Bark biorefinery: deconstruction and chemical potential of *Eucalyptus globulus* and *Picea abies* barks

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THESIS PRESENTED TO OBTAIN THE DOCTOR DEGREE IN

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Programa de doutoramento FCT (Sustainable Forests and Products, SUSFOR)

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Too all that decided to explore just a bit further

“That's the whole problem with science. You've got a bunch of empiricists trying to describe things of unimaginable wonder” – Bill Watterson in Calvin & Hobbes

“Somewhere, something incredible is waiting to be known” – Carl Sagan

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At the end of such an extensive trip, I have surely forgotten parts of the joy and despair that it took to make it, time makes sure of that. What I cannot forget is with whom I did it, for my success is not mine alone. The cries of joy, the "eureka" moments, the tears of sadness, the ever growing baldness were all supported by my community that shared the high peaks and low points, preventing me to fly like Icarus or sunk like Atlantis.

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Abstract

Eucalyptus globulus and *Picea abies* barks are huge industrial residues with upgradable potential. This research thesis aims at further the knowledge on these barks envisaging production of biomaterials, building blocks, chemicals and fuels.

Both industrial barks contained high wood and mineral extraneous contaminants, presenting higher extractives (10-20%) and ash (4-5%), but lower polysaccharides (52-61%) than their respective woods. *E. globulus* bark hemicelluloses (glucuronoxylan) and lignin (S/G=2.8, enriched in β-O-4', 83% of all inter-unit linkages elucidated by 2D HSQC-NMR) were similar to those of wood, while *P. abies* bark differed substantially in hemicelluloses (pectin, higher arabinan, lower mannan) and lignin (γ-OH significant acetylation, presence of hydroxystilbenes glucosides as "nonconventional" true lignin monomers, adding to the canonical established monolignols precursors). The presence of glucosides in lignin was reported for the first time with positive ramifications regarding possible design and bioengineering of polymers with special attributes (hydrophilicity, bioactivity)

Deconstruction pathways were tested considering the knowledge gathered.

Bleached kraft pulp was produced from *E. globulus* bark due to previously determined appropriate physical and chemical characteristics. Hydrothermal pre-treatment tested decreased extractives content allowing for lower active alkali (15%) usage in pulping process (resulting in 40% yield) with subsequent bleached pulp and respective handsheets showing similar characteristics to those produced with *E. globulus* wood. Bark proved to be a possible fiber source feedstock for pulp and paper production.

Crude extracts recovered with different solvents were analyzed for neutral monosaccharides and phenolic composition, antioxidant activity, antimicrobial and quorum-sensing potential. Polar extracts showed good or very good antioxidant activity. Gram positive and *Candida* strains had their growth highly impaired when exposed to n-hexane and ethanol extracts concentrations above 0.04 mg/mL.

Extractive-free barks were autohydrolysed and the solid residues saccharified with commercial enzymes (Saczyme and Ultimase), resulting in xylooligosaccharides/arabinooligosaccharides enriched liquors from autohydrolysis, glucose rich streams from enzymatic saccharification and lignin enriched solid residues.

This thesis evidences that these abundant industrial residues are interesting materials to be upgraded within a biorefinery concept of full biomass utilization with potential to generate several products and streams with different end-uses.

Keywords: Bark fractionation, Enzymatic hydrolysis, Lignin characterization, Extractives bioactivity, Kraft pulp

Resumo

As cascas de *Eucalyptus globulus* e *Picea abies* são resíduos industriais abundantes cujo aproveitamento pode e deve ser melhorado. Esta tese visa ampliar o conhecimento destes materiais e estudar a sua possível utilização na produção de biomateriais, moléculas elementares, químicos e combustíveis.

As correntes industriais de cascas encontram-se severamente contaminadas com madeira e detritos minerais, apresentando composições químicas com teores de extractivos (10-20%) e cinza (4-5%) superiores às respectivas madeiras e mas inferiores em polissacáridos. As hemiceluloses (glucuronoxilanas) e lenhina da casca de *E. globulus* (S/G=2.8, enriquecida em β-O-4', 83% do total de inter-ligações monoméricas determinadas por 2D HSQC-NMR) são semelhantes às da madeira, contrastando com a casca de *P. abies* cujas diferenças em relação à respectiva madeira são substanciais nas hemiceluloses (presença de pectinas, aumento das arabinanas e decréscimo da mananas) e lenhina (acetilação significativa em γ-OH e presença de hidroxistilbenos glucosilados como verdadeiros monómeros "não-convencionais", acrescendo aos percursores monolignol já estabelecidos). A presença de glucósidos na lenhina, reportada aqui pela primeira vez, pode ter implicações na bioengenharia de polímeros, possibilitando a inclusão de características/atributos especiais (hidrofilicidade, bioactividade).

Com base no conhecimento adquirido, vários processos de desconstrução das cascas foram testados.

Devido às caracteristicas físicas e químicas apropriadas para esse fim, produziram-se pastas kraft branqueadas de casca de *E. globulus*. Os pré-tratamentos hidrotérmicos testados eliminaram até 2/3 dos extractivos, permitindo a utilização de menores cargas alcalinas na deslenhificação. As folhas produzidas apresentaram características idênticas às obtidas com madeira, demonstrando a possibilidade de inclusão desta casca como matéria-prima fibrosa na produção papeleira.

Analisaram-se extractivos obtidos com diferentes solventes quanto à composição de monossacáridos neutros e compostos fenólicos, assim como à actividade antioxidante, e potencial anti-quórum e antimicrobiano. Os extractivos polares apresentaram boa ou muito boa capacidade antioxidante. Alguns extractos impediram o desenvolvimento de bactérias Gram-positiva e *Candida* quando aplicados com concentrações superiores a 0.04 mg/mL.

Processaram-se hidrotermicamente cascas previamente extractadas e os resíduos sólidos obtidos foram sacarificados com enzimas comerciais (Saczyme e Ultimase), obtendo-se licores ricos em xilooligosacáridos/arabinooligosacáridos provenientes da auto-hidrólise, licores ricos em glucose provenientes da sacarificação enzimática e um resíduo sólido enriquecido em lenhina.

Esta tese evidencia o elevado potencial de valorização destes abundantes resíduos industriais em contexto de biorrefinaria com utilização integral da biomassa para obtenção de diversos produtos.

Palavras chave: Fraccionamento da casca, Hidrolise enzimática, Caracterização da lenhina, Bioactividade de extractivos, Pastas Kraft

Resumo alargado

O desenvolvimento de uma indústria baseada em bio-hidrocarbonetos, técnica e economicamente viável e o desinvestimento nos combustíveis fósseis são um requisito imperativo para as próximas décadas se a humanidade quiser manter simultaneamente o nível de vida e a integridade da biosfera. Das possíveis matérias-primas, os materiais lenhocelulósicos estão entre os recursos naturais mais promissores para serem explorados em biorefinarias. As cascas produzidas pelas fileiras da madeira e da pasta para papel constituem um dos exemplos de materiais lenhocelulósicos cujo aproveitamento pode e deve ser melhorado. Consideradas como resíduos, estas cascas são utilizadas quase exclusivamente para produção de calor/electricidade por combustão directa, não sendo aproveitado o seu potencial químico.

Duas das espécies florestais mais exploradas pelas indústrias madeireiras e da pasta para papel europeias são a *Eucalyptus globulus* (Eg) e a *Picea abies* (Pa) com incidência principalmente no Sudoeste mediterrânico e no centro e norte da Europa, respectivamente. Esta tese teve como objectivo ampliar o conhecimento sobre as cascas destas duas espécies, determinando as suas características físicas, químicas e energéticas, e dessa forma possibilitar uma escolha adequada dos processos de desconstrução com vista a potenciar o aproveitamento integral dos seus constituintes, visando a obtenção de produtos, compostos químicos e combustíveis.

As cascas industriais continham um teor elevado de madeira (16-18%) e de contaminantes minerais. Estes últimos ocorrendo maioritariamente na *E. globulus*, provavelmente resultantes das condições do processo de transporte e processamento. Estes contaminantes aumentam a heterogeneidade da casca afetando negativamente os processos de transformação e qualidade dos produtos obtidos.

