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**HYDROLYSIS OF LIGNOCELLULOSIC BIOMASS  
BY A MODIFIED ORGANOSOLV METHOD ON  
A BIOREFINERY PERSPECTIVE – EXAMPLE OF  
*MISCANTHUS × GIGANTEUS***

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Thesis Submitted to  
The University of Birmingham  
for the Degree of  
DOCTOR OF PHILOSOPHY

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March 2013

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# Abstract

Concerns about climate change and our awareness on energy security have risen during the last decades leading the search for new forms of energy to reduce the world's dependence on fossil fuels. Bioenergy has been proposed as one route to contribute significantly to meet global energy demand by using renewable sources of energy. Several types of biomass have been studied and utilised over the years but the use of lignocellulosic biomass on a second generation biofuels brought advantages compared to the food crops on the first generation. Lignocellulosic biomass eliminated issues related to food competition and originated the opportunity to be processed under the biorefinery concept thus creating the opportunity to produce not only bioenergy but also a platform of high value chemicals.

The overall objective of this work was to study and optimise a hydrolysis treatment of lignocellulosic biomass but particularly of *Miscanthus  $\chi$  giganteus* under the biorefinery concept. A modified organosolv method using subcritical water, ethanol and carbon dioxide on a high--pressure batch reactor was proposed and tested for its efficacy on the hydrolysis and fractionation of *Miscanthus* into its lignocellulose main components, hemicellulose, cellulose and lignin. Temperature (80–200 °C), reaction time (5–60 min), ethanol concentration (0–70%), carbon dioxide initial pressure (10–55 bar) and load size (2.5–15 g) were the parameters studied and respective ranges. Optimisation models for solubilisation and delignification were obtained and validated using a central composite design based on a response surface methodology. According to both models temperature is the parameter that affects hydrolysis the most obtaining the highest hydrolysis solubilisation and delignification at 200 °C. On the other side CO<sub>2</sub> initial pressure was not significant, what should be further investigated in the future at higher pressures.

Reducing sugars quantification obtained a maximum concentration of 2g/10g *Miscanthus* by DNS assay with an optimal temperature to hydrolyse hemicellulose from 140 to 180 °C. FTIR analysis of each fraction confirmed a successful separation of the biomass main components with a reduction in the cellulose fibres crystallinity. Temperature was considered the most significant parameter to fractionate biomass with the highest temperature (200 °C) being the one that produced a better quality fibres, supernatant and lignin in terms of contamination by the other fractions. However, results also showed that higher temperature tends to oxidise lignin. Fibres analysis by scanning electron microscopy showed that fibre structure was preserved but presented lignin-type globules on their surface indicating lignin reprecipitation.

On a separate study, the extraction of phenolic compounds from the hemicellulose fraction by subcritical water modified with CO<sub>2</sub> yielded 10g/Kg of *Miscanthus* containing 10% in *p*-coumaric acid for the optimal hydrolysis condition at 150 °C for 30 min.

Dedicated to:

My beloved wife Jawaher,

And my family for their unconditional support.

# Acknowledgments

I owe my deepest gratitude to my main supervisor Dr. Regina Santos for her guidance and support during my PhD and teaching me how to be independent and able to link science to life. I am indebted to my industrial supervisor Dr. Steve Bowra for his understanding, guidance, and remarkable ability to translate my data into industrial setting. His great knowledge and commitment to the project have been an inspiration to me over the last few years. I would also like to thank my co-supervisor Dr. Gary Leeke for his support and contribution to the work.

My appreciation goes to my colleagues and friends at the School of Chemical Engineering especially Dr. Muhammad Baig and Dr. Tiejun Lu for their endless support and fruitful discussions throughout my PhD. I am also grateful for the kindness and help of Professor Alessandro Piccolo and Mr Davide during my work at the Università Federico II in Naples, Italy.

Many thanks go to my friends Mr. Fábio and Mr. João for their continuous encouragement and wisdom.

This work would have not been possible without the generous funding by the EPSRC and Phytatec, Ltd. and the help of the support staff at the School of Chemical Engineering at the university.

Finally, I cannot find the words to express my great thanks to my family, my loving parents, grandparents and brother for being on my side and encouraging me to pursue my dreams. It leaves me to thank my dear wife Jawaher for her endless understanding and support and her family for wishing me the best of luck.

## List of abbreviations

AFEX	Ammonia fibre explosion-method
ANOVA	Analysis of variance
ARP	Ammonia recycle percolation
BBI	Broad band Inverse
CCD	Central composite design
DNS	3,5-Dinitrosalicylic acid
DP	Degree of polymerisation
DRIFT	Diffuse reflectance Fourier transform infrared spectroscopy
Et-OH	Ethanol
FID	Free Induction Decay
GAE	Gallic acid equivalent
GHG	Greenhouse Gas
HCW	Hot compressed water
HMF	5-hydroxymethylfurfural
HMF	Hydroxyl-methyl-furfural
HPLC	High performance liquid chromatography
IEA	International Energy Agency
LCB	Lignocellulosic biomass
LHW	Liquid hot water
mid-IR	Mid-infrared
NMR	Nuclear Magnetic Resonance
NREL	National Renewable Energy Laboratory
RSM	Response surface methodology
SAA	Soaking in aqueous ammonia
SEM	Scanning electron microscopy
Sub-CW	Subcritical water
TPC	Total phenolic content

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# **1 INTRODUCTION AND OBJECTIVES**

## **1.1 Introduction**

As a consequence of the climate change there is the need to find new sustainable biofuels to replace our dependence on oil and other fossil fuels. At the same time, there is the need to develop methods and create environmental benign sources to produce those sustainable biofuels. This is the backbone of the present research study. With the need for new biofuels, ethanol production has been broadly studied for decades; however the ethanol for fuel utilisation started to be obtained from starch fermentation originated from food crops during the first generation of biofuels. Therefore, there was the need to look for new sources of biofuels and the solution came by using non-edible crops such as agricultural residues, industrial and municipal organic wastes also called lignocellulosic biomass due to their main components, lignin, hemicellulose and cellulose. But, with this new type of feedstock came the issue of their recalcitrancy to fermentation. So, there was the need to develop suitable pretreatments prior to the fermentation. These pretreatments have the ability to simplify the lignocellulosic biomass macromolecule to make cellulose accessible for hydrolysis and further fermentation into ethanol. Studies on these different pretreatments show the effectiveness of using physical, chemical, combined physicochemical and biological processes to enhance ethanol production.

The present research project is part in line with the bioethanol production and it is focused in the treatment of lignocellulosic biomass but more precisely in *Miscanthus  $\chi$  giganteus*. However, bioethanol production alone is not viable economical and environmentally. Bioethanol production needs to be seen under the biorefinery concept where lignocellulosic

biomass (LCB) follows a succession of treatments until bioethanol is produced as well as other high-value compounds, by-products derived from LCB. This in fact is the main guideline for the present work, by developing and optimising a biomass treatment that enables the fractionation and recovery of lignocellulose main components.

Until now, LCB pretreatments are seen as methods to prepare biomass, or more precisely the cellulose fraction, to enzymatic hydrolysis that in turn will convert it to fermentable sugars. Therefore, the present research created the opportunity to modify / aggregate several pretreatment methods to study the feasibility of the called modified organosolv method to hydrolyse and fractionate LCB but particularly *Miscanthus  $\chi$  giganteus* that will enable to eliminate the cellulose enzymatic hydrolysis step reducing time and costs associated to it.

In summary, the proposed modified organosolv method uses the basis of an organosolv pretreatment by using an aqueous ethanolic solution with the application of Sub-CW and catalysed by the addition of CO<sub>2</sub> which will create the acidic conditions for an enhanced hydrolysis yield. It was demonstrated that the addition of sulphuric acid improved either organosolv or Sub-CW pretreatments of LCB. [1] However, the use of sulphuric acid generates several environmental concerns. Sulphuric acid is highly corrosive and its neutralisation results in abundant production of solid wastes, which can be costly to dispose of and causes an environmental load. For example, the calcium sulphate resulting from neutralisation has problematic solubility characteristics in that it becomes less soluble at higher temperatures. For applications where landfill costs are high, calcium sulphate disposal costs can prevent from the use of dilute sulphuric acid. [2]

For that reason in this research CO<sub>2</sub> was tried as a replacement entrainer that produces carbonic acid in contact with water aiding in the hydrolysis of LCB process. The pH of carbonic acid is determined by the partial pressure of carbon dioxide in contact with water, and thus it can be neutralised by releasing the reactor pressure. Carbonic acid is referred to by van Walsum et al. [2, 3] has a relatively mild and hence does not offer the same hydrolytic capability of sulphuric acid. However, after his work, van Walsum et al. has demonstrated that at temperatures of the order of 200 °C, carbonic acid does exhibit a catalytic effect on hydrolysis of xylan. [2, 3] In another work undertaken by Pasquini et al. [4], the use of water-ethanol-carbon dioxide mixture is also referred to enhance the delignification process of *Pinus taeda* wood chips and sugar cane bagasse. In addition, temperature proved to have a much greater influence than pressure, although the catalysing effect of CO<sub>2</sub> in starch and cellulose hydrolysis is referred by Rogalinski et al. [5] to be reduced with the increasing of temperature.

## **1.2 Aims and objectives**

Reducing the world's dependence on oil and other fossil fuels opened new research routes to create more environmental friendly sources of energy which in turn led to the production of biofuels. Several types of biomass were then tested and used for bioethanol production but the environmental concern around food competition guided the research in biofuels to look for non-edible crops leading their studies to the use of lignocellulosic biomass. The use of this new type of feedstock originated the development of new technologies and processes to deal with its conversion to energy though, the search for new and more environmental friendly technologies and processes does not stop and the overall purpose of the present

work is to advance in the development of new more environmental technologies that can be used in abundant and renewable LCB resources to produce biofuels but at the same time being able to create a platform of high valuable chemicals that are by-products of the biofuel industry and in turn it will enhance the economic and environmental viability of the LCB.

With this concept in mind, the present work main objective was to study the behaviour of the *Miscanthus  $\chi$  giganteus* a LCB under a modified organosolv hydrolysis treatment at a range of temperatures and pressures, load sizes with ethanol and CO<sub>2</sub> as modifiers high-pressure batch reactor.

Within the integrated biorefinery concept, the output of the research was the discussion on the feasibility of this hydrolysis method to solubilise, delignify and fractionate the LCB and assess the use of Sub-CW to produce high-value phenolic compounds that will contribute to enhance the economic viability of the biomass.

The diagram outlining this research project is presented in Figure 1.1.



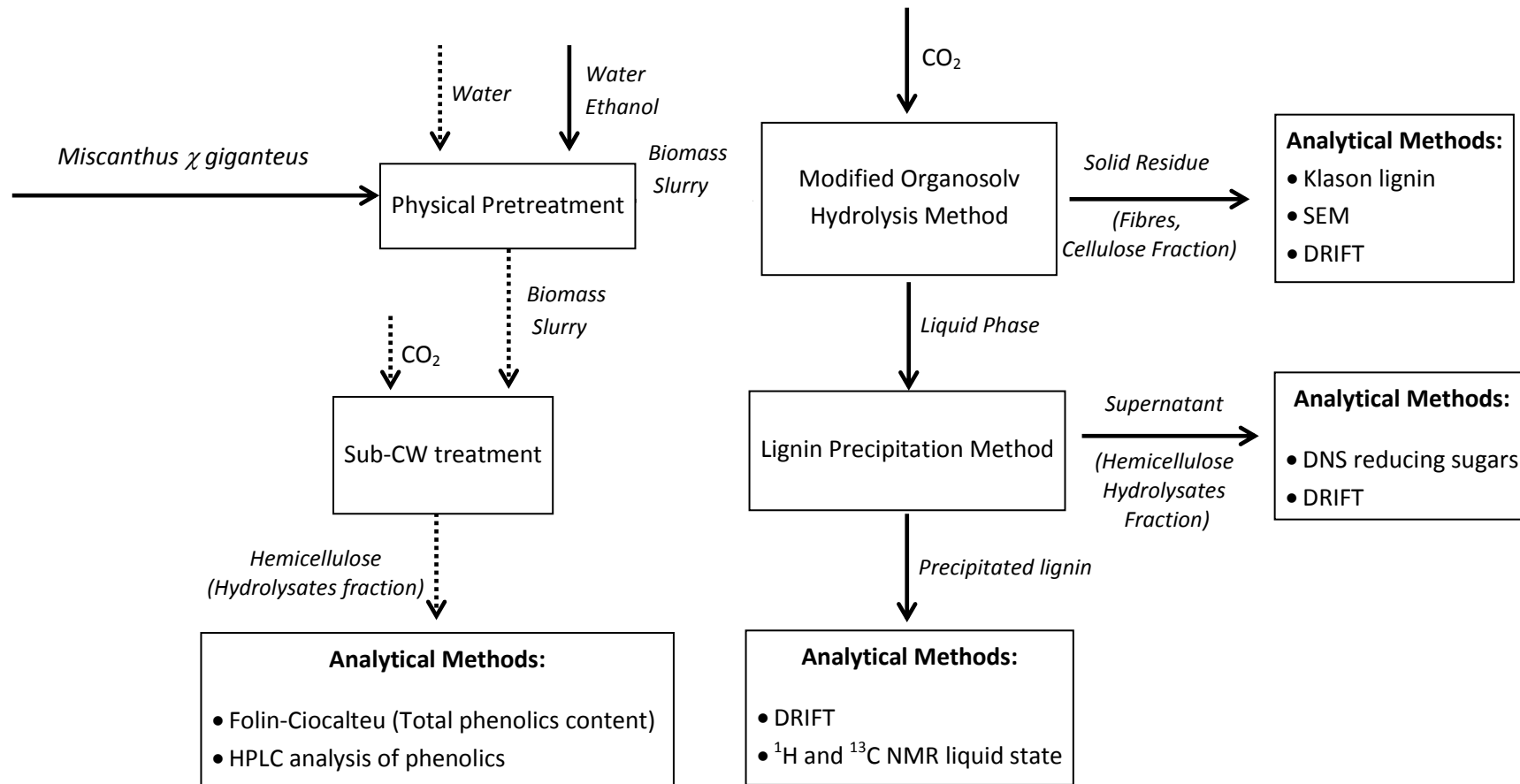


Figure 1.1. Diagram outlining this research.

## **1.3 Layout of the thesis**

The structure of this research work is presented in eight separate chapters as following

Chapter 1: Introduction and Objectives

Chapter 2: Literature review

Chapter 3: Materials and Methods

Chapter 4: Biomass physical pretreatment to improve particle size homogeneity

Chapter 5: Evaluation and optimisation of a modified organosolv method for solubilisation and delignification of Miscanthus biomass

Chapter 6: Characterisation of the liquid and solid fractions obtained by the modified organosolv hydrolysis method

Chapter 7: Evaluation of subcritical water mediated extraction of phenolic compounds from the hemicellulose fraction of Miscanthus

Chapter 8: Final conclusions and future work.

The main objectives of this research are defined and summarised in Chapter 1. Chapter 2 presents a literature review on biomass, energy crops/lignocellulosic biomass and their contribution to the current demand on renewable sources of energy with a focus on the integrated biorefinery concept and the treatments available to deconstruct the lignocellulosic biomass polymeric structure. A full description on the materials and methods used during this study are presented in Chapter 3, followed by the description and results analysis of the biomass physical pretreatment method developed to homogenise the raw

biomass particle size throughout study in Chapter 4. Chapter 5 and Chapter 6 represent the core of this research study. The optimisation results of the modified organosolv hydrolysis method to determine the optimal experimental conditions for solubilisation and delignification are presented in Chapter 5. In Chapter 6 the characterisation of both liquid and solid fractions obtained during the hydrolysis experiments on Chapter 5 are analysed. Chapter 7 presents the results of a separated study on the extraction and quantification of phenolic compounds using subcritical water modified with carbon dioxide. Finally Chapter 8 concludes the thesis with the main concluding remarks and suggestions on future plans regarding the lignocellulosic biomass hydrolysis treatments.

## 1.4 Publications

Part of the results of this research work have been published in peer reviewed journals and presented in conferences.

### 1.4.1 Publications

- R.M.N. Roque, M. N. Baig, G.A. Leeke, S. Bowra, R.C.D. Santos, “Study on sub-critical water mediated hydrolysis of *Miscanthus* a lignocellulosic biomass” *Resources, Conservation and Recycling*, 2012. 59(0): p. 43-46
- R.M.N. Roque, M. N. Baig, G.A. Leeke, A. Piccolo, S. Bowra, R.C.D. Santos, “Sub-critical water mediate hydrolysis and fractionation of lignocellulosic biomass (*Miscanthus  $\chi$  giganteus*)”, In: Bridgwater AV, editor. *Bioten Conference on Biomass Bioenergy and Biofuels 2010*. Birmingham, UK: CPL Press; 2010.

### 1.4.2 Conferences

- R.M.N. Roque, M. N. Baig, G.A. Leeke, S. Bowra, R.C.D. Santos; Study on Solubilisation and Delignification of *Miscanthus* – Modelling and Validation, 11th International Chemical and Biological Engineering Conference, Lisbon, Portugal, 5-7 September 2011. (Poster presentation)
- R.M.N. Roque, M. N. Baig, G.A. Leeke, A. Piccolo, S. Bowra, R.C.D. Santos, “Sub-critical water mediate hydrolysis and fractionation of lignocellulosic biomass (*Miscanthus  $\chi$  giganteus*)”, Chemistry Innovation Associate's Event 2010, 15/16 September 2010. Lancaster, UK. (Oral and poster presentations awarded 2nd place in the event)
- R.M.N. Roque, M. N. Baig, G.A. Leeke, A. Piccolo, S. Bowra, R.C.D. Santos, “Sub-critical water mediate hydrolysis and fractionation of lignocellulosic biomass (*Miscanthus  $\chi$  giganteus*)”, *Bioten Conference on Biomass Bioenergy and Biofuels 2010*, 21 – 23 September 2010. Birmingham, UK. (Oral and poster presentations, and peer-reviewed paper)

## **2 LITERATURE REVIEW**

### ***2.1 Biomass / Energy crops***

The world's need for sustainable fuels and chemicals to replace our dependence on oil and other fossil fuels is an ever-increasing issue at the top of many political groups, energy companies and other organisations' agendas. The use of fossil fuels is both detrimental to the world's climate as well as only being a finite solution as reported by the Intergovernmental Panel on Climate Change [6]. One of the biggest contributors to greenhouse gases is the transport sector, showing the highest rate of growth in emissions over the past 10 years. It is also highly dependent on oil [7].

Cost-effective opportunities exist today to conserve energy, switch to low-carbon alternative fuels, and reduce greenhouse gas emissions, though a number of emerging technologies might deliver more substantial benefits in the future. The utilisation of biomass, a renewable source, as raw material in a biorefinery is a promising alternative to fossil energy for the production of energy carriers and chemicals, as well as for enhancing energy security and mitigating climate change. [3] According to the EU Renewable Energy Directive, it was agreed the use of 20% renewable energy by 2020 with a UK-specific target of 15% by 2020. Therefore, the UK needs to radically increase its use of renewable energy to meet the target proposed. [4]

In light of this, an area of research that has gained significant interest is the use of specific energy crops such as *Miscanthus* and switchgrass.

Miscanthus refers to a genus of many different species. The particular species that is of interest here is *Miscanthus × giganteus*, a hybrid between *Miscanthus sinensis* and *Miscanthus sacchariflorus*, which is being planted commercially across the world [8]. A study by Heaton et al., [9] demonstrated the potential land and mineral efficiency of Miscanthus and Switchgrass over other options such as maize. In the same study Miscanthus was shown to achieve yields three times greater than Switchgrass, thus demonstrating its potential above other energy crops.

Research into the potential of Miscanthus as a source of fuel has been carried out in two main ways. One area has been looking into the possibility of direct combustion of the biomass [10], whilst the other area is more interested in converting it into bioethanol [11]. However, an integrated biorefinery concept is being developed lately to try to produce a complete range of marketable products instead of prior to obtaining the final product of one of those areas. This integrated biorefinery concept is indeed the main goal of this research project using Miscanthus.

Miscanthus is dominated by lignocellulosic biomass, which is made up of three main components: cellulose, hemicellulose and lignin. It is important to note that Miscanthus is made up of many other constituents such as proteins, starches, lipids, waxes etc. [12]. However in the biorefinery context, it is the lignocellulose which is of interest, in particular the cellulose which is used in fermentation to produce bioethanol.

Whilst the science and technology necessary to produce fuel from biomass is available and understood, there are still concerns over poor energy balances and land displacement which has slowed down policy on biofuels being deployed [13]. These factors inevitably affect the

economic feasibility of energy crops as a potential solution. However, through efficient and environmentally benign processing it may be possible to make full use of Miscanthus, to obtain a wide range of high-value chemicals as well as the biofuels to contribute further to its economic feasibility. This led this entire research project.

## ***2.2 Biobased economy, bioenergy***

As the world population will keep growing also the demand for food, energy and raw material will steadily grow. Combined with the need to strongly reduce emissions of greenhouse gases in the coming decades, consequently a biobased economy should be followed. Renewable resources have potential as alternative raw materials for chemical industries, considering that ecologically agricultural production and processing technologies are available and applied.[14] Agriculture is the core to the bio-based economy, providing source materials for commodity items, e.g. liquid fuels and value-added products such as chemicals and materials.

According to the European Commission, fostering a biobased economy will bring benefits and risks. On the plus side, a lower carbon economy and sustainable primary production will reduce CO<sub>2</sub> emissions, resource and land-use efficiency. It will create new business opportunities, higher potential for value creation through cascading use of biomass and reuse of waste materials, and EU global market leadership. A biobased economy will produce a resilient and sustainable food chain that contributes to the global food security, new agricultural practices to avoid competition between food and non-food use of biomass, and improved animal health and welfare. Finally, it will develop the European science base and stimulate high-skilled jobs by creating new integrated structures between researchers

and research funds, further research and innovation excellence in Europe, European leadership through knowledge and technology transfer, and Economic and employment incentive to rural and regional development. On the other hand, potential risks could arise at the level of food, agriculture and the environment, particularly if policies are developed and implemented in a disintegrated way. Risks include competition between food supply and biomass production, reindustrialisation and centralisation of the agri-food production, relocation of innovative industry actors, over-exploitation of natural resources and loss of biodiversity, and loss in consumer trust. [15]

Considering a biobased economy, the energy production will be guided by the use of renewable and sustainable sources, i.e. fostering bioenergy. Bioenergy is a collective term for gaseous, liquid and solid sources of energy derived from biomass [16] also referred as biofuel. Biofuels are considered as a promising substitute for fossil fuels when considering the possible reduction of greenhouse gases emissions. The first-generation biofuels which have attained commercial level production in several countries have generally been produced from food and oil crops, as well as animal fats using conventional technology. The most well-known first generation biofuels are ethanol made by fermenting sugar extracted from crop plants and starch contained in maize kernels or other starchy crops, and biodiesel produced from vegetable oils.[17]

However, first-generation biofuels production creates the conflict with food and fibre production for the use of arable land, high water and fertiliser requirements, a lack of well managed agricultural practices in emerging economies, biodiversity conservation and regionally constrained market structures. Consequently, the search for non-edible biomass (e.g. agricultural residues, industrial and municipal organic wastes) for the production of



biofuels is favoured. Second-generation biofuels are generally produced by two fundamentally different approaches, i.e. biological or thermochemical processing, from agricultural lignocellulosic biomass (non-edible crop residues or whole plant biomass) and industrial or municipal organic waste. Forestry, short rotation crops e.g. willow and specific energy crops e.g. Miscanthus and Switchgrass are being developed to meet the solid fuel/co-firing requirements for existing power generation. [16]

The lignocellulosic biomass is dominated by three naturally occurring polymers, hemicellulose, cellulose and lignin, which are found in close association and make up the cell walls. The three polymers are collectively referred to lignocellulose. All plant biomass contains a proportion of lignocellulose, but crop residues (husks) and specific energy crops have a higher proportion of these natural polymers.[16] Which is the case of the biomass in this study, *Miscanthus  $\chi$  giganteus*.

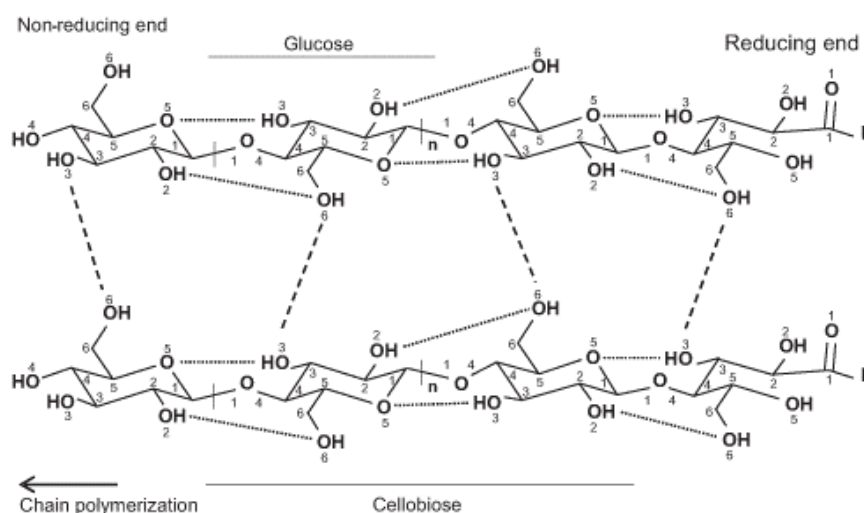
### **2.3 Lignocellulosic biomass**

In the plant-cell wall, the cellulose molecules are interlinked with hemicellulose a polymer primarily composed of pentosan and xylose. Another compound called lignin is also present in significant amounts and gives the plant its structural strength. [16]

Cellulose is the most abundant organic material on earth, with an annual production of over 50 billion tons. It is a polysaccharide with a glucose monomer unit and  $\beta$ -1,4 glycoside linkages. The basic repeating unit of the cellulose polymer consists of two glucose anhydride units, called a cellobiose unit (Figure 2.1). Cellulose can be considered as a condensation polymer of glucose, similar to starch, but the links between the glucose monomers are slightly different. Cellulose has an average molecular weight range of 300 thousand to 500

thousand, depending upon the length of the cellulose chain, which is often quantified by the degree of polymerisation (DP). Values for the DP range from 7 thousand to 10 thousand for wood to as high as 15 thousand for cotton. [18] Cellulose is insoluble in water because of its low-surface-area crystalline form held together by hydrogen bonds. The degree of crystallinity of cellulose varies with their origin and treatment. Strong acids and alkalis can swell and disperse or even dissolve the cellulose, breaking up the highly ordered crystallites. [19-21]

Industrially, cellulose has been used for paper, paperboard, and card stock production and for textiles made from cotton, linen, and other plant fibres. [22] Secondly, cellulose is also used as raw material in diverse hydrolysis processes to obtain glucose for further fermentation and consequently bioethanol production.



**Figure 2.1. The structure and the inter- and intra-chain hydrogen bonding pattern in cellulose. [23]**

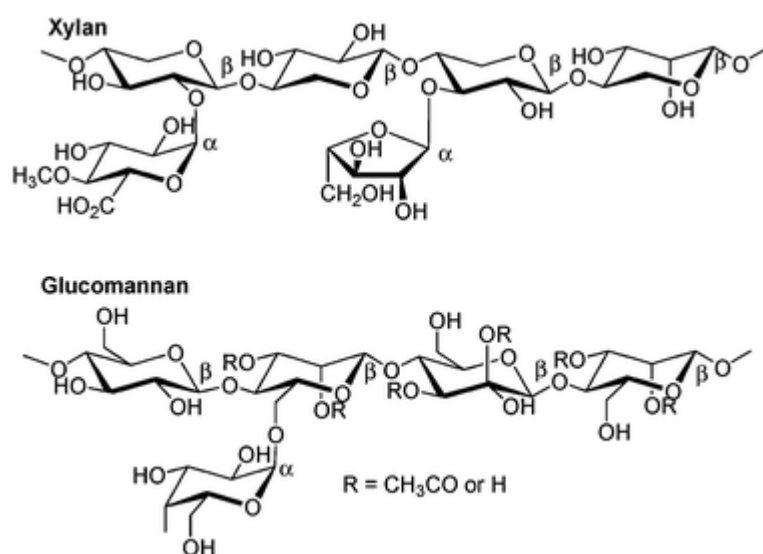
In relation to hemicellulose, it consists of various polymerised monosaccharides including 5-carbon sugars (usually xylose and arabinose), 6-carbon sugars (galactose, glucose, and

mannose), and 4-*O*-methyl glucuronic acid and galacturonic acid residues. It exists in association with cellulose in the cell wall. The most abundant building block of hemicellulose in the majority of grasses and hardwoods, such as, in corn stover, Miscanthus, switchgrass, and poplar is xylan, which consists of xylose monomer units linked at the 1 and 4 positions. Other occurring hemicellulose's building blocks are mannans, arabinans, and galactans among which most important in softwoods are galactoglucomannans and arabinoglucurinoxylans. Figure 2.2 shows the example of the two most abundant hemicellulose's building blocks in softwoods xylan and glucomannan. Despite lignin described next in this chapter being considered the major contributor to the phenolic content in the lignocellulosic biomass, hemicellulose has also been reported by Xu et al. [24] and Ou et al. [25] to have mainly *p*-coumaric and ferulic acids, they are covalently linked by ester bonds to the hemicellulose macromolecule and to lignin by ester or ether bonds in the case of sugarcane bagasse. Their work was focused on the phenolic compounds removal from the LCB by using an alkaline hydrolysis. In addition to that, Culhaoglu et al. [26] also mentioned that ferulic and *p*-coumaric acids are the major *p*-hydroxycinnamic acids found in grass cell-walls giving the example of maize in their study. This fact will be important on chapter 7 in the present research study as the quantification of *p*-coumaric acid will be used to analyse the effect of the hydrolysis of the Miscanthus but more precisely the hemicellulose fraction.

Hemicellulose is usually branched with DP ranging from less than 100 to about 200 units. Because of its structure and branched nature, hemicellulose is amorphous and relatively easy to hydrolyse to its monomer sugars compared to cellulose. [19-21]

Hemicelluloses most important applications are as gels, films, coatings, adhesives, and gelling, stabilising and viscosity-enhancing additives in food and pharmacy. Particularly the oligomeric hemicelluloses were shown to serve as biodegradable components in composites with synthetics or as pre-polymers for production of new functionalised polymeric materials.

[27]

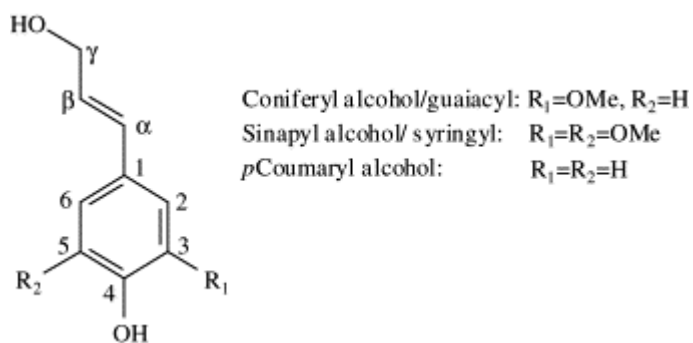


**Figure 2.2. Xylan and glucomannan, the two most abundant hemicellulose's building blocks in softwoods.**

Lignin is important for structural integrity of the cell wall, rigidity and strength of the plants stems, contributing as a barrier to the entrance of destructive enzymes into the cell wall offering natural defence against degradation.[28, 29]

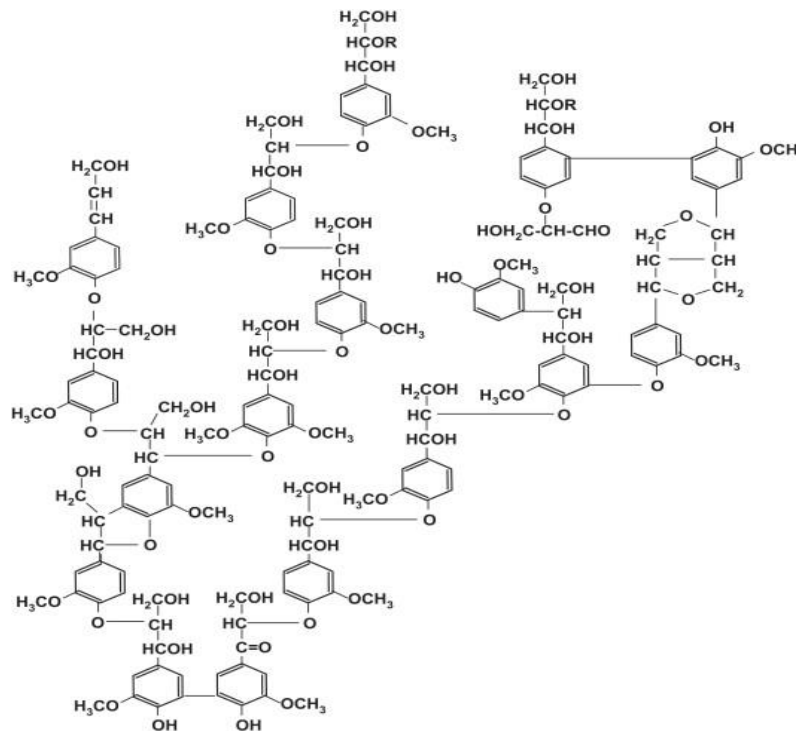
Lignin is a high molecular weight heterogeneous polymer based on a complex racemic aromatic heteropolymers derived mainly from three phenylpropane units or also known as hydroxycinnamyl alcohol monomers linked in a three-dimensional structure and differing in

their degree of methoxylation. [30] It has a structure and monomer distribution pattern dependent on factors such as sampling, plant family, location, and cultivation conditions. Plant lignins are usually divided into three main classes, softwood (gymnosperm), hardwood (angiosperm) and grass or annual plant lignin. For example, the most abundant lignin phenylpropane unit precursor in conifers (gymnosperm) is coniferyl alcohol (3-methoxy-4-hydroxycinnamyl alcohol), the mainly composition of guaiacyl lignin. In the case of hardwoods and grasses different proportions of sinapyl alcohol (3,5-dimethoxy-4-hydroxycinnamyl alcohol) and *p*-coumaryl alcohol units (4-hydroxycinnamyl alcohol) make part of the lignin polymer.[20, 31] In summary, gymnosperm lignins show predominance of guaiacyl groups, woody angiosperms lignins contain guaiacyl-syringyl groups and lignins from grasses contain guaiacyl-syringyl-*p*-coumaryl groups. [20, 32] Figure 2.3 shows the molecular structure of these three main building blocks of lignin.



**Figure 2.3. The three main building blocks of lignin. [33]**

As it is presented in Figure 2.4, lignin has an amorphous structure, which leads to a large number of possible interlinkages between individual units. Ether bonds predominate between lignin units, unlike the acetal functions found in cellulose and hemicellulose. Covalent linking also exists between lignin and polysaccharides.[19]



**Figure 2.4. Example of lignin structure from softwood.** [34] Adapted from Erich Adler(1977).[35]

Lignin has great potentials industrially it can generate materials such as wood adhesives, paper additives, phenolic resins polyolefins and epoxies. It has also been studied to be included in biocides and biostabilisers formulations, as well as to be added to soil substrates for crops cultivations.[36, 37]

Finally, biomass may also contain a wide range of organic compounds. Example includes fats, waxes, alkaloids, proteins, phenolics, simple sugars, pectins, mucilages, gums, resins, terpenes, starches, glycosides, saponins, and essential oils. These compounds function as intermediates in metabolism, energy reserves, and protective agents against microbial and

insect attack. Biomass also contains a small content of inorganic species, such as potassium, sodium, calcium. [19, 21].

Figure 2.5 shows a typical plant cell wall where it is possible to see the close association of cellulose, hemicellulose and lignin.

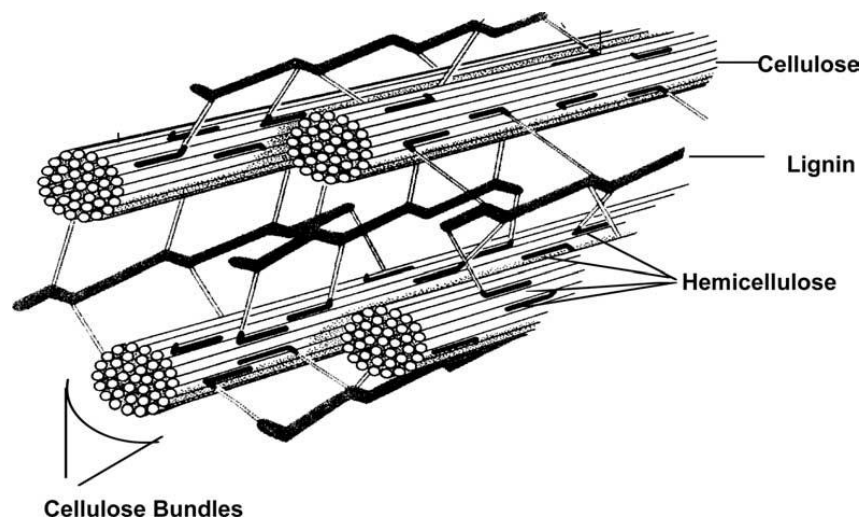


Figure 2.5. A typical plant cell wall.[16]

## 2.4 *Miscanthus x giganteus*

Choosing a biofuel crop is a major factor for the consequent biofuel conversion process and for the energy yield. However, just as important is the huge difference in environmental impacts of the different crop types due to variations in nutrient use efficiency, water use, pesticide demand and soil carbon impact. The choice between annual and perennial crops is the first dilemma to be faced, with the perennials usually providing the best environmental profile due to a lower fertiliser and pesticide requirement, a more efficient nutrient use, soil carbon storage, and a higher greenhouse gases mitigation and biodiversity.[38-42]

Here in the present study *Miscanthus  $\chi$  giganteus* was the lignocellulosic biomass chosen as a feedstock for the modified organosolv hydrolysis method, an environmentally benign yet efficient process developed. The main aim in this research was then to try to increase economic viability of *Miscanthus* by separating and recovering its principle components, i.e. cellulose, hemicellulose and lignin. In particular, efficient separation and purification of lignin, fermentation by product, will potentially further enhance the economics of bioethanol production.

In Europe *Miscanthus* has been studied and is now used commercially for bedding, heat, and electricity generation.[43] England but also Spain, Italy, Hungary, France, and Germany are the biggest producers of this lignocellulosic biomass but recently, Japan and China have taken renewed interest in this native species and started multiple research and commercialisation projects, in the United States, research began at the University of Illinois at Urbana-Champaign in 2001 [44] and has expanded rapidly to other U.S. universities due to the growing yields of *Miscanthus  $\chi$  giganteus* being reported by Heaton et al. [45] to be far greater than those of switchgrass and maize. Dohleman et al. [46] reported that *Miscanthus* had the highest leaf photosynthetic capacity in early and late season, while maize was more efficient in the warm midseason. Due to longer leaf duration the annual light interception was 61% higher in *Miscanthus*, which was reflected in a similar yield difference.[46]

Although strongly wanted for cellulosic ethanol, due to its composition and morphology giant *Miscanthus* has behaviour that likely make it better suited for thermochemical conversion processes over biological fermentation, at least under existing technology. On that view, giant *Miscanthus* has been proposed for use in combined heat and power generation, as a supplement or on its own.[47, 48]



*Miscanthus x giganteus* is a sterile hybrid horticultural genotype that was brought back to Denmark from Japan in 1935 as ornamental plant of the East Asian genus *Miscanthus Anderss* (Andropogoninae: *Poaceae*). From Denmark the plant was distributed to other European countries. More recently it has attracted attention as an alternative agricultural crop for dry biomass and fibre production.[49]

Around 15 perennial species of grasses make up the genus *Miscanthus*. Because of their ability to thrive on marginal, non-crop lands and their fast growth rates, a number of these species have been considered for their biomass potential, including *Miscanthus sinensis* and *Miscanthus x giganteus*, also known as giant *Miscanthus*. Giant *Miscanthus*, which has shown greater promise as a bioenergy crop, is a rhizomatous grass, the rhizomes are the below ground storage and perennating organ. It is triploid sterile hybrid cultivar of two *Miscanthus* species, *Miscanthus sinensis* and *Miscanthus sacchariflorus*.[50] Because *Miscanthus* a sterile hybrid it is seen as an advantage over the other crops, for example *Miscanthus sinensis*, the parent species of the giant *Miscanthus*, is listed as invasive species in Connecticut as well as *Miscanthus sacchariflorus*, the other parent species of giant *Miscanthus*, is listed on the Massachusetts prohibited plant list. Giant *Miscanthus* has been cultivated in Europe for over 30 years, and there have been no documented cases of the plant unintentionally spreading or escaping cultivation.[51]

As described by Beale et al. the perennial C4-grasses of the genus *Miscanthus* attracted much interest as a potential biomass crop in Europe during the 1990s due to their high productivity even in cool North European conditions.[52] It is commonly known, there are two different types of photosynthesis C3 and C4. However, C4 plants have bio-chemical, physiological and morphological adaptations to facilitate CO<sub>2</sub> concentration in a particular

site within the leaf. They can achieve higher rates of photosynthesis with less nitrogen than their C3 counterparts. C4 plants usually need higher temperatures than C3 plants to grow. That is the reason why maize is the only generally used C4 plant in UK all the others are C3 photosynthetic.

