Universidade de Aveiro
Ano 2013/2014

Departamento de Química

Rute das Neves Sarabando Carlos

Desconstrução de biomassa vegetal por auto-hidrólise

Deconstruction of plant biomass by autohydrolysis

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Química no ramo de Química Analítica e Qualidade realizada sob a orientação científica do Investigador Luís Jorge A. Chorão de Q. Duarte, Investigador Auxiliar da Unidade de Bioenergia do LNEG e coorientação do Doutor Artur Manuel Soares da Silva, Professor Catedrático do Departamento de Química da Universidade de Aveiro

Dedico este trabalho aos meus Pais e ao meu Mano

O júri

Presidente

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Palavras-chave Auto-hidrólise; Biorrefinaria; Hemiceluloses; Materiais lenhocelulósicos; Oligossacarídeos

Resumo

A procura pelo melhor aproveitamento da biomassa vegetal tem vindo a despertar o interesse no uso racional de todos os seus componentes, tendo de se dar especial atenção à valorização das hemiceluloses, uma das frações mais desafiantes.

Neste trabalho selecionaram-se dois resíduos vegetais, a biomassa do Cipreste-Português ou Falso-Cedro-do-Buçaco (*Cupressus lusitanica Mill.)* tendo-se estudado os seus principais constituintes (madeira, casca, folhas e gálbulas) e as cascas de melancia (*Citrullus lanatus)*, principal resíduo da potencial utilização industrial deste fruto.

Estudou-se a composição química dos diferentes materiais e otimizou-se o processo de auto-hidrólise para a produção de oligossacarídeos com potenciais novas funcionalidades.

Os resultados demonstram a elevada seletividade do processo de auto-hidrólise para a remoção das hemiceluloses, principalmente para a estilha do Cipreste-Português, tendo-se obtido elevadas concentrações de oligossacarídeos. Os diferentes oligossacarídeos foram caracterizados relativamente à sua composição e estabilidade química sob diferentes temperaturas e valores de pH e discute-se a sua potencialidade para aplicações na indústria alimentar.

Por forma a perspetivar uma valorização integrada dos materiais no conceito da biorrefinaria, estudou-se ainda a utilização da biomassa de Cipreste-Português como fonte de óleos essenciais e a digestibilidade enzimática da celulose presente na biomassa residual obtida do processo de auto-hidrólise como método de recuperação de glucose para fins fermentativos.

Keywords Autohydrolysis; Biorefinery; Hemicelluloses; Lignocellulosic materials; Oligosaccharides

Abstract

The search for better technologies for upgrading biomass wastes has received increasing attention in the recent years. Special consideration has to be given to the hemicellulosic fraction, one of the more challenging fractions.

In this work, two biologically different waste materials were iorefinery concept: the materials derived from the forestry processing of the cypress cedar of Goa (*Cupressus lusitanica Mill.*), namely chips, bark, leaves and cones, and the wastes of the watermelon (*Citrullus lanatus*) industrial processing (rinds).

The chemical composition of all materials was characterized taking special attention to the extractives content and to the structural polysaccharides and lignin and the autohydrolysis process was optimized aiming for the production of novel oligosaccharides.

The autohydrolysis process presented a high selectivity towards hemicelluloses, yielding oligosaccharides in high concentrations, especially for the chips of cypress cedar of Goa.

The oligosaccharides were characterized regarding their chemical composition and their stability under different temperature and pH values. Their potential industrial applications, namely for the food industry, are also presented and discussed.

As a preliminary evaluation of the integrated upgrade of these materials, the use of biomass from cypress cedar of Goa as a source of essential oils and the digestibility of the cellulose present in the residual biomass derived from the autohydrolysis treatments as a source of glucose for fermentation were also tested and are discussed.

Abbreviations

Ac	Acetyl groups
AOS	Arabino-oligosaccharides
Ara	Arabinose
Arn	Arabinan
Ash	Ash
CZE	Capillary Zone Electrophoresis
DAD	Diode Array Detector
Furf	Furfural
Glc	Glucose
Gn	Glucan
GOS	Gluco-oligosaccharides
H	Moisture
HAc	Acetic acid
HFor	Formic acid
HLev	Levulinic acid
HMF	5-Hydroxymethylfurfural
HPLC	High Performance Liquid Chromatography
LK	Klason lignin
LNEG	Portuguese National Laboratory for Energy and Geology (Laboratório Nacional de Energia e Geologia[1])
LSR	Liquid-to-solid ratio
NREL	National Renewable Energy Laboratory[2]
OS	Oligosaccharides
Xn	Xylan
XOS	Xylo-oligosaccharides
Xyl	Xylose

[1] www.lneg.pt
[2] www.nrel.gov

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Framework

This work was carried out at the Bioenergy Unit of the National Laboratory of Energy and Geology (LNEG), under the project "SSAD – Deconstruction of Biomass using solid superacids" (PTDC / AGR-ALI / 122261/2010) co-funded by the Foundation for Science and Technology and FEDER Program (Programa Operacional Fatores de Competitividade – COMPETE).

In this work, the process of autohydrolysis of two biomass residues, *Cupressus lusitanica Mill.* and *Citrullus lanatus* (watermelon), is studied for the production of different classes of oligosaccharides (OS) with new features. Also special attention is given to the chemical characterization and the reactivity of the OS produced.

In the first chapter of this dissertation the concept of biorefinery as an alternative to the valorization of plant biomass is presented. Also, the importance of OS as value-added products derived from hemicelluloses is discussed and autohydrolysis is presented as the main process to obtain these.

The second chapter describes the preparation of the materials, the experimental methods used in the processing of autohydrolysis and the main analytical methods.

Finally, the autohydrolysis optimization results are presented for three residues from the *C. lusitanica* and *C. lanatus* and their future potential is discussed.

1 Introduction

1.1 Biomass as a source of renewable raw materials

Biomass is the renewable source with the largest potential for energy, biofuels and chemicals production, and its use is strongly encouraged by the Europe Union (1,2). According to Directive 2009/28/CE, biomass is the biodegradable fraction of products and residues from agriculture (including substances of vegetal and animal origin), forestry and related industries, as well as, the biodegradable fraction of industrial and municipal wastes (3). The main advantages associated with its use, compared with "fossil resources", are reflected in the decrease of environmental and economic concerns, the increasing security of supply by a multiplicity of sources and possibilities of use and its great potential when used as source of raw materials for the production of renewable biofuels, biomaterials and chemical products (1). For example, many biofuels can be produced from biomass, such as methanol, ethanol, hydrogen, dimethyl ether, synthetic natural gas, synthetic diesel, bio-oil and biodiesel, among others (1,2,4). Biofuels have a high potential to reduce greenhouse gas emissions, and hence are an important means to fulfil CO_2 emission targets and gradually reduce the dependence on oil importation.

Currently, agricultural and forestry systems exploit only part of their production, i.e. "primary" products, while significant quantities of "residues" are not exploited. The use of both primary and residual resources through integrated and sustainable pathways should be promoted. It is also necessary that currently discarded biomass fractions are utilized and to make the best use of the whole plant.

1.2 Biorefinery

Allied to the full utilization of plant biomass, the concept of biorefinery arises. A biorefinery is a facility that integrates biomass conversion processes, such as bioengineering, polymer chemistry, food science and agriculture, and equipment to co-produce value-added chemicals, fuels, heat, and power from various biomass sources, with minimal waste generation and emission of harmful gases (1,5) . The range of products of a biorefinery includes not only the products produced in a petroleum refinery, but also products that are not accessible to petroleum refineries (Figure 1) (6,7).

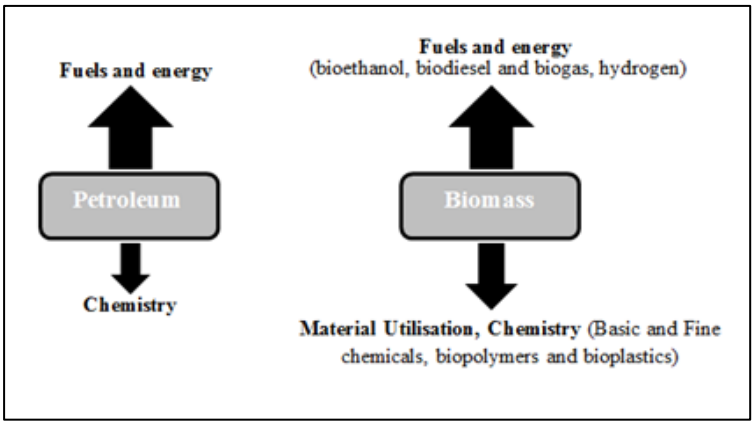


Figure 1 Comparison of the basic-principles of petroleum-refinery and biorefinery.

(adapted from (1))

1.3 Chemical and structural composition of plant biomass

The plant biomass can be divided into three broad categories, depending on their biological origin: resinous plants ("softwoods"), broadleaf plants ("hardwoods") and herbaceous (grasses and cereals) (8-10).

The softwoods belong to the group of gymnosperms and present as distinctive features the foliage in the form of needle and absence of fruits (11). At the level of the wood, they are considered "non-porous" woods because they do not contain porous vessels. In structural terms, the softwoods are relatively simple and consist essentially of a single type of elongated cells (2 to 5 mm), called tracheids. Pines emerge as a typical example of softwoods (8,9).

The hardwoods belong to the group of angiosperms, whose distinctive features are broad leaves and seeds enclosed in fruits. The angiosperms have "porous" woods because they have porous vessels. The hardwoods, in addition to being structurally complex, still exhibit a greater diversity of cellular standards organization. Oaks appear as a typical example of hardwoods (8,9).

The grasses are a very extensive family of annual and perennial herbs whose distribution is widespread, occurring in different regions, from sea level to mountain areas. They are characterized, in general, as monocotyledonous small herbs, stoloniferous, with stems usually hollow and articulated by solid knots, rarely modified and more or less woody. Most species have linear leaves, flowers and usually fascicular roots (8,9).

1.3.1 Ultrastructure of plant cell wall biomass

The plant biomass consists of tissues composed of cells with thick cell walls, whose shapes and sizes vary with the species of origin of the material. Their ultrastructure, the internal organization of the cell wall of different cells, is complex (Figure 2) (8,9,12). The layers that make up the cell have a concentric arrangement, resulting from differences in the chemical composition or orientation of the structural elements. Through this arrangement, the components are subdivided into structural components (cellulose) and substructural components (hemicelluloses and lignin) (8).

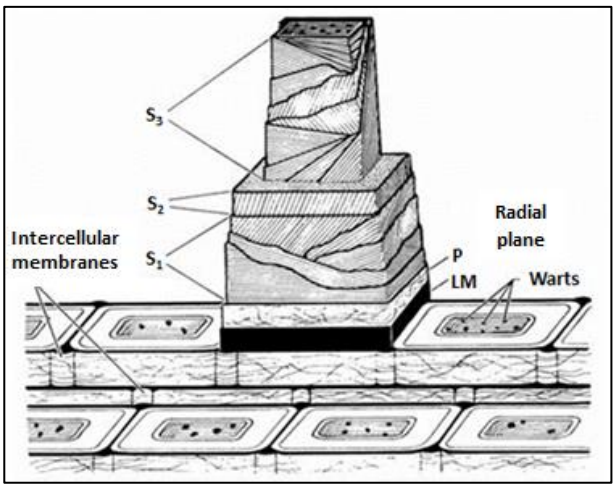


Figure 2 The ultrastructure of the plant cell wall.
(adapted from https://fp.auburn.edu/sfws/sfnmc/class/tangwood.htm)

The structural integrity of plants cells results from the existence of different layers which differ from each other both in structure and chemical composition: middle lamella (ML), primary wall (P), secondary wall, and W layer (Warts) (8,9,12). At each layer, the cell wall consists of three major components, cellulose microfibrils, hemicelluloses and either pectin in the primary wall (or growth wall) or lignin in the secondary wall (8,9).

Between individual cells there is a thin layer, called the middle lamella, which has the function to connect the cells together, forming the tissue. Although the middle lamella is, in principle, free of cellulose, it can be crossed by cellulosic fibrils. It contains a high content of pectin, especially in the initial stage of formation of the cell. Its thickness, except for the corners of the cells, ranges between 0.2 to 1 µm (8,9).

The primary wall (P) consists of cellulose microfibrils, hemicelluloses, pectins and proteins. It is a thin layer, whose thickness ranges from 0.1 to 0.2 µm. The cellulosic fibers are arranged in thin crossing layers, giving an aspect of mesh. This is the first layer

deposited during cell development, enabling its growth. As a consequence of cell expansion, the microfibrils at the outermost layer have a more oblique orientation. The middle lamella, together with the primary wall, is often referred to as middle lamella composite (8,9).

The secondary wall is the thickest layer of the cell wall, and it is deposited in the primary wall after its growth is complete. It has a high content of cellulose and lignin in comparison with the primary wall, which gives a dense, parallel arrangement, depending on the fibrils. Its thickness can vary from 1 to 10 µm. The secondary cell wall is subdivided into three layers: the outer (S_1), medium (S_2) and internal (S_3) layers (8,9).

In the outer secondary layer (S_1), whose thickness ranges from 0.2 to 0.3 µm, cellulosic fibrils have a very marked helical orientation. The angle formed between the fibrils and the axis of the cell may vary between 50 and 70º (8,9). Due to its high lignin content, it is more resistant to fungal attack than the medium secondary layer (S_2) (9). There are several sublayers, which are extremely thin and overlap each other, and thus the helical arrangement of the fibrils can be seen as a crossover arrangement in certain species.

The medium secondary layer (S_2) is the thickest layer of the cell wall, constituting the most important portion of the cell wall, whose thickness ranges from 1 to 9 µm. In this layer the fibrils are arranged perpendicularly to the axis of the cell and can vary between 10 and 30º, decreasing with increasing length of the cell (8,9).

In the inner secondary layer (S_3) the cellulose fibrils have a gentle slope, but not in a strictly parallel manner. It is a thin layer (0.1 µm) and contains a higher concentration of non-structural substances (hemicelluloses and lignin), which gives the surface a more or less flat appearance (8,9).

The W layer (Warts), also known as rough inner zone, is the name given to an amorphous, thin membrane located on the inner surface of the layer S_3, which consists of small quantities of carbohydrates, pectins and lignin similar material (8,9).

The system of arrangement and the arrangement of the cellulose fibrils in combination with non-structural substances gives the cells of the vegetal biomass a strong but flexible constitution, which is resistant to a variety of forces that act upon it (8).

1.3.2 Chemical constituents of plant biomass

Plant biomass carries a complex structure, consisting mainly of polysaccharides – cellulose (35-50 %) and hemicelluloses (20-35 %) – and lignin (10-25 %) (Figure 3), whose percentages depend on the biological origin of the raw material (11,13-15). In smaller proportions, other constituents are present, such as pectin and low molecular weight compounds such as fatty acids, vitamins, essential oils and minerals (especially calcium (Ca), potassium (K) and magnesium (Mg)) (11,16).

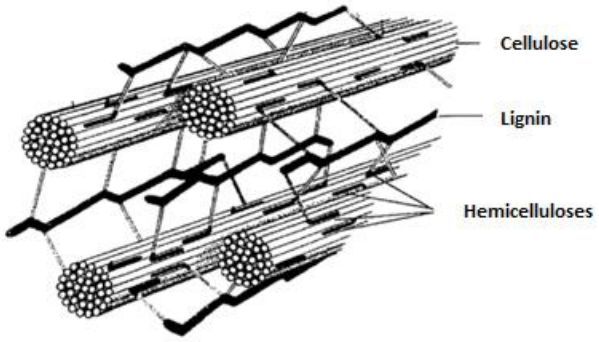


Figure 3 Structural components of plant biomass.
(adapted from http://www.intechopen.com/books/ionic-liquids-new-aspects-for-the-future/applications-of-ionic-liquids-in-lignin-chemistry)

Table 1 shows the chemical composition of various materials of plant origin. In general, agricultural materials are richer in hemicelluloses, hardwoods are those with the highest percentage of cellulose, while softwoods are richer in lignin.

Table 1 Chemical composition (structural components) of various materials of vegetable origin.

Type	Raw material	Cellulose[a]	Hemicelluloses[a]	Lignin[a]	Ref.
Herbaceous	Sorghum straw	35.1	24	25.4	(17)
Herbaceous	Wheat straw	33-40	20-25	15-20	(14,18)
Herbaceous	Corn cobs	34.3	39.0	14.4	(19)
Herbaceous	Sugarcane bagasse	35-42.8	26.2-35.8	16.1-25.2	(14,20)
Herbaceous	Rice husks	36.7-37.7	16.7-17.3	21.3-22.1	(14,21)
Hardwoods	Eucalypt	38-54.0	15-30	23.1-37	(22-24)
Hardwoods	Oak	38.9-44	18.7-23.8	21.5-24.7	(25)
Hardwoods	Red maple	46.0	22.5	24.0	(26,27)
Softwoods	Pine (*Pinus pinaster)*	39.2	18.4	26.3-30.2	(28,29)
Softwoods	Douglas-fir	44.0	21.2	32.0	(30)
Softwoods	Lobolly pine (*Pinus taeda*)	42.38-43.6	19.33-21.2	26.8-27.99	(31,32)
Softwoods	Red Pine (*Pinus resinosa)*	36.7	19.8	34.1	(33)

[a] values expressed in g/100g dry material

1.3.2.1 Cellulose

Cellulose $(C_6H_{10}O_5)_n$ is the most abundant biopolymer in the world and the main component of the primary wall of plant cells. Cellulose is a linear homopolysaccharide of D-glucose (Glc), with high molecular weight, formed by glycosidic bonds of the type β-(1→4) (11-13,34,35).

Between the glucose units numerous intra- and intermolecular hydrogen bonds are established. The intramolecular bonds are responsible for the rigidity and linearity, and the intermolecular bonds result in the formation of microfibrils, highly ordered structures that associate forming cellulose fibers (12). The microfibrils have areas of high degree of crystallinity, in which the Glc chains are firmly connected in parallel, and regions with a lower degree of order, referred to as amorphous regions (13). In the crystalline region, the cellulose fibers have higher tensile strength and elongation and are insoluble in water, compared with the amorphous region, where the fibers have greater flexibility (13).

Depending on the source of plant material, the length of the cellulose chains can have a degree of polymerization (DP) between 1.000 to 15.000, where DP is the number of repeated units of Glc (34). However, its structural analysis shows that its repeating unit is in fact cellobiose (molecular structural unit), as evidenced in Figure 4 (13).

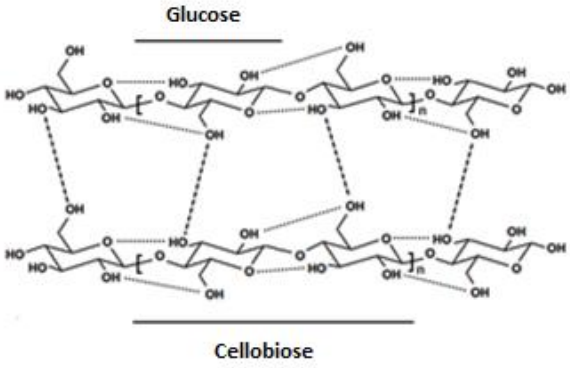


Figure 4 The chemical structure of cellulose.
(adapted from http://www.scielo.br/pdf/bjpp/v19n1/a01v19n1.pdf)

1.3.2.2 Hemicelluloses

Hemicelluloses are the second most common type of polysaccharides in the cell wall of plant biomass (20-35%) (13,14,34). They consist of a heterogeneous group of branched short-chain polysaccharides, formed by different biosynthetic pathways, resulting from the association of different sugar units, arranged in different proportions. These sugar

units include mostly aldo-pentoses (β-D-xylose and α-L-arabinose) and aldo-hexoses (β-D-glucose, α-D-galactose and β-D-mannose), and their hydroxyl groups (-OH) can be replaced by phenolic compounds, uronic groups or acetyl groups (Ac) (13,14,34,36). They also contain small amounts of hexuronic acids and deoxy-hexoses (Figure 5) (37).

Hemicelluloses have essentially a role of support and cohesion of the cell wall of plant biomass, linking the lignin to the cellulose by covalent bonds. Large variations may be verified in hemicellulosic content and chemical structure, depending on the type of biomass, and even among different components of an individual organism (stem, branch, seeds and bark) (14,38).

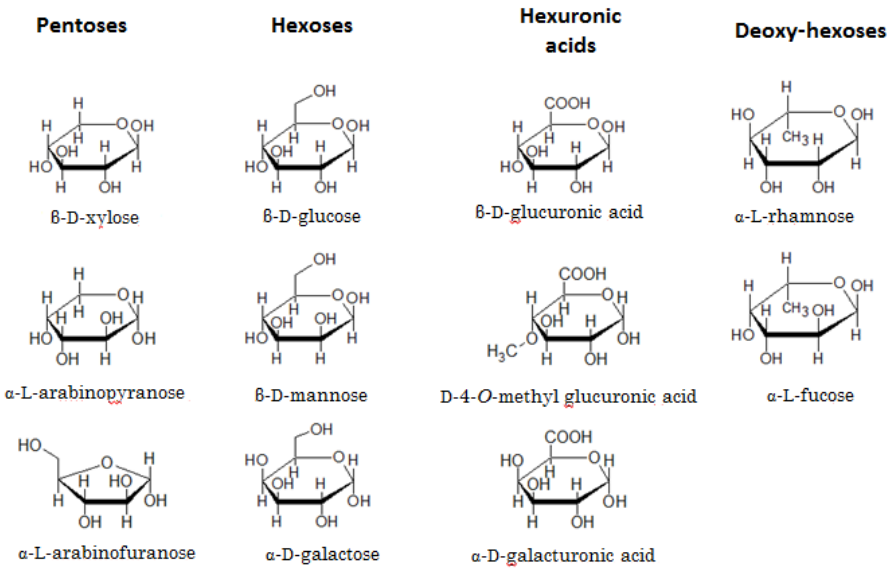


Figure 5 Diversity of monosaccharides and their derivatives present in the hemicelluloses. (adapted from http://www.scielo.br/img/revistas/rarv/v29n3/a14fig02.gif)

Table 2 presents some examples of raw materials of plant origin and their hemicellulose composition.

Depending on the biological origin of the plant, the hemicelluloses differ in their composition and monomeric structure and can be classified into four main types: xylans (Xn), mannans, β-glucans and xyloglucans, the first two being the most important (12,13,38).

Unlike cellulose, hemicelluloses are not chemically homogeneous and have low molecular weight, because their degree of polymerization varies only between 80 to 200

monosaccharide units, and do not contain crystalline regions (14,38). They are also defined as polysaccharides that can be extracted with aqueous alkaline solutions from plant tissue, unlike cellulose (13,38).

Table 2 Composition of the hemicelluloses obtained from different raw material.

Raw material		Hemicelluloses					Ref.
		Xyl[a]	Man[a]	Ara[a]	Gal[a]	Rha[a]	
Herbaceous	Wheat straw	19.2-21.0	0-0.8	2.4-3.8	1.7-2.4	-	(39)
	Rice husks	14.8-23	1.8	2.7-4.5	0.4	-	(40,41)
	Sugarcane bagasse	23.2-25.6	0.5	2.3-6.3	1.6	-	(42,43)
	Oak	21.7	1.0	2.3	1.9	-	(44)
	Eucalypt	14-19.1	1-2.0	0.6-1	1-1.9	0.3-1	(23,45,46)
	Willow	11.7-17.0	1.8-3.3	2.1	1.6-2.3	-	(26)
	Birch	18.5-24.9	1.8-3.2	0.3-0.5	0.7-1.3	0.6	(27)
Softwoods	Pine	5.3-10.6	9.4-15.0	1.0-1.2	1.9-4.3	0.3	(39,47,48)
	Firs	5.3-10.2	9.4-15.0	1.0-1.2	1.9-4.3	0.3	(39,47,49)

a values expressed in g/100g dry matter

Xylans correspond to the main hemicellulosic fraction found in the cell wall of hardwoods, although a small percentage of glucomannans can also be found (12,14,34-36). In some tissues of herbaceous, xylans can represent up to 50 % of their dry weight (13,14,38). Depending on the primary structure, xylans present in different plant tissues can be divided in glucuronoxylans, (arabino)glucuronoxylans and arabinoxylans (12,14,35,38).

Mannans (galactoglucomannans and glucomannans) are the major hemicellulosic components present in the cell wall of softwoods, but xylans can also be found (11,12,14,34-36). Softwoods have a high fraction of mannose and galactose units, unlike hardwoods that have a large amount of xylose (Xyl) units and Ac. In addition to the difference in the type of sugars that constitute the hemicelluloses, these are present in different proportions in softwoods (25 to 35 %) and hardwoods (24 to 40 %) (12).

β-glucans are defined as branched polysaccharides consisting of D-glucose units where 70 % are joined by glycosidic type β-(1→3) bonds and 30 % by type β-(1→4) bonds (38). They are present in cereal grains and softwoods, and are the key molecules associated with cellulose microfibrils during cell growth (38).

Finally, xyloglucans consist of linear chains of D-glucose units, whose glycosidic bonds are also of the type β-(1→4), and Xyl residues are found at position 6. These molecules are present in softwoods (12,38).

Below the major classes of xylans and mannans are discussed in greater detail.

1.3.2.2.1 Glucuronoxylans

The glucuronoxylans (4-*O*-methyl-D-glucuro-D-xylan) represent about 15 to 30 % of the dry weight of hardwoods and consist of linear polysaccharides composed of Xyl units linked by glycosidic type β-(1→4) bonds (38,50). Most of the residues of Xyl contain an Ac in carbons 2 and 3 (about seven residues of acetic acid (HAc) per every ten units of Xyl) and one out of ten molecules has attached an uronic acid group (4-*O*-methylglucuronic), linked by glycosidic type α-(1→2) bonds (38,50,51). The percentage of Ac varies with the hardwoods origin. In addition to these structural units, glucuronoxylans may also contain small amounts of L-rhamnose and galacturonic acid. The average degree of polymerization is between 100 and 200 (50).

1.3.2.2.2 (Arabino)glucuronoxylans

The (arabino)glucuronoxylans (L-arabino-4-*O*-methylglucuronoxylans) are present mainly in cereals and softwoods in smaller amount (5 to 10 % of dry weight) (52). Their main chain presents Xyl units, joined by glycosidic type β-(1→4) bonds with α-(1→2) branches of 4-*O*-methyltglucuronic acid and α-(1→3) branches of L-arabino-furanose (38,50,52). Compared with Xn from hardwoods, (arabino)glucuronoxylans in softwoods have lower amounts of Ac or even none, but may contain small amounts of L-rhamnose and galacturonic acid (50,52). The degree of polymerization varies between 50 and 185 (50).

1.3.2.2.3 Arabinoxylans

Arabinoxylans have been identified in several of the main commercial cereals, such as wheat, rye, barley, oat, rice and corn, as well as in other plants such as rye grass. They

represent the major hemicellulosic component of cell walls of the starchy endosperm (flour) and outer layers (bran) of the cereal grain (38).

Arabinoxylans have a linear backbone that is, in part, substituted by α-L-arabino-furanose residues positioned either on *O*-3 or *O*-2 (monosubstitution) or on both *O*-2 and *O*-3 (disubstitution) of the D-xylopiranose monomer units. In addition, phenolic acids such as ferulic and coumaric acid have been found to be esterified to O-5 of some L-arabino-furanose residues in arabinoxylans (38).

1.3.2.2.4 Glucomannans

Glucomannans represent 2 to 5 % of softwoods, although thet are also present in smaller amounts in the secondary wall of hardwoods (50). They consist of units of Glc and D-mannose joined by β-(1→4) glycosidic bonds, with an average degree of polymerization that varies between 40 and 70 (35). The relationship between the monomers of D-glucose and D-mannose may vary depending on the type of biological source of glucomannans (12,35,50).