As cascas apresentaram um maior teor de extractivos (10-20%) e de cinzas (4.5%) do que as respectivas madeiras (<1%) e um menor conteúdo em polissacáridos (52-61% vs. 71%). A casca da *E. globulus* apresenta características das hemiceluloses (maioritariamente xilanas) e da lenhina (S/G=2.8, enriquecida em ligações β-O-4', 83% do total de inter-ligações monoméricas determinadas por 2D HSQC-NMR) semelhantes às da madeira, contrastando com a casca da *P. abies,* que apresenta diferenças substanciais em relação à madeira nas hemiceluloses (presença de pectinas, maior conteúdo de arabinanas e menor de mananas) e na lenhina (acetilação significativa em γ-OH e presença de monómeros nunca antes associados a este polímero, hidroxistilbenos glucosilados, parcialmente incorporados por ligações β-éter).

A identificação de hidroxistilbenos glucosilados intrinsecamente ligados à lenhina comprovam a sua elevada plasticidade e complexidade, possivelmente ampliando o número de monómeros "não tradicionais" associados a este polímero para além dos canónicos monolinhois. A presença de glucósidos em determinadas lenhinas era já equacionada, embora os mecanismos da assimilação de um açúcar no polímero fenólico nunca tivessem sido esclarecidos satisfatoriamente. Esta identificação dos hidroxistilbenos glucosilados como monómeros da lenhina apresenta uma solução viável, elegante e plausível para o mecanismo bioquímico que permite a integração do açúcar a partir do aglicona que, contendo mais do que um grupo fenólico livre, se pode ligar à lenhina por uma reacção de acoplamento

de radical. Esta descoberta pode ter implicações importantes, permitindo modificar e criar através da bioengenharia, polímeros de base fenólica com atributos específicos (e.g. hidrofilicidade, actividade antioxidante, entre outras).

O estudo das propriedades térmicas das cascas evidenciou limitações da *E. globulus* na sua utilização para queima directa, devido a uma baixa densidade energética (maioritariamente resultante de baixa densidade do material e não tanto do seu poder calorífico), elevado teor mineral e elevado teor de cloro, que tem consequências para os equipamentos de queima assim como para o ambiente.

No fraccionamento mecânico estudado (moagem e peneiração) obtiveram-se diferentes classes de tamanho de partícula com composições químicas distintas. A fracção "finos" ficou enriquecida em cinzas e extractivos e empobrecida em polissacáridos quando comparada com as fracções de partículas de maior dimensão. No entanto, as variações não aparentam ser suficientemente impactantes para compensar os gastos energéticos e económicos deste processo de fracionamento mecânico.

Com base nas especificidades químicas e físicas determinadas para cada casca, foram equacionados vários processos de desconstrução com vista a uma utilização integrada de todo o seu potencial.

As características físicas e químicas da casca da *E. globulus* evidenciaram uma possível utilização como fonte fibrosa para produção de pasta Kraft branqueada, nomeadamente a sua elevada proporção de fibras, alto teor de polissacáridos (61%) e um baixo teor de lenhina (22%) com uma razão S/G elevada, 2.8, composta maioritariamente por ligações do tipo β-O-4'. Testou-se a utilização de um pré-tratamento hidrotérmico (autohidrólise) para redução da quantidade de extractivos e componentes minerais, avaliando a sua influência no processo de deslenhificação (com diferentes cargas alcalinas no licor branco) e nas características finais das pastas produzidas. Foi possivel obter pastas com número Kappa 17 utilizando baixa carga alcalina (15%) e as folhas laboratoriais produzidas (após branqueamento e refinação da pasta) demonstraram ter características mecânicas e ópticas semelhantes às das pastas obtidas com madeira da *E. globulus.* O menor conteúdo em polissacáridos relativamente à madeira (61 *vs.* 71%) explica o menor rendimento em pasta obtido (40% *vs*. 50%). Deste modo, a casca da *E. globulus* pode ser considerada como uma possível fonte de fibra para pasta para papel, podendo ser utilizadas baixas cargas alcalinas aquando da etapa de deslenhificação, se o processo for complementado com um pré-tratamento hidrotérmico prévio. No entanto, para além do menor rendimento em relação à madeira, outra das desvantagens que se pode apontar à matéria-prima deriva do elevado teor em minerais que inviabiliza a sua utilização para alguns fins, como por exemplo na produção de pastas químicas (*dissolving pulps*).

Ambas as cascas estudadas podem ser fraccionadas selectivamente, visando o aproveitamento integral de todos os seus componentes lenhocelulósicos (extractivos, hemiceluloses, celulose e lenhina).

Os extractivos, presentes nas cascas em quantidades mais apreciáveis do que na madeira, constituem uma fração interessante a ser obtida na primeira fase de um processo de fracionamento sequencial.

Várias extracções em Soxhlet foram realizadas com solventes de polaridade crescente (n-hexano, etanol e água) tendo-se analisado os extratos obtidos quanto à composição de monossacáridos neutros (após hidrólise ácida), compostos fenólicos totais, flavonoides e taninos condensados. Os rendimentos obtidos nas extracções com n-hexano foram menores (<4%) do que com etanol (3-9%) e água (9-15%). Os extractos em etanol apresentaram, em relação à água (ambos polares), menores teores de açúcares (63-85 mg/g_{ext} vs. 168-248 mg/g_{ext}) e flavonoides (18-19 vs. 21-31 mgCE/g_{ext}) e maiores teores de compostos fenólicos totais (208-375 vs. 172-242 mgGAE/g_{ext}) entre os quais taninos condensados (13-61 vs. 2.2-13.1 mgCE/g_{ext}).

Analisaram-se as actividades antioxidantes dos extractos polares (FRAP- potencial antioxidante de redução do Fe, BCB- branqueamento do β-caroteno, DPPH- inactivação de radicais livres) e os potenciais anti quórum e antimicrobiano de todos os extractos (testado em várias estirpes patogénicas de bacterias e leveduras). Os extractos polares mostraram ter bom ou muito bom índice potencial antioxidante (DPPH), correspondendo a metade do potencial FRAP do trolox (antioxidante sintético análogo à vitamina E). A inibição de crescimento de bactérias Gram-negativas foi fraca, em geral, embora alguns extractos tenham mostrado um bom potencial inibidor contra algumas estirpes Gram-positivas e Candidas. A concentração mínima inibitória ao desenvolvimento de bactérias e leveduras foi de 40 µg/mL, o que é 80 vezes mais elevada do que para padrões antibacterianos (tetraciclina) e antifúngicos (anfotericina B). A inibição do efeito quórum comprovou-se fraca ou inexistente para a generalidade dos extractos, tendo os melhores resultados sido obtidos com extractos de *E. globulus* (7 mm de raio de inibição pelo teste de difusão no agar).

O fraccionamento sequencial prosseguiu nas cascas extractadas recorrendo-se a dois processos: autohidrólise e hidrólise enzimática. Foram testadas várias condições de autohidrólise (factores de severidade 3.4-4.7) e os resíduos sólidos foram sacarificados usando cocktails de enzimas comerciais (Saczyme Yield e Ultimase BWL40). As melhores condições de autohidrólise permitiram um rendimento máximo de açúcares provenientes das hemiceluloses da casca de *E. globulus* de 11 g/100 $g_{casca\ extractada}$ (maioritariamente xilooligossacáridos) e de 14 g/100 $g_{casca\ extractada}$ (maioritariamente arabinooligossacáridos) partindo da casca de *P. abies*. Os resíduo sólidos provenientes das auto-hidrólises foram sacarificados enzimaticamente, com uma conversão quase total dos polissacáridos para a casca de *E. globulus* (98% de rendimento de açúcares no reactor) e até 75% para a casca de *P. abies*. A utilização da enzima Ultimase permitiu obter melhores resultados que a da Saczyme em todos os ensaios.

O rendimento total de açúcares do processo (contabilizando autohidrólise e sacarificação) atingiu um máximo de 73% e 51% para as cascas de *E. globulus* e *P. abies* respectivamente. Contabilizando os açucares nos licores da autohidrólise e da hidrólise enzimática, a obtenção máxima de açúcares foi de 540 kg (*E. globulus*) e 440 kg (*P. abies*) de açúcares monoméricos por tonelada de casca extractada. Os xilooligosacáridos/arabinooligosacáridos do licor da autohidrólise têm uma potencial utilização nas indústrias alimentares e farmacêuticas, embora também possam ser transformados biotecnologicamente em compostos de valor acrescentado tais como xilitol e arabitol, ou ser fermentados para produção de

etanol. Por outro lado, a solução rica em glucose proveniente da hidrólise enzimática pode ser uma fonte de açúcares facilmente fermentáveis para etanol ou para produção de compostos químicos (e.g. ácido láctico, ácido levulínico). Após esta sequência de processos, resta um sólido altamente enriquecido em lenhina que poderá servir de matéria prima em processos para obtenção e utilização de compostos fenólicos.