Examples of C3 species are poplar, willow, wheat and most other cereal crops. While the perennial grass, sweet sorghum, maize and artichoke, all use the C4 route as described by McKendry et al. [53] Miscanthus is unusual in that, despite using the C4 process. Its optimum temperature range seems to lie between the usual values for C3 and C4. Therefore, Miscanthus benefits from the C4 photosynthesis advantages as C4 plants use nutrients, light and water more efficiently than C3 plants and hence, in suitable climates, tend to have a higher yield.

Species from the genus Miscanthus are amongst the most cold-tolerant C4-species and maintain a high CO<sub>2</sub>-assimilation at temperatures below 15°C.[54, 55]

Annual yields across Europe, from established trials depending on soil type and climate, range from 10 to around 40 oven-dried tonnes ha<sup>-1</sup> from plants attaining 3m in height.[56] A recent long-term study done by Clifton-Brown et al. [57] in Ireland reported an average yield of 13.4 tonnes ha<sup>-1</sup> year<sup>-1</sup>.

A report presented in 2009 by the Centre for Ecology & Hydrology and Rothamsted Research in UK described the Miscanthus plantation method in UK where the usual method of propagation is to use pieces of rhizome, harvested from crops two to three years old. The pieces are planted at 10 000 – 20 000 ha<sup>-1</sup>, usually in March-April. At the end of the first year after planting there is insufficient growth for economic harvesting, so the aerial biomass is

mown and left in the field. This is done to add to the layer of litter and crop mulch that builds up on the soil surface, and which is added to in subsequent years by the *Miscanthus* leaves which drop off in the late summer and autumn before each harvest. The mulch helps to suppress weed growth and conserve water. The first proper harvest is taken at the end of the second year, and the crop is then harvested annually, usually around March. The report also mentioned that there is an ongoing experiment that in 2009 was on its sixteen year and the *Miscanthus* did not show sign of losing vigour which confirms the expectancy of 20 years per crop with yearly harvest (see Figure 2.6). It was also added that the crop in question in UK uses herbicides at establishment and only occasionally in mature crops and more important does not need fertilisation which is an advantage in the economic point of view as well as in the environmental side.[58]



**Figure 2.6. *Miscanthus x giganteus* growing at Rothamsted Research Ltd.[59]**

Although, *Miscanthus x giganteus* presents all the qualities and characteristics of an ideal lignocellulosic biomass for bioenergy production, there are other routes being researched and developed that also aim to create other sources of biomass as feedstock for energy

production. The third generation of biofuel represents this new route and it uses algae and sea weeds as a feedstock for biofuels production.

## **2.5 Third generation biofuels**

A third generation of biofuels is also being studied all over the world using as feedstock algae and sea weeds that can be converted into various biofuels using the different types of conversion methods available such as liquefaction, pyrolysis, gasification, extraction and transesterification, fermentation, and anaerobic digestion processes.[60-64] According to Tsukahara and Sawayama [61], Wang et al. [65] and Brennan and Owende [66] biofuels conversion technologies for algal biomass can be divided into four basic categories: biochemical conversion; thermochemical conversion; chemical reaction; and direct combustion. Each category can be further subdivided, for example the biological treatment for algae conversion into fuels can be either an alcoholic fermentation as described by Harun et al. [67] and Choi et al. [68], an anaerobic digestion to produce hydrogen or methane as shown by Sialve et al. [69] and Yang et al. [70] or a photobiological hydrogen production studied by Burgess and Fernández-Velasco [71] or Amutha and Murugesan [72]. On the other hand gasification, liquefaction and pyrolysis belong to the thermochemical conversion category which by using thermal decomposition either fuel gas, bio-oil or charcoal can be produced as shown by the work of Hirano et al. [73], Miao and Wu [74]; Chiaramonti et al. [75] and Minowa and Sawayama [76]. Oil extracted from algal biomass has also been studied by Chisti [77] and Huang et al. [78] to produce biodiesel by transesterification reaction. Finally, a less acceptable technology due to its environmental implications is the direct combustion

where the dried algal biomass can be burnt directly to provide heat and power as studied by Bruhn et al. [79]

Although the cells of microalgae are naturally able to bio-synthesise and store lipids similar to those types present in vegetable oils, there is still the need to achieve and develop commercially viable level of biofuels, either by genetic modifications of algal strains to get more efficient forms, accumulating higher quantities of lipids/carbohydrates or by develop strains that use organic waste, flue gases and industrial effluents as feeding which will also reduce GHG emissions and waste disposal problems and will contribute to the sustainability and market competitiveness of the microalgal biofuel industry. [17]

## ***2.6 Integrated biorefinery***

As discussed previously, biomass is a flexible resource that can fulfil a substantial share of the growing demand for renewable energy and products. Today biomass is mostly used for generation of electricity and heat, while application as feedstock for renewable transportation fuels is growing rapidly, due to ambitious targets in the EU and other parts of the world.

As the first generation biofuels were produced from sugar, starch and from vegetable oils crops, concern has risen over the net Green House Gas (GHG) mitigation effect of these fuels and other issues such as potential competition with food production. [14, 80] In this context the European Commission is designing environmental and socioeconomic criteria for biofuels production.

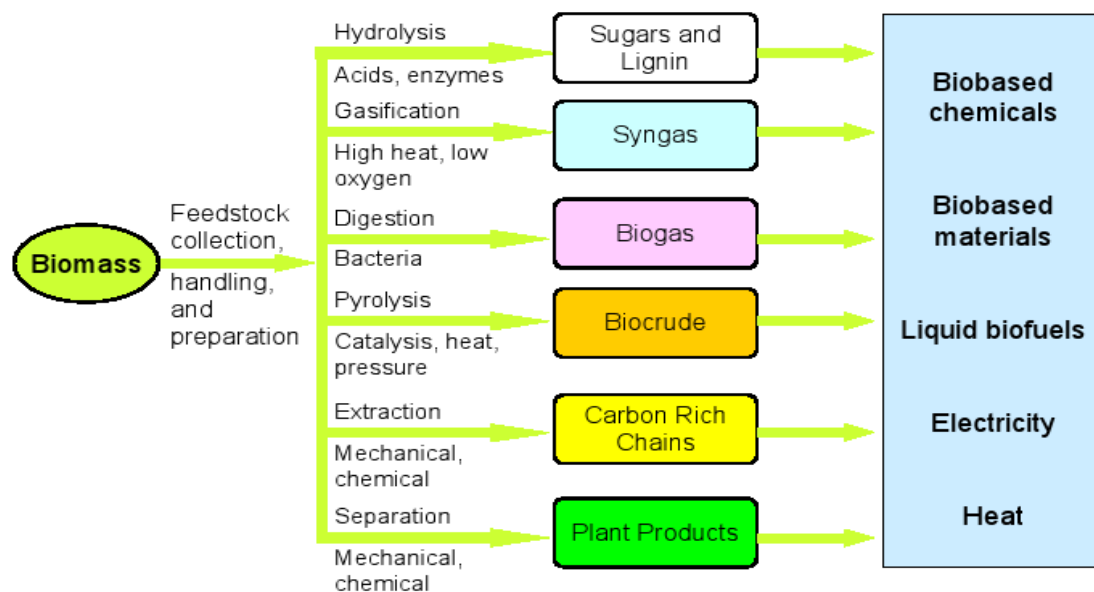
With the second generation biofuels such as bioethanol the use of abundant and low-cost lignocellulosic biomass (grass, wood etc.) and waste streams as feedstock allows application

at large scale due to the absence of competition with food production and a high net reduction of GHG emissions.

Although the view to develop cellulose biofuel looks promissory encouraging the technology development in this industrial field, lignocellulosic biomass needs to be also considered as feedstock for chemicals and other products to substitute for petrochemicals in order to improve both the economics and the ecological benefits of biomass processing. This new view brings up to discussion the integrated biorefinery concept i.e. the sustainable processing of biomass into multiple products that are separately marketable. In a biorefinery, a range of biomass types is converted (through a selection of processes) into multiple products including transportation fuel e.g. bioethanol, other bio-based products such as solvents, plastics, resins, surfactants, and electricity and heat for internal use and for export. One good definition of integrated biorefinery is described by Wertz et al. as “The integrated biorefinery is to biomass what the traditional refinery is to oil”.<sup>[81]</sup> In the same way as the oil refinery, biorefinery is understood as a further stage in the development of technologies based on biomass as feedstock. Therefore, in a broader definition, the International Energy Agency (IEA)–Bioenergy task 42 describes integrated biorefinery as “A sustainable combination of biological, thermo-chemical, and chemical processes, aimed to produce a complete range of marketable products, using a wide range of feedstock, and getting advantage of synergies between technologies.”<sup>[82]</sup>

Based on this integrated biorefinery concept, Figure 2.7 shows the major technologies available to treat biomass and the different routes to obtain a complete range of marketable products from biomass. By starting with a generic biomass as feedstock and after handling and preparation, it can be submitted to hydrolysis, gasification, digestion, pyrolysis,

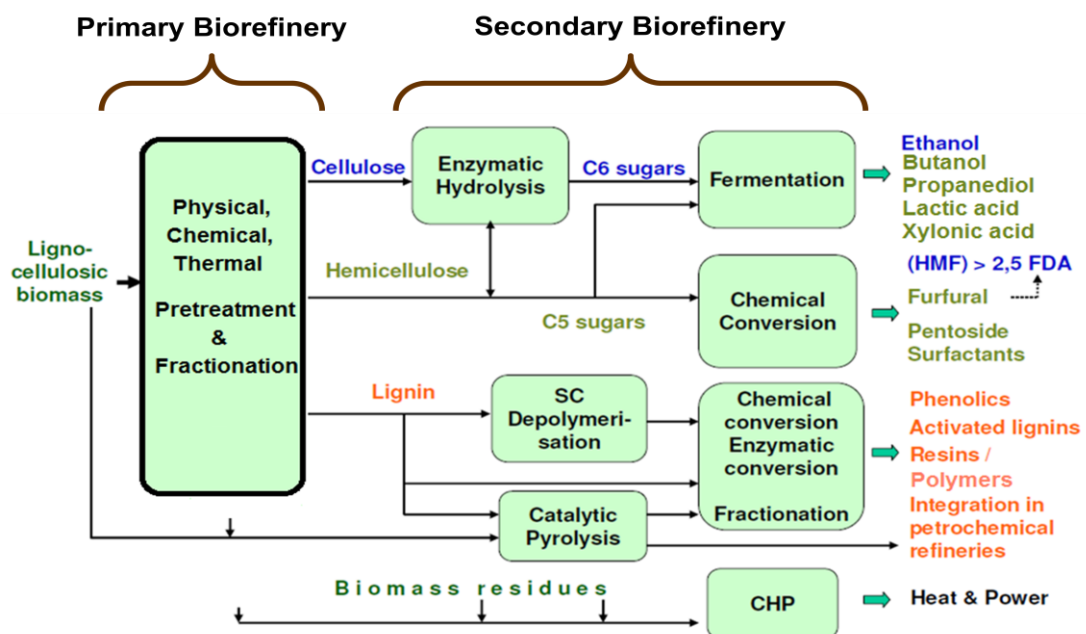
extraction or separation by using different techniques available, such as the use of acids, bases, enzymes, heat, bacteria, catalysis and mechanical methods. By doing this biomass can be fractionated in major platform products like sugars and lignin, syngas, biogas, biocrude, carbon rich chains chemicals or other plant products that later can be sub-fractionated or converted by other technologies to biobased chemicals and materials, liquid biofuels, electricity and heat. As it was demonstrated from a simple source of biomass it is possible to create an array of products that will enhance the economic value of that biomass despite, of course, being able to produce cellulosic biofuels as well. In this case, the cellulosic biofuel is not considered the main goal for the biomass but simply one of the products obtained.



**Figure 2.7. Integrated biorefinery technologies and the different output products.** Adapted from McMillan, JD. [83]

## 2.7 Integrated biorefinery applied to lignocellulosic biomass

Looking into the integrated biorefinery concept regarding the type of biomass focused in this project, the lignocellulosic biomass, Figure 2.8 shows a diagram where different technologies are applied in association in order to achieve a broad range of chemicals and therefore increase the economic value of the lignocellulosic biomass.



**Figure 2.8. Lignocellulosic biomass biorefinery concept.** The figure shows some possible products that can be obtained from a single source of lignocellulosic biomass. Adapted from Brink, et al. [84].

According to the diagram, biorefinery can be divided in primary or secondary treatment. The primary biorefinery is usually described as the biomass pretreatment and fractionation, including either physical, chemical or thermal approaches. In this first step the main lignocellulosic biomass compounds are separated, namely cellulose, hemicellulose and lignin. After that, these fractionated main compounds can both be used as they are, which is



the case when cellulose fibres are needed or these main compounds can be directed to a secondary biorefinery step. In the secondary biorefinery step cellulose can be further hydrolysed to glucose that can be fermented to produce a list of products, of course the most known product is ethanol as cellulosic biofuel but others such as butanol, lactic acid and HMF are also possible products with high economic value. Regarding hemicellulose, as a secondary biorefinery step can be submitted either to fermentation or chemical conversion to produce furfural and pentoside surfactants in addition to the products from fermentation. At last, lignin can be submitted to depolymerisation and further conversions to produce a huge amount of high value products such as phenolics, resins and polymers. And in the end as an integrated biorefinery concept should achieve, the residues coming from each biorefinery step can ultimately be used to produce heat and power in power generation stations.

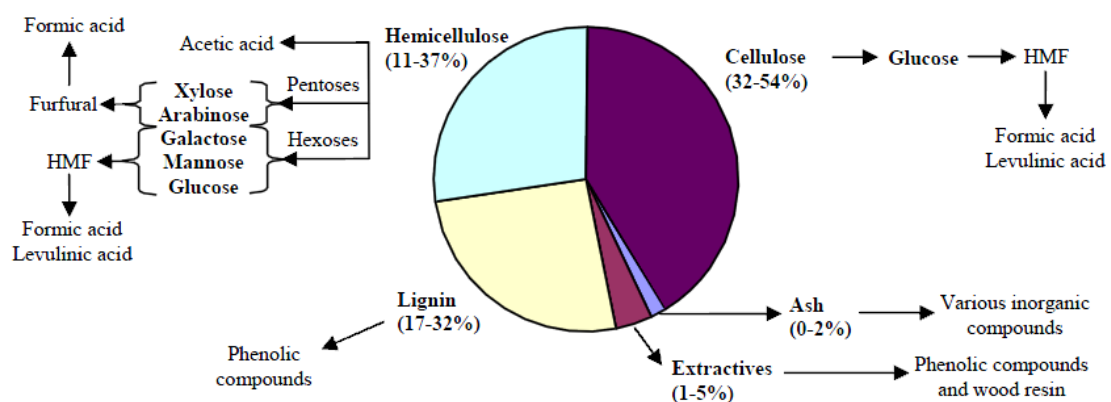
## ***2.8 Biomass pretreatment methods***

As discussed on Figure 2.7 in section 2.6 and according to the integrated biorefinery concept there are several technologies available for biomass pretreatment that can work alone or in combination with others to obtain a desired product or a platform of products from biomass. Therefore, the complete biomass degradation as a first step in biomass treatment is often not favoured but mainly chosen as a final step in an integrated biorefinery plant to get an additional and final value to the biomass by its complete fractionation. A complete biomass degradation method could include gasification or pyrolysis, for instance. On the other hand, to avoid the complete biomass degradation, a milder form of biomass

degradation should be used as a primary biorefinery treatment / pretreatment which will lead to a partial breakdown of the biomass by fractionating it on many components.

This pretreatment step will then open new routes for further biomass treatments that will create a range of new products.

One of the reasons to consider lignocellulosic biomass so appealing is the possibility to use the cellulose that is “locked up” on this type of biomass which represents an abundant and cheaper source of carbohydrate that can be used to produce bioethanol and at the same time increase overall economic viability. However, as it is known lignocellulosic biomass is not easily fermented by microorganisms due to its complex structure however, after pretreatment some of the products generated (eg. cellulose and hemicellulose) can be used as feedstock for microbial conversion into ethanol but not only. The products of biomass pretreatment can also originate after microbial conversion other value products such as hydrogen or butanol among others (Figure 2.8, section 2.7). Regarding the lignocellulosic biomass pretreatment Taherzadeh et al. [85] also presented a diagram where it shows the average composition of lignocellulosic biomass and its potential hydrolysis products (Figure 2.9).



**Figure 2.9. Lignocellulosic biomass composition and its potential hydrolysis products.[85]**

In addition, the pretreatment also endeavour to remove and modify lignin structure as well as depolymerise and remove hemicellulose and create disruption of the crystalline structure of cellulose causing the increase of the surface area and porosity. By doing this the lignocellulosic biomass pretreatment will lead to a rapid and greater yield cellulose and hemicellulose hydrolysis.

Most important of all is that biomass pretreatment methods should: [86, 87]

1. Be economic
2. Be suitable for a range of biomass types
3. Increase the amount and quality of fermentable sugars
4. Avoid the degradation of sugars (mainly pentoses) including those derived from hemicellulose
5. Reduce the formation of inhibitors which could affect downstream processing
6. Be part of an integrated biorefinery concept with potential for co-product production, for example by recovering lignin for conversion into valuable products.
7. Be suitable for industrial upscale production

In summary, biomass pretreatment can be performed under several conditions and methods that can include physical, chemical, combined physicochemical and biological processes.

The next section will describe the most important pretreatments studied to prepare different lignocellulosic biomass for further treatments but mainly the enzymatic hydrolysis for bioethanol production. Naturally there is a vast selection of lignocellulosic biomass available and for that reason it is important to emphasise that is not always possible to transfer the results of pretreatment from one type of biomass to another type of biomass. In

addition, one kind of technology considered efficient for a particular type of biomass might not work to another. In summary the efficacy of each method depends greatly on the biomass composition.

## **2.9 Physical pretreatment methods**

### **2.9.1 Mechanical comminution**

Usually reduction of particle size is carried out at the beginning of an integrated biorefinery process with the view to make material handling easier, to increase surface/volume ratio and to reduce the degree of polymerisation (DP).[88]

Mechanical pretreatment methods include chipping, milling or grinding and the resulting particle size is highly dependent either on the mechanical methods used, in the biomass physical properties and more important on the subsequent methods demands. The milling causes also shearing of the biomass.

The size of the materials is usually 10-30 mm after chipping and 0.2-2 mm after milling or grinding. [87] By using the combination of chipping, grinding, and/or milling cellulose crystallinity is reduced which will improve further hydrolysis results due to the reduction in crystallinity and will improve mass transfer characteristics from reduction in particle size.

The increase in specific surface area, reduction of DP, and the shearing, are all factors that increase the total hydrolysis yield of the lignocellulose in most cases by 5–25% (depends on kind of biomass, kind of milling, and duration of the milling), but also reduces the digestion time by 23–59% which translates in an increase in hydrolysis rate.[89, 90].

According to the study on hydrolysis of switchgrass with lime to improve enzymatic digestibility done by Chang et al. [91] it was found that a particle size reduction below 40 mesh (400  $\mu\text{m}$ ) has little effect on biomass digestibility thus with little effect on hydrolysis yield as well as hydrolysis rate of the biomass. Other groups have also demonstrated similar results for other biomasses as for the example of pure cellulose [92], newspaper and cardboard [93], ryegrass straw, [94] bagasse [95] and corn fibre [96]. For mechanical pretreatment factors like capital costs, operating costs, scale-up possibilities are very important.

## **2.10 Chemical pretreatment methods**

Several chemical based methods for biomass pretreatment have been studied over the years and they involve the contact of lignocellulosic biomass with a chemical for a specific period of time and temperature converting the hemicellulose and cellulose polymers into sugar monomers. These chemical methods include the use of acid, basis, organic solvents, subcritical water (Sub-CW), and oxidants to cause disruption of the lignocellulose molecule and therefore aid on the fractionation of the lignocellulosic biomass into its basic components so they can be further processed. As an example it is the case of conversion of lignocellulosic biomass to fermentable sugars to be converted into bioethanol. In this section each one of the methods will be described and their advantages and disadvantages will be discussed.

### **2.10.1 Acid Hydrolysis**

Acid hydrolysis has been successfully developed and normally used to hydrolyse lignocellulosic biomass and it can be divided into concentrated acid hydrolysis and diluted

acid hydrolysis. Concentrated acids commonly used in lignocellulosic biomass hydrolysis are sulphuric and hydrochloric acids that disrupt the bonding of hydrogen among cellulose chains converting it to a completely amorphous state which causes an improvement of further enzymatic hydrolysis by facilitating the production of fermentable sugars.

Concentrated acid is generally used at concentrations 30 to 70% and low temperature (around 40 °C) delivering low sugar degradation and yields in the order of 100%. [85]

Despite the low temperatures used and the reduced sugar degradation are seen as advantages of the method, the high acid concentration necessary imposes a high material corrosion risk which requires either expensive alloys or vessels built with non-metallic materials such as ceramics or carbon brick lining raising the economic issue to set up an industrial scale plant. Also there are environmental issues regarding the neutralisation products disposal from the sugar hydrolysates mixture after hydrolysis. Moreover, the use of high concentrated acid requires a high energy-demanding acid recovery step to reduce the costs and to make the process economically feasible by reusing the acid.

Regardless of all the disadvantages this method is still being used and developed by in North America by Masada Resource group and by Arkenol Inc in North America and Japan where it is operating since 2002.[85, 97, 98]

Dilute acid hydrolysis is one of the oldest methods of biomass pretreatment for bioethanol production, with the first trial to commercialise a process from wood recorded in Germany in 1898. Compared to the concentrated acid hydrolysis has been considered a useful approach to hydrolyse lignocellulosic biomass as it is less expensive but still effective by using acid concentrations normally below 4%. In this method usually sulphuric acid is

generally used but other acids have also been applied to this method like the case of such as hydrochloric acid, phosphoric acid and nitric acid. [99]. Dilute acid hydrolysis of biomass has started to be developed in two different ways. The first method consisted in one step hydrolysis that converts lignocellulosic biomass to glucose with yields of about 50-70% using  $\text{H}_2\text{SO}_4$  (< 3%) and at high temperatures (250-300 °C). However, this one step hydrolysis method due to the high temperatures applied causes degradation of the sugars produced during hydrolysis producing inhibitors for further fermentation. The main inhibitors for the fermentation step are recorded by Taherdazeh et al. in 2007 [85] as furfural, 5-hydroxymethylfurfural (HMF) levulinic acid, acetic acid, formic acid, uronic acid, 4-hydroxybenzoic acid, vanilic acid, phenol, cinnamaldehyde, formaldehyde among others. As proposed by Harris et al. in 1985, [100] to avoid this fermentation inhibition issue, the hydrolysis is run in two stages to grasp the differences between hemicellulose and cellulose recalcitrance and maximise sugar yields from the hemicellulose and cellulose fractions of biomass. Another study published by Taherdazeh et al. previously in 1997 [101] showed that more than 80% of hemicellulose could be hydrolysed with dilute acid at temperatures lower than 200 °C but cellulose gave a better yield at temperatures higher than 220 °C. Therefore, in the two steps dilute acid hydrolysis of lignocellulosic biomass the first stage is operated under milder conditions i.e. lower temperatures (<200 °C), which maximise yield from the easier hydrolysed hemicellulose. The second stage aims the hydrolysis of the more recalcitrant cellulose fraction at higher temperatures (200–300 °C). The liquid hydrolysates are recovered from each stage and fermented to alcohol. As an example of the 2 step hydrolysis process is the study done by the National Renewable Energy Laboratory (NREL) in U.S. where the conditions for the dilute acid hydrolysis of softwoods were:

- Stage 1: 0.7% sulphuric acid, 190 °C, and a 3-minute residence time
- Stage 2: 0.4% sulphuric acid, 215 °C, and a 3-minute residence time

These conditions after fermentation of the hydrolysates delivered yields of 89% for mannose, 82% for galactose and 50% for glucose from both hydrolysis steps. Liquid hydrolysates are recovered from each stage, neutralised, and fermented to ethanol. Fermentation with *Saccharomyces cerevisiae* achieved ethanol conversion of 90% of the theoretical yield.[102]

With these results dilute acid hydrolysis has the advantage to solubilise hemicellulose to xylan but also producing fermentable sugars. Even though, the two steps hydrolysis method reduces the amount of fermentation inhibitors, i.e. sugar degradation products, Saha et al. [103] detected furfural, HMF and aromatic lignin degradation compounds reducing fermentation yield. Also, Yang et al. [104] studied the dilute acid pretreatment of switchgrass germplasms for bioethanol production and indicated that using 1.5% sulphuric acid at 121°C for 60 min removed approximately 80% of the hemicelluloses, facilitated complete cellulose hydrolysis by cellulase, and produced ethanol from enzymatic hydrolysates with a 60% theoretical ethanol yield after yeast fermentation.

In summary, acid hydrolysis represents environmental and corrosion issues that lead to high operating costs with acid production and neutralisation, high power demand and high running costs when using elevated temperatures [19].

### **2.10.2 Alkaline hydrolysis**

During alkaline hydrolysis pretreatment, the  $\text{OH}^-$  ion attacks the anomeric carbon atom, therefore cleaving the ether bond. With the uptake of water and liberation of the  $\text{OH}^-$  ion,



glucose in the cellulose polymer is formed. This causes a swollen state of the biomass and makes it more accessible for enzymes and bacteria. Fengel et al. [105] as also mentioned that at higher alkali concentrations dissolution, peeling of end-groups, alkaline hydrolysis and degradation and decomposition of dissolved polysaccharides can take place.

As a pretreatment, alkaline hydrolysis aims to prepare biomass for bioethanol production by improving its digestibility in a final fermentation step. Therefore, other important role of an alkaline hydrolysis is the removal of lignin from the biomass, thus improving the reactivity of the remaining polysaccharides. In addition, alkali pretreatments remove acetyl and the various uronic acid substitutions on hemicellulose that lower the accessibility of the enzyme to the hemicellulose and cellulose surface [106]. Sun et al. [107] have also stated that alkaline hydrolysis mechanism is based on solvation and saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and other components such as lignin.

As a drawback for using this method is that mono- and dimeric carbohydrates, such as glucose, fructose, or cellobiose produced are severely attacked by  $\text{OH}^-$  at temperatures below 100 °C affecting sugar yields considerably. Glucose degradation also produces organic acids whose reaction with the alkali is also a problem. [19, 107] When used in lignocellulosic biomass the effect of alkaline hydrolysis depends on the lignin content of the biomass. The mechanism of alkaline hydrolysis usually leads several problems associated with high cost, environmental degradation and product degradation.

Calcium or sodium hydroxide and ammonia are the usual chemicals used to perform the alkaline hydrolysis pretreatment. When using calcium or sodium hydroxide there is a formation of salts which need to be removed or recycled.[108] In this type of hydrolysis, the

biomass is pretreated with calcium or sodium hydroxide and water under different conditions of temperature and pressure. Depending on the biomass lignin content oxidative conditions may be used by adding oxygen to the reaction medium under pressures around 10-15 bar. Nonoxidative conditions are effective at low lignin contents (below ~18% lignin), whereas oxidative conditions are required for high lignin contents (above ~18% lignin). [106]

The range of temperatures used for hydrolysis goes from 55 to 160 °C however, temperature will affect the rate of the reaction, taking up to 6 hours for higher temperatures but up to 8 weeks for the lowest temperature.

The other way to proceed with alkaline hydrolysis is by using aqueous ammonia.

Pretreatment of biomass with aqueous ammonia reduces lignin content and removes some hemicellulose while decrystallising cellulose. Ammonia pretreatment techniques include the ammonia fibre explosion-method (AFEX), soaking in aqueous ammonia (SAA) and ammonia recycle percolation (ARP).

Soaking in aqueous ammonia (SAA) at low temperature removes efficiently the lignin in the biomass by minimising the interaction with hemicellulose resulting in an increase of surface area and pore size. Consequently, retained hemicellulose and cellulose can be hydrolysed to fermentable sugars by most commercial xylanase and cellulase mixtures. Kim et al. [109] tested SAA as a pretreatment method of biomass. In their study, destarched barley hull was treated with 15-30% aqueous ammonia, at 30-75 °C for 12h to 77days with no agitation and a solid-to-liquid ratio 1:12. After soaking, the solids were recovered by filtrating, washed and analysed. With this treatment it was obtained 66% of lignin solubilisation and observed

saccharification yields of 83% for glucan and 63% for xylan when treating biomass with 15% aqueous ammonia at 75 °C during 48 h.

With ARP the biomass is pretreated with aqueous ammonia in a flow-through column reactor. The liquid flows at high temperature through the reactor column packed with biomass. To prevent evaporation the reactor system is slightly pressurised as applied by Kim et al., 2003 [110] and Kim and Lee, 2005 [111] which pressurised the reactor with nitrogen at 23 bar to study the pre-treatment of corn stover by ARP.

Aqueous ammonia reacts primarily with lignin but not cellulose causing depolymerisation of lignin and forcing cleavage of the lignin-carbohydrate linkages. A degree of delignification in a range from 23–63% was obtained in tests with hardwood by Yoon et al., [112] where an ammonia solution (~15%) was passed through a column reactor packed with biomass at temperatures of 160 to 180 °C and fluid velocity of 1 ml/(cm<sup>2</sup>.min) with residence times of 14 min.

After reaction the solid fraction rich in cellulose and hemicellulose is separated from the liquid. The liquid fraction is evaporated for ammonia recovery and lignin precipitation and sugar separation. Ammonia is then recycled to the reactor inlet and the separated fraction is sent into a crystalliser. After crystallisation a washing step is carried out in order to extract the sugars that have been retained in the solid matrix.

Overall the successful use of aqueous ammonia hydrolysis methods as pre-treatment for bioethanol production is highly dependent on the cost of ammonia and especially of ammonia recovery, however biomass pretreatment economics are also strongly influenced by total sugar yields achieved as stated by Holtzaple et al.. [113]

Regarding AFEX method it will be described later on the combined chemical and mechanical pre-treatment section. (Section 2.11)

In summary, a study presented by Pallapolu et al. compared switchgrass digestibility when pretreated by different methods and the results showed that for a fixed level of enzyme loading, dilute sulphuric acid and lime pretreatments exhibited higher digestibility than the SAA and AFEX.[114]

### **2.10.3 Organosolv**

Biomass hydrolysis by organosolv method is characterised by the use of an organic solvent or mixtures of organic solvents with water for removal of lignin before enzymatic hydrolysis of the cellulose fraction.

The organosolv process was originally developed as an alternative pulping process for paper making. [115] Recently, due to the popularity of second generation biofuels, the organosolv process has been studied in the context of bioethanol production. Cellulose from the organosolv process is susceptible to enzymatic hydrolysis into glucose followed by fermentation to dilute ethanol. At the same time hemicellulose is hydrolysed leading to an enhancement of the enzymatic digestibility of the cellulose fraction.

Usually the solvents used in an organosolv hydrolysis are ethanol, methanol, acetone, and ethylene glycol but many more options are available. In addition organic acids were also presented as options as organosolv catalysts for example oxalic, acetylsalicylic and salicylic acid. [107]

The range of temperatures used can go up to 200 °C in the presence of water as a co-solvent, but lower temperatures can be enough depending on the type of biomass and the

use of a catalyst as described by Ghose et al. [116]. This is required to thermochemically break the bonds within the lignin-cellulose-complex. During the process, also hemicellulose is hydrolysed. The part that remains of the biomass after organosolv pulping is cellulose fibres that can be used for paper production. Other route for the organosolv method is to obtain the cellulose fraction that can be made more susceptible for enzymatic hydrolysis to fermentable sugars.

In general organosolv hydrolysis of lignocellulosic biomass requires temperatures of 120-200 °C for effective biomass fractionation. At higher temperatures (higher than 230 °C) the cellulose fraction of the biomass will be depolymerised/degraded. Reaction times vary between 30 min and several hours for biomass fractionation. At the organosolv working conditions lignin is removed although complete delignification is hard to be achieved. Hemicellulose is hydrolysed and the cellulose recovered is more susceptible for enzymatic hydrolysis.

The addition of catalysts has also been studied in association with the organosolv process. As Ghose et al. [116] describes the uncatalysed organosolv at standard conditions can pulp only low-density hardwoods and agricultural residues. For the pulping of softwoods or high-density hardwoods, more severe process conditions (e.g. in the form of a catalyst) are required. The most usually applied catalyst is sulphuric acid but also others have been proposed such as aromatic acids as tested by Ghose et al. [116]. Previous studies of organosolv hydrolysis using sulphuric acid showed great ability to remove lignin (delignification). The use of sulphuric acid as catalyst in an organosolv method using different organic solvents proved to be able to remove at least 85% of lignin with some studies getting results higher than 90% in temperature range from 160-180 °C. The

delignification results were presented for the hydrolysis of corn stover by Lee et al., [117] and also O'Connor [118] that presented the results and patent the method in 2007. Lower delignification results were also obtained in the case of hardwoods as described by Pan et al. [119] where 74% of lignin was removed from poplar chips. The use of uncatalysed organosolv method also proved to be able to remove lignin. Carioca et al. [120] was able to remove the 70% of the lignin from elephant grass by using 50% aqueous ethanol at 180 °C for 3 hours. Moreover, with this method Carioca et al. [120] noticed that delignification occurred at a higher rate for the first 2 h slowing down considerably after that noticing lignin condensation. Hemicellulose was removed up to 90% also in the first 2 h. Regarding cellulose fibres, this study also reported that the quality of the fibres for enzymatic hydrolysis improves with longer hydrolysis time. Other example of using uncatalysed organosolv method as a pre-treatment for the enzymatic hydrolysis is the work done by Sun et al. [1]. In their work the hydrolysis of wheat straw was hydrolysed with aqueous glycerol at a higher temperature of 240 °C and it was reported a delignification of 70%. Comparing the delignification results in literature, it seems that the catalysed organosolv method delivers higher delignification results than the uncatalysed method special for hardwoods.

In summary, organosolv offers the opportunity to remove and recover lignin before fermentation making it able to obtain a better quality lignin to be used as a platform for chemical production as opposed to other methods such as the case of acid and alkaline hydrolysis, already described, where lignin remains until the end of the conversion process thus creating a lignin containing unhydrolysed sugar polymers as well as other organics. In comparison to the acid mediated hydrolysis the organosolv method reduces the amount of waste produced during neutralisation of sulphuric acid as well as it reduces the formation of

inhibitor compounds due to the absence of a strongly acidic environment. On the other hand, by using organic solvents, organosolv hydrolysis has a high cost associated to solvent recycling hydrolysis which might be compensated by the production of lignin chemical platform. In this case there is the need to remove the solvent as it can be an inhibitor for the enzymatic hydrolysis and fermentation steps. Removal and recovery of the solvent is also required for reducing its cost and environmental impact as well.

## 2.10.4 Hydrothermal

Hydrothermal pre-treatment also known as hot compressed water (HCW), liquid hot water (LHW), sub or supercritical water treatment is an alternative pretreatment method to the acid or alkaline hydrolysis of lignocellulosic biomass. Sub-CW pretreatment uses water at high temperatures that is kept under pressure to maintain it in the liquid state and is able to penetrate cell's structures, hydrates cellulose, depolymerises hemicellulose (to oligomers and monomers) as well as other highly polymeric materials such as starch and lignin as reported by Rogalinski et al. [5] where the hydrolysis kinetics of cellulose and starch was studied at temperatures from 240 to 310 °C. Other Study run by Hashaikeh et al. [121] where willow was used as biomass and it was treated with Sub-CW in a range of temperatures 200-350 °C at 100 bar demonstrated that lignin and hemicellulose were fragmented and dissolved at a temperature as low as 200 °C and cellulose dissolved in the 280–320 °C temperature range.

The effectiveness of the water at these conditions of temperature and pressure to pretreat biomass has distinctly different behaviour compared to water at ambient conditions which is due to the dramatic changes in physical properties, namely dielectric strength and ionic

product, which in turn can easily be altered by changing temperature and pressure. In water at high temperatures (150–230 °C), the H-bonding starts weakening, allowing autoionisation of water into acidic hydronium ions ( $\text{H}_3\text{O}^+$ ) that will act as catalysts. In addition, in the subcritical region (100–374 °C) the ionisation constant ( $K_w$ ) of water increases with temperature. However, when exceeding its critical point (374 °C and 22.1 MPa), the values of dielectric constant, ionisation constant ( $K_w$ ) and ionic product of water drop drastically. The ionic product ( $K_w$ ) of water changes as the temperature changes.  $K_w$  reaches the maximum value,  $6.34 \times 10^{-12}$ , at around 250 °C and then decreases to  $1.86 \times 10^{-16}$  at the critical point (374.15 °C, 22.120 MPa).[122] Furthermore, hydronium ions are generated from organic acids, mainly acetic acid from acetyl groups and uronic acid [123]. Acetyl groups are present in LCBs and as they are associated with hemicellulose, the hydration of the acetyl groups leads to the acidification of the liquor and thus, formation of hydrogen ions that act as catalysts in the hydrolysis of LCB.

Therefore, sub-critical water can be considered to be well-suited for the hydrolysis process that will aid enzymatic conversion of cellulose to glucose by solubilising hemicellulose fraction of the biomass, while leaving the cellulose more reactive and accessible to cellulase enzymes. Sub-CW pretreatment conditions for 15% (wt/vol) slurry of hybrid poplar were found by Kim et al. [124] to be 200 °C with 10 min reaction time, which resulted in the highest fermentable sugar yield with minimal formation of sugar decomposition products during the pretreatment. The LHW pretreatment solubilised 62% of hemicellulose as soluble oligomers.

Another example of the efficacy of Sub-CW pretreatment on lignocellulosic biomass was presented by Mok et al. [125] where six woody and four herbaceous biomass samples were



submitted to treatment in a tubular percolating reactor with Sub-CW for 0-15 min at 200-230°C and the results showed between 40% and 60% of the total biomass was dissolved, with 4–22% of the cellulose, 35–60% of the lignin and all of the hemicellulose being removed.

In summary, using sub-critical water/liquid hot water to mediate biomass hydrolysis process has numerous advantages because it utilises water as a reactant in the hydrolysis medium as well as solvent of the different components of LCB. Therefore, it is seen as environmentally benign. This hydrolysis process has a promising path for 'green chemistry' by providing alternatives to corrosive acids and toxic solvents. It is also seen as a way to optimise energy usage, since it does not require extra energy for a subsequent water evaporation step. [121]

On the other hand, Sub-CW pre-treatment of LCB showed at temperatures above 230°C to cause re-condensation reactions from the dissolution products of lignin and hemicellulose. These re-condensation reactions result from the fact that the dissolution products of lignin and hemicellulose tend to react internally and precipitate over the solid residue which will block the water access to the cellulose that will tend to dehydrate and form char-like precipitates inside the reactor. This issue was demonstrated by Hashaikeh et al. [121] when working with willow.

Moreover, acetic acid is formed during the treatment and acts as a catalyst for polysaccharide hydrolysis resulting in the formation of monomeric sugars that may further decompose to furfural (fermentation inhibitor) [125]. In addition to this issue, Ando et al. [122] used a flow type reactor with Sub-CW to hydrolyse three different types of LCB, bamboo, chinquapin (hardwood), and Japan cedar (softwood). A flow rate of  $10\text{ml}\cdot\text{min}^{-1}$  and

a pressure of 98 MPa were kept constants and temperature was increased in a rate up to above 300 °C. The results showed that free sugars and most of hemicellulose were the first to be solubilised followed by part of the lignin and finally cellulose started to decompose at 230°C. The issue presented by Ando's group was that part of the lignin could not be solubilised which could mean that Sub-CW alone might not be enough to break the LCB into its main compounds and probably some catalysts should be added as it happens with other pretreatments.

## ***2.11 Combined chemical and mechanical pretreatment***

These methods combine mechanical and chemical pretreatments and are described below.

### **2.11.1 Steam explosion**

With this method high-pressure saturated steam is injected into a batch or continuous reactor filled with biomass. During the steam injection, the temperature rises to 160-260°C. Subsequently, pressure is suddenly reduced and the biomass undergoes an explosive decompression resulting in substantial breakdown of the lignocellulosic structure, hydrolysis of the hemicellulosic fraction, depolymerisation of the lignin components and defibration. Therefore, the accessibility of the cellulose components to degradation by enzymes is greatly increased and so improving the overall fermentation process afterwards. Steam explosion can be uncatalysed or catalysed by the addition of acids or alkali. And the hydrolysis results are dependants on residence time, temperature, particle size and moisture content as described by Sun et al. [107]. One study done by Cara et al. [126] used steam explosion hydrolysis of olive tree pruning and the results showed that enzymatic hydrolysis was improved however the resulting material from the steam explosion hydrolysis was

submitted to delignification before fermentation. Later, the same group reported another study with the same biomass but found out that pretreatment by steam explosion of the olive tree pruning was enhanced by soaking the biomass with 1% sulphuric acid prior to steam explosion. Pretreatment conditions for maximum ethanol yield were 1% sulphuric acid impregnation and steam explosion pre-treatment at 230°C.[127]

However, the presence of lignin in the hydrolysate mixture after steam explosion pre-treatment leads to the presence of degradation products that may inhibit further fermentation process.[128] To avoid this issue, similar methods involving steam explosion are used in order to fractionate the biomass by separating the fermentable sugars fraction from the lignin fraction these methods apply the use of ammonia or carbon dioxide and are described below.

