I am CELISE

Sustainable production of Cellulose-based products and additives to be used in SMEs and rural areas

Deliverable D1.1. Recommendations about characterisation methods in lignocellulose biomass

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Executive Summary:	This document is issued in the framework of the CELISE project under the Marie Sklodowska-Curie Grant Agreement number 101007733 of the Horizon 2020 research and innovation programme of the European Union. It presents an overview of the physico-chemical methods recommended for the selected biomass and the used methods of the partners of the projects. Not only cellulose, but also the rest of fractions of the lignocellulosic biomass are included. In addition, not only the traditional methods, but also novel methods as HPLC, or FTIR.
Keywords	Characterization, lignocellulosic biomass, lignocellulosic fractions, analysis, HPLC, FTIR

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4. LIST OF ABBREVIATIONS

Table 2. List of abbreviations

Acronym	Definition
AES	Auger electron spectroscopy
AFM	Atomic force microscopy
ASTM	American Society for Testing and Materials
BET	Brunauer-Emmett-Teller
BrdU	Bromideoxyuridine/5-bromo-2'-deoxyuridine
BWI	Bond Work Index
CCSEM	Computer-controlled scanning electron microscopy
CD	Cationic demand
CHNOS	Carbon hydrogen nitrogen oxigen sulfur
CNC	Cellulose nanocrystals
CNS	Cellulose nano-spheres
CrI	Crystallinity index
D	Deliverable
DIN	Deutsches Institut for Normung
DDLS	Depolarized dinamic light scattering
DLS	Dinamic light scattering
DMA	Dynamic mechanical analysis
DMF	Dimethylformamide
DP	Degree of polymerization
DMSO	Dimethylsulfoxide
E	European
EA	Elemental analysis
EDS (EDX)	Energy dispersive X-rayspectroscopy
EI	Electronic ionization
EMC	Equilibrium Moisture Content
EPR	Electron spin resonance
FID	Flame ionization detector
FTIR	Fourier transform infrared
G	Guaiacyl
GH	Gas chromatography

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Acronym	Definition
GP	Gel point
GPC	Gel-permeation chromatography
H	Hydroxyphenyl
HGI	Hardgrove Grindability Index
5-HMF	5-hydroxymethylfurfural
HPAEC-PAD	High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection
HPLC	High Performance Liquid Chromatography
IC	Inverse gas chromatography
ICP-MS	Indusctively coupled plasma mass spectrometry
ICP-AES	Inductively coupled plasma atomic emissionspectroscopy
IRE	Internal Reflection Element
LAP	Laboratory Analytical Procedure
LCB	Lignocellulosic biomass
LC	Liquid chromatography
LCA	Life-cycle assessment
MALLS	Multi-angle laser light scattering
MB	Methylene blue
MCC	Microcrystalline cellulose
MFC	Microfibrillated cellulose
MMD	Molar mass distribution
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide
MWL	Milled wood lignin
NC	Nanocellulose
NFC	Nanofibrillated cellulose
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
NR	Neutral red
NREL	National Renewable Energy Laboratory
OM	Optical microscopy
PDMAAC	Poly(dimethyldiallyl-ammonium)chloride

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Acronym	Definition
PPUs	Phenylpropanoid units
PTFE	Polytetrafluoroethylene
RI	Refractive index
RID	Refractive index detection
S	Syringyl
SAXS	Small angle X-ray scattering
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
SIMS	Secondary ion massspectrometry
SSA	Specific surface area
SSNMR	Solid state nuclear magnetic resonance spectroscopy
TAPPI	Technical Association of Pulp and Paper Industry
TCC	Total carbohydrate contet
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
TGA	Termal Gravimetric analysis
TMDP	2–chloro–4,4,5,5– tetramethyl–1,3,2–dioxaphospholane
UV-vis	Ultraviolet-visible
VSOS	Vitreous Silica Outlet Splitter
WRV	Water retention value
XRD	X-ray diffraction
XRF	X-ray fluorescence spectroscopy
XRP	X-ray photoelectron
XTT	2,3-bis (2-methoxy-4-nitro-5-sulfenyl)-(2H)-tetrazolium-5-carboxanilide
ZP	Zeta Potential

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5. INTRODUCTION

5.1. General purpose of the document

The global trend for exploiting ~~of~~ natural renewable sources of our planet is expanding at an immeasurable rate in order to solve and prevent problems caused by environmental pollution and climate change. Replacement of fossil and synthetic substances with those made of natural sources is one of the main issues for today's scientists, manufacturers and politicians.

Lignocellulosic biomass is the most abundant and renewable raw material in the world. It is the main constituent of wood as well as wild and agricultural plants. The term "lignocellulosic" itself reveals the main constituents of the resource - cellulose and lignin, however they are complemented also with other important ingredients such as hemicelluloses, extractives, proteins, and other organic and inorganic substances significant for the formation and growth processes of different plants. All these components of biomass form various plant structures and are responsible for the visual appearance and properties of the plants.

Complete understanding of the lignocellulosic biomass matter in general as well as each of its components is the key for its smart and most effective utilization. Biomass as a raw material is being used in countless ways and for wide ranging applications. While solid wood can be used, for instance, for construction and furniture, processed wood and other lignocellulosic biomass can be used for numerous biorefinery concepts in different industries and for different products, such as biofuels, biochemicals and biomaterials. It is important to have full information of the properties of the biomass as a whole, as well as each of the biomass's components in order to find the right application, or to predict and design the properties of the final products.

The manuscript/paper/document offers a review of existing and the most advanced as well as most useful methods for characterisation of lignocellulosic biomass.

5.2. Role and contribution from partners

The D1.1 is part of WP1 activities and LS-IWC the lead company. LS-IWC has had the main responsiblity for this deliverable; however, the deliverable unites all of the activities related to the characterisation methods of biomass and the following partners are involved in these activities: UC (Universidad de Cantabria), UPM (Universidad Politécnica de Madrid), LS-IWC (Latvian State Institute of Wood Chemistry), SGGW (Warsaw University of Life Sciences), WUT (Warsaw University of Technology), IChF (Institute of Physical Chemistry, Polish Academy of Sciences), BANGOR University, AUTH (Aristotle University of Thessaloniki), TWI, INOCURE, CCC (Carbon Compost Company Ltd), ECORESOURCES, UCC (Universidad Cooperativa de Colombia), UNACH (Universidad Nacional de Chimborazo), UNL-FICH (Universidad Nacional del Litoral) and Latitud (Latitud-Fundación Laboratorio Tecnológico del Uruguay).

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6. PHYSICAL PROPERTIES OF LIGNOCELLULOSIC BIOMASS

6.1. Moisture content

Lignocellulosic biomass is plant-based dry matter, which mainly consists of cellulose, hemicelluloses and lignin. As biomass is hygroscopic matter, its behaviour in contact with water, in liquid or vapour form, is important during the processing into materials. Moisture is absorbed into the cell walls by the hydroxyl groups which hold water molecules through hydrogen bonding. Water is an essential molecule to sustain life as it acts as major cell liquid and plays a fundamental role in photosynthesis forming biomass matter [1]. Moisture content is a significant property for calculations of technological processes, e.g. the correct dosage of chemicals per dry unit of biomass.

Methods of biomass moisture content determination are based on the fact that water is removed from a sample by different procedures, and the amout of removal needs to be determined. If speaking about wood, the main methods of determining the moisture content are: (i) Drying in an oven at a temperature of 101 to 105°C, (ii) use of a moisture analyser, (iii) use of a moisture meter that measures electric properties of wood that are affected by moisture content, (iv) chemical methods - particularly Karl Fischer titration, distilling off the water and measuring its volume, (v) hygrometric methods [2]. Most of the methods can also be used for any other biomass.

In science, the most common method of biomass water content determination is the gravimetric method with drying, where drying can be performed by simple oven-drying, microwaves or infrared waves. The method involves weighing a moist sample, drying until there is no further change in weight, and calculating the mass of water lost as a percentage of the mass of the dried biomass. Drying methods also involve shredding or cutting the biomass into the smallest possible pieces in order to have fast and precise results.

Oven-drying is the most widely used general reference method. It has the disadvantage of being relatively slow, and errors can occur if the biomass contains volatile material, other than water, that evaporates during drying. The same drying principle is used in the more advanced moisture analysers, where faster drying is provided by infrared radiation or microwave, which also makes it suitable for industry and online measurement processes.

Moisture meters based on electric properties or optical moisture detection are quick and convenient, and are suitable for use in most commercial situations. They are widely used in the wood and biomass industry. However, they are generally not as accurate as gravimetry methods.

Less commonly used methods: The Karl Fischer titration, using iodine, pyridine and methanol, is probably the most accurate for all situations. However, it is more appropriate to science and research than commerce and is rarely used in practice. The distillation method can be used to measure the moisture content of biomass that contains appreciable amounts of volatile material that would cause errors in the drying methods. It requires relatively complicated apparatus, is slow, and may not be consistently accurate. It is not convenient for normal commercial practice but is used for moisture content determination as part of determining the retention of some organic preservatives in biomass. Hygrometric methods measure the relative humidity of air that has come to equilibrium with the biomass. They are slower and less convenient than moisture meters and are rarely used in practice.

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6.2. The shape and size of biomass feedstock particles

The shape and size of biomass feedstock particles are important properties affecting physicochemical processes during different processing steps, such as surface area for heat and mass transfer amongst others. In general, lignocellulosic biomass is irregular in shape because of its different origins, mechanical pre-treatment and intended use. However, the accurate characterization for biomass particle size and shape is essential for designing the handling, storage, and processing facilities [3]. Sieving analysis and imaging particle analysis are two major particle size characterization methods [4]. In sieving analysis, a set of sieves with different opening sizes and a suitable screen shaker are usually used. Sieves should be arranged vertically in a stack with gradually reduced opening size from top to bottom. Biomass is divided into fractions by particle size, and the amount of the fraction is determined by mass. The shape of biomass particles is determined by digital imaging analysis.

Since lignocellulosic biomass pre-treatment usually includes particle size reduction, grindability is an important physical property. The grindability of a material is a measure of its resistance to grind. The lignocellulosic components of biomass, especially cellulose and lignin, are very fibrous and difficult to grind [3]. Grinding biomass is an energy-intensive process. Energy consumption for grinding depends on the initial and final biomass particle size, moisture content, material properties, mass feed rate and machine variables such as screen size and type of grinding equipment [5]. Currently, there is no standard grindability test for biomass; however Hardgrove Grindability Index (HGI) and Bond Work Index (BWI) tests can be used when appropriate [6].

6.3. Density

Lignocellulosic biomass can be characterized using two densities: the particle density and the bulk density [3].

For a group of biomass particles, the particle density is the mass of all particles divided by the volume which the particles occupy excluding the pore space volume. The particle density of biomass is usually used in the computational fluid dynamics simulation of biomass thermochemical conversion reactors [7].

