1}$  regions. The band at 1500 $\text{cm}^{-1}$  was more enhanced in the pre-treated materials especially in SE3, while it was fairly reduced in the control sample. This was due to the presence of pseudo-lignins that are formed as a result of condensation reactions between lignin and acetic acid generated (Hu et al. 2012). The band at 1240 $\text{cm}^{-1}$  was less pronounced in the pre-treated materials as a result of the removal of lignin, in contrast to the untreated material where it was more pronounced. The reduction, absence and presence of these bands indicate the presence or removal of lignin. In SE1 and SE2; lignin was removed while for SE3 there was a high formation of pseudo-lignin that could affect the downstream processing, that is enzymatic conversion of the material.

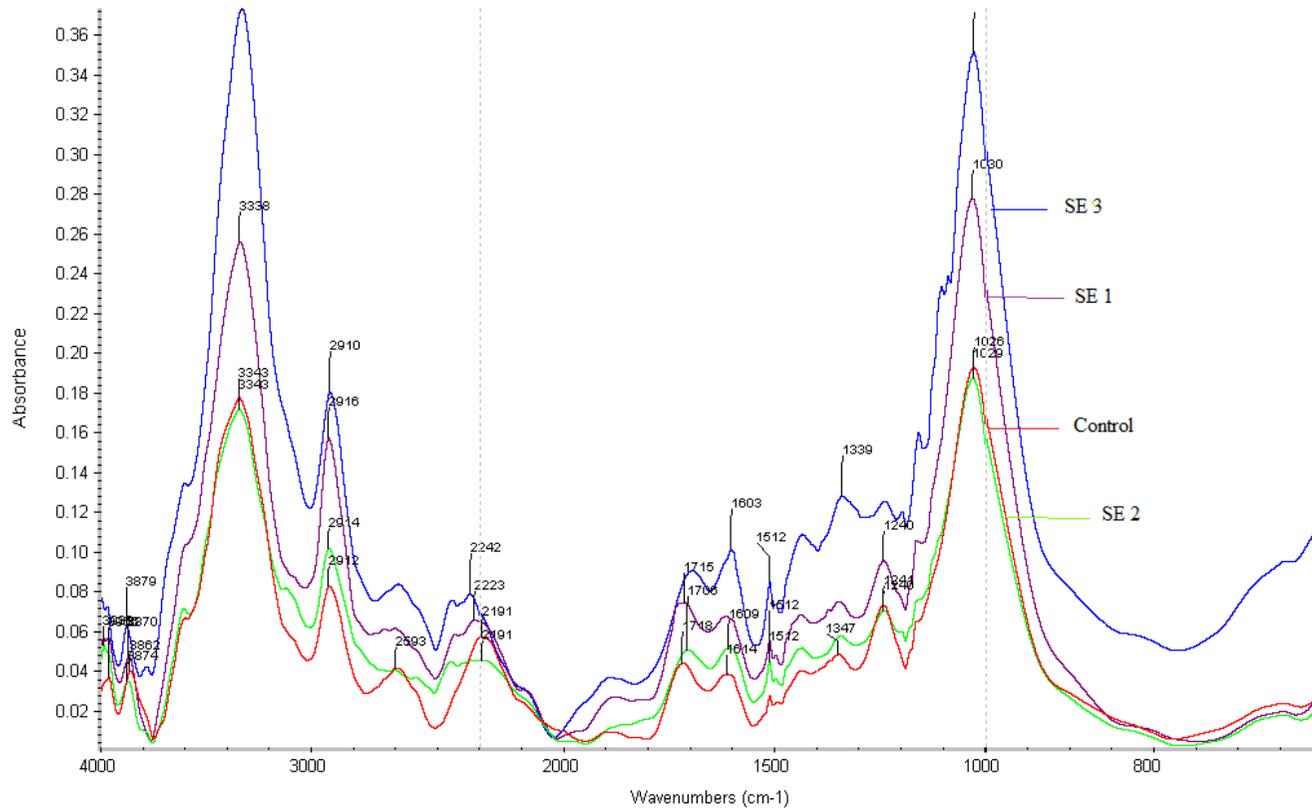


Figure 4.2: FTIR spectra of steam exploded sugarcane bagasse

*Ionic liquid fractionation*

A comparison study was conducted between the untreated sugarcane bagasse to ionic liquid fractionated sugarcane bagasse at different condition as presented in Figure 4.3. Also a major focus was the absorption bands at 1512 and 1200 $\text{cm}^{-1}$ . For all the fractionated materials, the peak at 1512 $\text{cm}^{-1}$  was absent compared to the untreated material. The 1241 $\text{cm}^{-1}$  peak was fairly reduced in all the pre-treated materials compared to the untreated material. This phenomenon describes the removal of lignin by ionic liquids resulting to a slightly “pure” cellulose material with less abundant xylose present.

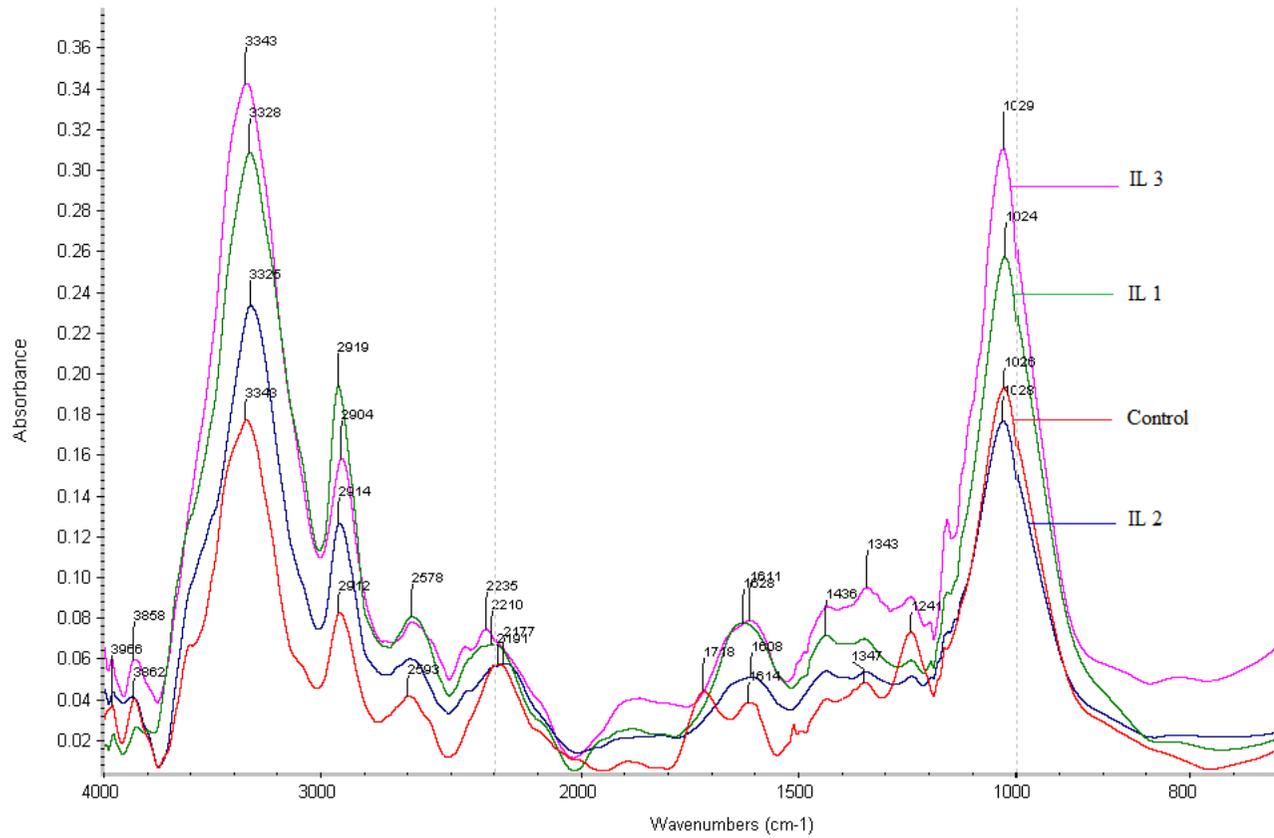


Figure 4.3: FTIR spectra of ionic liquid fractionated sugarcane bagasse

In general, the FTIR data completely supported the inverse relationship between the S/G ratio and the lignin content in the materials. For the steam explosion process, the lignin peaks were observed in the region of  $1500\text{cm}^{-1}$  whereas for the ionic liquid processed materials were fairly reduced compared to the control material. This means that lignin was removed in later processed materials compared to the former supporting the chemical composition of the materials in Tables 4.5 and 4.6. The sharp cellulose peaks ( $\sim 1000\text{cm}^{-1}$ ) were observed for all the processed materials compared to their control materials, this suggested high glucose concentrations as indicated in the Tables 4.5 and 4.6. Therefore, coupling chemical composition with FTIR analysis, verifies any data that might have some discrepancies.

#### **4.1.2 Comparison between the lignin content and the S/G ratio of the pre-processed (pre-extracted, steam exploded and fractionated) materials in relation to the xylan content**

From Tables 4.7 and 4.8, the quantity of lignin present in the material is inversely related to the ratio of the lignin monomers i.e. the higher the lignin content, the lower the S/G ratio. It is evident that the residual lignin is mostly linked to xylan as the structure of the lignocellulosic material fibres have been loosened by pre-extraction (Lawoko, 2005, Li et al. 2011 and Liu et al. 2012). According to the literature, the lignin that is linked to xylan is more resistant when the material is subjected to processing; it slows down the delignification process (Lawoko, 2005, Lawoko et al. 2006 and Li et al. 2011). The high resistance is reported to be due to the  $\beta$ -O-4 bond type between lignin and xylan which results to a linear coupling mode structure (Lawoko, 2005). Studies conducted by Santos et al. (2012c) have established a correlation between the structural forms of  $\beta$ -O-4- structures found in lignin and S/G ratio, indicating that the syringyl/guaiacyl composition is the key factor governing the proportion of  $\beta$ -O-4- structure of lignin. S/G ratio is a key factor affecting the rate of processing being pulping, enzymatic hydrolysis or pre-treatment (Santos et al. 2011). It also seems that the S/G ratio appears to control the  $\beta$ -O-4- content and the degree of condensation (Santos et al. 2012c).

**Table 4.7:** Relationship representation between the lignin content and the S/G ratio of the *E. grandis* pre-extracted materials

<b>Component (%)*</b>	<b>Control</b>	<b>Alkaline pre-extracted</b>	<b>Dilute acid pre-extracted</b>
<b>Xylan</b>	17.61	12.01	10.52
<b>S/G ratio</b>	1.10	7.70	1.12
<b>Klason lignin</b>	25.23	10.12	14.39

**Table 4.8:** Relationship between the lignin content and the S/G ratio of sugarcane bagasse pre-processed materials

<b>Component (%)*</b>	<b>Control</b>	<b>AE</b>	<b>DAE</b>	<b>SE 1</b>	<b>SE 2</b>	<b>SE 3</b>	<b>IL 1</b>	<b>IL 2</b>	<b>IL 3</b>
<b>Xylan</b>	24.04	21.7	13.68	57.5	51.15	12.80	71.2	67.7	72.17
<b>S/G ratio</b>	2.35	3.93	3.15	10.15	10.04	10.02	10.03	10.11	10.28
<b>Klason lignin</b>	18.8	11.17	16.63	n.d	0.10	0.20	0.10	0.10	0.01

\*Based on oven dry mass

The *E. grandis* material that was alkaline pre-extracted had a lower xylan content than the control material (*E. grandis* = 12.01 and control = 24.94%). This was due to the peeling reactions that are detailed in the literature in Section 6.2. When lignocellulosic materials is subjected to mild alkali conditions the aforementioned reactions occur and causes dissociation of the linkages between lignin itself and lignin that is associated with polysaccharides and resulting to high content of free lignin in which it is determined to be monolignols (Sun et al. 2000).

The ionic liquid fractionation method removed approximately 99% of lignin in the biomass materials. This will make the material being more accessible to enzymes during enzymatic hydrolysis process (Diedericks et al. 2011). The materials had high syringyl and hydroxyphenyl contents compared to guaiacyl content, therefore, the materials will be more reactive (Wang et al. 2012) and the residual lignin will be easily hydrolysed and will not limit the action of enzyme on the substrate as explained in Section 2.5 (Qin, 2010). The guaiacyl-rich lignin has a more branched structure and a higher degree of polymerisation than syringyl-rich lignin (Stewart et al. 2009), it has therefore been speculated that syringyl-rich lignin may have a lower softening point and is more easily relocated on the surface than guaiacyl-rich lignin. This phenomenon results to improved enzyme digestibility (Li et al. 2010). Furthermore, the high S/G ratio indicates that the lignin present in the materials is degraded (or very low) (Studer et al. 2011).

#### **4.2 Enzymatic hydrolysis of pre-treated sugarcane bagasse**

The solid residues of sugarcane bagasse from steam explosion pre-treatment and ionic liquid fractionation were enzymatically hydrolysed using a blend of enzymes i.e. Accelerase 1500 and Novozyme 188 cellulase. The sugar yields gave estimation of how much of the bound sugars were liberated during hydrolysis as a result of the pre-treatment process or ionic liquid treatment. . The processing conditions are detailed in Section 3.6.

The two biomass processing methods resulted in materials with different visual appearances. The pre-treated material was turned into slurry containing solid particles. The condition regarded as a severe condition, gave much darker and more fragmented slurry compared to the poor condition (Table 3.4). The condition that was regarded as severe i.e. (SE3) gave a higher digestibility of 77.9% as shown in Table 4.9 for the steam exploded material. The condition regarded as a good condition i.e. (SE2) gave 39.5%, while the IL1 condition was regarded as poor gave higher digestibility of 77.5% compared to the untreated (control) material that had a yield of 20.3%. This might be due to less condensation reactions occurring

between the ionic liquid and the lignocellulosic material. The limited enzymatic hydrolysis of untreated and SE1 samples are due to the limited or no modification of the crystalline structure of cellulose and the recalcitrance of the lignin (Chandra et al. 2007).

**Table 4.9:** Sugar analysis of enzymatic hydrolysed pre-processed sugarcane bagasse

<b>Component (%)*</b>	<b>Control</b>	<b>SE 1</b>	<b>SE 2</b>	<b>SE 3</b>	<b>IL 1</b>	<b>IL 2</b>	<b>IL 3</b>
<b>Xylan</b>	10.70	13.90	14.70	9.30	24.60	14.90	16.10
<b>Glucan</b>	9.60	12.50	24.70	68.60	51.30	28.40	31.10
<b>Arabinan</b>	0.00	0.50	0.10	0.00	1.60	0.90	1.00
<b>Total soluble sugar</b>	20.30	26.90	39.50	77.90	77.50	44.20	48.20

SE = steam explosion based on conditions on Section 3.5.3; IL = ionic liquid based on conditions in Section 3.5.2

By comparing the pre-treated material with untreated material yields, the pre-treated materials showed higher sugar yields, while the SE1 material was in the same range as the control material. On average, more than twice the glucose yields the pre-treated materials was obtained compared to the untreated material. In general, SE3 and IL1 have the highest sugar yields that are 77.9 and 77.5% respectively. It was observed that only IL1 with 24.6% doubled the xylose content compared to the untreated material with 10.7% while all other materials included the steam exploded pre-treated material were in the same range as the untreated material (9.3 to 16.1%). It can be observed that the removal of lignin by either method was effective since the sugar yield in enzymatic hydrolysis were doubled.

When incorporating the pre-treatment steps in calculating the yields, thus obtaining the overall yields of sugars, the combination effect of both pre-treatment and enzymatic hydrolysis can be clearly observed. The sugar yield is regarded as the most important information since it deals with all the processing steps from raw lignocellulosic material to fermentable sugars. According to the description of conditions in Section 3.5.3, it can be observed that even though the sugars were regarded as degraded during the pre-treatment steps, it was observed that they did not affect enzymatic hydrolysis; in fact their yields were much higher. Also the close association and complexity of the carbohydrates with lignin is the main obstacle in lignocellulosic degradation (Lee et al. 2009 and Studer et al. 2011).