O conhecimento sobre as características químicas e energéticas das cascas da *Eucalyptus globulus* e da *Picea abies,* assim como sobre as possíveis rotas de desconstrução e de aproveitamento integral das fracções, evidencia que estes resíduos industriais podem ser matérias primas muito interessantes no âmbito de uma biorefinaria, mostrando um potencial elevado para gerar múltiplos produtos com diversos fins.

Contributions

Published articles

Neiva, D.M., Araújo S., Gominho, J., Carneiro, A.C, Pereira, H. 2018. Potential of *Eucalyptus globulus* industrial bark as a biorefinery feedstock: Chemical and fuel characterization. *Ind. Crop. Prod.* 123: 262-270. https://doi.org/10.1016/j.indcrop.2018.06.070

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Conferences

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4º Encontro do Colégio de Química da Universidade de Lisboa, Lisbon, Portugal, July 2019.

Encontro com a Ciência e Tecnologia em Portugal, Lisbon, Portugal, July 2018

15th European Workshop on Lignocellulosics and Pulp (EWLP), Aveiro, Portugal, June 2018

3ª Encontro do Colégio de Química da Universidade de Lisboa, Lisbon, Portugal, June 2018

1º Encontro do Colégio de Química da Universidade de Lisboa, Lisbon, Portugal, july 2017

14th European Workshop on Lignocellulosics and Pulp (EWLP), Grenoble, France, June 2016

7th International Colloquium on Eucalyptus Pulp (ICEP), Vitória, Espirito Santo, Brasil, May 2015

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Rationale and objectives

The past two centuries saw the rise and establishment of fossil fuels as the main civilizational propulsor with many aspects of human society relying totally or partially on them. The civilizational leap was indubitably tremendous and mostly positive, although with an important and ever-growing cost to the health of the planet. With nature carbon stockpiles reserves being consumed at an increasing rate, the side effects will eventually reach the tipping point, with man realizing an undeniable worldwide increase effect on temperature, pollution and environmental disasters. The low cost and still large reserves have kept the transition to a cleaner industrial paradigm at bay, although society is slowly rising its concerns and desires for alternative routes to maintain its living standards while at the same time decreasing their impact at a regional and global scale. One thing is inevitable... the age of fossil fuels will pass, probably within the next century.

Biorefinery is the wide term that coins the present and future processing complexes that try to mimic the oil refinery and petrochemical associated industries but using natural and renewable feedstocks of biomass for the integrated production of a wide spectrum of chemicals, fuel and materials. The aim of these processes is to decrease, and if possible substitute, the fossil fuel demand by producing equal or similar products while trying to grasp the full potential of each biomass, decreasing to a minimum the residual streams and whenever possible using them as feedstock for other processes. Only by creating integrative full resource processes with a zero waste philosophy can they be competitive and economically appealing.

Being the most abundant and widespread form of biomass on the planet, lignocellulosic materials are without a doubt one of the most promising candidates as feedstock for these new green processes. Biomass has been used by men since the dawn of times but it has never reached its full potential, as happened with oil, probably due to its high recalcitrance, variability and oxygen content that required higher processing and costs and returned lower energetic yields.

With all this in mind, plenty research has been produced in the last decades aiming at new processes and raw materials that might be suitable, both technically and economically, for a viable biorefinery. Although there are few true biorefineries implemented yet, some industrial processes are very close to that, with the pulp and paper industry probably being among those that most resembles a fully functional biorefinery, producing both energy and products. Nevertheless, they are still a proto-biorefinery with many challenges and possible upgradable pathways to produce the last missing piece: fuels, fine chemicals and building blocks at full scale.

One of the residual streams of forest operations and of the timber and pulp industries is the bark fraction that is usually discarded prior to wood processing and burned for energy production. Barks are non-wood lignocellulosic products that account to 11-21% of the tree bole weight (depending on the species) which means that a substantial amount of bark is present at the industrial site for the most commonly used species.

The rationale underlying this thesis is the valorization of barks as a feedstock to biorefineries, namely of those that constitute industrial residues. The objective is to study two of these industrial residues regarding their chemical and energetic potential and developing fractionation pathways to separate the main lignocellulosic components (extractives, lignin, cellulose and hemicelluloses), aiming at possible integrated specific end-uses and products, thereby changing these barks status from residues to feedstock and integrating them in the timber and pulp industries in a biorefinery context.

Two wood species were chosen as the main focus of this work: a hardwood mostly used in the southern European Mediterranean countries for pulp production (*Eucalyptus globulus*) and a softwood exploited in the northern European countries (*Picea abies*) by the timber and pulp industries. It must be stated that these industrial barks might have different characteristics from the stem barks, mostly due to the handling, transport and processing in the field and at the industrial site.

The objective of this thesis is to further the knowledge on these residual barks and determine some of the possible ways to deconstruct and use the obtained fractions, trying to use the most economical and eco-friendly solvents, chemicals and processes.

The thesis is organized in four tasks and its main body is presented in the form of research papers, whether already published or still in processing.

- The first task regards the full chemical and fuel characterization of each raw material and the possible use of a mechanical size reduction process to obtain chemically different fractions (papers 1 and 2). Additionally, a fine chemical analysis targeted specifically the lignin of both barks to better understand its structural characterization, from monomeric composition to inter-unit linkages and the possible applications that would be most adequate to each specific species lignin (papers 3 and 4). The results of this task comprise four manuscripts.
- The second task regards the use of *E. globulus* bark as a possible source of fibers for the production of bleached kraft pulp with similar physical and mechanical characteristics of the pulps produced with eucalypt wood. Hot water pre-treatment was tested to address the higher ash and extractive content of the bark, and its influence on the pulping and pulp characteristics. The results of this task comprise one manuscript (paper 5)
- The third task focus on the obtainment of several extracts by using different solvents and the study of these crude extracts regarding their possible antioxidant, antimicrobial (against human pathogenic bacteria and fungi strains) and anti-quorum sensing abilities. The results of this task comprise one manuscript (paper 6)
- The fourth task addresses the fractionation of the extractive-free bark and a sequential processing of this solid residue by first applying hot water pre-treatments (autohydrolysis) to remove part of the hemicelluloses as oligosaccharides, and afterwards an enzymatic hydrolysis to obtain monomeric sugar rich liquid streams and a lignin enriched solid streams. The results of this task comprise one manuscript (paper 7)

State of the art / Literature review

Humanity has been growing exponentially since the beginning of the industrial age from less than 1 billion (19th century) to 7.7 billion (2019) [1]. This population shift was associated to the exploitation of fossil fuels at a world scale with ever-growing energetic and material needs, resulting in better human life conditions and higher life expectancy. However, the detrimental effects of utilizing the world reserves of fossil fuels to such extent and rate has also grown, leading to a point where the scientific community and society at large have little doubts that possible catastrophic consequences will occur in the future. Predictions of the world reserves depletion of such feedstocks in the near future, and the consequent escalation in the price of these commodities, have led to an increased search and scientific research for alternative, more environmentally friendly, raw materials and production processes. This search aims at inverting the potential environmental catastrophe, while maintaining our way of life in terms of energetic and products needs.

Hence the birth and development of biorefineries.

Biorefinery

Biorefinery is the broad concept (although not entirely defined yet) of all the present, and yet to be, production pathways and technologies that can use all types of biomasses as feedstocks for the integrated production of biofuels, biomaterials, fine chemicals or building blocks and utilities within each specific industrial plant. This means that the biorefinery term can be applied to a general concept, a facility, a process, a plant or a cluster of facilities and multiple plants [2]. It is the biobased equivalent counterpart of the fossil fuels and petrochemical industries aiming at product substitution by direct means (searching and producing already available compounds or building block used by other industries, but starting from biomass) or by indirect means (searching for novel compounds and products that open new markets, that have unique characteristics although possibly similar to the fossil fuel derived counterparts) [3–5].