### **2.11.2 Ammonia fibre explosion (AFEX) and CO<sub>2</sub> explosion**

Both ammonia fibre explosion (AFEX) and CO<sub>2</sub> explosion use the advantages of the addition of catalysts in order to aid the hydrolysis of LCB by steam explosion. In the case of the AFEX the addition of ammonia reduces the lignin content and removes some hemicellulose while decrystallising cellulose. [129]

The cost of ammonia and especially of ammonia recovery drives the cost of the pre-treatment as described by Holtzaple et al. [113].

On the other hand the use of carbon dioxide is seen as advantageous as it is easier to handle and recover. In this method high pressure CO<sub>2</sub> is injected into the batch reactor and then liberated by an explosive decompression. During steam explosion pretreatment CO<sub>2</sub> forms carbonic acid that increases the LCB hydrolysis rate. Dale et al. [130] also studied this

method to pretreat alfafa by pressurising CO<sub>2</sub> at 5.62 MPa in a ratio of 4 kg CO<sub>2</sub>/kg fiber. The results showed that 75% of the theoretical glucose was released during 24 h of the enzymatic hydrolysis. Although the yields were relatively low compared to steam or ammonia explosion pretreatment, they were high compared to the enzymatic hydrolysis without pretreatment.

Zheng et al. [131] investigated CO<sub>2</sub> explosion with steam and ammonia explosion for pretreatment of recycled paper mix, sugarcane bagasse, and repulping waste of recycled paper, and reported that CO<sub>2</sub> explosion was more cost-effective than ammonia explosion and did not cause the formation of inhibitory compounds that could occur in steam explosion. And Mes-Hartree et al. added that AFEX pretreatment does not significantly solubilise hemicellulose compared to acid pre-treatment and acid-catalysed steam explosion [129].

## **2.12 Biological pretreatment**

Biological pretreatments rely on a microbial or enzyme treatment to modify the chemical composition of the biomass and improve the sugar release yield by cellulose. Usually microorganisms such as white, brown and soft rot-fungi are employed to degrade hemicellulose and lignin but leave the cellulose intact [107]. Lignin degradation occurs through the action of lignin degrading enzymes secreted by the fungi. Compared to other technologies biological pre-treatment involve mild conditions and are of low cost, the disadvantages are though the low rates of hydrolysis and long pretreatment times required.

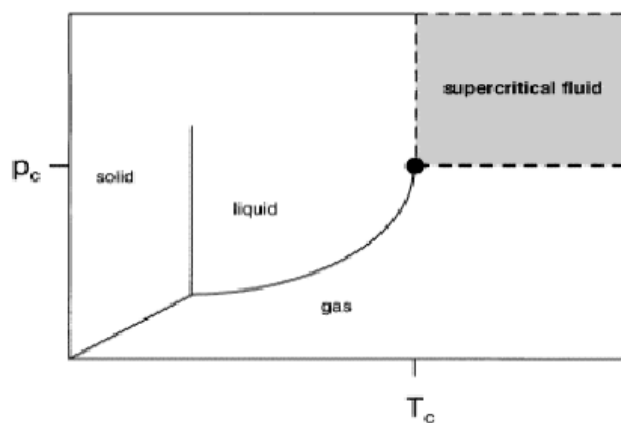
## **2.13 Proposed modified organosolv pretreatment**

The modified organosolv pretreatment method proposed on this work aims to associate the advantages of the organosolv pretreatment by removing lignin using ethanol as an organic solvent however this new method adds the advantage of not using organic acids and therefore reducing neutralisation products after pretreatment. Instead, the proposed method uses carbon dioxide that in presence of sub critical water, under pressure will form carbonic acid that will behave as a catalyst that can be removed in the end of the reaction by a simple depressurisation.

Taking into consideration that the hydrolysis of Miscanthus during this research project was done with a modified organosolv method using water under subcritical conditions it was thought that there was the need to define what are critical fluids and different applications that have been developed along the years.

## **2.14 Critical fluids**

The critical point of a pure substance is defined as the highest pressure and temperature ( $P_c$  and  $T_c$ ) that the substance can exist in liquid-vapour equilibrium (Figure 2.10). At pressures and temperatures higher than the critical point a homogeneous fluid is formed (supercritical fluid) which means only one phase is visible.[132]



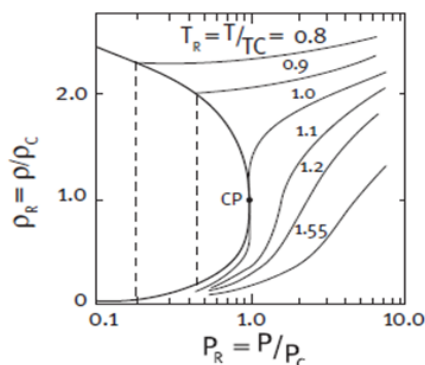
**Figure 2.10.** PT Phase diagram of a pure substance showing the state of aggregation.[132]

The critical point is known since the early XIX century when it was discovered by Cagniard de la Tour in 1822. At that time it was thought that all the substances above their critical points would be gases and therefore weak solvents. However, by the second half of the century Hannay and Hogarth discovered the ability of a supercritical fluid to dissolve solid substances with low vapour pressure while using supercritical ethanol to solubilise inorganic salts, leading to a new field of solvents. They also noted that the solubility increased with the increasing in the pressure. After Hannay and Hogarth several authors started investigating several other supercritical fluids such as hydrogen, water, helium, ammonia, nitrogen and nitrogen oxide but carbon dioxide was the one that showed the most interest. Since then several solutes have also been studied comprising organic and inorganic compounds and complex natural products. [133]

### 2.14.1 Properties of supercritical fluids

Supercritical fluid phase combines the properties of both gaseous and liquid phases, these fluids are liquid-gas like solvents combining diffusivity and dissolution of both phases and therefore enhancing their solvent/extraction power. The density of a supercritical fluid is extremely sensitive to minor changes in pressure and temperature near the critical point

making them tuning sensitive and therefore easily to separate the solvent from the solute as a result of the solvent power change.[134] The diagram on Figure 2.11 shows the dependency of the density in supercritical CO<sub>2</sub> as function of pressure and temperature.



**Figure 2.11. PT Density vs. pressure diagram for carbon dioxide.[1]**

As described on Table 2.1 by Brunner [134], the supercritical fluid state presents high density and low viscosity and diffusivity higher than the liquid state which explains the high and attractive mass transfer present in the supercritical fluids.

**Table 2.1. Physical parameters comparison in gases, liquids and supercritical fluids.[134]**

State of the matter	Density g/cm <sup>3</sup>	Diffusivity cm <sup>2</sup> /s	Viscosity g/cm.s
<b>Gas</b>			
P = 1 atm, T = 15-30°C	(0.6-2.0) × 10 <sup>-3</sup>	0.1- 0.4	(0.6-2.0) × 10 <sup>-4</sup>
<b>Liquid</b>			
P = 1 atm, T = 15-30°C	0.6 - 1.6	(0.2-2.0) × 10 <sup>-5</sup>	(0.2-3.0) × 10 <sup>-2</sup>
<b>Supercritical Fluid</b>			
P = P <sub>c</sub> , T=T <sub>c</sub>	0.2- 0.5	0.7 × 10 <sup>-2</sup>	(1-3) × 10 <sup>-4</sup>
P = 4P <sub>c</sub> , T=T <sub>c</sub>	0.4-0.9	0.2 × 10 <sup>-3</sup>	(3-9) × 10 <sup>-4</sup>

Different supercritical fluids have been investigated for many applications and each one has their characteristic critical point as showed on Table 2.2. Among the different substances

studied, carbon dioxide and water have been studied intensively due to their particular characteristics which will be mentioned in section 2.14.3, but mainly their reduced toxicity.

**Table 2.2. Critical points of different substances used as supercritical fluids solvents.[135]**

Molecule	Critical Properties		Toxicity and flammability
	T <sub>c</sub> (K)	P <sub>c</sub> (MPa)	
Argon	151	49	
Methane	191	45	Highly flammable
Carbon Dioxide	304	73	
Ethane	305	48	Highly flammable
Propane	370	42	Extremely flammable
Ammonia	406	111	Toxic
Water	647	218	

## 2.14.2 Supercritical fluids applications

The application of supercritical fluids is broad and it is in continuous development for example supercritical fluids have been applied in: [136]

- Supercritical fluid chromatography
- Particle formation
- Supercritical drying
- Reaction medium
  - Hydrolysis
  - Reactions where the product are soluble in the supercritical fluid
- Supercritical fluid extraction
  - Removal of fat from food
  - Decaffeination of coffee and tea
  - Removal of alcohol from wine and beer.



### 2.14.3 Carbon dioxide and water as critical fluids

Carbon dioxide has been investigated as the main supercritical solvent used in the extraction of natural compounds and production of pharmaceuticals because it is non-toxic, non-flammable and has mild critical conditions of 73MPa and 304K and can be acquired at low cost.[137] However, carbon dioxide has low polarity so there is a need to add co-solvents in order to increase its solvent power and therefore being able to use it as a solvent to extract more polar compounds. A co-solvent, should be used at low concentrations to a maximum of 10% v/v. Several co-solvents have been used in addition to supercritical carbon dioxide which is the case of ethanol, water, methanol, ethyl acetate and chloroform.[138]

Water has also been applied as a critical fluid. Its distinctly different behaviour compared to water at ambient conditions is due to the dramatic changes in physical properties, namely dielectric strength and ionic product, which in turn can easily be altered by changing temperature and pressure. Several biopolymers react with high-temperature water in short residence times and with high rates of conversion.[5] The ionic product ( $K_w$ ) of water changes as the temperature changes.  $K_w$  reaches the maximum value,  $6.34 \times 10^{-12}$ , at around 250°C and then decreases to  $1.86 \times 10^{-16}$  at the critical point (374.15°C, 22.120 MPa).[139]

Supercritical water has been applied to supercritical water oxidation as a way to destroy hazardous wastes at high temperature and pressure around 650°C and 235bar respectively. It has also been used in gasification of biomass where it converts aqueous streams of biomass into gases such as hydrogen, methane, carbon dioxide and carbon monoxide. In this case water is used as a reaction medium in combination with an oxidant such as oxygen or air to convert the organic compounds to carbon dioxide and water along with a release of energy. Heteroatoms, such as chlorine, that are bound to the organic will produce hydrochloric acid.

Sulphur and phosphorous will be oxidised to  $\text{SO}_4$  and  $\text{PO}_4$  producing sulphuric acid and phosphoric acid respectively. Metals will be oxidised to their highest oxidation state. [139] However, supercritical water represents some challenges mainly due to its high corrosion power, requiring corrosion, high pressure and high temperature reaction vessels and therefore increasing the cost of the whole process.

Other application of water is the use of subcritical water/liquid hot water to mediate biomass hydrolysis process which has numerous advantages because it utilises water as a hydrolysis medium and at subcritical conditions it is less corrosive than in the supercritical state. Therefore, it is seen as environmentally benign. This hydrolysis process has a promising path for 'green chemistry' by providing alternatives to corrosive acids and toxic solvents. It is also seen as a way to optimise energy usage, since it does not require extra energy for a subsequent water evaporation step. [121]

## **3 MATERIALS AND METHODS**

### **3.1 Aim**

This chapter describes the materials and methods used during the research project and it includes the feedstock preparation by a physical pretreatment method developed and described on chapter 4 that has the objective to reduce and homogenise particle size of the Miscanthus before hydrolysis treatment. The modified organosolv method used to hydrolyse the Miscanthus biomass, the backbone of this research study is also thoroughly described in this chapter. As a result of the hydrolysis method, lignin is removed and transferred to the liquid phase (hydrolysates) from the Miscanthus lignocellulosic structure and it was recovered by precipitation afterwards. Therefore the method of recovering lignin from the liquid phase is also presented here.

Several analytical methods were also used to study the efficacy of the hydrolysis treatment on fractionating the LCB main components, lignin, hemicellulose and cellulose.

The methods of analysis include the acid insoluble lignin determination by the Klason method that determines the amount of lignin still present in the solid residue after hydrolysis. This method allowed the calculation of the extent of delignification on each hydrolysis condition. Regarding to the cellulose fibres obtained after hydrolysis, they were analysed by Scan Electron Microscopy (SEM) giving a qualitative result of how the hydrolysis method affects the fibres structure.

Another important method used was the determination of the reducing sugar on the hydrolysates by the DNS (3,5-Dinitrosalicylic acid) method which gives the opportunity to

assess the efficacy of different hydrolysis conditions to produce a fermentable sugars rich mixture that can be used as a fermentation substrate for biofuel production, for instance bioethanol.

A comparative study on the effect of the different hydrolysis conditions on the chemical structure of the residual fibres, the recovered lignin by precipitation from the hydrolysates and the dried hydrolysates after lignin removal was carried out by diffuse reflectance Fourier transform infrared spectroscopy (DRIFT) and the results are presented on chapter 6. In addition to the DRIFT analysis,  $^1\text{H}$  and  $^{13}\text{C}$  NMR liquid-state analysis were also performed in four recovered lignin samples to study the effect of temperature, ethanol concentration and pressure on the lignin molecular structure.

Other point considered on this research was the evaluation of Sub-CW mediated extraction of phenolic compounds from the hemicellulose fraction of *Miscanthus* (chapter 7). This study was performed by treating *Miscanthus* biomass with water at temperatures lower than  $180^\circ\text{C}$  and addition of carbon dioxide at 55 bar initial pressure with the aim to attack the hemicellulose fraction and in turn to analyse its composition in terms of phenolic content. The phenolic content of the hydrolysates (liquid fraction obtained after hydrolysis) was studied by the Folin-Ciocalteu method, a more general method to determine the total phenolic content (TPC). In addition to the TPC determination method, a High Performance Liquid Chromatography (HPLC) method was also developed to analyse phenolic compounds on this hemicellulose fraction with a focus on the quantification of *p*-coumaric acid.

Overall, this chapter represents the methods used during the research project with the aim to assess the efficacy of the hydrolysis method to fractionate lignocellulosic biomass on their

major compounds, lignin, hemicellulose and cellulose that in turn will aid further conversions as part of the biorefinery concept as well as the recovery of high value compounds present in the biomass cell wall, such as the phenolic compounds.

### **3.2 Biomass feedstock**

The lignocellulosic biomass used in all the experimental work was air-dried *Miscanthus x giganteus* kindly provided by the Institute of Biological, Environmental & Rural Sciences (IBERS, UK) in collaboration with Phytatec (UK) LTD. It was harvested in February 2007 in Aberystwyth, Wales, UK and it was composed of 44% cellulose, 20% hemicellulose and 26% Klason lignin. The biomass was kept in bale in a cool, dry and dark place throughout the research project.

### **3.3 Biomass physical pretreatment**

Depending on the hydrolysis condition in study a determined amount of biomass was pretreated according to the method developed and described in chapter 4 to obtain an average particle size of 500 $\mu$ m. In summary, *Miscanthus* was immersed at 50°C for 20 min in the hydrolysis medium (250 ml) with varied concentrations in ethanol (Ethanol absolute  $\geq$  99.8%, Fisher Scientific) depending on the experimental condition, which softens the structure of the grass that was then grinded for 3 min in a blender (Moulinex Vitamix Y42, 400 W, 1.5 l).

### **3.4 Modified organosolv method**

The biomass slurry produced by the physical pretreatment was then transferred to the stirred high-pressure vessel (500 ml high pressure stirred reactor manufactured in Alloy C276

by Parr) and the pH of the mixture was measured. The reactor was then closed and pressurised according to the condition in study with carbon dioxide (vapour withdrawal, BOC, UK, analytical grade, 99.8%). After that, temperature was increased to the desired temperature where it was kept stable during the reaction time by checking it with the reactor 4836 Parr controller. To stop the reaction the high-pressure vessel was quenched both by removing the heating jacket and by opening the cooling system, which consists of a cooling coil inside the pressure vessel where a coolant flows at  $-7\text{ }^{\circ}\text{C}$ . When the temperature decreased below  $50\text{ }^{\circ}\text{C}$ , the reactor was depressurised slowly to release the gases and then it was opened. Heating and cooling times were dependent on the working temperatures (see Figure 3.1 and Table 3.1) Final pH was measured before the resulting insoluble fraction was recovered by vacuum filtration through a sintered disc, porosity 1, rinsed 3 times with 50 ml each with a solution with the same composition as the liquid phase, a mixture of water:ethanol from 0 to 70% in ethanol, (Ethanol absolute,  $\geq 99.8\%$  analytical grade, Fisher Scientific, UK). The final insoluble fraction was dried at  $65\text{ }^{\circ}\text{C}$  and weighed after cooled in a desiccator to room temperature until constant weight for calculation of the biomass solubilisation. In the end the obtained residue was stored in resealable polyethylene bags for later being analysed by the Klason lignin assay, DRIFT analysis, and SEM.

In the end of each hydrolysis experiment, two fractions were obtained, a solid residue fraction constituted by the cellulose-rich fibres and a liquid fraction resulting of the mixture of the hydrolysis medium with hemicellulose-rich hydrolysates and also the extracted lignin from the biomass.

Following the hydrolysis method, the liquid fraction was then processed to precipitate the lignin with the method described in section 3.5.

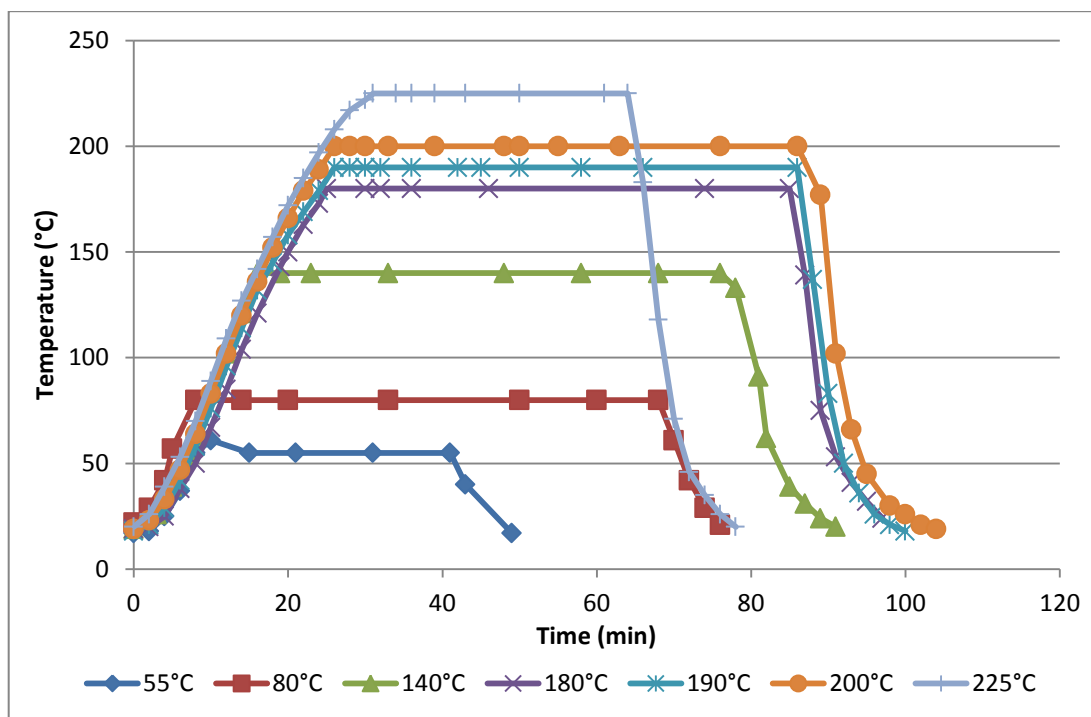
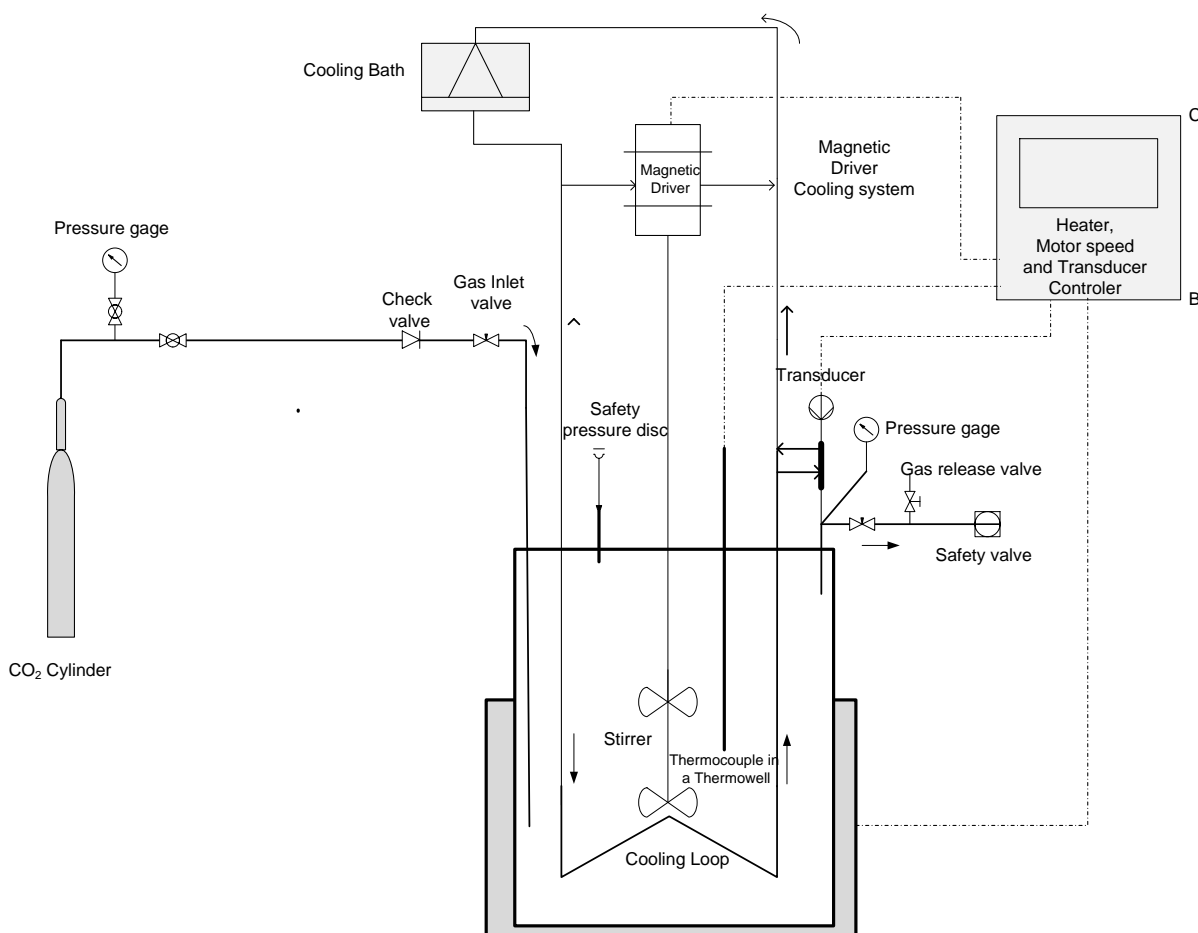


Figure 3.1. Heating and cooling curves depending on the hydrolysis temperature.

Table 3.1. Heating and cooling times needed to raise the hydrolysis medium temperature in the batch reactor up to the working temperature and later to quench it down to 50 °C.

Temperature (°C)	Heating time (min)	Cooling time (min)
55	8	2
80	9	4
140	16	7
180	23	7
190	25	7
200	25	7
225	31	8

The schematic diagram of the experimental set-up and the reactor system used in the modified organosolv experiments picture are shown next on Figure 3.2 and Figure 3.3. respectively.



**Figure 3.2. Schematic diagram of experimental Set-up A** Parr 500 ml high pressure stirred reactor manufactured in Alloy C276 is used for the hydrolysis experiments. The Hastelloy stirred reactor is able to work at maximum pressure of 345 bar and maximum temperature of 500 °C. The temperature, stirrer speed and pressure can be controlled electronically. It has a gas inlet valve so CO<sub>2</sub> can be added. The rig also has a cooling system where a pipeline is connected to a cooling bath to allow a faster quenching of the reactor. As a last feature, the reactor is also equipped with a gas release valve to depressurise the vessel in the end of the experiment.



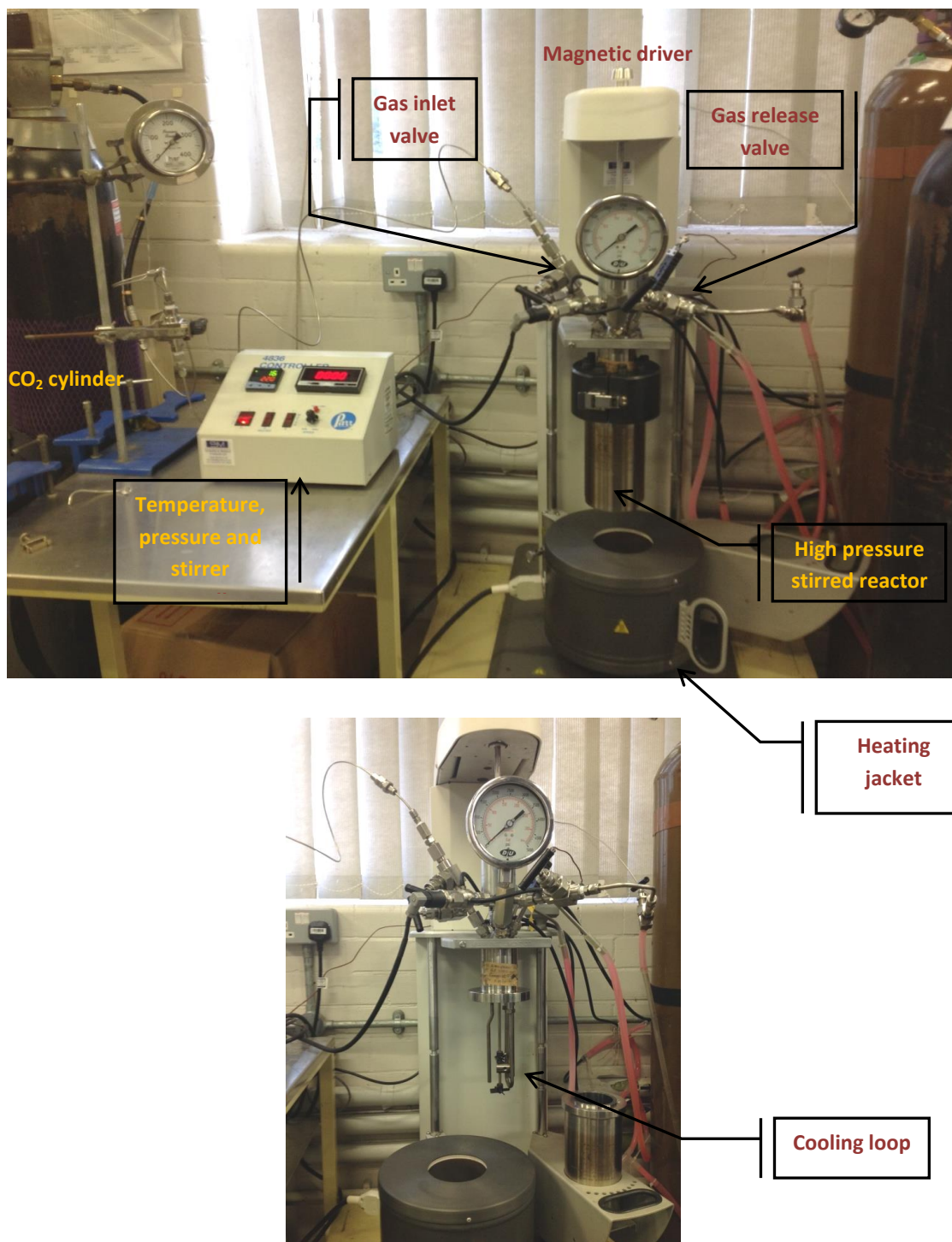


Figure 3.3. Reactor system used in the modified organosolv experiments with important parts labelled.

### ***3.5 Method for lignin precipitation from the liquid phase***

Lignin was precipitated according to the protocols described by Xu et al. [140] and García et al. [141] with the following modification. The filtrate obtained after vacuum filtration through a scintered disc, porosity 1, of the hydrolysed mixture was placed in a freezer at -20 °C for 2 h, after which the ethanol percentage was adjusted to 25% by adding water. The reduction on temperature and ethanol concentration reduces lignin solubility causing its precipitation as described by Xu et al. [140] and García et al. [141]. The suspension was then centrifuged at 4 °C and 10000 RPM (17695 g) for 10 min in a Beckman, model J2-21 centrifuge with a JA-10 rotor. The supernatant was decanted its volume recorded and samples were taken and stored in the freezer at -20 °C in 50 ml plastic centrifuge tubes until analysis of reducing sugars by the 3,5-Dinitrosalicylic acid assay, total phenolic content by Folin-Ciocalteu assay and HPLC analysis of phenolic compounds. The remaining supernatant was evaporated at 65 °C and then later analysed by DRIFT analysis. The resulting precipitated lignin was air-dried and stored in 10 ml glass storage vials at room temperature and later analysed by DRIFT and <sup>1</sup>H and <sup>13</sup>C NMR liquid-state.

### ***3.6 Klason lignin assay***

The amount of lignin present on Miscanthus biomass was determined and compared to the amount of lignin still in the residual insoluble fraction after each hydrolysis experiments to evaluate the efficacy of the proposed modified organosolv method on removing lignin from the lignocellulosic biomass. Therefore, the raw material and the dried insoluble fraction

obtained after hydrolysis were analysed for lignin content using the Klason assay following the ASTM Standard method E1721-01 (Determination of Acid insoluble Residue in Biomass)[142] and the amount of Klason lignin remaining in the insoluble fraction was compared to the amount present in the biomass giving the percentage lignin solubilised, also known as percentage of delignification.

The method undertaken is described below:

First, crucibles (Crucibles Gooch scintered borosilicate glass, porosity 4, 30 ml, Pyrex, Fisher Scientific, UK) were labelled and ignited at  $575 \pm 25$  °C in a muffle furnace (Carbolite, ELF 11/68) to achieve a constant weight of  $\pm 0.3$  mg. After that,  $0.3 \pm 0.01$  g of samples were weighed,  $W_1$ , to the nearest 0.1 mg, and placed in a test tube. The next step was the addition of  $3.00 \pm 0.01$  ml ( $4.92 \pm 0.01$  g) of 72%  $H_2SO_4$  (72%, Fluka) and stirring for 1 min or until mixed thoroughly with a glass rod. Then, the test tubes were placed in the water bath controlled to  $30 \pm 1$  °C, and hydrolysed for 2 h. Samples were stirred every 15 min to ensure complete mixing and wetting. Then, the hydrolysate was transferred to a glass bottle, and diluted to a 4% acid concentration by adding distilled water to bring the combined weight of sample, acid, and water up to  $89.22 \pm 0.04$  g. Bottles were then closed and the aluminium seals crimped into place. Afterwards, the samples went to the autoclave (Denley, Model: Majestic BA853) in their sealed bottles for 1 h at  $121 \pm 3$  °C. After completion of the autoclave cycle, samples were cooled for approximately 20 min at room temperature before removing the seals and stoppers. After hydrolysis in the autoclave, the hydrolysis solution was vacuum filtered through a previously ignited filtering crucible. With hot water, any particles clinging to the glass bottle were washed into the crucible and which allowed the filtered residue free

of acid, as well. Next, crucibles and its contents were dried at  $105 \pm 3$  °C overnight until a constant weight was achieved of  $\pm 0.3$  mg upon reheating. The crucibles are then cooled in the desiccator and the weight recorded to the nearest 0.1 mg,  $W_2$ , this weight was the weight of the crucible plus acid insoluble residue, and acid-insoluble ash. The crucible and its contents were placed in the muffle furnace, and ignited at  $575 \pm 2$  °C for 3 h until all the carbon was eliminated. Heating rate was of 10 °C/min to prevent flaming. The weight of the crucible with acid-insoluble ash was recorded to the nearest 0.1 mg after being cooled in a desiccator.

As a last step, the percent acid-insoluble residue on a 105 °C dry-weight basis was then calculated using the Equation 3.1 as followed:

$$\% \text{ acid insoluble residue} = \frac{W_2 - W_3}{W_1 \times \frac{T_{105}}{100}} \times 100 \quad \text{Equation 3.1}$$

Where:

$W_1$  = initial sample weight

$W_2$  = weight of the crucible, acid-insoluble residue, and acid-insoluble ash

$W_3$  = weight of the crucible and acid-insoluble ash

$T_{105}$  = % total solids determined at 105 °C in accordance with Test Method E 1756.

The percentage solids determined at 105 °C for the Miscanthus biomass were 90.3% and it was assumed as 100% for the residue as it was dried before analysis.

### **3.7 Scanning electron microscopy (SEM) images**

SEM images of Miscanthus fibres before and after the hydrolysis treatment are presented on chapter 6 with the aim to evaluate if the hydrolysis at 200 °C, reaction time of 60 min, biomass load of 15 g in 250 ml of 50% ethanol in water and carbon dioxide initial pressure of 50 bar would be enough to disrupt the cellulose fibres.

SEM plates were performed at the Centre for Electron Microscopy, University of Birmingham using a Philips XL30 FEG ESEM scanning electron microscope operating at 10 kV. Prior to imaging, samples were coated with gold for 120 s using a gold sputter coater, Polaron SC 7640.

### **3.8 DNS reducing sugars assay**

The DNS reducing sugars assay was adapted from King et al. [143] and it was developed according to Wood et al. [144]. This method gives the opportunity to assess the efficacy of different hydrolysis conditions to produce a fermentable sugars rich mixture that can be used as a fermentation substrate for biofuel production, for instance bioethanol. In addition, during this research, the DNS assay was used to evaluate the hemicellulose hydrolysis during the hydrolysis experiment by quantification of the mixture of xylose and arabinose in the hydrolysates since they are the main reducing sugars derived from Miscanthus hemicellulose.

In summary, DNS reagent was prepared by mixing 0.5 g of 3,5-Dinitrosalicylic acid (98%, Sigma-Aldrich) and 0.5 g of NaOH (>97% pearl Specified, Fisher Scientific). The solid mixture

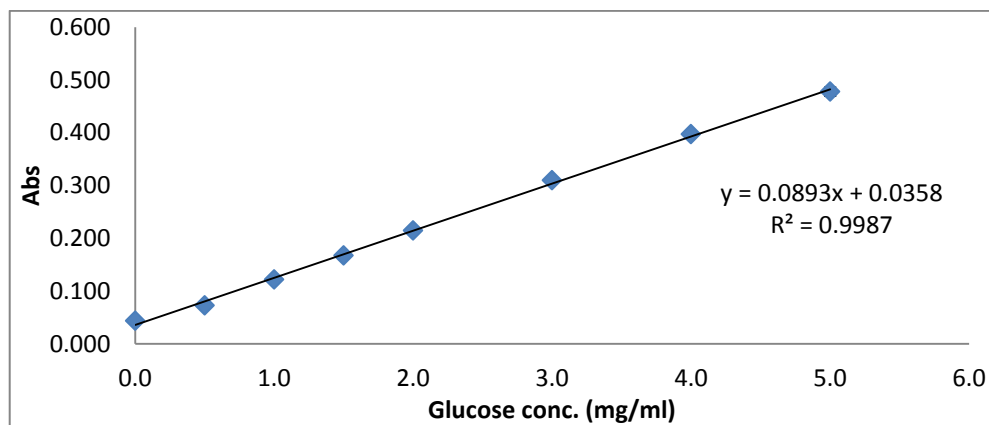
was then solubilised in distilled water up to a volume of 50ml to make a solution 1% in DNS and 1% in NaOH.

The DNS reagent is non-specific and reacts with both five and six carbon reducing sugars. Although this assay does not allow discrimination among specific carbohydrates, it can be used to quantify hydrolysis of a wide range of polysaccharide substrates. Glucose standards (D-(+)-Glucose, BioXtra,  $\geq 99.5\%$ , Sigma-Aldrich) of 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 mg/ml were prepared in 0.16 M sodium acetate buffer pH 5.5 ( $\geq 99.0\%$ , Sigma-Aldrich) and replicated three times per plate. 120  $\mu\text{l}$  DNS reagent were added to 60  $\mu\text{l}$  of standards or hydrolysis samples in a PCR 96-well microplate by using an 8-channel micropipette for a total reaction volume of 180  $\mu\text{l}$ . PCR plates were then sealed with aluminium sealant foil to avoid evaporation.

DNS reactions were carried out in PCR thermocyclers (Biorad iCycler, Hercules, CA and MJResearch Inc. PTC-100, Waltham, MA) by heating at 80 °C for 7 min followed by cooling to 4 °C for 1 min, and holding at 20 °C. With an 8-channel micropipette 36  $\mu\text{l}$  of the completed DNS reaction was then added to 160  $\mu\text{l}$  of distilled H<sub>2</sub>O in flat-bottom microplates (Corning Life Sciences 3370, Corning, NY) and absorbance was measured at 550 nm in a microplate spectrophotometer (Microplate Promega, Model 9301-010) and the concentration in reducing sugars was expressed in milligrams of glucose per millilitre of hydrolysates. By knowing the total volume of hydrolysates and the mass of Miscanthus used (load size) the concentration in reducing sugars was then expressed in milligrams of glucose per 10 g of Miscanthus. The results regarding the DNS assay will be presented in chapter 6.

As an example, Figure 3.4 shows a calibration curve obtained during one set of analysis.

Each calibration curve was run in triplicate per plate as well each hydrolysates sample.



**Figure 3.4. Example of a calibration curve used for reducing sugars quantification by DNS assay.**

### ***3.9 Diffuse reflectance Fourier transform infrared spectroscopy (DRIFT) of the precipitated lignin, fibres and dried hydrolysates after lignin removal***

The effect of the different hydrolysis conditions on the chemical structure of the residual fibres, the recovered lignin by precipitation from the hydrolysates and the dried supernatant after lignin removal was assessed by DRIFT and the results will be presented on chapter 6.

Diffuse reflectance infrared spectroscopy was carried out on a Perkin-Elmer Spectrum-One FTIR spectrometer. Mid-IR spectra of lignin samples were recorded between 600 and 4000  $\text{cm}^{-1}$ , using KBr as reference. To acquire all spectra, 8 scans were used for a high signal to noise ratio. The spectra were baseline corrected with automatic subtraction of water and smoothing achieved by the Perkin-Elmer Spectrum 5.0 FTIR software.

Samples of lignin and dried supernatant were mixed with dried potassium bromide powder (KBr, >99.9%, IR spectroscopy Spectroscopy grade, Fischer Scientific, Italy) in a small agate mortar and pestle and then reduced to powder, obtaining a homogeneous solid mixture sample used for analysis. The biomass fibres and the fibres residue obtained after hydrolysis were reduced to powder by an electrical agate-ball mill (Retsch PM 200, Retsch GmbH & Co., Haan, Germany), for 20 min after that the obtained powder was mixed with KBr in a mortar and pestle as described above.

### ***3.10 $^1\text{H}$ and $^{13}\text{C}$ NMR liquid-state experiments of precipitated lignin***

In addition to the DRIFT analysis, a  $^1\text{H}$  and  $^{13}\text{C}$  (Nuclear Magnetic Resonance) NMR liquid-state of four precipitated lignin samples obtained at different hydrolysis conditions was also performed to evaluate the effect of temperature, carbon dioxide initial pressure and ethanol concentration on the precipitated lignin molecular structure.

The analytical procedure started by solubilise 10 mg of precipitated lignin in 700  $\mu\text{l}$  of deuterated DMSO (Dimethylsulphoxide-D<sub>6</sub>, > 99.8% - Eurisotop) to obtain a concentration of around 14.3 mg/ml. The mixture was stirred to total sample solubilisation and finally transferred into a stoppered NMR tube (5 mm, 7", 507-HP-7 - NORELL) where the remaining void volume was gently degassed by a  $\text{N}_2$  flux.

A 400 MHz Bruker Avance spectrometer, equipped with a 5 mm Bruker Broad Band Inverse probe (BBI), working at the  $^1\text{H}$  and  $^{13}\text{C}$  frequencies of 400.13 and 100.61 MHz, respectively, was employed to conduct all liquid state NMR measurements at a temperature of  $298\pm 1\text{K}$ .