#### **4.3 Compositional analysis of water-insoluble-solids (WIS) from steam exploded and ionic liquid fractionated materials**

The chemical composition analysis for the water insoluble solids was done to determine the amount lignin that might have affected the rate of digestibility during enzymatic hydrolysis (Table 4.10) and in turn the effect the residual lignin that is associated with the carbohydrates in the WIS (the solid material that was not digested after enzymatic hydrolysis) generated after pre-treatment and enzymatic hydrolysis.

According to Table 4.10, the WIS generated from steam explosion and enzymatic hydrolysis was hydrolysed as the solid recoveries retained approximately 0.5% glucose and 0.2% xylose. The pre-treated values were in the same range as the WIS generated from the untreated material. The WIS generated from SE1 had higher acid insoluble lignin content (Klason) of 2.2% than any other material. This might be due to the formation of pseudo-lignins during steam explosion as a result of condensation reactions that might have occurred as a result of the acidic conditions which develop during steam explosion. Pseudo-lignins are detrimental

to the rate of enzymatic conversion (Hu et al. 2012) they affect the actual determination of lignin content in the material.

**Table 4.10:** Chemical composition of the WIS from steam exploded pre-treated sugarcane bagasse after enzymatic hydrolysis

<b>Component (%)*</b>	<b>Control</b>	<b>SE – 1</b>	<b>SE – 2</b>	<b>SE – 3</b>
Xylan	0.29	0.22	0.09	n.d
Glucan	0.49	0.45	0.46	n.d
Arabinan	0.02	0.01	0.01	n.d
Klason lignin	1.90	2.22	0.87	n.d
Ash	0.10	0.00	0.01	n.d

\*The components are based on the oven dry weight of the original material

n.d = Not detected

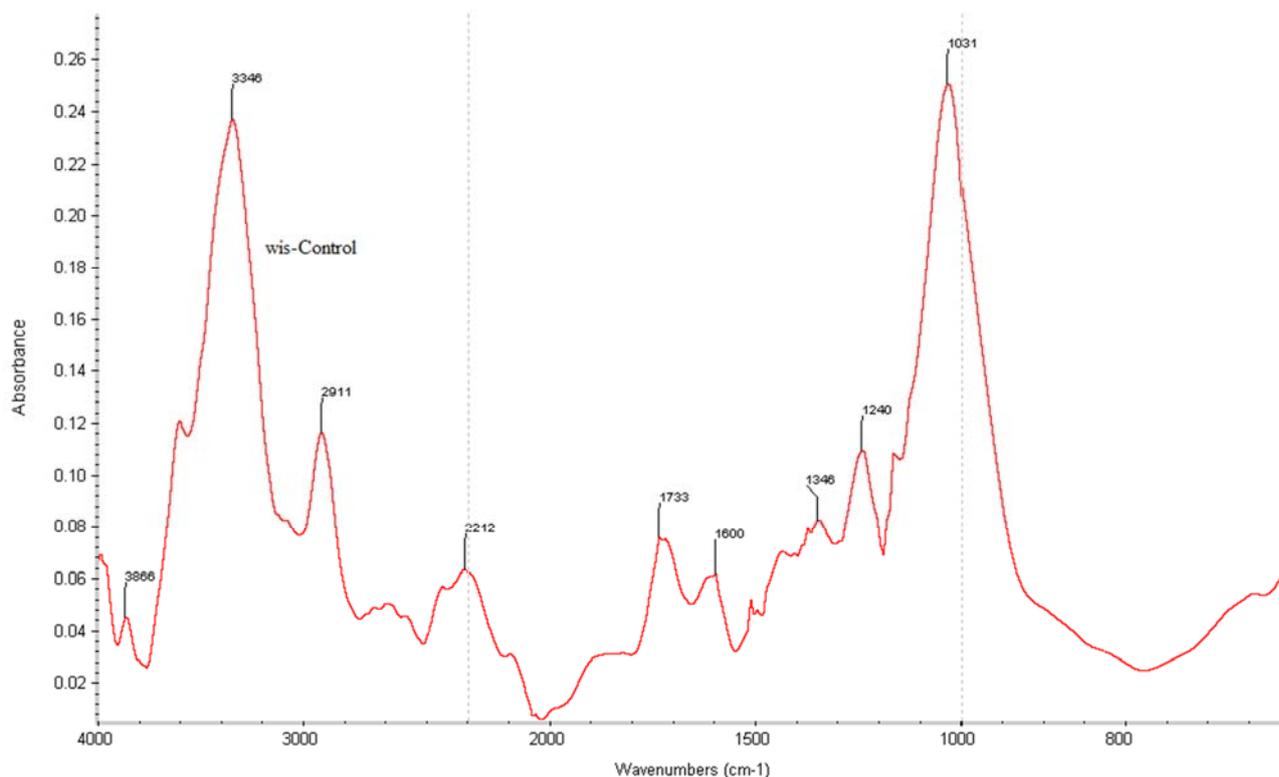
The proportion of acid insoluble lignin (Table 4.11) in WIS materials generated from ionic liquid fractionated then enzymatic hydrolysed material increased with the severity of the conditions (Table 3.4), while the proportions of the carbohydrates retained showed an inverse trend. In all the materials, there was no significant difference in the glucan and xylan content compared to the untreated material.

**Table 4.11:** Chemical composition of WIS from ionic liquid fractionated sugarcane bagasse after enzymatic hydrolysis

<b>Component (%)*</b>	<b>Control</b>	<b>IL – 1</b>	<b>IL – 2</b>	<b>IL – 3</b>
Xylan	0.29	0.15	0.17	0.17
Glucan	0.48	0.25	0.29	0.29
Arabinan	0.018	0.011	0.012	0.011
Klason lignin	1.90	0.33	0.31	0.40
Ash	0.0027	0.011	0.0021	0.0045

\*The components are based on the oven dry weight of the original material

FTIR analysis (Figure 4.4) of water-insoluble solid generated from raw sugarcane bagasse showed prominent peaks in the finger print region of  $1000\text{cm}^{-1}$ . The peaks were numbered and assigned to chemical compounds according to published literature (Takahashi et al. 1987). Most of the observed peaks in the biomass material represented major cell wall component such as hemicelluloses ( $1031\text{cm}^{-1}$ ) and lignin ( $1600\text{cm}^{-1}$ ); the shift in the peaks was observed as this meant there were fewer components in the material.



**Figure 4.4:** FTIR of the water-insoluble-solid generated from raw sugarcane bagasse material after enzymatic hydrolysis.

Closer inspection was done to determine the effect of pre-processing to enzymatic hydrolysis as the materials were subjected to ionic liquid fractionation and steam explosion (Figures 4.5 and 4.6) respectively.

Differences were observed between the raw and steam exploded sugarcane bagasse in the region from 3500 to 1000 $\text{cm}^{-1}$ . The absorption bands at around 1700, 1600 and 1500 $\text{cm}^{-1}$  were attributed to the functional groups present in the lignin that is associated with cellulose and hemicelluloses before and after the process of delignification (pre-treatment and enzymatic hydrolysis). Two major bands were also of importance i.e., the bands at around 1500 and 1200 $\text{cm}^{-1}$ . The band at 1500 $\text{cm}^{-1}$  was observed in the pre-treated materials more especially for SE3, while for the control it was fairly reduced. This was due to the formation of pseudo-lignins (Hu et al. 2012) that are formed as a result of condensation reactions resulting from acetic acid occurring generated during steam explosion. The 1240 $\text{cm}^{-1}$  band was less pronounced for the pre-treated materials in contrast to the untreated material indicating the removal of lignin. In samples SE1 and SE2; lignin was removed while in SE3 there was a high

formation of pseudo-lignin that could affect the downstream processing, that is enzymatic conversion of the material.

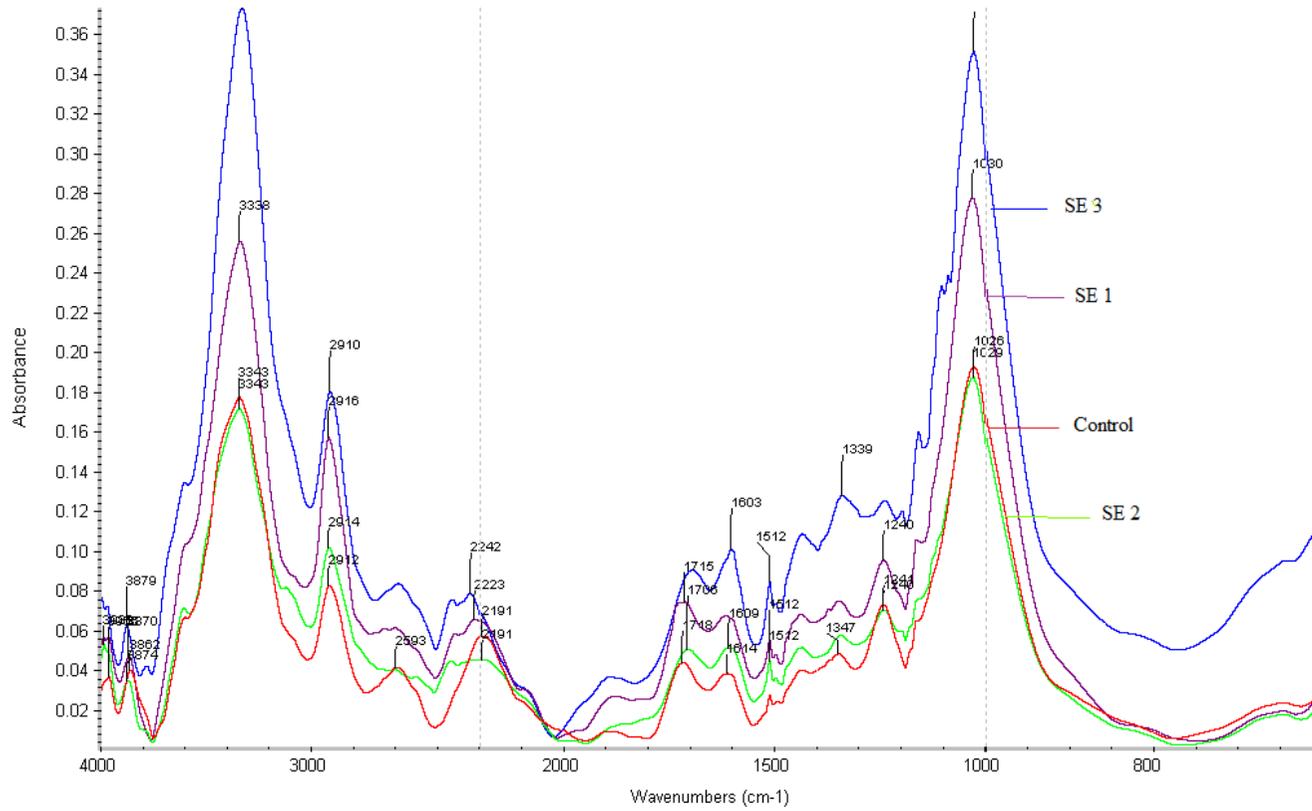
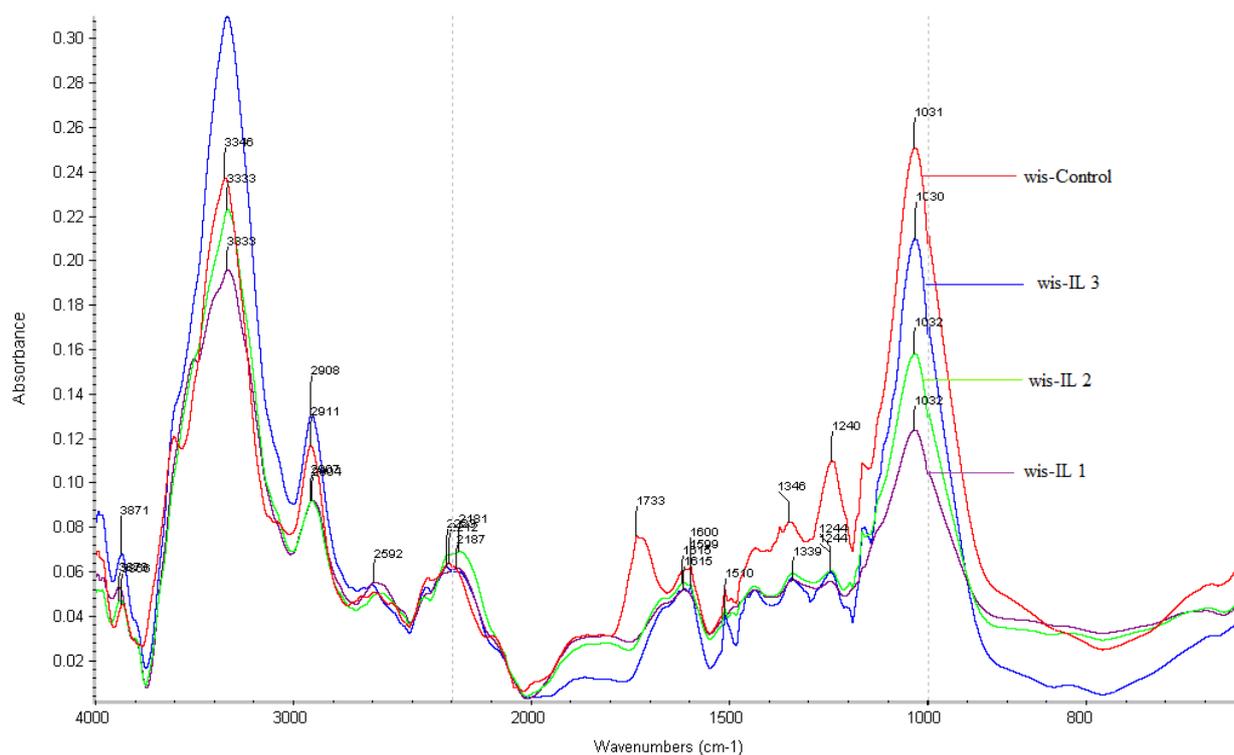


Figure 4.5: FTIR spectra of steam exploded sugarcane bagasse

The FTIR spectra of the WIS material generated from ionic liquid fractionated material with conditions detailed in Section 3.5.2 are illustrated in Figure 4.6. The aromatic skeletal vibrations for the materials were assigned in the 1500 $\text{cm}^{-1}$  region. The syringyl ring absorption was clearly seen at the 1300 $\text{cm}^{-1}$  region for all the materials. In contrast, the guaiacyl ring absorption appeared only as a shoulder at 1240 $\text{cm}^{-1}$  region. The absorption band around 835 $\text{cm}^{-1}$  originating from *p*-hydroxyphenyl units was not detected due to technical errors. The occurrence of the band at 1733 $\text{cm}^{-1}$  for the control material corresponded to carbonyl stretching, which could be attributed to the presence of lignin derivatives (pseudo-lignins).



**Figure 4.6:** FTIR spectra of water-insoluble-solids generated from ionic liquid fractionated materials.

In general, for both enzymatically hydrolysed materials (generated from IL and steam explosion prior to enzymatic hydrolysis), there was no significant amount of carbohydrates or lignin that was retained in all the samples. These results suggest that, very low to no lignin-carbohydrate complexes could be recovered or fractionated for the LCC study that will be detailed in following sections (Lawoko et al. 2011 and Li et al 2011).

#### 4.3.1 Comparison of the lignin content and S/G ratio of the pre-treated materials in relation to the sugar yields after enzymatic hydrolysis

The aim of this investigation was to determine the effect of lignin content and lignin structure i.e. lignin S/G ratio on monosaccharide sugar release from the selected biomass feedstocks. Also can a material with high or low sugar release be identified for LCC study analysis and draw conclusions on factors affecting enzymatic hydrolysis based on the outcomes of LCC study? It was important to understand the presence of the residual lignin by employing acidolysis analysis on the pre-treated material. It can be seen from monolignols that in general it would be difficult to process the materials and their yields would be affected significantly.

According to Table 4.12, all the steam exploded then enzymatic hydrolysed materials had high guaiacyl and hydroxyphenyl content than syringyl which is regarded as a highly reactive lignin building unit (Sun et al. 2000). This is due to the un-branched structure of S lignin that has a low degree of condensation and therefore, the syringyl lignin is easily hydrolysed during enzymatic hydrolysis compared to guaiacyl and *p*-hydroxyphenyl lignin.