The bulk density is the ratio of the mass of biomass particles to the total volume of biomass particles including the pore space volume between and within the biomass particles. It is a key physical property in designing the logistic system for biomass handling and transport [3]. Bulk densities of different biomass feedstocks show extreme variation, e.g., from lows of 15 –200 kg m^{-3} for cereal grain straws to highs of 280 – 480 kg m^{-3} for hardwood chips (Cheng, 2017). The bulk density of a lignocellulosic biomass can be determined in accordance with the ASTM standard E873 – 82 (2006).

6.4. Moisture sorption

The moisture sorption or hygroscopy caused behaviour of biomass is important for biomass harvest, handling, transport and storage. Therefore, proper drying and storage operations are required to preserve the quality of biomass feedstocks [3]. The Equilibrium Moisture Content (EMC) is a key parameter to characterize the water sorption behaviour of biomass, and it is defined as the moisture content of a material in equilibrium in a particular environment with respect to temperature and relative humidity [8]. EMC mainly depends on the biomass type and environmental humidity and several equations have been proposed for describing parameters for individual feedstock [3]. Moisture sorption methods usually are implemented on the basis of

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hygroscopicity experiments, where the sample is saturated with moisture under controlled temperature and humidity conditions with successive weighting and moisture content calculations [8].

6.5. Thermal properties

Lignocellulosic biomass is an anisotropic material, which means its thermal conductivity depends on heating direction, moisture, porosity, density and temperature. The choice of method is determined by the intended use of the biomass. For example, if the biomass is to be used for thermal insulation, the intended insulation material will most probably be formed from biomass and then tested by the heat flow meter technique.

However, if the biomass is to be used for thermochemical conversion processes, it is necessary to investigate thermal conductivity to predict its behaviour because biomass particles are subject to heat conduction along and across their fibers, which in turn influences their thermochemical conversion behaviour [3].

The heating value or calorific value is the property showing the energy that can be recovered during thermos conversion and is measured by calorimeters and is expressed in Mj per kg of dry biomass [4]. It represents the heat capacity of the material, and is an important thermal property from an energetics point of view, and it depends on the moisture content and temperature of lignocellulosic biomass [3].

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7. ASH

7.1. Ash content

The main component of biomass ash is the oxide form of silica, aluminum, iron, calcium, magnesium, titanium, sodium, and potassium [9]. Biomass ash can cause a number of operational problems during biomass processing, combustion, and emissions, therefore, the quantitative analysis of biomass's ash content is critical for process design [4].

Ash content is the quantity of the solid residue left after the lignocellulosic sample is completely burned. It can be measured by different methods and standards, for example by combusting the dried biomass sample in a furnace at 575±25 °C for 3 h, cooled and weighed, followed by a further 1 h at 575±25 °C until the sample mass varies by less than 0.3 mg from previous weighing in accordance with the ASTM standard E1755 – 01 (2015). Another method includes controlled combustion of the char left in the previous step and the determination of volatile matter at a temperature of 815°C (German Standard DIN 51719) [1].

Instrumental analysis also can be used, for instance, in the TGA analysis the flow gas is changed to oxidizing gas (air or oxygen), then a heating ramp begins until 800°C, followed by an 800°C isothermal hold for 3 minutes followed by a calculation of ash content [3].

The ash content is not exactly the same as the content of mineral matter because ash forms the residual part of the combustion process; i.e., not all inorganic elements are present in oxidic form. However, the ash content is a fairly good indicator of the ash yields that can be expected in industrial processes [1].

Ash content must be determined if it is necessary to express some biomass analysis results on a dry ash-free basis. Ash content is an important property for the estimation of the potential risk of slagging and fouling problems during biomass combustion or gasification [3].

7.2. Ash characterization

During the biomass thermochemical conversion, ash melting can cause an agglomeration of the reactor contents and slagging of heat exchangers, therefore ash melting behavior is an important issue [1]. The composition of the inorganic constituents in biomass and the ash composition in terms of quantities of major elements are usually characterized using X-ray fluorescence (XRF) spectroscopy, while X-ray diffraction (XRD) can be used to identify crystal structures of residues. Standard methods are typically related to analysis after controlled combustion, while other (more advanced) methods, such as chemical fractionation and computer-controlled scanning electron microscopy (CCSEM), are used to quantify the abundance, size, and association of inorganic compounds [1]. Metal content in ash, including heavy metals, can be determined via ICP-MS [10].

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8. EXTRACTIVES

The composition and amount of the extractives depend on the lignocellulosic plant species, morphological part of the plant, plant age, location, growing place and conditions, and many other factors. The extractives consist of mixtures of various components, from relatively low-molar-mass molecules to the higher molar-mass substances [11]. Extractives of lignocellulosic biomass can be divided into groups based on their chemical type – lipophilic or non-polar and hydrophilic or polar compounds. Each of the mentioned groups can be dissolved in different solvents – organic solvents or water. In order to eliminate all extractives, consecutive extraction is performed, using different solvents of increasing polarity, e.g. dichloromethane, acetone, toluene/ethanol and water [12].

The principle of content determination of extractives is based on the elimination of them from biomass by an extraction process followed by mass determination via evaporation of solvent or by determination of mass loss of biomass. Classic extraction techniques, such as maceration, Soxhlet extraction or Infusion, generally require long extraction times and large amounts of solvent. However, alternative techniques, such as microwave assisted extraction, ultrasound assisted extraction, pressurized liquid extraction, supercritical fluid extraction and others improve the efficiency of the extraction and reduced the environmental impact of the extraction process. The alternative techniques result in reduced extraction time, energy consumption, and the amount of solvent used, providing a high yield of extract recovery [13].

A qualitative analysis of extracts can be performed via advanced instrumental analysis, e.g. chromatography (GC), liquid chromatography (LC) using different detectors, such as flame ionization detector FID, mass spectrometric detector MS and others, followed by spectral libraries for identification of substances.

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9. DETERMINATION AND CHARACTERIZATION OF CELLULOSE

Cellulose is a major part of polysaccharides that are present in plants accompanied by hemicellulose and can be classified as the most abundant organic chemical on earth [14]. The content of cellulose in herbaceous plants is 30 to 40%, in woods 45 to 50%, in bast plants (flax, ramie, jute, etc.) 60 to 70% and in cotton fibers upwards of 90%. Major carbohydrate portion in woods is a combination of cellulose (40 - 45 %) and hemicelluloses (15 - 25 %) which so-called holocellulose and usually covered 65 - 70 % of dry basis biomass weight. Cellulose is linear, stereoregular, semicrystalline, and is an insoluble glucan polymer of D-glucopyranose units that link together by β-(1 → 4)-glucosidic bonds with a polymerization degree of about 10,000. The linear macromolecules joined by hydrogen bonds form a supermolecular structure of cellulose that consists of thread-like elementary nanofibrils and their bundles called microfibrils. The cellulose microfibrils form the layers and walls of plant cells (fibers). Cellulose is the main constituent of a plant cell wall, offering structural support and acting as a reinforcement element together with hemicellulose and lignin [14–16].

9.1. Determination of cellulose

Cellulose is the main fiber-forming component of biomass. Its amount in biomass is an important indicator in various production sectors, such as the paper industry, the production of fiber heat insulation materials, as well as the production of second-generation biofuels [17,18].

9.1.1. Kürschner-Hoffer

Kürschner-Hoffer cellulose is one of the most traditional methods for cellulose determination. For Kürschner-Hoffer cellulose amount determination, 2 g of an extracted biomass sample is placed in a 250 mL reaction vessel together with 150 mL of the mixture containing 30 mL of nitric acid and 120 mL of ethanol and then heated in a water bath at 92°C for 20 minutes. The solution is decanted or filtered leaving the biomass sample in the reaction vessel. 150 mL of the fresh mixture are added to the sample and heated in a water bath. The process is repeated for 5-7 cycles. Finally, warm deionized water is used to wash the fibers of the biomass sample. Kürschner-Hoffer cellulose content is determined gravimetrically as a percentage of oven dry treated sample vs. oven dry raw biomass sample [19,20]. It has been proven that in the Kürschner-Hoffer cellulose determination method, the optimal result is achieved after 4-5 cycles, when the lignin content in the sample rapidly decreases. In the following cycles (after cycle 5) the amorphous part of the cellulose is already dissolved and the crystallinity increases [21].

9.1.2. Hydrolysis

Acid hydrolysis is one of the most popular methods for the determination of chemical components in biomass. By properly preparing the sample for hydrolysis and High Performance Liquid Chromatography, it is possible to obtain a very precise chemical composition of the biomass. The most common methods are based on a two-step sulfuric acid hydrolysis, which has been used for over a century and modified for different objects and conditions [22]. Sluiter et al. [22] and Templeton et al. [23] reviewed the compositional analysis methods on the basis of sulfuric acid hydrolysis and their uncertainties. Many analogous versions of sulfuric acid hydrolysis methods have been reported [24–27]. The Laboratory Analytical Procedure (LAP) 'Determination of Structural Carbohydrates and Lignin in Biomass' published by the National Renewable Energy Laboratory (NREL) [28] is the most updated version. This method begins with an ethanol extraction step. It

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can interfere with the accurate quantification of polysaccharide sugars, Klason lignin, and acid soluble lignin, which can be separated and quantified. The extracted components then typically undergo a strong sulfuric acid hydrolysis step at room temperature and then a diluted sulfuric acid hydrolysis step at high temperature to break down to their monomeric forms analyzed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). The acid soluble lignin samples can be quantified using ultraviolet spectroscopy. The Klason lignin content can be gravimetrically obtained from the acid insoluble residue [3].

A High Performance Liquid Chromatography (HPLC) analysis of the obtained hydrolyzates is performed to determine the content of monosaccharides, 2-furaldehyde, 5-hydroxymethylfurfural (5-HMF), and organic acids using a HPLC with a refractive index detector. Glucose, xylose, cellobiose, arabinose, galactose, mannose, 2-furaldehyde, acetic acid, 5-HMF, levulinic acid, and formic acid are used as reference standards. Glucose, cellobiose, 2-furaldehyde, acetic acid, 5-HMF, levulinic acid, and formic acid are analyzed on a Shodex Sugar SH1821 column at 60 °C, with eluent 0.008 M H_2SO_4 at a flow rate of 0.6 mL·min^{-1}. Carbohydrate is determined on Shodex Sugar SP0810 column at 80 °C, with deionized water as the mobile phase under a flow rate of 0.6 mL·min^{-1}. $NaHCO_3$ are used for neutralization of sample to pH 5–7. Samples are filtered through a 0.45 µm membrane filter before injection [29,30].

If the biomass sample contains both cellulose and starch, both will be hydrolyzed to glucose, causing an inaccuracy of the two-step acid hydrolysis method and unacceptably large errors in the results. To eliminate these errors, enzymatic starch extraction by hydrolysis followed by filtration and washing has been developed. A two-step acid hydrolysis is then performed as described above [26].

9.1.3. Holocellulose

The hydrolysis method provides information on the amount of holocellulose (cellulose and hemicelluloses) in the sample; however, if it is necessary to isolate solid state holocellulose for further use, for example, as a filler in composite materials, or if it is necessary to determine the mechanical properties which may be affected by the presence of lignin, a holocellulose determination-extraction method is required. Cellulose content of a biomass sample can be determined by initially isolating the holocellulose (cellulose and hemicelluloses) and then dissolving the hemicelluloses from the holocellulose sample [31].