1.3.2.2.5 Galactoglucomannans

The galactoglucomannans are the major hemicellulosic heteropolysaccharides present in softwoods, representing 20 to 25 % of its dry weight (35,50). The main chain is composed by Glc and D-mannose units, joined by glycosidic type β-(1→4) bonds, partially acetylated at carbons 2 and 3 and substituted by D-galactose units linked to Glc and D-mannose linked by type α-(1→6) bonds (35,50,53). The average degree of polymerization varies from 40 to 100 (51,53).

There is also the possibility of some galactoglucomannans presenting high solubility in water, due to the high amount of galactose that they may have in its constitution compared to the insoluble polysaccharides (52).

1.3.2.2.6 Arabinogalactans

Arabinogalactans have a backbone of β-(1→3) linked D-galactose units and are highly branched at C-6. The side chains are composed of β-(1→6) D-galactose units, D-galactose and L-arabinose units or single L-arabinose units and single D-glucuronic acid units. Arabinose (Ara) is present in furanose and pyranose forms in an approximate ratio of 1:2. Softwoods contain only small quantities of arabinogalactans amounting to less than 1 % by weight of wood (12).

1.3.2.3 Lignin

Lignin is considered one of the most resistant macromolecular organic substances in nature and is present in all plant biomass (Figure 6) (12). It is associated with cellulose and hemicelluloses, preventing the degradation of these polymers, since it confers a rigid and impermeable surface which is resistant to microbiological attack (13).

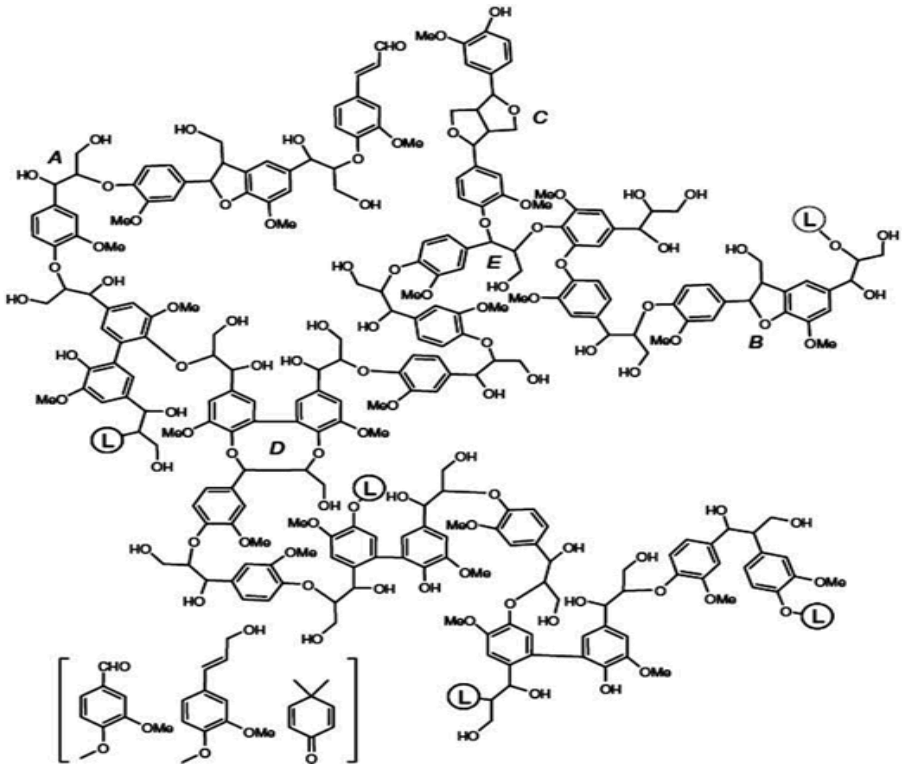


Figure 6 The chemical structure of lignin.
(adapted from www.intechopen.com/books/ionic-liquids-new-aspects-for-the-future/applications-of-ionic-liquids-in-lignin-chemistry)

The structure of lignin is very heterogeneous, with a three-dimensional shape formed by phenylpropane units, with methoxy substituents on the aromatic ring, linked together by ether type bonds and establishing cross-links between them (13,50). The main precursor of lignin in softwoods is *trans*-coniferyl alcohol (Figure 7) (12,13). In hardwoods, *trans*-sinapyl alcohol and *trans-p*-coumaryl alcohol are also lignin precursors (12,13) (Figure 7). The precursors are polymerized during lignin biosynthesis (12). In contrast to cellulose, lignin does not have a crystalline structure, and it is considered an amorphous polymer.

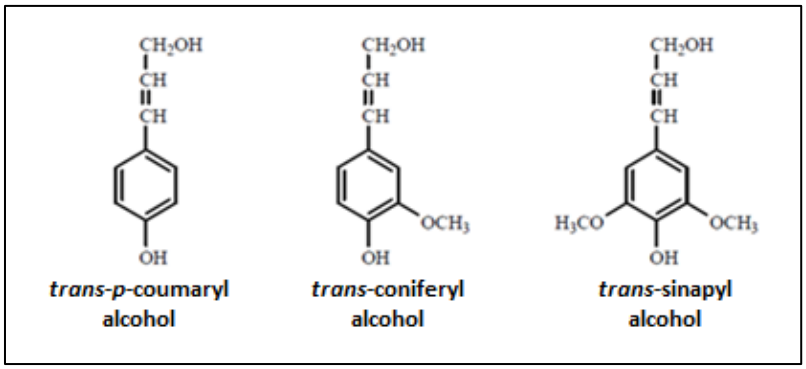


Figure 7 The alcohols precursors of lignin.
(adapted from (8))

Lignin does not contain sugars, and as such, it is usually considered as a waste of biofuel production, representing a major challenge to be converted into a value-added product. However, it is considered as a highly energetic component and it can be used to produce heat and electricity needed for biofuel production process (13).

1.3.2.4 Pectins

Pectins are the third group of polysaccharides of plant cell walls, existing in smaller amounts than cellulose and hemicelluloses (34,54). The structure of pectins is complex, being possible to recognize three structural polymers: homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII). HG is the most abundant type (about 60 % of the total pectin) and consists of a backbone of *R*-(1,4)-linked D-galacturonic acid residues. The structural unit can be esterified at C-6, and/or *O*-acetylated at *O*-2 and/or *O*-3. The RGI backbone is made of units of the dimer *R*-(1,2)-L-rhamnose-*R*-(1,4)-D-galacturonic acid. In this polymer, the rhamnosyl residues can be substituted at *O*-4 with neutral sugar side chains, mainly composed of galactosyl and/or arabinosyl residues. RGII is a region within HG, containing clusters of four different side chains (12,34,35,54). These structural polymers are highly susceptible to enzymatic hydrolysis, acid hydrolysis or hydrothermal treatments (54).

Pectin has found widespread commercial use, especially in the textile industry and in the food industry as a thickener, texturizer, emulsifier, stabilizer, filler in confections, dairy products, and bakery products, etc. (34).

1.3.2.5 Low molecular weight compounds

Plant biomass, as well as structural components of the cell wall, is also constituted by non-structural low molecular weight compounds. Though these represent a small

percentage of the total mass of biomass, they have a significant role in the properties and the quality of biomass (8,50). Regarding their chemical composition, the low molecular weight compounds belong to very different classes. Organic compounds are called extractables and inorganic compounds are commonly referred to as ash (8,12).

The extractables may be isolated with organic solvents, such as ethanol and dichloromethane, without inducing structural changes. They are classified into the following groups: terpenoids, phenolic compounds, alkaloids and esters of fatty acids (fats and waxes). Of the phenolic compounds, the most relevant are tannins, stilbenes, lignans, flavonoids and their derivatives (50). The extractable compounds correspond to 0-20 % of the dry mass of wood, being usually present in a smaller extent in softwoods than in hardwoods (55).

Relation to ash the main mineral components are potassium, magnesium and silicon (8,51).

1.3.2.6 Essential oils

Essential oils are reflected in the value presented by some herbs, present at concentrations lower than 2 %. Due to the pleasant and intense aromas of most of these volatile oils, they are also called essences (56). According to the International Standard Organization (ISO), an essential oil is a liquid consisting of complex mixtures of many volatile substances of plant origin, which are lipophilic and usually odoriferous. The designation of “oil” is due to some physicochemical characteristics as they are generally liquid and have an oily appearance, at room temperature. They are insoluble in water and slightly soluble in polar organic solvents, such as ethyl ether, receiving, therefore, also the name ethereal oils. However, their main feature is the volatility, distinguishing them from fixed oils (56).

Essential oils come from the secondary metabolism of plants and are commonly synthesized in the leaves and accumulated in the extracellular spaces, between the cuticle and the cell wall. These oils have a wide range of chemical constituents that result in different biological properties. Their chemical composition varies from hydrocarbons, alcohols, terpenes, ketones, aldehydes, phenols, phenolic esters, oxides to organic acids. These chemicals are present in different concentrations varying according to the nature of each plant (56).

They are retained in some plant organs such as the secretory organs, flowers, leaves, bark, trunk, roots, rhizomes or bulbs and seeds, or may even be present throughout the structure of plants, such as species belonging to softwoods. Although there is a great diversity of places where essential oils can be accumulated, their composition depends on their location. Thus, essential oils extracted from different parts of the same plant, despite having similar color and appearance, may have different physicochemical composition and odors (56).

The range of products incorporating essential oils in its composition is very diverse, ranging from the food industry, pharmaceuticals (products used in natural medicine) to cosmetics, nutraceuticals and, more recently, as insect repellents or insecticides (56).

1.4 Plant biomass fractionation

The first step for obtaining biofuels and other value-added products from plant biomass consists in a fractionation process. This should be an effective and selective separation of the main constituents of plant biomass – cellulose, hemicelluloses and lignin (13). However, this separation is compromised by several factors, both structural and compositional, that come from the close association between these chemical constituents (Figure 8) (13). Therefore, the application of a fractionation process, in addition to enabling to change or remove such structural barriers, increases the surface area of biomass, the porosity of the material and reduces the crystallinity of cellulose, to facilitate the access to other structural constituents (13,34).

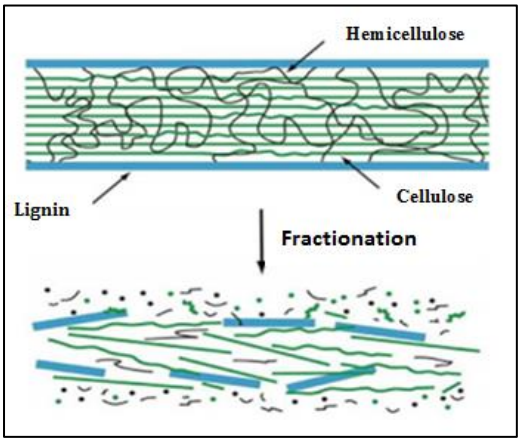


Figure 8 Schematic representation of the lignocellulosic biomass fractionation. (adapted from http://www.biomassmagazine.com)

There are a number of key features for the effective pretreatment of plant biomass. The determined fractionation process should have a low capital and operational cost. It

should be effective on a wide range of biomass loading and should result in the recovery of most of the structural components in usable separate fractions (13). In theory, the ideal fractionation process produces a disrupted, hydrated substrate that is easily hydrolyzed but avoids the formation of sugar degradation products and fermentation inhibitors. Assessment of biomass fractionation processes depends on a parameter called the "severity" factor, which is defined as the combined effect of temperature, acidity, and duration of fractionation (57). Fractionation process assessment is conducted by analyzing the sugars (both monomers and oligomers) released in the liquid and the carbohydrate content of the water insoluble solids after fractionation (13).

There are various processes of fractionation, with distinct yields and effects on plant biomass and consequent impact on subsequent steps. The conditions of these processes must be adapted to the desired chemical and structural composition of various biomass sources (13,34).

Physical, chemical or physicochemical processes have been developed to promote the separation or fractionation of various types of plant materials. However, it is worth noting that the same results are not always obtained in the fractionation of a certain type of material to another, i.e., a method that is efficient for a given material, may not be so for another.

Physicochemical methods are characterized by the use of heat, water and/or steam for fractionation of plant biomass and are therefore also known as hydrothermal processes (13,34,58). As a result of these operating conditions, the partial or complete solubilization of hemicelluloses is achieved (13,34,58). No chemical catalyst is added in these processes, making them economically and environmentally attractive (58). Of these processes, autohydrolysis is considered to present the greatest potential for industrialization and will be further discussed below.

1.4.1 Autohydrolysis

The term "autohydrolysis" is not the only one by which this fractionation method is known; there are also other terms, such as hydrothermolysis, hydrothermal pretreatment and aqueous fractionation (13 4246,58,59). It is simply based on the use of water and biomass as a reagents, using pressure to keep the water in liquid state (pressure above saturation point), at elevated temperatures (usually ranging between 150 °C to 230 °C) (13,37,58,60). The reaction time may vary from seconds up to hours, depending on the

temperature. In this way, there is a selective degradation of hemicelluloses, enabling the production of soluble chemical products, leaving a solid residue, composed mainly by lignin and cellulose (that are not degraded), which can be valued by subsequent processes (58). However, and depending on the used operating conditions, the main degradation of hemicelluloses yields the formation of a mixture of oligosaccharides (OS), monosaccharides, degradation products of monosaccharides and HAc, derived from the hemicellulose structure. Furthermore, 2-furfural (Furf) and 5-hydroxymethyl-2-furfural (HMF) resulting from the degradation of pentoses and hexoses, respectively (6,13,58,61,62) can also be present. These compounds can also be further decomposed, generating formic acid (HFor) and levulinic acid (HLev), respectively (62). The degradation of hemicellulosic monosaccharides, which results in the formation of hydrolytic inhibitors can be minimized if the fractionation process is conveniently optimized (61).

In summary, it is concluded that this fractionation method results in the solubilization of most hemicelluloses as OS, followed by the corresponding monosaccharides, whose percentages are dependent on the severity of the treatment. In several cases it is not possible to have a distinction between the percentages of recovered OS and monosaccharides, as total recoveries of saccharides are reported. Typical values reach around 65 to 75 % (22,62,63). The solubilization of the hemicelluloses is dependent on the biological feedstock, the type of reactors and the operating conditions. An almost complete solubilization of the hemicelluloses ($\geq$ 95 %) has been reported and usually the degradation of cellulose is not observed (43,48,62,64).

After solubilization, the recovery and purification of hemicellulosic sugars are also challenging because the resulting mixture of autohydrolysis contains not only OS, but also mono- and disaccharides, degradation products of monosaccharides and HAc. The low recoveries, compared to solubilization, are due to degradation reactions of the monossacharides (40,48,62,65,66).

1.4.1.1 Reaction mechanism

The hydrolytic degradation of hemicelluloses goes through the acid-catalysed cleavage of heterocyclic ether bonds linking monosaccharides units. The reaction is catalyzed by hydronium ions (H_3O^+) generated *in situ*, from water autoionization, followed

by the ionization of the HAc, generated from Ac (i.e. depolymerization of hemicelluloses), present in the feedstock or generated in the reaction medium (21,37,43,58,67) (Figure 9). In further reaction stages, the hydronium ions generated from the ionization of HAc also act as catalysts, improving reaction kinetics (67,68).

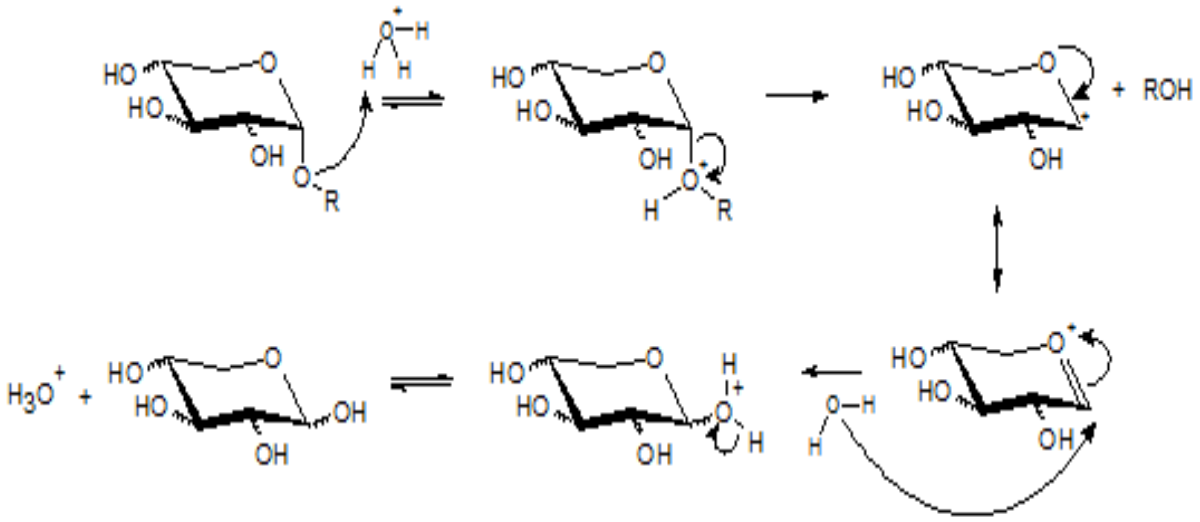


Figure 9 Reaction mechanism of the autohydrolysis process.

The mechanism of autohydrolysis of hemicelluloses is similar to that which occurs in dilute acidic medium and begins with the protonation of the oxygen from the glycosidic bond and subsequent formation of a carbocation. This results from the diffusion of H_3O^+ ions through the plant biomass generated from water (21,43,58). The formed carbocation subsequently breaks the main chain of the hemicelluloses, resulting in the formation of oligomers and monomers, depending on the position of the glycosidic bond in the polymer chain (21,43).

Finally, the carbocation formed at the beginning of the autohydrolysis process reacts with a water molecule, thus regenerating the H_3O^+ ion and the formation of a saccharide with lower degree of polymerization, depending on the position where the glycosidic bond was broken (21,43).

The advantages associated with this type of fractionation are based on the possibility of conversion of hemicelluloses in soluble oligomers with the separation of the remaining fractions (cellulose and lignin) and with high yields, in low formation of by-products (the reaction is carried out under mild pH conditions (4 to 7), limiting the

degradation reactions), reduction of corrosion problems (since no mineral acid is added to the reaction media), water and feedstock are the only reagents, and thus recovery of reagent is not needed, resulting in low operating costs. However, it is a process with high water and energy requirements, because of high operating temperatures (21,58,59,61,69).

1.4.1.2 Operating conditions of the autohydrolysis treatment

The fractionation of plant biomass by the autohydrolysis can be performed under certain operating conditions, especially the following parameters have to be carefully optimized: temperature, reaction time, particle size of the biomass and liquid-to-solid ratio (LSR). Autohydrolysis treatments intending a selective hemicellulose hydrolysis are usually carried out at temperatures within the range of 150-230 ºC. This variation range is fixed by both the little extent of hydrolytic reactions observed below 100 ºC and the fact that cellulose degradation reactions become important above 210-220 ºC. (59,64,70).

The reaction time for a given autohydrolysis process can vary from seconds up to several hours, and in certain cases, such as batch processes, the reaction time has an inverse relationship with temperature, i.e., the higher the temperature used, the lower the time required (58,65).The reaction time and temperature parameters can still be related through a factor, designated by severity factor (log R_0), which is directly related to the operational conditions and the extent of hydrolysis reaction (57).

A larger extension of hemicellulosic degradation process requires, in principle, reducing the particle size of plant biomass, i.e., increasing its surface area. However, this reduction in size is accompanied by high energy costs, and suggested to find a compromise between particle size and energy expenditure. In some cases, the reduction of particle size may not indicate a high solubilization of the hemicelluloses, while, in others, it may even result in a lower recovery of solubilized oligosaccharide easier due to their degradation during the process (48,71).

The LSR, also defined as the solids concentrations, is another important operating parameter, and can vary between 2 and 100 (w/w), although usually values around 10 are used. This operating parameter has a key role in the autohydrolysis process, since it has a direct influence on the acidity of the reaction medium. This influence can be seen in two ways, the first being related to the increasing concentration of solids, which causes an increased release of Ac, promoting depolymerization. On the other hand, there is a greater neutralization of the reaction medium with a higher concentration of solids, due to the

increased relative concentration of ash, which causes a reduction of the hydrolysis (6,66,72,73).

In Table 3, different operating conditions of autohydrolysis process are evidenced, applied to different materials.

Table 3 Diversity of operating conditions used in the autohydrolysis process.

Raw material	Reaction time (min)	Type of process	Temperature (ºC)	LSR (w/w)	Particle size (mm)	Ref.
Eucalyptus	30-66	isothermal	160	8	<8	(74)
Eucalyptus	94	Non-isothermal	<224	8	<8	(62)
Pinus pinaster		Non-isothermal	160-240	8	<1	(29)
Pinus pinaster	-	Non-isothermal	130-175	8	<8	(33)
Acacia dealbata	-	Non-isothermal	170-240	8	<8	(75)
Corn cobs	-	Non-isothermal	202	8	<1	(19,76)
Corn straw	-	Non-isothermal	150-240	10	<6	(77)
Brewery's spent	-	isothermal	150;170;190	8-10	>0,5	(68)
Rice rusk	-	isothermal	205	8	-	(36)
Barley husks	-	Non-isothermal	202-216	8	-	(78,79)
Barley husks	-	Non-isothermal	185-260	8	<0.071-2 mm	(80)
Almond shells	330	isothermal	150	8	-	(81)
Wheat bran	60	isothermal	160	10	-	(46)
Orange peel waste	40	Non-isothermal	140-200	2	-	(82)

1.4.2 Alternative methods

1.4.2.1 Steam Explosion

The steam explosion consists of a hydrothermal fractionation method, in which the biomass is subjected to the action of steam at high pressure (160-240 ºC and 0.7-4.8 MPa) for a period of time ranging from a few seconds to several minutes, followed by its mechanical rupture resulting from the sudden decompression to atmospheric pressure (13,20,58,83). The forces resulting from decompression cause the disintegration of the plant biomass matrix leading to breaking of intra- and intermolecular bonds (84). The structure of the cell wall of biomass thus undergoes an irreversible modification, culminating in the autohydrolysis of hemicelluloses, making them easily removable by aqueous extraction. A water insoluble fraction is also obtained, comprising lignin and cellulose, with most of the lignin being possible to be extracted using organic solvents such as ethanol (13,83).

The attractive features of this method include the low energy requirements, compared to mechanical fragmentation of the biomass, no need to recycle chemicals, low environmental impact and low operating costs (13,83).

The main difference between this physicochemical method and autohydrolysis is the amount of solubilized products and their concentrations, and in this method, a smaller amount of hemicellulosic sugars is obtained although its concentration is much higher. However, this high concentration enhances the formation of degradation products of hemicelluloses and lignin, such as the Furf and 4-hydroxybenzaldehyde, respectively, which is the main disadvantage of this type or treatment (58).

1.4.2.2 Acid Hydrolysis

This can be applied to a process of pre-hydrolysis with concentrated acid at moderate temperatures, or a pre-hydrolysis with dilute acid at high temperatures. The most suitable catalysts for this type of fractionation are sulfuric acid (H_2SO_4), hydrochloric acid (HCl), nitric acid (HNO_3) and phosphoric acid (H_3PO_4) (6,13,58). This possibility of applying different operating conditions maximizes the obtained amount of hemicellulosic sugars. The fractionation process performed under acidic conditions may require the use of a base to neutralize the hydrolyzate for subsequent utilization (13).

1.4.2.3 Hydrolysis with concentrated acid

This method is applied when seeking the solubilization of the hemicelluloses and cellulose fractions. Specifically, the fractionation applied to plant biomass consists in the cleavage of acetal linkages, promoted by protons released by the catalyst, present between the monomers of cellulose and hemicelluloses, thus leading to depolymerization of both fractions. This fractionation results in the production of the monomeric units constituting the cellulose and hemicelluloses (58).

The main disadvantage of the application this type of fractionation is the use of high concentrations of acid, which requires the use of corrosion-resistant equipment and the recovery of the used acid. Thus, the application of this method results in high energetic and economic requirements (6,58).

1.4.2.4 Hydrolysis with dilute acid

Hydrolysis with dilute acid is the most commonly used chemical fractionation method for hemicelluloses recovery. The fact that they present a lower degree of

crystallinity compared to cellulose, makes glycosidic bonds between Xyl monomers less stable than the existing bonds between the Glc monomers of cellulose. Thus, the fractionation of the pentoses from the hemicellulosic fraction is more likely to occur with this method. The activity of the catalyst is carried out at higher temperatures (120-220 ºC) compared to hydrolysis with concentrated acid, being hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) the most suitable (6,85,86). In comparison with the concentrated acid hydrolysis, this method has a greater recovery of hemicelluloses sugars (approximately 100 %), and a lower formation of inhibitors, which turns out to have a positive effect on the hydrolysis of the cellulosic fraction (6). Although it does not present so many problems of corrosion, the fact that this method operates at high temperatures, makes its application entail very high costs (85).

1.4.2.5 Alkaline Hydrolysis

Alkaline hydrolysis is another type of chemical fractionation and is based on the use of dilute bases. The main effect of this method on the materials of plant origin is the removal of lignin, making the carbohydrates more accessible (6,13,87). Among the bases suitable for this type of fractionation are sodium hydroxide (NaOH), potassium hydroxide (KOH), ammonium hydroxide (NH_4OH) and calcium hydroxide (CaOH) (6,13).

The effectiveness of this method is directly related to the content of lignin present in plant biomass, being more effective for the materials that contain a lower content of lignin (13). In addition to the use of different concentrations of base, this process is performed with moderate temperature and reaction time. Compared with other chemical methods, it is characterized by causing less degradation of sugars (6).

1.5 Oligosaccharides as commercial products

The derivatives of natural carbohydrates can be classified, according to their molecular size, as monosaccharides or OS, and originate especially from high molecular weight polysaccharides.

The OS are defined as a carbohydrate with medium or low molecular weight with monomeric units joined by glycosidic bonds. Although there is no specific distinction between the terms “polysaccharide” and “oligosaccharide”, OS are often defined as carbohydrates consisting of 3 to 10 monomer units, although sometimes other carbohydrates with a higher degree of polymerization are also designated OS (88-90).

1.5.1 Properties of oligosaccharides

The OS are water soluble and have a sweetening power between 30 to 60 % of the one of sucrose, and are therefore extensively applied in various foods where the use of this disaccharide is restricted (88) (90). The sweetening power is dependent on the chemical structure, the degree of polymerization and the levels of mono- and disaccharides present, decreasing with the length of the chain.

When compared to mono- and disaccharides, in terms of molecular weight, the OS can be also applied as fat substitutes in different products, since they can increase the product viscosity (90). They may also be used to alter the freezing temperature of frozen foods and as thickeners to enhance or mask certain flavors. They also provide a high moisture-retaining capacity, preventing excessive drying, and a low water activity, which is convenient in controlling microbial contamination (88,90).

The stability can greatly differ for the various classes of OS depending on the sugar residues present, their ring form and anomeric configuration and linkage types (88,90). Generally β-linkages are stronger than α-linkages, and hexoses are more strongly linked than pentoses. Nevertheless, as a whole, at pH lower than 4.0 and treatments at elevated temperatures or prolonged storage at room conditions, OS present in foods can be hydrolyzed resulting in loss of nutritional and physicochemical properties (90).