**Table 4.12:** S/G ratio analysis of WIS material generated from enzymatic hydrolysed steam exploded sugarcane bagasse in relation to the lignin content

Component (%)*	Control	SE – 1	SE – 2	SE – 3
Xylan	0.29	0.22	0.087	n.d
Guaiacyl	5.71	430.93	401.83	n.d
Syringyl	75.89	76.35	160.57	n.d
<i>p</i> -Hydroxyphenyl	1773.64	409.95	281.89	n.d
S/G	1.33	3.30	3.51	n.d
Klason lignin	1.90	2.22	0.01	n.d

\*The components are based on the oven dry weight of the original material

n.d = Not detected

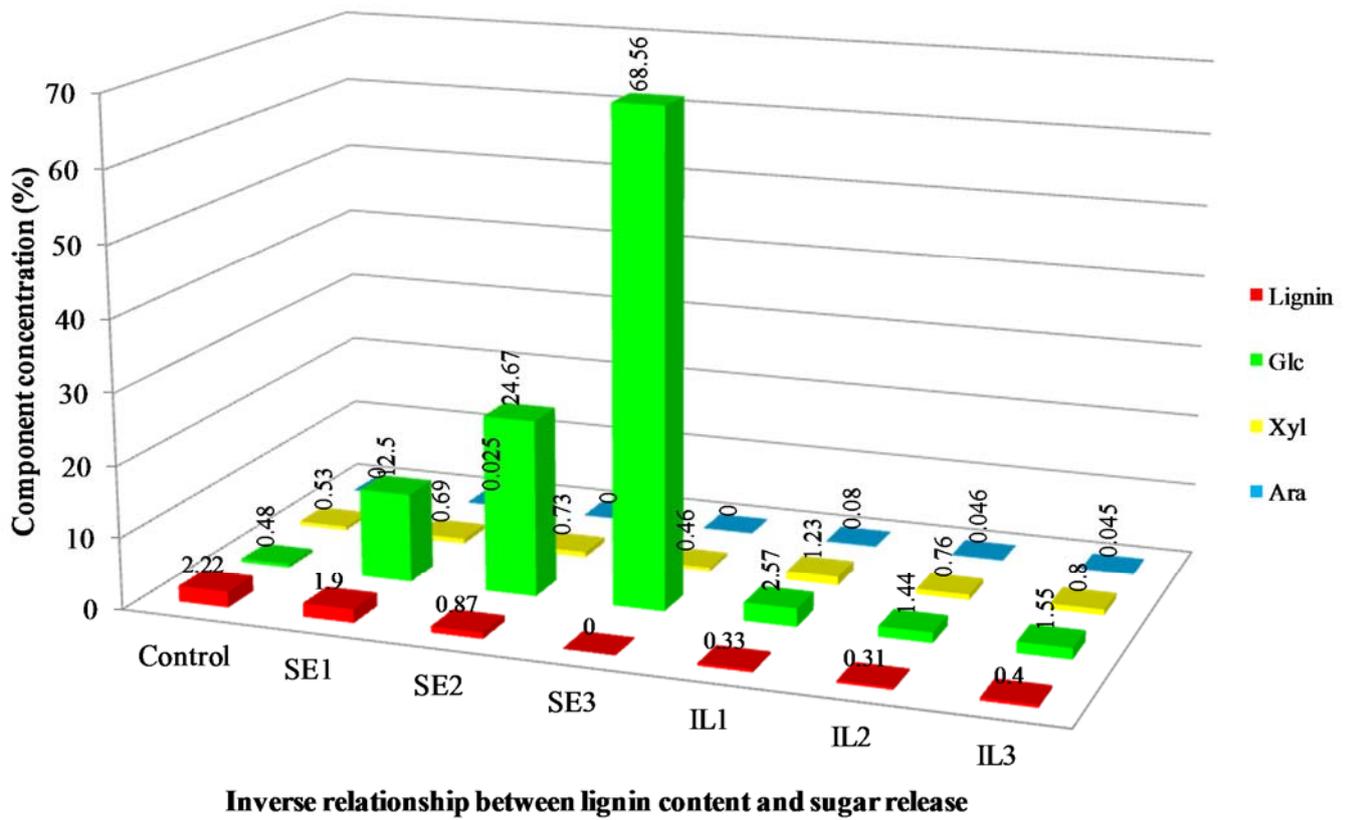
The WIS generated from ionic liquid fractionated then enzymatic hydrolysed material had a fairly low syringyl lignin compared to guaiacyl lignin. Even though the materials had high S/G ratio, suggesting that it be easy to process as presented in Table 4.13.

**Table 4.13:** S/G ratio analysis of WIS material generated from enzymatic hydrolysed ionic liquid fractionated sugarcane bagasse in relation to the lignin content

<b>Component (%)*</b>	<b>Control</b>	<b>IL – 1</b>	<b>IL – 2</b>	<b>IL – 3</b>
Xylan	0.29	0.15	0.17	0.17
Guaiacyl	5.71	160.58	517.13	550.17
Syringyl	75.89	706.81	22.37	235.66
<i>p</i> -Hydroxyphenyl	1773.64	78.45	386.16	4.11
S/G	1.33	9.27	3.29	3.54
Klason lignin	1.90	0.33	0.31	0.40

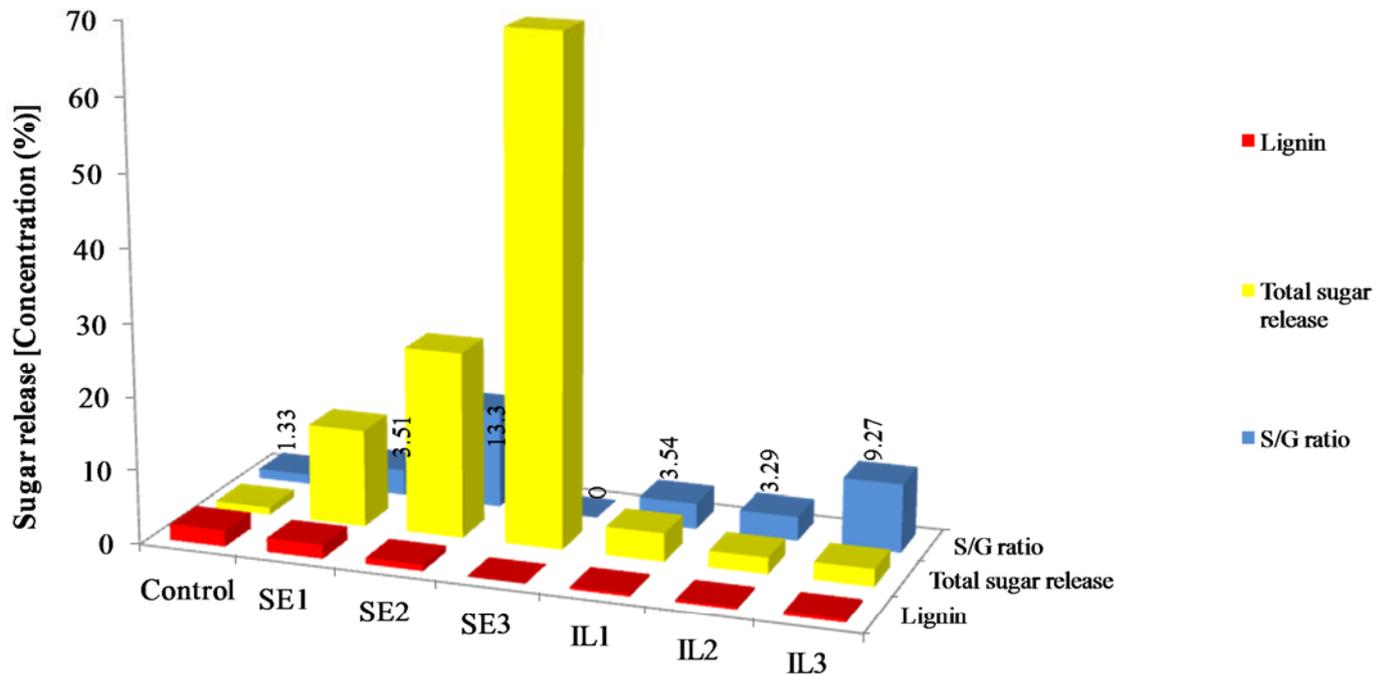
\*The components are based on the oven dry weight of the original material

In general this suggests that the materials generated in either condition had liberated most the sugars but the guaiacyl and *p*-hydroxyphenyl content were much higher than syringyl content slowing down the enzymatic hydrolysis process, resulting in not complete digestion of the lignocellulosic material. The efficiency of processing is directly proportional to the amount of syringyl units in lignin (Wang et al. 2012). The guaiacyl units have a free C-5 position available for C-C inter-unit bonds, which makes them fairly resistant to lignin depolymerisation, while S lignin is relatively un-branched and has a lower condensation degree and therefore easier delignification (Fengel and Wegener, 2003). Also, for all the materials, the residual lignin is likely associated with glucose, in which case it is easily hydrolysed compared to lignin that is associated with xylan (Lawoko et al. 2011 and Li et al. 2011). Figure 4.7 further shows that there was an inverse relationship between the sugar yield and the lignin content in the materials.



**Figure 4.7:** Inverse relationship between sugar release and lignin content after enzymatic hydrolysis.

For higher S/G ratio materials, the sugar release was much higher with an inverse relationship to the lignin content. Even though enzymatic hydrolysis on untreated material liberated a significant amount of sugars at low lignin content (below 20%), irrespective of the S/G ratio.



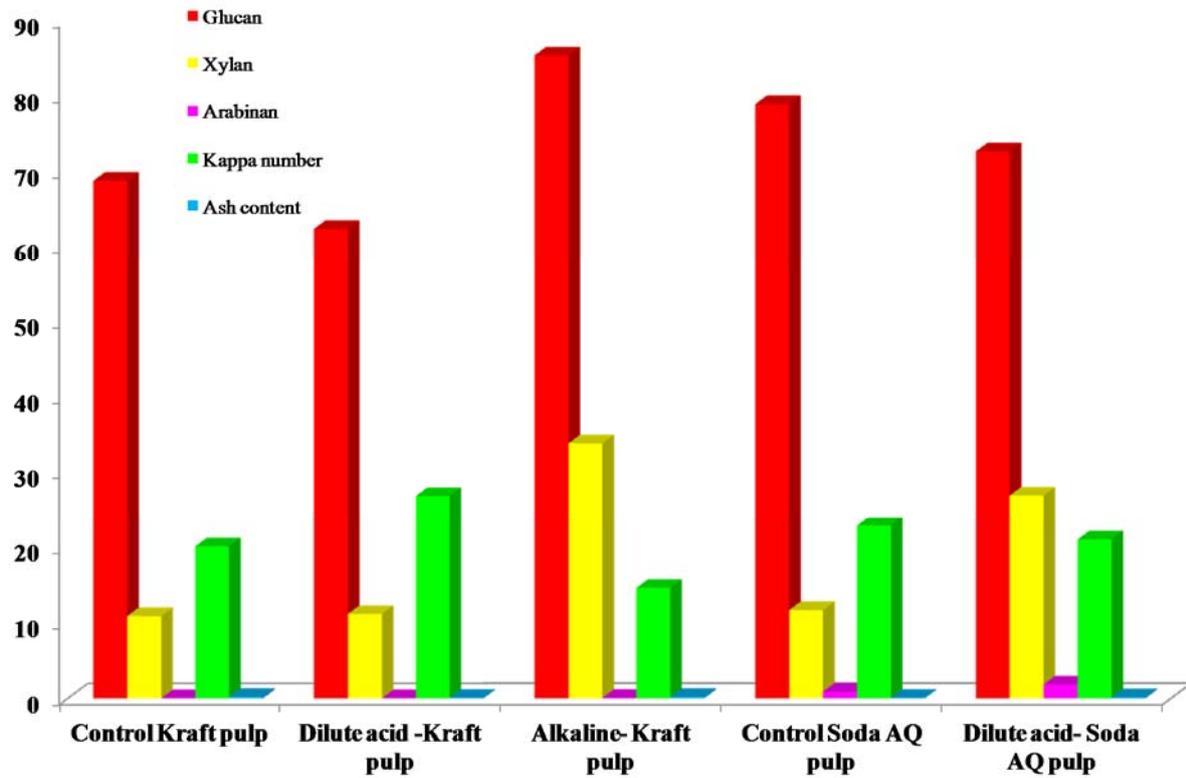
**Characterisation of EH material, relationship is shown between S/G ratio, lignin content and sugar release**

**Figure 4.8:** Relationship between sugar release and lignin content and S/G ratio in enzymatically hydrolysed material.

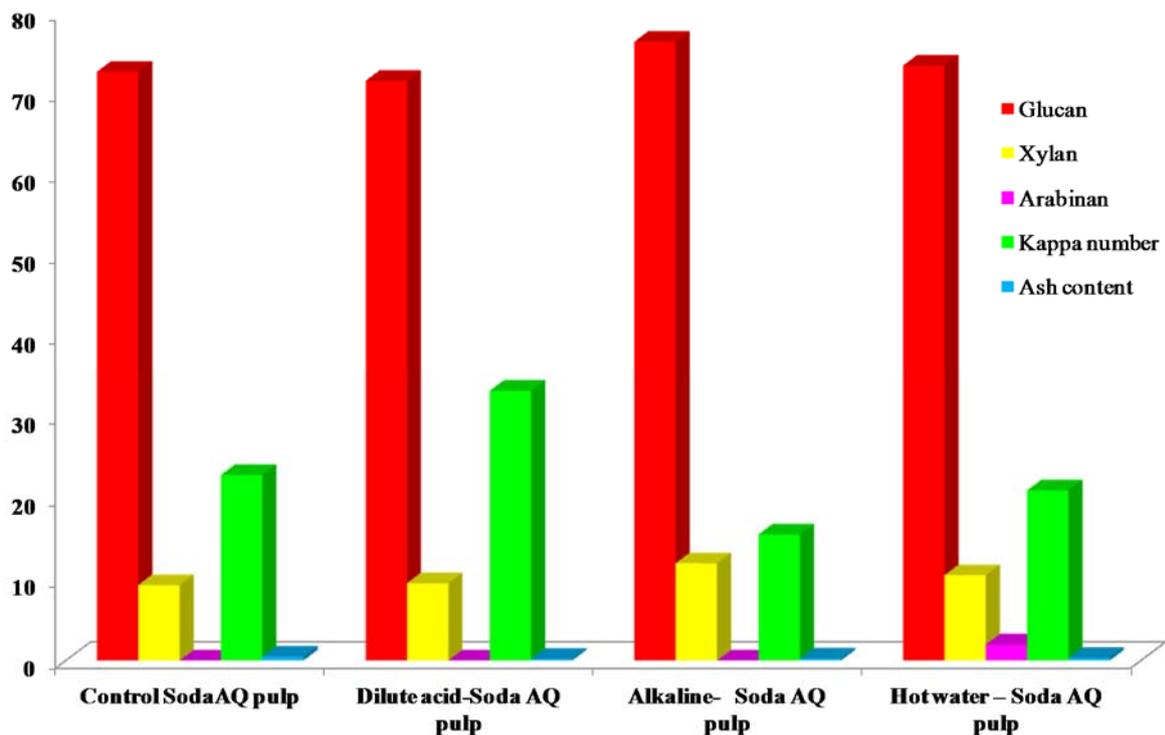
Sample SE1 (poor steam explosion condition – Table 3.4) in Figure 4.8 had a reasonable amount of lignin (1.9%) content and S/G ratio (3.51) and exhibited fairly reasonable sugar release compared to SE2 (regarded as a good steam explosion condition – Table 3.4). This suggests that factors beyond lignin and S/G ratio influence sugar release and deeper understanding on biological mechanism between enzymes and substrate is needed coupled with in depth chemistry of lignocellulosic materials before plants can be subjected to processing and this is in agreement with the literature (Studer et al. 2011).

#### 4.4 Compositional analysis of *E. grandis* and sugarcane bagasse pulps

The chemical composition of the pulp materials generated from *E. grandis* and sugarcane bagasse are shown in Figures 4.9 and 4.10.



**Figure 4.9:** Compositional analysis of pulps generated from *E. grandis* in various pre-extraction and pulping methods.



**Figure 4.10:** Compositional analysis of pulps generated from sugarcane bagasse pre-extracted in various methods and soda AQ pulped.