The determination method of holocellulose is based on Wise et al [32] method in which biomass samples are subjected to a number of acid chlorination treatment cycles using a mixture of sodium chlorite and glacial acetic acid [31]. The Wise method is implemented by placing 20 g of biomass sample in a round-bottomed flask and adding 1200 mL of distilled water. The flask is placed in a water bath at a temperature of 80°C and heated with continuous stirring. The delignification reaction is started by adding 8g of $NaClO_2$ and 1.6 mL of glacial acetic acid to the suspension. The mixture is continuously heated and stirred for 1h. The process is repeated 7 times. Finally, the mixture is filtered and washed several times with distilled water until the pH is neutral. The yield of holocellulose on the filter is determined gravimetrically [33]. Later, a standard method for the determination of holocellulose (ASTM International, 1978) [34] is developed based on the Wise method. 1 g of $NaClO_2$ and 0.2 mL of acetic acid are added to 150 mL of distilled water with 2 g of biomass sample and heated for 1 h at 70-80°C. Every consecutive hour, the next dose of 1g of $NaClO_2$ and 0.22 mL of acetic acid is added to the mixture. Total heating time is 5h [31]. Over time, the method of holocellulose determination and isolation has been modified by pre-extraction [35,36] or ash-correction [37] and adapted to different needs like analyzing different non-wood biomass [22,31] or optimizing the time, energy and amount of

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reagents and sample used for analyses [36]. The Jayme-Wise method uses a benzene/ethanol mixture for pre-extraction before determination of holocellulose. The method was developed for a larger amount of sample and requires special equipment and several days to complete [35]. Leavitt and Danzer modified the method for a smaller amount of sample and used a toluene/ethanol mixture for extraction [38].

Subsequently, alpha-cellulose content is determined by extraction with 17.5 % aqueous sodium hydroxide of the holocellulose powder and hemicellulose content is evaluated by the difference between holocellulose and alpha-cellulose content [37]. Alpha cellulose or in other studies the so-called crude [16,36,39] cellulose is determined by treating 2g of holocellulose samples with 100 mL of 17.5% sodium hydroxide solution for 30 minutes at room temperature. The mixture is filtered and washed with deionized water and 15 mL of 10% acetic acid is added. After oven drying the sample, alpha cellulose is determined gravimetrically [40].

9.1.4. Cellulose purification

With the hydrolysis method, it is possible to accurately determine the amount of cellulose in a sample, but for certain studies, such as C^{13} isotope analyses, it is necessary to obtain the pure cellulose in a solid state [35,39,41]. In the so-called Brendel method, there is a faster way to produce alpha-cellulose compared with the Jayme-Wise method. A biomass sample (50 mg) is treated with a mixture of 2 mL of 80% acetic acid and 0.2 mL of concentrated (69%) nitric acid at a temperature of 120°C for 20 minutes. The sample is washed with deionized water, ethanol and acetone, filtered and dried. The yield of cellulose is determined gravimetrically [41]. Later, other authors modified Brendel's method by adding a treatment with 17.5% NaOH to wash out the remaining hemicelluloses and an additional water treatment to wash out the water-soluble salts [35]. Bian et al. have modified Wise and Brendel's method for alpha-cellulose purification. In the first step, the biomass sample is delignified by a sodium chlorite and acetic acid treatment at 75°C for 2h, filtrated and washed. The second step includes crude cellulose extraction from holocellulose by 10% KOH treatment at 10-50°C for 10h. The last step is crude cellulose (0.5 g) purification by 10 mL of 80% acetic acid and 1 mL of 68% nitric acid at 120°C for 15 minutes [39].

9.2. Characterization of cellulose

9.2.1. FTIR analysis

Due to its technological advantages, a Fourier Transform Infrared (FTIR) spectroscopy is frequently used in both qualitative and quantitative studies of lignocellulosic biomass. It is a rapid analytical method, requires a very small amount of sample (2 mg), uncomplicated sample preparation and comprehensive multi-component analysis [27,42]. The FTIR analysis method is increasingly used in cellulose research as it offers a quick and easy way to obtain direct information about chemical changes of cellulose processed during chemical treatments [43,44]. Another advantage of the FTIR analysis method is that most organic compounds absorb the electromagnetic waves of IR light. FTIR measurements are usually carried out in a region with a wavelength between 4000 and 600 cm^{-1} [42]. FTIR measurements are performed on Internal Reflection Element (IRE) crystal material or by preparing KBr pellets with sample/KBr ration 1:100 [44]. FTIR has been successfully used for qualitative characterization of lignocellulose and it has become an everyday analysis method, meanwhile, the use of FTIR for accurate quantitative analysis is more complex [45–47]. There are also studies on the use of FTIR to determine the degree of cellulose crystallinity [48,49]. The crystallinity index can be calculated by applying ratios of proper

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FTIR band heights (H1429 cm^{-1}/H895 cm^{-1}, H1370 cm^{-1} / H2900 cm^{-1}) or surfaces (A1370 cm^{-1}/A670 cm^{-1}) [48]. Table 3 shows the peacks of the cellulose characteristic groups.

Table 3. FTIR peaks of the cellulose characteristic groups [43,50–52]

Wavenumber (cm^{-1})	Assigment
3280-3480	Intramolecular and intermolecular O-H group stretching
2900-2925	Symmetric C-H stretching
1430-1440	CH_2 bending of pyranose ring
1300-1370	CH bending
1150-1165	C-O-C asymmetric stretching
1030-1060	C-O stretching
890-895	C1-H deformation in β-glycosidic linkage

9.2.2. Crystallinity of cellulose by XRD analysis

With X-ray diffraction (XRD) it is possible to reveal a wide range of structural information of materials - chemical composition, crystal structure, deformations, crystal shape, dimensions and orientation. The structural information that can be obtained includes composition, strain, chemical structure, crystal size and orientation, and layer thickness. XRD provides the ability to characterize various materials including nanomaterials and various biomass components. [42]

Cellulose has six polymorphic crystalline forms (I, II, III, IV, V and VI). The most common polymorphic form is I and it is the only naturally occurring form. The polymorphic form I can be formed from two crystalline phases - the allomorph Iα, which has 1-chain triclinic units, or the allomorph Iβ, which has 2-chain monoclinic units. Taller plants, such as wood and grasses, have the Iβ crystalline structure, while bacterial and algal celluloses consist of the Iα crystalline form [39].

Miller indices for the cellulose crystalline planes of reflection are $(1\bar{1}0)$, (110), and (200) for cellulose I and $(1\bar{1}0)$, (110), and (020) for cellulose II. Main reflection peaks for taller plant cellulose at 2θ are about 16,5°, 22,6°, and 34,2° [53]. Depending on the cellulose origin, modification and processing affecting allomorphic cellulose structures, the reflection peak at 2θ may be slightly shifted [40,49].

The crystallinity of cellulose can be expressed as a crystallinity index (CrI) and can be calculated by various methods. According to the Segal method (1959) [54], the CrI can be calculated by the following equation:

$$CrI = 100\times[(I_{200}-I_{am})/I_{200}] \quad \text{(for cellulose I) or}$$

$$CrI = 100\times[(I_{110}-I_{am})/I_{110}] \quad \text{(for cellulose II)}$$

where CrI is the crystallinity index, while I_{200} (or I_{110}) represents the overall peak intensity and I_{am} the baseline intensity [42].

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The crystallinity index (CI) of the celluloses can be determined using the following equation:

$$CrI(\%) = \frac{A_{crystalline}}{A_{crystalline} + A_{amorphous}}$$

where $A_{amorphous}$ is the area under the amorphous curve, and $A_{crystalline}$ is the area under the sample curve.

The cellulose crystallite size is estimated using Scherrer's equation:

Crystal size L = kλ/βcosθ

where λ = 0.1540 nm, k is the correction factor of 0.91, θ is the diffraction angle in radians and β is the full width at half máximum [40].

9.2.3. SSNMR analysis

The solid state nuclear magnetic resonance spectroscopy (SSNMR) is an ideal technique to analyze biomass materials. This technique allows detailed structural composition of the main biomass components. SSNMR is characterized by a wide range spectrum of orientation-dependent interactions that provides detailed information on the structural, dynamic and chemical characteristics of materials in the solid state [42]. Like FTIR and XRD, SSNMR can also be used to determine the crystallinity of cellulose [39].

Advanced in-situ NMR experiments can be highlighted as promising for cellulose-based materials. 1D ^{13}C and 2D ^{13}C-^{13}C CP MAS based experiments are used to detect the proximity and accessibility of water to the different cell wall components; pulse field gradient NMR experiments are used for investigating the dissolution process and ion dynamics in different cellulose structures; and the comprehensive multiphase NMR technique, which is basically a combination of solid and solution state NMR techniques, is used to follow the processes involving the conversion of solid to liquids and vice versa, including drying, swelling, conformational changes, and dissolution processes in cellulose-based materials. Solid state NMR advanced technologies and high-tech opportunities beyond the conventional methods are able to provide deep insights, not only into the primary structure of cellulose, but also into the reaction mechanisms occurring in the structure, such as detecting intermediates appearing during oxidation and functionalization of cellulose. These methods include: (i) advanced hyperpolarization techniques (especially Dynamic nuclear polarization); (ii) sensitivity boosting techniques via isotopic enrichment; (iii) paramagnetic doping and the use of CryoProbes; (iv) magnetic resonance imaging and microimaging; (v) NMR relaxometry; (vi) NMR diffusometry; (vii) ultra-high magnetic fields; (viii) and ultra-fast MAS spinningIdeally, the pursuit of integrated methods, such as the combination of solid state NMR advanced techniques and computational approaches can provide the most valuable information and deep insights in studying cellulose-based materials [55].

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9.2.4. Degree of Polymerization

The two most commonly used techniques to measure the DP of cellulose are the viscometry and the gel-permeation chromatography (GPC) methods. The determination of the DP of cellulose begins with its dissolution. The two most practiced techniques to dissolve cellulose are: (i) dissolving cellulose in metal complex solutions; or (ii) forming cellulose derivatives by nitration or tricarbanilation.

The viscometry technique involves dissolving cellulose in 0.5 M of cupriethylenediamine solution. According to the TAPPI T 230 om-08 method, 0.25 g of dry cellulose is added to 25 mL of distilled water and 25 mL of cupriethylenediamine solution, with continuous nitrogen flushing. Shaking and heating might be required to solubilize the cellulose. The intrinsic viscosity is then measured for the resulting homogeneous solution. It appears that viscometry is more popular in determining DP of cellulose in lignocellulosic biomass.

Measuring the DP of cellulose viscometrically after nitration is a technique developed in the 1940s, in which extractive-free wood meal is treated in a mixture of nitric acid, phosphoric acid, and phosphorous pentoxide in the weight ratio of 64:26:10 at 17°C for ~40 h, resulting in the isolation of cellulose nitrates, which is then solubilized in either acetone or ethyl acetate. Today the nitration methodology is rarely used due to the uncertainty arising from possible acid hydrolysis of the cellulose chain during derivatization as well as the instability of the derivative.