When compared to fossil fuels, the major advantages of using biomass within a biorefinery concept for fuel, chemicals, materials and energy production are the following: renewable and globally widespread feedstocks; CO_2 neutral conversion; transition from (fossil derived) hydrocarbon to carbohydrate and hydrogen resources; biodegradable resources with great reactivity, low ignition point and combustion temperatures; cheap resources; reduction or possible use of residues and wastes as raw material; decrease of hazardous emissions (NO_X, SO_X); possibility to use both water and land biomass as feedstock; possibility to use low-value and degraded soils; revitalization of the rural areas with job creation; higher focus on rural land use and profitability from forest biomass which can have beneficial effects on fire prevention; increased carbon sequestration with consequent reduction of greenhouse gas effects; as opposed to petroleum where functionality is added to the hydrocarbon compounds, biomass already has

available functionality or-pre functionality within its chemical components mostly due to the higher content of oxygen; species can be genetically modified and tailor-made according to specific uses [6–8].

On the other hand, there are also several negative aspects and constraints regarding biomass use, as follow: if not correctly planned, it can pose a competition to edible biomass growth; growth of intensive single crops can decrease biodiversity and damage natural ecosystems (e.g. deforestation, land use changes); increases the use of pesticides and fertilizers (which currently are predominantly produced from fossil fuels); high costs of harvesting, collection, transportation and storage; normally low bulk density; high intra and interspecies variability; variation in quantity and seasonal availability; as opposed to petroleum, biomass is not homogenous; is susceptible to plagues, infestations and wildfires; biomass has high water and oxygen content which decreases its energetic value (low energy density); it has higher recalcitrance; overall production costs are higher with lower economic viability; in need of new and competitive production pathways and equipment [6,9,10].

In order to be economically and environmentally sound, biorefineries also aim at rendering the waste streams obsolete, searching for integrative ways to convert the initial biomass to its fullest and, whenever possible, to optimize the production pathways so that previously waste streams can be useful for another downstream process or product. This is important since not only it will help the viability of the plant by reducing the costs associated with waste and disposal management, but also due to the increasingly growing attention and public perception regarding the end-products production negative environmental impacts, since industrialized societies are taking more attention to these problems, culprits and possible solutions. Therefore the biorefinery concept works within a zero waste philosophy.

As with fossil fuels refineries, each specific biorefinery should work to produce high value low volume (HVLV) products or compounds and low value high volume (LVHV) material, compounds or commodities with the HVLV products increasing the profitability of the industry while the LVHV support the energy and fuel global demand [11]. A good example to understand disparities between HVLV and LVHV can be seen by the USA consumption of petroleum between the chemical production segment (3% of total oil) and the fuel/transportation segment (70% of total oil) and their revenue that was almost the same, around 380 billion $ (2007 values)[3]. Since there are several viable alternatives for the production of clean renewable energy, biorefineries should try to envisage biomass utilization more in the direction of biofuels, products or building blocks than for direct energy production by combustion since it is the only primary renewable resource available for this kind of end-uses [12].

With a large pool of different possible feedstocks, and due to the complex nature of each specific biomass, a multi-step process approach with hybrid technologies is needed through a combination of knowledge from different fields such as polymer chemistry, bioengineering, membrane separation, catalysis, etc. This large combination of feedstocks, processes and outputs makes the classification of biorefinery types difficult. Nevertheless efforts have been made to try to catalog and simplify some of the most important and possible viable platforms and processes.

According to the International Energy Agency (IEA) [2], biorefineries can be classified according to four main features: platforms (intermediates link feedstocks and final products), products, feedstock (dedicated or residues) and processes (thermochemical, biochemical, chemical, or mechanical/physical).

Platforms

- Syngas (Synthesis gas) – mixture of carbon monoxide and hydrogen produced by thermal degradation (gasification) with low oxygen input. It can be used for energy or as building block (through Fischer–Tropsch or fermentation) for alcohols, fuel and chemical products.
- Pyrolysis oil – thermal decomposition of the biomass in absence of oxygen to obtain a gaseous, liquid and solid stream depending on the conditions. The liquid stream (biooil) is often the target and the oils can be further processed to obtain chemical compounds, building blocks, fuels.
- Sugar – C6 or C5 monomeric moieties can be obtained through hydrolysis of starch, hemicelluloses, cellulose, oligosaccharides (depending on the biomass used) and converted through chemical or biological processes.
- Oil – triacylglycerol derived from seeds, algae or animal fat can be transesterified to alkyl esters (biodiesel among others) or to fatty acids. Both processes also produce glycerol, which had a low market value but recently has been used as building block material for conversion to higher value propylene glycol or other building block molecules [13].
- Biogas- anaerobic digestion of biomass (using waste streams from lignocellulosic biomass or food industries) and resulting in a gaseous stream rich in methane and CO_2 and a solid residue composed of digestate. The residual streams from other industries can be converted into methane which can be used for energetic or building block purposes.
- Organic solution- mechanical processing (pressing) of fresh biomass to obtain liquid (rich in sugars, proteins, organic acids, enzymes, etc) and solid stream (lignocellulosic rich material). Both streams can be further processed to obtain potentially interesting molecules and the cake can be used as cattle feed.
- Lignin- can be obtained from lignocellulosic biomass by thermochemical, chemical or biochemical processes. Depending on the pathway, it can be used for energy, building blocks, chemicals, used as it is for product production, among many other possible end-uses.
- Hydrogen- production of hydrogen through several methods (steam methane reforming, water-gas shift using CO) that can be applied after both Syngas and Biogas processes.
- Power and heat- Burning of biomass for energy and utilities production

Outputs

The output or products list is enormous, depending on both feedstock and processes applied. They can go from:

- Molecules obtained directly or indirectly from the biomass: phenolic compounds, flavonoids, stilbenes, lignans, monosaccharides, alcohols, carboxylic acids, alkyl esters, fatty acids, proteins, enzymes, among many others.
- Building blocks obtained directly or indirectly from biomass processing such as these top 30 obtained from 300 potential candidates studied by the National Renewable Energy Laboratory (NREL, USA) from the sugar and syngas pathways or other sources [14,15]: C1: carbon monoxide and methane, C3 molecules- glycerol, 3 hydroxypropionic acid, lactic acid, malonic acid, propionic acid, serine; C4-acetoin, aspartic acid, fumaric acid, 3-hydroxybutyrolactone, malic acid, succinic acid, threonine; C5- arabinitol, furfural, glutamic acid, itaconic acid, levulinic acid, proline, xylitol, xylonic acid; C6- aconitic acid, citric acid, 2,5 furan dicarboxylic acid, glucaric acid, lysine, levoglucosan, sorbitol.
- Polymers, either directly from the biomass or produced from it: cellulose, hemicelluloses, lignin, tannins, polyols, cellulose acetate, cellulose nitrate, cellophane, polylactic acid (PLA), poly-hydroxyalkanoates (PHA), polymers derived from lignin degradation monomers, among others [15–17].
- Human food, livestock feed or microorganism feed
- Biofuels: biodiesel, ethanol, butanol, bio jet fuel [15]
- Materials either totally or partially produced from biobased materials: pulp, paper, solid wood and associated products, polyurethanes, resins, dyes, glues, fragrancies, etc.
- Energy and utilities: electricity, steam, heat

Feedstocks

The feedstock of a biorefinery can derive from multiple biomass sources normally gathered under the following categories [2,18,19]:

- Dedicated feedstock
    - Agricultural feedstock based on sugar crops (e.g. sugarcane, beet, sweet sorghum)[20]
    - Agricultural feedstocks based on starch crops (e.g. wheat, corn, rice, potato)[20]
    - Oil based crops (e.g.: soya, palm, castor, rapeseed)[21]
    - Lignocellulosic crops (short rotation trees, miscanthus, cynara, arundo)
    - Aquatic biomass (algae and seaweeds)
- Residues
    - Agricultural and food industry residues (e.g. oils and fats from food processing, bagasse, leaves and straws from dedicated crops)
    - Forest residues (e.g. barks, stumps, leaves, uncooked material from pulping industries, saw mill residues)
    - Other residues (e.g. organic urban wastes, wastewaters, municipal solid wastes)

The processes applied in biorefinery can be multiple and sequential, being divided in several groups:

- Mechanical (e.g.: size fractionation, pressing)
- Chemical (e.g.: extractions, hydrolysis, pulping, water gas shift, esterification, hydrogenation)
- Thermochemical (e.g.: combustion, pyrolysis, gasification, torrefaction, liquefaction)
- Biochemical (e.g.: enzymatic hydrolysis, fermentation, anaerobic/aerobic digestion)

The concept of biorefinery gives ample space for multiple other classifications. Figure 1 shows a theoretical overview of the biorefinery possibilities and potentials outputs and possible applications.