1D  $^1\text{H}$  spectra were acquired with 2 s of thermal equilibrium delay, a  $90^\circ$  pulse length ranging between 8.06 and 8.17 ms, 200 transients and 15 ppm (5.995 KHz) as spectral width.

Partial structural identification of molecules was conducted by means of 2D experiments: homo-nuclear  $^1\text{H}$ - $^1\text{H}$  COSY (Correlation Spectroscopy), TOCSY (Total Correlation Spectroscopy), and hetero-nuclear  $^1\text{H}$ - $^{13}\text{C}$  HSQC (Hetero-nuclear Single-Quantum Correlation) and HMBC (Hetero-nuclear Multiple Bond Coherence), adopting, for  $^{13}\text{C}$ , a pulse length ranging between 17.21 and 17.7 ms, and 250 ppm (25.152KHz) as spectral width.

All homo-nuclear and hetero-nuclear 2D experiments were acquired with 48 and 80 scans, respectively, 16 dummy scans, a time domain of 2k points (F2) and 256 experiments (F1). In addition, TOCSY experiment was conducted with a mixing time of 80 ms while HSQC and HMBC experiments were optimised for 145 Hz (short range) and 10 Hz (long range)  $J_{\text{CH}}$  couplings, respectively. All executed 2D experiments were gradient enhanced.

$^1\text{H}$  DOSY (Diffusion-Ordered Spectroscopy) experiments were conducted adopting a stimulated echo bipolar gradient pulse sequence. The acquisition was executed by setting 1400 ms length sine-shaped gradients, linearly ranging from 0.674 to 32.030  $\text{G cm}^{-1}$  in 128 increments, and selecting a delay of 0.27 s between the first and the second gradient.

No apodisation and zero filling were applied to Free Induction Decay (FID) of  $^1\text{H}$  mono-dimensional spectra. All spectra were baseline corrected and processed by using both Bruker Topspin Software (v. 2.1) and MestReC NMR Processing Software (v. 4.9.9.9).

Moreover, the signal of solvent DMSO was employed, for all experiments, to calibrate both  $^1\text{H}$  and  $^{13}\text{C}$  frequency axes at 2.49 and 39.5 ppm, respectively.

### **3.11 Total phenolic content analysis of the hydrolysates samples by Folin-Ciocalteu method**

A mixture of Sub-CW and carbon dioxide was used to extract phenolic compounds that are mainly bound to the hemicellulose fraction of the biomass. As studied by Xu et al. [24], Ou et al. [25] and Culhaoglu et al. [26] the presence of ferulic and *p*-coumaric acids accounts for the majority of phenolic compounds in grass cell-walls and bound to the hemicellulose macromolecule. As hemicellulose requires lower temperatures than lignin and cellulose to undergo on hydrolysis, a set of hydrolysis experiments of *Miscanthus* at temperatures lower than 180 °C was then performed and the liquid phase (hydrolysates) was analysed for total phenolic content (TPC) that were extracted from the biomass during the hydrolysis.

The Folin-Ciocalteu method was used to determine the concentration of phenolic compounds in each hydrolysates sample. The micro-method described by Waterhouse [145] was followed, which includes each sample reacting with Folin-Ciocalteu reagent, producing a blue colour. The darker the colour the higher the phenolic content. The colour was measured using a colorimeter detecting at 750 nm and a calibration curve was constructed for each set of analysis using known concentrations of gallic acid (0, 50, 100, 150, 250 and 500 mg/l), so all results are reported as mg gallic acid equivalent (GAE). One example of a calibration curve obtained is shown on Figure 3.5 with a correlation of 0.9988.

In summary, 20 µl of calibration standard (gallic acid), hydrolysates liquid sample or distilled water for blank were mixed in a test tube with 1.58 ml water, followed by 100 µl Folin-Ciocalteu reagent and allowed to stand for 6 min. Sodium carbonate solution was then added (300 µl) and the mixture was incubated for 30 min at 50 °C in a water bath. Finally,

200  $\mu\text{l}$  of the reacted mixture was transferred to flat-bottom microplates (Corning Life Sciences 3370, Corning, NY) and absorbencies were measured at 750 nm. All the samples were analysed in triplicate.

Folin-Ciocalteu's phenol reagent, gallic acid (99%) and sodium carbonate (>99%) used had analytical-grade and were purchased from Fisher Scientific, UK.

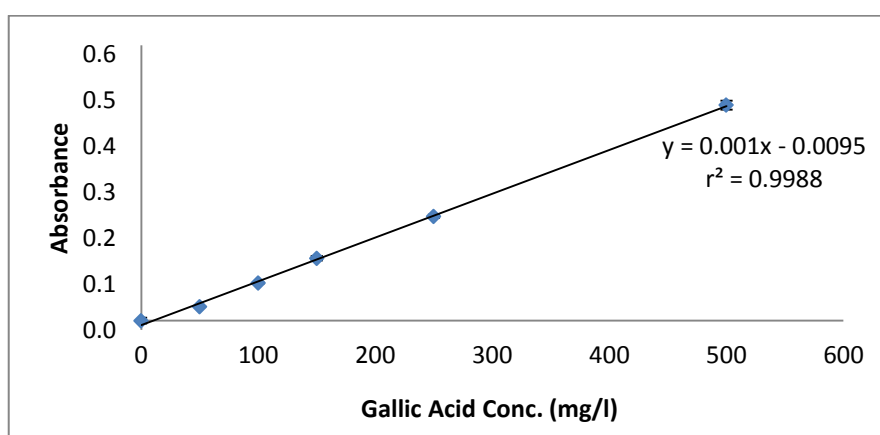


Figure 3.5. Example of a calibration curve used to determine TPC by Folin-Ciocalteu assay.

### ***3.12 Determination and quantification of phenolic compounds by High Performance Liquid Chromatography (HPLC) on the hemicellulose fraction.***

In addition to the results obtained by the Folin-Ciocalteu method on the TPC extracted from the biomass to the hemicellulose fraction, an HPLC method was also developed to analyse particular phenolic compounds of interest on this hemicellulose fraction with a focus on the quantification of *p*-coumaric acid.

The HPLC analysis was performed by reverse-phase chromatography on a Phenomenex Prodigy 5  $\mu\text{m}$  ODS-3 100 Å LC Column 250 x 4.6 mm. The HPLC system was a Shimadzu HPLC system and it was composed by several modules, a SIL-10AD auto injector, a binary pump, a DGU-14A degasser, a CTO-10A column oven set at 30 °C, and a SPD-10AV UV-Vis detector set at 280 and 320 nm wavelengths.

The flow rate was maintained at 1.2 ml/min and the injection volume of each sample and standards was 10  $\mu\text{l}$ . Two eluents were used: Eluent A was a mixture of acetic acid and water (20:980 v/v); Eluent B was a mixture of acetonitrile (95% v/v) in eluent A.

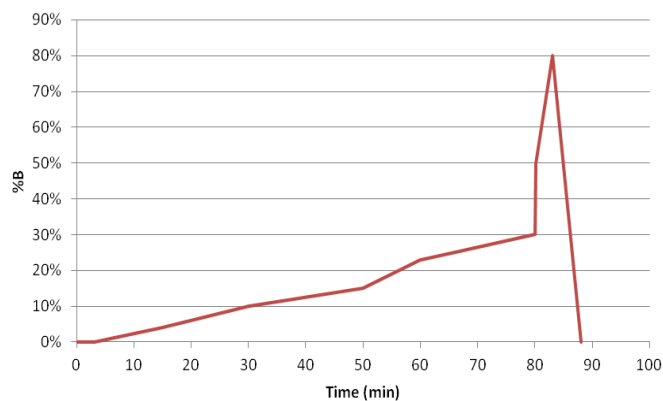
Acetonitrile (CHROMASOLV® Plus, for HPLC,  $\geq 99.9\%$ ), acetic acid (ACS reagent,  $\geq 99.7\%$ ) and HPLC-grade water (CHROMASOLV®) were used in the HPLC analysis and were purchased from Sigma-Aldrich, UK.

The phenolic acids were detected at either 280 or 320 nm by comparison to the retention times of standards. Several peaks were detected on the chromatograms but only the presence of *p*-coumaric acid and ferulic acid was identified with identical retention times to *p*-coumaric acid ( $\geq 98.0\%$ , Sigma-Aldrich) and the ferulic acid (99%, Sigma-Aldrich) standards purchased at 43.5 and 50.5 minutes respectively.

The gradient method used is illustrated by the Table 3.2 and Figure 3.6.

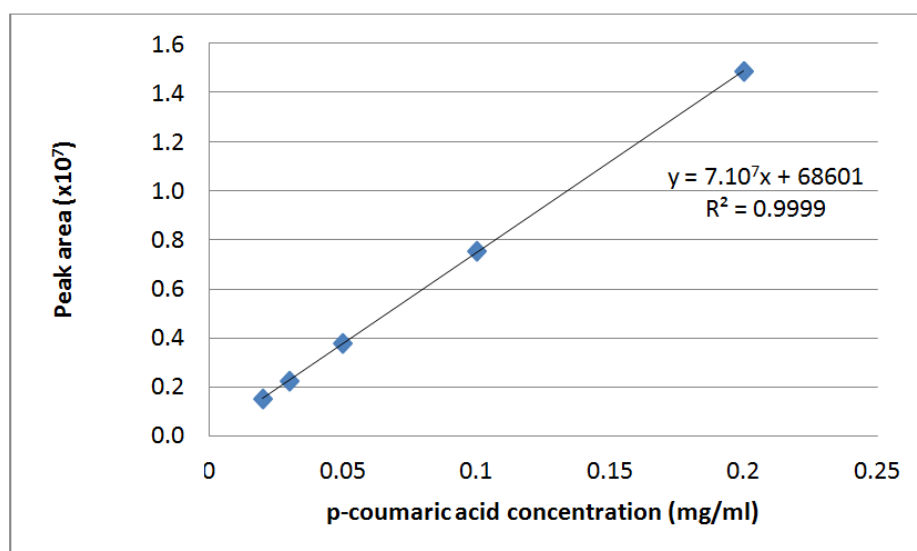
**Table 3.2. Gradient method used for resolving phenolic compounds by HPLC.**

Method	
time (min)	% B
0	0%
3	0%
15	4%
30	10%
50	15%
60	23%
66	25%
80	30%
80.1	50%
83	80%
88	0%
95	0%



**Figure 3.6. Gradient method used for resolving phenolic compounds by HPLC**

The phenolic compound *p*-coumaric acid was quantified by comparing the peak area of the samples with the peak area of the calibration curve presented on Figure 3.7 with a correlation of 0.9999.



**Figure 3.7. *p*-coumaric acid calibration curve used to determine the concentration of the compound in the hydrolysates**

## **4 BIOMASS PHYSICAL PRETREATMENT TO IMPROVE PARTICLE SIZE HOMOGENEITY**

### **4.1 Objective**

Biomass particle size can be considered another variable to the hydrolysis process. However, it has been reported by Rogalinski et al. [146] and also supported by Gilarranz et al. [147] that when using an organosolv hydrolysis method the biomass particle size does not have an important role on the delignification results obtained due to the low viscosity and hence high penetration of the organic solvent used. Based on that, it was decided to pretreat the biomass physically with a chosen grinding method so it would be possible to produce a homogeneous raw material to be hydrolysed throughout the research project. With this homogeneous biomass as raw material, it was possible to remove the particle size from the list of variables to be analysed in this study. Therefore, this chapter has the objective to present the results obtained during the physical pretreatment method development.

### **4.2 Introduction**

Reduction of particle size is often needed to make material handling easier and to increase surface/volume ratio. Mechanical pretreatment by comminution is usually carried out prior to following processing step, and the desired particle size is usually dependent on these subsequent steps. The material obtained with grinding has higher density, while process parameters selected properly allow for obtaining particles of defined size. A mechanical comminution of lignocellulosic materials through a combination of chipping, grinding, and/or

milling can be applied to reduce cellulose crystallinity. The size of the materials is usually 10-30 mm after chipping and 0.2-2 mm after milling or grinding [87].

Depending on the character and the properties of the biomass material, the grinding process will be different, and it will require the use of suitable devices. Vibratory ball milling is found to be more effective than ordinary ball milling in reducing cellulose crystallinity of spruce and aspen chips and in improving their digestibility [87].

For the grinding of wood and wood waste biomass which have anisotropic properties, i.e. are non-homogeneous materials, mills with spinning elements and appropriate separation sieves are used. The spinning elements may be beaters or multi-lobe rotors.

Miscanthus like switch grass (straw, reed) and other straw type biomass requires grinding before further processing. Due to its character these type of biomass needs grinding devices that perform the size reduction process mainly by cutting. Different work described in the literature uses either cutting mills or Wiley mills to perform an efficient particle size reduction that ranges from 50  $\mu\text{m}$  up to 3 mm. [146, 148, 149]

### **4.3 Methodology**

The process of choosing the best grinding method had to take into consideration several factors, first the equipment available in the department and second the method that could create the most homogeneous samples. Regarding the equipment available in the department, there are a roller mill, a coffee grinder and a blender. The three equipments were tested to check which one would give the most homogeneous samples. Starting with

the roller mill, it was denoted that some parts of the Miscanthus straw were being grinded to a homogeneous particle size but others, the softer parts of the plant, were just crushed but not cut. This problem caused by a difference of the plant hardness along the straw made impossible to use the roller mill as a pre-treatment method for this type of biomass. As a second option, a coffee grinder was tried. However, due to the hardness of the Miscanthus it was impossible to use it. Finally, a blender was tried. In this equipment, the same way as in the coffee grinder it was impossible to grind Miscanthus due to its rigidity. However, it was thought that by soften the biomass prior grinding would help. Therefore, hydrating the biomass with the hydrolysis solution for a period of time before the grinding would soften the biomass straw. A hydrating method was included then in the grinding method creating the physical pre-treatment of the Miscanthus biomass. This pre-treatment process was after that optimised to give a homogeneous and with reproducibility biomass samples throughout the research project.

Therefore, a set of experiments was designed to optimise the grinding conditions.

The experimental set up has included the addition of water and water:ethanol mixture to biomass and hydrating it for a period of time before grinding it in a blender. (Moulinex Vitamix Y42, 400 W, 1.5 l).

Each condition was run in triplicate, dried at 105 °C and the mass fraction of grounded material on each sieve was calculated after sieving, as well as, the standard deviation among replicates. The average of the particle size of the grounded material was also calculated, therefore it was possible to compare among different conditions used.



The optimal grinding condition was established after running the experiments, which are presented in Table 4.1.

**Table 4.1. Series of experiments with different grinding conditions.**

Experiment	$m_{\text{biomass}}$ (g)	$V_{\text{water}}$ (ml)	$\frac{m_{\text{biomass}}}{V_{\text{water}}}$ (g/l)	Hydration time (min)	Grinding time (min)	Grinding method
<b>GB01</b> <sup>a</sup>	50	500	100	20	2	5sec. grind.+ 5sec stay.
<b>GB02</b>	50	650	76.9	20	2	10sec. grind.+ 10sec stay.
<b>GB03</b>	25	325	76.9	20	2	10sec+10 sec
<b>GB04</b>	25	325	76.9	40	2	10sec+10 sec
<b>GB05</b>	25	325	76.9	20	3	10sec+10 sec
<b>GB06</b>	25	325	76.9	40	3	10sec+10 sec
<b>GB07</b>	25	250	100	20	3	10sec+10 sec
<b>GB08</b>	25	250 ml Water:Et-OH (1:1), 100 (g/l)		20	3	10sec+10 sec

a: GB – Abbreviation for grinding using a blender.

In order to improve the effect of hydrating on the grinding process, hot water (50 °C) was added to soak the material prior to grind it.

Table 4.1 shows a series of experiments run in triplicate where 25 g and 50 g of material were hydrated in a volume of water ranging 250-650 ml for a period of 20 or 40 min at 50 °C

After that time, the biomass/water mixture was grounded in a blender.

The measurement of grinding time included several stops during the process to protect the blender from overheating. Consequently, a pattern of grinding time followed by a stoppage time was done until the total time of grinding steps was reached.

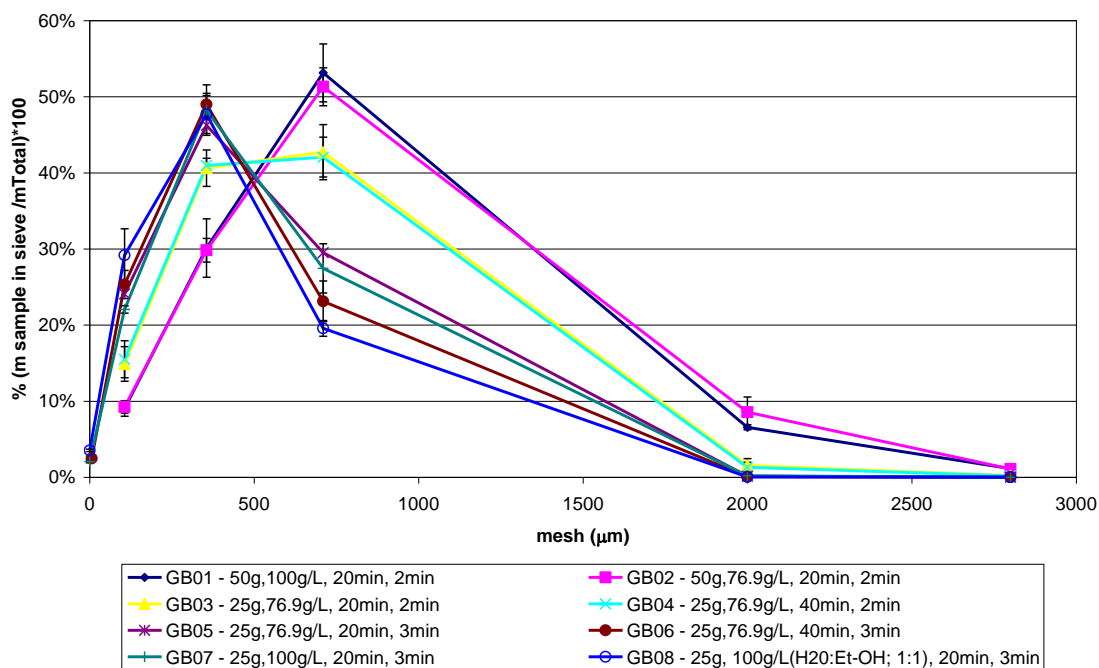
After that, the grounded material was filtered under vacuum and dried at 105 °C.

Finally, all the grounded material was sieved using a sieve shaker (Fritsch, analysett 3 Pro) in combination with a set of sieves with different mesh sizes to check the uniformity of the sample.

A set of 5 sieves were used, 2.80 mm, 2.00 mm, 710 µm, 355 µm and 106 µm. The sample was sieved during 2 cycles of 5 min each with 2.0 mm amplitude.

## ***4.4 Results and Discussions***

With the results obtained after weighing each fraction of biomass on each sieve, a graph (Figure 4.1) showing the particle size distribution of the biomass through the sieves was drawn. It shows the variation of the particle size among different grinding conditions for every conditions and replicates.



**Figure 4.1. Particle size distribution of grounded biomass through the sieves based on the average result from replicates and standard deviation for the conditions in study (GB01-GB08).**

Figure 4.1 shows the results of particle size dispersion, obtained at different grinding conditions. Since particle size distribution for each condition was obtained, the average of particle size of the ground biomass on each condition was calculated by the Equation 4.1.

$$P_s = \frac{\sum x_i \cdot d_i^4}{\sum x_i \cdot d_i^3}$$

Equation 4.1

here:

$x_i$  is the fraction of ground Miscanthus retained on each sieve (g);

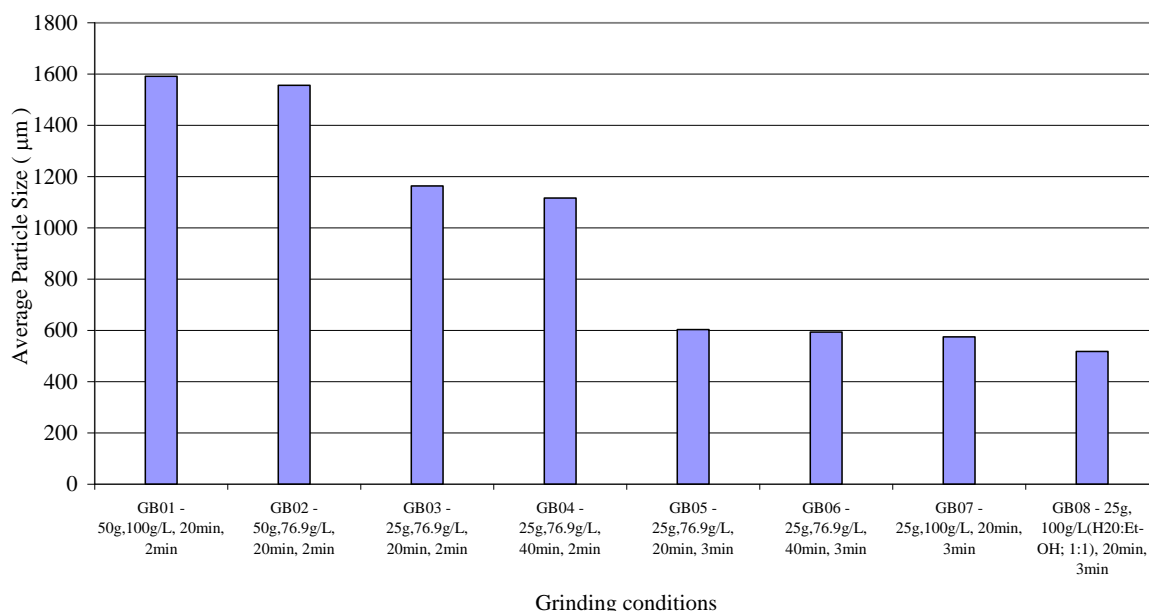
$d_i$  is the average particle size retained on each sieve ( $\mu\text{m}$ ).

Therefore, based on data shown on the previous graph and with the previous equation, the average of particle size of the ground biomass was calculated for each condition and the results are shown in Table 4.2.

**Table 4.2. Average of particle size of the ground biomass on each condition.**

Conditions	Ps ( $\mu\text{m}$ )
GB01 - 50g,100g/l, 20min, 2min	1591
GB02 - 50g, 76.9g/l, 20min, 2min	1556
GB03 - 25g,76.9g/l, 20min, 2min	1163
GB04 - 25g,76.9g/l, 40min, 2min	1116
GB05 - 25g,76.9g/l, 20min, 3min	603
GB06 - 25g,76.9g/l, 40min, 3min	593
GB07 - 25g,100g/l, 20min, 3min	574
GB08 - 25g, 100g/l(H <sub>2</sub> O:Et-OH; 1:1), 20min, 3min	518

With the results shown in Table 4.2 a bar chart (Figure 4.2) of the average of the particle size versus grinding condition was drawn.



**Figure 4.2. Average of the particle size versus grinding condition (GB01-GB08).** (Calculated by the equation 4.1 therefore no error bars are displayed).

Finally, with the results shown in Figure 4.2, it is possible to conclude that the hydrating time does not affect the results of grinding to a large extent (GB03/GB04 and GB05/GB06), as well as the mass of biomass per volume of water during hydrating and grinding procedures (GB01/GB02 and GB05/GB07). On the other hand, the graph shows a reduction on particle size when the amount of grinding biomass decreased (GB02/GB03). In addition, grinding time is also important to obtain smaller particle sizes. This effect can be observed comparing conditions GB03 and GB04 to conditions GB05 and GB06, where increasing the grinding time by 1 min led to a reduction 50% of the particle size.

## **4.5 Conclusions**

To conclude, this study has delivered interesting results concerning the hydrating time and the proportion of biomass per volume of water. On the first variable, increasing the hydrating time from 20 min to 40 min did not make a significant effect, approximately 4% between GB03 and GB04 and approximately 2% between GB05 and GB06.

In relation to the ratio between biomass and water, decreasing biomass concentration to 23% led to only 2% decreasing in the particle size for GB01/GB02 conditions. Based on this, run GB07 condition was carried out in order to prove that lack of influence on particle size.

Therefore, comparing the results obtained when using GB05 and GB07 conditions, it is possible to conclude that despite of the particle size being slightly smaller (less than 5%) when the amount of water was reduced, this does not represent an important change. Thus,

it proves that the ratio between biomass and water is not a variable of the grinding procedure.

Concerning to the change of the composition of the hydrating medium, if GB07 and GB08 results are compared it is possible to say that modifying it from water (GB07) to a mixture of water and ethanol (1:1) (GB08) led to a reduction of the particle size around 10.4%. This means that regardless of small improvement when hydrating medium was changed, GB08 condition delivered the optimal condition of this study.

All things considered, the best condition obtained GB08 is composed by 25 g of biomass, 250 ml of 50% ethanol in water, 20 min of hydrating and 3 min of grinding with an average particle size obtained is 500  $\mu\text{m}$ .

During the hydrolysis experiments different biomass loads were used. However, to keep the same conditions of the grinding method the biomass:solvent ratio was kept constant. In addition, the solvent used to grind the biomass was added to the reactor as part of the hydrolysis medium.

## **5 EVALUATION AND OPTIMISATION OF A MODIFIED ORGANOSOLV METHOD FOR SOLUBILISATION AND DELIGNIFICATION OF MISCANTHUS BIOMASS**

### **5.1 Aim**

The basis of an integrated biorefinery is the deconstruction of the lignocellulosic biomass macromolecule into multiple products that are separately marketable. On this view, this chapter has an overall aim to present the results of a modified organosolv hydrolysis process on a batch reactor and to discuss its feasibility to deconstruct *Miscanthus  $\chi$  giganteus* biomass but more precisely to study the solubility and delignification behaviours of the biomass at different hydrolysis conditions.

This chapter is divided in two different sections the first section presents the results of preliminary hydrolysis tests that allowed conceiving a broad idea on the effect of different hydrolysis parameters on the solubilisation and delignification of the *Miscanthus*. With the results from the preliminary tests, several experimental parameters were chosen and a central composite design (CCD) based on a response surface methodology (RSM) was implemented to determine the optimal conditions for solubilisation and delignification of *Miscanthus* by the proposed organosolv hydrolysis method and the results will be presented on a second part of this chapter.

In this work, the parameters in study were temperature, carbon dioxide initial pressure, reaction time, ethanol concentration and load size.

## **5.2 Background**

Lignocellulosic biomass is mainly composed by hemicellulose, cellulose and lignin but also a variety of other compounds which are water and ethanol soluble, the two solvents used in the experiments.

Several parameters were chosen for this study, temperature, carbon dioxide initial pressure, reaction time, ethanol concentration and load size. Based on these parameters their working range was selected according to the compounds present in the lignocellulosic biomass and their solubility in the hydrolysis medium.

Taking into consideration the parameters in use, the first compounds to be extracted are the water:ethanol soluble compounds, followed by the hemicellulose then the lignin and finally the cellulose fibres, which is in agreement with previous studies using lignocellulosic materials as feedstock in sub-critical water hydrolysis. In general it is agreed that temperature is the parameter that plays the most important role in biomass hydrolysis. In these studies it was found that hemicelluloses dissolve above 120 °C, lignin begins to dissolve at around 180 – 190 °C and cellulose degrades at temperatures greater than 230 °C. [150-152]. Therefore, regarding temperature, it was chosen a range from 80 to 200 °C. This maximum temperature was set to avoid cellulose degradation but allowing the recovery of the remaining compounds in the biomass leaving the insoluble cellulose fibres. In relation to carbon dioxide initial pressure, previous studies show hydrolysis results obtained at higher pressures of 190 to 230 bar. [4] However, in this research it was chosen a range from 10 to 55 bar initial CO<sub>2</sub> pressure, being the maximum pressure chosen according to the maximum pressure in a CO<sub>2</sub> cylinder. The use of lower CO<sub>2</sub> pressures has been demonstrated to be



enough to hydrolyse xylose and furan compounds in corn stover [153]. A range from 5 to 60 min reaction time was selected based on the principle that this research was trying to find a new modified method that is environmental benign and energy sustainable. Therefore, the energy spent in the process would be related to the reaction time as its major contributor side by side with the temperature. In line with this it was decided to keep the maximum 60 min reaction time. Which is also supported by Sreenath et al. [154] that used sub critical water at 220 °C to dissolve hemicelluloses completely and remove lignin partially within 2 min with no other chemicals used. Other parameter studied was the ethanol concentration in the medium. According to Xu et al. [140] lignin solubility increases considerably for ethanol concentrations higher than 42% Based on this information it was decided during the current work to study the influence of ethanol concentration in a range from 0 to 70%.

The last parameter in study was the load size, which is the mass of biomass per 250 ml hydrolysis medium. The load size range studied was from 2.5 to 15 g of *Miscanthus* per 250 ml hydrolysis medium.

Some scoping tests were performed by fixing all the other parameters and varying the load size. The results for the load size effect on *Miscanthus* solubilisation and delignification will be presented in the next section with the scoping experiments results but in summary these experiments showed that there was not a significant effect of the load size from 2.5 to 15 g either for solubilisation or delignification. Previous studies presented a ratio of biomass:liquid medium higher than the ones in this study, for example, Macfarlane et al. [155] and Zhou et al. [156] used 1:10 ratio on *Salix schwerinii* (willow) and Aspen chips respectively, also Villaverde et al. [157] used 1:12 ratio for *Miscanthus* however this study

was using an organosolv method with acetic and formic acid with hydrochloric acid as a catalyser and the hydrolysis mixture was simply refluxed with stirring under atmospheric pressure. In the same view the present study was also extended to higher load sizes of 20 and 25 g but at these conditions it was produced a viscous mixture in the reactor that made impossible a good homogenisation with inconclusive results. Therefore, 10g of biomass in 250 ml of medium was chosen to be the most suitable load size to avoid issues regarding sample homogeneity and on the other hand to reduce errors associated to mass losses during the experimental work by using reduced load sizes. In summary, despite other studies presented results with higher biomass:liquid medium ratio, this parameter has shown that it is highly dependent on the biomass type and hydrolysis method used. In this case, 1:10 (25 g) and 1:12 (20 g) were completely impossible ratios to work with the batch reactor.

The volume of the hydrolysis medium was kept constant at 250 ml throughout the all study as well as the Miscanthus particle size was kept constant at 500  $\mu\text{m}$ .

### ***5.3 Preliminary study on the effect of different hydrolysis parameters on solubilisation and delignification of Miscanthus to be included in the RSM***

Preliminary tests were performed to quick assess the effect of possible parameters to be chosen during the modelling study using RSM.

The processing parameters investigated were temperatures of 180, 190 and 200 °C, load sizes of 2.5, 5, 10 and 15 g of biomass mixed in 250 ml of 50:50 ethanol:water mixture with

carbon dioxide added at initial pressure of 55 bar. Each condition was run for 1 hour.

Ethanol and carbon dioxide were used as modifiers in this work.

During the experiments solubilisation and delignification of the lignocellulosic biomass were determined.

Table 5.1 presents the results of solubilisation obtained during the experiments with all the experiments being run at least in triplicate.

**Table 5.1. Solubilisation results obtained during preliminary tests.**

Experiment	Parameters (T, load, cEt-OH, t, p)	% Solubilisation	
		Average	stdev
1	180°C, 2,5g, 50%, 60min, 55bar	40%	3%
2	190°C, 2,5g, 50%, 60min, 55bar	47%	3%
3	200°C, 2,5g, 50%, 60min, 55bar	53%	2%
4	180°C, 5g, 50%, 60min, 55bar	39%	3%
5	190°C, 5g, 50%, 60min, 55bar	46%	4%
6	200°C, 5g, 50%, 60min, 55bar	51%	3%
7	180°C, 10g, 50%, 60min, 55bar	36%	3%
8	190°C, 10g, 50%, 60min, 55bar	44%	4%
9	200°C, 10g, 50%, 60min, 55bar	50%	4%
10	180°C, 15g, 50%, 60min, 55bar	35%	3%
11	190°C, 15g, 50%, 60min, 55bar	41%	3%
12	200°C, 15g, 50%, 60min, 55bar	48%	2%

As shown in the results in Table 5.1 the range of biomass solubilisation obtained was from 35% to 53% according to temperature. To determine whether temperature or load size affect the solubilisation extent the analysis of variance (ANOVA) was calculated.

The temperature effect on biomass solubility was shown to be significant,  $p = 9 \times 10^{-5}$  with a confidence level of 95%, while the effect of load size ratio was not significant  $p = 0.78$ .

Different studies report the greater effect of temperature on biomass solubilisation

comparatively to other parameters, which is also in accordance to the scoping experiments results in Table 5.1 The research presented by Pasquini et al. [4] and the work of Li and Kiran [151] are also in line with the efficacy of temperature on biomass solubilisation, in addition, Li and Kiran [151] has shown a maximum solubilisation for red spruce wood of 19.3% at 190 °C and 290 bar, the *Miscanthus* solubility in our research varies between 35 and 53%. A recent study on *Miscanthus  $\chi$  giganteus* done by Ligeró et al. [158] also showed the influence of temperature on the biomass hydrolysis but his study presents lower biomass solubility results, from 31 to 37% for the same range of temperatures from 180 to 200 °C however, this study used an autohydrolysis treatment which means only Sub-CW was used without any addition of ethanol or CO<sub>2</sub> like it happened in this study. This is likely to explain the difference in the results since the use of ethanol and CO<sub>2</sub> as entrainers is focused on aiding lignin solubilisation. Therefore, adding to the mass of biomass solubilised. So, the difference between this work results and the ones presented by Ligeró et al. [158] are seen as the effect of the ethanol and CO<sub>2</sub> in the medium.

Table 5.2 presents the results of delignification obtained during the preliminary experiments.

**Table 5.2. Delignification results obtained during the scoping tests.**

Experiment	Parameters (T, load, cEt-OH, t, p)	% Delignification	
		Average	stdev
1	180°C, 2,5g, 50%, 60min, 55bar	67%	2%
2	190°C, 2,5g, 50%, 60min, 55bar	78%	3%
3	200°C, 2,5g, 50%, 60min, 55bar	86%	2%
4	180°C, 5g, 50%, 60min, 55bar	66%	4%
5	190°C, 5g, 50%, 60min, 55bar	76%	5%
6	200°C, 5g, 50%, 60min, 55bar	83%	3%
7	180°C, 10g, 50%, 60min, 55bar	58%	3%
8	190°C, 10g, 50%, 60min, 55bar	70%	6%
9	200°C, 10g, 50%, 60min, 55bar	78%	8%
10	180°C, 15g, 50%, 60min, 55bar	58%	5%
11	190°C, 15g, 50%, 60min, 55bar	67%	7%
12	200°C, 15g, 50%, 60min, 55bar	74%	5%

The percentage of delignification achieved under different temperatures (Table 5.2) was in the range 58% to 86%. To determine whether temperature or load size affect biomass delignification the ANOVA was calculated.

The effect of temperature on biomass delignification was significant  $p = 0.002$ . Therefore, it is possible to consider if the load size is fixed the biomass delignification increases on average 18% when the temperature changes from 180 to 200 °C. The effect of the load size was not significant  $p = 0.48$ .

While the effect of load size did not have a significant impact on biomass delignification, there was slight tendency for percentage delignification to decrease with increased biomass load.

The results are in accordance with those reported by Pasquini et al. [4] where a delignification extent of 93.1% for *P. taeda* wood chips and 88.4% for sugar cane bagasse. However, with *Miscanthus* the extent of delignification was obtained at lower pressures.

## **5.4 Optimisation of a modified organosolv method for solubilisation and delignification of *Miscanthus* biomass**

Optimisation of a method has the goal to find the values of controllable factors (variables) that determine the behaviour of a system (responses). The RSM is used as a potent tool extensively in scientific research for experimental design, building efficient models, determine optimum conditions and evaluate the influence of factors and variables in the experimental responses.

## **5.5 Methodology**

### **5.5.1 Experimental Design DoE**

A central composite design (CCD) based on a response surface methodology (RSM) was applied to determine the optimal conditions for solubilisation and delignification of *Miscanthus* by the proposed organosolv hydrolysis method.

The optimisation development of the modified organosolv process was based on a series of experiments designed by applying a response surface method with central composite using the Design Expert 8 software. This software allows a strong statistical analysis with a generation of model equations based on different parameters ranges.

Based on the scoping tests, it was decided that the parameters in study were temperature, CO<sub>2</sub> initial pressure, reaction time and ethanol concentration in the hydrolysis medium. As the scoping test demonstrated, for the ratio biomass:liquid phase (load size) it did not have a significant effect on the solubilisation and delignification results. Therefore, it was decided to keep the load size constant and out of the chosen parameters in study.

RSM was used to analyse the percentage of Miscanthus solubilisation and delignification based on the effect of four main factors, hydrolysis temperature and initial CO<sub>2</sub> pressure and hydrolysis time and ethanol concentration in the hydrolysis medium. The responses are solubilisation (Y<sub>1</sub>) and delignification (Y<sub>2</sub>) both in percentage.

The ranges (low and high values) of the variables were as follows on Table 5.3.

**Table 5.3. Hydrolysis factors studied and their ranges.**

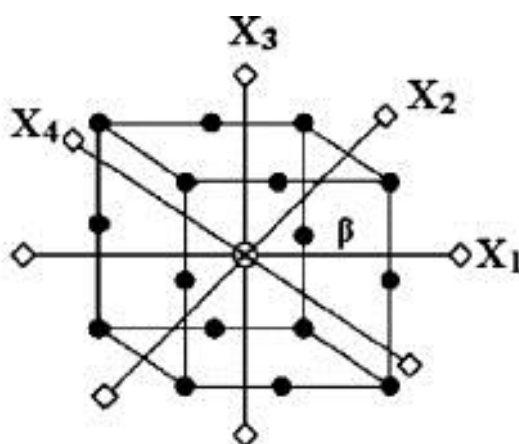
Factors	Range
Temperature	80 - 200°C
CO <sub>2</sub> pressure	10 - 55 bar
Time	5 - 60 min
Ethanol conc.	0 - 70%

The load size was kept constant at 10g of biomass per 250ml of medium.

All the factors were coded at three levels: -1, 0, and +1 in order to be optimised, un-coded and coded levels are illustrated in Table 5.4. The experimental domain of the extraction process for a 4 factors and 4-level CCD is illustrated in Figure 5.1 There are total of 30 experiments consisted of, 6 central points, and 24 factorial points. Six replicates of the central points were used in order to estimate the experimental error.

Table 5.4. Independent factors and their values for CCD.

Factors	Symbol	Factor levels		
		-1	0	1
Temperature, °C	$X_1$	80	140	200
CO <sub>2</sub> pressure, bar	$X_2$	10	33	55
Time, min	$X_3$	5	33	60
Ethanol conc., %	$X_4$	0	35	70

Figure 5.1. Graphical representation of factors levels (●), center point (⊗) and star points α-level (◇) of the CCD of 4 variables ( $X_n$ ). [159]

## 5.5.2 Statistical analysis

To predict the responses of optimisation of both solubilisation and delignification of *Miscanthus*, Design Expert 8 software was used and a quadratic polynomial regression model was chosen. The model proposed for both responses  $Y$  was according to the following equation:

$$Y = A_0 + \sum A_i X_i + \sum A_{ii} X_i^2 + \sum A_{ij} X_i X_j$$

Equation 5.1



Where  $Y$  represents response,  $X_i$  and  $X_j$  are independent variables,  $A_0$  is constant,  $A_i$  is linear coefficient of the model,  $A_{ii}$  representing quadratic coefficient and  $A_{ij}$  is interactive coefficient of the model. The model was evaluated by comparing the experimental and predicted responses and analysis of variance (ANOVA).

### **5.5.3 Design of experiment - full type design**

Hydrolysis experiments were designed statistically using the Design Expert 8 software after defining the main variables and their levels (see Table 5.4). As a result, a total of 30 experiments were designed for full design type with alpha (axial point) value of 1.414. The four factors values for each run are represented in Table 5.5. Centre point was designed to be at hydrolysis temperature, initial  $\text{CO}_2$  pressure and hydrolysis time and ethanol concentration in the hydrolysis medium at their zero level. Axial points designed by the software represented an issue to be accomplished experimentally. On these conditions it was decided to adjust the factor value to the closest experimentally possible. For example for the condition 10 in Table 5.5 the ethanol concentration was adjusted from “-14.50%” to 0%, the reaction time on condition 11 was adjusted from “-6.39” min to 5 min and at last, for the condition 12 and 13 the initial  $\text{CO}_2$  pressure were adjusted from 0.68 and 64.32 bar to 5 and 55 bar respectively, since that was the maximum pressure supplied by the  $\text{CO}_2$  cylinder.