Cellulose tricarbanilate is the most utilized derivative for GPC studies due to the following advantages: (i) complete substitution; (ii) no depolymerization occurs during derivatization; (iii) stability of the derivative; (iv) solubility and stability in THF. Cellulose tricarbanilation is commonly performed by reaction of the cellulose with phenyl isocyanate in either dimethylsulfoxide (DMSO) or pyridine as the solvents. Therefore, pyridine is the most commonly used solvent for the derivatization. Typically, a dried cellulose sample (15 mg) is derivatized by adding anhydrous pyridine (4.00 mL) and phenyl isocyanate (0.50 mL, 0.00462 mols), and kept at 65 °C with stirring until the cellulose is completely dissolved. Afterwards, methanol (1.00 mL) is added to the reaction mixture to eliminate the unreacted phenyl isocyanate. The mixture is then poured into a 3:7 water-methanol mixture (100 mL) to precipitate the cellulose tricarbanilate. The derivatized cellulose is finally purified by repeated washing with water-methanol (3 × 100 mL) followed by water (2 × 100 mL).

Viscometry measurements are relatively quick and convenient. However, it has three limitations: (i) it provides only average molecular weight, which is not an absolute average since it depends on the solvent/temperature conditions; (ii) it provides no information concerning the molar mass distribution (MMD); and (iii) the complex metal solutions used along with the method can degrade cellulose. On the other hand, GPC provides the MMD. Like viscometry, GPC does not give the absolute molar mass because it is calculated based on the molecular weight of a set of standards which is frequently a set of well-defined polystyrene standards with varying molecular weights.

In order to obtain absolute molecular weight of cellulose and thus DP, light scattering techniques are usually applied. Size exclusion chromatography (SEC) coupled with a multi-angle laser light scattering (MALLS) is the system typically used for the light scattering measurement. SEC-MALLS analysis has been mostly performed on cellulose-rich samples, because dissolving native wood is still difficult to achieve [56].

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9.2.5. Functional carboxyl groups

Traditionally, the neutralization titration method is used for the determination of carboxyl groups in cellulose.

Both alkyl and acid neutralization reactions can be carried out. The method is based on the reaction of cellulose with potassium acetate or sodium hydroxide, when a sample is soaked in the corresponding solution for 24 hours. The unreacted solution is then titrated with NaOH or HCl, respectively. The equivalence point of the titration can be determined in different ways - using potentiometric or conductometric methods, or using the appropriate acid-base indicators [57–60]

A spectroscopic method can be used to determine the carboxyl content on solid cellulose fibers, based on measuring the absorbance (at the wavelength of 664 nm) of the remaining methylene blue (MB) in the filtrate (after the adsorption with fiber sample). After subtracting the absorbance from the blank solution (i.e., before the fiber' adsorption experiment), the net absorbance of MB is found to be proportional to its concentration [61].

There are also methods available for the determination of carboxyl group in cellulose based on the different instruments, such as headspace gas chromatography [62,63], nuclear magnetic resonance (NMR) [64], fluorescence labeling [65] and complexometric titration by using photometric identification of equivalence point [59], as well as polyelectrolyte titration using polyelectrolytes like Poly(dimethyldiallyl-ammonium)chloride PDMAAC [58].

9.2.6. Morphology

The size characterisation of cellulose fibres and morphology can be analyzed by different kinds of microscopy - Light, optical, scanning electron, transmission electron and atomic force microscopy. Another method of analyzing the dimensions of cellulose fibers is optical measurements by a fiber tester.

9.3. Characterization of cellulose micro and nano structures

Due to its fibrillar structure and presence of amorphous and crystalline regions, cellulose can be broken down into different micro and nano particles, which in general represent cellulose chemical composition; however, they have unique physical and mechanical properties. Although in micro or nano scale, those particles or materials are still a cellulose, therefore, practically all the methods related to characterization of chemical and some of the physical properties of cellulose are applicable.

Fibrillar structures of cellulose are obtained by defibrillation, where opening into their sub-structural microfibril occurs. A mechanically induced breakdown of cellulose fibers into micro- or nano-scale fibrils usually demands a huge energy input, therefore most of the fibrillar forms are produced with an enzymatic or chemical pretreatment step [66]. Two main terms associated with fibrillated forms of cellulose are microfibrillated cellulose (MFC) and nanofibrillated cellulose (NFC). MFC usually consists of aggregates of cellulose microfibril, while NFC – of nanofibril, however, it should be noted that in literature, these terms are often applied to the same material and it is not clearly distinguishable where "micro" ends and "nano" starts, furthermore, MFC can contain a significant number of nano-size fibrils. Most of NFCs have lengths in the micron scale and widths ranging from 10 to a few hundred nanometers [67]. NFC or highly fibrillated cellulose suspensions, even at a low concentration, usually appear as a viscous gel-like substance due to

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a substantial increase in the accessibility of rich hydroxyl groups with water molecules on the cellulose surface as the specific surface area greatly increases after nano-fibrillation.

When subjected to acid hydrolysis, cellulose fibres and/or microfibrils undergo transverse cleavage along the amorphous regions, which results in a rod-like material with a relatively low aspect ratio referred to as ''cellulose whiskers''. The typical diameter of these whiskers is around 2–20 nm, but there is a wide length distribution from 100 to 600 nm and in excess of 1 μm in some cases [68]. The size of the rod-like whiskers is the main criteria for definition - microcrystalline cellulose (MCC) or cellulose nanocrystals (CNC). MCC dimensions are in the range of 10-50 micron, while CNC is 3-5 nm in width and 100-200 nm in length [69]. While CNCs are largely rod-like, they also exist in the form of spherical structures, called CNS. Therefore, the structural morphology of CNC is typically classified as either nanorods or nano-spheres, where they are differentiated by the aspect ratios (length--to-width ratio) that span greater than or equal to unity, respectively.[70].

Summarizing the information mentioned earlier, quite often inseparable mixtures of cellulose particles of micro and nano size are used and analyzed, and most authors do not distinguish size-specific methods. Therefore, the term "nanocellulose" will be used further, however we also mean cellulose microforms under this term.

Nanocellulose (NC) itself has been one of the most popular biobased nanomaterials for the last two decades. There are at least three main reasons for this fact: (1) high demand for biobased materials in general; (2) the abundance of cellulose and (3) unique properties and functionalities of NC – small size, morphology, big surface area, wide area of high-performance applications – films, cosmetics, paints, nanocomposites, fluids, paper and cardboard products, adhesives, membranes, flexible electronic components, textiles, biomedical and tissue engineering materials, food coatings etc [71].

In order to characterize the obtained NC and adjust the target properties of the selected final product, many characterization methods have been developed and are still being developed for the needs and demand of both scientific society and industry. An individual particle can be analyzed as well as particle suspension with direct methods, furthermore, properties of NC can be assessed by analyzing and characterizing it indirectly - after forming films (also called nano-paper). In general, the characterization of nanoparticles often requires state-of-the-art technologies based on various scientific techniques such as optical, physical, mechanical, and rheological methods, with each analysis giving information on the suspension from a different perspective [72]. The most used NC characterization methods in science are rarely present in industry, moreover, industry needs low cost, convenience, and quick results. In order to meet industry needs several articles and reviews [67,72–76] have been dedicated to summarizing the characterization methods of NC, however, the most comprehensive compilation along with highlighting the main drawbacks and limitations in the current characterization methods is given in the work of Balea et al [71]. This article presents a nearly complete compendium of methods, and Table 4 reflects the findings of authors, accenting suitability of method for the industrial environment. Furthermore, the most significant methods of NC characterization are explained as follows.

The methods used for the general characterization of cellulose e.g., Physical properties, Chemical properties such as Elemental analysis or Surface charge and chemistry are explained in the section on Cellulose characterization.

Optical microscopy (OM) is used to get an overview of the micro-scale celluloses, for example, of the sample homogeneity, size and morphology. Microscopy enabling imaging with higher

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resolution such as scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) provide details of the nano scale dimensions. The samples can be prepared by air-drying, freeze drying or by critical point drying. At the moment, the high-resolution electron microscopy or atomic force microscopy can be considered as the state-of-the-art methods for measuring the particle size. SEM imaging can also be used to study the branching of the nanofibrils, but the determination of length is difficult due to the entanglement of the fibrils and their micrometer-scale length. However, in most cases, the resolution provided by SEM is not adequate for evaluation of details of nanoscale cellulose fibrils and microscopic techniques with higher resolution, TEM and AFM, are utilized. AFM can be used for topography measurements of cellulose particles, revealing morphological characteristics at nano-scale. To obtain the best and most accurate information on the morphology and dimensions of cellulose micro and nanostructures, a combination of the microscopic methods, such as FE-SEM and TEM or cryo-TEM and AFM, has usually been utilized [73]. From literature, the determination of NC dimensions from image analysis has been reported using different methods, with different levels of complexity that vary from a simple and basic measurement of a few nanoparticles from an image to a complex statistical analysis performed on all nanomaterial present in a large quantity of microscopy images [77]. Many other techniques based on light interactions, electrical properties, sedimentation, sorting, classification and polymer, and dye adsorption could be also used to determine the size of the nanoparticles.

The method that has often been used for nanocellulose - dynamic light scattering (DLS) -has values which cannot be directly correlated to the particle length or cross-section of nanocellulose because NC particles have high aspect ratio, rod or fibrillated materials with differing translational diffusion constants parallel and perpendicular to the particle axis, therefore, although light scattering techniques are faster than microscopy, their simplifying assumptions are not especially accurate in the case of nanofibers or nanocrystal particles [71].

The amount of nanomaterial is an important property for polydisperse fibrillated celluloses, since the size of the majority of the material may well be outside the nanoscale. The property can be determined by estimating the particle size distribution using mechanical fractionation or ultracentrifugation [73] or by determination of the nanoscale particle quantity in the suspension by a gravimetric method. The turbidity of the NC suspensions is measured with a turbidimeter (specific for fibrillated NC) and linked to the shape, size, and refractive index of the suspended matter. This test measures the scattered light at an angle of 90◦ to the incident light. If the suspension is only composed of nanoscale materials, the turbidity value is close to zero. However, the presence of poorly fibrillated fibers in the suspension results in an increase of the turbidity [72]. The capacity of CNFs to transmit or scatter visible light can also be related to the fibrillation degree both in suspensions or films. Dynamic light scattering, light transmittance analysis, ultraviolet-visible (UV-vis) spectrophotometry, or turbidity measurements are often used to compare CNF quality [72].

Due to the strong interfibrillar forces of cellulose fibrils, CNF and MFC have gel l forming properties, already observed at low concentrations. The rheology of the cellulose fibril suspensions has been studied using various types of rheometers, measuring in a steady state, stepped flow or under dynamic conditions as well as under low and high shear. Optical measurements have been used to obtain complementary information of the behavior of fibrils under shear [73]. The surface chemistry of NC is linked to its colloidal stability, rheological properties, and interactions with other chemical species [71]. Zeta Potential of NC suspension is related to the surface potential and surface charge density and is used to assess NC colloidal stability. Zeta Potential (ZP) can be measured using an electrophoretic mobility analyzer. In

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general terms, suspensions with absolute ZP values above 20 mV are considered stable. Common values for CNC are -20 to -50 mV and ZP values for NFC can be near to -60 mV.