Since several of the feedstocks are produced for food or in plantations that can be otherwise used for food growth, research is focusing more and more on those that minimize the food-feed-fuel conflicts. The competition regarding the feedstock within these first generation biorefineries (based on the sugar and oils dedicated crops) raises several ethical, political, social, environmental and long term sustainability problems that reduce their possible success [18]. The major advantages of using agricultural feedstocks in a biorefinery is the very high sugar and oil content of the crops, which is complemented by the fairly easy fractionation processes and conversion to monomers (monosaccharides, glycerol, fatty acids) or final product (e.g. ethanol, biodiesel, building blocks). Nevertheless several studies have found that when regarding life cycle assessment with broader environmental aspects such as air pollution, acidification, ozone depletion, land use, among others, the substitution to biofuels might not be beneficial when compared to fossil fuels, even if the global warm emissions are reduced [22].

Therefore, research has lately shifted its focus to second (lignocellulosic-based) and third generation (agricultural and organic waste streams and residues or aquatic-based biomass) biorefineries.

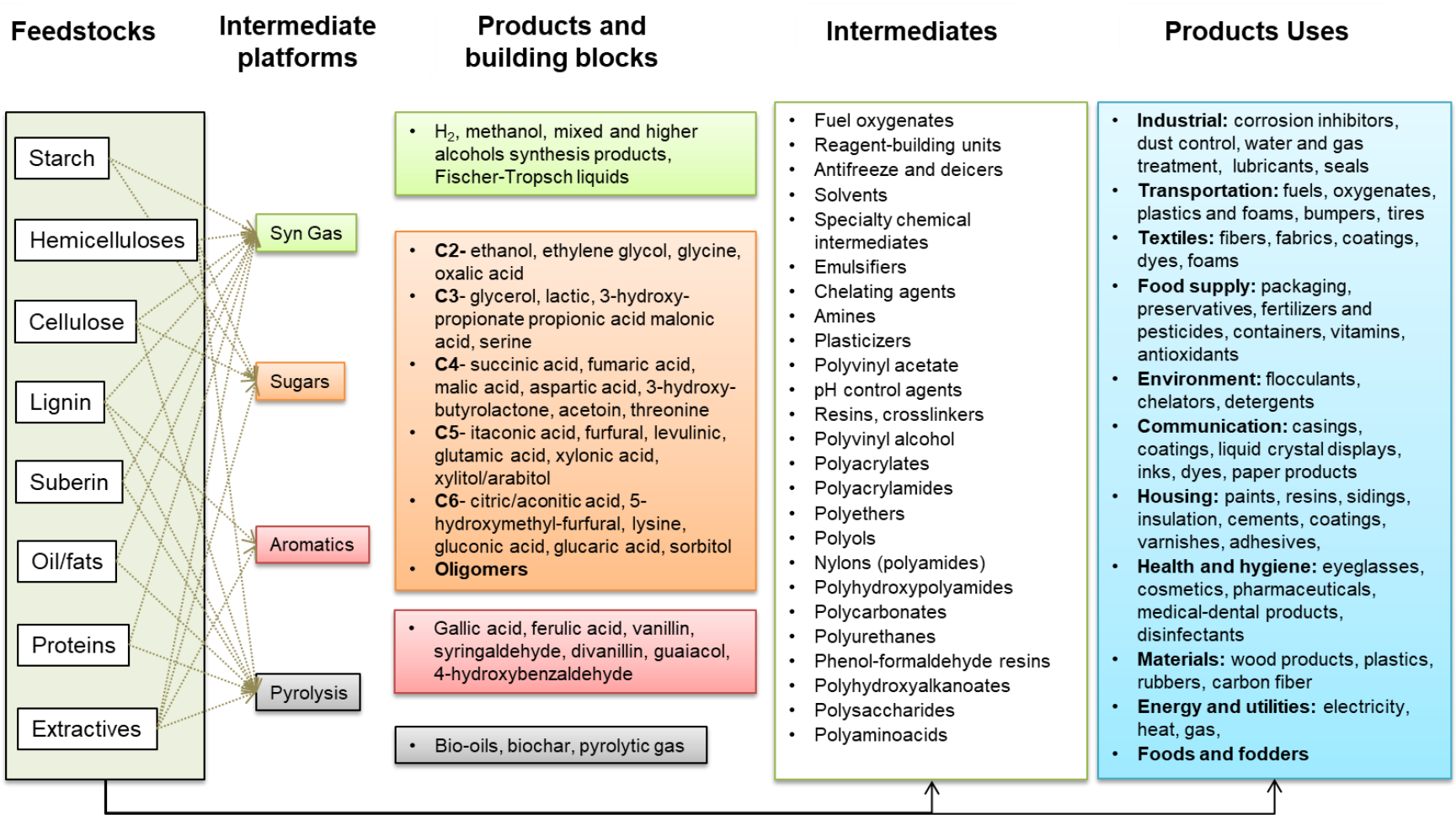


Figure 1. Broad range overview of the biorefinery potential adapted from several sources [14,17,18,23–25]

Figure 2 shows the location and type of biorefineries operating in Europe as of 2017. Of the 224 industrial sites, 63 are sugar/starch based for bioethanol and other chemicals production and 118 are oil/fat based for biodiesel and oleochemistry products, leaving only 43 second and third generation industrial sites: 25 wood based for pulp, bio-based chemicals, fuels and energy production (excluding pulp for paper production only); 5 lignocellulosic (other than wood) biorefineries for pulp/fibers, proteins, chemicals, fuels and energy; and 13 with wastes as principal feedstock for products and energy production [26]. Portugal's only industrial site considered as a biorefinery is located in Caima (Altri) and falls within the wood-based (excluding pulp for paper only) type, producing 115000 tonnes/year of dissolving pulp mostly for rayon production.

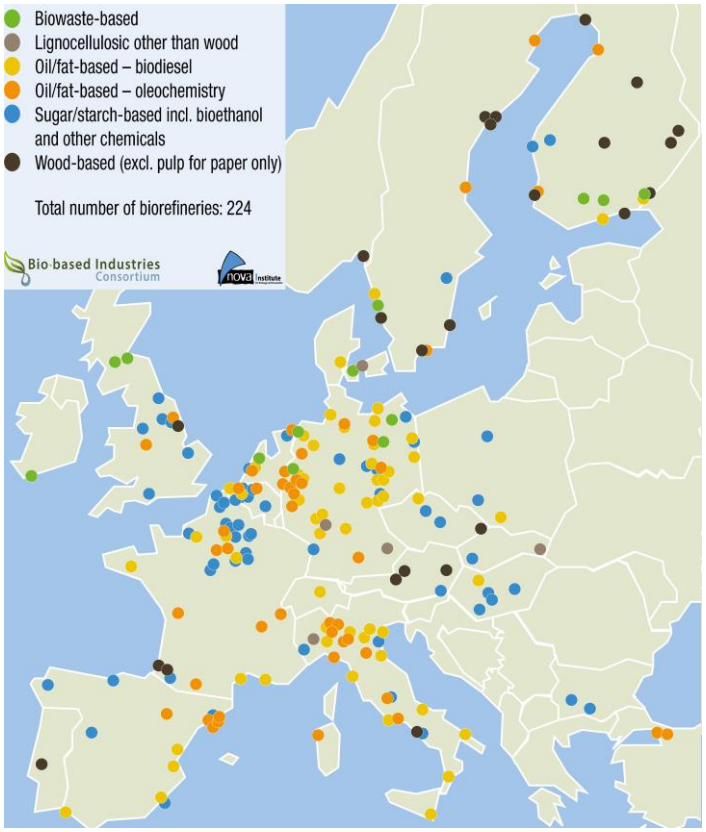


Figure 2. Biorefineries functioning in Europe in 2017 (adapted from [26]**)**

The number of biorefineries in Europe using non-food crop biomass is only 19% of the total biorefinery industrial sites, meaning that we are still in the infancy of a biobased sustainable economy. Nevertheless the efforts are in this direction with regulation being imposed to decrease the share of food-crop based biofuel to 3.5% off all biofuels by 2030 [6]. Scientific research has focused on discovering and improving new viable (both technically and economically) ways to use non-edible biomass with particular interest being given to the lignocellulosic biorefinery (LCB).