Based on their functional properties, OS can be classified as digestible or non-digestible being distinguished by the glycosidic bond at the anomeric carbon displayed, α-(1,4) and β-(1,4), respectively (88,90). The non-digestible OS are not found in great abundance in nature and have several applications as food ingredients. The major scientific and commercial interest in these compounds is due primarily to the identification of a number of advantageous properties, both for human consumers's health (prebiotic effect, anti-infective properties, stimulating the growth of beneficial bacteria in the gut, protection against colon cancer bacteria) or technological point of view (food, pharmaceutical and cosmetics industries) (88,90).

1.5.2 Different classes of oligosaccharides and methods of current production

Although the OS market is dominated by Japan, their production has been intensified in Europe and the United States. The diversity of OS with food application has been rising, especially with increasing knowledge of their functional properties.

The lactose-derived OS (Table 4) are the ones that are most produced worldwide. In addition to those presented in Table 4, the production of other OS has been described from various polysaccharides, using microbial enzymes, including the pectate OS, β-gluco-OS, agaro-OS, manno-OS and chitin and chitosan OS (91-93).

Table 4 Different classes of OS, synthesis method and their classifications. (Adapted from (88))

Type	Synthesis	Link/bond type
Galacto-oligosaccharides	Enzymatic synthesis from lactose (transglycosylation by β-galactosidase)	α-D-Glc-(1→4).(β-D-Gal-(1→6-)*n*, *n*=2-5
Lactulose	From the lactose (alkaline isomerization)	β-D-Gal-(1→4-)-β-D-Fru
Lactosucrose	Enzymatic synthesis from lactose and sucrose (transglycosylation by β-fructofuranosidase)	β-D-Gal-(1→4-)-α-D-Glc-(1→2)-β-D-Fru
Malto-oligosaccharides	Enzymatic hydrolysis of starch (pullulanase, isomaltase, amylase)	(α-D-Glc-(1→4)-)*n*, *n*=2-7
lsomalto-oligosaccharides	Enzymatic hydrolysis of starch Conversion maltose (α-amylase, β-amylase) Transglycosylation (α-glucosidase)	(α-D-Glc-(1→6)-)*n*, *n*=2-5
Cyclodextrins	Enzymatic hydrolysis of starch Digestion (pullulanase, α-amylase) Transglycosylation (ciclomaltodextrina-glucanotransferase)	(-α-D-Glc-(1→4)-)*n*, *n*=6-12
Soybean oligosaccharides	Extraction from the soybean whey	(α-D-Gal-(1→6)-)*n*,-α-D-Glc-(1→2)-β-D-Fru, *n*=1-2
Xylo-oligosaccharides	Extraction of xylan from corn cobs Enzymatic hydrolysis (endo-1,4-β-xylanase)	(β-Xyl-(1→4)-)*n*, *n*=2-9
Gentio-oligosaccharides	Acidic or enzymatic hydrolysis of starch Enzymatic synthesis from glucose (transglycosylation)	(β-D-Glc-(1→6)-)*n*, *n*=2-5
Glucosyl sucrose	Enzymatic synthesis from sucrose and maltose (glucanotransferase ciclomaltodextrina)	α-D-Glc-(1→4)-α-D-Glc-(1→2)-β-D-Fru
Fructo-oligosaccharides	Enzymatic synthesis from sucrose (transglycosylation by β-fructofuranosidase) Enzymatic hydrolysis of inulin (inulinase)	α-D-Glc-(1→2)-(β-D-Fru-(1→2-)*n*, *n*=2-4; β-D-Fru-(1→2-)-(β-D-Fru-(1→2)*n* , *n*=1-9 α-D-Glc-(1→2-)-(β-D-Fru-(1→2)*n* , *n*=2-9
Palatinose (isomaltulose) oligosaccharides	Enzymatic synthesis from sucrose (isomaltulose synthase)	(α-D-Glc-(1→6)-D-Fru)*n*, *n*=2-4

1.6 Potential alternative sources of hemicellulosic oligosaccharides

1.6.1 *Cupressus lusitanica Mill*.

The cypress cedar of Goa, Mexican cypress, or Kenya cypress is a tree of the genus *Cupressus*, this name being applied to a wide variety of species of coniferous trees of the family *Cupressaceae* (Figure 10) (94,95).

The *C. lusitanica Mill*. is a species similar to *C. lindleyi* or *C. benthamii*, originated from Central America, specifically from Mexico and Guatemala. The name "*lusitanica*" refers to a possible Portuguese origin, however it does not, as it comes from the fact that its initial classification has been made from existing specimens in Portugal, more specifically in the woods near the Convent of Buçaco, where they were introduced in the XVII century from Central America (94,95). The formal classification was done by Philip Miller in 1768, assigning in the specific name "*lusitanica*"(94).

Figure 10 *Cupressus lusitanica Mill.*.
(photo credits: http://www1.ci.uc.pt/nicif/santuario/botanico/especies/80_cedro%20do%20bucaco.JPG and http://www1.ci.uc.pt/nicif/santuario/botanico/especies/80_cedro%20do%20bucaco.JPG)

Due to the interesting features of the species, some of its specimens were cultivated across many parts of Europe, justifying its numerous designations such as Cypress-Portugal, Mexican Cypress, Cypress-Buçaco. Cedar-of-Goa or Cedar-of-cypress (as it is better known in Brazil), more commonly known as Cedar Buçaco and constituting the ex-libris of the forest of Buçaco (94).

Regarding its general characteristics and morphology, it is presented as a tree of medium height, 25-30 m tall, fast growing, with a dense, conical crown and a trunk surrounded by a reddish-brown bark. It produces clear, light and low density wood, but with fine texture and high dimensional stability. It also produces small cones, 1 to 1.5 cm in diameter, blue-grey when young, which acquire a brown and shiny tone with age. *C. lusitanica Mill.* is also distinguished from other cypresses by the sharp vertices and bluish-green color of their leaves. It is considered a very adaptive species with respect to environmental conditions, yet presenting a special preference for soil with neutral to slightly acidic or basic pH. It grows mostly on humid climates and it presents high resistance to urban air pollution.

1.6.1.1 Commercial use

The tree *C. lusitanica Mill* has an outstanding ornamental use, justified by its beauty and pleasant odor, and as a source of wooden chips and cellulose, mainly in southern Europe. In Portugal, its presence is signaled in the forest of Buçaco and as an ornamental, all over the country (94,95). It is also used as a raw material for obtaining essential oils (94).

1.6.1.1.1 As a source of essential oils

The chemical composition of the essential oils from the *C. lusitanica* presents significant differences depending on their region of origin. This can be related to the different conditions of climate and soil where it originated, as these have a direct influence on plant metabolism. The diversity of chemical components can be identified by applying gas chromatography coupled to mass spectrometry and are essentially terpene compounds (monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20) and oxygenated derivatives) (56,94).

1.6.1.1.2 Methods of extraction of essential oils from *C. lusitanica*

The essential oils of the *C.lusitanica Mill* can be obtained by applying various methods. Among these are the hydrodistillation, the hidrodifusion, the extraction with organic solvents, and supercritical fluid extraction, pressing, among others (56,94,96). Of these, the most common industrial method is hydrodistillation or extraction with water vapor due to its versatility. This extraction method is the entrainment of volatile compounds by water vapor obtained in a suitable generator, followed by the passage of steam through a condenser, where the mixture of oil and water vapor condenses. Subsequently, due to the difference of densities, the aqueous phase separates from the essential oils. The distillate therefore consists of essential oils and flavored water (decoction water), since there is always a small percentage of essential oils which dissolve in water. This method of separation of essential oils is considered to be the most economically advantageous (56). Currently, it seems that the use of supercritical fluid extraction (SFE) with CO_2 has significant advantages over hydrodistillation, not only on the full recovery of the odor, but also on the technological side (97).

At lab-scale, the standard method encompasses the use of a “Clevenger” apparatus (98). In this case, the plant material is kept in contact with boiling water and steam that

causes the cell walls to rupture to break-loose the oil, that is evaporated along with the water that goes to the condenser. There the condensation of steam occurs, followed by phase separation by density difference. The essential oils obtained after the separation of the aqueous phase can be dried with sodium sulfate (Na_2SO_4) (56).

1.6.1.2 New applications

The essential oils industry generates waste that can be reused as substrates in biotechnological processes. In this work, the solid waste generated after extraction of essential oils from leaves, bark and wood of *C. lusitanica Mill.* was subjected to the autohydrolysis process to obtain hemicellulose-derived OS.

1.6.2 *Citrullus lanatus*

Watermelon, whose botanical name is *Citrullus lanatus var lanatus*, belongs to the family of *Cucurbitaceae*, that also includes melon, cucumber and pumpkin (99-101). It is native from the dry regions of tropical Africa, and also thrives in regions such as Central Asia and the Mediterranean (99). It derives from the variety *Citrullus lanatus citroides* that exists in Central Africa, where its cultivation has begun (99).

1.6.2.1 Chemical and morphological characteristics

The watermelon, considered as an herbaceous plant with an annual growing cycle, can take a diversity of forms, such as round, oblique or elongated, and may reach 60 cm.

The rind (pericarp) is thick, varies between 1 and 4 cm and may have light or dark green color, a single tone, scratched or smudging. The pulp (mesocarp) is usually red, or yellow, orange, white or green (Figure 11). The seeds are included in the tissue that forms the edible part and contain high fat (99).

Figure 11 Diversity of watermelons.
(Photo credits: http://upload.wikimedia.org/wikipedia/commons/a/a2/Citrullus_lanatus.jpg, http://www.prlog.org/10760591-watermelon.jpg, http://cuke.hort.ncsu.edu/cgc/cgcgenes/wmgenes/wmgn-stripe1000.jpg)

The cultivation of watermelon requires light and fertile soils, rich in organic matter. It tolerates a pH of 5, but the optimum pH values are between 6 and 7 (99). It is a non-climacteric fruit which must be harvested ripe. The main indicators are crop size, fruit color, and the color of the area that is in contact with the soil (99).

The quality of the *Cucurbitaceae* fruits is determined by their sweetening power. In the case of the watermelon, the sweetening power is determined by the content of total sugars and the proportion between the main accumulated sugars, such as Glc, fructose and sucrose (102). The proportions of sucrose and Glc range between 20-40 % of total sugars while the proportion of fructose is around 30-50 % (102).

Watermelon is also a fruit with high natural antioxidant capacity, which translates into a qualitative factor, important in the choice of foods. It is a source of various minerals (K, Mg, Ca and Fe), vitamins (A, B, C and E) amino acids (citrulline and arginine) and phenolic compounds (e.g. carotenoids, as lycopene), that are present in the rind, pulp and seeds (100,103). Lycopene is the main carotenoid synthesized and stored in the mesocarp of watermelon (70-90 %) and is responsible for its typical red color (103).

All compounds listed above justify the high antioxidant power, high anti-inflammatory capacity and vasodilatory properties of the watermelon, which makes it recommended to reduce the risk of certain diseases such as cancer, cardiovascular diseases and pathological degenerative diseases (100,103).

1.6.2.2 Production and commercial uses

The watermelon is cultivated for its fruit. The fruits are usually consumed raw, and because of its high water content, are ideal to be consumed as a refreshing dessert in summer (99,103). In some areas, the seeds are also consumed, like in India, where bread is made from watermelon seed flour, and in the Middle East, where the seeds are roasted. The watermelon rinds are also used for the production of sweets (e.g. jellies) (99).

Its global production has been increasing steadily during the last century and its cultivation corresponds to 6.8 % of the global vegetable production in 2011 (101).

1.6.2.3 New applications

The full utilization of foods is an environmentally friendly and sustainable practice that meets a more efficient use of natural resources. Products from the watermelon are

examples of products derived from fruits with great acceptance by consumers, being obtained from industrialization processes. However, these processes generate large quantities of waste, often dumped into the environment without any recovery, which contributes to increasing the environmental pollution.

1.7 Aims

In this work, we intend to study, within the concept of biorefinery, the process of autohydrolysis of biomass residues from the previously presented materials for the production of different classes of OS with new features. Special attention is given to the chemical characterization and reactivity of produced OS. That being said, the following steps were taken:

- Summative chemical characterization of different materials
- Quantification of extractable residues of different *Cupressus lusitanica Mill*
- Study of autohydrolysis process to produce OS
- Chemical characterization of liquors obtained during autohydrolysis
- Evaluation of liquors stability during storage
- Evaluation of purification methods for OS
- Evaluation of OS stability to pH and temperature
- Enzymatic hydrolysis of residual solids obtained during autohydrolysis

2 Materials and Methods

2.1 Feedstocks

The *Cupressus lusitanica Mill.* biomass (chips, branches and bark) used in this work was kindly provided by Silvapor, Lda. (Figure 12).

Figure 12 The processing of the *Cupressus lusitanica Mill*. tree used in this work

(photo credits: Silvapor, Lda.)

Upon receiving, the biomass was stored in plastic bags at room temperature until further use. The sample characteristics are described in the following table.

Table 5 Characteristics of the *Cupressus lusitanica* tree used in this work.

Cupressus lusitanica Mill.	
Height	12.50 m
Diameter at breast height	0.66 m

The watermelons (*Citrullus lanatus*) used in this work were kindly provided by Hortas da Idanha, Lda. and correspond to typically rejected fruits, not fitted for regular commercial applications.

2.1.1 Feedstock preparation and storage

2.1.1.1 *Cupressus lusitanica Mill.*

The chips of *C. lusitanica Mill*. had a typical size of 50 x 50 x 1 mm. As such, they did not have the appropriate size to be directly applied to the autohydrolysis process. Thus, it was necessary to use a knife mill (Waringg, Snijders Scientific, Netherlands) for their

commination. As this mill does not have a screen, the milling resulted in a very heterogeneous sample in terms of grain size and it was therefore subjected to particle size characterization (see below). Particles with a size greater than 1.66 mm are not suitable for autohydrolysis processes because they may cause diffusional limitations on mass and heat transfer and the fractions below 1 mm require more careful handling and are more likely to suffer degradation during the process of autohydrolysis. Therefore, the fractions between 1.00 and 1.60 mm were chosen for subsequent work and were stored in plastic containers at room temperature.

The branches were separated manually to obtain leaves, cones and small branches. No milling was carried out and the separated materials were stored in plastic containers at room temperature, without occurring problems biological degradation.

2.1.1.2 Watermelon rind

Watermelon is divided into four layers: pericarp, epicarp, mesocarp and endocarp (Figure 13). As the endocarp and mesocarp have industrial use (e.g. for fruit pulp or production of juice), the junction rind (pericarp) and epicarp are the remaining materials and can be considered residues.

Upon getting in the laboratory, the watermelons were sliced into small sizes using a kitchen knife. The rinds were removed and ground in a blender to yield a paste (Figure 14). Finally, they were stored in plastic containers and frozen.

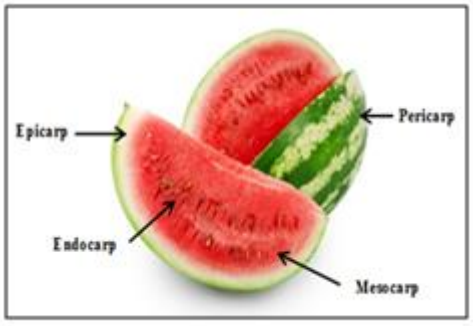


Figure 13 Watermelon morphology.

Figure 14 Processed watermelon rind.

2.2 Extraction of essential oils

The essential oils were extracted from leaves, barks, cones and chips of *C. lusitanica*. The materials (150 g of sample) were suspended in 1500 mL of distilled water and

subjected to extraction, during approximately 3 hours, using a Clevenger-type apparatus (Figure 15). At the end of the extraction process, the essential oils were collected and weighed. Decoction waters were recovered after pressing (up to 200 bar) to separate them from the residual biomass using a manual hydraulic press (Sotel, Portugal).

The essential oils were frozen and stored until further (external) analysis. Decoction waters were frozen and later analyzed by High Performance Liquid Chromatography (HPLC) and Capillary Zone Electrophoresis (CZE) (section 2.6.3.1 and 2.6.3.3). The residual biomass was also frozen and later analyzed for the quantification of structural components (section 2.6.2.4).

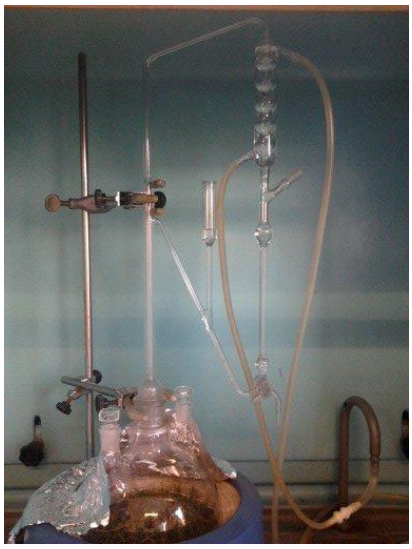


Figure 15 The Clevenger-type apparatus used in this work for the extraction and quantification of essential oils.

2.3 Autohydrolysis process

Autohydrolysis was performed in a laboratory scale stirred Parr reactor. The reactor had a total volume of 2 L and an external electric heating mantle. The temperature, agitation and pressure were controlled through a Parr (Parr Instrument Company, USA) PID controller, model 4842 (Figure 16). Cooling water was circulated through an internal serpentine coil to rapidly cool the reactor content at the end of each run.

Figure 16 The Parr reactor used for the optimization of the autohydrolysis processes.

All autohydrolysis treatments used 1.2 kg of total mass (biomass and water) and were carried out at an agitation speed set at 150 rpm, under non-isothermal conditions: the reactor was heated to final temperatures ranging between 150 and 230 ºC, and upon reaching the desired temperature rapidly cooled down to 100 ºC. The reactor was then removed from the heating jacket and the vessel was introduced in a cold water bath.

For all autohydrolysis processes performed, heating and cooling profiles, as well as pressure profiles, were plotted and the data were used to calculate the severity factor (log R_0) (57).

For chips, several LSR were assayed, ranging from 7 to 13, using a final temperature of 200 ºC. The LSR was then set at 10 for subsequent studies to evaluate the temperature effects for all *C. lusitanica* biomass samples.

The reaction mixture was filtered for solid and liquid separation using filter paper (Machinery-Nagel 1342). Due to the high hygroscopicity of chips and leaves, this separation was firstly performed by pressing (up to 200 bar) using the hydraulic press.

The solid phase was washed with distilled water, at ambient temperature, and filtered/pressed again.

In the case of the autohydrolysis of the watermelon rind, the produced paste described above was well mixed and used without any addition of excess water. Upon completion of the autohydrolysis process, the separation of the solid and liquid phases was carried by filtration.

The liquid fractions (hydrolyzates) were weighed, stored frozen and later analyzed for sugars, aliphatic acids and sugar-degradation products. The remaining solids (after

washing with the same amount of water as used in the treatment) were also weighted, sampled for moisture determination and also stored frozen and later analyzed for the quantification of structural components (section 2.6.2.4).

2.4 Products characterization and purification

2.4.1 Evaluation of the storage stability of the hydrolyzates

To evaluate the storage stability of liquor, 200 mL of liquor of each hydrolyzate obtained under optimized conditions were used. The liquors were filtered through 0.45 µm membranes (Millipore) to universal bottles (Schott) and left at room temperature in a dry and dark place. At regular time intervals, aliquots were taken for analysis. Then, the hydrolyzates were subjected to quantitative acid hydrolysis in order to determine the amount of OS present (section 2.6.3.1 and 2.6.3.2).

2.4.2 OS purification

2.4.2.1 Freeze-drying

Samples (200 mL) of the hydrolyzates obtained at optimal conditions were filtered through 0.45 µm membranes (Millipore) to 500 mL wide-mouth bottles. Then, the samples were placed in a freeze-drier at a temperature of -50 ºC and under vacuum (maximum pressure of 0.200 mbar).

2.4.2.2 Vacuum evaporation

Samples of liquors obtained at optimal conditions were again filtered through 0.45 µm membranes (Millipore) and evaporated.

Vacuum evaporation was carried out in an evaporation system (Büchi, Flawil, Switzerland) that comprises a vacuum pump VAC® v-500 and a vacuum controller B-721 set at 70 mbar and a water bath set at 40 ºC. Sample volume was 100 mL. Under these conditions, the process was terminated in about 3 to 5 h. Attention had to be paid so that no foaming was formed.

2.4.3 Evaluation of the stability of oligosaccharides in relation to pH and temperature

The stability of the produced OS was determined by applying a method that had previously been developed in our laboratory, which is based on some of the methods described in the literature, that it is here briefly described (104,105).

The purified OS obtained from vacuum evaporation, as described above, were used for all tests. The "buffer" solutions used are presented in Table 6. The samples were dissolved in "buffer" solutions, resulting in a mixture of concentrations approximately equal to 100 g/L.

Table 6 Preparation of the "buffer" solutions for the evaluation of the stability of OS and respective solutions used in their preparation.

pH	Solution A	Solution B	Vol. Sol. A (mL)	Vol. Sol. B (mL)	Total Vol. (mL)	Ref.
1*	0.2M KCl	0.2 M HCl	50.0	97.0	200.0	(106)
3	0.1M $C_6H_8O_7$	0.2 M Na_2HPO_4	39.8	10.2	100.0	(106)

*****This solution is not a true buffer solution, but for ease of writing, is thus considered in this work, similar to what is described in the literature.

To determine the stability of OS, the prepared solutions were placed in a thermostated oil bath at a temperature of 100 ºC, during 1 hour, being subsequently analyzed by HPLC.

2.5 Enzymatic hydrolysis of the solids residues

The enzymatic digestibility was performed both in the untreated and autohydrolysis treated biomasses and was carried out based on the NREL/TP-510-42629 protocol (107). This procedure consists in using a sample of biomass of 0.15 g that is digested in the presence of 5 mL of sodium citrate 0.1 M pH 4.8 buffer solution, 100 µL of a solution of sodium azide (2 % w/v), as anti-microbial agent and enzymes *Celluclast* 1.5 L (56 µL) and *Novozyme* 1.88 (Novozymes, Denmark) (8 µL). Finally, the volume was adjusted with distilled water to 10 mL considering that the biomass has a density of 1.

Blank assays were also prepared to account both the sugars that may arise from the enzyme solution (carried out without added biomass) and the sugars derived from non-

enzymatically hydrolysis of the biomass (carried out without added enzymes) in order to correct the results for the free saccharides present in the biomass and products that could possibly be formed in the absence of enzymes.

Thereafter, the samples were placed in an orbital incubator (Comecta), at 50 ºC, for 72 h under orbital shaking (250 rpm). After 72 h, the samples were placed in a boiling water bath, for 5 min, to inactivate the enzymes. Finally, the samples were filtrated using Millipore membranes (0.45 µm), under vacuum, and analyzed by HPLC (section 2.6.3.1) for the released sugars. All assays were performed in small capped-plastic jars (40 mL), at least, in duplicate.

The enzymatic digestibility was determined by the ratio of digested cellulose to the initial cellulose loaded.

2.6 Analytical methods

2.6.1 Physical characterization of the materials

2.6.1.1 Granulometric analysis

Granulometric characterization was performed using a sieve shaker and seven sieves (Retsch, Germany ASTM E11) with different pore sizes (dust particles, 250 µm, 0.500, 1.00, 2.00, 2.36 and 3.55 mm) arranged in series according to the pore diameter (Table 7).

Table 7 Pore dimensions for the sieves used in granulometric analysis.

Sieves	Diameter pore (mm)	Diameter pore (Mesh)
1	dust particles	-
2	250 µm	63
3	0.500 mm	35
4	1.00 mm	18
5	2.00 mm	12
6	2.36 mm	8
7	3.55 mm	6

To carry out the analysis, at least 3 samples (circa 100 g each), were screened for at least 20 min, after which the pre-weighed sieves containing the biomass were weighed in a precision balance to the nearest 0.01 g.

2.6.2 Chemical characterization of raw materials and processed solids

2.6.2.1 Determination of moisture content

Porcelain crucibles were oven dried in the muffle furnace (Heraeus D-6450, Germany) at 550±1 ºC for at least 5 h and transferred into desiccators to cool down, for at least 1 h before weighing. For each sample (feedstock or solid residues resulting from Clevenger extraction or autohydrolysis), 2 g were weighed on the analytical balance (Mettler HK160, Switzerland), and then oven dried at 105 ºC, at least for 18 h, after which they were cooled down in the desiccator and weighed again. This procedure was repeated whenever necessary, being performed always in duplicate.

2.6.2.2 Determination of ash content

Ash content was determined on the dry biomass obtained from the moisture quantification using a muffle furnace set to 550±1 ºC for at least 5 h. After the crucibles were cooled down in the desiccators, they were weighed again on the analytical balance. This procedure was repeated whenever necessary, being performed, at least, in duplicate. Appendix A describes the mathematical formulae used.

2.6.2.3 Determination of extractives content

The determination of extractives present in the samples was performed according to the protocol of NREL (108), with an adapted Soxhlet extraction system (85 mL). The sample (circa 2 g) was added to a pre-weighed extraction thimble (oven dried at 105±5 °C, for a at least 18 h) and weighed in an analytical balance. The samples were successively extracted with water and ethanol for 16 h each, with a reflux of 4 to 5 cycles/h. After the extractions, the flasks were placed on a rotary evaporator to evaporate the solvent and then placed in the oven until constant weight.

2.6.2.4 Quantification of structural polysaccharides and lignin

The chemical composition of the raw material and solids residues was determined according to the NREL Laboratory Analytical Procedure NREL/TP-510-42618 (108).

Whenever necessary, the samples were ground in a knife mill (IKA MF10 Werck, Staufen, Germany) to a particle size smaller than 0.5 mm.

Milled samples were weighed (500±1 mg) into test tubes (2 cm diameter) and 5.00±0.01 mL of H_2SO_4 72 % was added to each test tube. A Teflon stir rod was used to mix for 1 min, or until the sample was thoroughly mixed. The tubes were placed in a water bath (Memmert, Germany) at 30±3 ºC for 60±5 min. Occasional stirring (typically, every 5 to 10 min) was done, without removing the sample from the bath. Stirring is essential to ensure even acid to particle contact and uniform hydrolysis.

Upon completion of the incubation period, samples were transferred from the test tubes into 250 mL universal bottles (Schott, Germany). Water was used to help to transfer all solids and acid, and to dilute the acid to a final concentration of 4 %. Then the bottles were placed in autoclave (Uniclave, Portugal), at a temperature of 121 ºC for 1 hour. After completion of the autoclave cycle, the hydrolyzates were slowly cooled down to room temperature. After cooling, the reaction mixture was filtered through pre-weighed muffle-oven dried filtering crucibles (porosity 3). The liquid phase was characterized using HPLC (section 2.6.3.1 and). The solid residue deposited in the filtering crucibles was washed with distilled water to remove residual sugars. The filtering crucibles were dried for 18 h at 105 ºC, cooled down in the desiccator, weighed, and then ashed in the muffle-oven for 5 h at 550 ºC. The dry mass corrected for the ash content was considered to be the Klason lignin (KL). Each sample was processed, at least, in duplicate. Appendix A describes the mathematical formulae used.

2.6.3 Chemical characterization of liquors

2.6.3.1 Determination of monosaccharides, aliphatic acids and furans

The liquors obtained from autohydrolysis, the hydrolyzates from the quantification of structural polysaccharides procedure, the decoction waters and the enzymatic digests were filtered through 0.45 μm membranes (Millipore) and directly analyzed by HPLC using an Agilent 1100 Series, equipment (Germany) described in the following table.

Table 8 HPLC equipment used in this work.