The fibrillation degree of cellulose micro or nano particles can be likened to the water retention value (WRV), as the WRV increases due to an increase in surface area. On the basis of this fact, the so-called "yield of fibrillation" is determined through nanoparticle isolation, thanks to the centrifugation steps [72].

Mechanical properties are also very important characteristics of fibrillated celluloses, and some efforts have been taken towards measuring the strength and elastic moduli of individual cellulose fibrils by AFM [78]. However, a more common approach for evaluating the mechanical properties of fibrillated celluloses has been carried out through measuring the strength properties of CNF films or so-called nano-papers, with the same principle.

9.3.1. Health and safety characterization

Nanomaterials, which have nanosized dimensions and a large surface area, have high reactivity and potential effects on natural systems. As they can have a negative biological impact, their toxicological risks must be established. The toxicity of NC is highly influenced by their physicochemical properties, primarily their dimensions, aggregation degree, and surface chemistry. In order to evaluate health and safety risks, different tests have been designed to assess cytotoxicity, the inflammatory response, the oxidative stress status of the biological system, genotoxicity etc. [71]. For example, the cytotoxicity of NC has been determined by many different testing methodologies, which include MTT assay, XTT assay, BrdU assay, neutral red solution N6264 Sigma (NR) cytotoxicity procedure, observation of the morphology of the biological test system with light microscopy, evaluation of apoptosis/necrosis or cell-cycle via flow cytometry [71].

Life-cycle assessment (LCA) studies of NC are also needed to identify and analyze possible high and low risk scenarios and, therefore, evaluate the potential risks of NC towards human health and the environment [79]. Table 4 shows the main NC properties and characterization techniques.

Table 4. Main NC Properties and Characterization Techniques [71]

Property	Characterization techniques	Acronym	Advanced research	Industrial environment
Size and morphology: Length (L), Width (w) Diameter, Aspect ratio (L/w), Average particle size / size distribution	Atomic force microscopy	AFM	X	
	Scanning electron microscopy	SEM	X	
Size and morphology: Length (L) Width (w) Diameter Aspect ratio (L/w) Average particlesize / size distribution	Atomic force microscopy	AFM	X	
	Scanning electron microscopy	SEM	X	
	Transmission electron microscopy	TEM	X	
	Optical microscope	OM		X
	Fiber analyzer			X
	Fractionators			X
	Gel point (for aspect ratio)	GP	X	X
	Viscosity (for aspect ratio)		X	X
	Light scattering techniques: Dynamic light scattering Depolarized DLS	DLS	X	X
		DDLS		

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Property	Characterization techniques	Acronym	Advanced research	Industrial environment
Physical properties: Crystallinity (mainly for CNC) Degree of polymerization (DP) Specific surface area (SSA) Density	Crystallinity and dimensions of cellulose crystals: X-ray diffraction Raman spectroscopy Infrared spectroscopy ^{13}C nuclear X-ray diffraction magnetic resonance Solid-state nuclear magnetic resonance	XRD	X	
		^{13}C NMR		
		ssNMR		
	Viscosity method		X	X
	Determination of SSA: N2 adsorption, Brunauer-Emmett-Teller isotherms Congo Red method Small angle X-ray scattering	BET	X	
		SAXS		
	Network density (on nanopapers): Air permeability (Vase de Mariotte, Bendsen) Porosity (calculation from basis weight and thickness) Transparency		X	X
Elemental analysis (EA)	Carbon hydrogen nitrogen elemental analysis	CHN CHNS CHNSO CHNSOX	X	
	Secondary ion mass spectrometry	SIMS	X	
	X-ray photoelectron	XPS	X	
	Auger electron spectroscopy	AES	X	
	Energy dispersive X-ray spectroscopy	EDS (orEDX)	X	
	Inductively coupled plasma: Mass spectrometry Atomic emissionspectroscopy	ICP-MS ICP-AES	X	
Water retention	Water retention value	WRV	X	X
Fibrillation degree(for NFC)	Atomic force microscopy	AFM	X	
	Scanning electron microscopy	SEM	X	
	Transmission electron microscopy	TEM	X	
	Optical microscope (for low fibrillation degree)	OM		X
	Mechanical fractionation by combination of sieves and membranes		X	
	Field/tube flow fractionation		X	
	Centrifugation		X	X
	Transmittance by UV-vis spectroscopy		X	X
	Turbidity		X	X

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Property	Characterization techniques	Acronym	Advanced research	Industrial environment
Dissolved and colloidal substances (amount and quality)	Atomic force microscopy	AFM	X	
	Scanning electron microscopy	SEM	X	
	Transmission electron microscopy	TEM	X	
	Gel permeation chromatography	GPC	X	
	Size-exclusion chromatography	SEC	X	
	High-performance liquid chromatography	HPLC	X	
Surface charge and chemistry: Surface modifications Charge determination	Surface chemically modified by adsorption: Fourier-Transform infrared spectroscopy Elemental analysis	FTIR	X	
		EA		
	Surface chemically modified by covalent bonding: Fourier-Transform infrared spectroscopy Solid-state nuclear magnetic resonance X-ray photoelectron spectroscopy Elemental analysis	FTIR	X	
		ssNMR		
		XPS		
		EA		
	Inverse gas chromatography	IC	X	
	Conductimetric titration		X	X
	Charge determination: Cationic demand Zeta potential	CD	X	X
		ZP		
Rheology: Viscosity Yield stress Gel point Aspect ratio	Low shear viscosity by viscometers		X	X
	Gel strength, viscoelastic properties by rheometers		X	X
Mechanical and thermal properties	Tensile testing		X	X
	Flexural testing		X	X
	Compression testing		X	X
	Dynamic mechanical analysis	DMA	X	
	Raman spectroscopic		X	
	Thermogravimetric analysis	TGA	X	X
Other properties	Health characterization (i.e., eye irritation, skin irritation, genotoxicity, toxicokinetic testing, systemic testing, Ecotoxicity)		X	
	Safety characterization (i.e., deflagration index)		X	

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10. HEMICELLULOSE

Hemicellulose is calculated as the difference between holocellulose (Wise method) and Seifert cellulose. Another way to determine the hemicellulose content is the summative analysis considering all the monomers derived from hemicellulose, sugars, and other decomposition products. To quantify all the monomers, a standardised two-step acid hydrolysis method, T249 cm-00 (TAPPI, 2000-a) followed by chromatography analysis (as follows in section 10.1), using stoichiometric factors calculations (explained in section 10.2) is carried out.

Figure 1 shows the structure of hemicellulose and the sugar derivatives, formed because of pretreatment and hydrolysis of lignocellulosic biomass.

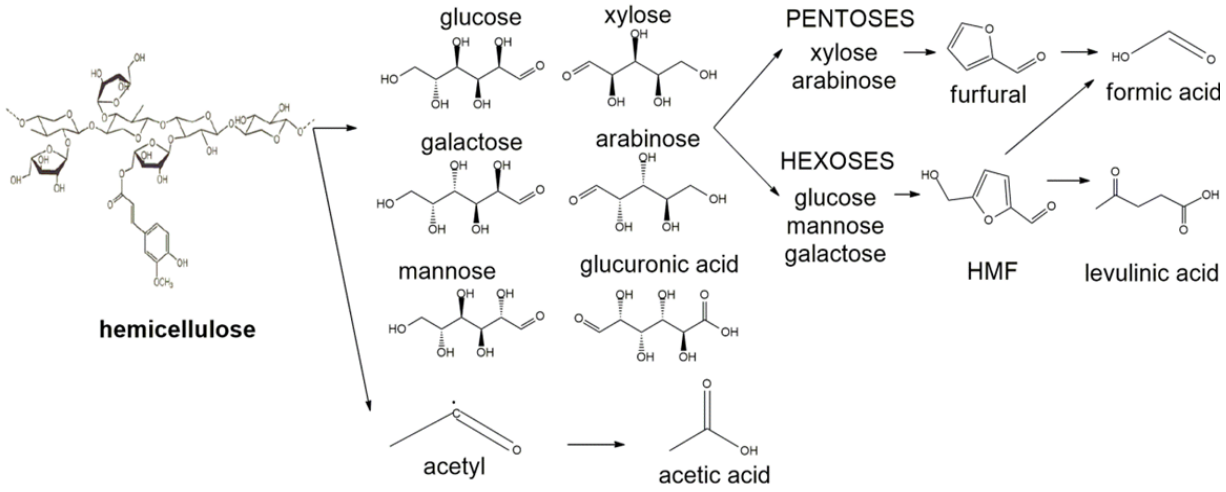

Figure 1. Hemicellulose constituents formed after hydrolysis.

10.1. Carbohydrate quantification by HPLC/RID

Monosaccharides, aliphatic acids, and furan aldehydes are determined by means of HPLC/RID technique. Four chromatographic methods are developed. The HPLC system used is a Shimadzu Prominence LGE-UV equipped with a control system CMB-20A, an inline degasser channel DGU-20-A5, an isocratic pump LC20AD, and auto sampler SIL-20AHT with thermostatic cooling (samples held at 4ºC), a column oven CTO-20ASVP and a refractive index detector RID-10A.

The methods developed are successfully applied within different LCB samples: wood, pulp and bleached pulps, detoxified liquors, weak and thick industrial liquors, synthetic sulfite spent liquors, paper and dissolving grade liquors, and hydrolyzates from the kinetic study.

Details regarding the operating conditions and column assayed and methods validation are shown in Table 5.

The steps involved in pulp and wood samples depolymerisation, hydrolysis and sugar quantification are widely described in section 9.1.2. Two-step acid hydrolysis in accordance with the T249 cm-00 standard followed by HPLC analysis and stoichiometric calculations [80,81] are carried out.

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Table 5. Standards and method conditions [83]

Column	Components	Standards (gL^{-1})	Retention times (min)	Method conditions
HPX-87P	Sugars	0.5-5	25.01-33.07	0.3 $mL \cdot min^{-1}$, ultrapure water, 79 ºC, 20 µL of sample volume injection
HPX-87H	Sugars	0.1-10	9.21-13.66	0.5 $mL \cdot min^{-1}$, H_2SO_4 5mM, 79 ºC, 20 µL of sample volume injection
	Acids	0.2-10	17.48-21.64	
	Alcohols	0.2-10	22.67-25.26	
CHO-782 Pb	Sugars	0.2-10	22.07-35.70	0.3 $mL \cdot min^{-1}$, ultrapure water, 68 ºC, 20 µL of sample volume injection
SH-1011	Sugars	0.2-10		
	Acids	0.2-10		
	Alcohols and Furfurals	0.5-5		

Briefly, the main steps regarding qualitative and quantitative total carbohydrate content (TCC) determination by HPLC techniques of solid biomass or liquid hydrolyzates are shown in Figure 2.