Lignocellulose Biorefinery (LCB)

Lignocellulosic biomass is the most abundant biomass on the planet, and widely spread throughout the globe, contrary to what happens to fossil fuels. In the EU-28, 43% (182 million hectares) of the total land area are forests and other woodlands, covering a slightly higher proportion than agriculture (41%), reaching over 75% of woodland in some countries (Finland and Sweden) and around 55% in Portugal and Spain. Biomass used as an energy source (wood/other biomass/municipal wastes) accounted for nearly two thirds (64%) of the gross energy consumption of renewables, with wood, pellets and briquettes representing 45% of all organic, non-fossil material of biological origins.[27] Taking in consideration that the European Union only accounts for 5% of the total world forests, the worldwide potential of these resources is immeasurable [27]. However the use of the non-food lignocellulosic material by the bio-based industries is well below its growing potential in Europe, e.g. by 2030 only almost half of the available resources will be required (476 million vs 1 billion tons)[9], meaning that this biomass has yet to reach its full potential as feedstock.

But what is a lignocellulosic material?

Lignocellulose is the entangled result of three main polymers (lignin, cellulose and hemicelluloses) that create the matrix of plant cell walls. In broad terms it refers to plant crops, forest material or residues deriving from their processing. Although these three polymers are the main constituents of plant biomass several other components exist in (normally) lower percentages such as suberin (also structural when present), inorganics, proteins or extractives [19]. These components might be low or inexistent in stem wood, but can have a very high predominance in other lignocellulosic material (e.g. bark)

Briefly these chemical components may be characterized as follows:

- **Cellulose**. The most abundant natural polymer composed by glucose molecules as a linear sequence of anhydro-β-D-glucopyranose linked by β-(1→4) glycosidic bonds that can have up to 10000 units. The high number of hydroxyl groups (-OH) and linear structure allows strong intra and inter-molecular hydrogen bonds producing a macrostructure by connecting cellulose molecules into microfibrils that group into fibrils and these into fibers that can present crystalline and amorphous forms. This strongly chemically bonded polymer results in a very resistant material regarding both chemical and enzymatic degradation.

- **Lignin**. The second most common natural polymer. It is a phenolic based heteropolymer derived mainly from the oxidative coupling of three monolignols (*p*-coumaryl (H), coniferyl (G) and sinapyl (S) alcohols)[28], although several other phenolic compounds similar to these (p-hydroxybenzoates, p-coumarates, ferulates, caffeyl alcohol, 5-hydroxyconiferyl alcohol, hydroxycinnamaldehydes) can be found in the lignin structure and are therefore considered to act as true lignin monomers[29–33]. Recently other phenolic monomers from beyond the monolignol biosynthetic pathway (tricin, hydroxystilbenes) have also been discovered to act as

true lignin monomer [34–37]. Lignin is an amorphous polymer that forms a web structure through ether, C-C linkages or both (β-O-4'alkyl-aryl ether, β-5' phenylcoumarans, β-β' resinols, 5-5' dibenzodioxocins, β-1' spirodienones), with the alkyl-aryl linkages being the most easily broken. This polymer links to cellulose and hemicelluloses through covalent bonds and is largely responsible for the recalcitrance nature of lignocellulosic biomass.[19] The lignin monomeric composition is of great importance since it will play an important role in any biomass deconstruction process.

- **Hemicelluloses**. Polysaccharide heteropolymers composed of monomeric sugars (glucose, xylose, mannose, arabinose, galactose, rhamnose, and sometimes their acetylated counterparts) and uronic acids (galacturonic and glucuronic acids) linked through glycosidic bonds. Hemicelluloses are amorphous branched polymers with 50-200 polymerization degree [38,39] that along with lignin "glue" the cellulose fibers, bonding the entire structure together. In softwood trees, hemicelluloses are usually of two types, galactoglucomannans (~20% of the biomass) and arabinoglucuronoxylans (5–10%), while in hardwoods they are composed of a backbone mainly of glucuronoxylans (15–30%) and glucomannans (2–5%) [40]. When compared to the other structural components, these polymers are the most easily assessed and degraded either by chemical or enzymatic hydrolysis.

- **Suberin**. A non-linear polyester polymer formed by esterification of glycerol molecules with saturated or unsaturated long chain fatty acid, ω-hydroxyacids, α,ω-diacids with small amounts of aromatic monomers (mainly ferulic acid). Suberin is specific to the phellem (cork) of bark periderms that in a limited number of species may reach important proportions. This hydrophobic polymer serves as insulant for fluids such as water and air, and has thermal insulation properties [41].

- **Extractives**. This highly heterogenic group of non-structural compounds is defined only by their possible extraction from biomass through solvent dissolution without chemical reaction. Depending on the solvent, several families of compounds can be retrieved according to their polarity and chemical affinity. Usually sequential extractions with different solvents are needed to fully remove all extractives, leaving only the structural components of the biomass [38,42]. The amount and chemical composition of the extractives vary drastically between species, type of tissues (e.g. heartwood, sapwood, bark) [42–44], within tree location (stump, bole, branch) [45–47] and are also very susceptible to edaphoclimatic conditions, season, health of the individual, among other parameters. Extractives may comprise several chemical families such as alkanes, fats, waxes, fatty acids, fatty alcohols, terpenes, steroids, resin acids, simple phenolic compounds, flavonoids, stilbenes, condensed tannins, hydrolysable tannins, monosaccharides, polysaccharides, lignans, amino acids, among others [48,49].

- **Inorganics.** They comprise the mineral salts and other inorganic matter present in the lignocellulosic biomass, usually referred to as ash after total combustion. The content varies significantly depending on the biomass, with very low contents in wood (<1%), while much higher values are found in barks (<13%)[48], energy crops (e.g. cynara <30%)[50] and agro-industrial residues (e.g. rice straw- 46%, cotton stalk- 7%, corn stover- 7%)[23]. This fraction is very important when considering certain processes (e.g.: energy, pulping) since it can have a detrimental effect on the global value, machinery or the final product quality.

Figure 3 shows a representation of a possible lignocellulosic biorefinery (LCB) with the main structural cell wall components fractionation and end-uses.

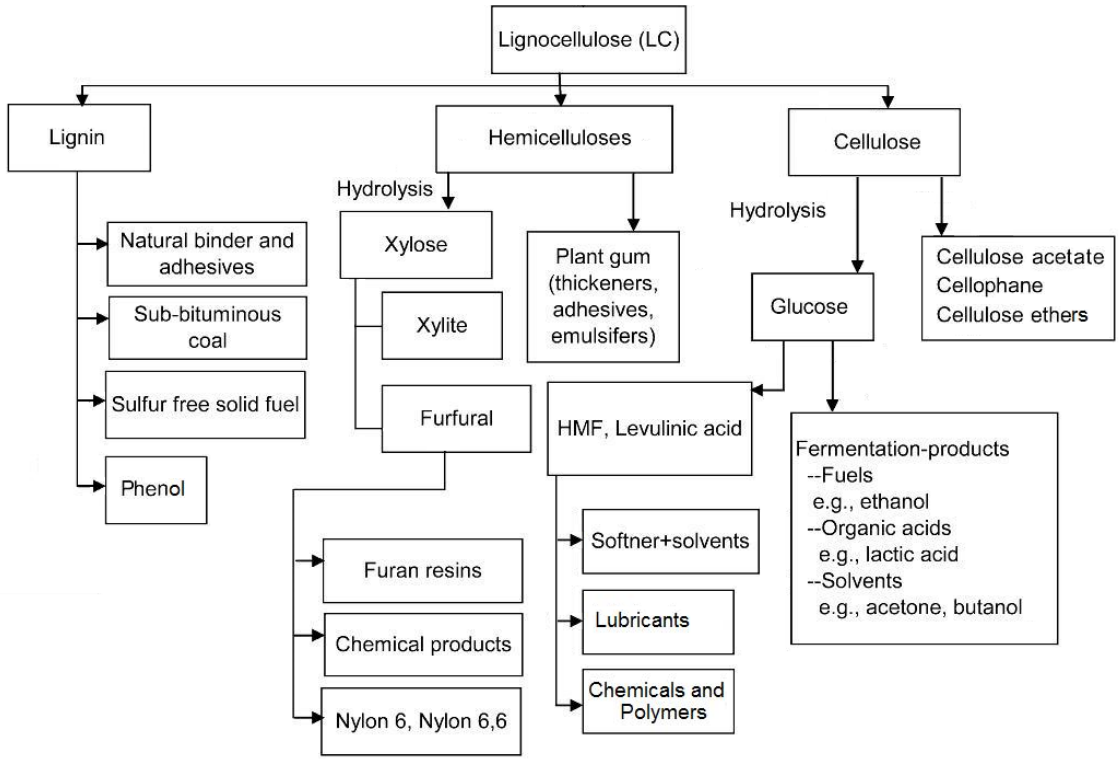


Figure 3. Main scheme of possible lignocellulosic biorefinery and products (adapted from [51]**)**

Many feedstocks are available for an LCB and each has positive and negative aspects on its use, deconstruction pathways and resulting end-uses. One of the most available lignocellulosic feedstock apart from wood and non-edible crops is the bark fraction of the trees. This non-wood material has specificities that may impact on its valorization or upgrade, thereby requiring an interested look and attention in a biorefinery context.