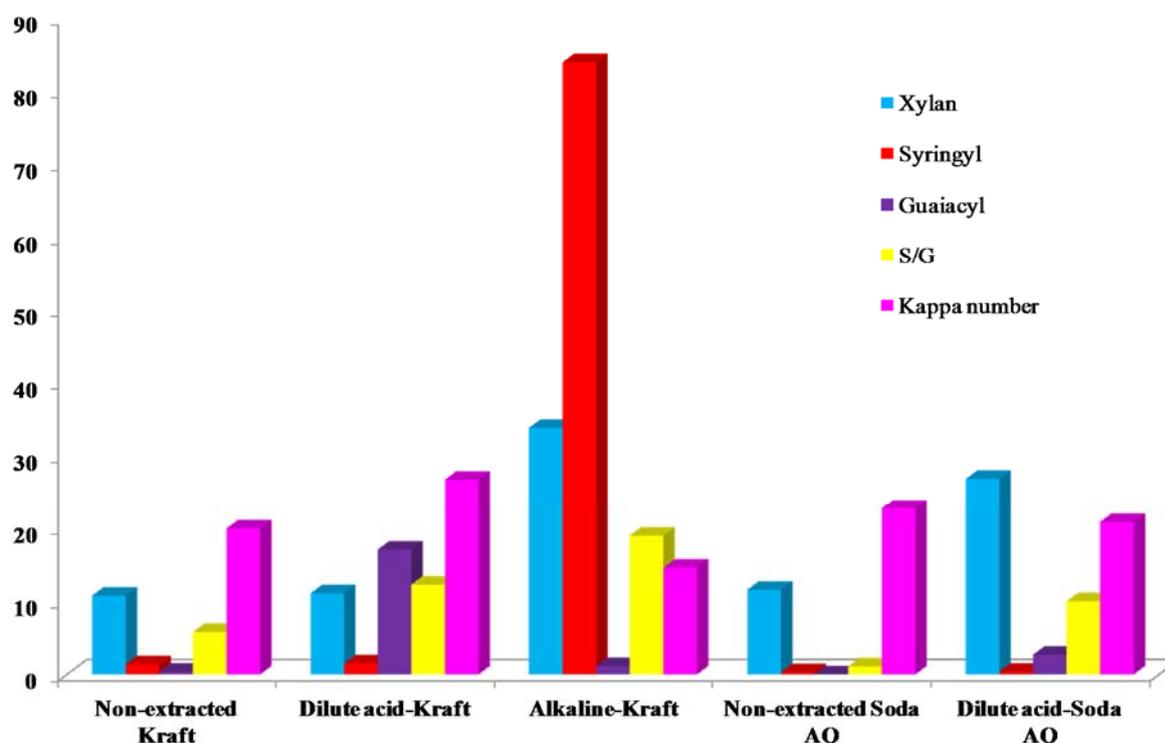
Figures 4.9 and 4.10 show that the alkaline pre-extracted then pulped *E. grandis* and sugarcane bagasse and materials had a low Kappa numbers of 14.6 and 15.51 respectively than any other pulp materials. This is the result of the cleavage reactions that lignin undergoes when a lignocellulosic material is subjected into alkali conditions. Water pre-extracted then pulped SCB had a Kappa number 20.96 which was close to the control material with Kappa number 22.85 for sugarcane bagasse. This is due to the fact that water becomes acidic as acetic acid is liberated during pre-extraction and condensation reactions occur and pseudo-lignins are formed. This mechanism is similar to the reactions with dilute acid pre-extraction where dilute acid then pulped material had a Kappa number of 33.22 for sugarcane bagasse while *E. grandis* had Kappa numbers of 20.95 and 22.81 for the pre-extracted and soda AQ control pulps respectively. The Kraft pulped *E. grandis* behaved totally different from its well-known chemistry. From the study, it was expected that the Kappa numbers be lower compared to the soda AQ pulped material which was not the case as the dilute pre-extracted pulp had a Kappa number of 26.84 while the control that had 20.03. This suggests that the dilute acid extracted then Kraft pulped material has high contents of LCCs compared to the soda AQ pulped material. In general, high LCC content should be extracted in reasonable quantities from *E. grandis* dilute acid pre-extracted pulps compared to the sugarcane bagasse pre-

extracted pulps as Figures and the discussion above suggest the presence of pseudo-lignins due to high Kappa number content determined.

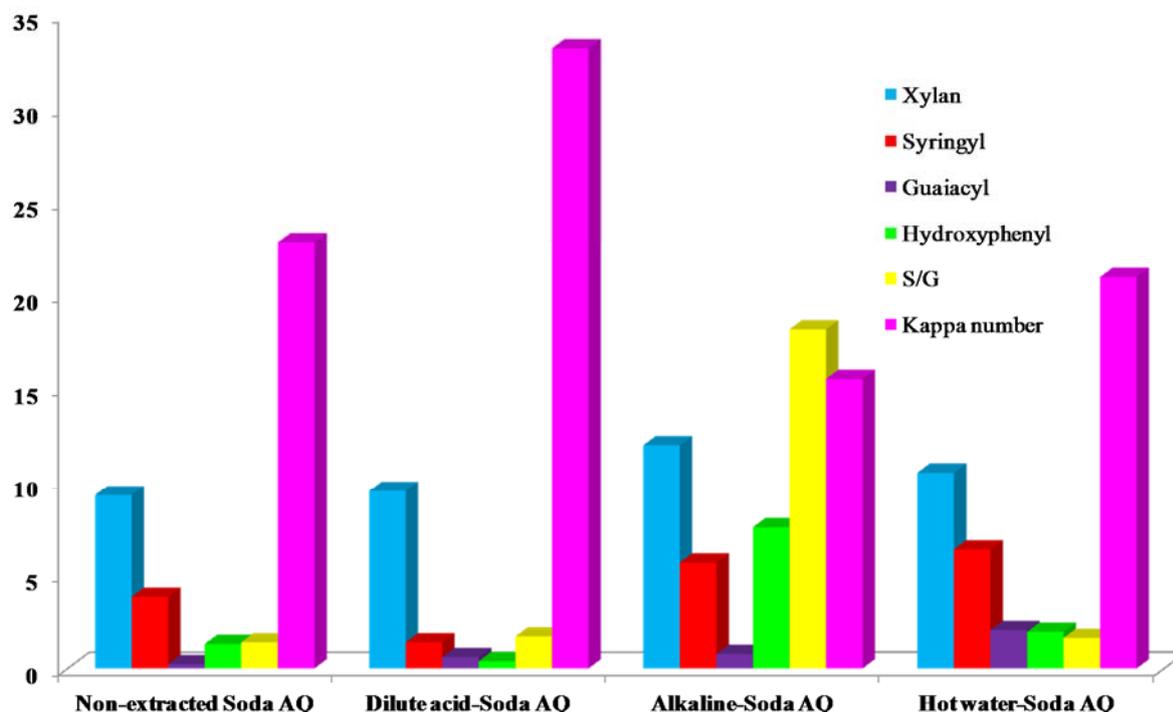
#### 4.4.1 Characterisation of the Kappa number and the S/G ratio of the pulp in relation to the residual xylan content recovered

The residual lignin in the xylan pre-extracted pulp was determined to establish the effect of xylan removal on the structure of the lignin. Knowledge of the structure of the lignin, in particular the contents of the basic structural lignin units i.e. syringyl and guaiacyl units could allow for an opportunity to improve the pulping conditions in combination with pre-extraction.

For both feedstocks, in Figures 4.11 and 4.12 it can be observed that the alkali-pre-extracted then pulped samples had higher S units than G units which could determine the extent of bleaching that is required in downstream processing of the pulp (Tsutsumi et al. 1995). In general, the  $\beta$ -aryl-ether of syringyl lignin is easily cleaved than guaiacyl lignin when lignocellulosic material is subjected to mild alkali conditions (Bose et al. 2009). Therefore, the lower content of the G unit as observed means that milder bleaching condition would be required downstream.



**Figure 4.11:** Comparison between lignin and S/G ratio in relation to the residual xylan in *Eucalyptus grandis* pulps



**Figure 4.12:** Correlation between Kappa number of sugarcane bagasse pulps and S/G ratio in relation to residual xylan.

#### 4.5 Compositional analysis of the lignin-carbohydrate complexes (LCCs)

Compositional analysis of LCCs generated in variously processed lignocellulosic material was done to understand and quantify the lignin that is associated with polysaccharides after pulping and enzymatic hydrolysis. The quantity and type of sugars associated with the residual lignin give an idea on how resistant is the lignin-polysaccharide association during delignification phase.

##### 4.5.1 Compositional analysis of the extracted LCCs from raw materials

According to Table 4.14, less than 85% of lignin was extracted from both feedstocks. Li et al. (2011) recovered 80 and 90% for the raw material and pulps respectively and Lawoko et al. (2011) recovered 70.56% for the raw materials. In this study, the recovery was better than what Lawoko et al. (2011) recovered even though the same method was used. However, a lower recovery was observed for the sugarcane bagasse feedstocks, even though it was in close range with what Lawoko et al. (2011) recovered. This suggests that for a successful

LCC extraction, a milder method must be applied for the sugarcane bagasse material compared to the hardwood *E. grandis*. The analytical data that is presented in Table 4.11 shows that the residual lignin is both associated with glucan and xylan. Approximately 7% for both raw lignocellulosic materials are associated with xylan while less than 30% glucan is directly linked to lignin. A similar phenomenon was observed by Lawoko (2005) and Lawoko et al. (2011).

In general, both lignocellulosic materials would be easily processed as the polysaccharide association with lignin is denoted as lignin-glucan. This type of linkage is easily hydrolysed as compared to xylan-lignin association (Lawoko et al. 2003 and Li et al. 2011).

**Table 4.14:** Chemical composition of the isolated LCCs from raw materials

Component	<i>Eucalyptus grandis</i> (%)*		Sugarcane bagasse (%)*	
	LCC 1	LCC2	LCC 1	LCC 2
Lignin	30.9	51.7	15.5	29.9
Glucan	9.5	7.8	11.6	9.6
Xylan	1.3	1.4	1.9	4.1
Arabinan	n.d	n.d	0.1	0.3

\*The components are based on Oven Dry weight of the original material

n.d = Not detected; LCC 1 = lignin carbohydrate complex that is a glucan-lignin fraction

LCC 2 = lignin carbohydrate that is a xylan-lignin-(glucan) fraction

#### 4.5.2 Compositional analysis of LCCs from pre-processed materials

The lignin content of the extracted and fractionated lignin-carbohydrate complexes (Table 4.15) from pre-extracted *E. grandis* materials was less than 85% that has been reported to be a reasonable yield (Lawoko, 2005). Dilute acid pre-extracted material had higher lignin than alkaline pre-extracted material. This is due to the lignin condensation reactions under acidic conditions and lignin cleavage reactions that occur under alkaline conditions (Santos et al. 2012). The syringyl-to-guaiacyl ratio content of the materials was 10 times higher for the alkaline pre-extracted material compared to the control this might be due to the positive syringyl-lignin type cell wall digestibility although the mechanism is still unclear (Xu et al. 2010)

and Santos et al. 2013). The dilute acid pre-extracted material was in the same range as with the control. This implied that when the alkaline pre-extracted material is further processed e.g. pulping, it would be easy to be further processed than the control and dilute acid pre-extracted materials. Even though the dilute acid pre-extracted material had the same range S/G ratio as the control, it had high guaiacyl content of ~23.3 than 2.9 of the control in the range as the 2.7 of the alkaline pre-extracted material (this is the content of both LCCs combined). This implies that the dilute acid pre-extracted material will be more resistant to delignification compared to the control and the alkaline pre-extracted material.

In general, dilute acid pre-extraction is not an ideal pre-extraction method as high guaiacyl contents are measured. However, this also depends on the intended product. Table 4.15 also shows that the extracted lignin is more associated with glucan for the alkaline pre-extracted material than the dilute acid pre-extracted which is more associated with xylan. As it has been reported in many studies (Lawoko, 2005; Lawoko et al. 2011; Li et al. 2011 and Lawoko et al. 2003), the lignin that is associated with glucan is easily hydrolysed than the lignin that is associated with xylan. Therefore, this statement supports that the material that is dilute acid pre-extracted will be difficult to delignify.

**Table 4.15:** Compositional analysis of LCC extracted from *E. grandis* pre-extracted materials prior to pulping

Component (%)*	Control		Alkaline pre-extracted		Dilute acid pre-extracted	
	LCC 1	LCC 2	LCC 1	LCC 2	LCC 1	LCC 2
Lignin	30.90	51.70	1.30	53.50	57.80	74.90
Glucan	9.50	7.80	33.60	29.90	1.80	0.70
Xylan	1.30	1.40	1.00	4.20	1.10	9.40
Arabinan	n.d	n.d	0.30	0.20	n.d	n.d
Syringyl	2.90	3.60	1.30	0.30	3.10	2.70
Guaiacyl	1.00	1.90	1.50	1.20	13.20	10.10
p-Hydroxyphenyl	n.d	n.d	0.01	0.00	n.d	n.d
S/G ratio	1.70	2.10	10.10	16.10	2.10	5.90

\*The components are based on Oven Dry weight of the original material

n.d = Not detected

Tables 4.16 and 4.17 present the chemical composition of LCCs from pre-treated sugarcane bagasse. All the extracted lignin-carbohydrate complex fractions showed fairly reduced syringyl content with slightly higher guaiacyl and p-hydroxyphenyl contents compared to the control material. This was due to the fact that syringyl unit is easily hydrolysable than the other monolignols.

The xylan content in both tables is slightly higher than the glucan content. This could significantly affect further processing of these materials as xylan is not easily hydrolysable as glucan. Consumption of chemicals and enzymes will be higher for these pre-treated materials even though the S/G ratios are significantly high.

**Table 4.16:** Compositional analysis of LCC extracted from sugarcane bagasse pre-extracted materials prior to pulping

Component	Control (%)*		Alkaline pre-extracted (%)*		Dilute acid pre-extracted (%)*	
	LCC 1	LCC 2	LCC 1	LCC 2	LCC 1	LCC 2
Lignin	29.70	15.50	3.40	94.50	4.80	90.30
Glucan	11.60	9.60	0.70	0.50	0.60	0.60
Xylan	1.90	4.10	6.90	14.90	3.30	9.90
Arabinan	0.10	0.30	0.90	1.10	0.20	0.50
Syringyl	3.20	6.00	0.03	2.20	2.00	4.00
Guaiacyl	6.30	3.50	0.40	3.00	11.70	19.80
p-Hydrox	n.d	n.d	1.60	2.30	5.10	12.00
S/G	3.10	2.00	0.01	0.09	0.12	0.13

\*The components are based on Oven Dry weight of the original material

**Table: 4.17:** Compositional analysis of LCCs extracted from steam exploded and ionic liquid fractionated sugarcane bagasse prior to enzymatic hydrolysis

Components (%)*	Control		SE 1		SE 2		IL 1		IL 2	
	LC1	LC2	LC1	LC2	LC1	LC2	LC 1	LC2	LC 1	LC2
<b>Lignin</b>	29.7	15.5	59.9	76.7	55.7	93.4	n.d	4.30	n.d	68.1
	0	0	0	0	0	0				0
<b>Glucan</b>	11.6	15.5	32.4	12.5	31.2	32.1	n.d.	21.1	n.d.	11.5
	0	0	0	0	0	0		0		0
<b>Xylan</b>	1.90	9.60	14.6	14.7	6.23	12.8	n.d.	8.50	n.d	18.6
			0	0		0				0
<b>Arabinan</b>	0.10	0.30	0.60	0.80	0.10	0.80	n.d.	0.40	n.d	1.70
<b>Syringyl</b>	3.20	6.00	0.10	0.03	0.03	0.03	n.d.	0.09	n.d.	0.03
<b>Guaiacyl</b>	6.30	3.50	1.40	0.10	0.06	0.30	n.d	1.10	n.d	0.20
<b>p-Hydroxyphenyl</b>	n.d	n.d	1.70	0.40	0.30	0.70	n.d.	1.80	n.d	0.60
<b>S/G ratio</b>	3.1	2.0	15.5	5.7	3.4	9.0	n.d.	12.8	n.d	7.1

\*The components are based on Oven Dry weight of the original material

LC = lignin carbohydrate fraction

n.d. = Not detected

#### 4.5.3 Compositional analysis of LCCs from pulps

Unbleached pulps are composed mainly of cellulose and smaller amount of hemicelluloses and lignin. However, the enzymatic removal of lignin has been reported to results in a different composition of the polysaccharides associated with residual lignin than that in the pulp (Hortling et al. 2001). However, application of enzymatic hydrolysis on hardwood pulps has

been reported to result in low LCC fractions that are difficult to quantify and high protein contamination has been reported to further complicate this (Capanema, 2004). Therefore, extraction of LCCs from *E. grandis* hardwood pulps using inorganic solvent method was used. Tables 4.18 and 4.19 are summaries of the compositional analysis of the LCCs extracted from *E. grandis* and sugarcane bagasse pulps respectively.