Equipment	Model
Quaternary pump	G1311A
Automatic Injector	G1313A
Column oven	G1316A
Diode Array Detector	G1315B
Refractive index detector	G1362A
Software	Agilent Chemstation for LC 3D Rev. B.01.03

Elution took place using an Aminex HPX-87H column (Bio-Rad, Hercules, USA) operating at 50 ºC with H_2SO_4 5 mM as mobile phase, at a flow rate of 0.4 mL/min or 0.6 mL/min and an injection volume of 20 or 5 µL, respectively. Monosaccharides (Glc, xylose (Xyl) and Ara) and aliphatic acids (HAc, HFor and HLev) were detected using the Refractive Index detector; the furan derivatives, Furf and HMF, were detected with the UV/Vis photodiode detector (DAD) set at 280 nm (Table 9).

Table 9 Analysis conditions for the chromatographic columns HPX-87H and HPX-87P.

Characteristics	Columns	
	HPX – 87H	HPX-87P
Column dimensions	300 × 7.8 mm	300 × 7.8 mm
Mobile phase	H_2SO_4 0.5 mM	H_2O
Flow	0.6 mL/min, 0.4 mL/min [a]	0.6 mL/min
Injection volume	5 µL, 20 µL	20 µL
Temperature of the column	50 ºC	80 ºC
Temperature of the detector IR	50 ºC	55 ºC
Wavelength UV	280 nm	-

[a] **Chemical characterization of the feedstock and solid waste resulting from autohydrolysis.**

Due to the partial overlap of peaks corresponding to galactose, mannose and xylose, a second column was used, HPX-87P, to carry out a complementary analysis, whenever possible, to more accurately calculate the concentrations of these monosaccharides.

The concentrations of the studied compounds were calculated from the calibration curve plotted from the standard solutions. These standards were analyzed daily.

Appendix A describes the mathematical formulae used to calculate the yields for polysaccharides, monomers, acetyl groups and lignin.

2.6.3.2 Quantification of oligosaccharides

The OS were measured by an indirect method based on quantitative acid hydrolysis according to NREL/TP-510-42623 (109).

Concentrated sulfuric acid (72 %) was added to an aliquot of liquor resulting from the autohydrolysis treatments, to reach a final H_2SO_4 concentration of 4 %. The mixture was placed in an autoclave and hydrolyzed at 121 ºC for 1 h. After completion of the autoclave cycle, the hydrolyzates were slowly cooled down to room temperature. After cooling, a sample was collected, filtered using 0.45 μm membranes (Millipore) and analyzed by HPLC (section 2.6.3.1) The OS concentrations were calculated from the increase in sugar monomers. This procedure was always performed, at least, in duplicate.

2.6.3.3 Identification of phenolic compounds

The presence of phenolic compounds in the hydrolyzate and decoction waters, obtained during autohydrolysis and extraction of essential oils, respectively, was analyzed by CZE, according to (110). An Agilent Technologies CE system (Waldbronn, Germany) equipped with a diode array detector (UV-DAD) was used. An uncoated fused silica extended light-path capillary from Agilent, i.d.=50 mm, total length 62 cm (56 cm to the detector) was also used. A buffer EDTA solution (20 mM) pH 9.3 was used. The operating conditions are summarized in Table 10.

The Agilent 3D-CE ChemStation data software (Rev B.04.01) was used to perform qualitative analysis of the electropherograms.

Table 10 Conditions used for the CZE analysis.

Parameters	Operating conditions
Temperature	25 ºC
Offset	8 mm from the bottom of the vial
Injection	50 mbar x 15 s. at the anode (+)
Current	120 µA
Migration time	30 min
Voltage	25 kV with 0.5 min uphill ramp
Wavelength detector DAD	200, 280, 375 nm
Preconditioning	3 min washing with 0.1 M NaOH followed by 3 min run buffer

Phenolic compounds were identified by electrophoretic comparisons (migration times and UV spectra) with authentic phenolics standards. The analyzed standards include oleuropein, tyrosolm, hydroxytyrosol, catechol, 4-mehtycatechol, catechin, epicatechin, naringin, naringenin, hydroquinone, syringaldehyde, vanillin and 3-hydroxybenzoic, caffeic, ellagic, ferulic, syringic, *trans*-cinnamic, and vanillic acids.

2.6.4 Quantification of the enzyme activities

Filter Paper Cellulase Units (FPU) and β-glucosidase activities were previously measured according to Ghose,1987 (111) and Berghem and Pettersson, 1973 (112) procedures, respectively. All assays were performed, at least, in duplicate.

3 Results and discussions

3.1 *Cupressus lusitanica Mill.*

To better evaluate the upgrade potential of the *C. lusitanica* biomass it is important to have a quantification of its partitioning among its main constituents. The following table presents the results for the tree used in this work.

Table 11 Biomass partition for the *C. lusitanica* tree used in this work.

Fraction		Value
Total Weight (Kg)		263.0 Kg
Chips (wood)		57.8%
Branches		36.1%
Leaves	18.0 %	
Cones	10.0%	
Small branches	8.1%	
Bark		6.1%

Wood, as expected, is by far the most significant fraction, accounting for more than 57% of the total biomass. Branches are the second major constituent, but are also the most diverse, as they include leaves, cones and the small branches themselves. Finally bark is also a significant fraction.

Although these values are important to establish future potential yields it should be taken into consideration that these values may change according to forest management practices, site location, tree age and growth season (113,114).

Furthermore, this corresponds to an analysis of a single tree and much more data needs to be gathered in order to have significant values. Nevertheless, the reported data are important to define which of the different fractions should be preferably studied.

3.1.1 Chemical characterization

For a better understanding of the available materials, it becomes important in the initial phase of this work to chemically characterize them.

As described in the introduction, this species is a potential source of essential oils and, as such, this was evaluated. Subsequently, the materials were characterized in terms of

their structural macromolecules (cellulose, hemicelluloses and lignin), taking special attention to the hemicelluloses, because the composition of these influence their use for the production of OS.

3.1.1.1 Extractives

3.1.1.1.1 Quantification of essential oils

The following table shows the yield of essential oil resulting from the extraction of different parts of the *C. lusitanica* using the Clevenger apparatus.

Table 12 Essential oils yield (% of dry weigh) from the different parts of *C. lusitanica Mill.*.

Fraction	Essential oil (%)
Leaves	0.53
Cones	0.45
Bark	0.17
Chips	0.00

Leaves and cones present the highest essential oil content, conversely to chips from which it was not possible to recover any oil, which hence can be directly used for other applications. Bark also presents some oil, but at lower amounts, and its use as essential oil source should be carefully evaluated, also taking into consideration the possible qualitative differences among the recovered oils (currently ongoing work).

These values should be considered as maximum (laboratory) values for the sample, and will probably not be easily attained industrially. Furthermore it is also important to study the seasonal deviations that may imply significant variations.

3.1.1.1.2 Characterization of the decoction waters

Table 13 shows the chemical composition of decoction waters resulting from the extraction processes of essential oils of different parts of the *C. lusitanica*.

Table 13 Chemical composition of the decoction waters obtained using the Clevenger extraction procedure.

Compound	Decoction waters composition (g/L)			
	Bark	**Chips***	**Leaves**	**Cones**
Glc	0.10	n.a	2.48	1.76
Xyl	0	n.a	0	0
Ara	0.10	n.a	5.81	0
HFor	0.08	n.a	0	0
HAc	0.06	n.a	0.30	0.22
HLev	0	n.a	0	0
HMF	0	n.a	0	0
Furf	0	n.a	0	0

*n.a.: not available

Simple sugars are the main components present in the decoction waters, and these are specially relevant in the decoction waters produced from leaves and to some extent from cones, but not from bark, that only presents very small quantities of sugars, and aliphatic acids. The samples were not analyzed for oligomeric sugars. Furans are not identified in any sample.

The total concentration of free sugars (Glc and Ara) obtained in the decoction waters does not enable them to be assigned, for now, to any possible future applications, as they are not quantitatively significant, the exception possibly being the decoction waters from leaves, that might be used for fermentation purposes if it could be optimized to yield higher sugar concentrations, e.g. by studying the LSR of the extraction. Nevertheless, this would only be a solution if no significant inhibitors also exist.

In fact, it is now important to evaluate the presence of other microbial inhibitors, such as phenolic compounds, that on another side can conversely potentially be used as value-added bioactive compounds.

The decoction waters were then analyzed by CZE with the aim of detecting the presence of phenolic compounds.

Figure 17 shows an example of an electropherogram obtained after analysis of decoction waters resulting from the extraction of essential oils from the leaves of *C. lusitanica*.

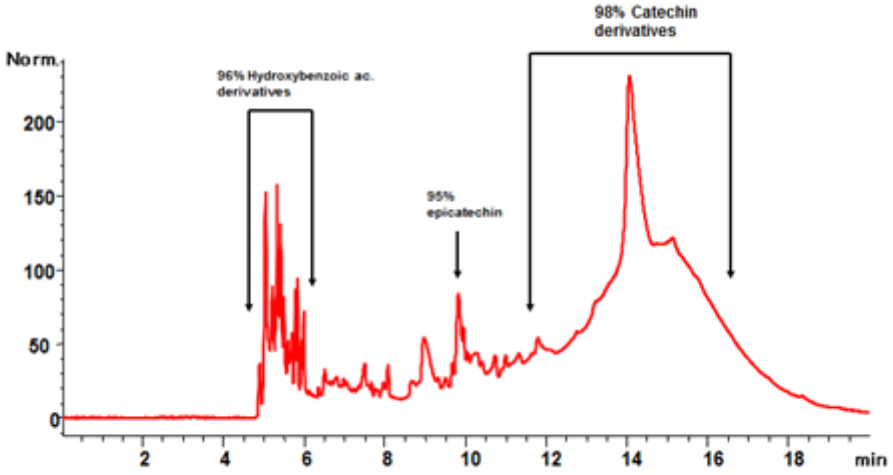


Figure 17 CZE phenolic profile of the decoction water from leaves of *C. lusitanica* at 200 nm.

As no definite separation (or identification) was, yet, possible, no quantification was carried out. Nevertheless, table 14 presents the values corresponding to the areas of the electropherogram of the phenolic compounds detected in the decoction waters resulting from extraction tests performed on different parts of *C. lusitanica*, as an indicative relative quantification of these compounds among these samples.

Table 14 Phenolic compounds detected in the decoction waters resulting from the extraction process of the essential oil from *C. lusitanicaMill.*.

Phenolic compound	Decoction water*			
	Bark	Chips	Cones	Leaves
Hydroxybenzoic acid derivatives	-	540	1465	3047
Epicatechin	-	-	5645	5438

*The reported values are the CZE peak areas

In the decoction waters from bark, no phenolic compounds were detected. Conversely, chips, and specially cones and leaves presented hydroxybenzoic acid derivatives and epicatechin (compound belonging to the class of the catechins) and catechin derivatives in cones and leaves.

If these compounds prove to be commercially interesting, they can be recovered e.g. by using membrane-based technologies. These processes would also enable the

recovery of the water back into the process, so that it could be reused in the extraction of essential oils.

From these data it is clear that the leaves possess the highest upgrade potential.

3.1.1.2 Chemical composition of the structural compounds of *C. lusitanica*

Advanced biomass fractionation depends on the specific internal structure. The chemical composition of the structural biomass compounds may vary depending on their taxonomic class, geographical and biological origin, growing conditions and even their industrial processing, if that is the case.

The chemical characterization of the used materials is of the utmost importance, specially the characterization of the hemicellulosic fraction, because its nature influences the possibility of use for the production of OS. Apart from a detailed knowledge of the composition of raw materials, the chemical characterization is also necessary for the calculation of theoretical yields in OS, monosaccharides and their degradation products.

Table 15 shows the average chemical composition of the different parts of *C. lusitanica* expressed on dry basis.

Table 15 Chemical composition (% of dry weight) of the different parts of *C. lusitanica* studied in this work.

Component	% of dry weight			
	Bark	**Chips**	**Leaves**	**Cones**
Gn	36.80	44.59	19.39	25.36
Hemicelluloses	21.97	29.60	22.11	25.07
Xn	11.48	21.75	10.54	13.73
Arn	9.15	3.71	10.04	9.88
Ac	1.34	4.14	1.53	1.46
KL	35.41	35.85	46.68	40.60
Ash	2.85	0.61	5.07	5.82
Others (by difference)	4.32	0	6.74	3.13

The bark, chips, leaves and cones of *C. lusitanica* differ significantly in their chemical composition. This is due to the heterogeneous nature of the cell wall and of its developing stage of the different parts of the raw material.

The total polysaccharides are higher for the chips, which account for more than 74 %. Conversely, cones and specially leaves present a smaller percentage (approximately 41 %). The cellulose, as estimated from the glucan (Gn) content, is present in higher

percentage in chips (approximately 44 %) and smaller in leaves (approximately 20 %). By comparing the amount of cellulose in the literature, where it varies between 36 % and 52 % for softwoods (e.g. pines), it is found that bark and chips are in the expected percentages, whereas for the leaves and cones there is no such agreement (10,30). Specifically the bark and chips have a similar percentage of Gn of Red pine and Fir or Lobolly pine, respectively (30-33).

The hemicelluloses, as estimated from Xn, arabinan (Arn) and Ac content accounte in general, to 13-28 % of the raw material, being again higher in chips and lower in leaves. According to the literature, the percentage of hemicelluloses in softwoods (e.g. pine, fir) varies between 13 and 26 % (10,30). Thus, the obtained results are within the expected range.

Xn is always the main component, but Arn follows close by, except for chips, where the highest ratio of Xyl to Ara exists. In fact, Xn is the major hemicellulosic component reaching 21.75 %. Based on these data, it is possible to predict that very different OS may be produced due to the different initial composition of the hemicelluloses. These differences are even further extended if the HAc content is also considered. In fact, chips present again a higher hemicellulosic content than all the other samples.

At this stage it is also important to highlight that the reported values for Xn can be somewhat overestimated as the used HPLC column does not enable to differentiate Xyl from galactose and manose, sugars that are typically described to be present in softwood-derived hemicelluloses, as described in the introduction chapter.

The KL content is similar between the bark and chips (approximately 35 %), and strangely lower than in the leaves (approximately 46 %) and cones (approximately 40 %). The values for bark and chips are in agreement with the literature, where typically percentages of 26 to 34 % for KL in softwoods are described (10). The high percentage of lignin found in leaves and cones must be further studied, and it has to be assured that this is real lignin and not any other compounds, e.g. precipitated proteins.

The ash, as estimated from inorganic matter content, has similar percentages in leaves and cones (approximately 5 %). The smaller amount in chips must also be highlighted.

3.1.1.2.1 Chemical composition of the different parts of *C. lusitanica* after the extraction process

Table 16 shows the average chemical composition of the different parts of *C. lusitanica* after the extraction process, expressed on dry basis.

Table 16 Chemical composition of the different parts of *C. lusitanica* after the extraction process with Clevenger apparatus.

Component	% of dry weight			
	Extracted Bark	**Extracted Chips**	**Extracted Leaves**	**Extracted Cones**
Gn	33.74	45.22	20.53	27.76
Hemicelluloses	18.83	30.36	20.46	23.63
Xn	12.15	22.86	11.74	14.67
Arn	5.67	5.28	6.90	7.25
Ac	1.01	2.22	1.81	1.71
KL	47.78	37.67	50.50	45.74
Ash	4.31	0.40	3.87	2.73
Others (by difference)	0	0	4.67	0.11

After the Clevenger extraction, *C. lusitanica* samples present lower amounts of extractives, as expected from the results described above for the oil yields and especially for the decoction waters.

The bark, after the extraction process, showed 52.56 % of total polysaccharides. This value is due to the decrease in percentages of Gn and hemicelluloses. The most significant change on hemicelluloses occurred in the percentage of Arn from 9.15 % to 5.67 %. There was no change in the percentage of Xn, which indicates that the bark, after the extraction process, may continue to enable the production of hydrolyzates rich in xylo-oligosaccharides (XOS). It is also important to note the significant increase in the KL content of 35.41 % to 47.78 %.

The chips showed 75.57 % of total polysaccharides, verifying that the extraction process has resulted in an increase of the percentages of Gn and hemicelluloses. In relation to hemicellulosic components, unlike what occurred in the bark, there was a slight increase in the percentage of Xn from 21.75 % to 22.86 % and the percentage of Arn from 3.71 % to 5.28 %. An increase in the percentage of KL from 35.85 % to 37.67 % was also reported.

For the leaves, there was a decrease of their hemicellulosic content, mainly due to the change in the percentage of Arn to 6.90 %, resulting in 40.99 % of total polysaccharides. Again, oddly, the percentage of KL increased from 46.68 % to 50.50 %.

Finally, the extraction process of cones resulted in a variation of total polysaccharides due mainly to the increase in the percentage of Gn from 25.36 to 27.76%. The percentage of hemicelluloses has also suffered a slight variation due to the increase of Xn to 14.67% and decreased Arn to 7.25%. The KL showed a final percentage of 45.74%.

After the extraction process, despite the variations in chemical composition of the different part of *C. lusitanica*, the results continue to indicate a strong potential for the production of hydrolyzates rich in OS, namely in XOS.

For the subsequent work, only three materials were chosen. Chips, as they are the most significant fraction and can be directly used without extraction of essential oils, and leaves, both before and after oil extraction, as these represent the most significant fraction containing essential oils, extractable sugars and phenolic compounds.

3.1.1.3 Granulometric characterization of chips

The particle size, as an indicator of the surface area, of raw materials has an effect on the pretreatment processes, because it has implications in heat and mass transfer (115).

Previously to the hydrothermal treatments, the chips of *C. lusitanica* were characterized regarding the particle size, in order to decide which fractions would be used in autohydrolysis processes.

A granulometric separation was performed, after size reduction of the chips of *C. lusitanica* to particles smaller than 4 mm, into seven fractions (Figure 18).

Based on Figure 18, it was determined that the fractions with particle size between 1 and 2 mm corresponded to 60.32 % of the total mass while larger fractions (≥ 2 mm) represented only 8.85 % of total mass, and the smallest fractions (≤0.5 mm) represent 30.64 %. In this work, it was decided to use the fraction corresponding to 1-2 mm, because it represents the major fraction and this particular diameter is more suitable for autohydrolysis processes (115). The fractions above 3.55 mm, due to their dimensions, are not suitable for hydrothermal studies, since they may impose diffusional limitations to mass and heat transfer. On the other hand, the fractions smaller than 1 mm are more likely

to be degraded during the treatments and, for that reason, are more suitable for thermochemical treatments (115).

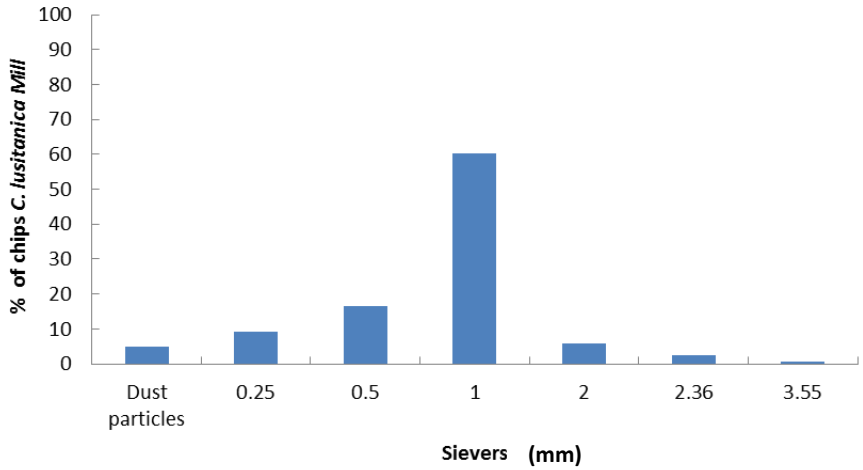


Figure 18 Mass distribution of different granulometric fractions of the milled chips of *C. lusitanica Mill*..

3.1.2 Autohydrolysis process

3.1.2.1 Characterization of the temperature profile

The autohydrolysis process was studied using non-isothermal conditions and, for each performed test, the temperature and pressure profiles were recorded over time. The heating profile of the reactor follows a linear trend, whereas the pressure variation follows an exponential trend.

Figure 19 shows, as an example, the heating profile for the test in non-isothermal conditions for chips of *C. lusitanica* (T=210 ºC, LSR=10 w_{water}/w_{chips}). All other profiles did not differ from the presented trend, showing a high degree of reproducibility.

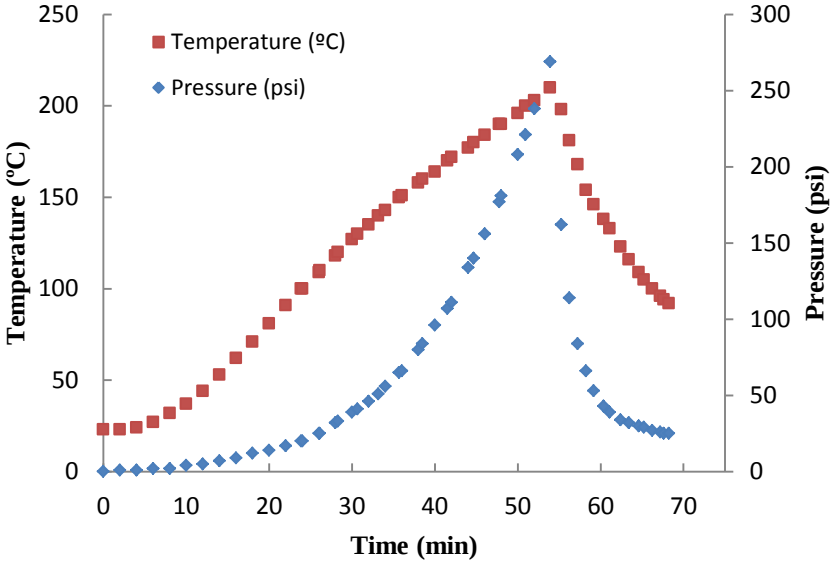


Figure 19 Typical temperature and pressure profiles for the autohydrolysis processes.

3.1.3 The effect of the liquid-to-solid ratio on the autohydrolysis of *C. lusitanica* chips

The relationship between the amount of water and biomass is one of the operating conditions that can determine the concentration of the resulting products and hence influence their purification efficiency. Furthermore, it strongly influences the installation capacity and the amounts of biomass able to be processed in a given time frame. Whether or not it also specifically influences the process performance is still subject to discussion in the literature, and this is here explored.

The autohydrolysis of the chips of *C. lusitanica* was assayed using different LSR between 7 and 13 w_{water}/w_{chips}, to a final process temperature of 200 ºC.

Figure 20 presents the gluco-oligosaccharides (GOS), xylo-oligosaccharides (XOS) and arabino-oligosaccharides (AOS) yields on the liquid phase profile as a function of LSR.

The data shows that the variation of the LSR had no significant influence on the obtained OS yields. The maximum OS yield was obtained for LSR of 13 w_{water}/w_{chips}, with yields of 1.03 g GOS/100g of dry biomass, 8.63 g XOS/100g of dry biomass and 0.42 g AOS/100g of dry biomass, with minimal difference to the other conditions. For instance,

XOS reached a maximum yield for LSR of 10 w_{water}/w_{chips}, which corresponded to 8.81 g XOS/100g of dry biomass.

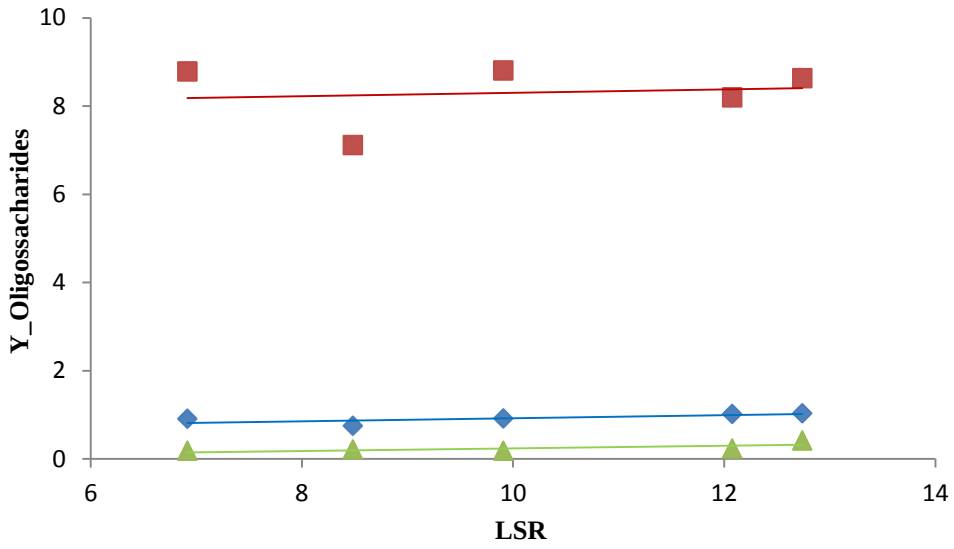


Figure 20 Effect of the LSR on the OS yield (g OS/100g of dry biomass) obtained after autohydrolysis of *C. lusitanica* chips up to 200 ºC
(XOS, GOS, AOS).

The fact that LSR did not cause significant yield changes in OS liquors indicates that the high concentrations of raw material used in the lower LSR processes do not impose any diffusional limitations to mass transfer. Moreover, the lower availability of hydronium catalyst (H_3O^+), derived from the autoionization of water, did not lead to decreased efficiency of the hydrolysis of hemicelluloses present in the raw material.

Along with the production of OS, the formation of the respective monosaccharides also occurred. The monosaccharides quantified in this study, as well as their yields, are represented in Figure 21.

As verified for the OS, the variation of the LSR did not have great influence on the production of monosaccharides, and a maximum yield of 4.48 g monosaccharides/100g of dry biomass was reached for LSR of 12. W_{water}/w_{chips}. The Xyl was the monosaccharide which was obtained with the highest yield (2.85 g Xyl/100g of dry biomass), followed by Ara and Glc, which corresponded to a maximum yield of 1.29 g Ara/100g of dry biomass and 0.33 g Glc/100g of dry biomass.

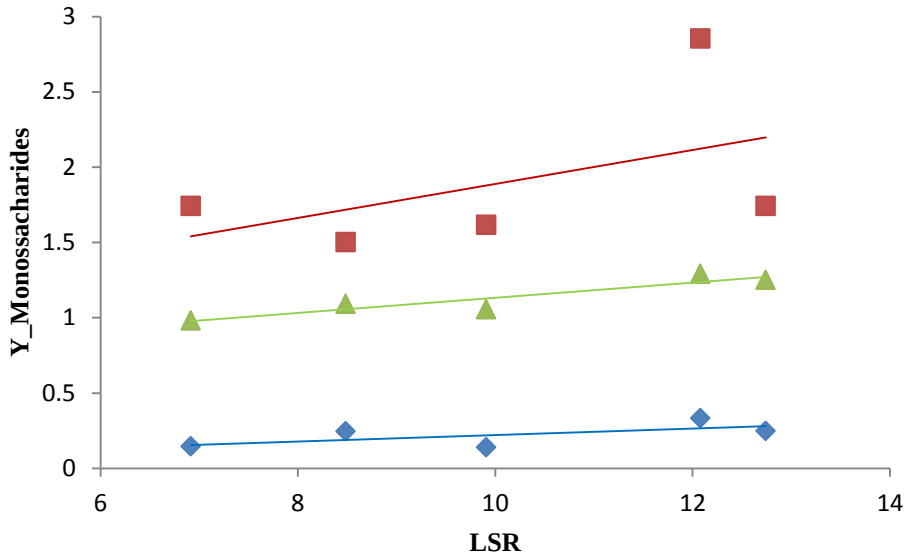


Figure 21 Effect of the LSR on the monosaccharides yield (g monosaccharides/100g of dry biomass) obtained after autohydrolysis of *C. lusitanica* chips up to 200 ºC (Xyl, Glc, Ara).