Bark Biorefinery (BB)

Bark comprises the outermost radial layers of tissues of the bole from the vascular cambium to the surface, representing 9-15% in volume [52] or 11-21% in mass [53,54]. It encompasses the phloem (functional and non-functional), periderm (phelloderm, phellogen and phellem) and rhytidome. The living (phloem, functioning phelloderm and phellogen) and non-living (dead periderm and rhytidome) layers are also commonly designated as inner and outer bark [55]. Figure 4 shows a typical bark transverse section.

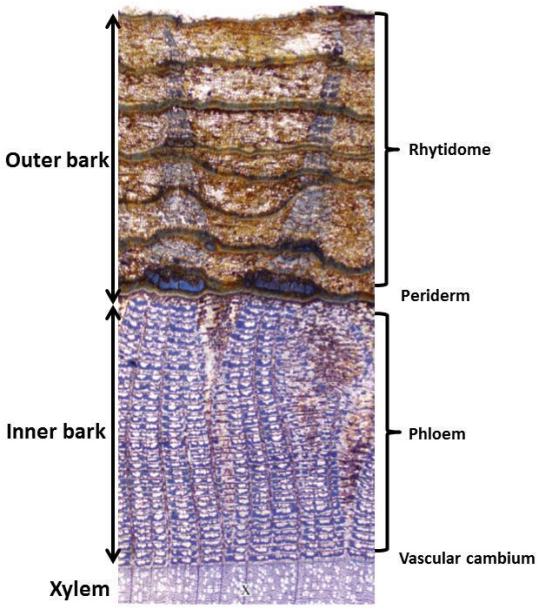


Figure 4. Bark transversal section showing the several typical tissues from vascular cambium outwards. Adapted from [56]

The chemical and physical characteristic of bark varies substantially among different species and even within the same species. This heterogeneous and complex material comprising physiologically active living and inert dead cells is the outermost fraction of the tree acting as the interface between the organism and the external media [52]. This means that bark will have to deal with numerous external aggressions, either biological (fungus, parasites, bacteria, animals) or environmental (weather conditions, fires, droughts, floods) leading to specific physical/chemical characteristics such as: suberin rich cell walls for air and water impermeability; lignin enriched cell walls for mechanical strength and biological protection, sap and resin veins as protective agents for healing wounds and insect attacks; polyphenolic rich cells for biological attacks; mineral encrusted cells (normally calcium oxalate crystals); secondary metabolites with diverse bioactivity [38,55,57,58]. These complex characteristics are what make barks especially interesting to be used under a biorefinery context.

Nevertheless, the physical and chemical variability is a two-edge sword. On one hand, the inter-species variability grants a wider range of possible deconstruction pathways and consequent products, chemicals and end use materials while, on the other hand, the intra-species variability introduces a heterogeneity that may lead to higher deconstruction and production problems, and a lower product specification. The problem is enhanced if the feedstock is to be comprised of mixed species barks.

Quantifying the global availability of bark is not an easy task, since just a small fraction is commercialized or used for further processing, with most of it being burned for energy, industrial utilities or simply incinerated or landfilled [53]. According to FAO [59], the world roundwood production was 3797 million m^3 in 2017 which should represent around 380 million m^3 of bark residues (considering an average 10% of bark in the stem volume) largely concentrated at wood processing industrial sites.

Although barks are mostly accounted for as residues, for some species this is not the case and the bark is highly profitable and commercially the most important part of the tree. The bark of the cork oak (*Quercus suber*) is probably the most impressive example of bark valorization, namely of the cork component of the periderm. This highly suberized cork is the center of an economic relevant industry with an annual production of 200000 tons that can be used for bottle stoppers (60% of its market) [60], production of agglomerates and composites with various end-uses [41].

Other barks have been used throughout human history for many purposes with additional possible alternative end-uses being tested throughout the years. Barks have been used for: obtaining tannins for leather curing, clothing, utensils, wares, livestock fodder, human food, flavoring, spices and fragrancies (e.g. cinnamon, camphor, vanillin), energy and fuel production (either by direct burn or through mixed addition with other materials for pellets and briquettes production), mulching, plant growing media, organic peat replacement, filler in resins and glues, adhesives formulations, dyes, natural rubber, pollutant absorbent (pesticides, heavy metals, etc.), in production of thermal and sound insulating boards, filler with strengthening properties in plastics, activated carbon, for obtaining bioactive compounds with various pharmacological (e.g. quinine, taxol, curare) and antioxidative properties, among other uses [61–64].

However the current utilization of barks is mostly for energy purposes, not because their chemical an physical nature makes them good solid fuels, but mostly due to lack of economically viable alternative processes and possible products. In fact, bark is considered a poor raw material for energy production with an energetic output of 70 % of that of bituminous coal and 50% of that of oil when completely dried, which usually is not the case, meaning an even lower energy output since part must be consumed to address its normal water content. With water contents between 30 and 60% (depending on the debarking process and season), the energetic value decreases falling to flat zero if the water content is around 90% [52]. Barks tend to be technologically inconvenient for burning due to a lower density compared to wood, and despite a similar mass energetic value (17-25 MJ/kg), their volumetric energy density is low (e.g. 13 vs 5 MJ/m^3 for *Robinia pseudoacacia* wood and bark respectively) [62].

Additionally, many barks have inherent high ash content, mostly due to the mineral encrusted cells, normally parenchyma cells rich in calcium oxalate crystals [38,57], that besides reducing its energetic content have a detrimental effect upon boilers due to fouling damage and also increase the cost of managing and dealing with the unburned material [53]. Barks also tend to have high ash content due to exogenous contamination such as dirt, sand and grit thrusted by the wind when still unfelled or directly from the ground after cutting down, transport, handling and storage [52]. Another negative aspect for energetic end-uses is the sometimes high chlorine and sulphur content of barks that might prevent their use for pellets and briquettes production (most markets require very low threshold values)[65], decreasing the lifespan of equipment due to corrosion and increasing the cost of dealing with higher environmentally harmful emissions.

Although barks received much less attention than wood regarding characterization and possible uses [64], more and more studies are addressing their rich nature. Of all the main bark components, the extractives fraction is probably the one that has gathered more interest, due to its usual high content, while on the other hand the carbohydrate fraction is comparatively lower making it less appealing if this fraction is the to be targeted. Nevertheless, the lower holocellulose is usually related to the decrease of cellulose (bark tends to have less fibers than wood) meaning that hemicelluloses can also be a targeted component. Lignin content is often similar to that of the respective wood although with different monomeric composition [48]. Table 1 presents the hardwood and softwood typical composition of both wood and bark regarding their main components. Comparing bark and wood, polysaccharides typical values for barks are significantly lower while extractives are much higher, and it is noticeable the wider range of variability observed for bark for each of the main components.

Table 1. Typical chemical composition (main components in dry weight %) of hardwood and softwood wood and bark [41,52,66–71]

	Softwoods		Hardwoods	
Component	Wood	Bark	Wood	Bark
Lignin	23-29	17-44	18-25	14-48
Suberin	-	0-36	-	0-43
Polysaccharides	60-71	23-53	70-78	10-60
Extractives	2-9	2-25	2-5	5-50
Ash	< 1	< 20	< 1	< 20

Caution is necessary when simplifying the information regarding such diverse materials (especially bark) as some species may fall outside the presented values. This high variability between species can propel different approaches and target diverse end-uses and should be looked with both caution and interest.