**Table 4.18:** Compositional analysis of LCCs extracted and fractionated from *E. grandis* pulps

Material	Carbohydrate		Acidolysis products from LCC lignin (g/100g)		
	relative	distribution			
	(%)				
	Xyl	Glc	S	G	S/G
untreated- sodaAQ pulp LCC 1	1.60	1.80	1.10	0.60	1.70
untreated - sodaAQ pulp LCC 2	6.00	1.20	2.80	1.50	1.80
Dilute acid - sodaAQ pulp LCC 1	2.10	2.80	1.70	2.30	0.70
Dilute acid - sodaAQ pulp LCC 2	0.50	0.05	1.60	3.80	0.40
Dilute acid - Kraft pulp LCC 2	1.70	2.90	2.20	1.00	2.20
Alkaline-Kraft pulp LCC1	3.30	24.60	0.01	0.00	1.80
Alkaline-Kraft pulp LCC 2	3.70	15.50	0.30	0.50	2.40
untreated- Kraft pulp LCC 1	0.60	0.20	2.20	1.20	1.30
untreated- Kraft pulp LCC 2	0.90	0.10	3.20	4.10	0.50

According to Tables 4.18, the S/G ratios of the LCCs from the alkaline pre-extracted materials and dilute acid Kraft pulps were not significantly different as compared to soda AQ pulping LCCs (above 1). This suggests that almost complete hydrolysis occurred during Kraft pulping than soda AQ (less than 1) even though both processes are alkaline pulping methods that result in complete cleavage of lignin.

The chemical composition of LCCs extracted from sugarcane bagasse is presented in Table 4.19. The S/G ratios of LCCs are less than compared to the control material, meaning that the residual lignin is associated with polysaccharides.

**Table 4.19:** Compositional analysis of LCCs extracted and fractionated from sugarcane bagasse pulps

Sample	Carbohydrate		relative	Acidolysis products from LCC lignin		
	distribution (%)			(g/100g)		
	Xyl	Glc	S	G	S/G	
untreated - sodaAQ pulp LCC 1	2.60	32.50	0.10	0.60	0.10	
untreated- sodaAQ pulp LCC 2	11.20	0.50	0.10	1.10	0.06	
Hot water - sodaAQ pulp LCC 2	9.40	0.50	0.10	0.20	0.30	
Alkaline - sodaAQ pulp LCC 1	6.10	0.60	0.10	0.10	0.70	
Alkaline - sodaAQ pulp LCC 2	3.70	0.10	0.10	0.20	0.30	
Dilute acid - sodaAQ LCC 2	3.00	2.80	0.30	0.40	0.50	

In general, this means that the pulps samples will need harsher bleaching conditions. The residual lignin in both feedstocks is more associated with xylan than glucan as the chemical composition of the materials indicated. This type of association slows down the delignification process. The residual lignin had high quantities of guaiacyl than syringyl; this is due to the high reactivity and easy hydrolysis of syringyl lignin than guaiacyl lignin. High guaiacyl group content slows down the delignification process because of the low reactivity of the phenyl ring as a result of the presence of methoxyl groups at position 3 and 5 (Fengel and Wegener, 2003), as a result to the side chain reaction that occur at C-5 position of the phenyl-glycoside chain.

#### 4.5.4 Compositional analysis of LCCs from WIS

The composition of the fractionated products from steam exploded WIS material is presented in Table 4.20. The sugars are presented in an anhydro form to permit quantification.

It was observed that the LCC fraction extracted from WIS-SE2 had the highest glucan content (76.63%) and a reduced xylan content compared to LCCs from WIS-control (24.5% glucan and 18.6% xylan) and SE1 (51% glucan and 10.6% xylan) respectively. This suggests that when the materials were subjected to steam explosion at higher temperatures, (SE2) most of the xylan was hydrolysed as a result of xylan reactions under steam explosion. However, the lignin content of LCC from WIS-SE2 was significantly higher than the LCC from WIS-SE1. This is due to the formation of pseudo-lignins reactions because of high generation of acetic acid as the temperature increased; steam explosion is known to fragment lignin.

**Table 4.20:** Compositional analysis of LCCs extracted from steam exploded WIS material

<b>Component (%)*</b>	<b>WIS-control LCC 2</b>	<b>WIS-SE 1 LCC 2</b>	<b>WIS-SE 2 LCC 2</b>
Glucan	24.53	50.78	76.63
Xylan	18.58	10.63	4.35
Arabinan	1.71	0.58	0.15
Lignin	94.50	50.80	76.60
Syringyl	0.04	0.04	0.04
Guaiacyl	0.29	0.136	0.40
<i>p</i> -Hydroxyphenyl	0.60	0.42	0.86
S/G ratio	7.82	4.87	10.40

\*Based on oven dry mass

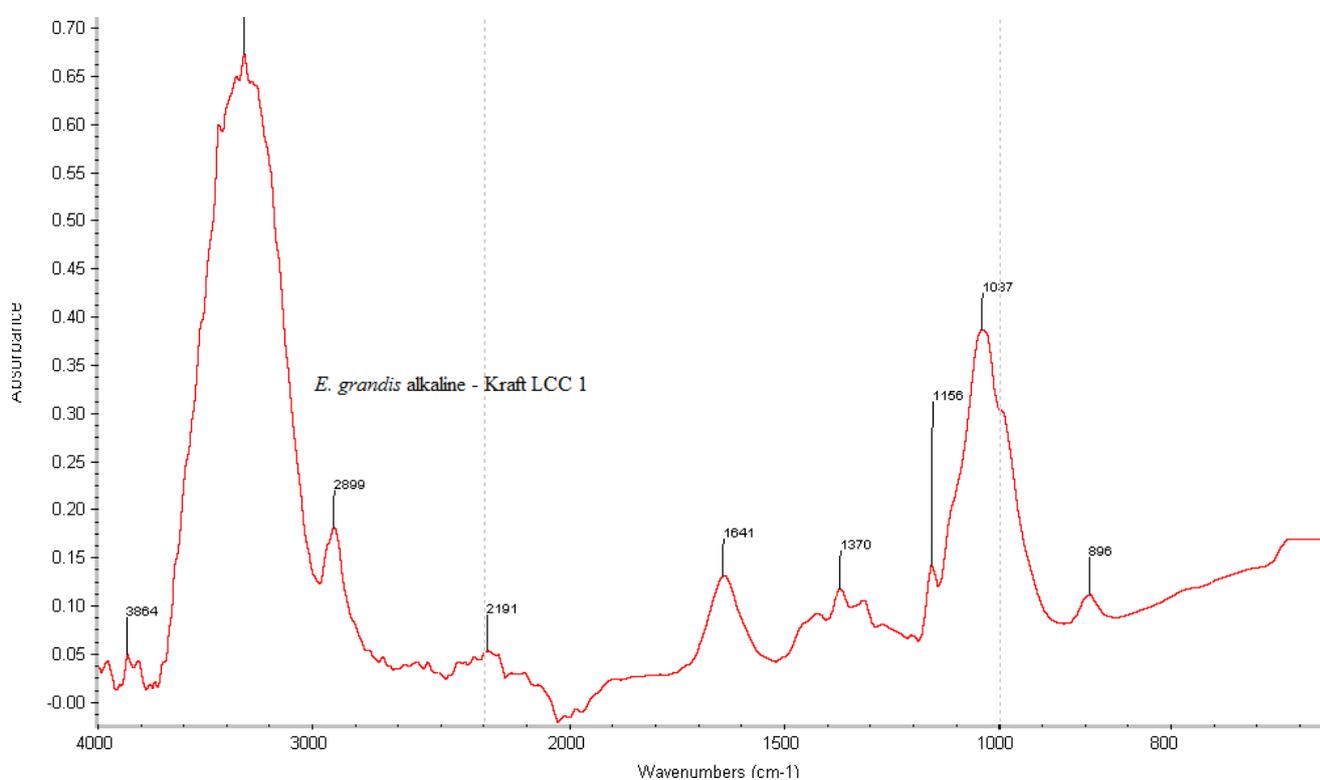
In general, it can be concluded that WIS-SE2 LCC2 fraction was easily hydrolysed because of the high S/G ratio and glucan content and low xylan content compared to WIS-SE1 LCC2. This is related to the -OCH<sub>3</sub> content in the lignin (Fengel and Wegener, 2003). Therefore, complete hydrolysis of the material pre-treated under the condition (SE2) described in the sections above can be achieved if the enzyme load dosages were increased.

## 4.6 Characterisation of LCCs from pulps and WIS materials

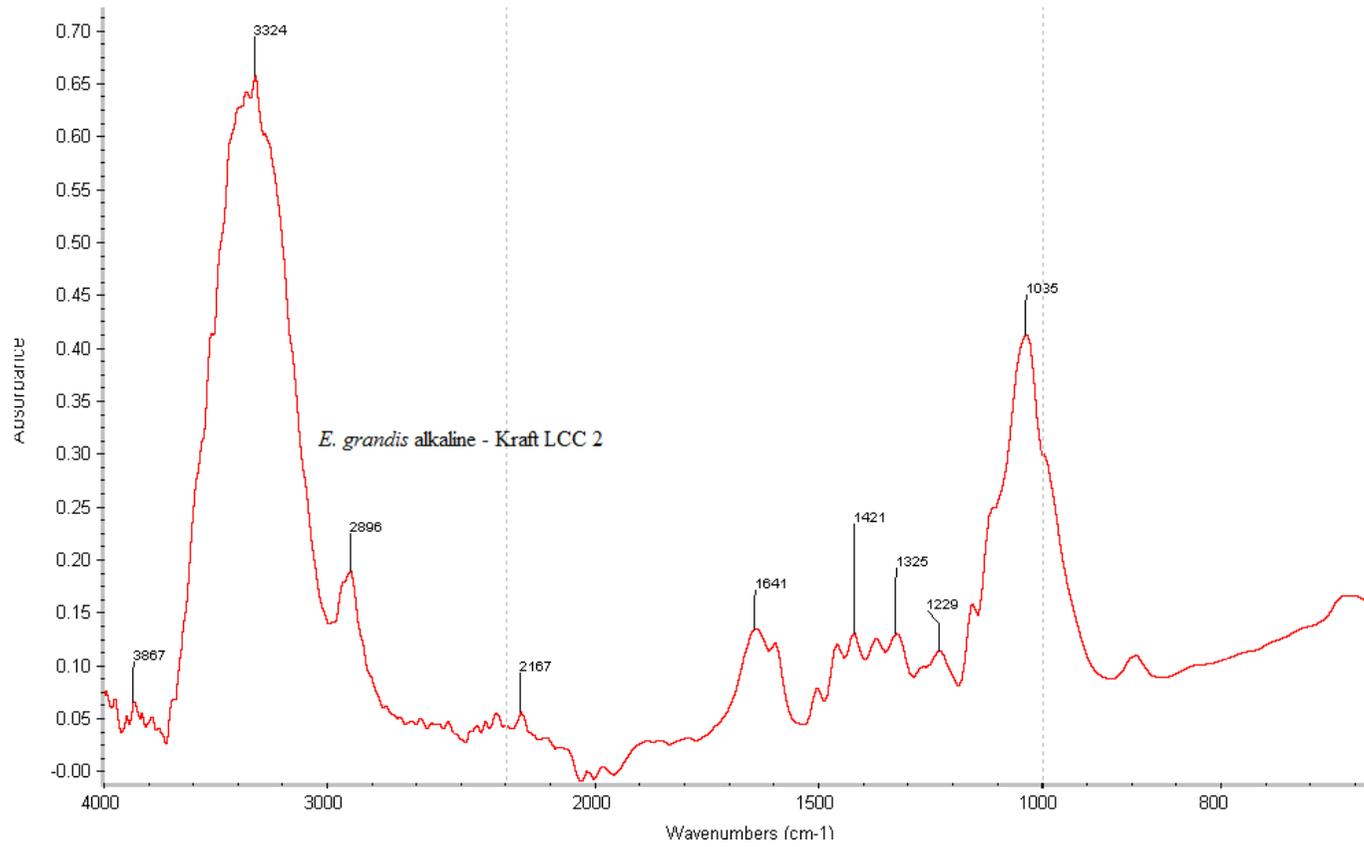
### 4.6.1 Characterisation of LCCs from pulps

Figure 4.13 shows the spectrum of the LCC fraction extracted from *E. grandis* under alkaline conditions intergrated with Kraft pulping. The LCC fractions indicated that there was slight degradation of cellulose and fragmentation of lignin structures. A stretching vibration of hydroxyl groups was observed at 3334cm<sup>-1</sup>. The absorption that was observed at 2915cm<sup>-1</sup> was due to C-H stretching. There were relative weak intensities of bands for aromatic skeleton vibrations assigned at around 1600cm<sup>-1</sup>. There were no peaks observed at around

1590 $\text{cm}^{-1}$  to be assigned to the carbon-carbon bonds of aromatics for the alkaline pre-extracted then pulped *E. grandis*. This phenomenon suggests that there was complete cleavage of the aryl-ether ester bonds in the ring structure of lignin. The band due to conjugated carbonyl groups ( $\alpha$ -carbonyl groups) was observed at 1641 $\text{cm}^{-1}$ . There were no guaiacyl or syringyl peaks detected at around 1200 and 1300 $\text{cm}^{-1}$  respectively. This suggests that the monolignols were completely washed and removed on the fibre surfaces of the pulp material. *E. grandis* alkaline-Kraft LCC1 – A weak signal was 1155 $\text{cm}^{-1}$  showed the presence of a *p*-coumaric ester group, typical of HGS lignins. An aromatic C-H out-of-plane bending was observed at 899 $\text{cm}^{-1}$ .



**Figure 4.13:** FTIR spectrum of LCC1 extracted from *E. grandis* alkaline – Kraft pulp



**Figure 4.14:** FTIR spectrum of LCC2 extracted from *E. grandis* alkaline pre-extracted – Kraft pulp

The spectra of other LCCs extracted from *E. grandis* pulps is presented in Figure 4.15. According to the spectra, all the LCCs were similar. They were only different in peak intensities indicating high/low component concentration in the material. In the 1500cm<sup>-1</sup> region there was a shift to the 1700cm<sup>-1</sup> band which indicated an ester type of linkage between lignin and the carbohydrate (Nelson et al. 1964b). The lignin peaks were low, whereas the syringyl and guaiacyl peaks were high at the regions of 1400 and 1100cm<sup>-1</sup> respectively (Qiu et al. 2012). This behaviour supported the inverse relationship between the lignin content in the material with its building units. The LCCs extracted from *E. grandis* Kraft pulp (control – Kraft pulp) had a higher ratio of S/G than any other material. This is due to the high cleavage reactions that occur during Kraft pulping. The LCCs extracted from dilute acid pre-extracted then soda AQ pulped material had a low S/G ratio for a pre-extracted material. This was due to the lignin condensation reactions that occurred when subjected to acidic conditions (Fengel and Wegener, 2003). The guaiacyl content was observed to be higher than the syringyl; this phenomenon would result on the material being not easily delignified during downstream processing (Mustajoki et al 2010). The dilute acid pre-extracted materials behaved similarly to the control with regard to the lignin content in material in the region of 1500-1600cm<sup>-1</sup>.