In Figures 22 and 23 the corresponding profiles for aliphatic acids (HFor, HAc and HLev) and furans (HMF and Furf), respectively, are represented as a function of the LSR.

Taking into account that the HAc results from the hydrolysis of the Ac present in the hemicelluloses, the expected yield would be 4.41 g HAc/100g of dry matter (value obtained with the determination of the chemical composition of the chips of *C. lusitanica* (Table 15)). However, the maximum yield shown in Figure 22 was 1.05 g HAc/100g of dry biomass (for LSR=12 w_{water}/w_{chips}), lower than the expected value.

The maximum yield of HFor was 1.01 g HFor/100g of dry biomass for LSR of 12 w_{water}/w_{chips}. The yields obtained for the HLev (maximum yield of 0.05 g HLev/100g of dry biomass) are negligible, being this a degradation product of HMF.

The yield profiles for furans (Furf and HMF), present in the liquor, reflect the degradation of monosaccharides, whose maximum yields reached 0.40 g Furf/100g of dry biomass and 0.11 g HMF/100g of dry biomass.

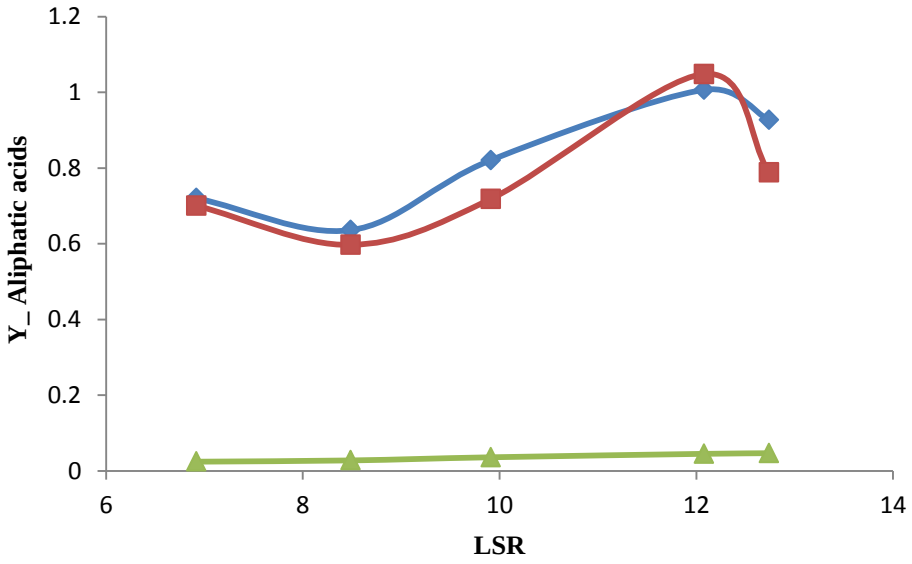


Figure 22 Effect of the LSR on the aliphatic acids yield (g aliphatic acids/100g of dry biomass) obtained after autohydrolysis of *C. lusitanica* chips up to 200 ºC
(HFor, HAc, HLev).

The analysis of the LSR is not complete without the evaluation of the impact of LSR on the solid fraction, as the analysis of the chemical composition of the residual solids can also contribute to verify the impact and efficiency of autohydrolysis process on the raw material. Figure 24 presents the composition in Gn, Xn, Arn, Ac and KL, as a function of the LSR.

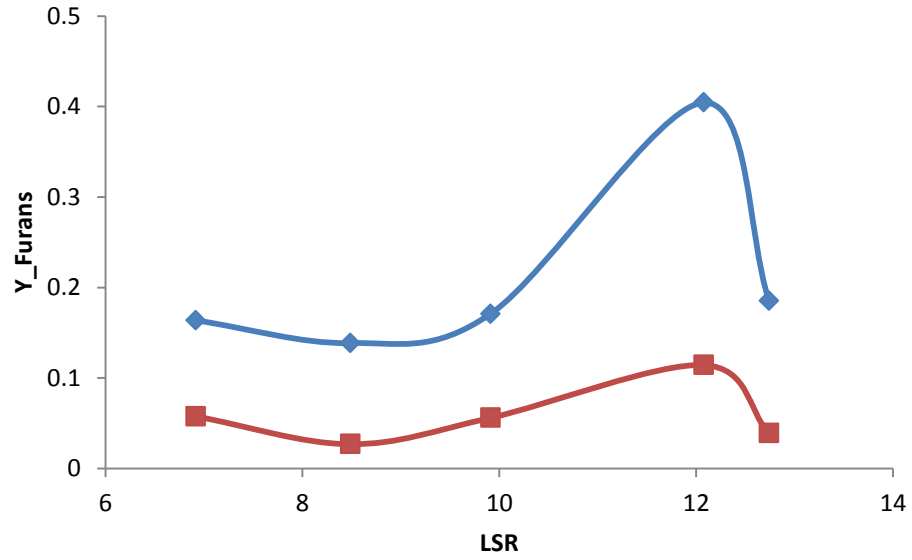


Figure 23 Effect of the LSR on the furans yield (g furans/100g of dry biomass) obtained after autohydrolysis of *C. lusitanica* chips up to 200 ºC
(HMF, Furf).

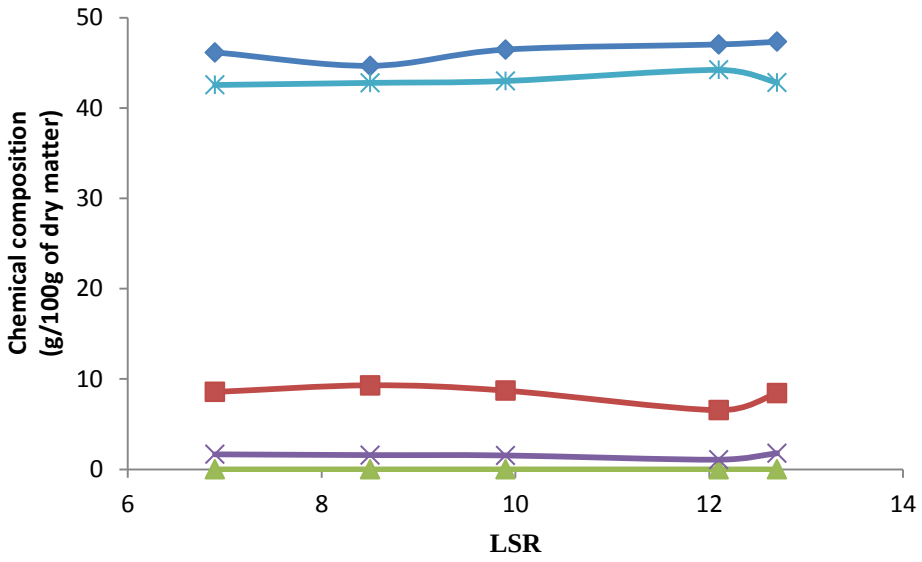


Figure 24 Effect of the LSR on the chemical composition of the solids resulting after autohydrolysis of *C. lusitanica* chips up to 200 ºC (Gn, Xn, Arn, Ac, KL).

As expected, the composition of the solid residue resulting from the autohydrolysis is not similar to the raw material, presenting a higher amount of Gn and lower hemicellulosic content that can be explained by the selective hydrolysis of the hemicellulosic polysaccharides. But, again, no significant variation among the tested conditions was found.

Regarding the KL, a similar trend to Gn is observed, but contrarily to Gn, which is partially soluble, the lignin is completely recovered on the solid phase.

As no significant difference between the tested LSR was identified, LSR=10 w_{water}/w_{chips} was established as the operational LSR for subsequent studies, as it is also one of the most common values used in literature (6,66,72,73). Nevertheless, it should be pointed out that, for larger scale operations, LSR=7 $w_{water}/w_{biomass}$ would be preferable.

After the determination of the optimal LSR conditions for the autohydrolysis of the chips of *C. lusitanica*, five tests were carried out at the optimal LSR, whose temperature was varied between 150 and 230 ºC.

3.1.4 The effect of temperature on the autohydrolysis of *C. lusitanica* chips

Figure 25 presents the composition of the obtained liquors in terms of OS for different temperatures for the chips of *C. lusitanica*.

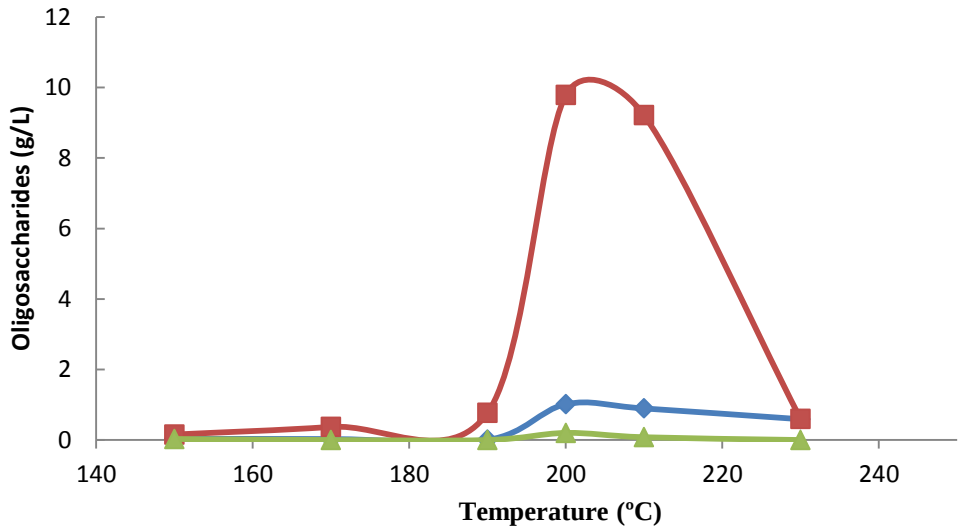


Figure 25 Effect of the temperature (ºC) on the OS concentration (g/L) obtained after autohydrolysis of *C. lusitanica* chips (XOS, GOS, AOS).

The produced OS reached a maximum concentration of 11 g/L (9.90 g OS/100g of dry biomass) at 200 ºC. This is then considered the optimal temperature for the production of OS from autohydrolysis of chips of *C. lusitanica*, because the sugars were recovered mainly in oligomeric form. They include the XOS, as oligomeric component produced in larger quantity, reaching a maximum concentration of 9.78 g/L (which corresponds to a maximum yield of 8.19 g XOS/100g of dry biomass), followed by the GOS with a maximum concentration of 1.01 g/L (maximum yield of 0.80 g GOS/100g of dry biomass). The AOS only reached a maximum concentration of 0.20 g/L (maximum yield of 0.07 g AOS/100g of dry biomass).

For temperatures higher than 200 ºC, there was a decrease in the concentration of OS due to the formation of monosaccharides and their degradation products. Figure 26 shows the concentration profiles of the monosaccharides present in the liquors for the different temperatures tested in the autohydrolysis process. There was an increase in the

concentration of Xyl from 190 ºC, reaching a maximum concentration of 3.98 g/L (maximum yield of 3.92 g Xyl/100g of dry biomass) at 230 ºC.

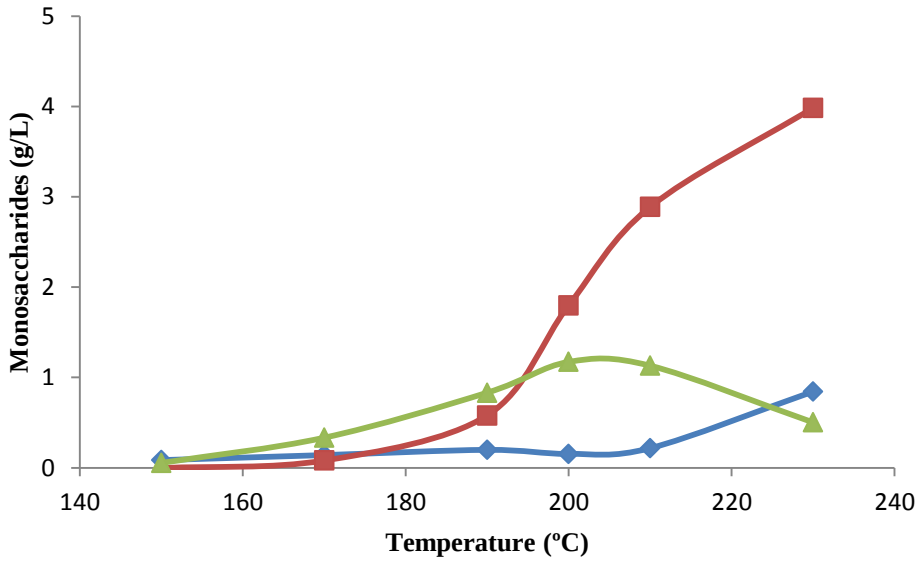


Figure 26 Effect of the temperature (ºC) on the monosaccharides concentration (g/L) obtained after autohydrolysis of *C. lusitanica* chips (Xyl, Glc, Ara).

The concentration profile of Ara showed a decrease for the highest temperature and a maximum concentration of 1.17 g/L (maximum yield of 1.00 g Ara/100g of dry biomass) was reached at 210 ºC.

Glucose has a concentration profile that is in agreement with the profile of GOS, with an increase in concentration from the moment that the concentration of corresponding oligomeric components begins to decrease, reaching a maximum concentration of 0.84 g/L (maximum yield of 0.83 g Glc/100g of dry biomass) at 230 ºC.

Figures 27 and 28 present the concentration profiles of aliphatic acid and furans respectively, as a function of temperature.

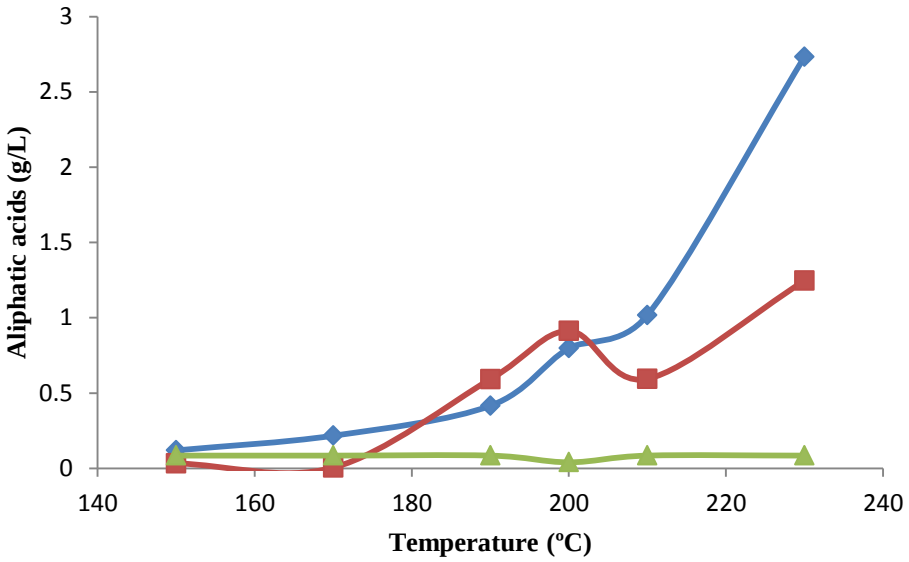


Figure 27 Effect of the temperature (ºC) on the aliphatic acids concentration (g/L) obtained after autohydrolysis of *C. lusitanica* chips (HForm, HAc, HLev).

The HAc, resulting from hydrolysis of the Ac, reached a maximum concentration of 2.73 g/L, at the highest assayed temperature (T=230 ºC). The yield corresponding to that maximum is 2.69 g HAc/100g of dry biomass. Comparing this value with the expected maximum yield (4.14 g HAc/100g of dry biomass (Table 15)), it is concluded that the experimentally obtained yield was lower, indicating that the temperature of 230 ºC did not induce a complete hydrolysis of the Ac present in the raw material. For others acids, there is a maximum production of 1.25 g/L of HFor (maximum yield of 0.53 HFor/100g of dry biomass), also at 230 ºC. HLev concentration remained negligible (maximum yield of 0.08 g HLev/100g of dry biomass).

The concentrations of furans, obtained at different temperatures, reflect a degradation of the corresponding monosaccharides. The increase of the concentration of Furf from 200 ºC coincides with the maximum production of XOS. From this point, the amount of Xyl, present in the liquor, tends to increase with increasing temperature, being also expected an increase in the corresponding degradation products, in this case, Furf. The maximum concentration reached by Furf was 1.77 g/L at 230 ºC (maximum yield of 1.74 g Furf/100g of dry biomass). Increasing concentrations of Xyl and Furf with decreasing concentration of XOS to 230ºC is justified by the increased HAc causing a decrease in Xyl. Degradation of XOS results in the formation of Xyl and consequently of the Furf. In the case of HMF, there is an increase in its concentration from 190 ºC, reaching a maximum of

2.17 g/L (maximum yield 2.14 g HMF/100g of dry biomass) at 230 ºC. As Glc did not present a significant decrease, it can be hypothesized that some of the HMF produced is not, in fact, derived from Glc, but rather from other hexoses, namely galactose and mannose that are being analyzed as Xyl as discussed above in the materials and methods chapter.

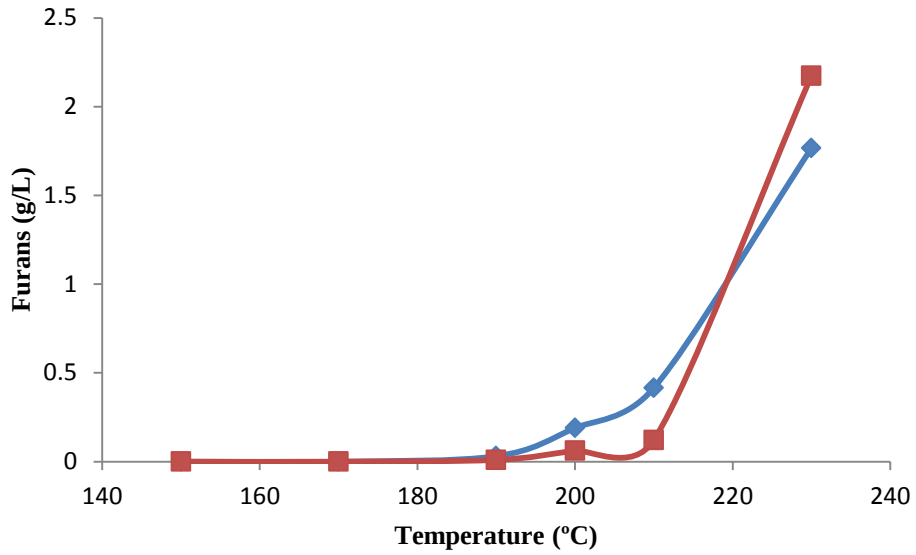


Figure 28 Effect of the temperature (ºC) on the furans concentration (g/L) obtained after autohydrolysis of *C. lusitanica* chips (HMF, Furf).

The liquors resulting from the autohydrolysis processes of chips of *C. lusitanica* were also analyzed by CZE, to verify if any specific phenolic compounds were present. Table 17 shows the phenolic compounds present in the liquors, as well as the respective integration values, obtained at different temperatures.

Table 17 Phenolic compounds identified by CZE on liquors resulting from the autohydrolysis of chips of *C.lusitanica* for the different temperatures tested.*

Phenolic compound	Temperature (ºC)					
	150	**170**	**190**	**200**	**210**	**230**
Hydroxybenzoic acid derivatives (λ=200nm)	226	426	509	345	347	365
Vanillin (λ=320nm)	-	-	25	55	89	210

* The reported values are the CZE peak areas

The phenolic compounds obtained from the liquors resulting from the autohydrolsis of chips were hydroxybenzoic acid derivatives and vanillin. Vanillin was only detectable in the liquors obtained from 190 ºC onwards, and the corresponding integrals indicate an increase of the vanillin content as the final temperature of autohydrolysis increased.

In addition to the liquors was also performed chemical analysis of residues solids arising from the autohydrolysis process Figure 29 represents the chemical composition of the residual solids obtained in each autohydrolysis process as a function of temperature.

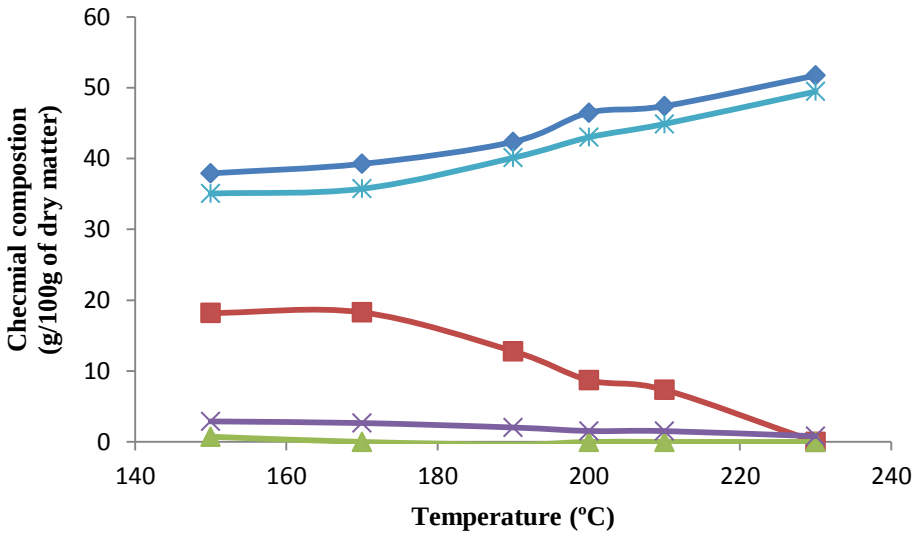


Figure 29 Chemical composition of the solids obtained from the autohydrolysis process of *C. lusitanica* chips as a function of temperature
(Gn, Xn, Arn, Ac, KL).

The residual solids obtained in the autohydrolysis process up to 170 ºC, have a similar chemical composition to that of the raw material, which is justified by the low levels of produced OS, especially XOS.

For temperatures above 170 ºC, the level of residual Xn begins to decrease, reaching minimum concentrations for the highest temperatures. A similar trend is observed for the content of Ac. It is also important to note that Arn has been totally removed, although the Arn content is smaller than Xn, as can be seen on the description of the initial composition (Table 15).

The concentration of KL and Gn in the residual solids increased with temperature, which is justified by the decrease of the content in the Xn, Arn and Ac. Lignin was totally recovered in the solid phase, whereas Gn was partially solubilized.

In view of these data, the efficiency and selectivity of the autohydrolysis process is confirmed, for the hydrolysis of hemicelluloses present in the chips of *C. lusitanica* resulting mainly in the production of OS.

3.1.5 The effect of temperature on the autohydrolysis of *C. lusitanica* leaves

The leaves of *C. lusitanica* were also subjected to various autohydrolysis processes, whose final temperature was varied between 150 ºC and 230 ºC, for a constant LSR of 10 w_{water}/w_{leaves}. The main aim was to verify the effect of temperature variation and, consequently, to establish an optimum temperature for the production of OS for this material.

Figure 30 shows the profile presented by the various OS produced according to different temperatures applied in the autohydrolysis process. The maximum production of OS occured at a temperature of 190 ºC, being the AOS produced in greater quantities (4.28 g/L, whose maximum yield corresponds to 3.89 g AOS/100g of dry biomass), followed by the XOS (3.34 g/L, with yield of 3.04 g XOS/100g of dry biomass) and lastly the GOS (1.77 g/L, whose maximum yield corresponds to 1.60 g GOS/100g of dry biomass). Despite establishing 190 ºC as the optimum temperature for the production of OS, it is noteworthy that the XOS only reached their maximum (4.13 g/L) at 210 ºC (maximum yield of. 3.51 g XOS/100g of dry biomass).

After 190 ºC, there was a decrease in the concentration of AOS and GOS, which should correspond to an increase in concentration of the monosaccharides Ara and Glc, respectively. The same should happen for the XOS but only after 210 ºC.

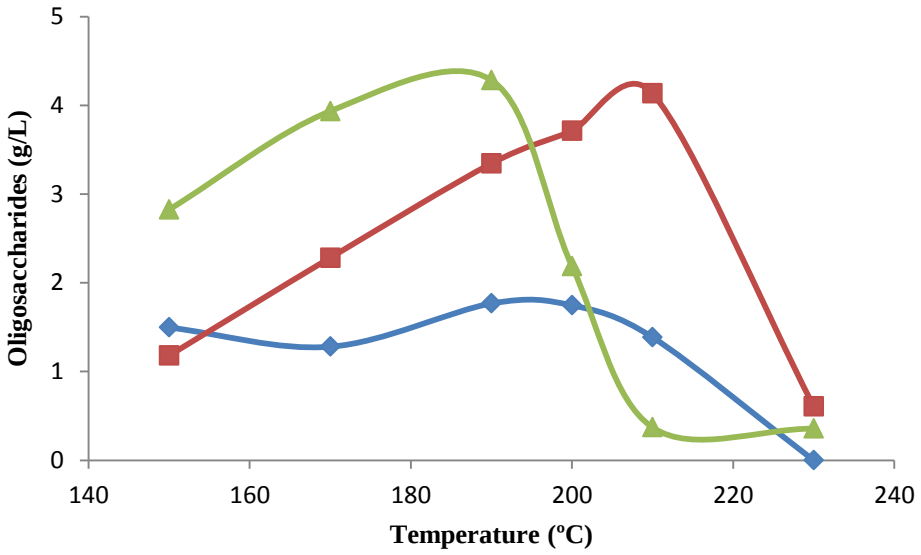


Figure 30 Effect of temperature (ºC) on the OS concentration (g/L) obtained after autohydrolysis of *C. lusitanica* leaves (XOS, GOS, AOS).

Analyzing the concentration profiles of the monosaccharides, represented in Figure 31, it is found that the concentration profile of Ara was consistent with the profile of the AOS, reaching a maximum concentration of 7.12 g/L (maximum yield of 6.74 g Ara/100g of dry biomass) at 200 ºC, at which temperature the production of AOS started decreasing. The concentration profiles of Xyl and Glc also occurred as expected because there was a maximum concentration of 1.67 g/L (maximum yield of 1.55 g Xyl/100g of dry biomass) and 3.12 g/L (maximum yield of 2.88 g Glc/100g of dry biomass), respectively, for the temperatures at which the minimum concentration of the corresponding oligomeric components was reached (T=210 and 230 ºC).

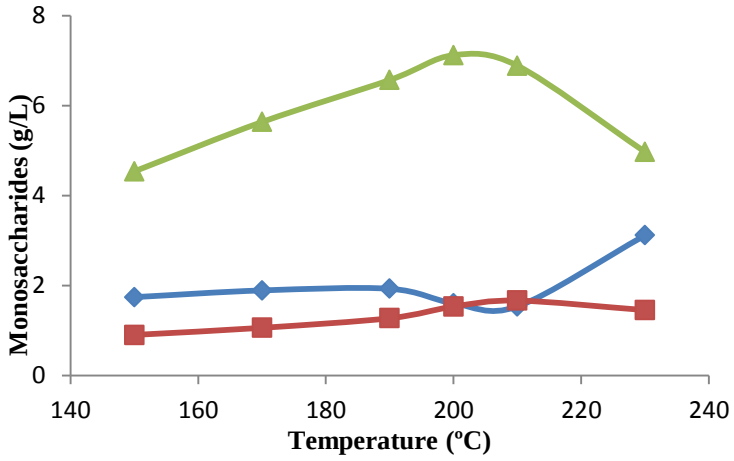


Figure 31 Effect of temperature (ºC) on the monosaccharides concentration (g/L) obtained after autohydrolysis of *C. lusitanica* leaves

(Xyl, Glc, Ara).