**Table 5.5. Full experimental design for optimisation of solubilisation and delignification of Miscanthus biomass.**

Condition	Factor 1	Factor 2	Factor 3	Factor 4
	Temperature °C	Ethanol Conc. %	Time min	CO <sub>2</sub> Pressure Bar
1	55	35	33	33
2	80	0	5	10
3	80	0	5	55
4	80	0	60	10
5	80	0	60	55
6	80	70	5	10
7	80	70	5	55
8	80	70	60	10
9	80	70	60	55
10	140	0	33	33
11	140	35	5	33
12	140	35	33	5
13	140	35	33	55
14	140	35	33	33
15	140	35	33	33
16	140	35	33	33
17	140	35	33	33
18	140	35	33	33
19	140	35	33	33
20	140	35	71	33
21	140	85	33	33
22	200	0	5	10
23	200	0	5	55
24	200	0	60	10
25	200	0	60	55
26	200	70	5	10
27	200	70	5	55
28	200	70	60	10
29	200	70	60	55
30	225	35	33	33

## **5.6 Results and Discussions**

### **5.6.1 Modelling results of Miscanthus solubilisation and delignification by full type design of experiments**

With the full experimental design set and after weighing the oven-dried residual insoluble fraction obtained on each experiment the percentage of biomass solubilised (solubilisation) was calculated. The percentage of biomass solubilised was determined by calculating the ratio between the weight of the residual insoluble fraction and the initial weight of biomass. In addition the insoluble fraction of the hydrolysis was analysed by the Klason lignin method and the amount of soluble lignin was estimated by subtracting the insoluble lignin from the initial lignin content in the biomass and the results were compared to the amount of Klason lignin in the raw material, which is 26.5%.

The solubilisation and delignification responses results obtained experimentally for each condition are represented in Table 5.6.

**Table 5.6. Solubilisation and delignification responses at each condition of the full type design.**

Condition	Factor 1	Factor 2	Factor 3	Factor 4	Response1	Response 2
	T °C	Et-OH Conc. %	Time min	P CO <sub>2</sub> Bar	Solubilisation %	Delignification %
1	55	35	33	33	13.40	18.81
2	80	0	5	10	13.19	15.22
3	80	0	5	55	13.33	11.85
4	80	0	60	10	13.11	13.30
5	80	0	60	55	11.96	13.07
6	80	70	5	10	13.82	19.67
7	80	70	5	55	13.58	18.50
8	80	70	60	10	14.08	19.44
9	80	70	60	55	12.48	16.45
10	140	0	33	33	18.91	19.02
11	140	35	5	33	15.73	21.41
12	140	35	33	5	17.87	27.32
13	140	35	33	55	17.57	27.63
14	140	35	33	33	17.70	27.60
15	140	35	33	33	17.60	27.64
16	140	35	33	33	17.53	27.66
17	140	35	33	33	17.55	27.65
18	140	35	33	33	17.58	27.62
19	140	35	33	33	17.62	27.67
20	140	35	71	33	18.97	29.19
21	140	85	33	33	12.96	27.73
22	200	0	5	10	35.38	30.83
23	200	0	5	55	35.37	28.85
24	200	0	60	10	41.46	21.57
25	200	0	60	55	32.63	21.48
26	200	70	5	10	24.25	44.59
27	200	70	5	55	24.60	44.48
28	200	70	60	10	39.94	75.31
29	200	70	60	55	42.20	73.63
30	225	35	33	33	51.84	54.40

The results on Table 5.6 allowed the software to create a statistical model equation that describes the effect of the different parameters in study. The software analysed different models and tested the lack of fit of the each model in order to find the best fit model for the tested design. The Design Expert 8 software suggested a quadratic behaviour for the best fit to describe solubilisation obtaining an  $r^2$  of 0.97 for the model suggested. A summary of the model statistics for solubilisation response is shown on Table 5.7.

**Table 5.7. Model summary statistics for the solubilisation response**

	<b>Std.</b>		<b>Adjusted</b>	<b>Predicted</b>	
Source	Dev.	R-Squared	R-Squared	R-Squared	
Linear	5.85	0.7531	0.7136	0.6419	
2FI	5.96	0.8058	0.7036	0.5043	
<u>Quadratic</u>	<u>2.80</u>	<u>0.9661</u>	<u>0.9345</u>	<u>0.7549</u>	<u>Suggested</u>
Cubic	1.10	0.9979	0.9898		Aliased

The analysis of variance (ANOVA) of the statistic model is presented on Table 5.8.

**Table 5.8. ANOVA for response surface quadratic model of the solubilisation response.**

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
<b>Model</b>	3353.44	14	239.53	30.56	< 0.0001	significant
<b>A-Temperature</b>	2523.21	1	2523.21	321.88	< 0.0001	significant
<b>B-Ethanol conc</b>	16.88	1	16.88	2.15	0.1629	
<b>C-Time</b>	77.29	1	77.29	9.86	0.0067	significant
<b>D-CO2 pressure</b>	4.58	1	4.58	0.58	0.4565	
<b>AB</b>	16.44	1	16.44	2.1	0.1681	
<b>AC</b>	94.67	1	94.67	12.08	0.0034	
<b>AD</b>	0.71	1	0.71	0.091	0.7669	
<b>BC</b>	58.37	1	58.37	7.45	0.0155	
<b>BD</b>	7.05	1	7.05	0.9	0.358	
<b>CD</b>	5.71	1	5.71	0.73	0.4067	
<b>A2</b>	478.33	1	478.33	61.02	< 0.0001	
<b>B2</b>	5.11	1	5.11	0.65	0.4319	
<b>C2</b>	0.74	1	0.74	0.095	0.7627	
<b>D2</b>	0.3	1	0.3	0.038	0.8472	
<b>Residual</b>	117.59	15	7.84			
<b>Lack of Fit</b>	117.57	10	11.76	3241.76	< 0.0001	significant
<b>Pure Error</b>	0.018	5	3.63E-03			
<b>Cor Total</b>		3471.03	29			

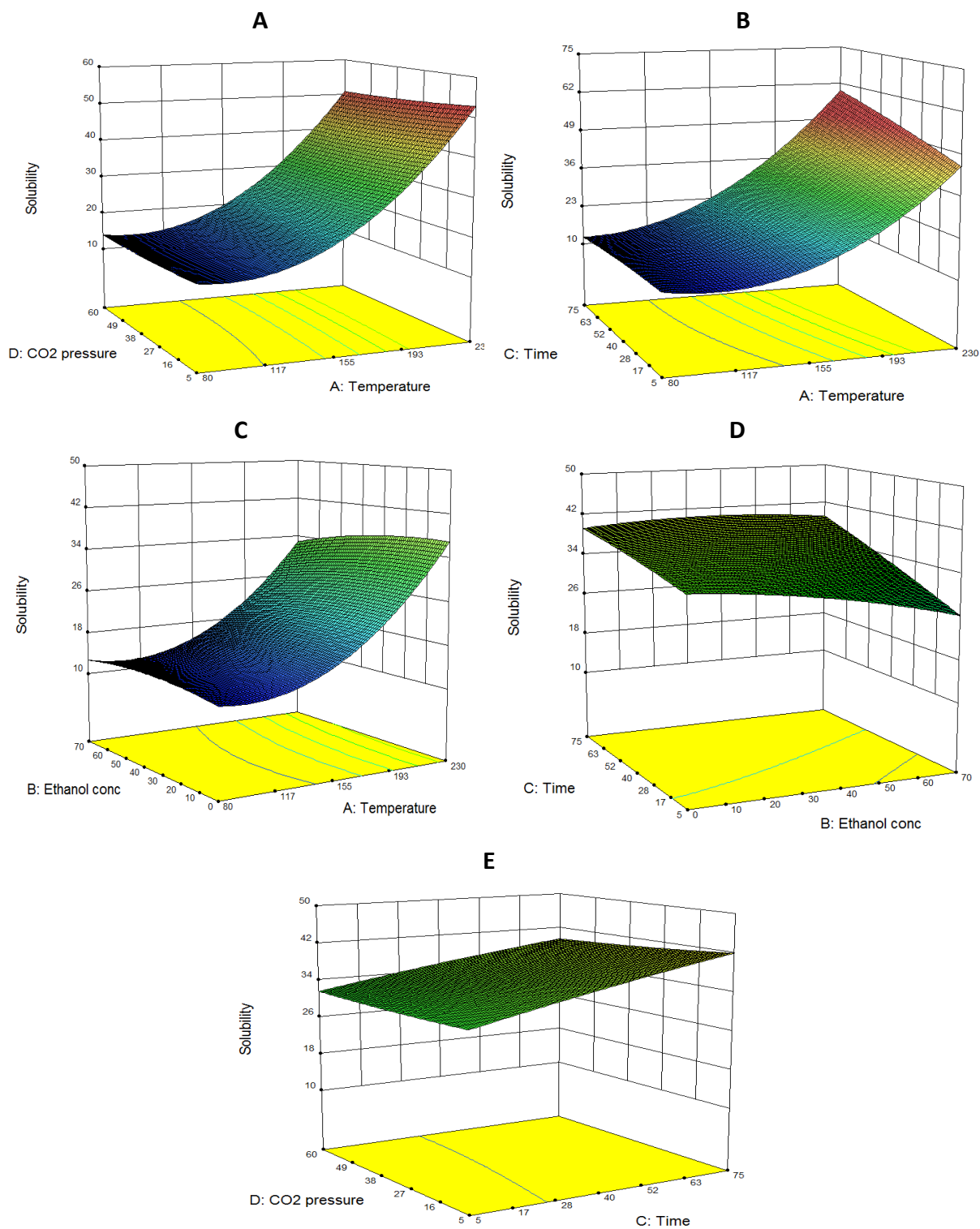
The ANOVA calculations on Table 5.8 confirmed that the solubilisation model is significant, with temperature and reaction time being the most significant parameters with  $p$ -values of <0.0001 and 0.0067 respectively with a confidence level of 95%. However, the ethanol concentration and CO<sub>2</sub> pressure showed not to be significant.

Therefore the model equation based on the quadratic model obtained for biomass solubilisation within the parameters range is:

$$\begin{aligned} \text{Solubilisation (\%)} = & 36.80729 - 0.43331 \cdot \text{Temperature} + 0.01871 \cdot \text{Ethanol Conc} - \\ & 0.13818 \cdot \text{Time} - 0.03304 \cdot \text{Pressure} - \\ & 4.83 \times 10^{-4} \cdot \text{Temperature} \cdot \text{Ethanol Conc} + 1.47 \times 10^{-3} \cdot \text{Temperature} \cdot \text{Time} - \\ & 1.56 \times 10^{-4} \cdot \text{Temperature} \cdot \text{Pressure} + 1.98 \times 10^{-3} \cdot \text{Time} \cdot \text{Ethanol Conc} + \\ & 8.43 \times 10^{-4} \cdot \text{Pressure} \cdot \text{Ethanol Conc} - 9.66 \times 10^{-4} \cdot \text{Time} \cdot \text{Pressure} + \\ & 2.12 \times 10^{-3} \cdot \text{Temperature}^2 - 1.01 \times 10^{-3} \cdot \text{Ethanol Conc}^2 - \\ & 4.90 \times 10^{-4} \cdot \text{Time}^2 + 5.33 \times 10^{-4} \cdot \text{Pressure}^2 \end{aligned}$$

#### Equation 5.2

From the model equation (Equation 5.2), solubilisation response was plotted in five different combinations to show the effect of the several variables in study. On each plot solubilisation is represented against two factors, keeping the remaining factors fixed.



**Figure 5.2. Graphic model 3D surfaces of the *Miscanthus* solubilisation.** For each graph 2 parameters were kept fixed. (A) - Solub. vs. pCO<sub>2</sub> initial vs. T, with t = 33min, Et-OH= 35%, (B) - Solub. vs. t reaction vs. T with pCO<sub>2</sub> initial = 33 bar, Et-OH=35%, (C) - Solub. vs. Et-OH vs. T, with t = 33 min, pCO<sub>2</sub> initial = 33 bar, (D) - Solub. vs. t vs Et-OH, with T = 200 °C, pCO<sub>2</sub> initial = 33 bar, (E) - Solub. vs. t vs pCO<sub>2</sub> initial, with T = 200 °C, Et-OH = 35 bar.

Temperature has an important role on biomass solubility as it is denoted from graphs A, B and C in Figure 5.2 when temperature increases, solubility increases sharply. Reaction time showed to have some effect but less pronounced as the temperature. (Graphs B, D and E), On the other side, carbon dioxide initial pressure and ethanol concentration have a less significant effect on biomass solubilisation at the working conditions (Graph E) which had been predicted before by the ANOVA. This result confirms the behaviour already seen during the preliminary tests in and it is generally accepted by different authors. The research presented by Pasquini et al. [4] and the work of Li and Kiran [151] are also in line with the efficacy of temperature on biomass solubilisation.

Delignification modelling was done in the same way as for the solubilisation response. According to the experimental data shown on Table 5.6 the Design Expert 8 software suggested a quadratic behaviour for the best fit to describe delignification obtaining an  $r^2$  of 0.94 for the model suggested. A summary of the model statistics for delignification response is shown in Table 5.9.

**Table 5.9. Model summary statistics for the delignification response.**

	<b>Std.</b>		<b>Adjusted</b>	<b>Predicted</b>	
Source	Dev.	R-Squared	R-Squared	R-Squared	
Linear	8.89	0.721	0.6764	0.5295	
2FI	5.94	0.9055	0.8558	0.5602	
<u>Quadratic</u>	<u>5.48</u>	<u>0.9363</u>	<u>0.8769</u>	<u>0.5235</u>	<u>Suggested</u>
Cubic	0.15	1	0.9999		Aliased



The analysis of variance (ANOVA) of the delignification statistic model is presented in Table 5.10.

**Table 5.10. ANOVA for response surface quadratic model of the delignification.**

Source	Sum of Squares	df	Mean Square	F Value	<i>p</i> -value Prob > F	
<b>Model</b>	6633.64	14	473.83	15.76	< 0.0001	<i>significant</i>
<b>A-Temperature</b>	3473.51	1	3473.51	115.5	< 0.0001	<i>significant</i>
<b>B-Ethanol conc</b>	1505.36	1	1505.36	50.05	< 0.0001	<i>significant</i>
<b>C-Time</b>	130.39	1	130.39	4.34	0.0549	
<b>D-CO<sub>2</sub> pressure</b>	6.22	1	6.22	0.21	0.6558	
<b>AB</b>	821.68	1	821.68	27.32	0.0001	
<b>AC</b>	133.52	1	133.52	4.44	0.0524	
<b>AD</b>	0.95	1	0.95	0.032	0.8613	
<b>BC</b>	350.81	1	350.81	11.66	0.0038	
<b>BD</b>	4.90E-03	1	4.90E-03	1.63E-04	0.99	
<b>CD</b>	0.17	1	0.17	5.59E-03	0.9414	
<b>A<sup>2</sup></b>	206.41	1	206.41	6.86	0.0193	
<b>B<sup>2</sup></b>	25.19	1	25.19	0.84	0.3746	
<b>C<sup>2</sup></b>	4.02	1	4.02	0.13	0.7197	
<b>D<sup>2</sup></b>	2.26	1	2.26	0.075	0.7878	
<b>Residual</b>	451.12	15	30.07			
<b>Lack of Fit</b>	451.12	10	45.11	66341.11	< 0.0001	<i>significant</i>
<b>Pure Error</b>	3.40E-03	5	6.80E-04			
<b>Cor Total</b>	7084.76	29				

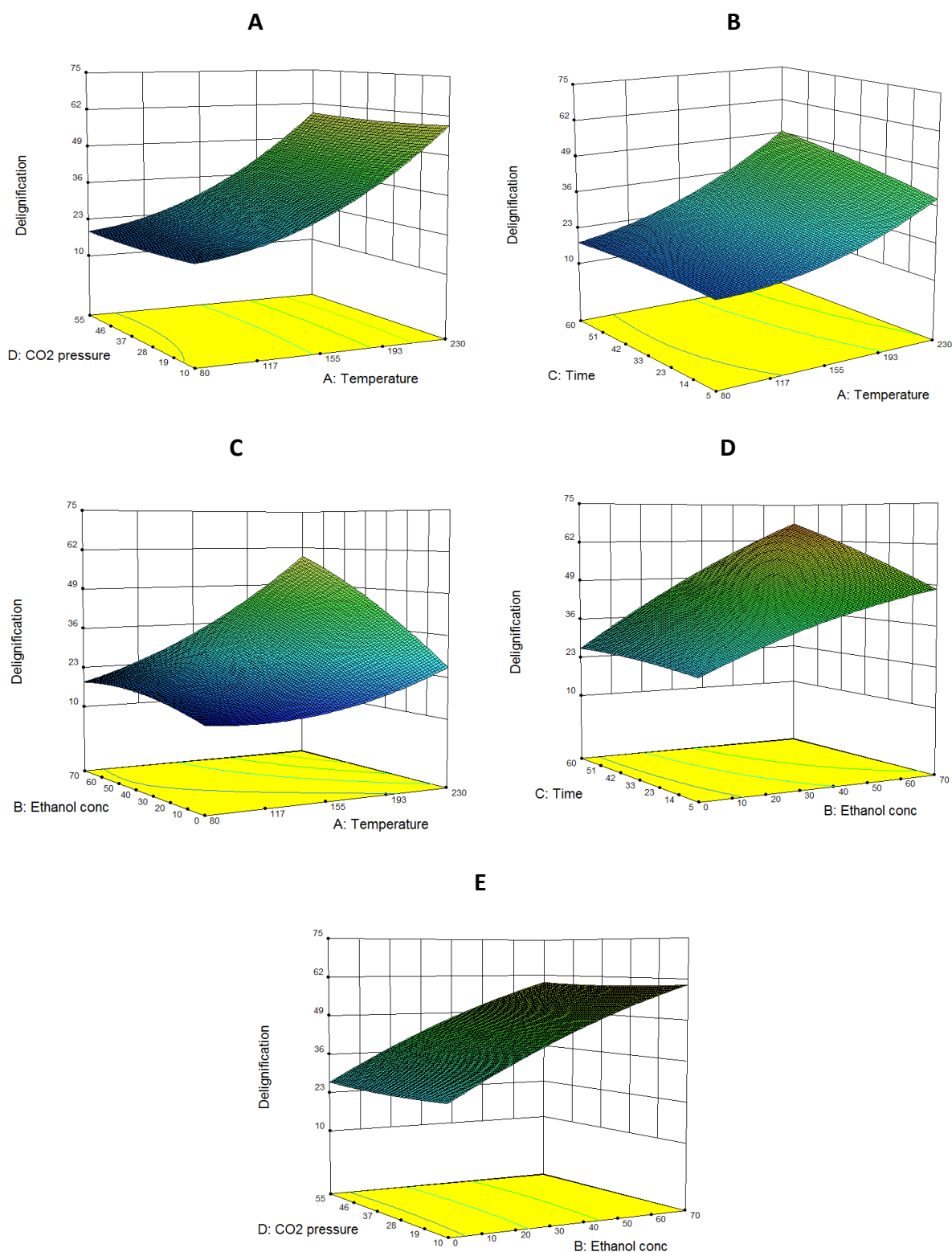
The ANOVA calculations on Table 5.8 confirmed that the delignification model is significant, with temperature and ethanol concentration being the most significant parameters with *p*-values of <0.0001 with a confidence level of 95%. However, the reaction time and CO<sub>2</sub> pressure showed not to be significant at the range of hydrolysis conditions in study.

Therefore the model equation based on the quadratic model obtained for biomass delignification within the parameters range is:

$$\begin{aligned}
 \text{Delignification (\%)} = & 40.9206 - 0.35315 \cdot \text{Temperature} - 0.21699 \cdot \text{Ethanol Conc} - \\
 & 0.24989 \cdot \text{Time} - 0.15055 \cdot \text{Pressure} + \\
 & 3.41 \times 10^{-3} \cdot \text{Temperature} \cdot \text{Ethanol Conc} + \\
 & 1.75 \times 10^{-3} \cdot \text{Temperature} \cdot \text{Time} + 1.81 \times 10^{-4} \cdot \text{Temperature} \cdot \text{Pressure} + \\
 & 4.86 \times 10^{-3} \cdot \text{Time} \cdot \text{Ethanol Conc} - 2.22 \times 10^{-5} \cdot \text{Pressure} \cdot \text{Ethanol Conc} + \\
 & 1.66 \times 10^{-4} \cdot \text{Time} \cdot \text{Pressure} + 1.39 \times 10^{-3} \cdot \text{Temperature}^2 - 2.24 \times \\
 & 10^{-3} \cdot \text{Ethanol Conc}^2 - 1.14 \times 10^{-3} \cdot \text{Time}^2 + 1.46 \times 10^{-3} \cdot \text{Pressure}^2
 \end{aligned}$$

**Equation 5.3**

From the model equation (Equation 5.3), delignification response was plotted in five different combinations to show the effect of the several variables in study. On each plot delignification is represented against two factors, keeping the remaining factors fixed.



**Figure 5.3. Graphic model 3D surfaces of the *Miscanthus* delignification.** For each graph 2 parameters were kept fixed. (A) - Delig. vs.  $p\text{CO}_2$  initial vs. T, with  $t = 33$  min, Et-OH= 35%, (B) - Delig. vs.  $t$  reaction vs. T with  $p\text{CO}_2$  initial = 33 bar, Et-OH=35%, (C) - Delig. vs. Et-OH vs. T, with  $t = 33$  min,  $p\text{CO}_2$  initial = 33 bar, (D) - Delig. vs.  $t$  vs Et-OH, with  $T = 200$  °C,  $p\text{CO}_2$  initial = 33 bar, (E) - Delig. vs.  $t$  vs  $p\text{CO}_2$  initial, with  $T = 200$  °C, Et-OH = 35 bar.

The graphic model 3D surfaces that represent the delignification response (Figure 5.3) show that temperature is the most effective parameter to cause biomass delignification (graphs A, B and C). When temperature increases, delignification increases as well.

Ethanol concentration in the medium has also an important role on the lignin removal from the biomass as represented on the graphs C, D, and E, but for lower temperatures, it seems that there is an optimal concentration in ethanol for delignification of around 50% (graph C) which is in correlation with the research done Pasquini et al. [4] on the delignification of sugarcane bagasse.

On the other side, pressure does not have an effect on biomass delignification (Graph A), fact that was mentioned when the ANOVA results were calculated as this factor was showed that it is not significant.

According to the results, the reaction time only has a significant effect on lignin removal for higher temperatures and higher ethanol concentration (Graphs B and D).

## **5.7 Conclusions**

Preliminary tests allowed a quick analysis of the different factors to be included in RSM. They suggested that load size does not play an important role on the biomass solubilisation and delignification. Therefore, it was decided it would not be included in the broader modelling.

Temperature has been shown to be the most important factor either in *Miscanthus* solubilisation or delignification which is in accordance with the literature.

ANOVA analysis of the solubilisation model equation showed that initial carbon dioxide pressure and ethanol concentration at the hydrolysis conditions are not significant.

However, on the delignification model carbon dioxide pressure and reaction time are the non-significant parameters with the ethanol playing an important role side by side with the temperature on lignin removal.

Finally results have shown that there is a preferable ethanol concentration around 50% in ethanol that enhances the delignification of Miscanthus.

Due to the fact that the carbon dioxide initial pressure was considered non-significant on the solubilisation and delignification models, the effect of carbon dioxide in the hydrolysis could not be verified. However, results showed that among the experiments done based on the RSM it was obtained a solubilisation range from 12 to 52% and delignification between 12 to 75%. In addition, preliminary tests on particular conditions hydrolysis obtained higher values of solubilisation (53%, at 200 °C, 2,5g, 50%, 60 min, 55 bar) and delignification (86% at 200 °C, 2,5 g, 50%, 60 min, 55 bar). These results are in accordance with the literature so the presence of carbon dioxide should catalyse the hydrolysis. Nevertheless, more tests at higher CO<sub>2</sub> pressures by connecting a CO<sub>2</sub> pump to the reactor should be done.

## **6 CHARACTERISATION OF THE LIQUID AND SOLID FRACTIONS OBTAINED BY THE MODIFIED ORGANOSOLV HYDROLYSIS METHOD**

### **6.1 Aim**

Under the biorefinery concept, the main guideline for this research study, this chapter has the aim to analyse how efficient the proposed organosolv method is to fractionate lignocellulosic biomass into its main components, lignin, hemicellulose and cellulose. On this view, several analytical methods were used targeting the analysis of these main components.

After the hydrolysis experiments at different conditions of temperature, biomass loading, ethanol concentration, reaction time and CO<sub>2</sub> initial pressure the pH of the final hydrolysis medium was compared to the initial values and the results will be presented in this section. Then the cellulose-rich solid residue obtained was separated by vacuum filtration from the liquid phase (hemicellulose and lignin fraction). Lignin was then recovered by precipitation from the liquid phase obtaining a supernatant (hemicellulose-rich fraction) and precipitated lignin. The hemicellulose fraction (supernatant) was then analysed for reducing sugars quantification by the DNS assay. As mentioned in the literature review, at the range temperatures used in this research (55-225 °C) only hemicellulose and lignin would be hydrolysed from LCB. On this view, by using DNS assay to quantify the reducing sugars in the supernatant the results could be used to quantify only the reducing sugars originated by the

hemicellulose, i.e. xylose and arabinose which in turn the reducing sugars can work as a marker for the hydrolysis of hemicellulose in the LCB.

A comparative analysis of the chemical structure of the residual fibres, the recovered lignin and the dried supernatant was also assessed by DRIFT which focused on the determination of which hydrolysis parameters affect the most the quality of each lignocellulosic component in terms of contamination and oxidation of the compounds. In addition, four samples of recovered lignin were also analysed by  $^1\text{H}$  and  $^{13}\text{C}$  NMR liquid-state to evaluate the effect of temperature, carbon dioxide initial pressure and ethanol concentration on the precipitated lignin molecular structure. And finally, one sample of fibres residue obtained after hydrolysis was compared to the raw material by SEM to show the effect of the hydrolysis method on the physical structure of the fibres.

## **6.2 Background**

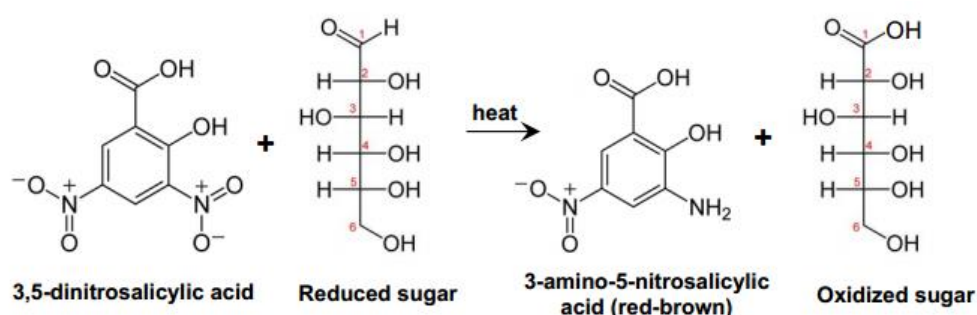
Before fermentation of sugars to ethanol several LCB pretreatments can be used in association to deconstruct LCB by removing lignin, hydrolysing hemicellulose and cellulose to fermentable sugars. These pretreatments can use physical, chemical, combined physicochemical and biological processes. Research on the bioethanol production from LCB has always seen organosolv hydrolysis as a pretreatment step prior to an enzymatic hydrolysis however with the modified organosolv method proposed in this research it was the aim to show that it is possible to fractionate LCB into its main components by using different hydrolysis condition. In general it is agreed that temperature is the parameter that plays the most important role in biomass hydrolysis. In these studies it was found that hemicelluloses dissolve above 120 °C, lignin begins to dissolve at around 180 – 190 °C and

cellulose degrades at temperatures greater than 230 °C. [150-152]. However, it was showed by Hashaikeh et al. [121] when working with willow that at temperatures above 230 °C to cause re-condensation reactions from the dissolution products of lignin and hemicellulose. These re-condensation reactions result from the fact that the dissolution products of lignin and hemicellulose tend to react internally and precipitate over the solid residue which will block the water access to the cellulose that will tend to dehydrate and form char-like precipitates inside the reactor. Therefore, the fractionation of the LCB prior to complete hydrolysis of cellulose to ethanol is mandatory to avoid these issues. The aim of this research allowed tackling the fractionation of the LCB, particularly *Miscanthus* by optimising the hydrolysis conditions for removal of lignin and hemicellulose and by leaving cellulose fibres ready for further treatment at higher temperatures for cellulose conversion to glucose. Therefore, looking to the hemicellulose behaviour under the hydrolysis conditions this chapter presents the results of the amount of reducing sugars extracted/hydrolysed from the biomass determined by the DNS reducing sugars assay at different hydrolysis conditions. The reducing sugars quantification was used to evaluate the hemicellulose hydrolysis during the hydrolysis experiment by quantification of the mixture of xylose and arabinose in the hydrolysates since they are the main reducing sugars derived from *Miscanthus* hemicellulose.

The DNS reducing sugars assay was adapted from King et al. [143] and it developed according to Wood et al. [144] to reduce errors associated to the analysis, namely the use of 8 channel micropipette instead of single channel to add DNS reagent showed that this method is highly time-dependent improving considerably the calibration curves correlation.



Moreover, covering the PCR plates with aluminium seal avoided evaporation and improved the replication results. In the same way to avoid evaporation heating temperature and time needed to be adjusted from 95 °C for 5 min suggested by King et al. [143] to 80 °C for 7 min. These changes in the conditions proved to be mandatory especially for samples with ethanol as they were more prone to evaporation. DNS assay, it uses 3,5-dinitrosalicylic acid that is reduced to 3-amino-5-nitrosalicylic acid with the concomitant oxidation of an aldose or ketose sugar, turning into a red-brown colour (Figure 6.1).



**Figure 6.1. Reduction of DNS reagent reaction basis of the DNS assay to determine reducing sugars.**

The use of DNS method has been broadly applied to LCB to evaluate how efficient hydrolysis treatments are for production of glucose-rich solutions for fermentation to bioethanol. However, normally the assay is used to evaluate different hydrolysis pretreatments indirectly for instance, the DNS assay is applied to the enzymatic hydrolysates that in turn will determine how good the hydrolysis pretreatments are to deconstruct the LCB polymer so the cellulose can be used as a feedstock to enzymatic hydrolysis. With this aim, Ibrahim et al. [160] compared alkaline pulping with steam explosion pretreatments on rice straw by analysing by DNS assay the glucose resulting from the enzymatic hydrolysis of the alkaline pulping and steam explosion pretreatments, the results showed a range from 28.9–58.4 g/l

in glucose with the steam exploded hydrolysis the less effective pretreatment compared to the alkaline pulping but the highest concentration in glucose was obtained when using a combination of alkali-extraction and steam explosion.[160] In the same way Zhang et al. [161] reported the use of the DNS assay to evaluate different enzymatic hydrolysis conditions from alkaline pretreated wheat straw and sugar cane bagasse with NaOH.[161] Other study done by King et al. [143] also uses DNS assay as a quick method to analyse the polysaccharide hydrolysis by fungal extracts, incorporated in different LCB substrates during a 10 days period. With the same aim the present research on *Miscanthus* used the DNS assay method as a quick assessment of the reducing sugars formation at different hydrolysis conditions.

In addition to the DNS method, infrared spectroscopy (DRIFT) was also used to analyse the three biomass fractions obtained after hydrolysis at different conditions, i.e. the solid residue (cellulose-rich fibres), the recovered lignin by precipitation and also the dried supernatant were analysed qualitatively by DRIFT analysis to evaluate how efficient is the proposed hydrolysis method on fractionating the biomass main components. The method allowed targeting differences in special molecular vibration bands assigned for each type of compound.

Mid-infrared (mid-IR) spectroscopy, called FT-IR has been used to analyse LCB for many years. The mid-IR spectral region is the Wavenumber range from 4000 to 400  $\text{cm}^{-1}$  and the majority of lignin fundamental molecular vibrations fall in this range. DRIFT spectra are obtained when IR radiation is incident on a scattering sample at a specific angle and is reflected at all angles. The diffuse reflectance process involves transmission, scattering, and

reflection, and samples can be analysed directly (as a non-destructive analysis) or dispersed in KBr as a finely divided powder to minimise the scattering effect.[162]

To complete the characterisation of the recovered lignin after hydrolysis further analysis on its polymeric structure was performed by using 1D  $^1\text{H}$  and 1D  $^{13}\text{C}$  NMR and partial structural identification of molecules was conducted by means of 2D experiments:

- Homo-nuclear  $^1\text{H}$ - $^1\text{H}$  COSY (Correlation Spectroscopy),
- TOCSY (TOtal Correlation Spectroscopy),
- Hetero-nuclear  $^1\text{H}$ - $^{13}\text{C}$  HSQC (Hetero-nuclear Single-Quantum Correlation)
- $^1\text{H}$  DOSY (Diffusion-Ordered Spectroscopy)

In the literature, different studies have been using 1D  $^1\text{H}$  and 1D  $^{13}\text{C}$  NMR to determine relative proportions of structures in lignin. Xu et al. [163] reported that lignin  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra obtained by an organosolv hydrolysis of wheat straw using acetic and formic acids showed an increase of guaiacyl units and lignin condensation for higher acid concentration. On the other hand, lignin obtained from an organosolv hydrolysis using ethanol and methanol without added acid as catalyst contained almost equal amounts of non-condensed guaiacyl and syringyl units with fewer p-hydroxyphenyl units. On other study Timilsena et al. [164] mentioned that the relative abundance of syringyl, guaiacyl and p-hydroxyphenyl moieties is very important in delignification process. The presence of less guaiacyl units results in a less branching lignin structure which is thought to aid carbohydrates accessibility, enzyme adsorption, and hydrolysis. In addition, guaiacyl lignin is known to facilitate recondensation reactions during delignification process. This research group used  $^{13}\text{C}$  NMR to quantify the syringyl, guaiacyl and p-hydroxyphenyl ratios ethanol

organosolv hydrolysis lignin originated from three different biomasses, Miscanthus, empty palm fruit bunch and typha grass.

Finally, Scanning electron microscopy was used to compare the residual fibre physical structure after treatment to the initial raw material. The sample chosen was obtained after hydrolysis at 200 °C, reaction time of 60 min, biomass load of 15 g in 250 ml of 50% ethanol in water and carbon dioxide initial pressure of 50 bar. Some studies have mentioned that SEM can qualitatively illustrate the lignin re-precipitation over the fibres. This phenomenon happens when lignin that has been previously solubilised re-precipitates during the cooling step after reaction or during fibres washing due to a change in lignin solubility, in agreement with this Xu et al. [140] also studied the effects of washing organosolv pulp and the temperature effect on the lignin precipitation over the fibres and the results showed that during pulp washing due to lowering the ethanol concentration. Adding to that, Hewson et al. [165] mentioned before that lignin undergo in condensation reactions during the cooling step that causes lignin precipitation over the residual fibres. Finally some studies undertaken by both Micic et al. [166] and Xu et al. [140] applying SEM analysis to capture lignin precipitates over fibres added that when lignin precipitates over the fibres it does in a form of spheres ranging in size from 0.02–1000 nm.

## **6.3 Results and Discussions**

### **6.3.1 Study on how the medium pH values are affected by different hydrolysis conditions.**

The present section aims to add the information over how the pH value of the hydrolysis medium changes during the hydrolysis reaction. The modified hydrolysis method presented in this thesis applied the use of carbon dioxide as the way to produce carbonic acid and therefore creating the acidic environment necessary to catalyse the hydrolysis reaction instead of using of strong acids in the common organosolv methods already discussed in the literature review. Therefore the pH value of the medium was measured before and after each hydrolysis.

As described on Chapter 3 in the Materials and Methods, section 3.4 regarding the modified organosolv method used, pH was measured before and after each hydrolysis condition and the results recorded. Table 6.1 shows all the hydrolysis conditions done during the Experimental Design DoE with the respective pH values obtained before and after hydrolysis. The results were sorted by the final pH value obtained and show that initial pH value of the medium is around 6.2 however final pH varies from 3.3 to 6. Lower values of pH hence more acidic medium was obtained for higher temperatures but more importantly for conditions without ethanol. On the opposite side for lower temperatures and higher concentrations in ethanol in the hydrolysis medium delivered a higher pH values (around 6) with almost no change in the pH value compared initial value.

**Table 6.1. Change in pH value during the hydrolysis reaction at different conditions**

Condition	Factor 1	Factor 2	Factor 3	Factor 4	pH <sub>initial</sub>	T <sub>initial</sub> °C	pH <sub>final</sub>	T <sub>final</sub> °C
	T °C	Ethanol Conc. %	Time min	CO <sub>2</sub> Pressure bar				
<b>24</b>	200	0	60	10	6.1	21.4	<b>3.3</b>	22.8
<b>25</b>	200	0	60	55	6.2	24.7	<b>3.5</b>	29.5
<b>30</b>	225	35	33	33	6.2	22.5	<b>3.7</b>	26.8
<b>22</b>	200	0	5	10	6.1	25.0	<b>3.9</b>	24.1
<b>23</b>	200	0	5	55	6.1	26.7	<b>3.9</b>	26.8
<b>10</b>	140	0	33	33	6.2	27.0	<b>4.6</b>	22.2
<b>2</b>	80	0	5	10	6.1	23.0	<b>4.7</b>	17.2
<b>4</b>	80	0	60	10	6.2	21.3	<b>4.8</b>	20.3
<b>3</b>	80	0	5	55	6.2	21.5	<b>4.9</b>	17.9
<b>5</b>	80	0	60	55	6.2	25.0	<b>4.9</b>	20.6
<b>28</b>	200	70	60	10	6.3	22.4	<b>4.9</b>	25.6
<b>12</b>	140	35	33	5	6.3	24.6	<b>5.0</b>	22.7
<b>15</b>	140	35	33	33	6.1	23.1	<b>5.0</b>	20.1
<b>18</b>	140	35	33	33	6.2	22.4	<b>5.0</b>	22.4
<b>20</b>	140	35	71	33	6.2	25.1	<b>5.0</b>	20.1
<b>29</b>	200	70	60	55	6.3	21.3	<b>5.0</b>	25.5
<b>13</b>	140	35	33	55	6.1	23.5	<b>5.1</b>	21.0
<b>14</b>	140	35	33	33	6.2	22.2	<b>5.1</b>	19.4
<b>16</b>	140	35	33	33	6.1	22.6	<b>5.1</b>	21.3
<b>19</b>	140	35	33	33	6.3	22.3	<b>5.1</b>	20.1
<b>11</b>	140	35	5	33	6.3	25.3	<b>5.2</b>	20.4
<b>17</b>	140	35	33	33	6.3	21.3	<b>5.2</b>	20.2
<b>26</b>	200	70	5	10	6.3	21.5	<b>5.3</b>	25.1
<b>27</b>	200	70	5	55	6.3	20.8	<b>5.3</b>	21.8
<b>1</b>	55	35	33	33	6.3	20.7	<b>5.4</b>	17.1
<b>21</b>	140	85	33	33	6.1	21.0	<b>5.6</b>	23.0
<b>6</b>	80	70	5	10	6.5	23.2	<b>5.9</b>	17.1
<b>7</b>	80	70	5	55	6.3	24.7	<b>5.9</b>	13.9
<b>8</b>	80	70	60	10	6.4	22.9	<b>5.9</b>	19.2
<b>9</b>	80	70	60	55	6.4	23.6	<b>6.0</b>	20.0

In the literature there is not information on the pH obtained during organosolv hydrolysis using ethanol and carbon dioxide but the results are thought to be related to the lower activity of the hydronium ion in ethanol compared to water.

In conclusion, a variation in the pH values at the end of the hydrolysis was denoted especially for higher temperatures and without ethanol in the medium. At the beginning of the hydrolysis reaction the average pH of the medium was around 6.2 however, in absence of ethanol and at higher temperatures the pH value was reduced down to 3 with little change for conditions with higher concentrations in ethanol. The literature review does not offer information of other studies where pH was measured in an organosolv hydrolysis without addition of acid like in this case but it is thought that these variations in the final acidity of the medium is due to the lower activity of the  $H^+$  ion in ethanol compared to water.

### **6.3.2 Evaluation of the modified organosolv method on the hemicellulose hydrolysis from Miscanthus biomass by using the DNS reducing sugars assay.**

The quantification of reducing sugars is an important tool to evaluate how viable is the hydrolysis medium to be used as a pretreatment for the fermentation step on bioethanol production. However, the DNS reagent is non-specific and reacts with both five and six carbon reducing sugars. Although this assay does not allow discrimination among specific carbohydrates, it can be used to quantify hydrolysis of a wide range of polysaccharide substrates. Therefore, the DNS assay was used during this research with the aim to quantify

the mixture of reducing sugars originated by the hydrolysis of hemicellulose namely xylose and arabinose and thus allowing predicting optimum conditions for LCB fractionation.

To analyse the effect of the different parameters on the production of reducing sugars, the liquid phase obtained after filtration of the hydrolysis material and lignin precipitation (supernatant) was submitted to the DNS assay by using a calibration curve with glucose standard and therefore the results will be represented in terms of mass of glucose per mass of biomass used (mg glucose / 10 g biomass). Temperature (55, 80, 140, 180, 190, 200 and 225 °C), load size per 250 ml of hydrolysis medium (2.5, 5, 10 and 15 g), ethanol concentration in the medium (33, 50 and 67%), reaction time (5, 33 and 60 min) and CO<sub>2</sub> initial pressure (10, 33 and 55 bar) were the hydrolysis parameters tested over 29 hydrolysis conditions.