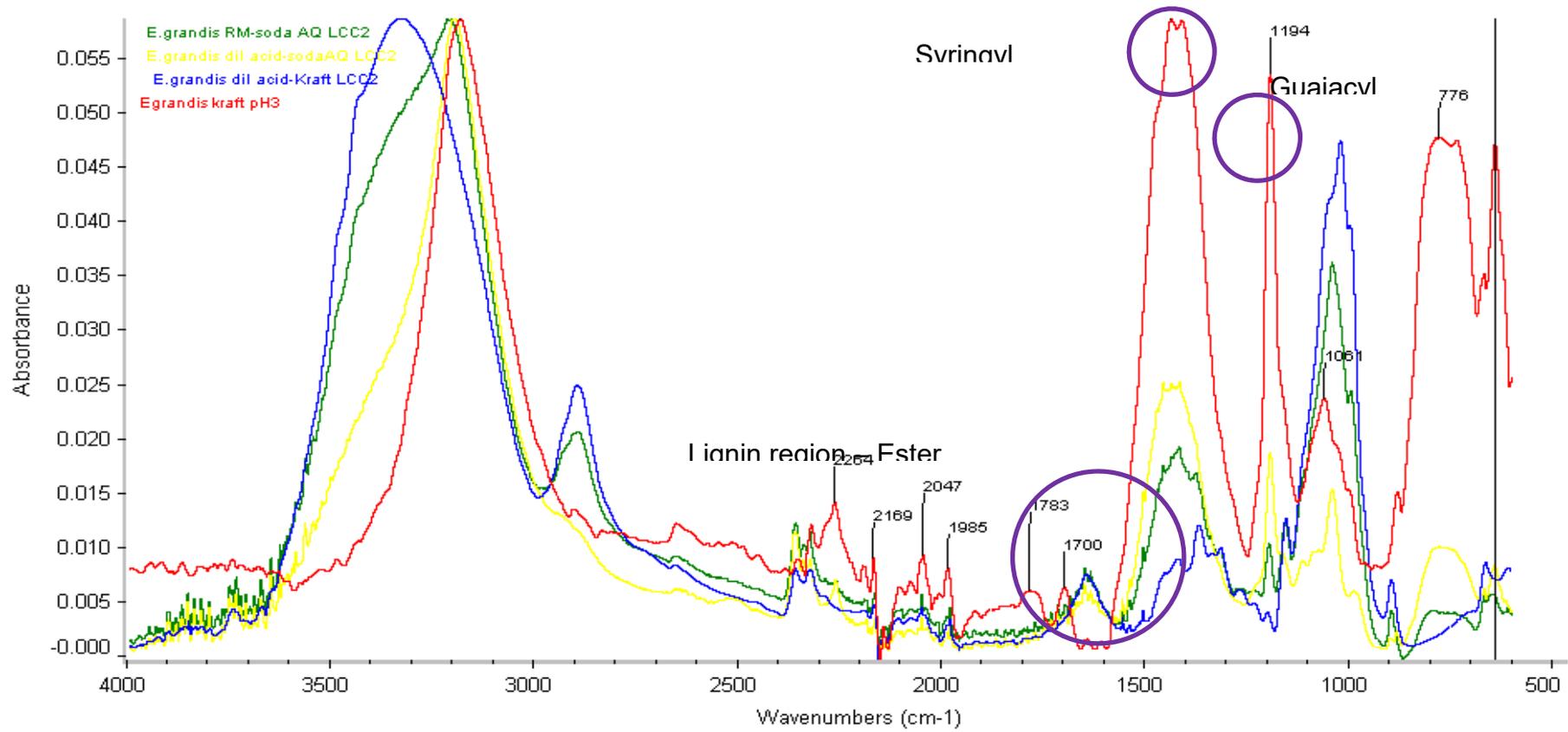
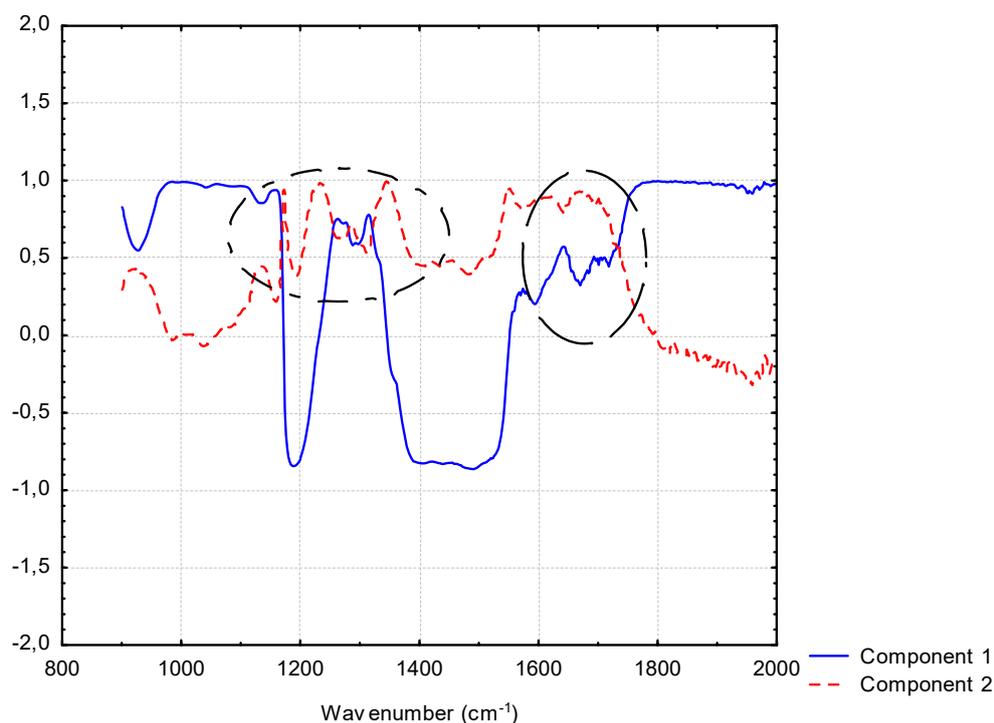


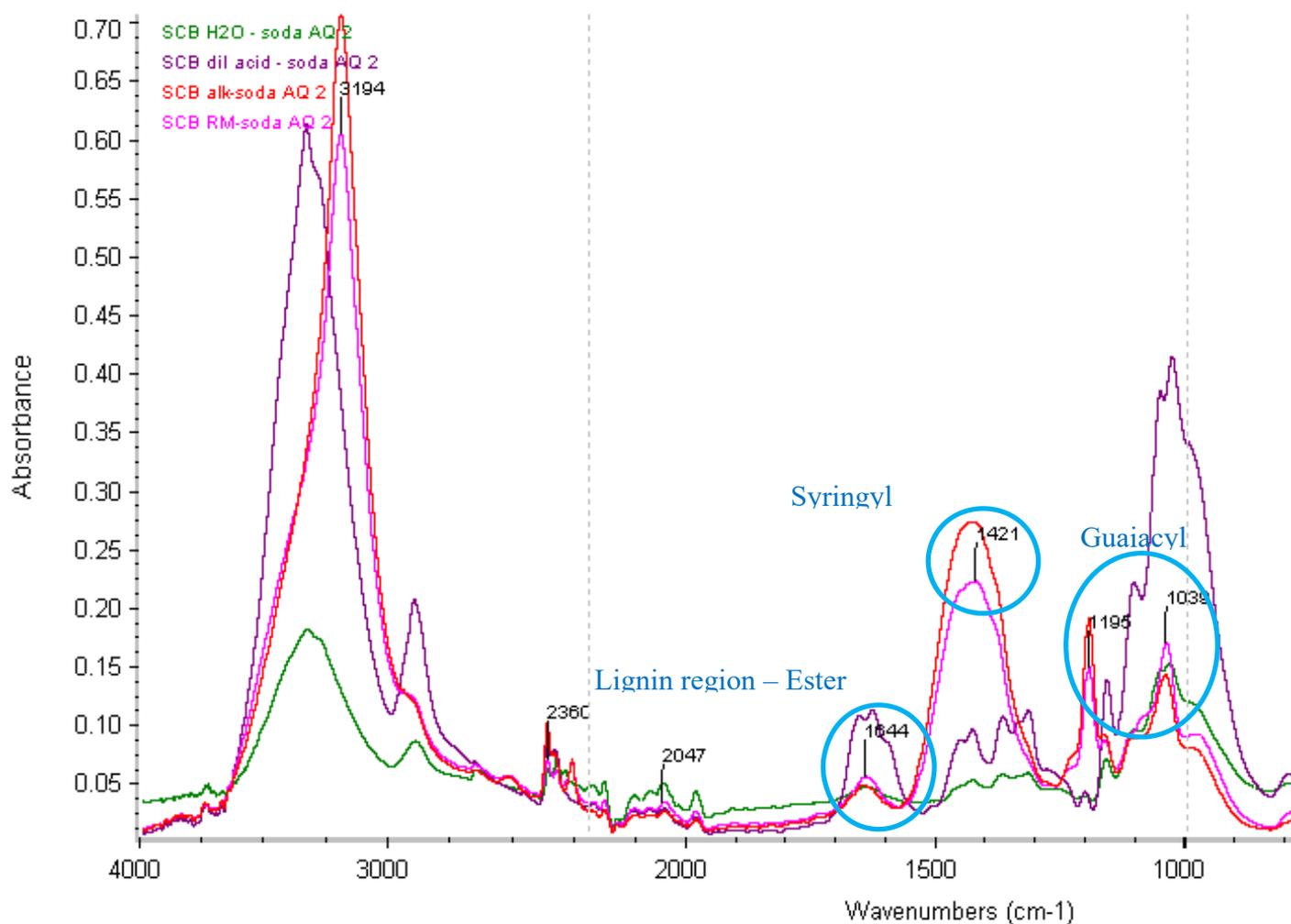
Figure 4.15: FTIR of LCCs extracted from *E. grandis* pulps

Because there was no clear separation on where the reactions occurred in the extracted LCCs, principal component analysis (PCA) was employed. For *E. grandis* LCCs, it can be seen in Figure 4.16 that their differences are in wavenumbers are attributed to syringyl and guaiacyl units (1200 and 1350 $\text{cm}^{-1}$ ). However it can be seen that the aromatic wavenumber (1510 $\text{cm}^{-1}$ ) is not so much different from lignin. It can also be observed that the differences in *E. grandis* are attributed to an oxidative effect (region more than 1600 $\text{cm}^{-1}$ ). Therefore, *E. grandis* suffered more oxidative reaction when it was subjected to pulping.

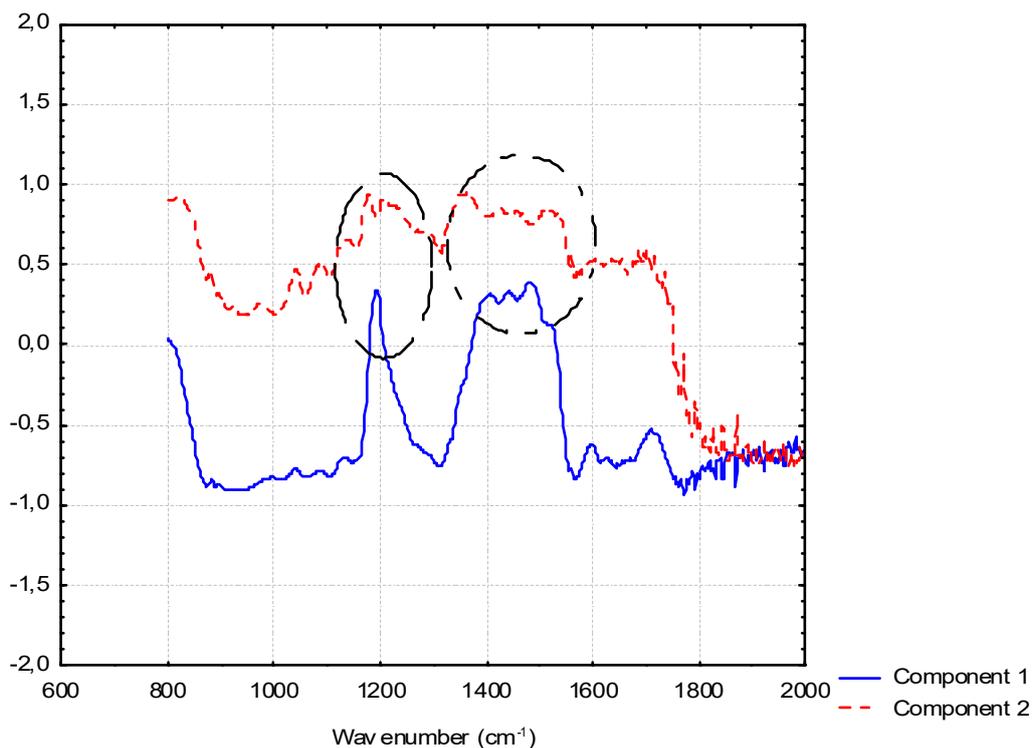


**Figure 4.16:** Load plot of LCCs extracted from *E. grandis* pulps

The differences in LCCs extracted from sugarcane bagasse pulps (Figures 4.17 and 4.18), tare can be attributed to Syringyl and Guaiacyl (1200 and 1350 $\text{cm}^{-1}$ ) and differences attributed to aromatic ring and C=O (1400 to 1600 $\text{cm}^{-1}$ ).



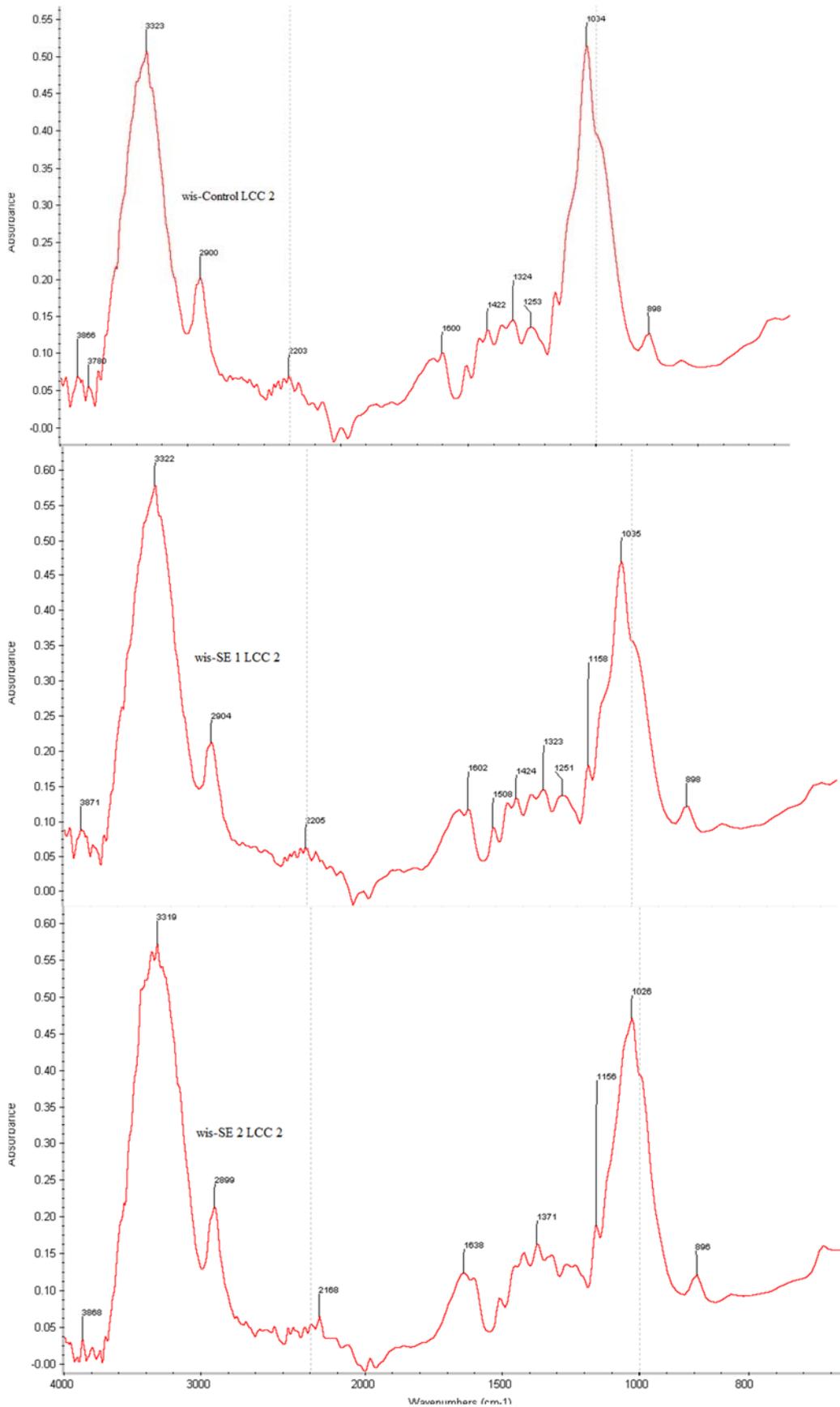
**Figure 4.17:** FTIR of LCCs extracted from sugarcane bagasse pulps



**Figure 4.18:** Load plot for the LCCs extracted from sugarcane bagasse pulps

#### 4.6.2 Characterisation of LCCs from WIS

The spectrum of the LCC (Figure 4.19) extracted from SE1 was similar to the spectrum of the control. Most of the peaks were not detected from the LCC fraction extracted from SE2. This is due to the degradation of hemicelluloses and lignin during pre-treatment. For the LCCs from WIS-control and WIS-SE1 the relative intensities of the bands for the aromatic skeleton vibrations were assigned at 1600, 1500 and 1400 $\text{cm}^{-1}$ . An intensive band at 1508 $\text{cm}^{-1}$  was assigned to the C-C bonds of the aromatic rings detected for the WIS-SE1 LCC2 fraction. An intensive band at 1708 $\text{cm}^{-1}$  assigned to non-conjugated carbonyl groups was not observed for all the materials. This was supposed to indicated the presence of non-conjugated ketones and aldehydes in the lignin fraction partially/ or degraded during processing either being pre-processing of steam explosion or ionic liquid fractionation. The syringyl band was observed at 1324 $\text{cm}^{-1}$  for all the materials while guaiacyl was observed at 1253 $\text{cm}^{-1}$  for the WIS-Control and SE1 LCC fractions. The band at 1158 $\text{cm}^{-1}$  detected for the WIS-SE1 LCC2 fraction indicated an aromatic C-H in-plane deformation of syringyl whereas for the guaiacyl it was observed at 1034 $\text{cm}^{-1}$  was the aromatic C-H in-plane deformation together with C-O in primary alcohol for all the LCC fractions.