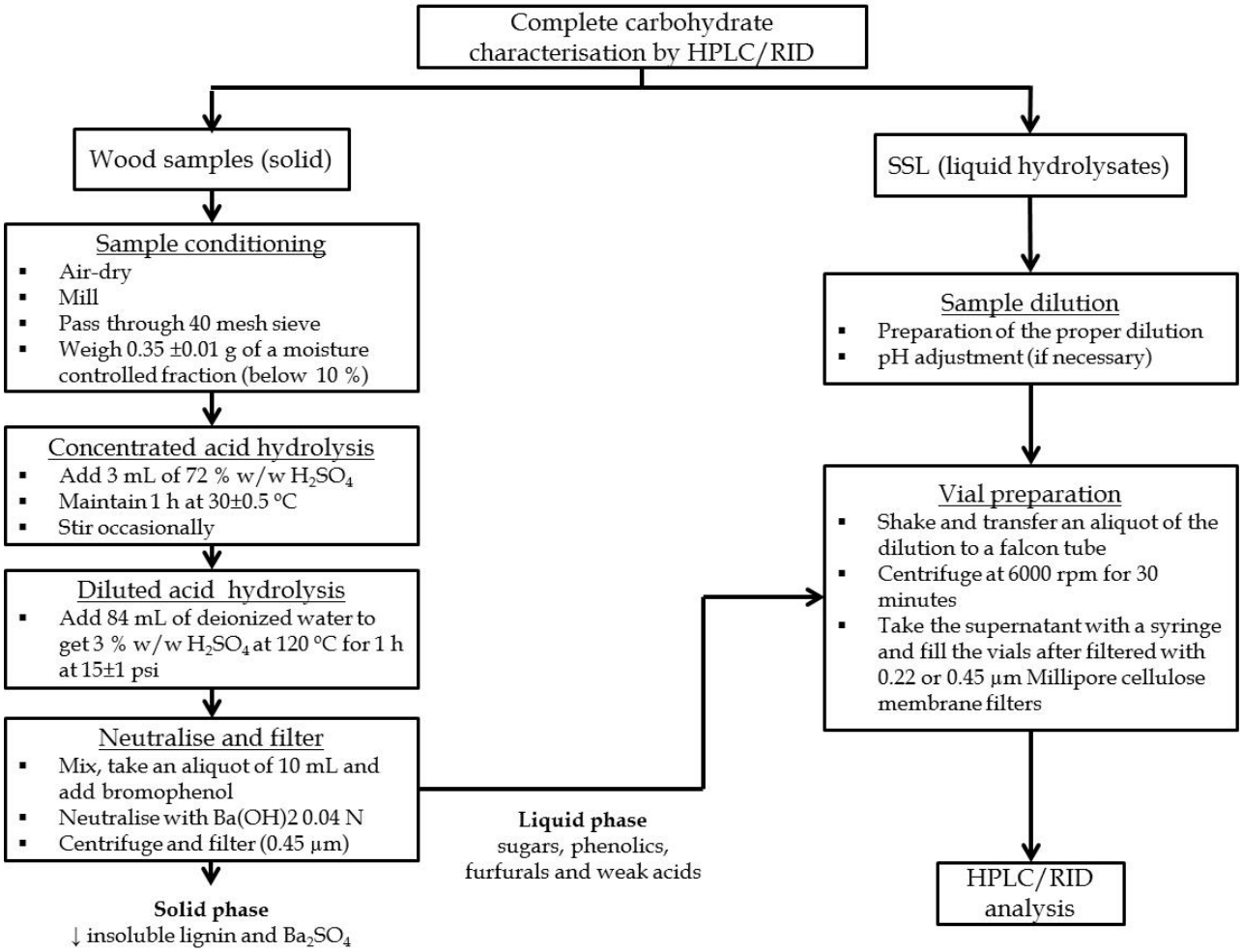

Figure 2. Stages that should be implemented working with solid biomass (left) or liquid hydroyzates (right).

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10.2. Total carbohydrate content by means of a summative analysis

A complete methodology of how to determine carbohydrates (cellulose and hemicellulose) of wood, pulp and liquid hydrolyzates is described in Llano et al. [82], as well as the chromatographic methods in Llano et al. [83]. Each monomer can be reported in the summative analysis as its pure theoretical homopolymer [81]. The weight of each constituent, determined quantitatively after the hydrolysis, has to be multiplied by a factor to calculate its contribution to the original wood component (as a theoretical homopolymer). Calculations are made by using the stoichiometric factors obtained in literature [80,81,84]. These factors consist of molecular mass of an anhydrous unit divided by the molecular mass of the isolated substance. Table 2.3 shows all the conversion factors used in this work. It should be noted that carbohydrate summative analysis calculations are based on models previously applied in wood samples [80,81]. The percentage of each component in the extractives-free hydrolyzates is obtained as follows in equations:

$$C^* = C_{HPLC} \cdot D_f = C_{HPLC} \cdot \frac{V_{Ba(OH)_2}+10}{10}$$

$$C_w = \frac{C^* \cdot V_F}{P_S \cdot 1000} \cdot 100$$

Where:

C^* (gL^{-1}) = Real concentration of the component in the hydrolyzate.

C_{HPLC} (gL^{-1}) = Concentration of the component giving by the HPLC peak integration.

$V.Ba(OH)_2$ (mL) = volume of barium hydroxide added into the hydrolyzate.

Df = Dilution factor.

Cw (% w/w) = Concentration of the component referred to the dry weight of the woody hydrolyzate.

VF (mL) = final volume of the hydrolyzate.

PS (g) = dry weight of the initial woody sample, before hydrolysis.

To calculate the concentration of each homopolymer from their monomers according to Table 6, not only the monosaccharides but also the degraded compounds derived from carbohydrates are considered, e.g., cellulose is the sum of cellobiose, glucose, HMF and levulinic acid multiplied by their stoichiometric factors.

The individual contribution of carbohydrate-derived compounds to the final cellulose or hemicellulose content depends on the chemical structure of the macromolecules forming the cell wall. In this work, all the glucose is assumed to generate from the cellulose [81]. Simultaneously, it is also assumed that formic acid is an inhibitor mostly produced from pentose sugars, the formation of formic from hexoses being a remainder against the levulinic acid conversión [85]. Acetic acid which is the major weak acid is not associated with pentoses or hexoses because it is considered a co-product (secondary reaction) from hydrolysis formed at the same time as the monosaccharides by the degradation of acetyl groups presented in polysaccharides [86]. Once the concentration of the main polymers is obtained, the macrocomponents and the total mass balance of the sample are calculated considering the stoichiometric factors in Table 6.

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Table 6. Stoichiometric factors to calculate the percentage of theoretical homopolymer.

Hydrolyzed constituents	Homo-polymer	Carbohydrate contribution	Conversion factor	Ethanol factor g.EtOH/ g.monomer
Glucose	Glucan	Cellulose	162/180	0.511
HMF	Glucan	Cellulose	162/126	-
Levulinic acid	Glucan	Cellulose	162/116	-
Cellobiose	Glucan	Cellulose	324/342	-
Xylose	Xylan	Hemicellulose	132/150	0.511
Furfural	Xylan	Hemicellulose	132/96	-
Formic acid	Xylan	Hemicellulose	132/46	-
Arabinose	Arabinan	Hemicellulose	132/150	0.511
Galactose	Galactan	Hemicellulose	162/180	0.511
Mannose	Mannan	Hemicellulose	162/180	0.511
Acetic acid	Acetyl	Hemicellulose	43/60	-

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11. LIGNIN

Lignin is the most abundant biopolymer after cellulose, corresponding to 15–30% of the wood weight of softwood and approximately 20-25% of that of hardwood [87]. Its structure consists of phenyl propane (C_6–C_3) units joined together through various interunit linkages, e.g., ether linkages (C-O-C) or carbon-carbon bonds (C-C). These interunit linkages impart heterogeneous complex structures to the lignin. Lignin is an amorphous polyphenolic irregular polymer generated through the polymerization of three monomers, coniferyl, sinapyl and p-coumaryl alcohols, creating a heterogeneous composition of phenylpropanoid units (PPUs), namely p-hydroxyphenyl(H), guaiacyl (G) and syringyl (S) PPUs, differing in the degree of OCH_3 substitution on the phenolic ring. Lignin *in situ* is covalently linked with hemicelluloses in the lignin-carbohydrate complex [88,89].

The biological function of lignin, together with polysaccharides, is to generate resistant plant tissue structures, providing plants with mechanical strength and efficient water-conducting systems. Lignin also plays a significant role in plant defense against biodegradation. During plant biomass processing, the significant *in situ* modification of the lignin structure occurs and the chemical structure of "technical lignins" (functionality, molar mass, cross linking density, etc.) depends not only on the botanical plant origin and the environmental conditions of growth but also on the conditions of isolation from plant tissue [90].

11.1. Lignin determination:

11.1.1. Acid Hydrolysis of lignocellulosic or plant biomass

A substantial proportion of the methods falling into this category consist of the hydrolysis and solubilization of the carbohydrate component of the lignified material, leaving the lignin as a residue which is determined gravimetrically. Hydrolysis of the polysaccharides is catalyzed by a variety of strong mineral acids, applied either individually or in combinations.

11.1.2. Klason lignin

The most widely applied variation of the acid hydrolysis approach employs sulfuric acid to promote carbohydrate hydrolysis. The lignin isolated in this treatment is referred to as acid-insoluble lignin, or more commonly as Klason lignin, in recognition of the wood chemist who pioneered the development of the isolation method. The procedure described below is essentially the Official Test Method T222 om-83 of TAPPI (Technical Association of the Pulp and Paper Industry 1983) for the determination of lignin in wood and pulp. The amount of acid insoluble lignin and acid-soluble lignin is determined by standard TAPPI T-222. 0.5 grams of ground (2 mm) extract-free-sample is treated with 7.5 mL of 72% H_2SO_4 and treated for two hours at 25 ºC. The sample is then diluted with 260 mL deionized water and refluxed for 4 hours. The sample is then filtered through A P100 glass filter. The precipitate is washed with hot deionized water till neutral pH. The precipitate is dried at 105 ºC till constant mass.

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11.1.3. Hydrolysis lignin

Hydrolysis of cellulosic biomass in mineral acid is one of the major methods employed to separate lignin and monosaccharides from lignocellulosic biomass; however, this process is strongly dependent on the acid concentration and temperature. It has been reported that at low mineral acid concentrations, high temperature is required to achieve significant hydrolysis, thus requiring high energy loading. Although a high mineral acid concentration can significantly reduce temperature or the energy requirement, > 20% concentration of mineral acids would be needed.