Figure 32 shows the concentration profiles of aliphatic acids present in the liquors resulting from the autohydrolysis process. It is seen that all acids reached maximum concentrations at 230 ºC, being 1.82 g/L for HFor (1.68 g/100g of dry biomass), 2.35 g/L for HAc (2.17 g HAc/100g of dry biomass) and 0.18 g/L for HLev (0.17 g HLev/100g of dry biomass.

For the HAc, an increase of the concentration until a maximum of 2.48 g/100g of dry matter (corresponding to the total solubilization of Ac present in the hemicelluloses) would be expected, after which its concentration should stabilize. The maximum experimental yield was not very different from the expected value.

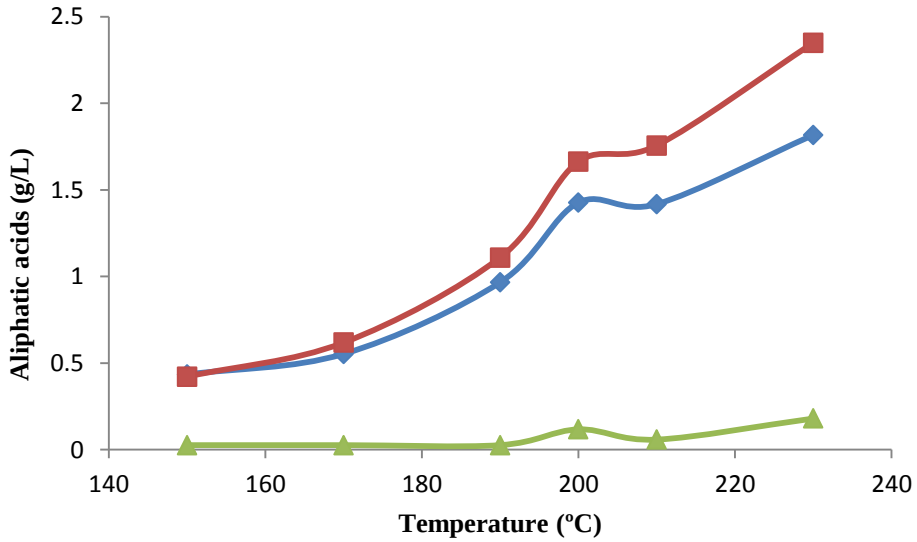


Figure 32 Effect of temperature (ºC) on the aliphatic acids concentration (g/L) obtained after autohydrolysis of *C. lusitanica* leaves
(HAc, HForm, HLev).

The profiles depicted in Figure 33 show an increase in the concentration of furans between 190 and 210 ºC, temperatures at which the maximum production of OS (AOS and XOS) occurred. The increase in concentration of furans from these temperatures is due to the increase of monosaccharides, which in turn motivates the production of degradation products. The maximum concentrations were 0.51 g/L for Furf (maximum yield of 0.47 g Furf/100g of dry biomass) and 0.46 g/L for HMF (maximum yield of 0.42 g HMF/100g of dry biomass).

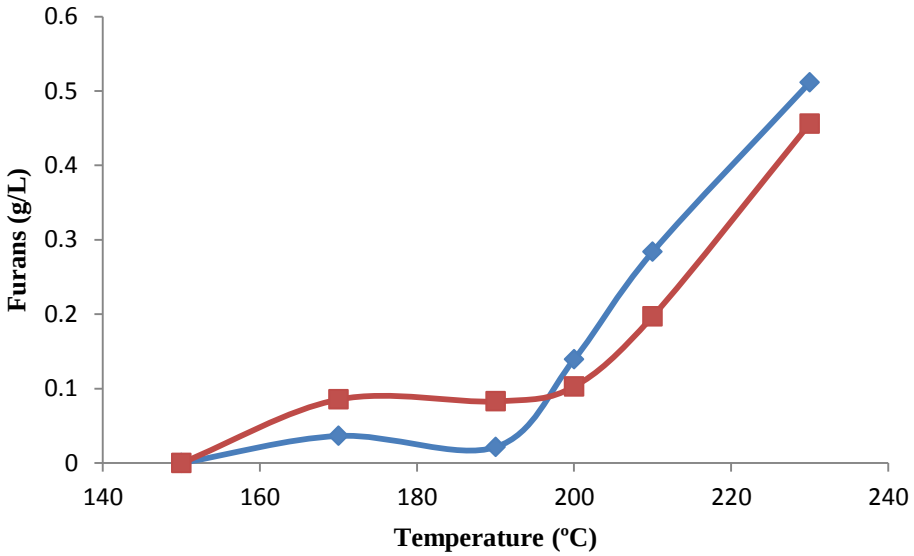


Figure 33 Effect of temperature (ºC) on the furans concentration (g/L) obtained after autohydrolysis of *C. lusitanica* leaves (HMF, Furf).

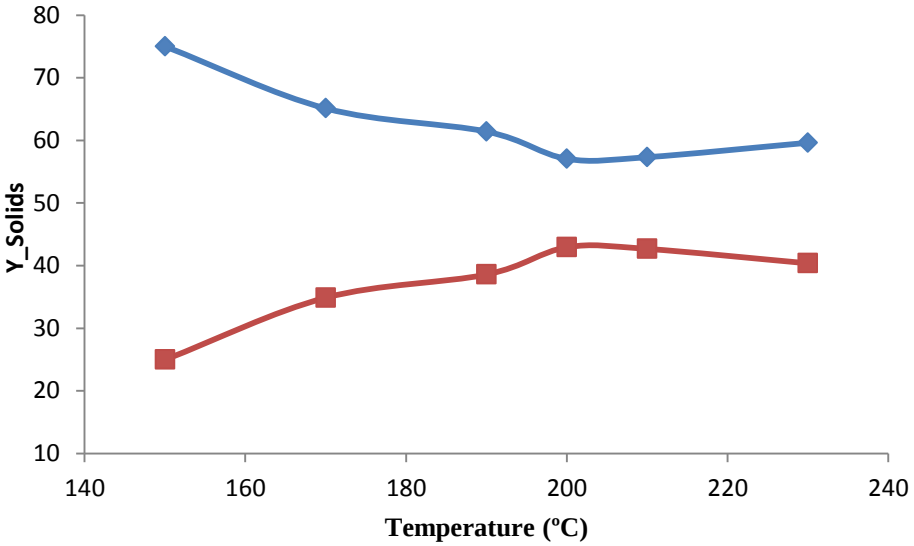


Figure 34 Change of the solid yield (Y_Solids) and solubilized solids as a function of temperature (ºC) for autohydrolysis of *C. lusitanica* leaves (solid yield; solubilized solids).

The previous data indicate an increase of solubilization of hemicelluloses in leaves with increasing temperature of the autohydrolysis process. The profiles of the yield variation of solids and solubilized solids, shown in Figure 34, reinforce these data, showing a progressive decrease of the collected solids from raw material with the temperature increase.

The liquors resulting from the autohydrolysis processes of leaves of *C. lusitanica* were analyzed by CZE, to verify what specific compounds were present. Table 18 summarizes the data.

Table 18 Phenolic compounds identified by CZE on liquors resulting from the autohydrolysis of leaves of *C. lusitanica Mill.* for the different temperatures tested.*

Phenolic compound (λ=200nm)	Temperature (ºC)					
	150	**170**	**190**	**200**	**210**	**230**
Hydroxybenzoic acid derivatives	5678	5116	4541	4035	3981	2658
Catechol	381	393	414	421	463	774
Epicatechin	2894	2768	1546	1192	985	446

* The reported values are the CZE peak areas

The phenolic compounds detected in the liquors resulting from the autohydrolysis processes of leaves were hydroxybenzoic acid derivatives, catechol and epicatechin. As the final temperature of the autohydrolysis process increased, there was a gradual decrease of the corresponding integrals of hydroxybenzoic acid derivatives and epicatechin. The reverse situation happened with catechol, in which there was an increase in the concentration of this compound in the obtained liquors.

Taking into account the profiles of the OS resulting from the autohydrolysis of the leaves (Figure 30) and the data on the phenolic compounds, it is concluded that at the highest temperatures, there is a decrease of the OS and phenolic compounds contents, except for catechol.

Figure 35 shows the variation of the chemical composition of residual solids with the temperature, resulting from the various autohydrolysis processes tested for the leaves.

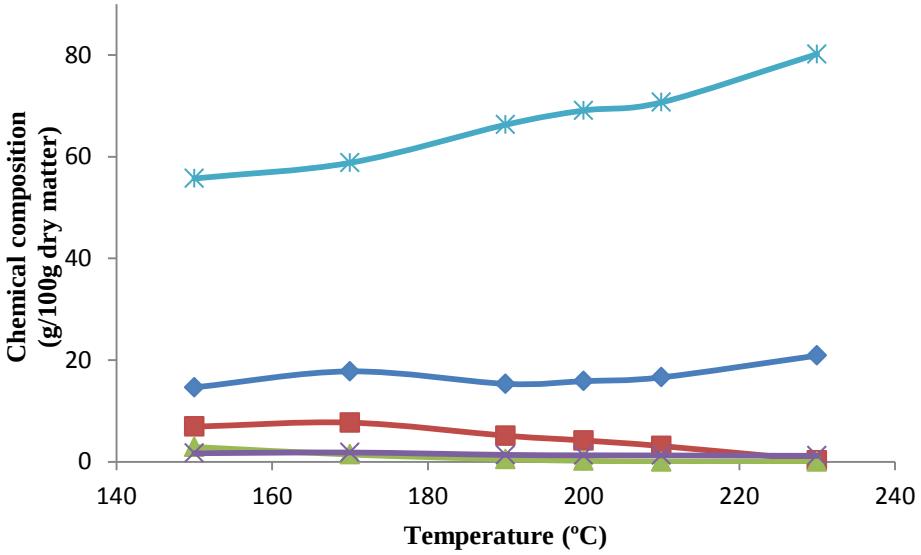


Figure 35 Chemical composition of the solids resulting from the autohydrolysis of *C. lusitanica* leaves (Gn, Xn, Arn, Ac, KL).

The similarity in chemical composition presented by the raw material (Table 15) and the residual solids, resulting from the various processes of autohydrolysis, justifies the low content in OS and high yield in solids observed for the lower temperatures. For more severe conditions, specifically for temperatures higher than 190 ºC, there is a decreased concentration of Arn and Xn in the composition of solids, which corresponds to an increase in the concentration of the corresponding oligomeric components (AOS and XOS).

The decrease of the concentration of Ac is justified by the production of HAc. For a temperature of 230 ºC it was expected that the Ac reached a concentration close to zero, taking into account the concentrations presented by HAc (Figure 32), and indeed this was observed.

For the different temperatures applied in the autohydrolysis process an increase in the concentration of lignin was also visible, which was later recovered in the solid phase. The variation of the concentration of Gn, in the composition of the residual solids, is justified by the fact that a partial solubilization occurred during the autohydrolysis process.

After this analysis, it is concluded again that the autohydrolysis process has proved a high selectivity for the hydrolysis of hemicelluloses present in the leaves of *C. lusitanica* resulting mainly in the production of OS.

3.1.6 The effect of temperature on the autohydrolysis of *C. lusitanica* extracted leaves

As leaves have a significant essential oil content, it is relevant to study the autohydrolysis process after the extraction process. As such, the leaves of *C. lusitanica* were subjected to various processes of autohydrolysis at temperatures of 190 ºC, 200 ºC and 210 ºC using a LSR of 10. The utilization of this biomass within the biorefinery concept and the detection of possible differences in yield and in the type of produced OS from non-extracted and extracted leaves were the main objectives of this study.

Figure 36 represents the concentration profiles of the different OS produced from the extracted leaves, as a function of temperature.

The maximum yield of OS occurred at 190 ºC, corresponding to a total of 7.55 g/L. At this temperature, the OS produced in the largest quantity were the XOS (3.59 g/L, whose yield corresponds to 4.04 g XOS/100g of dry biomass), followed by the AOS (2.89 g/L, whose yield corresponds to 3.26 g AOS/100g of dry biomass) and GOS (1.07 g/L, whose yield corresponds to 1.21 g GOS/100g of dry biomass).

The effect of temperature variation was found mainly in the concentration of XOS and AOS because, for temperatures above 190 ºC, a marked decrease in their concentrations occurred. This decrease is explained by the increase of the concentration of the corresponding monomeric components (Xyl and Ara, respectively), as can be seen in Figure 37. GOS showed no significant changes with the temperature variation, the same being verified on the Glc concentration profile in Figure 37.

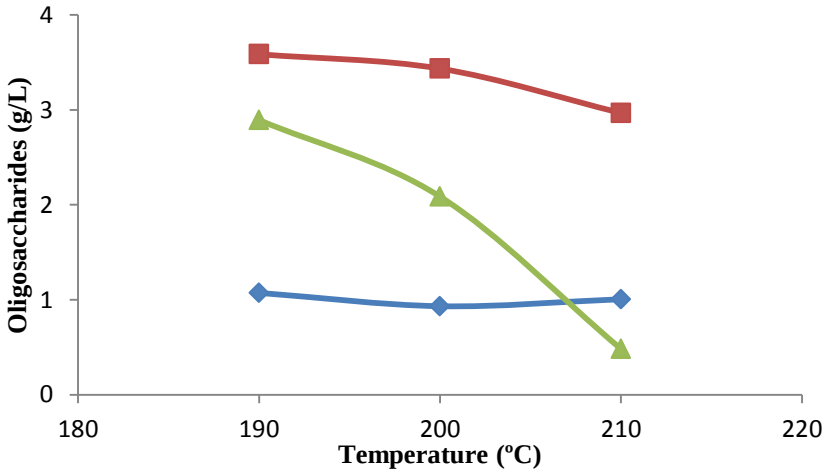


Figure 36 Effect of the temperature (ºC) on the OS concentration (g/L) obtained after autohydrolysis of *C. lusitanica* extracted leaves (XOS, GOS, AOS).

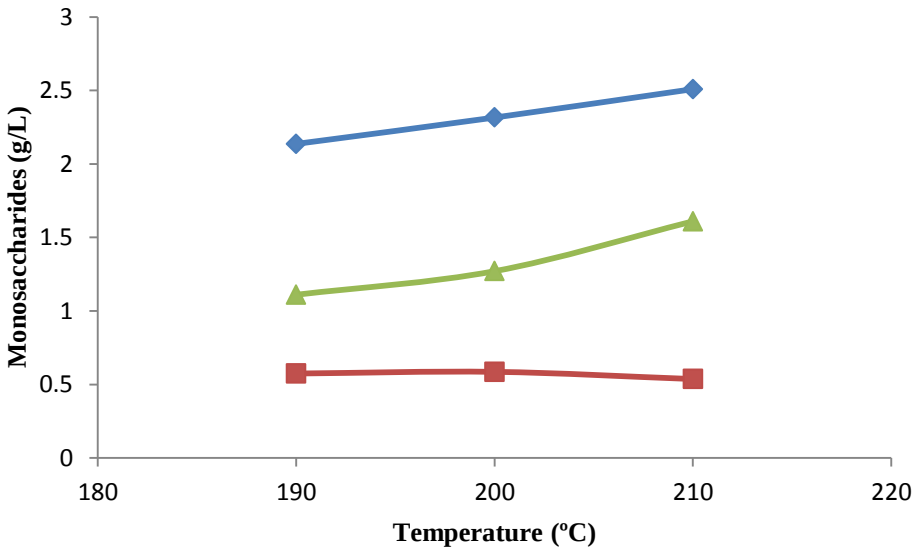


Figure 37 Effect of the temperature (ºC) on the monosaccharides concentration (g/L) obtained after autohydrolysis of *C. lusitanica* extracted leaves (Glc, Ara, Xyl).

Xyl and Ara concentrations increased from the moment that there was a degradation of the corresponding OS. Xyl presented a maximum concentration of 1.61 g/L (maximum yield of 1.83 g Xyl/100g of dry biomass) and Ara of 2.51 g/L (maximum yield of 2.86 g Ara/100g of dry biomasss) at a temperature of 210 ºC.

Figure 38 shows a progressive increase of the concentrations of HAc and HFor with increasing temperature, and a maximum concentration of HAc (1.31 g/L) and HFor (1.20 g/L) was observed at 210 ºC. The maximum production of HFor corresponds to a total of 1.37 g HFor/100g of dry biomass. In the case of HAc, the maximum yield was 1.49 g HAc/100g of dry biomass. Compared with the expected maximum yield (3.02 g/100g of dry matter), it is concluded that there was no hydrolysis of all Ac present in the raw material. The HLev concentration remained near zero (0.02 g/L) indicating that the degradation of HMF did not occur.

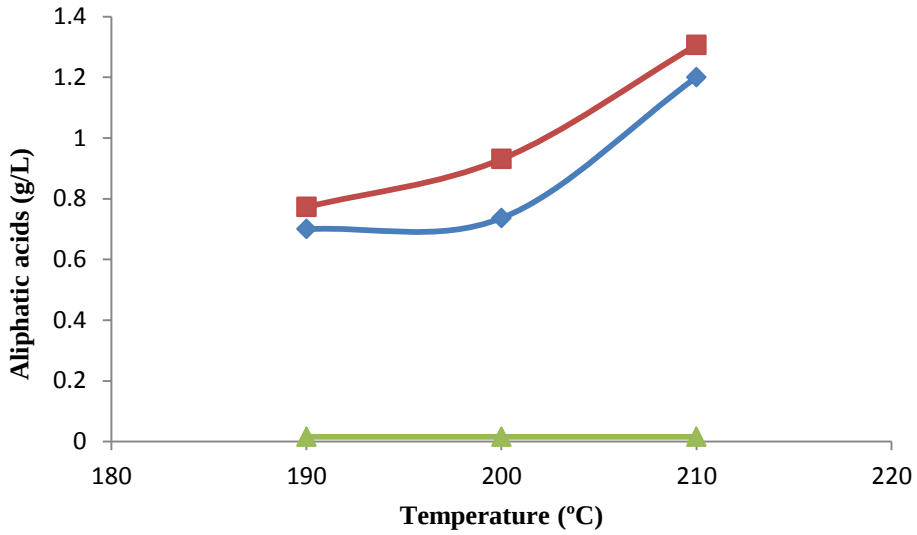


Figure 38 Effect of the temperature (ºC) on the aliphatic acids concentration (g/L) obtained after autohydrolysis of *C. lusitanica* extracted leaves (HAc, HFor, HLev).

The maximum concentration of furans was reached at a temperature of 210 ºC and it was 0.17g/L for Furf (maximum yield of 0.20 g Furf/100g of dry biomass) and 0.14 g/L for HMF (maximum yield of 0.16 g HMF/100 of dry biomass) (Figure 39).

As in the chips and non-extracted leaves of *C. lusitanica*, liquors resulting from the autohydrolysis of the extracted leaves were also analyzed by CZE. However, no phenolic compounds were detected. This may be explained by two facts that were previously presented. On the one hand, there are extractable phenolic compounds in the decoction waters, and on the other hand no significant lignin removal was observed, meaning that the phenolic compounds produced during the autohydrolysis of the leaves were in fact extractables and not structural phenolic compounds derived from lignin.

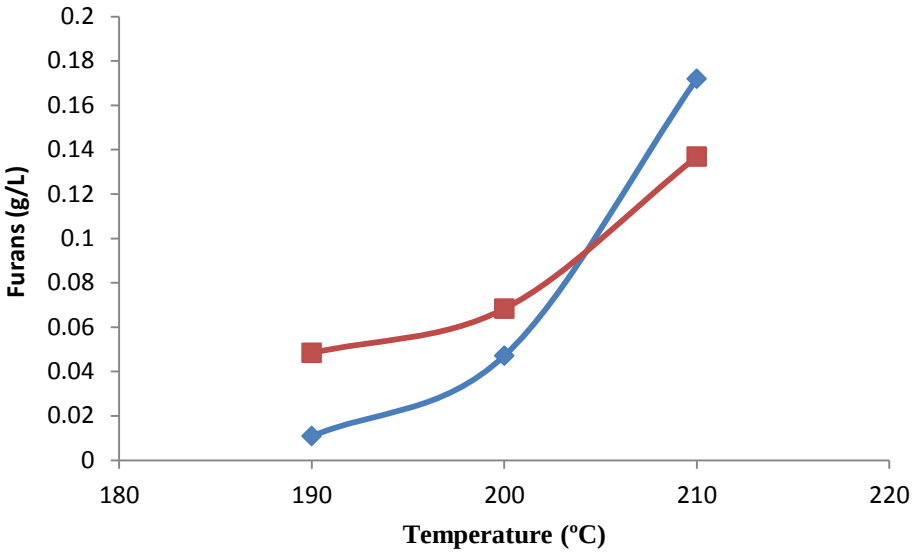


Figure 39 Effect of the temperature (ºC) on the furans concentration (g/L) obtained after autohydrolysis of *C. lusitanica* extracted leaves (HMF, Furf).

Figure 40 represents the chemical composition of the residual solids resulting from the three autohydrolysis processes performed for extracted leaves.

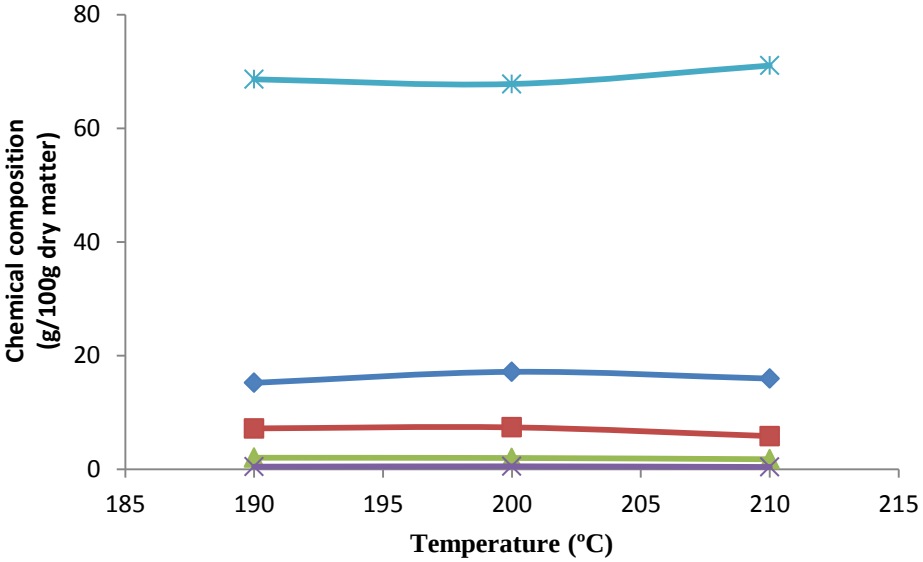


Figure 40 Chemical composition of the solids resulting of autohydrolysis process (Gn, Xn, Arn, Ac, KL).

It is found that the solids resulting from the first autohydrolysis process (T=190ºC) presented a different chemical composition from that presented by the untreated extracted

leaves (Table 16). This difference is explained by the high yields of XOS and AOS, resulting in a decrease in the content of Xn and Arn in solids. In the following treatments, their content showed a slight decrease with increasing final temperature, which was not very significant.

Lastly, the Gn and KL showed an increase in their concentration in residual solids, with increase the temperature of the autohydrolysis process, being the lignin totally recovered in the solid phase, further supporting the discussion on the phenolic compounds described above.

Once again, the autohydrolysis process proved to be a selective fractionation process for hydrolysis of hemicelluloses, essentially culminating in the formation of the OS.

3.1.7 Comparative study of oligosaccharides production from *C. lusitanica*

Figure 41 shows the profile of GOS, XOS and AOS yields in liquors resulting from the autohydrolysis process of all of the *C. lusitanica* fractions tested in this work as a function of temperature.

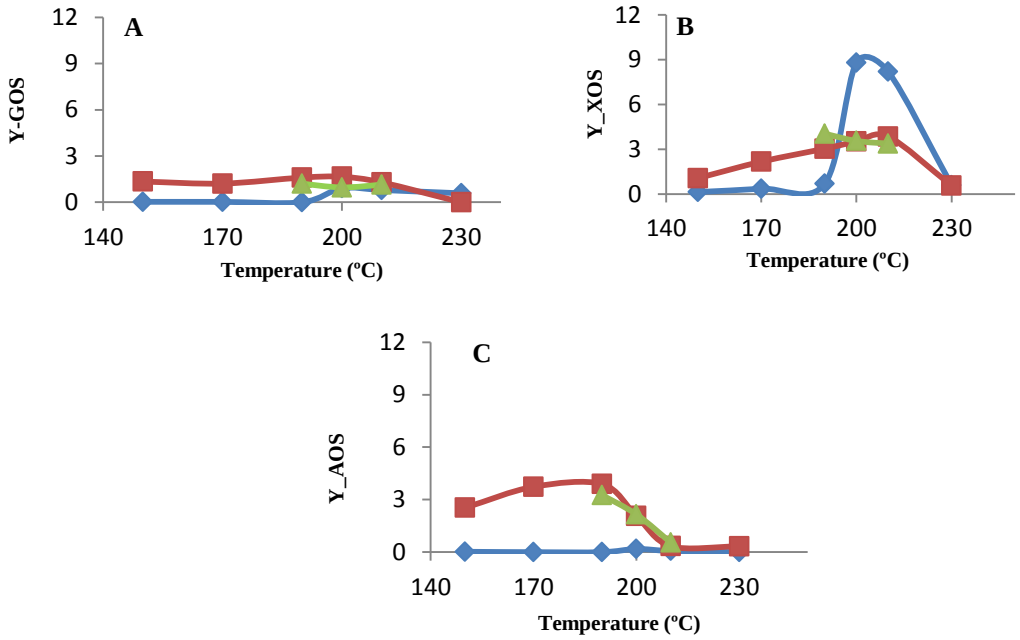


Figure 41 Profile of GOS (A), XOS (B), and AOS (C) yields resulting from the autohydrolysis process of the chips, non-extracted and extracted leaves of *C. lusitanica* as a function of temperature. (non-extracted leaves, extracted leaves, chips).

The profiles shown in Figure 41A indicate that the non-extracted leaves originated a greater yield of GOS, in comparison with the chips, at 200 ºC. The maximum yield corresponds to 1.65 g GOS/100g of dry biomass for non-extracted leaves and 0.91 g GOS/100g of dry biomass for chips. These values were not as expected, considering the data present in Table 15, indicating that the chips have a higher content of Gn relatively to the leaves. The non-extracted leaves also showed a higher yield of GOS as compared to those produced by the extracted leaves (1.21 g GOS/100g of dry biomass). This may be explained by the presence of some easily extractable Gn as it can be noted from table 16, where it is shown that the leaves after the extraction process had a lower content of Gn.

Contrarily to the GOS, the XOS (Figure 41B) were produced in greater quantity from chips (8.81 g XOS/100g of dry biomass), which was expected because it corresponded to the part of *C. lusitanica* that had a higher Xn content (Table 15). This high yield can also be explained by the higher susceptibility to hydrolysis of xylan as compared to glucan (cellulose) present in the chips.

The AOS were mainly produced by non-extracted leaves, followed by the extracted leaves and finally by the chips, whose yields corresponded to 3.89, 3.30 and 0.18 g AOS/100g of dry biomass, respectively (Figure 41C). Taking into account that the non-extracted leaves showed the highest content in Arn and the chips the smallest (Table 15 and 16), it is concluded that AOS yields occurred within the expected. The temperature at which the maximum yield in AOS was reached was 190 ºC for non-extracted and extracted leaves and 200 ºC for the chips, indicating that the later are less prone to hydrolysis.