**Figure 4.19:** FTIR spectra of the LCC fractions extracted from WIS material

## 4.7 Gel permeation chromatography of thioacidolysed lignin-carbohydrate complexes

### 4.7.1 GPC analysis of LCCs extracted from WIS material

The extracted lignin-carbohydrate complexes from WIS material were acetylated as described in the methodology section. The acetylation was done to improve the lignin solubility as well as to prevent the hydrogen bonding type interaction of the phenolic hydroxyl groups in the lignin with THF (Choi and Faix, 2010). The relative molecular weights of the LCCs were determined based on the calibration curved obtained from the polystyrene standard (Himmel et al. 1995). The GPC data for the LCCs generated from pre-treated and corresponding WIS-material is presented in Table 4.21 as determined by the refractive index (RI).

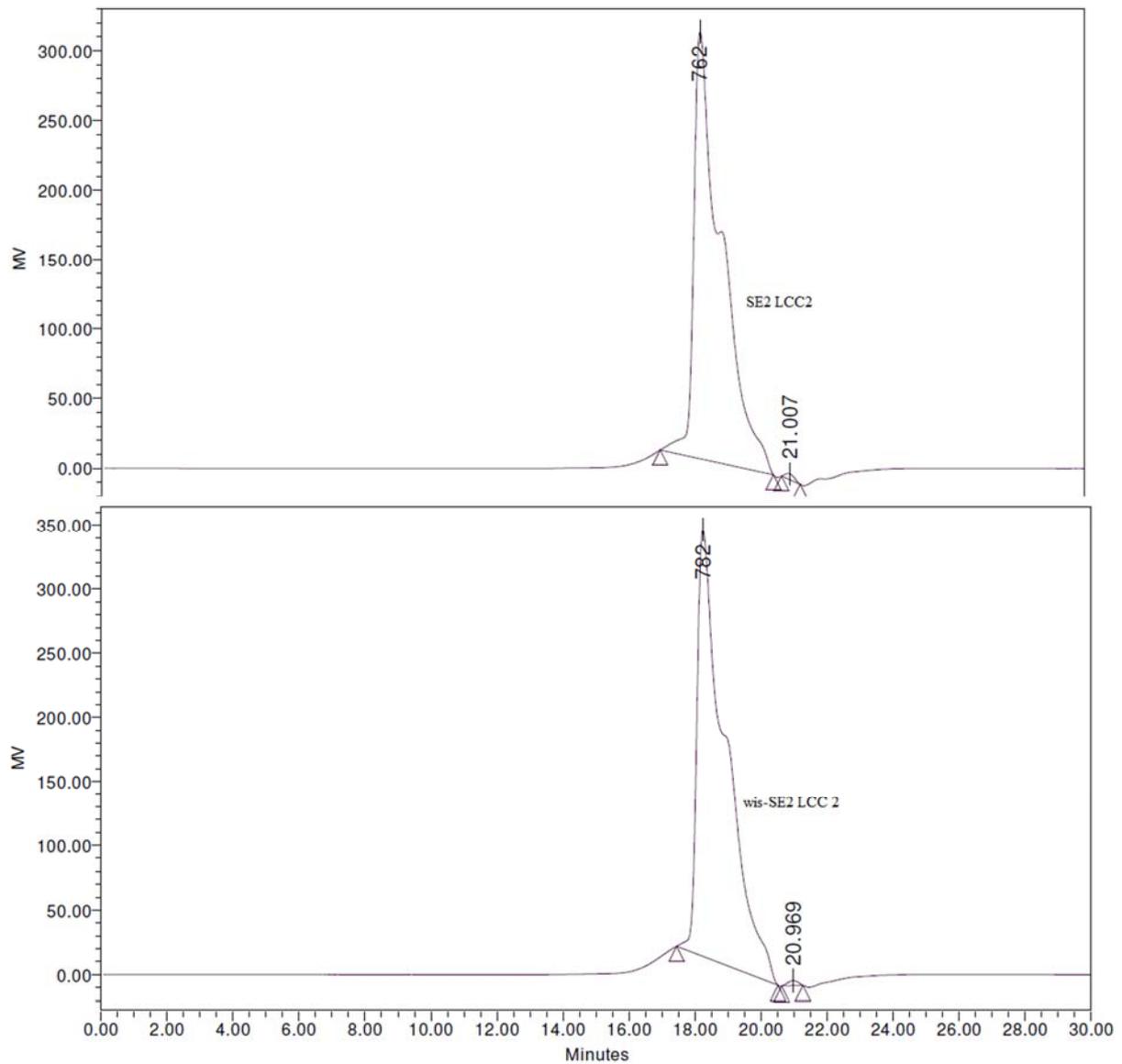
**Table 4.21:** Determination of the average molecular weight and polydispersity of the LCCs

LCC fractions	RI detector		
	<i>M<sub>w</sub></i>	<i>M<sub>n</sub></i>	<i>M<sub>w</sub>/M<sub>n</sub></i>
Raw SCB (control) LCC2	890	477	1.87
SE 1 LCC2	530	394	1.36
SE2 LCC2	564	411	1.37
WIS-Control LCC2	829	443	1.87
WIS-SE1 LCC2	510	376	1.36
WIS-SE2 LCC2	547	401	1.37

SCB = sugarcane bagasse

*M<sub>w</sub>/M<sub>n</sub>* = polydispersity

When the elution differences were determined between RI and UV detectors, there was no significant difference on the average molecular weights of the extracted lignin-carbohydrate complexes. The elution profile of the pre-treated materials had average molecular weights and polydispersity of 890 and 1.87, 530 and 1.36, and 564 and 1.37 for raw control, SE1 and SE 2 respectively. However, the profiles of the LCCs from enzymatically hydrolysed material were transformed and shifted to lower average molecular weight regions LCCs from SE1 and its corresponding WIS as typical examples as shown in Figure 4.20.



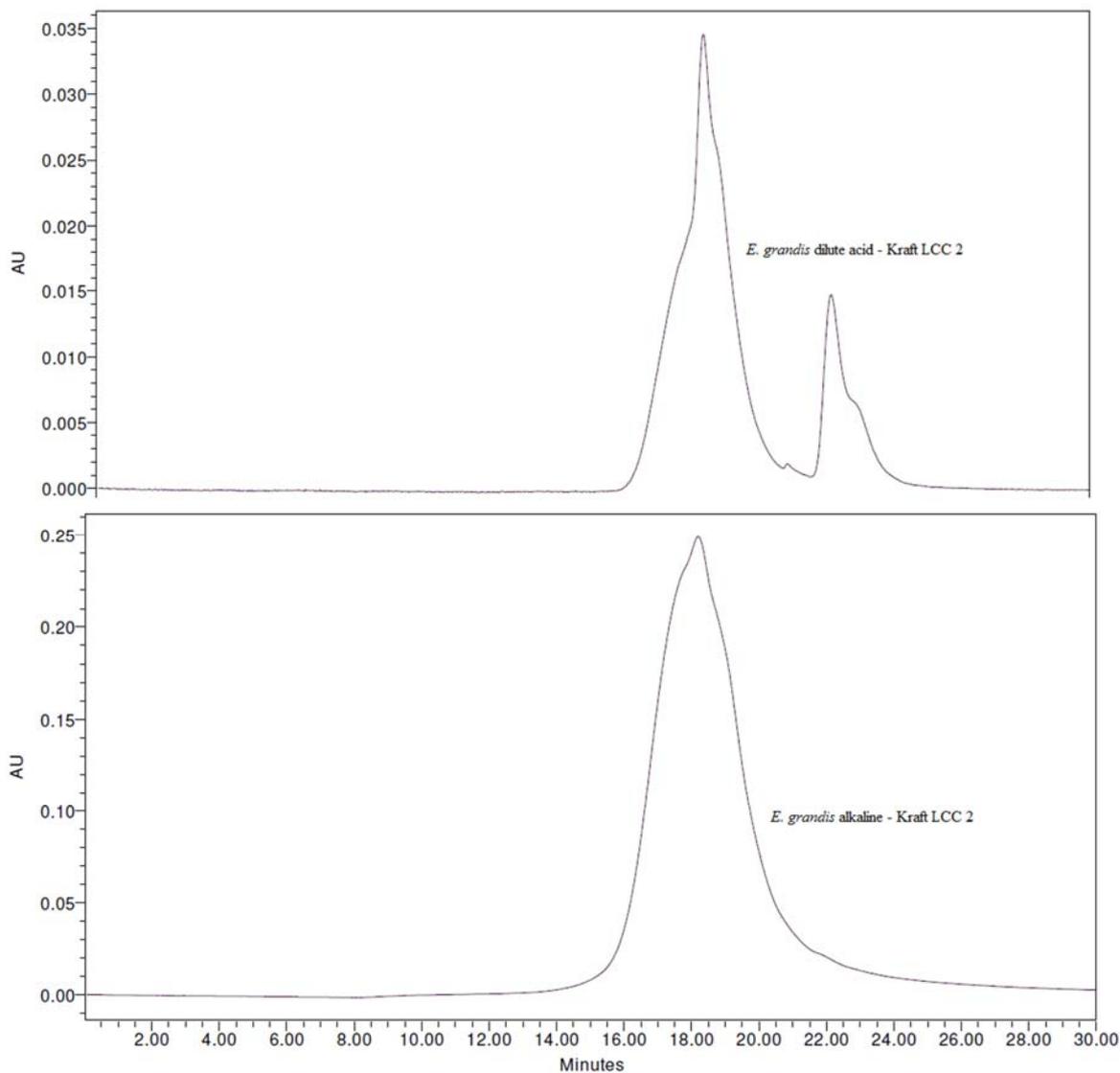
**Figure 4.20:** Average molecular weight profile of LCC2 from SE2 with corresponding WIS material.

The average molecular weights of the LCCs from WIS- control, SE1 and SE2 were reduced to 829, 510 and 547 respectively. A significant finding was that the estimated average molecular weight was approximately 1.7 and 1.6 folds lower in SE1 and SE2 LCCs as compared to the *Mw* of the control (raw sugarcane bagasse). This shows that the lignin bonds were cleaved during the steam explosion pre-treatment process.

In general, the marked decreased of molecular weights in LCCs from pre-treated and WIS material may be explained by the chemical reactions such as cleavage between the lignin fragments during physico-chemical and biological processing.

#### **4.7.2 GPC analysis of LCCs isolated from pulp material**

The lignin structures from Kraft pulps extracted by dilute acid and mild alkali were degraded by thioacidolysis into monomers by cleavage of all  $\beta$ -O-4 linkages as also observed by (Li et al. 2011; Onnerud and Gellerstedt 2003) before they were subjected to gel permeation chromatography. Dissolution of the degraded materials in THF showed the presence of monomers, dimmers and trimmers. There was also a possibility of the presence of oligomers with high molecular weight in the from Kraft pulps. The extracted LCCs degraded by thioacidolysis in combination with GPC were used to study the differences between the alkaline and dilute acid Kraft pulps in Figure 4.21.



**Figure 4.21:** Size exclusion chromatographic spectra of LCCs extracted from *E. grandis* dilute acid and alkaline – Kraft respectively.

It is evident that the LCCs from dilute acid – Kraft pulp had a higher number of components than the alkaline extracted Kraft pulp as there are peaks that were observed in the lower and higher molecular weight regions.

The xylan-lignin (LCC2) fraction from dilute acid Kraft pulp had substantial amounts of UV-absorbing materials. This shows that the lignin structures were more heterogeneous than any other biomass component (Li et al. 2011) and this was also due to the lignin reactions with an acid during the pre-extraction step (Table 4.22).

**Table 4.22:** Molecular weight of LCCs degraded by thioacidolysis extracted from Kraft pulp *E. grandis*

LCC fractions	RI detector		
	<i>M<sub>w</sub></i>	<i>M<sub>n</sub></i>	<i>M<sub>w</sub>/M<sub>n</sub></i>
Raw <i>E. grandis</i> (control) LCC2	890	477	1.87
Dilute acid - Kraft LCC2	564	394	1.36
Alkaline - Kraft LCC2	530	411	1.37

There was no significant difference in mw between LCC fractions extracted from *E. grandis* dilute acid and alkaline – Kraft pulps. The molecular weight (*M<sub>w</sub>*) of these fractions is less than 600 Da, with a difference of 300 Da to the control. This suggests there is a possibility that the extracted lignin was not associated with polysaccharides as the polydispersity of materials was less than 2. According to He et al. (2008), a molecule has a 3-dimensional network if the polydispersity is  $\geq 2$  and linear if the polydispersity is less than 2. Therefore, the polydispersity of the lignins in the dilute acid and alkaline – Kraft pulps was less than 2. This implied that the lignin was changed from a three-dimensional network structure to a fragmented structure (monomers). The change was due to the cleavage of linkages between the lignin units (Fengel and Wegener, 2003).