Table 6.2 shows the combination of the experimental parameters analysed.



**Table 6.2. Hydrolysis experimental conditions analysed by DNS assay.**

Condition	T (°C)	Load (g)	Et-OH conc (%)	t (min)	p (bar)
1	55	10	33	33	33
2	80	10	0	5	10
3	80	10	0	5	55
4	80	10	0	60	10
5	80	10	67	5	10
6	80	10	67	60	10
7	80	10	67	5	55
8	140	10	0	33	33
9	140	10	33	60	33
10	140	10	33	5	33
11	140	10	33	33	33
12	140	10	33	33	5
13	140	10	67	33	33
14	180	2.5	50	60	55
15	180	5	50	60	55
16	180	10	50	60	55
17	180	15	50	60	55
18	190	2.5	50	60	55
19	190	5	50	60	55
20	190	10	50	60	55
21	190	15	50	60	55
22	200	2.5	50	60	55
23	200	5	50	60	55
24	200	10	0	5	10
25	200	10	0	60	10
26	200	10	50	60	55
27	200	10	67	60	10
28	200	15	50	60	55
29	225	10	33	33	33

All the supernatant samples obtained were analysed at least in triplicate by the DNS method originating a relative standard error of less than 1%, except for the conditions with the smallest load sizes (2.5 g) where 3 to 16% relative standard error standard errors were obtained which is thought to be due to the experimental error on using such small amounts of biomass in the reactor which lead to mass losses and thus more variability in the results.

The reducing sugars results obtained are combined in the graphs in Figure 6.2 and Figure 6.3 allowed determining the influence of the experimental parameters used during hydrolysis.

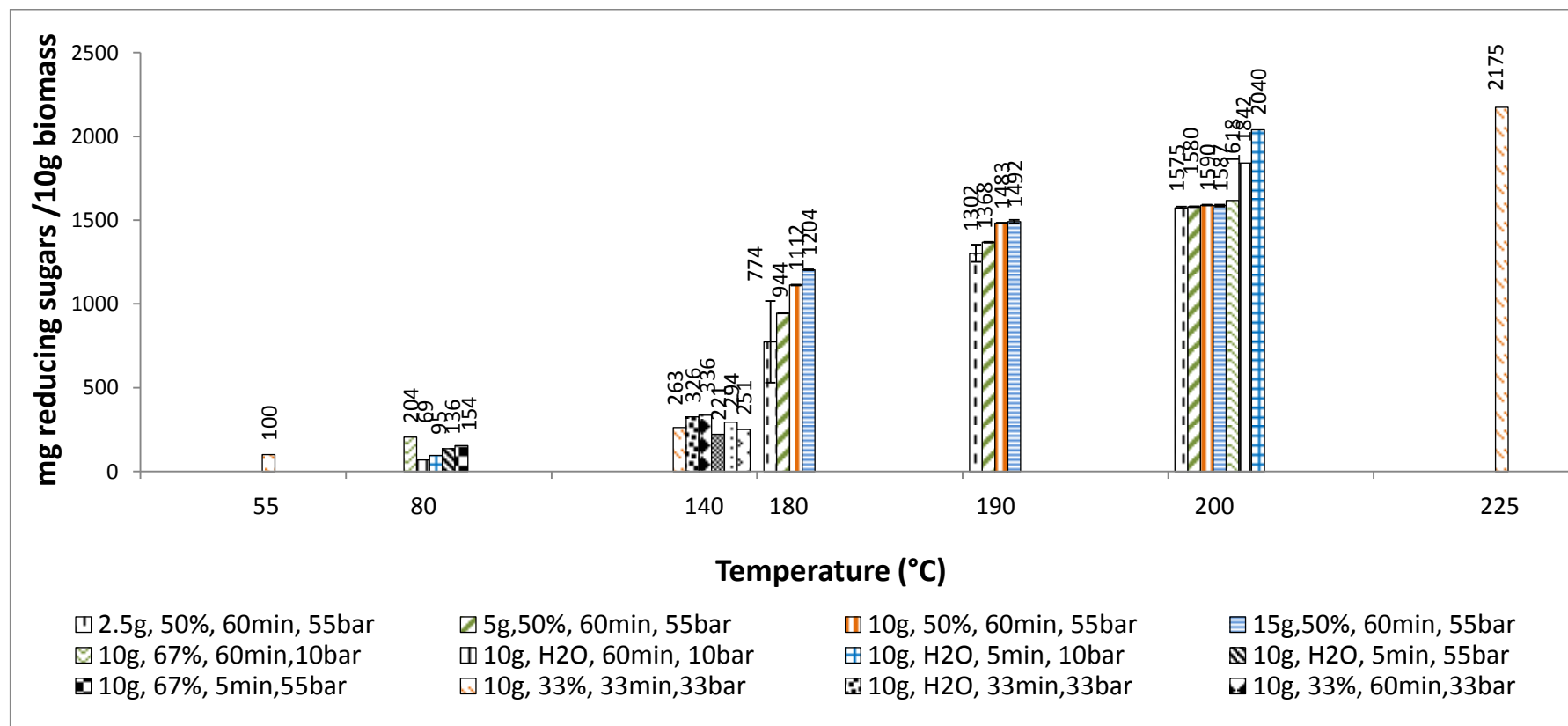
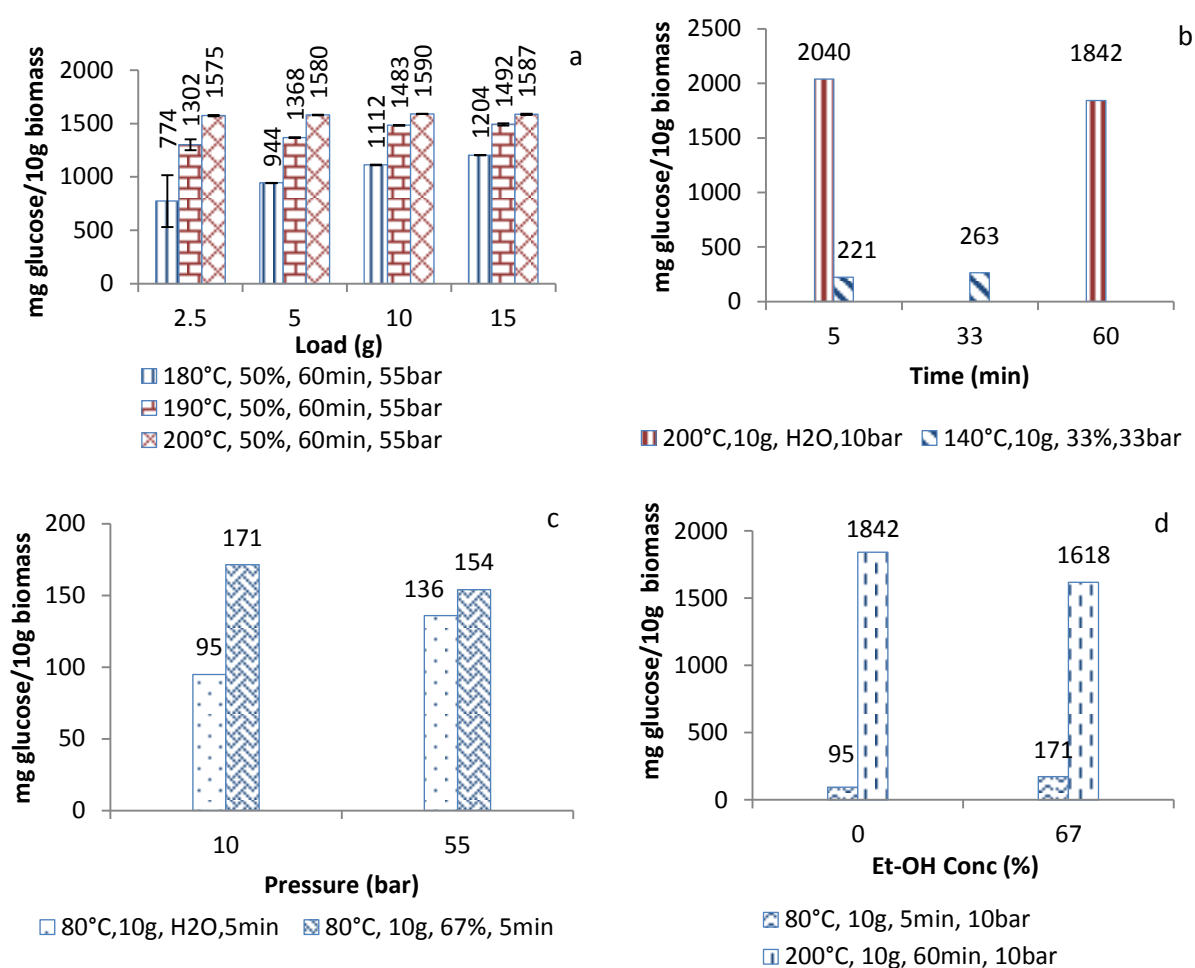


Figure 6.2. Effect of temperature on the reducing sugars quantification by the DNS assay on the hydrolysates after lignin precipitation.



**Figure 6.3. Reducing sugars quantification by the DNS assay on the hydrolysates after lignin precipitation.** a: effect of load size per 250ml medium, b: effect of reaction time, c: effect of pressure and d: effect of ethanol concentration in the medium.

From Figure 6.2 it is clearly seen that temperature represents an important role on the hydrolysis of the hemicellulose fraction. Results showed a sharp increase in reducing sugars concentration from 140°C to 180 °C with a smaller increase at 190 °C and then a stabilisation between 190 to 200 °C, which is in accordance with the literature that states that hemicellulose hydrolyses between 180 to 190°C as described by Leppänen et al. [150], Li et al. [151] and Borrega et al. [152]. However, the concentration in reducing sugars increased for conditions without ethanol at 200 °C, what can be due to the lower activity of the H<sup>+</sup> ion in ethanol compared to water [167] thus reducing the pH level of the medium and therefore

catalysing the hydrolysis. That might be confirmed by the graph on (Figure 6.3 c) where the effect of CO<sub>2</sub> pressure is more denoted in the hydrolysis medium with only water.

Regarding the effect of loading size, (Figure 6.3 a) results showed that for lower temperatures (180 °C) loading plays a role in the concentration of the reducing sugar, but what might be implicit here is the hydrolysis rate of the hemicellulose being too slow for lower temperatures and biomass concentration. In addition, the production of acetic acid from hemicellulose when high biomass concentration is used, was shown of lowering the pH, fact referred by Gilarranz, et al. [147] According to the results nothing can be commented on the graphs b (reaction time) and d (ethanol concentration).

### **6.3.3 Evaluation of the modified organosolv method on the quality of the recovered hydrolysis fractions assessed by DRIFT analysis**

The use of infrared spectroscopy allows the detection of vibration characteristics of chemical functional groups in samples. Therefore, this section presents the results of a qualitative DRIFT analysis of the solid residue (cellulose-rich fibres), the recovered lignin by precipitation and also the dried supernatant to evaluate if the different hydrolysis parameters in study could influence the outcome fractions in terms of purity or if there is any condition that delivers fractions with less contaminant products from the other fractions.

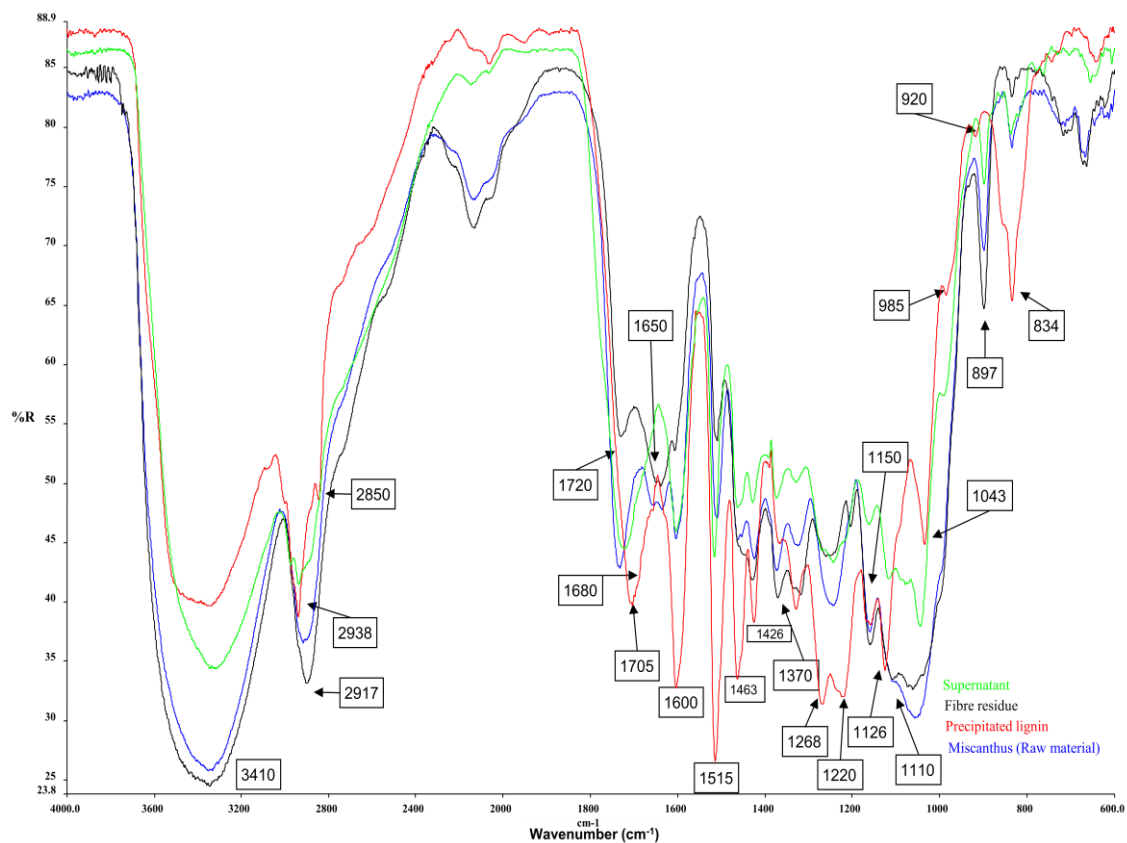
This set of analysis involved the evaluation of 16 conditions with IR spectra being analysed for three different samples per condition (fibres, lignin and supernatant). The parameters in study were temperature (180, 190, 200 °C), load size per 250ml of hydrolysis medium (2.5, 5, 10 and 15 g), ethanol concentration in the medium (33, 50 and 67%), reaction time (5, 60

min) and CO<sub>2</sub> initial pressure (10, 55 bar). The hydrolysis conditions used to obtain the biomass fractions in study are presented in Table 6.3.

**Table 6.3. Hydrolysis experimental conditions analysed by DRIFT**

Condition	Temperature (°C)	Load (g/250ml)	Et-OH conc. (%)	Reaction time (min)	CO <sub>2</sub> pressure (bar)
A	200	2.5	50	60	55
B	180	5	50	60	55
C	190	5	50	60	55
D	200	5	50	60	55
E	180	10	50	60	55
F	190	10	50	60	55
G	200	10	50	60	55
H	180	15	50	60	55
I	190	15	50	60	55
J	200	15	50	60	55
K	180	10	33	60	55
L	190	10	33	60	55
M	180	10	67	60	55
N	200	10	67	60	55
O	200	10	67	60	10
P	200	10	67	5	10

Figure 6.4. represents a comparative example of the FTIR spectra obtained in the 4000 – 600  $\text{cm}^{-1}$  region for three hydrolysis fractions (fibre, precipitated lignin and supernatant) and the untreated Miscanthus.



**Figure 6.4.** Example of a comparative DRIFT spectra on the 4000 – 600  $\text{cm}^{-1}$  region of untreated Miscanthus, the residual fibre, precipitated lignin and the supernatant. Spectra obtained after hydrolysis with the condition “G” at 200 °C, 10 g/250 ml, 50% in ethanol, for 60 min and  $\text{CO}_2$  initial pressure of 55 bar. The spectra also present the main reflectance bands labelled.

Based on the spectra obtained and on the literature, a table with the reflectance bands and their Wavenumber assignments, characteristic of lignocellulosic biomass was built in order to later compare the differences in chemical functional groups present on each fraction caused by different hydrolysis conditions. The bands assignments are presented in Table 6.4.

**Table 6.4. Reflectance bands and their Wavenumber assignments, characteristic of lignocellulosic biomass.**

Wavenumber(cm <sup>-1</sup> )	Short interpretation
<b>3410</b>	OH Stretching, aromatic and aliphatic hydroxyl groups [168, 169] (Present in lignin and cellulose)
<b>Bands 2938</b>	CH stretching in aromatic methoxyl groups and in methyl and methylene groups of side chains[169]
<b>2917 and 2850</b>	CH stretching in aliphatic methylene group [169]
<b>Weak to med bands 1705-1720</b>	Non-conjugated carbonyl / carboxyl stretching[168] Band Increases intensity in oxidised lignins [169] Ester carbonyl vibration in acetyl, feruloyl, <i>p</i> -coumaroyl, groups in lignin and hemicelluloses (ester bonds between hydroxycinnamic acids and lignin were cleaved during hydrolysis) [170]
<b>Shoulder 1680</b>	Conjugated carbonyl / carboxyl stretching[169]
<b>1650</b>	Carbonyl moieties (unconjugated C = O in xylans (hemicellulose)[171] stretching of conjugated carbonyl groups with the aromatic ring[168] (shows if the ester bonds between hydroxycinnamic acids and lignin were cleaved or not during treatment)[170]
<b>1600, 1515, 1426</b>	Aromatic phenylpropane skeleton vibrations are common for all lignins with the most characteristic bands for lignin in the fingerprint region [168, 169, 172], contamination of fibres by lignin bands at 1600 and 1515 [168]
<b>1463</b>	CH deformation combined with aromatic ring vibration[169]
<b>1370 - 1375</b>	Phenolic OH and aliphatic C-H deformation (symmetric) in methyl group[168, 169]
<b>1328</b>	Syringyl (S) ring plus Guaiacyl (G) ring condensed [169], S ring breathing with C–O stretching [168]
<b>1268 + 1150</b>	Vibrations characteristic of the G unit 1268 - G ring breathing and C=O stretch [168, 169] 1150 - CH in-plane deformation [169]
<b>1220</b>	C-C plus C-O plus C=O stretching[169], G ring with C-O and C=O[173] ether bridges[168] (This band is related to the presence of C=O groups formed after lignin oxidation) [173]
<b>1126</b>	Typical of S unit; also secondary alcohol and C=O stretching
<b>1110</b>	Glucose ring stretch (asymmetric)[168] (Present in cellulose and hemicellulose)
<b>1043</b>	Aromatic CH in-plane deformation for G units [168] complex vibration associated with the C–O, C–C stretching and C–OH bending in polysaccharides [169]
<b>920</b>	G-type C-H out of plane (aromatic ring)[168]
<b>897</b>	Vibration attributed to β (1 → 4) linkage (glucosidic bond between two glucose units) formation of β (1→ 4) glucopyranose macromolecules (cellulose) and hemicellulose.[168, 174] Increases with the increase of amorphous cellulose [160]
<b>834</b>	S type aromatic C-H out-of-plane vibration[168]

After the assignment of the main lignocellulosic biomass DRIFT bands to their Wavenumber in Table 6.4, it was possible to analyse the main characteristic bands of each hydrolysis fraction from the comparative DRIFT spectra in Figure 6.4.

By analysing the DRIFT spectrum on Figure 6.4 it is immediately noted that the major differences in the spectra are in the region  $1800 - 800 \text{ cm}^{-1}$ , the fingerprint region. However, among all the samples the precipitated lignin presents much more intense bands than the other fractions on the bands related to aromatic vibrations i.e. lignin characteristic vibrations ( $1705, 1600, 1515, 1463, 1426, 1268, 1200$  and  $834 \text{ cm}^{-1}$ ). On the other hand, the bands at  $1650$  and  $897 \text{ cm}^{-1}$  disappeared or were highly reduced indicating a successful fractionation of lignin from polysaccharide fractions (cellulose and hemicellulose). In relation to the fibre residue spectrum and by comparison to the raw material spectrum, it is denoted the lignin characteristic bands were highly reduced ( $1720, 1600, 1515, 1426, 1268$  and  $1220 \text{ cm}^{-1}$ ) but the bands at  $2917, 1650 \text{ cm}^{-1}$  as well as  $897 \text{ cm}^{-1}$  increased in intensity showing an increase on cellulose and hemicellulose peaks but more important the peak at  $897 \text{ cm}^{-1}$ , as described by Ibrahim et al. [160] shows that the amorphous type cellulose concentration in the fibres increased which is important as it will aid further hydrolysis of the cellulose to glucose if enzymatic hydrolysis is to be used. However, the main aim of this research is to fractionate LCB with the proposed hydrolysis method by removing lignin and hemicellulose prior to use the same hydrolysis method but at higher temperature (over  $230 \text{ }^\circ\text{C}$ ) to hydrolyse cellulose to glucose without the formation of fermentation inhibitors derived from the composition of hemicellulose and lignin fractions.



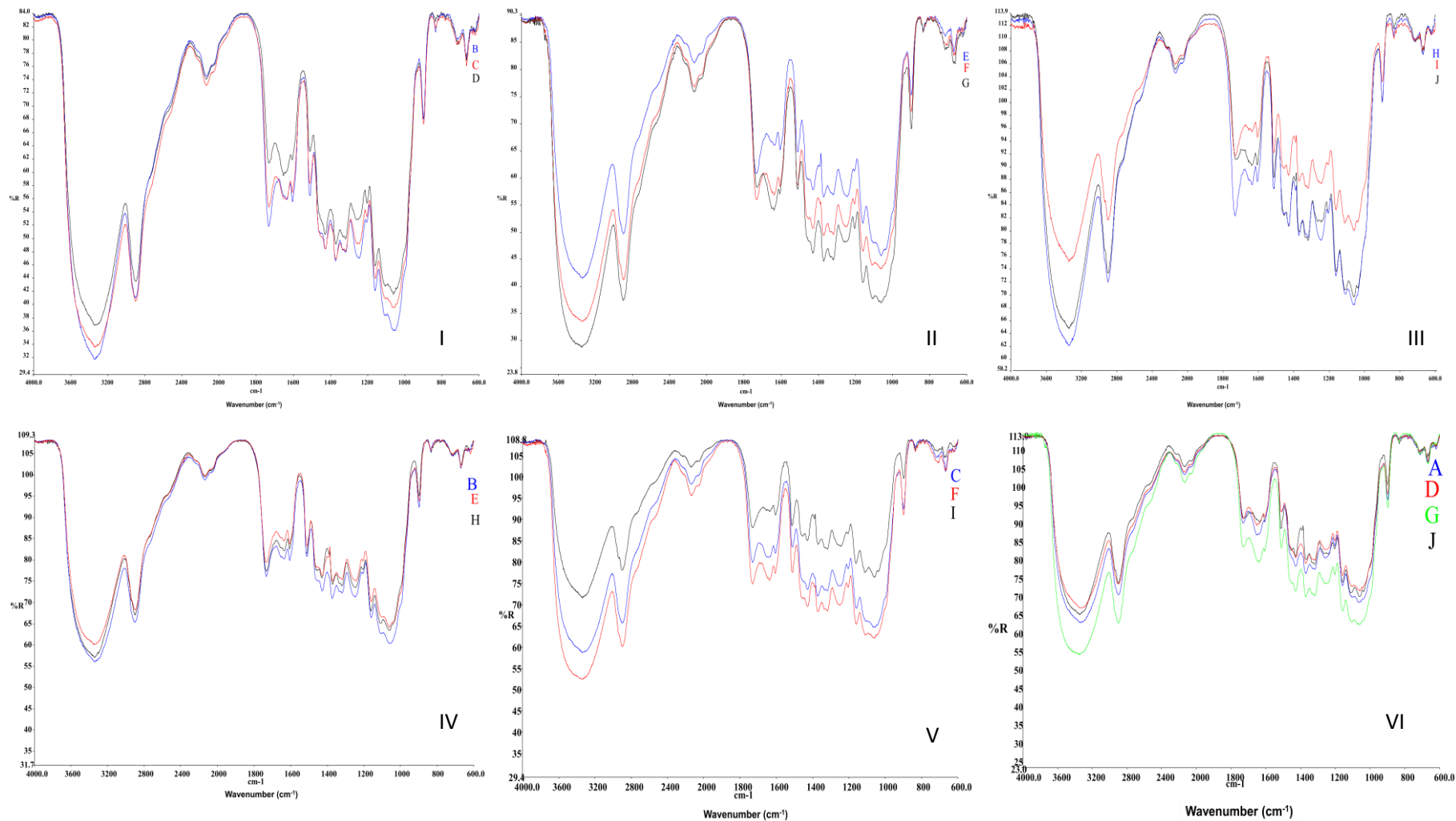
Finally the supernatant DRIFT spectrum showed that the peak at  $1650\text{ cm}^{-1}$  was eliminated indicating the cleavage between hydroxycinnamic acids and lignin and the peaks at  $1463$  and  $1110\text{ cm}^{-1}$  were reduced which can be seen as first the reduction on the aromatic content in the sample by lignin-related compounds. And second, the presence of glucose molecule was reduced due to only the glucose expected in the supernatant is the one derived from the hemicellulose fraction compared to the raw material with much higher concentration in glucose derived from the cellulose polymer.

Overall, each fraction indicated changes on its vibration spectra showing the increase in reflectance intensity on its characteristic fraction band, i.e. cellulose, hemicellulose and lignin.

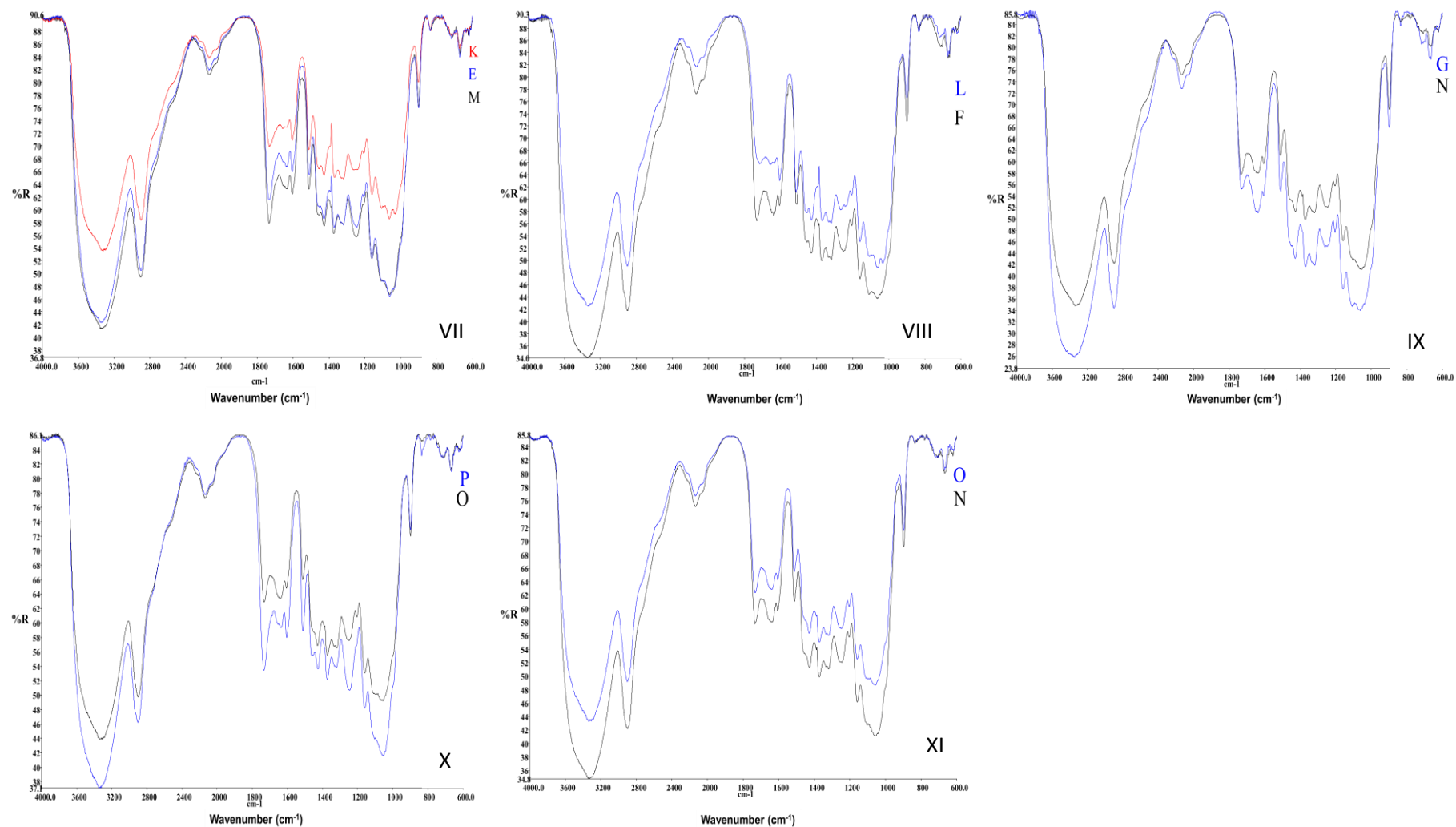
The spectra obtained by DRIFT analysis for all three hydrolysis fractions (fibres residue, precipitated lignin and supernatant) will be presented in the next sections (6.3.4, 6.3.5 and 6.3.6). Based on the hydrolysis conditions and parameters in study presented on Table 6.3 the obtained spectra were grouped and the differences in the spectra profiles were compared qualitatively in terms of intensity of peaks and bands characteristic of the LCB, and the creation or disappearing of any particular peak.

### **6.3.4 Evaluation of hydrolysis method on the fibre residue quality by DRIFT analysis**

Figure 6.5 in the next page represents the possible combinations of the DRIFT spectra obtained for the fibre residue based on the conditions on Table 6.3. It shows eleven different combinations possible to study the influence of the parameters on the fibre residue. The parameters studied were temperature (180, 190, 200 °C), load size per 250 ml of hydrolysis medium (2.5, 5, 10 and 15 g), ethanol concentration in the medium (33, 50 and 67%), reaction time (5, 60 min) and CO<sub>2</sub> initial pressure (10, 55 bar).



**Figure 6.5. Combinations of DRIFT spectra of the fibre residue.** Spectra obtained on the 4000–600  $\text{cm}^{-1}$  to compare the different hydrolysis parameters; temperature: I, II and III, and load size: IV, V and VI.

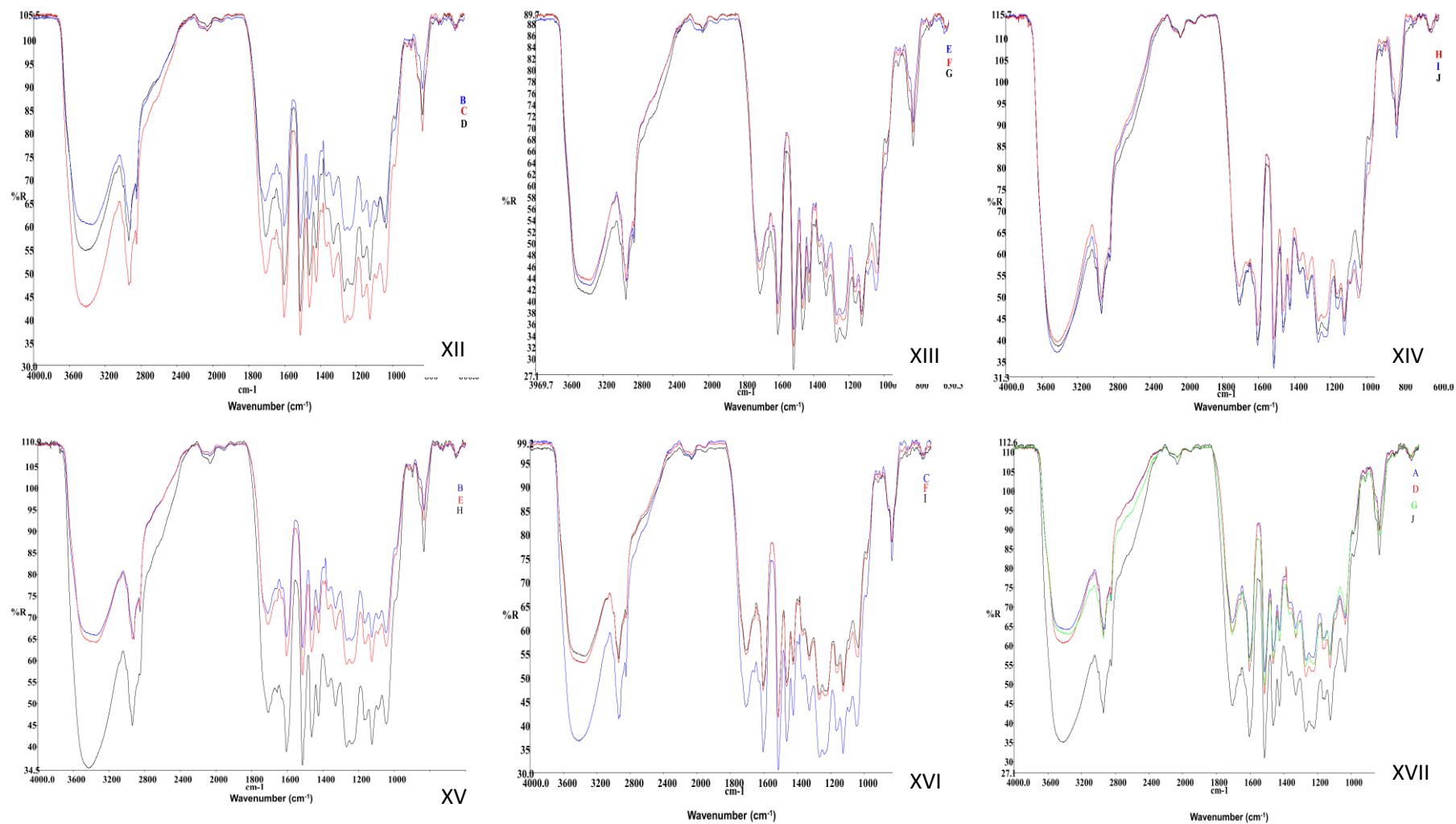


**Figure 6.5. (continuation): Combinations of DRIFT spectra of the fibre residue.** Spectra obtained on the 4000–600 cm<sup>-1</sup> region to compare the different hydrolysis parameters; ethanol concentration: VII, VIII and IX, reaction time: X and CO<sub>2</sub> initial pressure: XI.

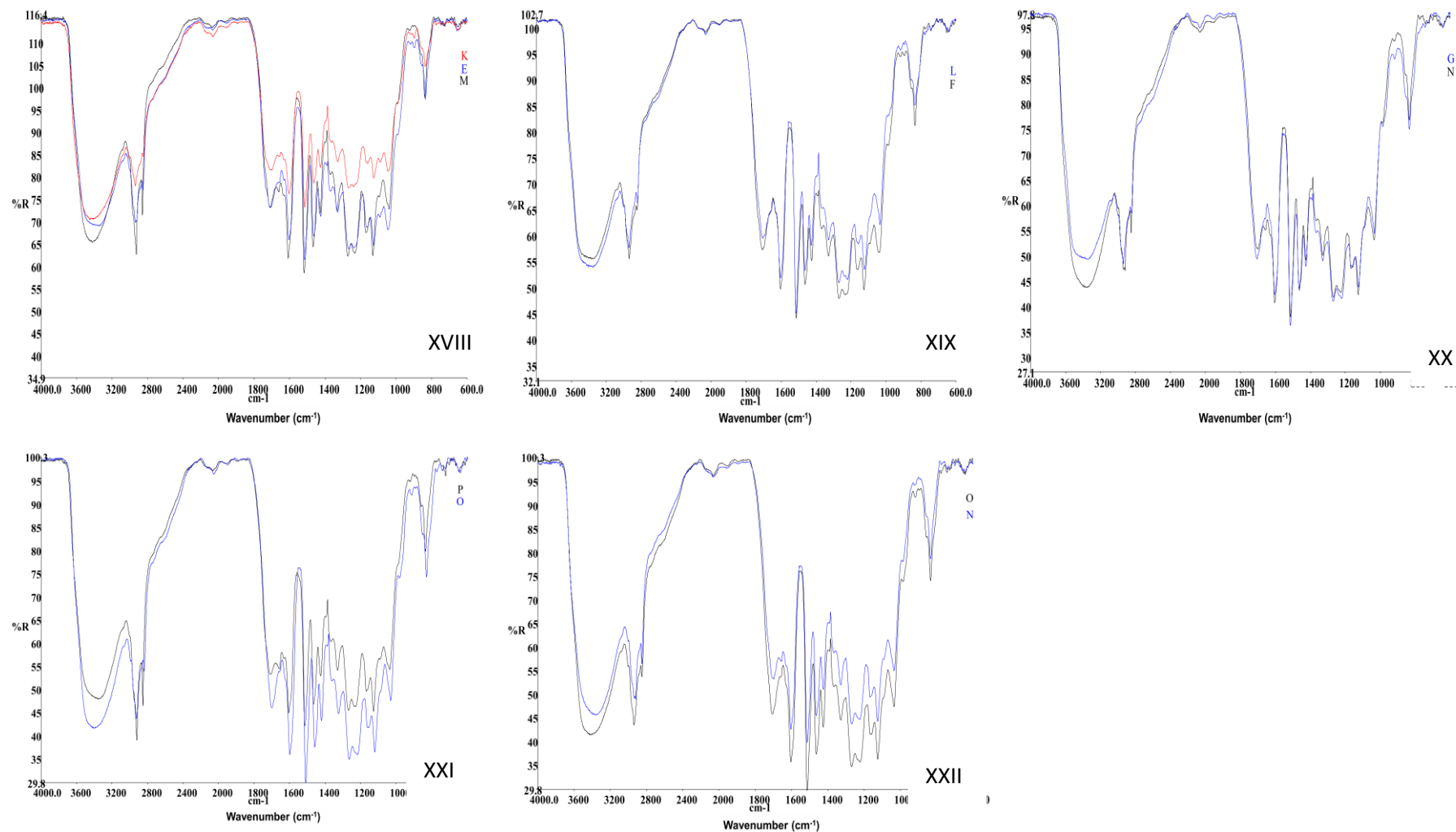
By comparing the DRIFT spectra results of the fibres residue on Figure 6.5 there are several peaks assigned to lignin that are still present and therefore contaminating the fibre residue. However, temperature plays an important role on reducing the lignin content in the fibres. In this way, the peaks at 1705, 1600, 1515 and 1426  $\text{cm}^{-1}$  characteristics of lignin decrease their intensity when the hydrolysis temperature increases. On the other hand the band at 1220  $\text{cm}^{-1}$  is assigned to the C=O groups formed after lignin oxidation, in this case band increases intensity for higher temperatures, thus causing lignin oxidation. Regarding the load i.e. the amount of biomass in a batch reaction per 250 ml of total medium volume, there are mainly two peaks affected by these experimental parameter, they are the bands at 1515 and at 1463  $\text{cm}^{-1}$  and both are characteristic of lignin-type vibrations. The results showed that the peaks intensity increases and therefore the lignin concentration in the fibres increases for higher load sizes but the band at 1515  $\text{cm}^{-1}$  suggests that the load only affects the increase of lignin concentration in the fibres at 200 °C what might be related to the higher amount of lignin removed during hydrolysis but due to solubility the lignin might re-precipitate back over the fibres. In addition, ethanol affects the bands at 1705 and 1650  $\text{cm}^{-1}$  also bands characteristic of lignin, in this case, the spectra shows that for lower ethanol concentration in the hydrolysis medium lower the bands, what was not expected as ethanol aid lignin solubilisation during hydrolysis. In relation to the hydrolysis reaction duration, it is clear that all the bands associated to lignin were reduced for longer reaction times which is the case for the bands at 1705, 1600, 1515, 1426 1463 and 834  $\text{cm}^{-1}$ ) however the band at 1220  $\text{cm}^{-1}$  increases for longer reaction times which is related to lignin oxidation. Finally  $\text{CO}_2$  initial pressure was not found to interfere with the fibres quality.

### **6.3.5 Evaluation of hydrolysis method on the precipitated lignin quality by DRIFT analysis**

Figure 6.6 in the next page represents the possible combinations of the DRIFT spectra obtained for the precipitated lignin based on the conditions on Table 6.3. The study on the effect of the different parameters on the lignin quality will be done in the same way as it was done previously for the fibres residue in section 6.3.4.



**Figure 6.6.** Combinations of DRIFT spectra of the precipitated lignin. Spectra obtained on the 4000–600cm<sup>-1</sup> region to compare the different hydrolysis parameters; temperature: XII, XIII and XIV, and load size: XV, XVI and XVII.