After this study, it is concluded that the GOS and the AOS are mainly produced from the non-extracted leaves, and the XOS from the chips. The different types and yields in OS obtained from these fractions that come from the same biomass can be related to differences presented at the level of their cellular tissues. The cellular tissues are formed by cells whose structural integrity results from the existence of different layers (middle lamella, primary and secondary wall and *W* layer) (Figure 2), which differ from each other either in structure or in chemical composition (8,9). In each of these layers, the cell wall is composed of three main components: the cellulose microfibrills, hemicelluloses and either pectins in the primary wall or lignin in the secondary wall (8,9). Therefore, it is concluded that different amounts of polysaccharides produced by chips and leaves of *C. lusitanica* (Table 15) result from the difference in chemical composition in the primary and

secondary layers which constitute the respective cellular tissue. More specifically, different amounts of hemicelluloses presented by chips and leaves (Table 15) justify the different obtained yields in OS, after the autohydrolysis process.

No studies were found in the literature related to the yield obtained for different parts of a given raw material. However, the yield of XOS obtained for this material, in particular for chips, is lower than the values given by corn cobs (25.1 g XOS/100g, for non- isothermal operation), wheat straw (10.6 g XOS/100g and 10.5 g XOS/100g, for non-isothermal operation), eucalypt wood chips (13.0 g XOS/100g, for non-isothermal operation) and brewery's spent grain (16.3 g XOS/100 g, for isothermal operation) (116,117). When compared with the oil palm (4.3 g XOS/100g), the yield of XOS obtained from chips of *C. lusitanica* was far superior (118).

3.2 *Citrullus lanatus*

3.2.1 Partitional characterization of *Citrullus lanatus*

To better evaluate the upgrade potential of the watermelon biomass it is important to have a quantification of its partitioning among their main constituents. The following table presents the results for the samples used in this work.

In Table 19 data on biomass partitioning for watermelon samples used in this work are presented.

Table 19 Biomass partition for the watermelon samples used in this work.

Fraction	Value
Average Total Weight (kg)	4.40 ± 0.89
Pulp (%)	64.74 ± 2.76
Rinds (%)	28.85 ± 2.90
Seeds (%)	1.94 ± 1.22

As expected, pulp is by far the most significant fraction, accounting for two thirds of the total biomass. Rinds are the second major constituent, accounting for almost all of the remaining biomass, with seeds accounting for less than 2 %. As such, this work dealt only with rind upgrade.

3.2.2 Chemical characterization

Table 20 shows the average chemical composition of the watermelon rinds, expressed on dry basis. The characterization was made with dry rind.

It is found that the total polysaccharides correspond to a total of 40.72 % of the chemical composition of watermelon rinds, of which Gn represent 23.36 % and 17.36 % are hemicelluloses (as estimated from Xn, Arn and Ac content).

Regarding the KL content, the experimentally obtained values correspond to a total of 3.16 %, one of the lowest reported in literature, as expected for this kind of material (14,18,20,42). The high content of extractives (here quantified by difference) is also worth notice.

Table 20 Chemical composition (% of dry weight) of the watermelon rinds studied in this work.

Components	% of dry weight
	Watermelon rinds
Gn	23.36
Hemicelluloses	17.36
Xn	13.12
Arn	3.19
Ac	1.05
KL	3.16
Ash	10.78
Others (by difference)	56.13

3.2.3 The effect of the temperature on the autohydrolysis of watermelon rinds

The watermelon rinds were subjected to various autohydrolysis processes with final temperatures varying between 150 ºC and 230 ºC. The main aims of this study consisted essentially in verifying the effect of temperature variation and, consequently, in establishing an optimum temperature for which the OS production is maximum. Finally, it was intended to make a comparison between the OS produced by watermelon rinds and different parts of the *C. lusitanica* which differ much on their composition.

Figure 42 shows the yield variation of OS in the liquors obtained by autohydrolysis as a function of temperature. It is concluded that the increase in temperature led to a degradation of AOS and an increase in XOS and GOS. The maximum yield in OS corresponded to a total of 1.46 g/100g of dry biomass, reached at 210 ºC. The AOS were

the OS produced in greatest quantity, but conversely to the previous results at the lowest tested temperature.

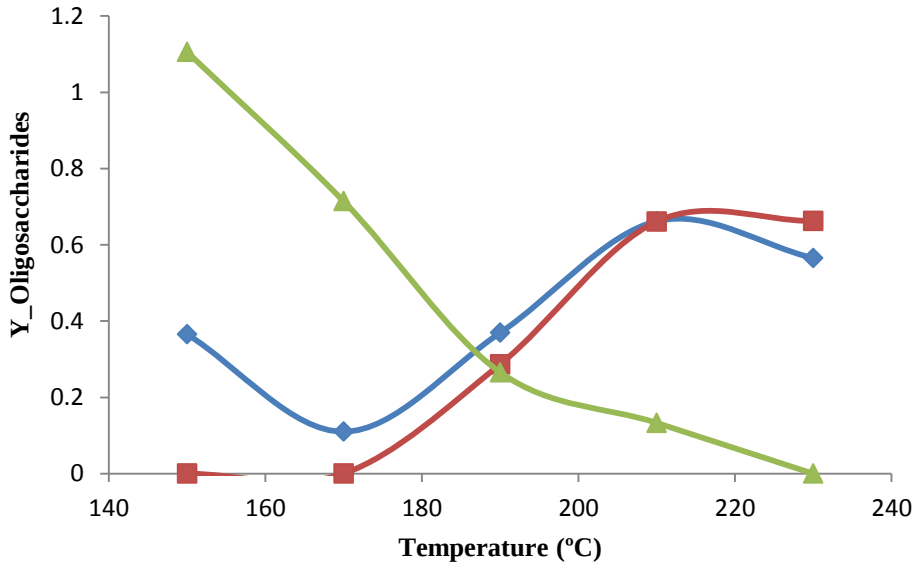


Figure 42 Effect of the temperature (ºC) on the OS yield (OS g/100g of dry biomass) obtained after autohydrolysis of watermelon rinds.
(Y_XOS, Y_GOS, Y_AOS).

Regarding GOS and XOS, the maximum experimentally obtained yield corresponded to 0.66 g/100g of dry biomass for both OS, and it was reached at higher temperatures (T=210 ºC). The presence of GOS in the different obtained liquors is justified by partial depolymerization of amorphous regions of cellulose, or other easily hydrolyzable glucans. In fact, the presence of starch is not excluded, and must be investigated in the future.

At temperatures higher than 150 ºC and 210 ºC, there is a decrease in the yields of different OS, justified by the hydrolysis in their monosaccharides and formation of the respective degradation products, as can be seen on Figure 43.

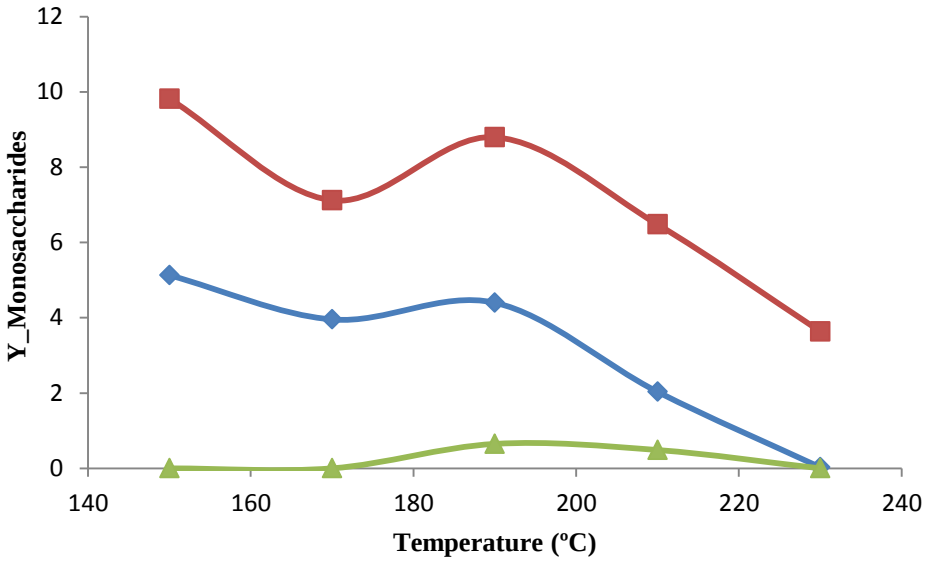


Figure 43 Effect of the temperature (ºC) on the monosaccharides yields (g OS/100g of dry biomass) obtained after autohydrolysis of watermelon rinds (Y_Xyl, Y_Glc, Y_Ara).

The yield profiles of monosaccharides as a function of temperature reflected the equilibrium between monosaccharide production from OS hydrolysis and the monosaccharide degradation. In the case of GOS and XOS, a decrease in the yields of their monomeric components was observed, reaching values of 5.13 g Glc/100g of dry biomass and 9.82 g Xyl/100g of dry biomass at 210 ºC.

For Ara there was an increase of its yield until 190 ºC, temperature at which a decrease of the production of AOS also occurred. The maximum yield was reached at 190 ºC, being 0.65 g Ara/100g of dry biomass.

After reaching their maximum yield, the different monosaccharides began to deteriorate resulting in the formation of their degradation products (aliphatic acids and furans). Figures 44 and 45 show the yield profiles of aliphatic acids and furans, respectively.

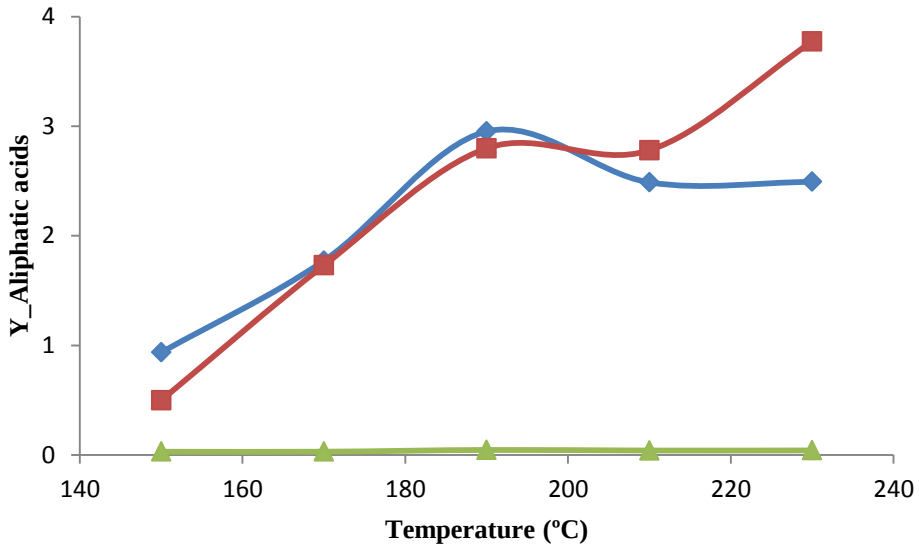


Figure 44 Effect of the temperature (ºC) on the aliphatic acids yields (g aliphatic acids/100g of dry biomass) obtained after autohydrolysis of watermelon rinds.
(Y_HAc, Y_HFor, Y_HLev).

For HAc, a product derived from the hydrolysis of Ac of hemicelluloses, an yield equal to or around 1.05 g/100 of dry matter would be expected (value obtained experimentally in the determination of the chemical composition of watermelon rinds), after which the HAc concentration should stabilize. However, the yields obtained in this study reached values of 3.78 g HAc/100g of dry biomass, i.e., were much higher than those corresponding to the total solubilization of Ac present in the raw material. In the case of HFor, the maximum obtained yield was 2.51 g HForm/100g of dry biomass, at a temperature of 230 ºC. The values corresponding to the yield of HLev are negligible.

Furf was the major furan produced, whose maximum yield was 1.70 g /100g of dry biomass, in comparison with the HMF (0.51 g/100g of dry biomass) (Figure 45).

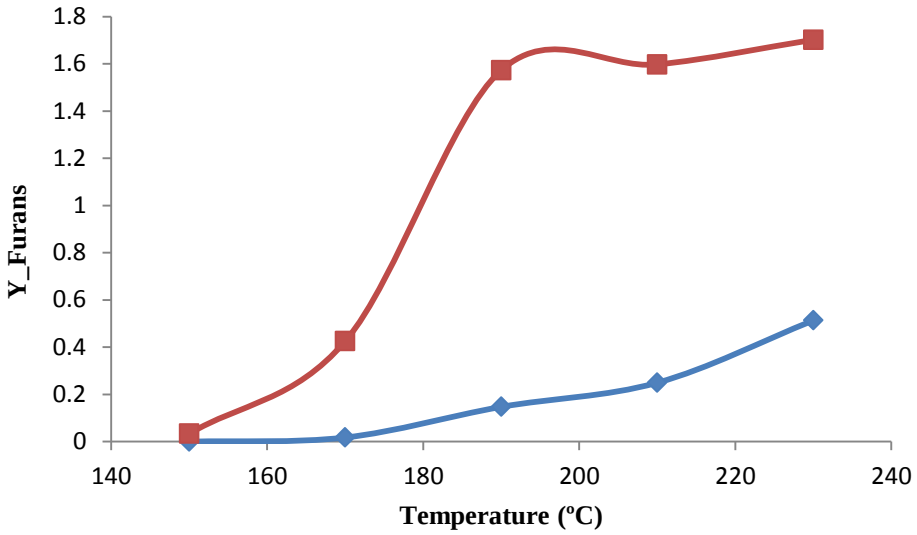


Figure 45 Effect of the temperature (ºC) on the furan yields (g furans/100g of dry biomass) obtained after autohydrolysis of watermelon rinds.
(Y_HMF, Y_Furf).

As already discussed, the furans originated from the degradation of monosaccharides and, as such, an increment in their concentrations was expected after the moment when the OS reached their maximum yield. The reason for this increase is the fact that the decay of the yield in the OS results from an increase of dissolved sugars in liquors, which in turn motivates the formation of degradation products. However, both furans reached the maximum yield at the highest temperature applied in the autohydrolysis process (T=230 ºC).

The liquors resulting from the autohydrolysis processes of watermelon rinds were also analyzed by CZE, to verify if specific phenolic compounds were produced. Table 21 shows the phenolic compounds present in the liquors obtained at the different temperatures tested in the autohydrolysis process.

Table 21 Phenolic compounds identified by CZE on liquors resulting from the autohydrolysis of rinds of *C. lanatus* for different temperatures tested.*

Phenolic compound (λ=200nm)	**Temperature (ºC)**				
	150	**170**	**190**	**210**	**230**
Catechol	357	383	314	287	455

* The reported values are the CZE peak areas

The only phenolic compound detected was catechol. However, the corresponding integrals did not show a trend concerning the effect of temperature variation. A possible

justification for this fact may be related to the difficulty in obtaining a homogeneous mixture of rinds and consequently the use of a constant LSR for all the performed autohydrolysis processes was not possible. In this way, it becomes difficult to establish a relation between the final temperature of the process and the concentration of the formed phenolic compounds. In addition to catechol, few other compounds were also identified, namely catechol derivatives, but putatively at very low concentrations.

The residual solid resulting from the autohydrolysis processes was subjected to an analysis of their chemical composition, in order to confirm the efficiency of the fractionation process and its impact on the raw material. Figure 46 represents the chemical composition profiles (in Gn, Xn, Arn, Ac and KL) relating to residual solids resulting from the autohydrolysis processes of watermelon rinds.

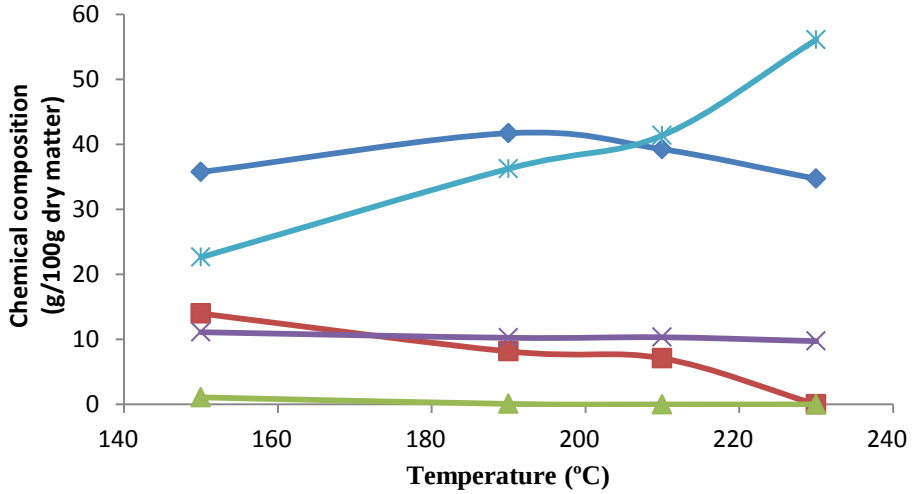


Figure 46 Chemical composition of the solids resulting of autohydrolysis process of watermelon rinds (Gn, Xn, Arn, Ac, KL).

For the lower temperature conditions (T<190 °C), it appears that, concerning the Xn content, the composition of solid residue resulting from the autohydrolysis is similar to that of the raw material, a fact confirmed by the low yields in XOS, recorded for these conditions (Figure 42). For the remaining components, this similarity is no longer present, because at these temperatures the production of AOS and GOS occurred (Figure 42). At higher temperature, it is noteworthy the total removal or Arn and the decrease of Xn content. At a temperature of 230 ºC there is a complete removal of the Xn in the chemical composition of the solid waste. Ac behavior is atypical, being expected a decrease in their content corresponds to the hydrolysis of hemicelluloses.

The KL presented a contrary behavior to the remaining components, as an increase of their content in solid residues is observed with increasing temperature of the autohydrolysis process. For a temperature of 230 ºC, the yield in KL was 56.08 g KL/100g of dry biomass and KL is completely recovered in the solid phase.

Regarding Gn, its content increases initially, but after 190 ºC it starts decreasing, meaning that it is being partially solubilized. As already put forward, either this is non-cellulosic Gn or it corresponds to a non-crystalline structure. A further hypothesis that must be studied is if the lack of lignin and, hence, of its protective effects induces a higher cellulose hydrolysis rate.

After this analysis concerning the chemical composition of the solid waste, it can be concluded that for this material autohydrolysis is not such a selective process for hemicellulose hydrolysis as described before for the *C. lusitanica* derived materials.

3.3 Comparative study of the *C. lusitanica* and watermelon autohydrolysis process

Table 22 summarizes the maximum obtained OS yields. The different materials presented very different optimal conditions and especially very different yields.

Table 22 Maximum OS yields obtained for the different studied materials.

Material	XOS		AOS		GOS	
	Yield	Max. Temp.	Yield	Max. Temp.	Yield	Max. Temp.
Chips	8.81	200	0.18	200	0.91	200
Leaves	3.85	210	3.89	190	1.65	200
Extracted leaves	4.04	190	3.30	190	1.21	190
Watermelon rinds	0.66	210	0.71	150	0.66	210

The highest yield in XOS was obtained for chips, followed by the non-extracted and extracted leaves of *C. lusitanica*, and finally by watermelon rinds. Regarding the maximum yield in XOS achieved for watermelon rinds, it appears that, in addition to being lower than that obtained for the *C. lusitanica* fractions, it was also lower than the values reported in literature (116-118).

The *C. lusitanica* (specifically non-extracted leaves) also presented a greater yield in AOS, at a temperature of 190 ºC. The yields from chips and watermelon rinds did not surpass 0.18 and 0.71 g AOS/100g of dry biomass, respectively. The GOS were also

produced in greater quantity by the non-extracted leaves and in smaller quantity by watermelon rinds.

It is important to note that the non-extracted and extracted leaves did not differ in the total yield in obtained OS (8.53 and 8.51 g OS/100g of dry biomass, respectively). The maximum yield of total oligosaccharides achieved by chips was 9.90 g OS/100g of dry biomass. The watermelon rinds were not beyond the 1.46 g OS/100g of dry biomass. Through this study, is it concluded that *C. lusitanica* achieved higher yields in OS, as compared with the watermelon rinds (*C. lanatus*). This is due to different taxonomic class to which *C. lusitanica* and *C. lanatus* belong. As previously mentioned, *C. lusitanica* is a softwood plant and *C. lanatus* is an odd herbaceous material. This difference in biological origin, results in different percentages of the polysaccharides present in the chemical composition of the studied materials (Table 15 and 20), culminating in the difference in the yields in obtained OS.

According to the literature, softwoods typically present higher cellulose and lignin content than herbaceous materials (14,19,20,29,32,42). This quantitative difference was also observed in the studied materials, since *C. lusitanica* showed higher percentages of polysaccharides and lignin than *C. lanatus* (Table 20). It is also important to remember that the hemicellulosic composition depends on the biological origin of the raw material, both in content and in the type of polysaccharides that constitute the hemicelluloses.

3.4 Comparative study of the liquors stability during storage

The liquors obtained under optimized conditions for different materials were studied regarding their storage stability. This study consisted in storing the liquors at room temperature, in a dark and dry place, without undergoing any type of sterilization to prevent microbial contamination.

Figure 47 presents the variation of the OS concentration (g/L) in the liquors of the different studied materials as a function of storage time.

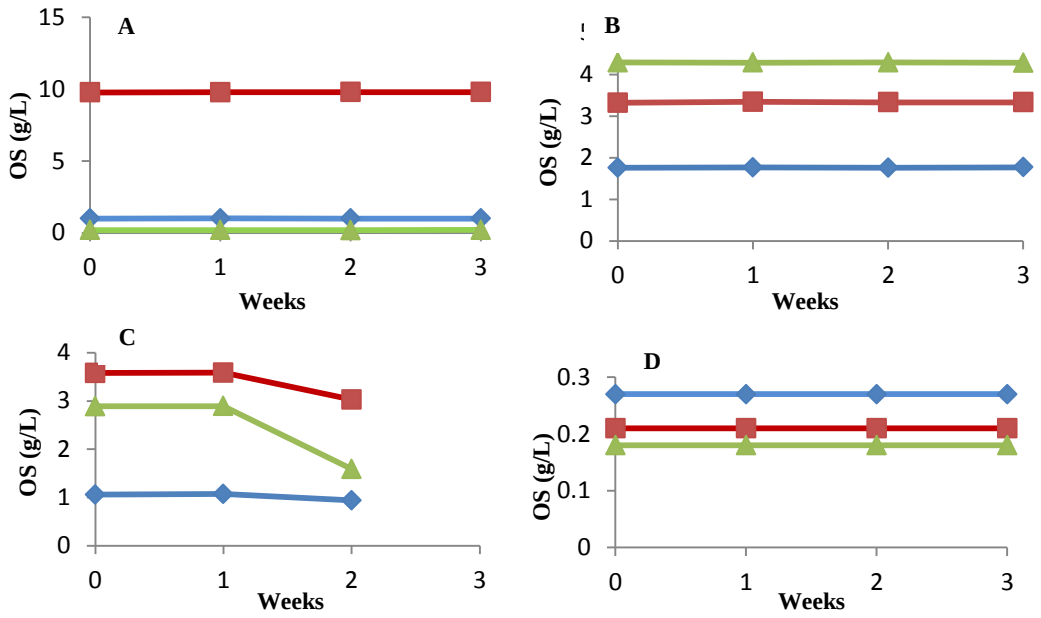


Figure 47 Profile of the concentrations of OS for autohydrolysis liquors of chips (A), leaves (B), and extracted leaves (C) of *C. lusitanica* and the watermelon rinds (D) along storage time. (GOS, XOS, AOS).

During the storage period, the OS produced by the chips and leaves of *C. lusitanica Mill.* were stable for, at least, 3 weeks. Throughout this storage period, no contamination was detected, except in the liquor resulting from the autohydrolysis of the extracted leaves. The absence of contamination in the liquors of the chips and non-extracted leaves of *C. lusitanica Mill.* and rinds of *C. lanatus* can be justified by the presence of significant concentrations of Furf and HAc, classified in the literature as inhibitors of microbial growth (119,120).

Associated with the presence of Furf some negative characteristics also arise, related with the fact that it is irritating to the skin, can cause severe burns and it is classified as teratogenic agent. For these reasons, foods containing Furf cannot be processed and ingested, thus there is a need to submit the liquors to a purification process.

3.5 Oligosaccharides purification

The liquors obtained at the optimum conditions yielding the highest OS concentrations were subjected to a freeze-drying process to purify the present OS.

Within the variety of methods than can be used in the purification process, in this work freeze-drying was chosen due to its mild operational conditions. In fact, this method is considered to be a good method for concentration and purification of biomolecules, whose structure must be maintained, as compared to others where high temperatures are

applied (121,122), e.g. spray-drying, which can destroy the produced OS. But, as high temperature based-processes are potentially faster and cheaper to operate, in this work the vacuum evaporation was also tested.

The results obtained for freeze-drying processes are presented in table 23. Similar results were obtained for the vacuum evaporation process (data not shown).

Table 23 HAc and Furf removal (%) for the freeze-drying process.

Materials		**HAc removal**	**Furf removal**
C. lusitanica	Chips	71.0	75.5
	Leaves	74.4	93.2
	Extracted leaves	63.2	83.6
C. lanatus	Rinds	73.4	75.4

As can be seen, Furf and HAc were both removed from the hydrolyzates in a very high extension.

Furthermore, vacuum evaporation, as well as freeze-drying, did not induce significant OS hydrolysis, as the ratio of Xyl to Glc remained fairly constant for all tested conditions (data not shown).

As there were no substantial differences between these two processes, and as vacuum evaporation was far faster than freeze-drying (up to 5 h, compared to more than 3 weeks), vacuum evaporation is considered to be the best choice for future industrial OS purifications.

3.6 Comparative study of oligosaccharides stability as a function of pH

After the purification process, the OS were studied for their stability as a function of pH, at a temperature of 100 ºC. Stability was evaluated by relating the concentration of monosaccharides (Glc, Xyl and Ara) before and after the stability tests . Figure 48 presents the obtained data.

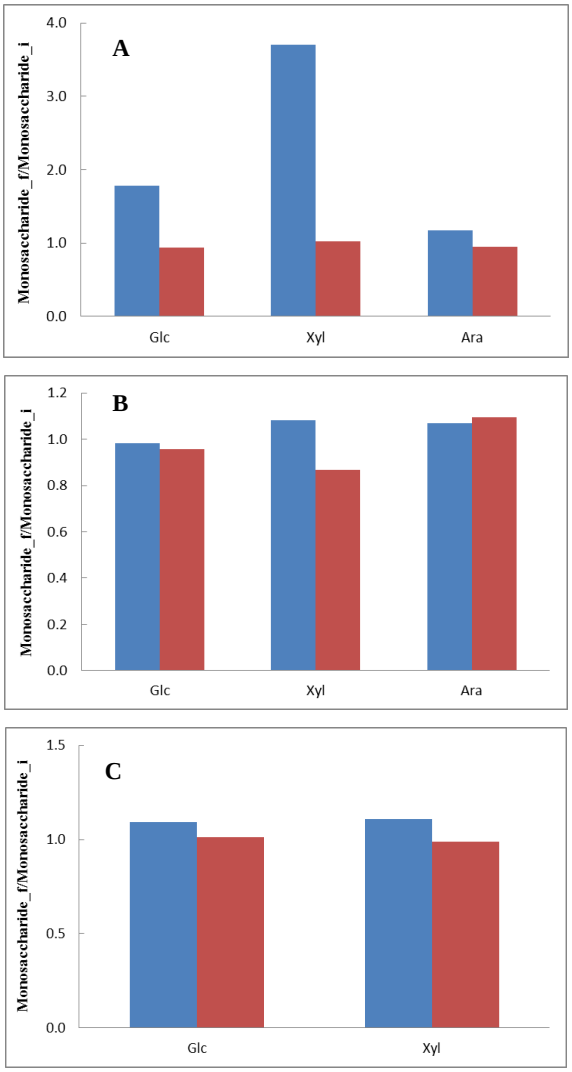


Figure 48 Stability at 100ºC of OS of chips (A), and leaves (B) of *C. lusitanica* and the watermelon rinds (C), as a function of pH
(pH=1, pH=3).