11.1.4. Formic acid hydrolysis

A hydrolysis reaction is carried out under atmospheric pressure. The dewaxed plant material powder (10.0 g) is added into 200 mL of 86.24 wt.% formic acid solution with 0.2 M H_2SO_4 as a catalyst under treatment conditions according to Sun et al [91]. After treatment, the residual solids are filtered with four layers of nylon cloth, then washed with hot formic acid (aqueous solution reagent grade) and finally with hot distilled water. The residual solids are extracted with dioxane + H_2O (9:1 in volume) mixture. The resultant products are obtained from the dioxane + H_2O mixture by precipitation with ether in volume. The precipitants are washed with hexane and water separately before being freeze-dried. H_2SO_4 in the hydrolyzates is neutralized with equal moles of HCOONa. The sugars (hydrolyzing products from cellulose and hemicelluloses) are isolated from the concentrated hydrolyzates at reduced pressure and 60 °C by precipitation with 3 volumes of 95% ethanol per volume of hydrolyzates at 20 °C for 12 h and the evaporated formic acid can be recycled and reused. The soluble lignin is obtained from the corresponding supernatants by reprecipitation after evaporation of all the organic solvents (ethanol and formic acid), ethanol and formic acid could be separated and reused by fractional distillation. The soluble lignin fractions are then washed with acidified water and then freeze-dried. The purified lignin from the residual solids of hydrolyzates is denoted as RL (residue lignin), and the lignin soluble in the hydrolyzates is denoted as SL (soluble lignin) [92,93].

11.1.5. MWL lignin

MWL as the control is obtained based on the Björkman method. The biomass is milled in a planetary ball mill at 300 rpm for 72 h. The milling is paused for 10 min every 20 min to prevent overheating. The wood meal is extracted by 1, 4–dioxane/water (96:4, v/v) with a solid/liquid ratio of 1/15 (g mL^{-1}) for 24 h. The mixture is centrifuged, and the solid is washed with 1, 4–dioxane/water (96:4, v/v) until the filtrate is clear. The filtrates are combined and concentrated to 50 mL in a rotary vacuum evaporator. After freeze–drying, the crude MWL is dissolved in acetone/water (20 mL; 9:1, v/v) and precipitated into water (200 mL). The precipitated MWL is collected and freeze-dried to produce the purified MWL [94,95].

11.1.6. Kraft process

Sulfate- or kraft lignins occur in the industrial pulp extraction process. Kraft (sulfate cooking) process is used inmore than 90% of all for the world's pulp mills. Kraft lignins can be obtained from coniferous and deciduous wood treating them with NaOH and Na_2S, while Na_2S yields as a result. Chopped wood is boiled under high pressure (bioreactor) and typically delignification requires several hours at 130 – 180 °C. After this cooking, condensation products of lignin and hemicelluloses are soluble in alkaline solution and this remaining solution is called "black liquor"

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(the name is borrowed due to the color of the solution) and it contains lignin, carbohydrates, hemicelluloses, Na_2SO_4, Na_2CO_3 and others inorganic salts [96,97].

Na_2S + NaOH aqueos solution

Na_2S obtained from Na_2SO_4 reaction with carbon:

$Na_2SO_4 + C \rightarrow Na_2S + 2CO_2$

11.1.7. Alkali lignin

Alkali lignin is obtained by boiling cellulose with a sodium aqueous hydroxide solution at elevated pressure and temperature >150 °C without using sulfur-containing chemicals. Practically all lignin and hemicelluloses pass into the solution. After the neutralization with acid (HCl, H_2SO_4 also CO_2), natron-lignin precipitates, the yield of which is not 100 % from protolignin due to significant lignin breakdown. The preparation is with increased content of phenolic –OH groups compared to other lignin preparations. Anthraquinone is used as a catalyst, which increases the yield of cellulose and lignin. This process mostly does not use woody plants, but cereal straw, sugarcane hemp, hemp, sisal, abaca, flax, jute, etc [98].

11.1.8. Lignosulfonates

The chemical process of sulfite pulping (delignification) involves the use of sulfite (SO_3^{2-}) or bisulfite (HSO_3^-) salts (typically sodium, magnesium, ammonium or calcium) at high temperatures (140–170 °C). During this process, lignin with a reduced molecular weight is formed because of the breaking of ester bonds that interconnect lignin units (fragmentation). At the same time, the introduction of sulfonic groups on the aliphatic chains makes the lignin water-soluble (sulfonation). The insoluble cellulose fibres are separated from LSs by filtration. The resulting by-product, called 'spent liquor', contains poorly sulfonated lignins of different molecular size, inorganic salts, extracts from wood and pentose and hexose sugars coming from the acidic hydrolysis of the hemicellulose. LSs for concrete applications are further modified to achieve the desired properties [99].

11.1.9. Organosolv lignin

The organosolv process is another important method for making sulfur-free lignin from biomass. To date, there is no commercial organosolv lignin on the market. Most efforts are still at laboratory scale. The principle of the organosolv method is to extract lignin by solubilization in organic solvent or solvent mixtures. Methanol, ethanol, acetone, ethylene glycol, and 1,4-dioxane have been among the most commonly used organic solvents for lignin extraction and isolation. Organosolv lignin is highly soluble in organic solvents but insoluble in water. Due to this hydrophobic characteristic, organosolv lignin can be easily precipitated and recovered by adding water to the solution [100].

11.2. Lignin characterization:

Currently, there are no analytical methods showing the complete characterization of lignins. Very often there is a need to characterize certain lignin structural properties, depending on the area in which it is intended to be used. To get as much as possible information about it, it is necessary to combine different methods. Analytical studies of lignins can be divided into several groups: determination of functional groups, elemental analysis, spectrometric methods, degradation

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experiments and thermal analysis. Lignin chemical and instrumental methods are used to study the structure.

The Ceizel-Fibeck-Schwapach method is most widely used for the determination of methoxyl groups, which involves quantitative cleavage of these groups with hydroiodic acid to form methyl iodide. The total nuclear magnetic resonance is used to determine hydroxyl groups, acetylation, methylation, reactions among others. Hydroxyl groups of phenol are determined using potentiometric and conductometric titration, hemosorption and methylation methods, differential UV spectrometry, etc. Aliphatic hydroxyl groups are determined as the difference between total and phenolic content of hydroxyl groups, as well as by NMR. The reaction with hydroxylamine hydrochloride is used for the most extensive determination of aldehyde and keto groups. Carboxyl groups are determined using mainly potentiometric and conductometric titrations. Elemental analysis, together with the detection of methoxyl groups, gives information about the C_9 unit average content in lignin. The most commonly used spectrometric methods are UV and FTIR spectroscopy, nuclear magnetic resonance spectroscopy (PMR and C^{13}-NMR), mass spectrometry combined with gas chromatography and electron spin resonance spectroscopy (EPR) [101,102].

Degradation methods include chemical degradation treatment techniques: ethanolysis, acidolysis, hydrogenolysis, mild hydrolysis, thioacetolysis, oxidation (with permanganate, nitrobenzene, copper (II) oxide), ozonation, as well as the thermal combustion method. These methods are based on splitting the lignin molecule into submolecular products, whose identification gives information about the structure of the macromolecule and the types of bonds in it.

11.2.1. Analytical pyrolysis

Analytical pyrolysis belongs to the most prospective destructive methods in wood chemistry, it is traditionally used in the study of the structure of lignins. The analytical pyrolysis method is fast because it does not require complex chemical treatment of the sample; the preparation procedure consists of drying and grinding the sample. Lignocelluloses in the process of analytical pyrolysis of materials, polysaccharide and lignin degradation products are broken down by gas chromatography and identified by mass spectrometry. In pyrolysis, the molecule is heated cleaved at specific sites with low bond energies. Under analytical pyrolysis conditions, the breaking of ether and carbon-carbon bonds occurs and, as a result, lignin breaks down to monomers and dimer compounds. The method is useful for various backgrounds for rapid comparison of lignins and is widely used to study lignin structure. Within the pyrolysis methods, it is possible to classify the samples based on the relative content of guaiacyl and syringyl derivatives of the products as guaiacyl-type or guaiacyl-/syringyl-type lignins [102,103]. The Py-GC/MS analysis is performed using a Frontier Lab (Japan) Micro Double-shot Pyrolyser Py-2020iD (pyrolysis temperature 500 °C, heating rate 600 °C s−1) directly coupled with the Shimadzu GC/MS-QP 2010 apparatus (Japan) with capillary column RTX-1701 (Restec, USA), 60m×0.25mm×0.25m film (the injector temperature 250 °C, ion source 523K with EI of 70 eV, the MS scan range m/z 15–350, carrier gas helium at the flow rate of 1mLmin^{-1} and the split ratio 1:30). The mass of a sample probe (residual moisture content < 1%) is 1.00–2.00 mg. The oven program is 1 min isothermal at 60°C, then 6 °Cmin^{-1} to 270 °C, and finally held at 250 °C for 10 min. The apparatus is modified by installation of the splitter of gas-carrier flow Vitreous Silica Outlet Splitter VSOS (SGE, Australia) in order to operate FID and MS detectors simultaneously. The mass spectrometer is operated in the electron impact mode using 70 eV electron energy. The identification of the individual compounds is performed on the basis of

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GC/MS chromatogram using Library MS NIST 147.LI13, whereas the relative area of the peak of individual compounds is calculated using the Shimadzu software on the basis of GC/FID data. The summed molar areas of the relevant peaks are normalized to 100%.

11.2.2. SEC/GPC

For determining the molecular weight of lignin, gel filtration chromatography is most widely used, which is denoted also SEC (size exclusion chromatography) or GPC (gel permeation chromatography). According to the basic principle of this method, with smaller eluent volumes, large molecular fractions are separated, with larger volumes - submolecular fractions. Polystyrene is usually used for calibration standards [104]. The molecular weight properties of lignin samples are determined by gel permeation chromatography (GPC) using a Waters instrument System. Two to five mg of dried lignin sample is dissolved in HPLC-grade DMF, and the solution is filtered through a 45 µm syringe filter. HPLC-grade DMF, filtered through a 45 µm PTFE membrane filter and degassed, is used as the mobile phase. The flow rate of the mobile phase is set to 0.3 $mLmin^{-1}$. Separation is achieved on Waters APC XT 450 2.5 µm; APC XT 200 2.5 µm and APC XT 45 1.7 µm columns. A sample volume of 20 µL is injected into the GPC instrument and the UV signal is recorded at different wavelengths 254 and 280 nm and RI detector at the same time. Calibration is performed using polystyrene standards with nominal MW's ranging from 480 to 130,000 Da. The final analysis is performed using the intensity of both detector signals with the help of Waters Empower 3 build software.

11.2.3. Methoxyl groups:

The Ceizel-Fibeck-Schwappach method is a modified IWC procedure. The Principles of the method: by processing the organic compound containing methoxyl group, a quantitative reaction according to equation takes place.