**Figure 6.6. (continuation):** Combinations of DRIFT spectra of the precipitated lignin. Spectra obtained on the 4000–600 cm<sup>-1</sup> region to compare the different hydrolysis parameters; ethanol concentration: XVIII, XIX and XX, reaction time: XXI and CO<sub>2</sub> initial pressure: XII.

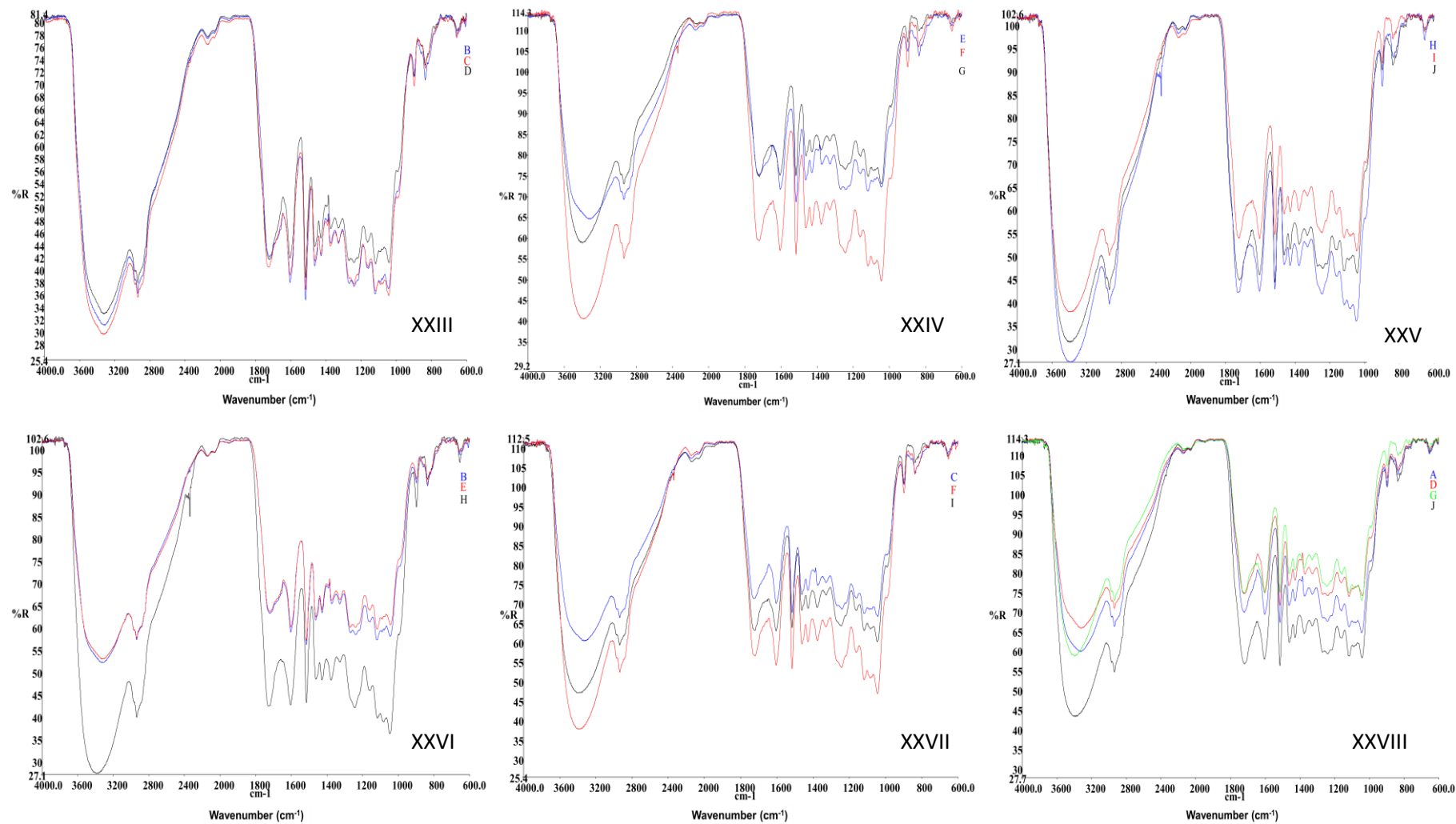


According to the DRIFT spectra analysis from the precipitated lignin, it is possible to immediately recognise that both peaks at 1650 and 1110  $\text{cm}^{-1}$  were removed. This is a clear indication that the hemicellulose linkages-type were not present in the precipitated lignin, leaving a suggestion that the combination of the hydrolysis method and the precipitation method work well together to produce lignin free of carbohydrate contaminants. Regarding the parameters in study, temperature once again plays an important role on delignification and in this case lignin purity, with the lignin characteristic bands at 1600, 1515 and 1426  $\text{cm}^{-1}$  increasing in intensity for higher temperatures and at the same time is clearly seen that for higher temperatures  $\beta$ -linked hemicelluloses are removed with the peak at 897  $\text{cm}^{-1}$  being completely removed at temperatures of 200 °C. On the other hand, higher temperatures represent an oxidation problem with the increase of the peak at 1220  $\text{cm}^{-1}$ . In addition, the reduction in guaiacyl units is detected at higher temperatures but not at 180 °C. (920  $\text{cm}^{-1}$ ). Regarding the effect of load size, only the band at 920  $\text{cm}^{-1}$  is affected, for higher loads, a higher concentration in guaiacyl units is obtained.

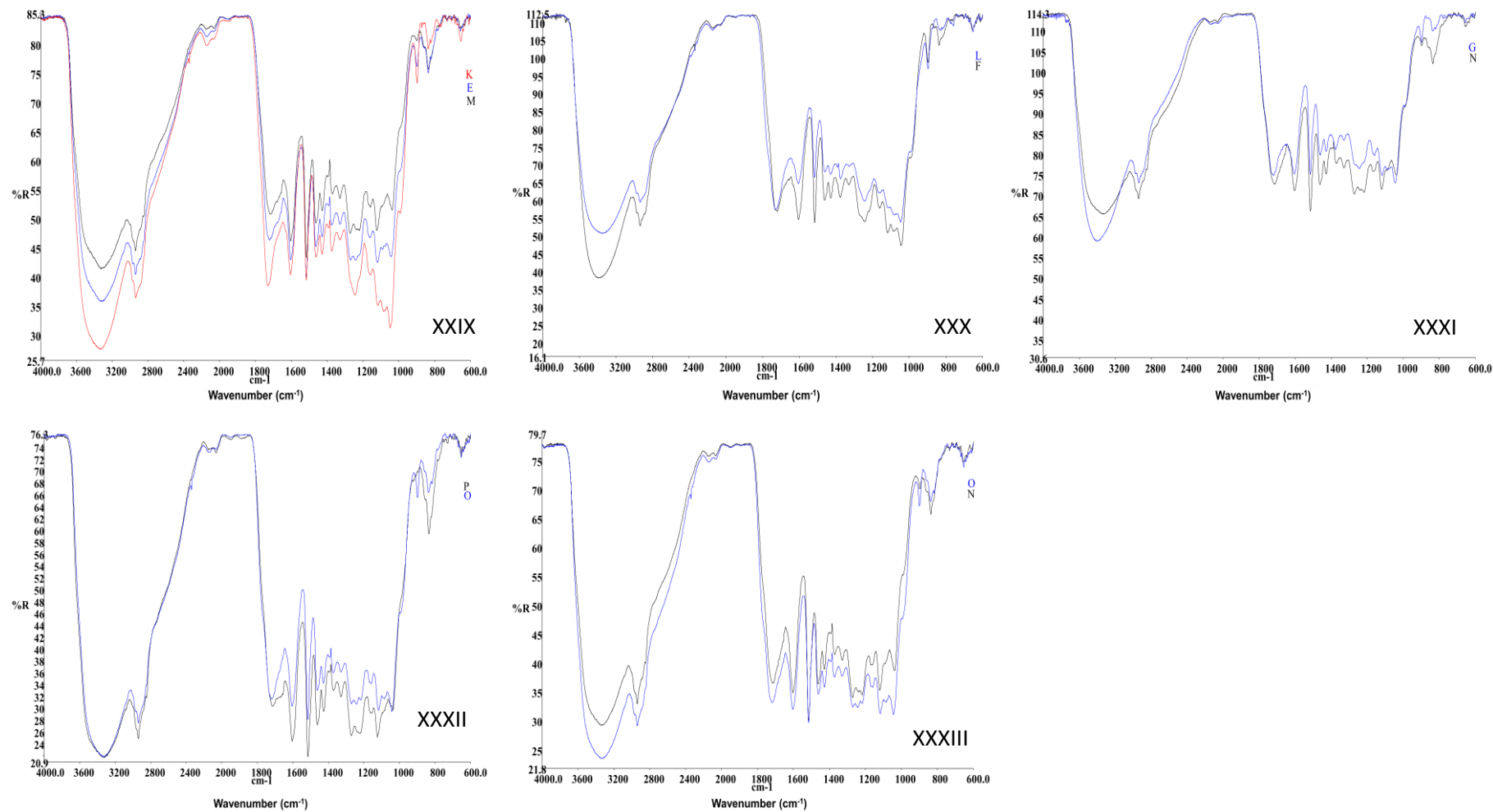
Ethanol concentration does also affect the presence of guaiacyl units, for higher ethanol concentration higher concentrations of this unit in the precipitated lignin (1268 and 1160  $\text{cm}^{-1}$ ) on the opposite side, longer reaction times proved to reduce guaiacyl units in the recovered lignin. Peaks at 1600, 1515, 1426 1463  $\text{cm}^{-1}$  belonging to the lignin vibration types also increase in intensity for longer reaction times. However, the peak at 1705 suggests that longer reaction time and higher pressure causes lignin oxidation.

### **6.3.6 Evaluation of hydrolysis method on the dried supernatant quality by DRIFT analysis**

Finally the DRIFT spectra obtained for the dried supernatant are presented on Figure 6.7.



**Figure 6.7. Combinations of DRIFT spectra of the dried supernatant.** Spectra obtained on the 4000–600 cm<sup>-1</sup> region to compare the different hydrolysis parameters; temperature: XXIII, XXIV and XXV, and load size: XXVI, XXVII and XXVIII.



**Figure 6.7. (continuation):** Combinations of DRIFT spectra of the dried supernatant. Spectra obtained on the 4000–600 cm<sup>-1</sup> to compare the different hydrolysis parameters; ethanol concentration: XXIX, XXX and XXXI, reaction time: XXXII and CO<sub>2</sub> initial pressure: XXXIII.

The third fraction obtained from the Miscanthus hydrolysis was the dried supernatant that is expected to be a hemicellulose-rich fraction. The effect of temperature was reflected in the supernatant quality, contamination with lignin was found to be higher for lower temperature (bands 1600, 1515 and 1426  $\text{cm}^{-1}$ ), which is believed that the lignin extracted from the biomass at lower temperature is harder to precipitate than at higher temperature and so it stays in the supernatant. In addition, the presence of hemicellulose-type vibration at 1110  $\text{cm}^{-1}$  showed that for higher loads, higher the temperature and higher ethanol concentration more intense is the hemicellulose type band. The spectra regarding the ethanol concentration showed contradictory results regarding bands 1705 and 1220  $\text{cm}^{-1}$  both bands give an idea of lignin oxidation but in this case 1705  $\text{cm}^{-1}$  band shows that lower ethanol concentration increases the band intensity and with the opposite, the band at 1220  $\text{cm}^{-1}$  shows that the band is reduced for lower ethanol concentrations.

Finally reaction time showed that hemicellulose concentration increase with reaction time represented by the bands at 1650 and 897  $\text{cm}^{-1}$  but shorter reaction times resulted in an increase of guaiacyl units showed by the bands at 1268 and 1150  $\text{cm}^{-1}$ .

Regarding the initial  $\text{CO}_2$  pressure it was found that for higher pressure lower the hemicellulose related peak at 897  $\text{cm}^{-1}$  which was not expected as the carbon dioxide works as a catalyst during the hemicellulose hydrolysis.

### 6.3.7 Characterisation of the recovered lignin by $^1\text{H}$ and $^{13}\text{C}$ NMR liquid-state experiments

As part of the integrated biorefinery concept, lignin is seen as a source of phenolic compounds that create a wide range of platform chemicals therefore it is considered a high value by-product of the LCB hydrolysis.

However, several parameters during the hydrolysis method were studied and there was the necessity to find how they would influence the polymeric structure of the lignin obtained. This section presents the results of the NMR characterisation of four different samples that allowed comparing major changes on the lignin structure by recovering it after hydrolysis at the parameters extreme conditions. In this sense, the results represent the effect of temperature in a range of 180-200 °C, ethanol concentration at 50 and 67% and CO<sub>2</sub> initial pressure of 10 and 55 bar.

Table 6.5 represents the hydrolysis conditions used to extract lignin from Miscanthus.

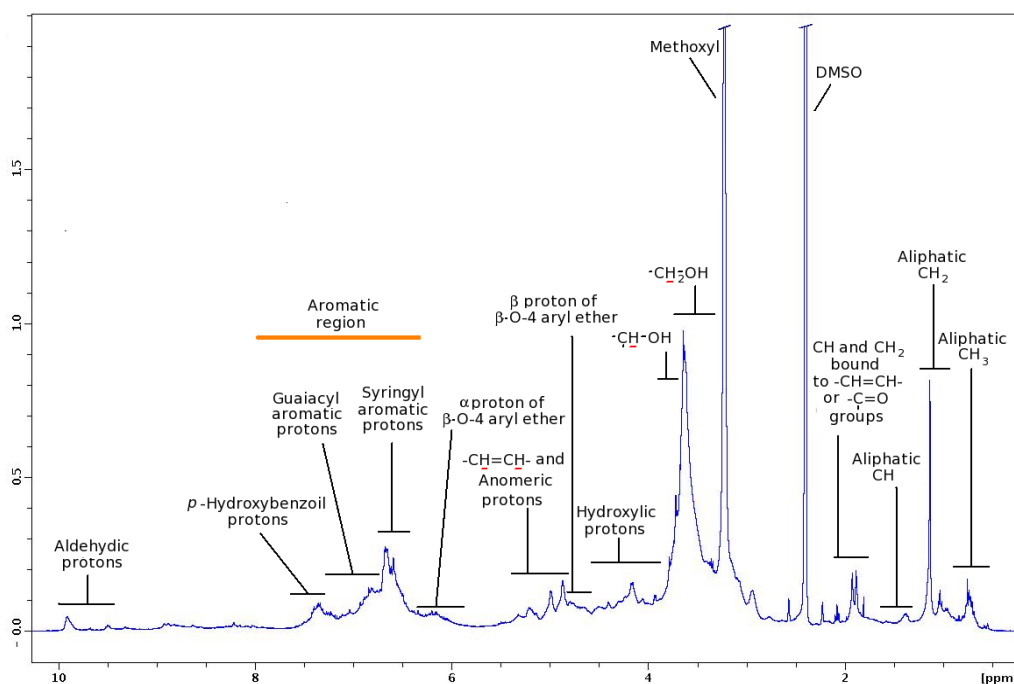
**Table 6.5. Hydrolysis experimental conditions analysed by  $^1\text{H}$  and  $^{13}\text{C}$  NMR liquid-state**

Condition	Temperature (°C)	Ethanol Conc. (%)	Pressure (bar)
A	180	50	55
B	200	50	55
C	200	67	55
D	200	67	10

Note: For all the conditions the load was maintained constant at 10 g of biomass per hydrolysis 250 ml medium

After hydrolysis, lignin was recovered from each hydrolysis condition on Table 6.5 and a sample of 10 mg of precipitated lignin was dissolved in 700  $\mu\text{l}$  of deuterated in DMSO, and the solutions were analysed by  $^1\text{H}$  and  $^{13}\text{C}$  NMR.

Figure 6.8 represents the example of the  $^1\text{H}$  spectrum obtained for the condition A that allowed to illustrate the assignment of main protonic regions before any comparison among the different conditions.

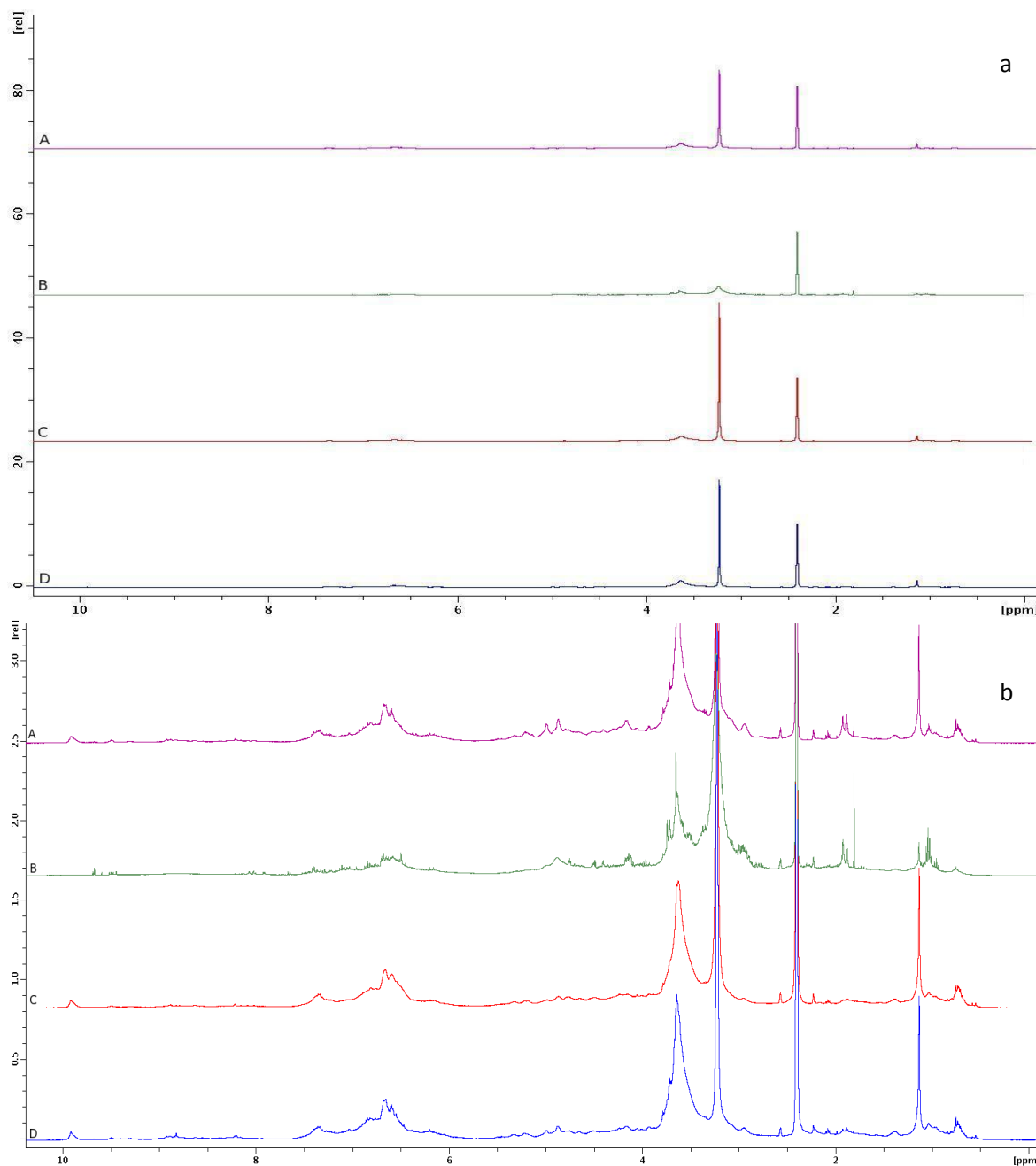


**Figure 6.8.** Illustrates the assignment of main protonic regions.  $^1\text{H}$  spectrum of sample A, the spectral width is included between 0.3 and 10.4 ppm and the assignment of the main protonic regions is indicated. The methoxyl and DMSO signals were graphically cut on top of plot to permit a magnified observation of spectrum.

Analysis of the  $^1\text{H}$  NMR signal intensity in the range of 8.0–6.2 ppm provides an indirect method of monitoring the level of substitution on the aromatic ring of lignin. The integrals of signals between 0.8 and 1.5 ppm were related to the aliphatic moiety in the lignin. Signal at 3.3 ppm was related to protons in water in DMSO. Protons in DMSO gave also an intense signal at 2.5 ppm which is not shown in the spectra. Signal at 3.8 ppm was attributed to

methoxyl protons ( $-\text{OCH}_3$ ) [168] The integrals of signals between 6.2 and 6.8 ppm were attributed to aromatic protons in syringylpropane and guaiacylpropane structures.

After assignment of the main protonic regions associated to lignin, the spectra in Figure 6.9 show the comparison among all the samples.



**Figure 6.9.** Comparison among  $^1\text{H}$  A, B, C and D spectra normalised to DMSO area. a: in a global view. b: zoomed view.



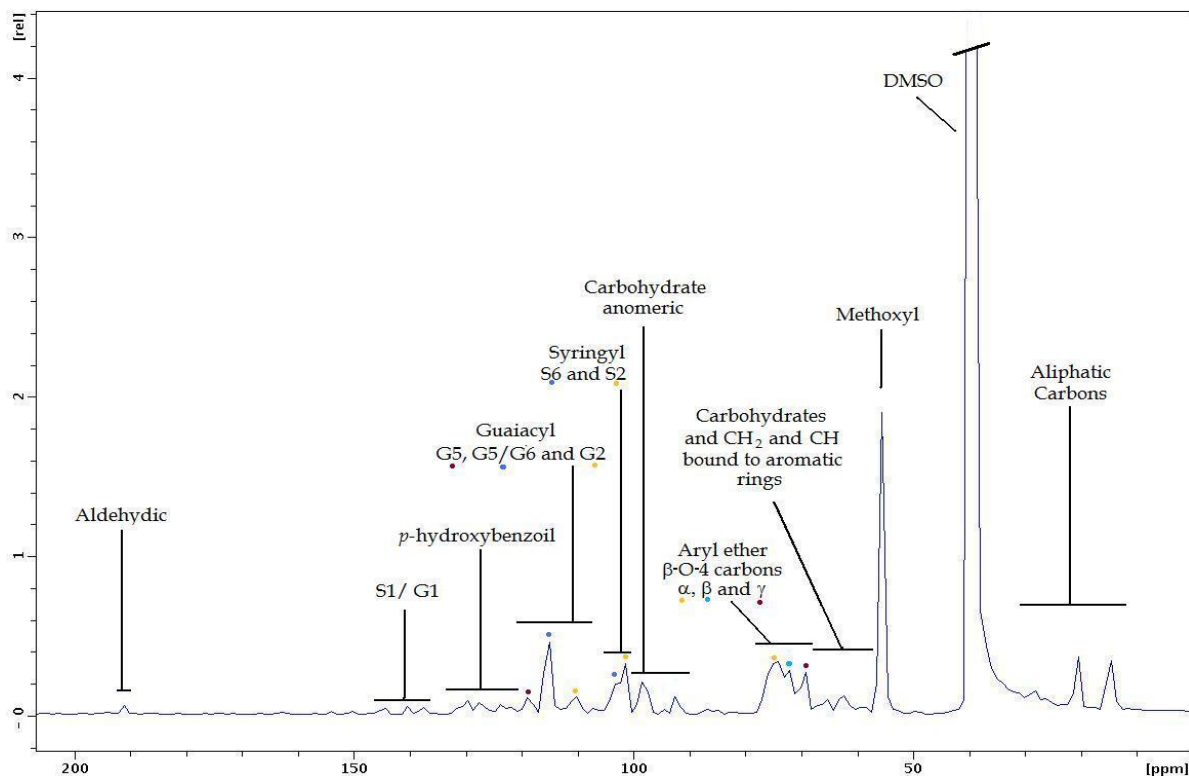
As it appears evident, sample B (200 °C, 50%, 55 min) exhibited more differences than other samples and the largest dissimilarities. At sample B hydrolysis condition, lignin spectrum exhibits narrower and more resolved peaks along the whole protonic spectral width, thus suggesting the presence of either smaller lignin macromolecular structure or less reticulated molecular network, probably due to lignin-block cleavage originated by hydrolysis.

There is the appearance of few peaks in the aliphatic region of methynes and methylenes within 1.75 and 2.2 ppm (slightly down-fielded than the saturated aliphatic signals, being them bound to chemical groups exerting low de-shielding effect, like olefinic protons, carbonyl groups or hydroxylated carbons) suggesting the occurrence of long- and short-chain both unsaturated and alcoholic domains (both verified by COSY, TOCSY and HSQC 2D spectra) probably derived also by the rupture of part of molecular lignin network.

The region included between 2.8 and 5.5 ppm exhibited more signals, thus denoting a larger heterogeneity in region of alcoholic and olefinic protons, above all in the more restricted hydroxylic region (3.8-4.6 ppm), explainable again by a smaller lignin macromolecular structure and by the possible presence of carbohydrate side domains;

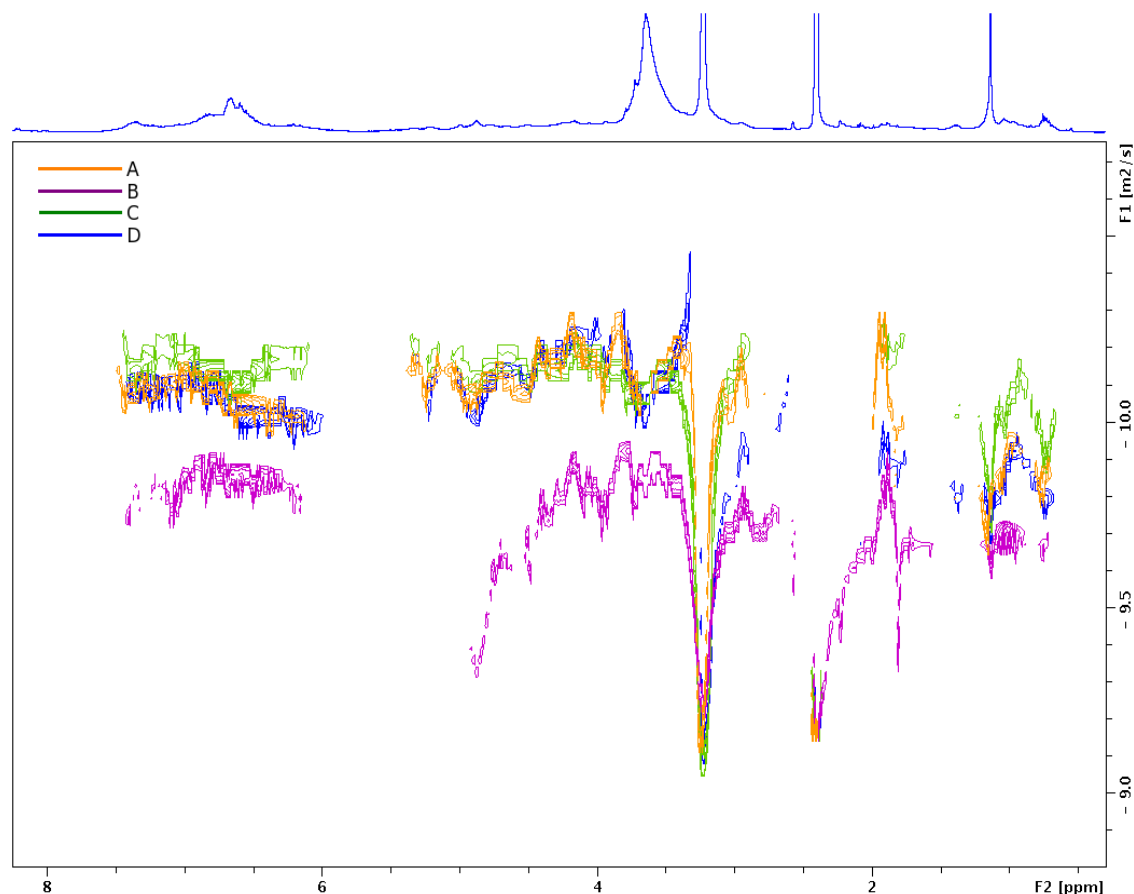
A lower intensity of all aromatic signals, and in particular the syringylic protons resonating at 6.7 ppm, was observed.

Moreover, the broaden aldehydic peak at 9.8 ppm was not detected in sample B, while few narrower aldehydic signals, equally bound to aromatic rings, as verified by 2D long range experiments, resonated in a slightly up-field region, included between 9.7 and 9.3 ppm.



**Figure 6.10. Sample A  $^{13}\text{C}$  1D projection of 2D HSQC experiment.** The assignment of main carbon regions is reported and the coloured points, distinguish different carbons within the same region.

In Figure 6.10 is reported the mono-dimensional projection of 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC experiment of sample A, where, nevertheless, only the protonated carbons are observable and the signal intensities not providing reliable quantitative information. This spectrum was enclosed with the aim to provide, at least, a qualitative low resolved reference of carbon signals, with the exception, obviously, of quaternary carbons.



**Figure 6.11.  $^1\text{H}$  DOSY spectrum within 1.2 and 8.2 ppm.** In the plot is exhibited the superimposition of A, B, C and D diffusion experiments and the correlation existing between the colours and the samples is indicated in the legend positioned in the up-left corner.

It is remarkable the fact that B diffusion spectrum showed different characteristics than A, C and D spectra, since its projections are all shifted to higher F1 values than the projections of other samples, thus indicating that the self-diffusion constants of lignin dissolved in this sample are smaller than A, C and D ones (Figure 6.11) and therefore the apparent size of sample B is smaller than the ones of the other samples.

As previously detected by narrowest lines and a highest resolution in 1D B spectrum, the sample B exhibited also different diffusion characteristics compared to other samples. This is presumably justified by larger T2 transversal time of peaks associated to sample B and, since longer T2 relaxation times are generally correlated to molecule with higher mobility, this

also suggests a lower size of B macromolecule, in accord with previously discussed diffusion results. Probably, the extraction conditions are responsible of partial cleavage of lignin macromolecules, thus producing smaller sub-fractions and therefore justifying the above mentioned narrower protonic lines and the different diffusion results.

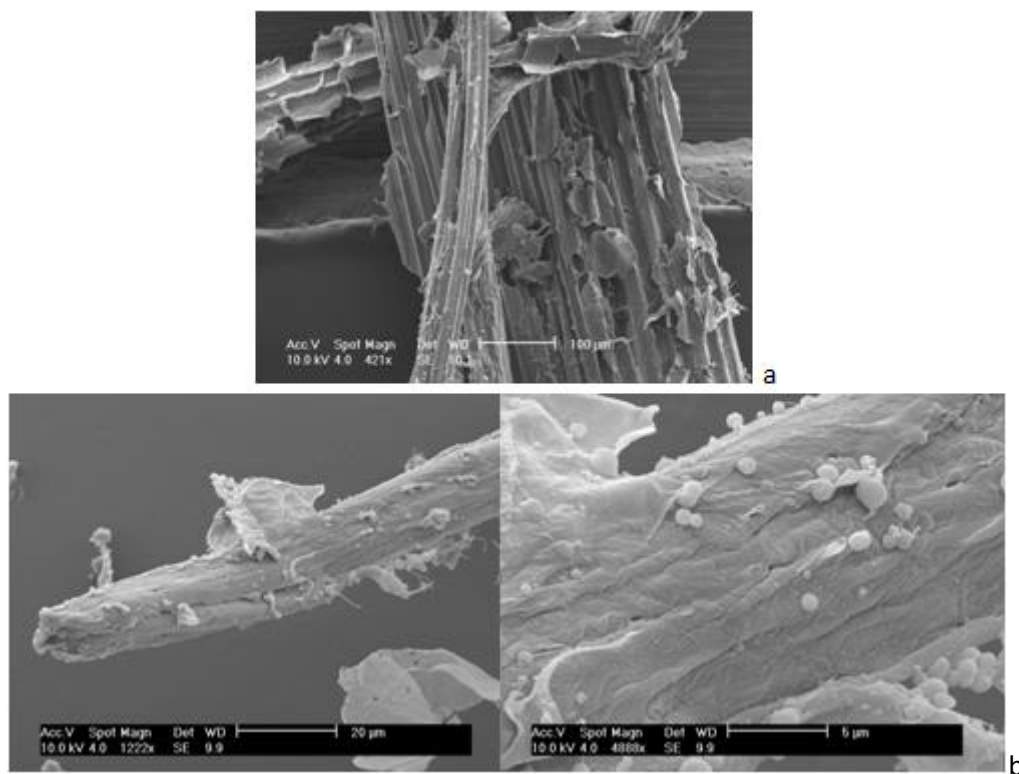
Instead, not consistent differences were appreciated when samples A, C, and D were comparable, however sample C with higher ethanol concentration exhibited a slightly higher apparent size than both samples A and D due to higher concentration of syringylic (S) protons (6.35-6.75 ppm), as observable in Figure 6.11 and in agreement with 1D  $^1\text{H}$  spectrum, thus suggesting that probably a higher amount of S-lignin-monomers is preserved on hydrolysis at higher ethanol concentration.

Small differences were further detected in the molecular rigidity of protons resonating in the whole aliphatic region, probably testifying the possibility to obtain differentiated aliphatic long-and short-chain both unsaturated and alcoholic domains.

### **6.3.8 Qualitative evaluation of the Miscanthus fibres by Scanning Electron Microscopy.**

The main objective for biomass hydrolysis is to fractionate it into its main components (hemicellulose, cellulose and lignin). On this view the present study aimed to indeed separate the three main compounds but also leaving cellulose fibres intact so in a biorefinery concept these cellulose rich fibres could be used in different ways. They would either suffer further hydrolysis to convert cellulose to glucose followed by fermentation for bioethanol production or they could be used as fibres in textiles or composite materials.

This section makes the use of SEM to analyse the external structure of Miscanthus fibres after one hydrolysis condition and compare it to the raw material.



**Figure 6.12. SEM plates** from (a) Miscanthus fibres before hydrolysis, (b) insoluble fraction fibres obtained after hydrolysis at 200 °C, reaction time of 60 min, load of 15 g, and pressure of 50bar and ethanol concentration of 50%. Pictures were obtained by Philips XL30 FEG ESEM microscope, operating at 10 kV acceleration voltage after coating by a gold sputter coater, Polaron SC 7640.

The plates of the fibres represented on Figure 6.12 show the comparison between Miscanthus fibres before and after this hydrolysis treatment. Comparing SEM pictures of Miscanthus fibres to the insoluble fibres after hydrolysis it is clear the structure of the fibres has been preserved and lignin has been removed although there appears to be some lignin globules that remains attached to the fibres as indicated in previous studies where [140, 166]. Each globule structure has a size between 0.6 µm and 1 µm. Consequently, it falls in the range for the molecular lignin structures referred by Micic et al. [166] of 0.02–1000 nm.

Therefore, the use of the modified organosolv under the operating parameters described supports biomass delignification without destroying the cellulose fibres. Fibres structure has been preserved.

## **6.4 Conclusions**

The use of several analytical methods allowed studying the behaviour of the *Miscanthus* main components (hemicellulose, cellulose and lignin) under different organosolv hydrolysis conditions. Results on the quantification of reducing sugars in the supernatant showed a high dependence of the hemicellulose hydrolysis on the temperature with a sharp increasing in the reducing sugars concentration when the temperature rises from 140 to 180 °C, stabilising for higher temperatures denoting a complete hydrolysis of hemicellulose at these the lower range of temperatures. However, lower concentration in ethanol produced higher reducing sugar content what is thought to be related to the lower activity of the H<sup>+</sup> ion in ethanol compared to water causing a reduction in the pH of the medium.

DRIFT spectroscopy of the different lignocellulosic fractions showed an increase of intensity of the bands assigned to each component (hemicellulose, cellulose and lignin) thus confirming the fractionation of the LCB and verify the reduction of cellulose crystallinity. Moreover, temperature is seen the parameter that affects the most the quality of each fraction, the lignin in the fibres is clearly reduced for higher temperatures, and  $\beta$ -linked hemicelluloses are removed from the precipitated lignin the same way, the higher temperatures seems to produce supernatant with less contamination in lignin. However, higher temperatures also compromise the lignin quality by causing its oxidation. Regarding the load, results suggest that lignin solubility in the medium might be an issue for higher load

sizes especially at higher temperatures. Fibres showed an increase of lignin  $\beta$ -type vibration bands for higher loads at higher temperatures. Regarding the precipitated lignin only the guaiacyl units vibration band increased intensity for higher loads and higher ethanol concentration. Longer reaction times allowed a higher extraction of lignin from the fibres and a higher quality precipitated lignin however lignin oxidation occurs for longer reaction times. Finally the addition of carbon dioxide proved to be the least effective on the quality of the fractions and further tests should be done to confirm it. Results showed that higher pressure would reduce the presence of hemicellulose related peak at  $897\text{ cm}^{-1}$  in the supernatant and increase oxidation of precipitated lignin.

Results of  $^1\text{H}$  and  $^{13}\text{C}$  NMR of precipitated lignin showed, that the hydrolysis condition at  $200\text{ }^\circ\text{C}$ , 50% in ethanol and 55 min reaction time demonstrated more differences than the others.  $^1\text{H}$  NMR spectra and  $^1\text{H}$  DOSY, diffusion spectra suggested the presence of either smaller lignin macromolecular structure or less reticulated molecular network, probably due to lignin-block cleavage originated by hydrolysis. On the other side, higher concentrations in ethanol produced lignin with a slightly higher apparent size.

Finally, SEM analysis of the fibres showed that lignin was removed but lignin globules precipitated back over the fibres with a size between  $0.6\text{ }\mu\text{m}$  and  $1\text{ }\mu\text{m}$  what is in accordance with the literature.

## **7 EVALUATION OF SUBCRITICAL WATER MEDIATED EXTRACTION OF PHENOLIC COMPOUNDS FROM THE HEMICELLULOSE FRACTION OF MISCANTHUS**

### **7.1 Aim**

This section presents the results of a study that evaluates the phenolic content of the hydrolysates for hydrolysis temperatures up to 180°C and without ethanol in the medium. These conditions with maximum temperature and absence of ethanol were chosen to avoid lignin solubilisation and therefore focus in the phenolic compounds that make part of the *Miscanthus* cell wall which can be extracted at lower temperatures. To evaluate the effect of the different parameters studied, temperature, loading size and reaction time after hydrolysis and recover the hydrolysates by filtration, it was analysed by two different methods. First, by total phenolic content, a non-specific method that determines the total amount of phenolics by using the Folin-Ciocalteu method and the other method, HPLC (High Performance Liquid Chromatography) method a more specific method that identifies compounds by comparing them to existing standards. As mentioned in Chapter 2 in the literature review, both *p*-coumaric and ferulic acids have been found to be the major *p*-hydroxycinnamic acids found in grass cell-walls. Moreover, Xu et al. [24] and Ou et al. [25] reported that *p*-coumaric and ferulic acids are covalently linked by ester bonds to the hemicellulose macromolecule and to lignin by ester or ether bonds in the case of sugarcane bagasse.



In this study the presence of *p*-coumaric acid was identified to be in the hydrolysates and therefore it was quantified for each hydrolysis condition. Both TPC and the amount of removed *p*-coumaric acid results were then used to identify the variables responsible for the removal of phenolic compounds from the LCB but more particularly from the hemicellulose fraction that is removed at lower temperature range and in absence of the organic solvent ethanol used in the modified organosolv method.

## **7.2 Background**

There has already been much research on the extraction of high-value chemicals from *Miscanthus* using a variety of methods. Villaverde, et al. [12] were some of the first to investigate and characterise various phenolics and sterols present in *Miscanthus*. They found, using alkaline hydrolysis that aromatic compounds represent about 829 mg/kg of dry *Miscanthus* core with vanillic acid, *p*-coumaric acid and vanillin being identified as the major components. These aromatic compounds are found in *Miscanthus* bound to cell walls via ester bonds causing cross-linkages between the walls, which also affect the accessibility of polysaccharides such as the cellulose and hemicellulose [175].

Villaverde et al. [12] recognised that it would only be worthwhile extracting these aromatic compounds if it was in the context of a larger biorefinery concept where they are extracted further upstream before the biomass is used as feedstock in the manufacture of biofuel. They also acknowledged the need for a more environmentally benign extraction process.

Another study carried out by Lygin et al. [175] looking at the cell wall composition of various *Miscanthus* grasses found that *p*-coumaric acid and ferulic acid were two of the most

abundant phenolic compounds present. Across the different *Miscanthus* genotypes they found the content of ester-bound phenolics was 23-27  $\mu\text{g}/\text{mg}$  of cell wall.

Therefore the study presented in this section aimed to show the effect of different hydrolysis parameters (temperature, loading size and residence time) on the extraction of phenolic compounds that are present in the biomass cell walls. This was possible, by focus the study at lower hydrolysis temperatures without the use of ethanol avoiding the hydrolysis of lignin and so eliminating the phenolic compounds on lignin biopolymer.

Table 7.1 shows the experimental conditions run in this study in order to obtain the liquid phase that was analysed for TPC by Folin-Ciocalteu method and also by HPLC.