The analysis of Figure 48 enables to conclude that the OS produced from different materials retained their stability for a pH equal to 3. Conversely, at pH 1, OS from *C. lusitanica* chips were not stable, specially the XOS and GOS, resulting in an increase of the monomeric sugars content in the liquor. The other OS were also not stable, but not so markedly.

The stability of the OS to pH higher than 1 and at 100 ºC is an assurance that they can withstand food processing conditions, and hence be available for the consumers if introduced in processed food products.

It is also important to assure that the OS retain their stability at 37 ºC and pH 1, as these conditions somehow mimic experimentally the digestion conditions (123). All

produced OS may present this feature, although this must be further validated for the OS produced from the *C. lusitanica* chips, that were not stable at 100 ºC and pH 1. Regardless of this chemical stability, it will also be important to study the OS enzymatic stability over the digestive enzymes. Only then it will be possible to assure that the produced OS will be able to reach the intestine, where they act as prebiotic agents (88,90).

3.7 Comparative study of the effect of autohydrolysis on the enzymatic digestibility of the obtained solid residues

In order to fully upgrade the available biomass resources, it is now important to test whether the remaining biomass can easily withstand further processing. The most usual upgrading route implies the recovery of Glc from the cellulose-enriched solid residues (124).

Therefore, the biomass resulting from the autohydrolysis processes was characterized according to its enzymatic digestibility. Thus, enzymatic assays were performed using *Celluclast* 1.5 L and *Novozyme* 188. This study was conducted with the objective of better shaping the recovery of materials in a framing of the biorefinery.

Table 24 shows the digestibility of Glc (%) obtained after enzymatic hydrolysis of each material without any autohydrolysis treatment. All materials presented a limited enzymatic digestibility, especially *C. lusitanica* chips.

Table 24 Enzymatic digestibility of the untreated materials.

Materials		Digestibility (%)
C. lusitanica	Chips	0.53
	Leaves	30.82
	Extracted leaves	29.72
C. lanatus	Rinds	45.83

Figures 49 and 50 present the graphics corresponding to the concentration profiles of of Glc and digestibility yields as a function of the tested severities. All solid residues obtained for each material, after the autohydrolysis process, presented increased cellulose digestibility.

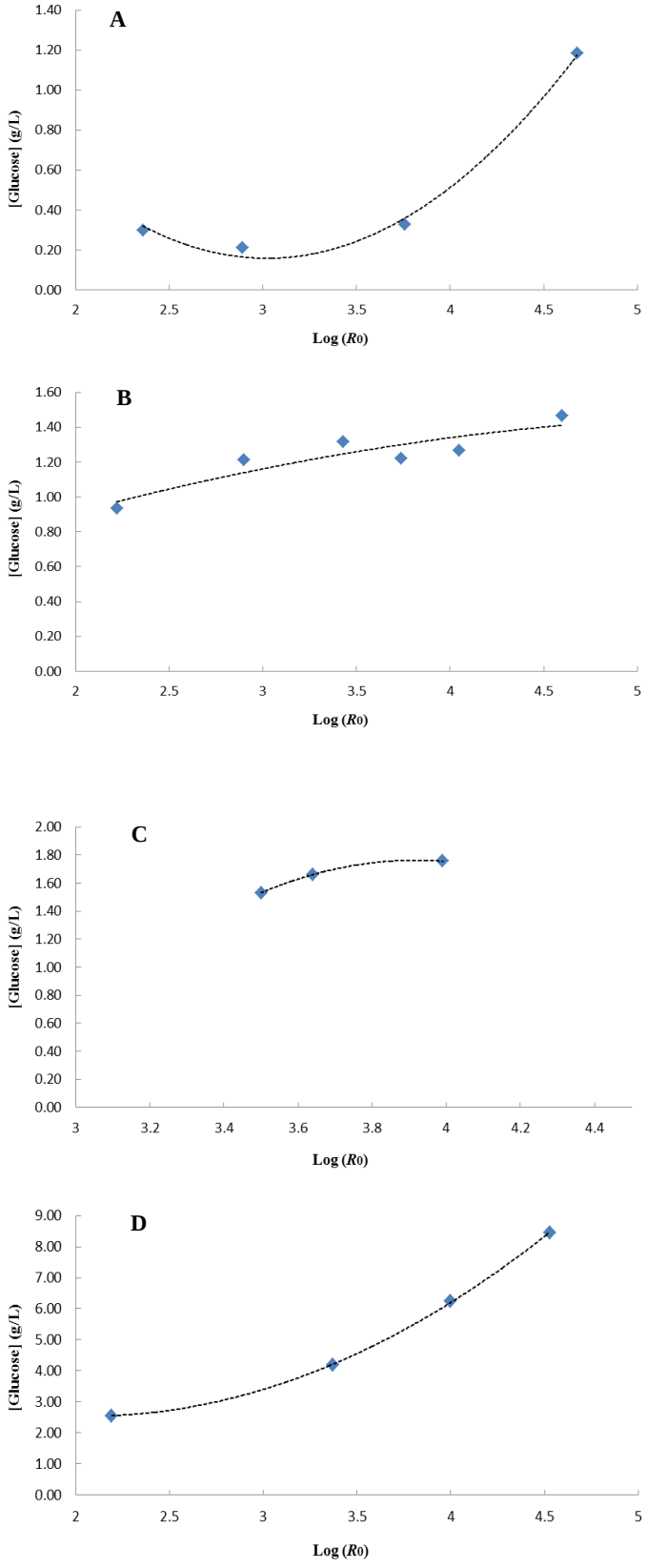
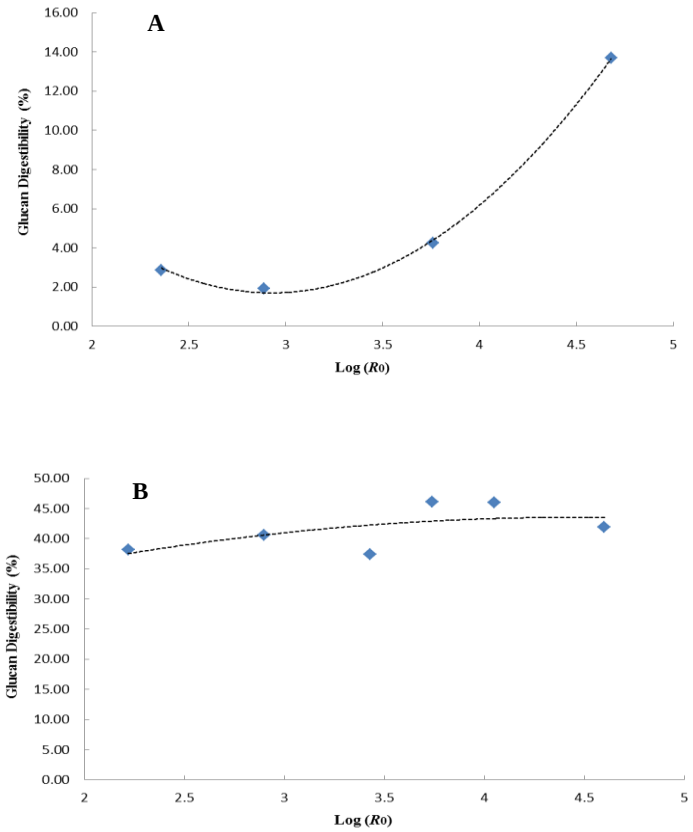


Figure 49 Glc concentration (g/L) obtained in the enzymatic hydrolysis assays for treated chips (A), non-extracted leaves (B), and extracted leaves (C) of *C. lusitanica* and watermelon rinds (D), as a function of log R_0.

The results indicate that for all materials there is a clear trend towards an increase of digestibility as the severity of the autohydrolysis process increases. However, in spite of this increase, low final concentrations of Glc were obtained for chips and for non-extracted and extracted leaves of *C. lusitanica*. In contrast, the watermelon rinds pretreatment enabled to reach very interesting final Glc concentrations. And, although Glc yields did increase, there is still room for further improvement, especially for *C. lusitanica* chips. Conversely, watermelon rinds are already well underway on the optimization path, as enzymatic hydrolysis reached its theoretical maximum yield for the highest tested severity. Furthermore, the cellulose digestibility found for the best extracted leaves treatment (66 %) was encouraging.



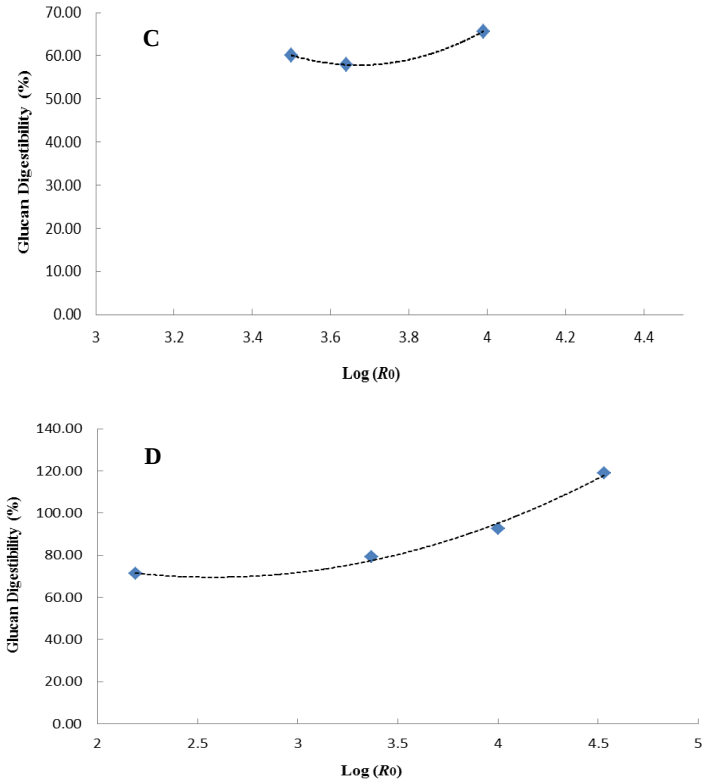


Figure 50 Percentage digestibility of cellulose (%) obtained in the enzymatic digestibility assays from chips (A), non-extracted leaves (B), and extracted leaves (C) of *C. lusitanica* and watermelon rinds, as a function of log R_0.

4 Conclusions

The plant materials studied in this work are widespread in Portugal, produced in large amounts and no effective valorization route exists. These reasons make them relevant to be potentially upgraded within the biorefinery framework.

As such, it is important to fully understand their chemical composition. For both materials, polysaccharides are a relevant fraction, in quantitative terms. Although *C. lusitanica* fractions present slight differences among them, they all show a great relevance of hemicelluloses representing more than 40% of the total polysaccharides. Contrarily, the extractives are quantitatively less relevant in *C. lusitanica* fractions, but they may play an important role on the valorization route as they can yield several value-added products, namely essential oils and potentially bioactive phenolic compounds, especially for *C. lusitanica* leaves and bark, but not for chips.

As the studied materials are quite distinct in terms of their biological origin and, hence, in their chemical structure, we expected to have also some distinct behavior during the autohydrolysis process and, in fact, some differences were found.

The autohydrolysis process has shown to be highly selective towards the hemicelluloses present in *C. lusitanica*, but not so selective for watermelon rinds, where glucans were also significantly removed, especially in the most severe treatments. This is putatively explained based on the low lignin content of watermelon rinds, which is, hence, not able to protect the cellulose. Nevertheless, hemicellulose-derived OS were always the main products.

The maximum OS production for *C. lusitanica* occurred in different conditions that ranged from 200 ºC for chips to 190 ºC for non-extracted leaves. The chips proved to be the main source of OS, being the XOS the most produced, whose total yield reached 8.81 g XOS/100 g of dry biomass. In turn, the leaves have enabled to obtain similar yields of XOS and AOS with maximum achieved values of 3.85 g XOS/100g of dry biomass and 3.89 g AOS/100g of dry biomass.

For non-extracted and extracted leaves, the operating conditions to obtain the highest yield of OS were the same, namely a temperature of 190 ºC. However, the non-extracted leaves enabled to obtain a higher total yield of OS (9.39 g OS/100g of dry biomass), as compared with extracted leaves whose maximum was 8.61g OS/100g dry biomass. Still,

the extracted leaves reached a higher yield of XOS (4.04 g XOS/100g of dry biomass) in relation to non-extracted leaves (3.85 g XOS/100g of dry biomass). That being said, it is assumed that the leaves of the *C. lusitanica* after the essential oils extraction are a possible source for the production of OS.

The type of OS produced in the largest quantities from watermelon rinds were the AOS (1.11 g AOS/100g of dry biomass), followed by XOS and GOS. But, compared to the data presented by *C. lusitanica*, watermelon rind is not such a good OS source.

The liquors obtained at the optimal conditions were studied regarding their storage stability at room temperature. The OS obtained from leaves and chips of *C. lusitanica* and watermelon rinds showed high stability. The same was not verified for extracted leaves-derived OS, where there was a decline in their concentrations and the occurrence of microbial contamination, which was attributed to the lower concentrations of HAc and Furf in the liquors.

Besides being classified as microbial inhibitors, HAc and Furf are also harmful to human health, and as such it is necessary to remove them from liquors if they are to be used for human consumption. For this, two methods were tested, freeze-drying and vacuum evaporation, and both enabled the removal of HAc and Furf, without destroying the valuable OS. The use of the simpler and more economical heat-based evaporation process can effectively substitute the more costly freeze-drying.

After OS purification, their stability at different pH values and at 100 ºC was studied. It was confirmed that all produced OS were stable at pH 3 and the majority was also stable at pH 1, which guarantees their potential application in the food industry. The exception were the OS derived from *C. lusitanica* chips, that were less stable at pH 1 and 100 ºC.

Concerning the upgrade of the solid residues remaining after autohydrolysis, it was shown that they can be further upgraded within the biorefinery framework, namely by the production of fermentative Glc-rich media. The autohydrolysis process was especially effective to increase the enzymatic digestibility of the watermelon rind.

The ability to easily produce value-added compounds, OS with potential nutraceutical activities, allied to their easy and cost effective operation, and their effect on the solid residues, makes autohydrolysis the process of choice for the initial pretreatment/fractionation of the studied biomasses in the biorefinery framework. In fact,

this work enlarges the boundaries to the use of autohydrolysis to other feedstock materials, besides the more usually reported hardwoods and herbaceous materials.

5 Future work

Although the results presented an interesting prospective, there are still some issues that must be addressed before the industrial application of the developed upgrade strategy for the studied residual raw materials.

A first step would be the economic evaluation of the proposed processes. The use of modeling software such as Aspen Plus or Super Pro Designer would be a useful tool. Here, it is important to notice that this evaluation can only be carried out after this work, as it requires the definition of the product yields and process conditions, which only were established now. Actually, this evaluation is also only relevant because the produced OS presented potential useful traits, such as their chemical stability.

An equally important issue is the refinement of the chemical analysis of the raw materials, especially for the quantification/differentiation of hemicelluloses and pectin and protein/lignin and also the advanced chemical characterization of the obtained products. The use of HPLC and CZE has shown to be effective, but the use of more advanced chemical analysis techniques, such as NMR and GC-MS, could help to gain further insight on the products characterization, especially for OS (see appendix B). Furthermore, the functional characterization of the produced OS is also a hot topic that must be looked into.

If the functional characterization of the OS reveals to be commercially relevant, further assays will also need to be carried out regarding the process development. As such, the use of pilot reactors is specially advised in order to take into consideration the issues that the use of larger reactors will impose. In fact, some industrial reactors present a faster heating rate and a lower cooling rate that is exactly the opposite of the lab-scale reactors. This probably implies different process kinetics and must be probably studied afterwards in a scale-up approach. A further development regarding the OS production, and specially their purification, can be the evaluation of the spray-drying technique to recover OS, as this technique is potentially faster and even more economical than the use of vacuum evaporation and freeze-drying.

The upgrade of the residual solids after the autohydrolysis process also requires further studies, as only their enzymatic digestibility was evaluated. In fact, the enzymatic step and the probable fermentation step must be further optimized and designed, respectively.

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Appendix

A - Mathematical Formulae

Quantification of structural polysaccharides and lignin in solid samples

The moisture content (H, %) of the samples was calculated using the following expression:

$$\boldsymbol{H = \frac{wet\ sample\ weight\ (g) - dry\ sample\ weight\ (g)}{wet\ sample\ weight\ (g)} \times 100}$$

The ash content (Ash, %) of the samples was calculated using the following expression:

$$\boldsymbol{Ash = \frac{Ash\ weight\ (g)}{dry\ sample\ weight\ (g)} \times 100}$$

The concentrations of Glc, Xyl, Ara and HAc in the liquors resulting from the quantitative acid hydrolysis of raw materials and solid wastes were used for the calculation of glucan, xylan, arabinan and Ac content (%), respectively. The acid-insoluble residue, after correction for the ash content, was quantified as KL.

During the quantitative acid hydrolysis, a significant percentage of the monosaccharides is degraded, so correction factors are introduced to account for the losses (125). Based on these percentages it is possible to calculate the correction factors (F).

$$Gn = F \times \frac{100}{1005} \times \frac{162}{180} \times \frac{Glc \times P_{sol}}{A}$$

$$Xn = F \times \frac{100}{1005} \times \frac{132}{150} \times \frac{Xyl \times P_{sol}}{A}$$

$$Arn = F \times \frac{100}{1005} \times \frac{132}{150} \times \frac{Ara \times P_{sol}}{A}$$

$$Ac = \frac{100}{1005} \times \frac{60}{61} \times \frac{HAc \times P_{sol}}{A}$$

$$KL = \frac{AIS - Ash}{A} \times 100$$

Where,

Gn, Xn, Arn, Ac and KL are the concentrations of glucan, xylan, arabinan, acetyl groups and Klason lignin (g per 100 g of dry solid) respectively;

Glc, Xyl, Ara and HAc are the concentrations of glucose, xylose, arabinose, and acetic acid in liquors (g/L), respectively;

The terms $\frac{162}{180}, \frac{132}{150}, \frac{60}{61}$ are stoichiometric conversion factors of monomers into polysaccharides;

F is the correction factor accounting for sugar degradation;

W_{sol} and A are the weights of the solution and dried sample used in the test, respectively (g);

AIS and Ash are the weight of the acid-insoluble residue of the sample and its ash content, respectively (g).

Polymer Recoveries

The recovery of each of the polysaccharides, Ac and KL, expressed as the percentage that remains in the solid residue after hydrolysis, was calculated according to the following equations:

$$Gn_R = \frac{Gn \times Y_S}{Gn_{RM}}$$

$$Xn_R = \frac{Xn \times Y_S}{Xn_{RM}}$$

$$Arn_R = \frac{Arn \times Y_S}{Arn_{RM}}$$

$$Ac = \frac{HAc \times Y_S}{Ac_{RM}}$$

$$LK_R = \frac{LKn \times Y_S}{LK_{RM}}$$

Where,

Gn_R, Xn_R, Arn_R, Ac_R, KL_R are the percentages of glucan, xylan, arabinan, acetyl groups and Klason lignin that remain in the residue after the hydrothermal processing (g/100g of the respective initial component);

Gn_{RM}, Xn_{RM}, Arn_{RM}, Ac_{RM}, KL_{RM} are the percentages of glucan, xylan, arabinan, acetyl groups and Klason lignin on the raw materials, respectively (g/100 g of raw material);

Y_s is the solid yield (g of recovered solid /100g of raw material).

The percentage of each of the polysaccharides and Ac solubilized into monomers, as well as the percentage of monomers converted into the respective degradation products was calculated according to the following equations.

$$Glc_S = \frac{162}{180} \cdot \frac{Glc \cdot W_H}{Gn_{RM} \cdot A.0{,}01.\rho_H} \cdot 100$$

$$Xyl_S = \frac{132}{150} \cdot \frac{Xyl \cdot W_H}{Xn_{RM} \cdot A.0{,}01.\rho_H} \cdot 100$$

$$Ara_S = \frac{132}{150} \cdot \frac{Ara \cdot W_H}{Arn_{RM} \cdot A.0{,}01.\rho_H} \cdot 100$$

$$HAc_S = \frac{60}{61} \cdot \frac{HAc \cdot W_H}{Ac_{RM} \cdot A.0{,}01.\rho_H} \cdot 100$$

$$Furf_S = \frac{132}{96} \cdot \frac{Furf \cdot W_H}{Xn_{RM} \cdot A.0{,}01.\rho_H} \cdot 100$$

$$HMF_S = \frac{162}{126{,}1} \cdot \frac{HMF \cdot W_H}{Gn_{RM} \cdot A.0{,}01.\rho_H} \cdot 100$$

Where,

Glc_s, Xyl_s, Ara_s, HAc_s $Furf_s$, HMF_s, are the percentages of glucose, xylose, arabinose, acetic acid, furfural and 5-hydroxymethylfurfural recovered in the hydrolyzate (g/100g of polysaccharides/HAc/Furf/HMF resent in raw material);

Furf and HMF are concentrations of furfural and 5-hydroxymethylfurfural in the liquors (g/L);

W_H is the mass of hydrolyzate obtained in the assay (g);

ρ_H is the density of hydrolyzate (g/L).

B - Structural characterization of hemicellulosic oligosaccharides

The increasing applicability of OS requires that these are better understood from the viewpoint of both their physical and chemical composition and their structure (126).

To quantify the OS present in the liquor resulting from the autohydrolysis, an hydrolysis process can be applied. This method enables an indirect quantification of OS present in the hydrolyzate, by using dilute sulfuric acid (127). Generally, acid concentrations of 3-7 %, temperatures of 100-121 ºC and reaction times of 2 h and 10 min are used (128-130). Factors such as speed, economy and a high recovery of monosaccharides (even if such recovery has to be optimized for each OS liquor) make the application of this method very advantageous. Nevertheless, it also has some disadvantages, such as the formation of some additional degradation products, in particular from the dehydration of monosaccharides.

For a more complete study of the OS a variety of analytical methods can be applied, among which mass spectrometry (MS), nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC) stand out.

MS has shown to be one of the most important analytical techniques in the analysis of biomolecules, because it enables a specific structural characterization. In the case of OS, it can help to elucidate their composition in terms of sugar residues, their monomer sequence and verify the absence or presence of branching and the type of glycosidic bonds present in their structure (131-133). It is a destructive technique, which has high sensitivity and is commonly used in the analysis of substances at low concentrations. The chemical characterization is performed by fragmentation of the ions formed from the OS, which are then expressed in a mass spectrum. The application of this technique results in obtaining a product ion spectrum of a given molecular ion that provides considerable information about the structure of the monomer components in the OS sequence, i.e., enables a detailed characterization of the extracted OS (131-133).

The number of studies involving the characterization of OS by MS has increased over recent years as a result of the achievements in the application of methods such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), combined with a time of flight (TOF) mass analyzer because these result in improved sensitivity of this analytical technique and simplicity of interpretation of data and also due to the use of fragmentation patterns (131,133). The ESI method also enables to decrease

the amount of required sample for analysis. This method is also easy to match with *tandem* mass spectrometry (MS^n), being quite useful in determining the order of the monomer residues in the OS. The MALDI-TOF technique in addition to the determination of the molecular weight of the OS also provides information about their fragments.

Studies involving structural characterization of OS often make use of ^1H and ^{13}C NMR spectroscopy and two-dimensional techniques (COSY, HSQC, HMBC) (126,132,134).

Techniques such as MS and NMR require purification of the sample containing the OS mixture. This purification consists essentially in removing salts and may be, for example, achieved by preparative paper chromatography (134). This purification technique enables the elimination of possible interferences and provides isolation of fractions of different OS. Subsequently, in order to obtain information as consistent as possible, the composition and structure of the different fractions can then be obtained by MS or NMR. It is sometimes difficult to obtain considerable amounts of sample with high purity, in the range (0.5-1 mg) required for NMR technique (134).

The chemical characterization can be made using HPLC which relies essentially on the separation through the different retention times. The quantitative analysis of these compounds is little time consuming and has a high resolution, efficiency, sensitivity and good reproducibility (135).

The HPLC technique may also be coupled to mass spectrometry (HPLC-ESI-MS^n) and has demonstrated broad applicability in the separation and characterization of OS due to the high quality of structural information. It has primarily been used in the identification and quantification of sugar residues present in the OS chain.

The technique of HPLC with anion exchange column (HPAEC) appears as another widely used technique for separation and analysis of a variety of structurally similar OS (131,136). This separation is achieved through specific existing interactions between the hydroxyl groups of the OS and the stationary phase of the column at high pH ($pH \geq 12$). Thus, the OS (in anionic form), by interacting with the stationary phase, are separated based on their size, composition and type of glycosidic bonds present. The obtained data also enable the quantification of OS present in the sample as well as the specific location of glycosylation.

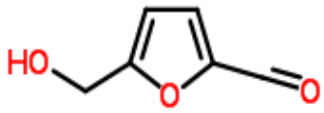
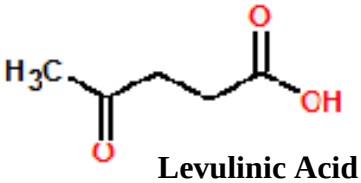
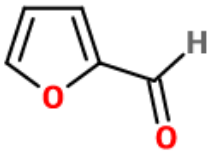
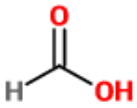
When it is desired to evaluate the distribution of molecular size, high efficiency size exclusion chromatography (HPSEC) may be applied. In response to the separation and analysis of OS based on differences in hydrophobicity, reversed phase chromatography can be used.

However, and given this diversity of analytical techniques, it should be considered that the behavior of elution of the various types of OS, present in a complex mixture, is quite unpredictable and corresponding structural patterns are not always available, making their identification more difficult. Thus the information obtained by this technique has to be combined with NMR spectroscopy or MS in order to obtain the exact structures of the separate OS.

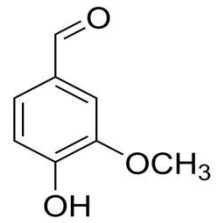
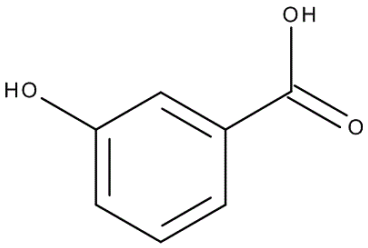
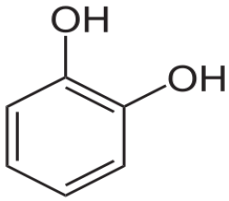
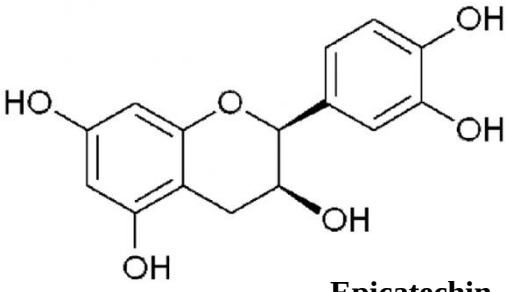
The Fourier Transform Infrared Spectroscopy (FTIR) is also applied to the study of the OS structures, specifically to identify the type of conformation that they may acquire. It is also quite useful in the study of their physicochemical properties.

C – Chemical Structures

Degradation products of monosaccharides (hexose and pentose)

 **5 – Hydroxymethyl – 2 - Furfural**	 **Levulinic Acid**
 **2 – Furfural**	 **Formic Acid**

Phenolic compounds

 **Vanillin**	 **Hydroxybenzoic acid**
 **Catechol**	 **Epicatechin**