$R\text{-}OCH_3 + HJ \rightarrow R\text{-}OH + CH_3J$

The formed methyl iodide as a volatile compound is quantitatively redistilled, taking in a mixture of sodium acetate and glacial acetic acid containing bromine. Bromine quantitatively reacts with methyl iodide according to the equation:

$CH_3J + Br_2 \rightarrow CH_3Br + JBr$

At the same time, the predominance of bromine oxidizes iodine bromide according to the equation:

$JBr + 2Br_2 + 3H_2O \rightarrow HJO_3 + 5HBr$

By adding an excess of potassium iodide and sulfuric acid to this solution, free iodine is released, according to the equation:

$HJO_3 + 5HJ \rightarrow 3J_2 + 3H_2O$

The released amount of iodine is equivalent to the content of methoxyl groups in the weight taken, and it is determined by titration with sodium thiosulfate according to the equation:

$3J_2 + 6Na_2S_2O_3 \rightarrow 6NaJ + 3Na_2S_4O_6$

~40 mg of analyte is weighed on an analytical balance and the reactions are transferred to a flask and ~0.5 g of phenol, 6 drops of acetic anhydride and 3 mL of 57 % of hydroiodic acid solution added. The plate is half filled with red phosphorus suspension in water. 15 mL of bromine solution is poured into the receiving tube through a funnel, a bubbler is inserted and attached to the device. The receiving tube is connected through a line to a washcloth containing 10%

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HCOOH solution. The reaction flask is connected to a N_2 and held for 30 minutes in a temperature-controlled bath at 140 °C, while simultaneously following a steady flow of N_2. The receiver solution is quantitatively rinsed into a 250 mL Erlenmeyer flask with a polished stopper into which 25 mL of 20% CH_3COONa had previously been poured. By vigorously rinsing, 4% HCOOH is added from the burette to the red-brown solution, immediately 4 mL, then the reagent is addeddrop by drop until the solution became colorless. Then 2 drops of methyl red solution are added and a color change is observed. When all the excess bromine is distributed, 20 mL is added to the flask 10% H_2SO_4 and 5 mL of 10% KI, the flask is capped and allowed to stand for 5 min. The released iodine is titrated with 0.05 M $Na_2S_2O_3$ to a light orange color, then 2 mL of starch is added to the solution and the titration is completed by rinsing vigorously.

11.2.4. Determination of total hydroxyl groups

$-OH_{phen.}$ and $-OH_{COOH}$. conductometric titration is used to determine the lignin functional groups (acidic $-OH_{COOH}$ and phenolic $-OH_{phen.}$). They are often analysed by an "acid - base" conductometric titration method. By introducing alkali (direct titration), the degree of ionization of acidic groups increases, and thus the total in the system increases electrical conductivity. When such a system is titrated with an acid, the opposite occurs (reverse titration), and, according to the acidity, first the weak acidic $-OH_{phen}$, then the most acidic $–OH_{COOH}$ groups are titrated. The total quantity of hydroxyl groups is determined by acetylation according to methodology developed by LSIWC.

The method is based on an aliphatic and phenolic hydroxyl group's reaction with acetic anhydride in the presence of a pyridine catalyst resulting in a free acetic ion. Approximately 10 mg of the sample is weighed into a chromatography bottle, 0.1 mL acetylating mixture prepared fresh on the day of analysis (4 mL pyridine and 4.7 mL acetic anhydride) is added. The added mass of the acetylating mixture is weighed. The bottle is closed and treated for 24 hours at 50 °C. Then it is rewashed quantitatively with acetone and water into a beaker, approximately 20 mL of water is added and titrated with 0.1 M NaOH solution.

11.2.5. Determination of acid hydroxyl groups

The hemosorption method - developed for the analysis of insoluble natural polymers. The method is based on ion exchange reactions between the acidic groups of the substance and $Ba(OH)_2$ or $(CH_3COO)_2Ca$. The predominance of $Ba(OH)_2$ is titrated with acid and the content of acidic groups in the preparation is calculated from the amount of bound Ba.

11.2.6. FTIR analysis

FTIR spectroscopy, which is a complementary and extensively used method, yields information about the molecular conformation and hydrogen bonding patterns. Some important peaks of the spectra are explained. The FT-IR spectra of absorption peaks at 3396 cm^{-1} denote O–H stretching vibration, and the absorption peaks at 2924 cm^{-1} represent C–H stretching vibration, while the absorption bands at 1727 cm^{-1} are the stretching vibration peaks of C=O, which are the characteristic absorption peak of xylan. The peaks at 1631 cm^{-1} are the skeleton vibration of lignin benzene ring with weak C=O vibration, and those at 1511 cm^{-1} are the skeleton vibration of aromatic ring. The absorption peaks at 1380 cm^{-1} are the C–O or OH bending vibration of lignin benzene ring, and the absorption bands at 1233 cm^{-1} denote the ether linkages between hemicellulose and lignin. The absorption bands at 1066 cm^{-1} are the C–O stretching vibration on C–O–C, and those at 890 cm^{-1} are a β-(1,4) glycosidic bond[105].

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11.2.7. Thermal analysis

The thermal degradation patterns of the lignin gives an evidence of their thermal stability. Among the lignocellulosic materials, lignin is the most thermo-stable component mainly due to the inherent structure of aromatic rings with various branches. Because the reactivity range of lignin is quite wide, the degradation of lignin occurs within a wide temperature range. The thermal degradation of lignin could take place in the following steps: 1) cleavage of α- and β-aryl-alkyl-ether linkages occurs between 150 °C and 300 °C; 2) aliphatic side chains start splitting off from the aromatic ring around 300 °C; 3) the carbon-carbon linkage between lignin structural units is cleaved at 370-400 °C decomposition or condensation of aromatic rings is believed to take place at 400-600 °C. Most lignin shows their maximum rate of weight loss between 300 °C and 400 °C [106].

11.2.8. ^{1}H–, ^{13}C–, and ^{31}P–NMR Spectroscopy for Lignin Analysis.

The analytical techniques of ^{1}H–NMR, ^{13}C–NMR, and ^{31}P–NMR are essential tools for the determination of lignin structural features impacting upon delignification and brightening reactions. Below is a brief review of the application of NMR spectroscopy for lignin analysis. Proton–NMR is able to quantify a number of important residual lignin structural features including: carboxylic acid (δ 12.6–13.5 ppm), aldehyde (δ 9.4–10.0 ppm), phenolic hydroxyl (δ 8.0–9.4 ppm), β–5 phenolic hydroxyl (δ 8.99 ppm), syringyl C_5 phenolic hydroxyl (δ 8.0–8.5 ppm), aromatic protons (δ 6.3–7.7 ppm), and aliphatic protons. Proton-NMR has also been used for the quantification of structures in lignin related humic acid and fulvic acid samples. The major advantages of ^{1}H–NMR are no modification of the residual lignin is required and the high intrinsic sensitivity allows for the use of a small sample size and a short acquisition time. ^{1}H-NMR of Acetylated Lignin samples provides improved spectral resolution of key lignin functionality [107]. Carbon(13)–NMR is a powerful technique capable of revealing a large amount of lignin structural information including the presence of aryl ether, condensed and uncondensed aromatic and aliphatic carbons [108]. Quantitative 13C–NMR analysis requires a number of conditions to be fulfilled. First, the lignin sample must be free of contaminants such as carbohydrates or extractives. Also, the lignin/solvent solution must be made as concentrated as possible to maximize signal– to–noise and minimize baseline and phasing distortions. Generally, 13C–NMR spectra of concentrated lignin/DMSO–d6 are acquired at 50ºC in order to reduce viscosity. Hydroxyl functional groups in isolated lignins have been identified by a ^{31}P–NMR technique that involves derivatization with the phosphorylating agent 2–chloro–4,4,5,5– tetramethyl–1,3,2–dioxaphospholane (TMDP). TMDP reacts with hydroxyl functional groups to give phosphite products which are resolvable by 31P–NMR into separate regions arising from aliphatic hydroxyl, phenolic, and carboxylic acids groups. The major advantages of the TMPD/31P–NMR is that the technique is well developed, and a database of model compound spectral information is available. An additional derivatizing agent, 2–chlor–1,3,2–dioxaphospholane, has been reported to allow for the discrimination between primary and secondary hydroxyl groups and also to differentiate between erythro– and threo–conformations.

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12. OTHER COMPOUNDS

12.1. Starch

12.1.1. Extraction of starch

Starch can be separated from biomass by various methods - water steeping[109,110], alkaline steeping[111,112], and acid steeping[111], by etanol method[113] or enzymatic[114–116]. Thermal, mechanical or microwave treatment can be used to improve the yield of the separated starch. The choice of the most appropriate method depends on the amount of starch in the raw material and its binding to other substances in the matrix.

12.1.2. Starch determination

There are two most popular methods for determining starch in biomass - colorimetry and HPLC. In the first one, iodine compounds are used, which color starch-containing samples, and the amount of starch is determined colorimetrically[117]. In the second case, enzymatic or acid hydrolysis of starch is performed first, so that it does not affect the content of hemicelluloses and starch, and then the amount of starch is determined by HPLC[118].

12.2. Determination of proteins

Protein in biomass is difficult to measure directly. In many cases the nitrogen content of the biomass sample is measured by combustion or Kjeldahl methods and the protein content is estimated using an appropriate Nitrogen Factor [119].

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13. CONCLUSIONS

Today, many different methods of separation and characterization of biomass components have been developed, starting with classical analytical chemistry methods (e.g., gravimetry and titration) and ending with precise instrumental methods, paying special attention to the main components of biomass - cellulose, lignin and hemicelluloses.

In order to choose the most appropriate separation or characterization method, the expected frequency of use of the method and the purpose of using the available equipment and method should be taken into account.

Table 6 shows a summary of the recommended analysis depending on the times of analysis, the application and the place (rural or industrial).

Table 7. Recommended characterisation methods.

Where	Times of analysis	Macro-components and individual components		Recommended analysis
Rural areas	Several times	Physical	Moisture	Drying
	Several times		Particle size	Sieving
	Few times		Thermal behaviour	Depends on the application
	Several times	Extractives		Extraction, depending on the raw material
	Several times	Cellulose		Kürschner-Hoffer method
	Several times	Hemicellulose		By difference between holocellulose and cellulose
	Several times	Lignin		Total lignin depending on the application
Rural areas with a central place for analysis	Few times	Physical	Moisture	Karl Fisher titration
	Few times		Shape and particle size	Grindability tests
	Few times		Thermal behaviour	TGA
	Few times	Ash		ASTM and TGA
	Few times	Cellulose	Total sugar content	Hydrolysis and HPLC
	Few times	Hemicellulose	Sugars and inhibitors	Hydrolysis and HPLC (depending on the application)
	Few times	Lignin	Toghether with the rest of fractions	FTIR

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Where	Times of analysis	Macro-components and individual components		Recommended analysis
SEMs (more specific app.)	Several times	Physical	Moisture	Drying and Karl Fisher titration
	Several times		Shape and size	Sieving and grindability tests
	Several times		Density	ASTM
	Few times		Moisture sorption	EMC
	Few times		Thermal	Depending on the application
	Few times	Ash	Ash content	ASTM
	Few times		Individual compounds	XRF, SRD, CCSEM
	Several times	Extractive	Total extractives	Extraction
	Few times		Individual extractives	LC
	Several times	Cellulose	Total cellulose	Kürschner-Hoffer method
	Few times		Individual components	Hydrolysis and HPLC/RID, FTIR
	Several times	Micro and nano cellulose		SEM, TEM, AFM, DLS, morphology
	Several times	Hemicellulose	Total hemicellulose	By difference between holocellulose and cellulose
	Few times		Individual components	Hydrolysis and HPLC/RID, FTIR
	Several times	Lignin	Total lignin	Total methods depending on the application
	Few times		Individual components	SEC/GPC, FTIR, NMR
	Few times	Others	Starch	Extraction
	Few times		Proteins	Kjeldahl method

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