**Table 7.1. Experimental hydrolysis conditions.**

Run	T (°C)	Load (g)	t (min)
1	120	10	30
2	130	10	30
3	140	5	30
4	140	10	0
5	140	10	10
6	140	10	30
7	140	10	60
8	140	15	30
9	150	10	30
10	160	10	30
11	160	10	75
12	170	10	30
13	180	10	60

The experiments conducted for 0 min were still heated up to the target reaction temperature, but immediately quenched, so as to gain an understanding of the effect of the heat-up and cool-down time.

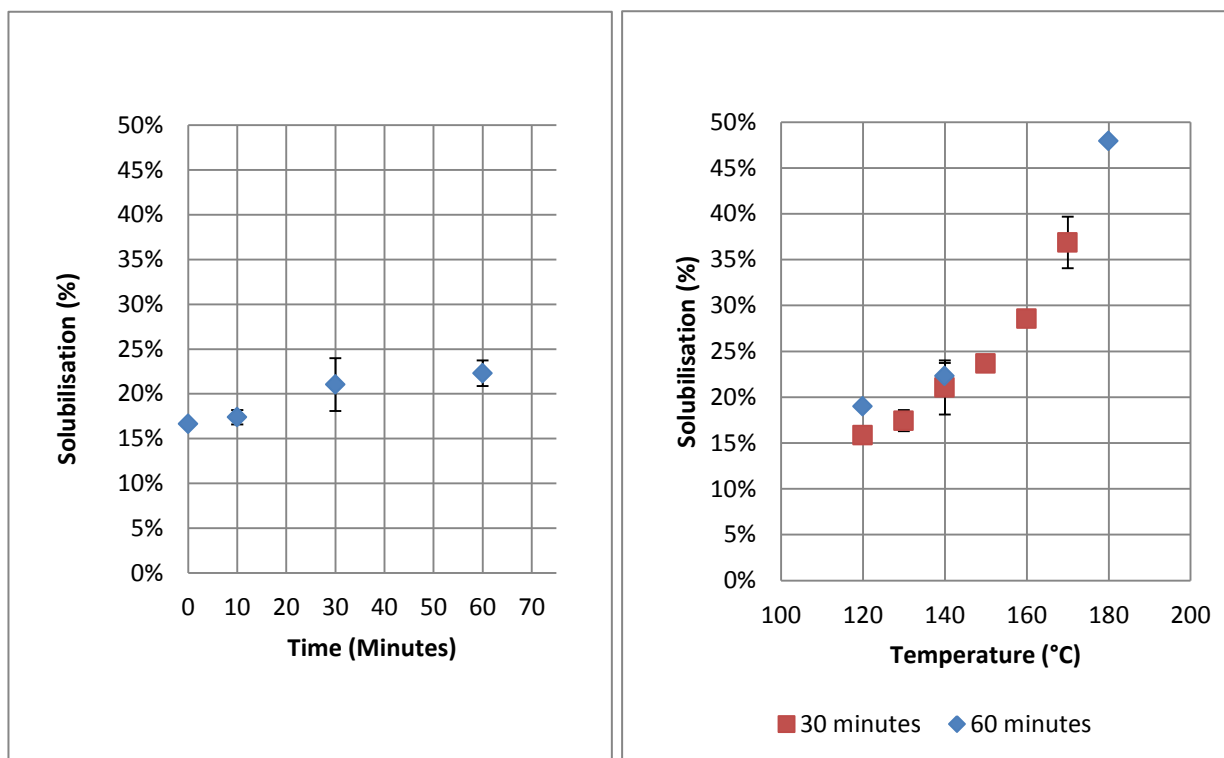
As pressure was found to be a minor factor for previous experiments performed, temperature, loading size and residence time were chosen as the only variables and initial pressure of CO<sub>2</sub> was kept constant at 55bar.

## ***7.3 Results and Discussions***

### **7.3.1 Effect of different hydrolysis conditions on solubilisation of Miscanthus.**

During this study the percentage of biomass solubilised for each experiment was also determined. These results were also important to determine if the solubility behaviour of Miscanthus under lower temperatures and without ethanol would be the same as verified before during the scoping tests and with the modelling results.

Both graphs in Figures 7.1 and 7.2 show the behaviour of Miscanthus solubility under the hydrolysis conditions presented in Table 7.1.



**Figure 7.1. Biomass solubilisation varies with time (m= 10 g at 140 °C)** **Figure 7.2. Biomass solubilisation varies with temperature.**

For those points with error bars displayed, three repeats have been used to calculate an average. Some of the experiments conducted for 60 min were not repeated. The data shows a high degree of repeatability, with the biggest standard deviation being  $\pm 3\%$ .

Figure 7.1 shows that during heat up the majority of the solubilisation has taken place, and there is little change in the amount of biomass solubilised after 30 min ( $\sim 22\%$ ). Further proof of the small effect residence time has on solubilisation is shown in Figure 7.2: there is little difference in the solubilisation trend with temperature using a 30 min residence time or a 60 min residence time. What can also be observed in Figure 7.2 is the strong temperature dependence of solubilisation: increasing reaction temperature from 120 °C to 150°C (with a 30 min residence time) sees a  $\sim 9\%$  increase in the percentage of *Miscanthus* solubilised to

24%; increasing temperature from 150 to 180 °C causes a further 24% increase in the percentage solubilised, to 48%. This represents almost 3 times as much of an increase as that caused by the first 30 °C ramp.

It is likely that only ~1% of each of the figures can be attributed to extracts of sterols, aromatics and fatty acids etc., as Villaverde et al. found [12]. As mentioned earlier, about 25-30% of the Miscanthus can be called hemicellulose [176], which dissolves readily in sub-critical water at these temperatures [150]. 10-15% is not lignocellulosic biomass, and it could be assumed that it is this material that is also dissolving at these temperatures, as lignin is unlikely to dissolve much in Sub-CW below 180 °C and cellulose will not dissolve until over 220 °C [150].

### **7.3.2 Total Phenolic Content (TPC)**

Liquid phase samples obtained after filtration of the hydrolysis medium were analysed by Folin-Ciocalteu method. The results are presented in Figures 7.3 and 7.4 and are expressed in terms of mg of gallic acid equivalents per kg of Miscanthus.

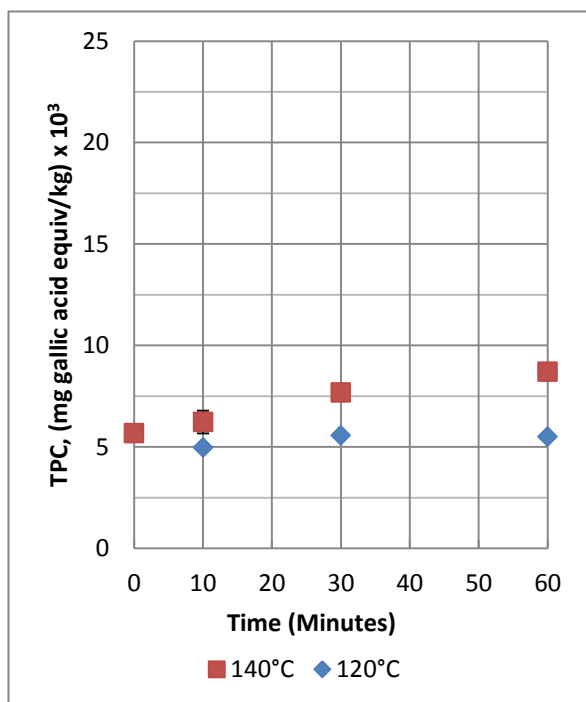


Figure 7.3. TPC vs Time

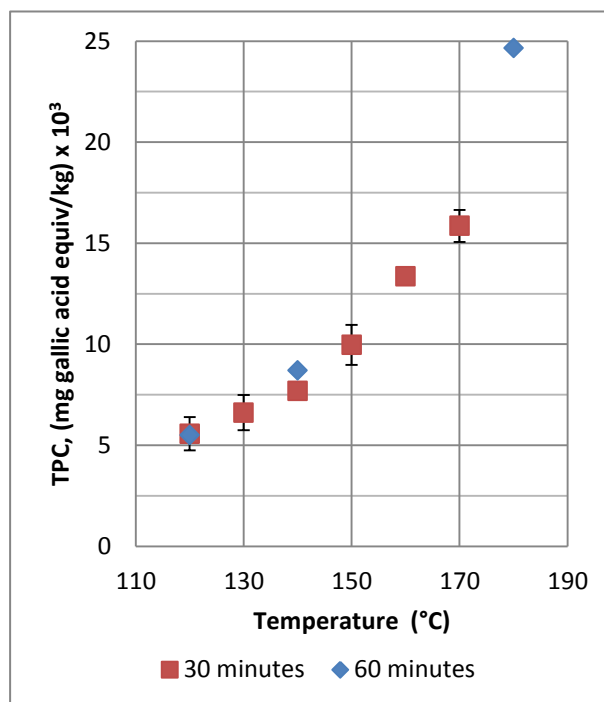


Figure 7.4. TPC vs Temperature

Figures 7.3 and 7.4 show how total phenolic content (assessed via the Folin-Ciocalteu method) varies with residence time and temperature. The error bars on the red points demonstrate the repeatability of both the experiments and the Folin-Ciocalteu method. At mid-range conditions (10 g *Miscanthus* at 150 °C for 30 min) approximately 10,000 mg of phenolics (expressed as gallic acid equivalent mass) were produced per kg of biomass reacted. This is an order of magnitude bigger than the total amount of aromatic compounds extracted from *Miscanthus* by Villaverde et al. [12] and of a similar order to the amount extracted by Lygin [175] using sodium hydroxide, and from rice-bran using sub-critical water [177]. About 9000 mg/kg of phenolics are produced after 60 min of reaction at 140 °C, but over half of that extraction appears to have occurred during the heat up period.

For reactions at 120 °C, there is a clear increase in the amount of phenolic compounds extracted as reaction time increases until a maximum is reached sometime around 30 min.

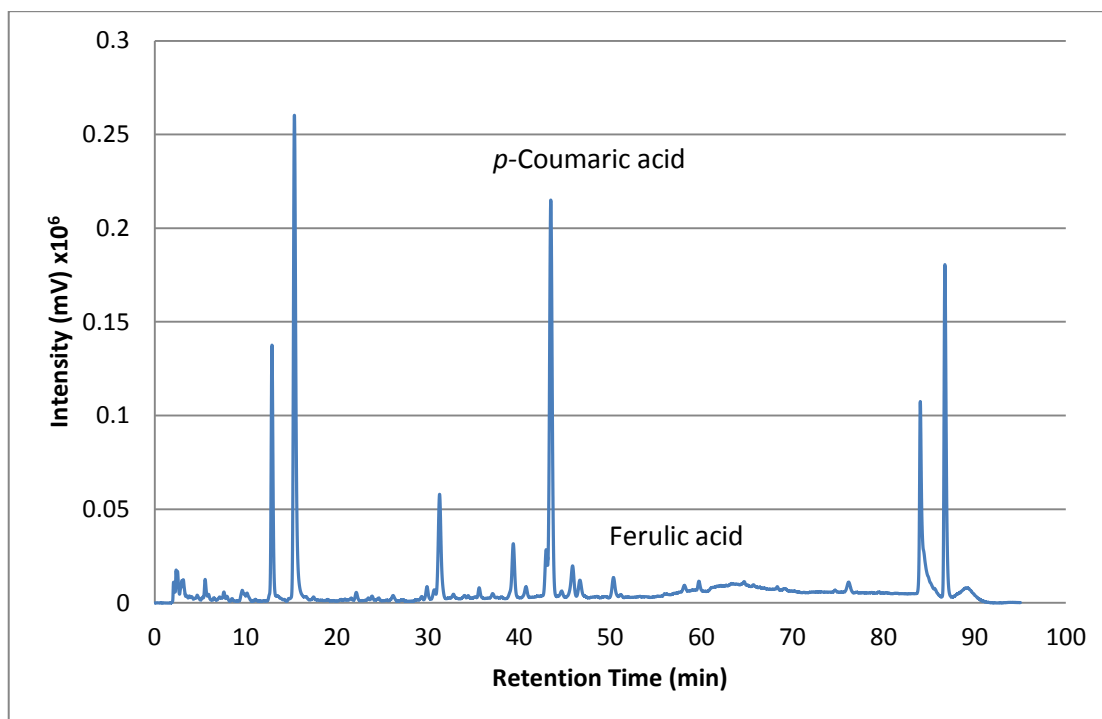
For reactions at 140 °C the TPC is still increasing at 60 min. The changes in temperature have a much bigger effect as demonstrated by the comparison of Figure 7.3 with Figure 7.4. Over 15,000 mg phenolic compounds per kg of biomass are produced after 30 min at 170 °C, double the amount produced at 140 °C. ~25,000 mg/kg are extracted after 60 min at 180 °C.

A further interesting point is that a significantly stronger relationship between TPC and temperature exists after ~150 °C, which is the same point at which the gradient of the data in Figure 7.2 (solubilisation vs temperature) also becomes steeper. This suggests that the plant material that breaks down and solubilises at temperatures greater than 150 °C perhaps releases a significant amount of phenolic compounds in the process. Another possibility is that at these higher temperatures, phenolic compounds are being created from reactions between phenolic and non-phenolic extracts. This is very similar to the results obtained by Pourali et al. [177] in their study of sub-critical water extraction of phenolics from rice bran; they attributed the increased phenolics production to the greater breakdown of cross-linking bonds, and an increase in the solubility of the phenolics in sub-critical water as the temperature gets closer to the critical point. Any of these theories, or a combination, could be true.

## ***7.4 Characterisation of the liquid phase in terms of phenolic compounds by HPLC***

Liquid phase samples that were analysed previously for TPC, were also analysed by HPLC analysis with the aim to identify and quantify phenolic compounds. Figure 7.5 represents a typical chromatogram for one of the hydrolysis conditions at 160 °C, m = 10 g, t = 30 min followed by a table with the main peaks detected and respective retention times. The

identification of some of the peaks was obtained by running standards at the same HPLC conditions.



**Figure 7.5.** Example of a HPLC chromatogram to resolve phenolics from a sample of the liquid phase. Sample obtained after hydrolysis of *Miscanthus* at  $T = 160\text{ }^{\circ}\text{C}$ ,  $m = 10\text{ g}$ ,  $t = 30\text{ min}$ .

**Table 7.2.** Retention time of the main peaks obtained by HPLC analysis of phenolics from a sample of the liquid phase. Sample obtained after hydrolysis of *Miscanthus* at  $T = 160\text{ }^{\circ}\text{C}$ ,  $m = 10\text{ g}$ ,  $t = 30\text{ min}$ .

Retention Time	Identified
13 min	Possibly HMF
15 min	Unidentified
31 min	Unidentified
39.3 min	Unidentified
42.9 min	Unidentified
43.5 min	<i>p</i> -coumaric acid
50.5 min	ferulic acid



Figure 7.5 shows a sample chromatogram from the HPLC analysis. The peak at 13 min matches the retention time for an HMF standard, but as HMF was not expected at such low temperatures it cannot be said with certainty that the compound produced is indeed HMF.

gallic acid, ferulic acid and *p*-coumaric acid standards were also run through the same method, and the latter two corresponded to the peaks at 50.5 and 43.5 min respectively.

The other peaks are yet to be identified. The identification of *p*-coumaric acid was confirmed by running a collected sample of the HPLC output at 43.5 min through a Mass Spectrometer.

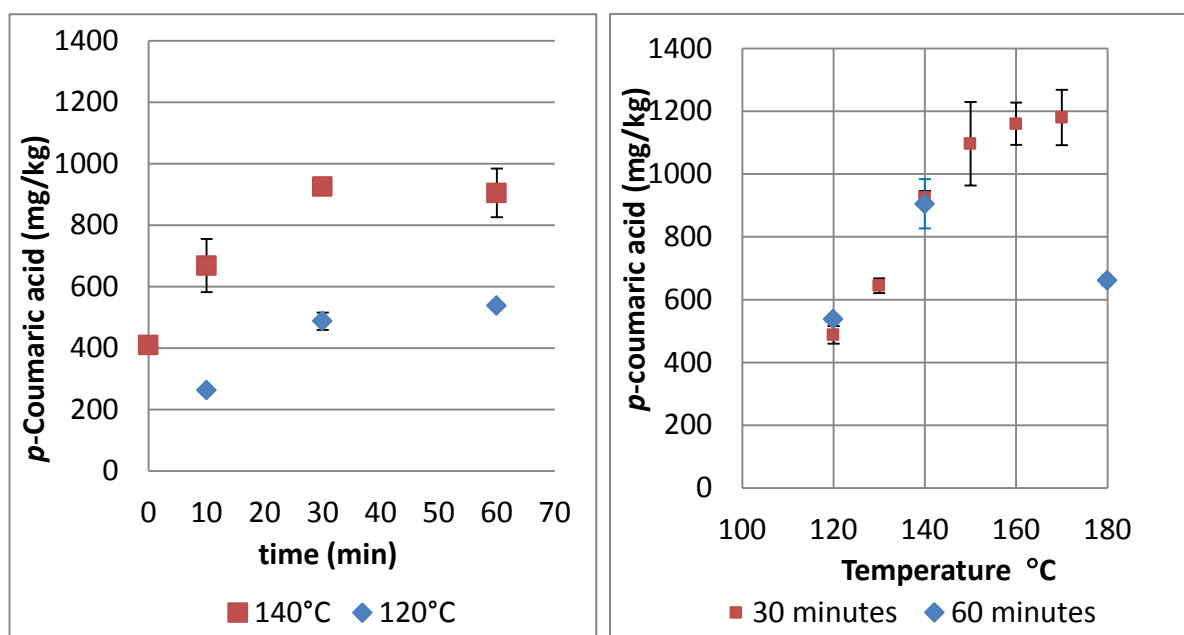
Ferulic acid and *p*-coumaric acid were both expected to be present as they have been extracted from *Miscanthus* before [12, 26, 175].

The next section will analyse the effect of the hydrolysis conditions on the *p*-coumaric acid extraction by quantifying the amount of *p*-coumaric acid in the liquid phase by using the HPLC method.

## ***7.5 Effect of the hydrolysis conditions on the p-coumaric acid extraction***

After confirmation the presence of *p*-coumaric acid by mass spectrometry a *p*-coumaric acid calibration curve was run through the HPLC with the same method used to analyse the liquid phases. The results obtained were expressed in terms of mg of *p*-coumaric acid per kg of *Miscanthus*.

Figures 7.6 and 7.7 demonstrate how the amount of *p*-coumaric acid removed from *Miscanthus* varied with temperature and time.



**Figure 7.6. *p*-coumaric acid amount varies with time**

**Figure 7.7. *p*-coumaric acid amount varies with temperature**

After a 30 min reaction at 140 °C, 930 mg *p*-coumaric acid/kg biomass was extracted. This is over 5 times the amount extracted through dichloromethane by Villaverde et al. [12] but much less than the 18,000 mg/kg extracted by Lygin et al. [175] (with NaOH) and 20,000 mg/kg extracted from maize by Culhaoglu et al. [26]. It is also a lot greater than the 110 mg/kg extracted from rice bran using sub-critical water by Pourali et al. [177].

There are several other similarities to that study. For example, the *p*-coumaric acid peak makes up 40% of the area under the chromatogram at these conditions, yet the total phenolic assay suggests that around 8000 mg of phenolic compounds were produced per kg, so just like Pourali et al. [177] it has to be concluded that many other phenolic compounds are being produced that are not detected by the HPLC method used, or that the Folin-Ciocalteu method for assessing total phenolic content is producing erroneous results.

Another similarity is that *p*-coumaric acid production was found to be optimum at around 150-160 °C for both this study on *Miscanthus* and the study on rice-bran[177], despite the fact that the actual maximum amount extracted was so different in both studies (1200 mg/kg compared to 110 mg/kg). This could mean that *p*-coumaric acid begins to break down at temperatures above 160 °C. Indeed, after 30 min at 180 °C in the *Miscanthus* experiments considerably less *p*-coumaric acid was present.

### 7.5.1 Effect of loading

The effect of load size in the extraction of *p*-coumaric acid was tested and compared to the results of TPC showed previously. The results of *p*-coumaric acid were obtained as in section 7.5 by comparing HPLC peak area results of the liquid phase samples to the results obtained by analysis of a *p*-coumaric acid calibration curve by the same HPLC method.

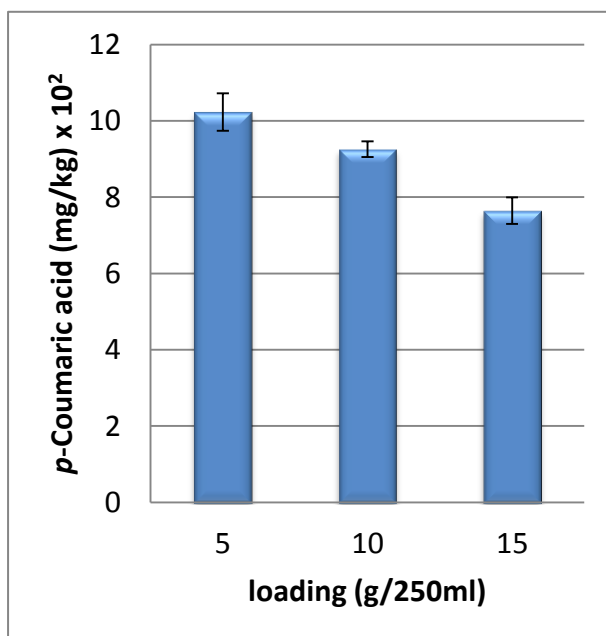


Figure 7.8. The relationship between the amount of *p*-coumaric acid produced and load size (T= 160 °C, t = 30 min).

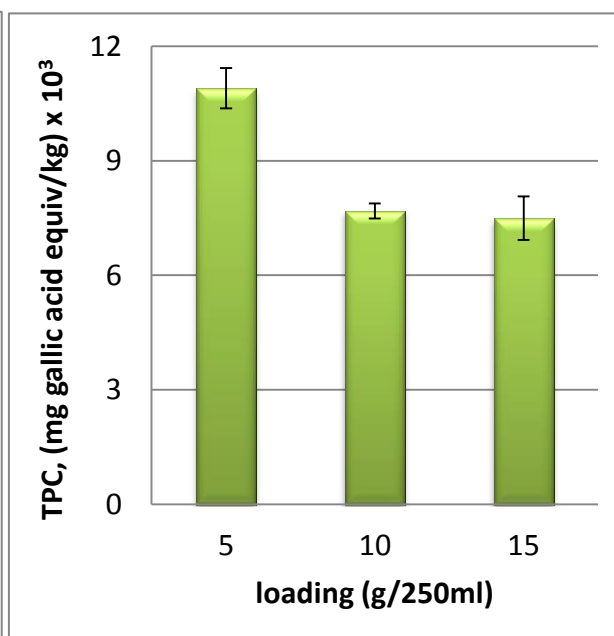


Figure 7.9. The relationship between total phenolic content and loading size (T= 160 °C, t = 30 min).

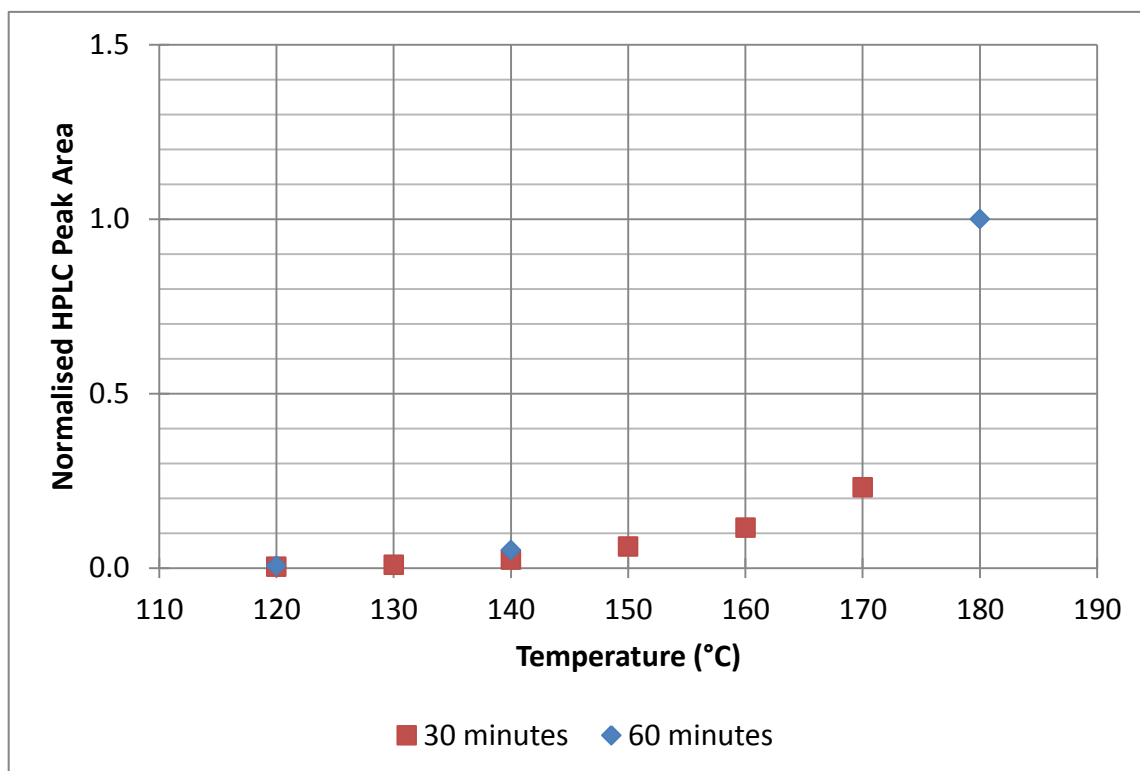
Figure 7.8 and Figure 7.9 show the effect that changing the amount of biomass loaded into the reactor had on amount of *p*-coumaric acid produced and the total phenolic content respectively.

It is clear that higher loadings reduce the efficiency of phenolics extraction, with approximately 30% less phenolic compounds per kg when 10 g of Miscanthus was used instead of 5 g. It is very unlikely that this is caused by the water reaching saturation as the solubility of phenolic compounds in Sub-CW, even at 140 °C, is very high [178-180].

The reasons for this phenomenon are not well understood. It could partly be attributed to lost homogeneity as biomass loading is increased, but that seems insufficient to explain such a reduction in extraction at higher loadings. Further work is required to explore this aspect of the findings, as it is vital for scale up.

### **7.5.2 Unidentified compound that is possibly Hydroxymethylfurfural (HMF)**

During the HPLC analysis of the liquid phase to determine phenolics some peaks were not identified (retention times: 13, 15, 31, 39.3 and 42.9 min) (see Table 7.2). On the other hand, the peak at 13 min matches the retention time for an HMF standard, but as HMF was not expected at such low temperatures it cannot be said with certainty that the compound produced is indeed HMF. In any case the effect of the hydrolysis temperature in this compound is shown in the graph in Figure 7.10, where the area of the peaks was normalised relatively to the highest peak at 180 °C.



**Figure 7.10. Relative amount of unknown compound produced at different temperatures.**

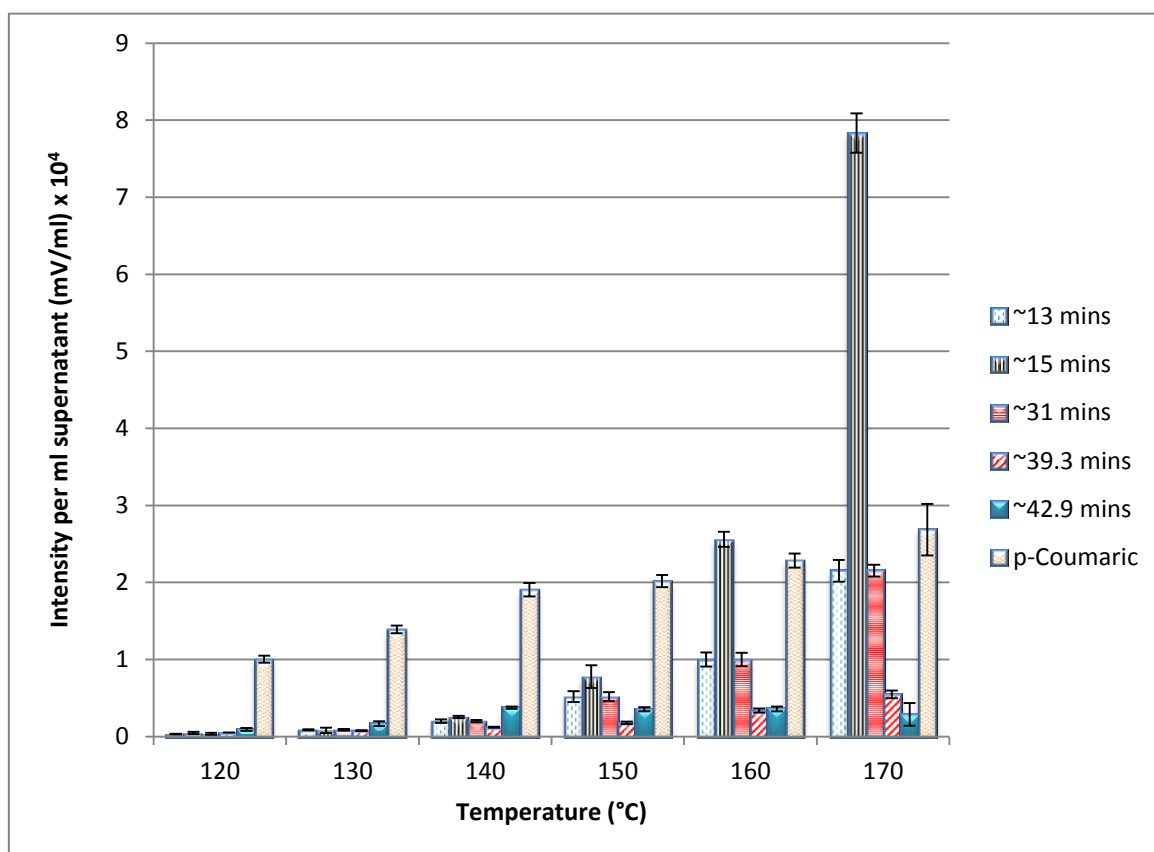
As briefly mentioned before, HMF was not expected as an extractive. It is not listed as an identified compound by any literature. It has also been concluded that the dissolution of hemicelluloses must account for a large proportion of the solubilised mass. Hemicelluloses decompose into hexoses such as mannose, glucose and galactose; and pentoses such as xylose and arabinose [181, 182]. Some starch is also present in *Miscanthus*, which will also yield monosaccharides after hydrolysis. The dehydration of the hexoses can produce HMF [183-185]; dehydration of the pentoses produces furfural [181]. Yoshida et al. [183] suggest that *Miscanthus* hemicelluloses are mainly composed of arabinoxylyan, a co-polymer of arabinose and xylose. If this is the case, HMF production is unlikely to be occurring. This suggests the possibility that it is furfural, not HMF that is showing on the chromatogram. Another possibility is that the larger, adjacent peak (~15 min) is furfural.

It has been suggested that pH strongly influences degradation pathways of these sugars [186]. Pourali et al. [177] hydrolysed rice bran biomass using sub-critical water at similar temperatures, breaking down hemicelluloses and producing monosaccharides, but neither HMF nor furfural were detected. If HMF and/or furfural are being produced, this could be due to the acidic conditions used in these experiments. Moderate yields of HMF from fructose have been produced in HCl-catalysed sub-critical water at 180 °C [187] and other similar reaction conditions have also produced moderate HMF and furfural yields that add weight to this theory.[184]

The possibility of the production of HMF and furfural is interesting for two reasons. The first is that both compounds, but HMF in particular, could be highly valuable chemical building blocks that fit well within the integrated biorefinery vision [184, 188], so their production is a lucrative prospect. On the other hand, a key purpose of these pre-treatment steps is to prepare the biomass for fermentation, and furfural and HMF are potential inhibitors to the cellulose fermentation process [189], although other research suggests that the inhibition effect may not be as strong as previously believed.[189]

### 7.5.3 Overall hydrolysis effect on the unidentified compounds extraction

Finally, the intensity of each unknown peak obtained by HPLC is presented in the Figure 7.11. to try to perceive the solubility and stability of the different unidentified compounds at different hydrolysis temperatures.



**Figure 7.11. Histogram showing Relative Abundance of Main HPLC Peaks.**

The histogram (Figure 7.11) illustrates how different conditions produce different mixtures of the main unidentified products. Some compounds, such as compound “42.9”, see their optimum production at moderate temperatures. For many others, higher temperatures encourage production. The dominance of the ~13 min and ~15 min peaks at the higher temperatures is also very evident from this graph. Nevertheless, without further

identification of these compounds the main conclusion is this: the optimum conditions for sub-critical water:CO<sub>2</sub> extraction of high-value compounds heavily depends on which compounds are desired, and how much simultaneous hemicellulose degradation should be encouraged.

## 7.6 Conclusions

This study has demonstrated that high-value phenolic compounds can be extracted from *Miscanthus* using Sub-CW and CO<sub>2</sub>. This is valuable information as such a process could be used as a pretreatment stage for biomass (*Miscanthus*) refining that would make the ultimate production of energy/biofuels/high-volume chemicals more economically viable.

Based on the results from the total phenolic assays, it can be concluded that at least 10,000 mg of phenolic compounds can be extracted from *Miscanthus* (per kg of biomass) using Sub-CW:CO<sub>2</sub> at temperatures between 120 and 180 °C. This is comparable to the amounts of phenolic compounds found in previous studies. Of this 10,000 mg, about 10% (~1000 mg per kg biomass) can be identified as *p*-coumaric acid, a valuable pharmacological chemical. The other phenolic compounds are yet to be identified. The optimum conditions for *p*-coumaric acid extraction, according to these experiments, are ~150°C for ~30 min, however a higher total amount of phenolic content is created at higher temperatures and longer residence times.

There is evidence to suggest that some of the unidentified compounds that are produced at the higher temperatures within this range may be related to furfural or hydroxyl-methyl-furfural (HMF). These compounds could be very valuable if separated from the mixture, but can also inhibit the later fermentation steps needed to produce bioethanol.



Further work is required in this investigation. Firstly, the unidentified compounds can be identified through more thorough analysis. This would allow better conclusions to be drawn about a) any chemical conversion of the extracted compounds, and b) make good progress towards understanding what the optimum conditions for this pre-treatment step might be.

## **8 CONCLUSIONS AND FUTURE WORK**

### **8.1 Main concluding remarks**

The use of a “greener” organosolv method using Sub-CW and ethanol modified with carbon dioxide to replace the use of inorganic acids such as sulphuric acid was tested for fractionation of lignocellulosic biomass but in particular *Miscanthus x giganteus*. Temperature, carbon dioxide initial pressure, reaction time, ethanol concentration and load size were the parameters tested experimentally to hydrolyse and separate lignin, hemicellulose and cellulose fractions from the biomass and the main conclusions are as follow:

#### **8.1.1 Optimisation study of modified organosolv method for solubilisation and delignification**

Preliminary hydrolysis tests allowed scoping the effect of different processing parameters on biomass solubilisation and delignification. The parameters tested were temperatures at 180, 190 and 200 °C, load sizes of 2.5, 5, 10 and 15 g of biomass mixed in 250 ml of 50% ethanol in water and carbon dioxide was also added at initial pressure of 55 bar. Results obtained showed a solubilisation range from 12 to 52% and delignification from 12 to 75%, with the best hydrolysis condition being at temperatures of 200 °C with a load of 2.5 g of *Miscanthus* in 250 ml of a 50% ethanol in water medium and a reaction time of 60 min, on this condition CO<sub>2</sub> was added at initial pressure of 55 bar.

Optimisation models for solubilisation and delignification were obtained using a central composite design based on a response surface methodology. Two statistical model

equations describing the effect of each hydrolysis parameter (factors) on the solubilisation and delignification (responses) were determined and validated by ANOVA statistical procedure. ANOVA also determined that ethanol concentration and CO<sub>2</sub> initial pressure were not significant for solubilisation and reaction time and CO<sub>2</sub> initial pressure were not significant for delignification. According to the results, carbon dioxide did not show a change on its catalytic power by changing its initial pressure despite the results obtained for both responses were in accordance to other work in the literature. This means that it is not possible to be sure if CO<sub>2</sub> is playing or not a role as catalyst in the hydrolysis reaction of Miscanthus at the working conditions use. Further work to establish the effect of CO<sub>2</sub> as a modifier should be done by connecting a pump to the reactor to get experimental data on the effect of CO<sub>2</sub> at higher pressures and therefore increasing the concentration in carbonic acid in the medium causing a drop in the pH value enhancing the catalyst effect.

### **8.1.2 Characterisation of the liquid and solid fractions obtained by the modified organosolv hydrolysis method**

The behaviour of the Miscanthus main components (hemicellulose, cellulose and lignin) under different organosolv hydrolysis conditions was assessed by means of several analytical techniques that tackled each one of them. Reducing sugars quantification in the supernatant evaluated the hydrolysis conditions to hydrolyse hemicellulose by quantifying its hydrolysis products (mainly xylose and arabinose, both reducing sugars). The results showed a highly dependence on the temperature, with the majority of the hemicellulose being hydrolysed between 140 and 180°C. At this temperature range DNS assay determined that 1.5 to 2 g of reducing sugars per 10 g of Miscanthus were extracted during hydrolysis. The results also

showed that conditions without ethanol produced higher quantity of reducing sugars, what might be related to the lower activity of the  $H^+$  ion in ethanol compared to water causing a reduction in the pH of the medium thus catalysing the hydrolysis reaction.

Infrared spectroscopy, DRIFT of the three LCB hydrolysis main components confirmed the fractionation by assigning specific bands to each component and by verifying the change on the bands intensity with the different conditions. DRIFT spectra also allowed perceiving that the cellulose in the residual fibres reduced its crystallinity. Moreover, temperature was considered the most significant parameter to fractionate biomass with the highest temperature (200 °C) being the one that produced a better quality fibres, supernatant and lignin in terms of contamination by the other fractions. However, as a negative side, higher temperature tends to oxidise more the lignin which might be an impediment for further lignin processing. Results of  $^1H$  and  $^{13}C$  NMR of precipitated lignin showed, that the hydrolysis condition at 200 °C, 50% in ethanol and 55min reaction time demonstrated more differences than the others.  $^1H$  NMR spectra and  $^1H$  DOSY, diffusion spectra suggested the presence of either smaller lignin macromolecular structure or less reticulated molecular network, probably due to lignin-block cleavage originated by hydrolysis. On the other side, higher concentrations in ethanol produced lignin with a slightly higher apparent size.

Finally, SEM analysis of the fibres showed that fibre structure had been preserved but some lignin-like globules precipitated back over the fibres with a size between 0.6  $\mu m$  and 1  $\mu m$  which might indicate lignin reprecipitation during cooling down the reactor and so reducing lignin solubility in the medium.

### **8.1.3 Evaluation of subcritical water mediated extraction of phenolic compounds from the hemicellulose fraction of Miscanthus**

Phenolic compounds were extracted from Miscanthus with Sub-CW catalysed by the addition of CO<sub>2</sub>. The hydrolysis conditions were set to hydrolyse only the hemicellulose fraction and therefore extract the phenolic compounds on this fraction. Folin-Ciocalteu assay results of the liquid phase showed that 10 g of phenolic compounds were extracted per kilogram of Miscanthus at temperatures from 120 to 180 °C. Moreover, about 10% of the total phenolics content in the hydrolysates was identified as *p*-coumaric and quantified by reverse phase HPLC. Nevertheless, further studies should be done to identify the remaining phenolics in the hydrolysates. Optimal extraction conditions of *p*-coumaric acid were found to be 150 °C for 30 min but the extraction of other phenolic compounds unidentified by HPLC also accounting for the total phenolics content is higher for higher temperatures and residence time.

In conclusion, the use of the modified organosolv method significantly disrupted the lignocellulose polymer and successfully fractionated the biomass not only into its main components, hemicellulose, cellulose and lignin but also high-value phenolic compounds could be extracted. However the role as a catalyst of carbon dioxide was not well understood and deserves further.

## 8.2 Future Work

During this research project several areas deserving further investigation were identified regarding hydrolysis of lignocellulosic biomass.

- The effect of carbon dioxide as a modifier on the LCB hydrolysis needs to be further investigated at higher pressures by adding a pump to the reactor. With the increasing in the pressure the concentration in carbonic acid in the medium will increase causing a dropping in the pH value and therefore increasing the catalyst effect.
- Other point of interest investigate would be the comparison of the cellulose residue fibres quality obtained in a batch reactor to the quality of the fibres obtained in a continuous flow reactor. This would be an important study as it might solve the issue of lignin reprecipitation during the cooling step in a batch reactor.
- Finally, the identification of the phenolic compounds extracted during hemicellulose hydrolysis is an interesting area to develop. The recovery of high value compounds before complete hydrolysis of the LCB is part of the biorefinery concept and it will certainly enhance the economic viability of the biomass.

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