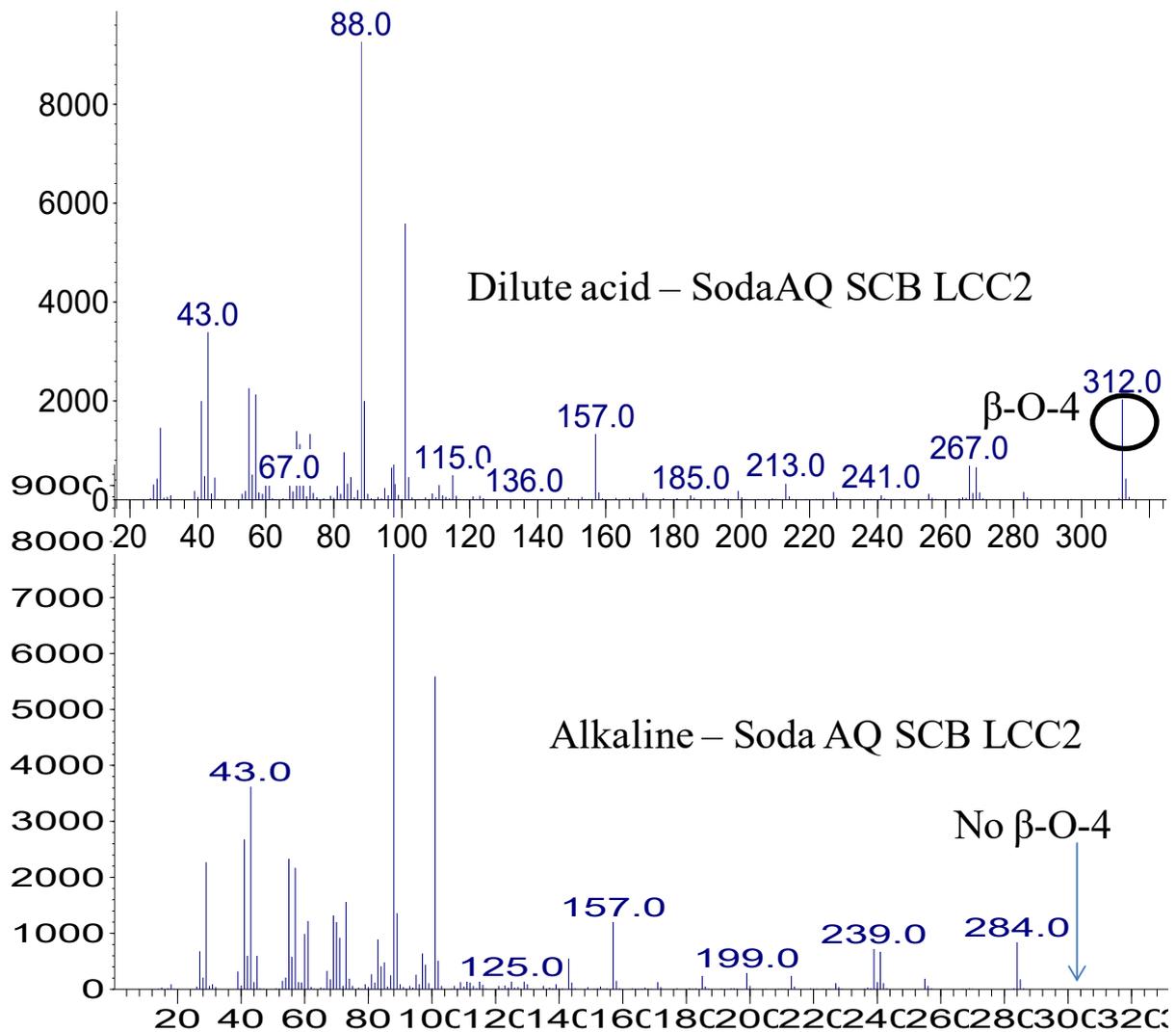
In general, this shows that alkaline pulping degraded the original lignin with a high molecular weight and three-dimensional network structure to small molecules. Therefore, the adsorption of the monolignols on the surface of pulp fibres greatly interferes with the true residual lignin (Kappa number) of the material.

## 4.8 GC-MS analysis of LCCs from biomass materials

### 4.8.1.1 GC-MS analysis of LCCs extracted from sugarcane bagasse pulps

Figure 4.22 shows the mass spectra of the LCCs extracted from sugarcane bagasse pulps. It was also possible to identify the covalent lignin linkages between its monomers. A comparative study was done between LCC 2 fractions generated from sugarcane bagasse pulps processed

in two different pre-extraction methods as detailed in the spectra. It was observed that the monomers analysed from LCCs extracted from alkaline pre-extracted prior to pulping sample did not have the  $\beta$ -O-4 linkage. This was due to the hydrolysis and cleavage (Lawoko et al. 2003) of lignin bonds when the sugarcane bagasse material was subjected to mild sodium hydroxide pre-extraction conditions. This also shows that less condensation reactions occurred and that the highly reactive syringyl monomer participated in the complete hydrolysis of the lignin whereas (Kang et al. 2012), this was not the case for the dilute acid pre-extracted material prior to pulping. This was due to the formation of pseudo-lignins that are formed as a result of condensation reactions that occur at position the C-5 of the guaiacyl monomer and C-3 and C-5 position of the *p*-hydroxyphenyl (Hu et al. 2012). The presence of these bonds suggest that when the pulp sample pre-extracted with dilute acid prior to pulping due to the presence of lignin-carbohydrate complexes.



**Figure 4.22:** Ion chromatography of LCCs extracted from sugarcane bagasse pulps

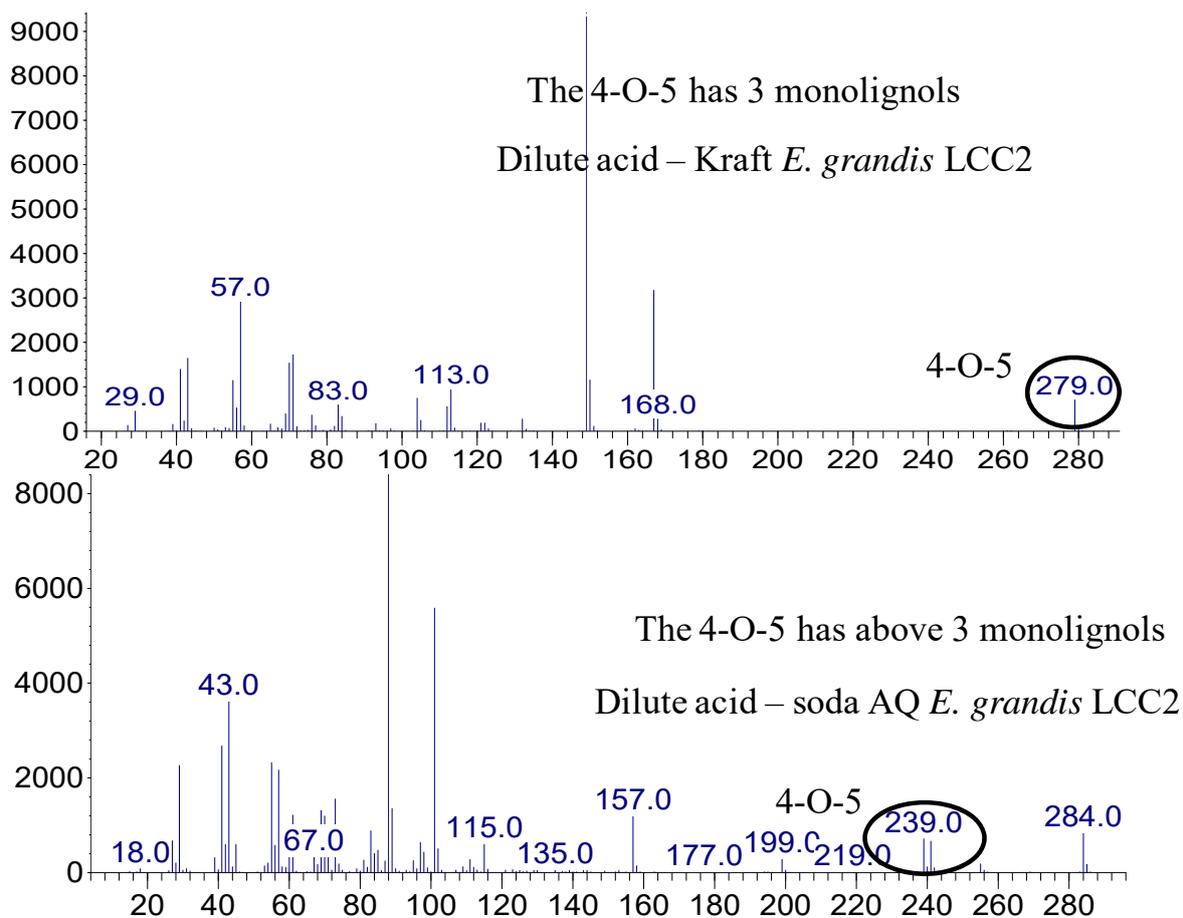
In general, pre-extraction of hemicelluloses prior to pulping has been reported to be a better approach in complete utilisation of biomass as they are lost in the black liquor during processing. Each process has its pros and cons. For pulping, dilute acid pre-extraction is not recommended as the delignification process is affected by the formation of pseudo-lignins that are associated with polysaccharides. However, alkaline pre-extraction is an ideal pre-extraction method integrated with pulping as sodium hydroxide used in pre-extraction is recycled during pulping and the pulps are much brighter in comparison to dilute acid pre-extracted pulp (Vena, 2013). Therefore, mild alkaline pre-extraction integrated with pulping resulted in less to none LCCs being extracted. The lignin therefore that was determined by

Klason method, according to S/G analysis shows that it was in monomeric fragment form than a 2 or 3D complex chain as this is supported by the polydispersity measurement.

#### **4.8.1.2 GC-MS analysis of LCCs extracted from *E. grandis* pulps**

A comparison study on dilute acid pre-extraction integrated with different pulping methods was done to determine the association of residual lignin with polysaccharides in the pulp material. According to the chemistry of Kraft and soda AQ pulping that has been discussed in detail in Section 2.6, Kraft pulping is harsh compared to soda AQ where the catalyst AQ protects the functional groups of the lignocellulosic material. In Kraft pulping there is no shielding of the carbohydrates such as xylan and glucose almost complete hydrolysis/delignification occurs.

The spectra presented in Figure 4.23 show the LCCs extracted in dilute acid-soda AQ pulp had a 4-O-5 type of bond so as for the LCC from dilute acid-Kraft. The difference between the extracted lignin-carbohydrate complexes is the number of monolignols linked together associate with the polysaccharide (Lawoko et al. 2003). In the dilute acid-soda AQ LCC2, there were more than 3 monolignols, linked together (Vanholme et al. 2010), this resulted on a big molecular structure and resulted on less interaction between the sample and the stationary phase of the column and was eluted earlier than LCC2 from dilute acid-Kraft that had few monolignols.



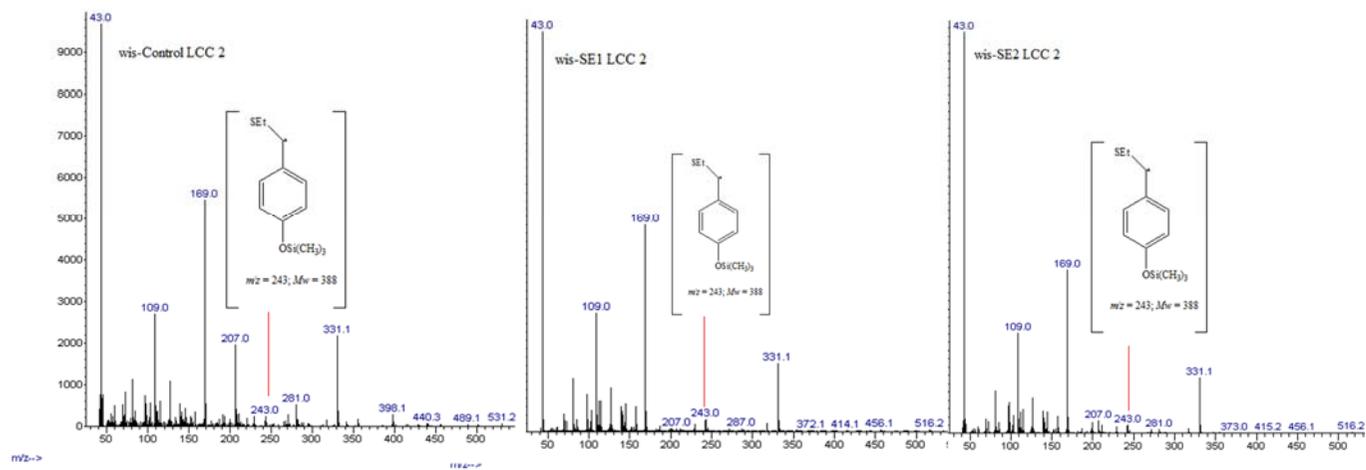
**Figure 4.23:** GC-MS spectra of LCCs extracted from *E. grandis* pulps

In general, integration of dilute acid hemicelluloses pre-extracted with Kraft pulping is ideal as compared to dilute acid hemicelluloses pre-extracted with soda AQ pulping because low molecular weight LCCs are formed. The concentration of 4-O-5 bonds are lesser compared to the LCCs extracted from dilute acid integrated with soda AQ pulping.

#### 4.8.2 GC-MS analysis of LCCs from WIS

A comparative study for the LCCs extracted from steam exploded – WIS material is presented in Figure 4.24. The cleavage of  $\beta$ -aryl ethers has been studied for decades (Lu and Ralph 1997 and Singh et al. 2005). It has been speculated that complete cleavage of the linkages will provide valuable and concrete information regarding native lignin based on the characterisation of the degraded monomers.

The total yields and relative distribution of monomers reflect the amount of and ring type of lignin units involved in the alkyl-aryl ether linkages (Lappiere et al. 1995). Also they provide information about the units involved in various lignin linkages. The knowledge of the residual lignin polymer will help maximise the amount of enzyme load for complete hydrolysis and improve the steam explosion pre-treatment conditions in order to reduce the generation of acetic acid that results to the formation of pseudo-lignins.



**Figure 4.24:** Mass spectra of LCC fractions extracted from steam exploded – WIS material

According to Figure 4.24, the H monomer was detected in lower frequencies for all the LCCs extracted from the WIS material. The S and G monomers were not detected. This is due to their higher reactivity compared to H monomer respectively. Although H monomer is generally less significant as compared to other monomers but its quantification is also important (Kishimoto et al. 2010). The characteristic fragment ion peak  $m/z$  243 with the molecular weight of 388 (Yue et al. 2011) was assigned to H lignin derived thioacidolysis monomer. According to Yue et al. (2011) report, the fragment ion peak  $m/z$  239 was detected. The slight shift was due to the different lignins used in the studies as they used synthesized model lignin compounds and this study used real lignin. This means that other lignin monomers were hydrolysed during processing compared to high resistance of H lignin. This was due to the high frequency of condensation reactions that occur.

In general, the limited monomeric sugar yield presented in section 4.2 can be explained by complete hydrolysis of the reactive monolignols (S and G lignins). The residual H lignin affected the enzymatic hydrolysis as more condensation reactions might have occurred on C-3 and C-5 positions of the phenyl ring. Also there might have been limited cleavage of the xylan molecules by hemicellulases at a location that liberated substantial LCCs. These lignin-carbohydrate complexes are resistant toward enzymatic hydrolysis because they are complex with xylose oligomers Table 4.21 and thus unreactive due to electron delocalisation within the many oxygen atoms present in the xylose oligomers.

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## **CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS**

Lignin-carbohydrate complexes (LCCs) were extracted in quantitative yields from *E. grandis* and sugarcane bagasse unbleached Kraft and soda AQ pulps and water-insoluble-solids after enzymatic hydrolysis of steam exploded sugarcane bagasse. From the dilute acid pre-extracted hemicelluloses then pulped, approximately 75% LCCs were extracted in quantitative yields from the method that was adapted from Lawoko et al. (2011).

The study has shown that from the non-pre-processed and dilute acid processed materials that lignin is covalently bonded to lignocellulosic polysaccharides, that is, glucan, xylan and arabinan.

According to the author's knowledge, a study on lignin-carbohydrate complexes from hemicelluloses pre-extracted lignocellulosic materials (*E. grandis* and sugarcane bagasse) prior to pulping has not been conducted also on the water-insoluble solids generated after enzymatic hydrolysis. Therefore, it is of great interest that further studies on bleached pulp materials be done to support the statements on conclusions.

The inter-unit linkages of LCCs and lignin with various S/G ratios were determined by acidolysis in combination with HPLC. The conclusions of the lignin structure and inter-unit linkages were highly dependent on the S/G ratio of each material. Similar tendencies were observed in variation of lignin structures deriving from differences in the S/G ratio although there were significant differences between dilute acid and alkaline pre-treated feedstocks. This suggests that similar lignin condensation and cleavage reactions occur regardless of lignocellulosic feedstock.

Lignin-carbohydrate complexes extracted from alkali pre-treated materials have S/G ratio compared to the dilute pre-extracted that is significantly lower, this was revealed by FTIR and acidolysis. Therefore, this could potentially result to less drastic process condition requirements, leading to carbohydrate preservation.

The low digestibility observed for SE 1 material implies that high lignin content in the biomass may be inhibiting the complete hydrolysis of  $\beta$ -glucan during enzymatic hydrolysis. It implies that irreversible adsorption of lignin on to crystalline  $\beta$ -glucan structure was occurring.