Bark is considered highly detrimental (either due to its physical or chemical properties) to most, if not all, wood transformation processes and final products. In both pulp and timber industries bark is a

contaminant for the production process and stripping it from the bole is probably the first major step in any industrial site. While pulping for some kinds of papers might still admit a low bark content as contaminant to the digester [52], for dissolving pulps the bark content fed to the digester must be close to nil so as not to block the nozzles upon fiber formation (the higher probability of shives and higher mineral contents leads to obstruction and calcification of the nozzle.

The amount of this residual stream is therefore proportional to the wood processed, resulting in considerable quantities at the industrial sites since most of the debarking currently takes place within the wood or pulp processing plant and not at the harvesting site). This means that there are some species for which the production, harvesting, management, transportation and initial processing steps are currently integrated and already incorporated in the costs of another production hub. From an economic point of view, these are probably the best candidates regarding a bark biorefinery, due to their availability and expected lower investment cost [72]. Nevertheless, the bark upgrade for anything other than energy/utilities must be proven as technically feasible, economically beneficial and also not to disrupt, in any way, the main wood production process. Even if used only for energy, bark has already a role within the biorefinery concept since this stream (at least when its end is not the landfill) serves a purpose, even if not the best one regarding its chemical potential.

The most important species for timber and pulping in Europe are birch, pine, spruce, poplar, Douglas-fir and eucalypt of which the correspondent barks will be widely available at pulp mills or industrial sawmills.

As with any lignocellulosic feedstock the biggest problem resides on how to mine the chemical richness of this recalcitrant material in such a way as not to degrade each fraction beyond further use and, if possible, to encompass the highest number of fractions for adequate end-uses. Many alternative or combined processes are, at least hypothetically, possible.

Biomass deconstruction

No “one approach” deconstruction pathway is viable for all barks, at least when considering the best use of each specific bark. Some processes can be more or less “blind” regarding raw material, while others will make sense only for specific species. Two approaches can be considered for biomass fractionation, one being focused solely on a single product or process and the other being a multi-product, multi-process to generate value from each fraction. The higher the separation and purification steps, the higher the production cost, while on the other hand the isolation of each component or the obtainment of pure compounds will probably increase their profitability and utilization possibilities [40]. The best pathway for deconstruction and utilization of the raw material will be between these two and might shift with time, depending on raw material costs, development of processes or final product value.

A brief discussion of the most important deconstruction methods and pre-treatments will follow organized in their four main types: mechanical, chemical, thermochemical and biochemical. Most, if not

all, derive from already tested deconstruction methods applied to wood, thereby being more or less appealing when applied to other raw materials such as barks. Sometimes the processes are called pre-treatments if the purpose is to enhance some material characteristics making it more prone to subsequent processing (mostly used for enzymatic hydrolysis and subsequent fermentation to ethanol).

Mechanical treatment encompasses those processes where no chemical or biological reaction takes place, using mainly physical processes to separate fractions or compounds within the biomass. The most common are size reduction, solvent extractions and pressing.

Size reduction- mechanical downsizing of lignocellulosic material is almost always necessary, either to better manipulate the material, rupture the cell wall structure, decrease cellulose crystallinity and increase particles superficial area and impregnability by reagents and solvents. In mainly homogenous materials this step will only create smaller particles with the same composition than the original, while on heterogeneous materials such as barks (with highly differentiated tissues and specialized cells) it might serve the purpose of obtaining chemically and physically sized fractions, richer in specific compounds. Many studies have focused in different fractions of bark, mainly showing the chemical, physical and energetic value variations between the tissues, or more commonly between inner and outer bark [42,47,73–75]. Some research focused on using a mechanical downsizing in order to obtain substantially different fractions of barks that can be used as raw material [76–81]. The variation in density and friability between tissues results in finer fractions normally richer in ash and extractives, while the coarser fractions are normally enriched in suberin (in barks containing this polymer) and sugar polysaccharides. Grinding is a costly operation but most of the times unavoidable and using the right milling and particles size might be a first step toward a simple and better optimization biomass processing.

- Extraction- The soluble fraction of barks can be highly relevant due to its significant content. Targeting extractives from barks can have two purposes (not mutually exclusive): to obtain a valuable fraction of compounds or to remove them prior to structural components fractionation. Solvents are chosen mostly based on polarity following the rule “equal dissolves equal”, meaning that apolar solvents will solubilize mostly apolar compounds and the equivalent will happen with polar ones. Unfortunately, further steps of isolation and purification are needed if single compounds are to be acquired in this way.

  Several extraction methods have been applied such as cold extraction, soxhlet, soxtec, microwave or ultrasound assisted, supercritical and pressurized extraction [82]. Each method has its own pros and cons regarding solvent volume, required time and temperatures, extraction

yield, selectivity, equipment cost and complexity, degradation of compounds and solvent recuperation.

Extracts can be detrimental to some processes (e.g. pulping, enzymatic hydrolysis, fermentation) [83–86] but can also include very interesting bioactive components known to have antioxidant, anti-inflammatory, analgesic, antidiabetic, cardioprotective, antineurodegenerative, antitumural, antimutagenic, antibacterial, antifungal, antiviral, antiprotozoal, antihelminths, anti quorum-sensing, anti-HIV, among other effects [45,87–92]. Although not all components of the crude extracts will have the same (or any) bioactive action, some synergetic or antagonism effects may occur. These extracts and individual components are therefore very enticing natural products for food, polymers, cosmetic, nutraceutical and pharmaceutical Industries. Table 2 presents examples of extracts (with their respective main compounds) from several barks and their bioactivity showing the wide possibility of compounds that can be generated form this material.

Table 2. Bark extract composition of different woody vascular plant and their respective biological action (adapted from [90])

Composition of Extract	Action/Application	Ref.
dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, *p*-hydroxybenzoic acid, proanthocyanidin b2, catechin, epicatechin, syringic acid, taxifolin, quercetin, homovanillic acid, epigallocatechin	antioxidant	[93]
gallic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, ferulic acid, sinapic acid, resveratrol, myricetin, quercetin, cinnamic acid	antioxidant in food, cosmetics and pharmaceutical industry	[94]
gallic acid, catechol, caffeic acid, vanillin, *p*-coumaric acid, ferulic acid	anti-inflammatory, antioxidant, anti-rheumatic	[95]
procyanidin, epicatechin, coumaric acid, coniferin, quercetin, taxifolin-*o*-hexoside, coumaric, acid-di-*o*-hexoside, syringic acid-di-*o*-hexoside, coniferyl alcohol-*o*-hexoside-*o*-pentoside	antioxidant	[96]
gallic acid	Analgesic, anti-inflammatory topical	[97]
apigenin, luteolin, vitexin, apigetrin, cymaroside	anti-inflammatory	[98]
quinic acid, gallic acid, protocatechuic acid, catechin, chlorogenic acid, ellagic acid, taxifolin, quercetin, mearnsetin, naringenin, ellagic acid-rhamnoside	antioxidant	[99]
gallic acid, ellagitannin, ampelopsin, gallotannin, epigallocatechin gallate, ellagic acid derivative, punicalagin, corilagin, ellagitanninellagic acid glucuronide, gallotanninmethylellagic acid glucuronide, methyl-(*s*)-flavogallonate and its isomers	antibacterial	[100]
procaynidin dimer b2, procyanidin trimer c1, epicatechin, lupeol, betulinic acid	cancer prevention chemoprevention	[101]
quercetin, *o*-coumaric acid, ferulic acid, gallic acid	antioxidant antiviral, cytotoxic	[102]
α-hydroxyerysotrine, 4'-methoxy licoflavanone (mlf), alpinumisoflavone, (aif), wighteone	antitumoral, cytotoxic effect on HL-60 cells	[103]
flavanocoumarin epiphyllocoumarin, epiphyllocoumarin-[4β→8]-(−)-epicatechin	anti-inflammatory, antioxidant	[104]

rhaponticin, rhapontigenin, piceatannol, taxifolin	antioxidant	[105]
catechin, procyanidin, epicatechin, apocynin e, cinchonain i, 3-methoxybenzoylquinic acid	antioxidant, anti-inflammatory, antidepressant, neuroprotective	[106]

- Pressing - compression is applied to separate biomass into a rich liquid stream (rich in sugars, proteins, organic acids, enzymes, etc) and solid stream (lignocellulosic rich material) or simply to disrupt the physical properties of the biomass, making it more susceptible to further processing. Pressing can be used in combination with chemicals (acid or alkali) to improve results. This process is applied mostly to green biomasses and grasses but can also be used with woods [107] or similar lignocellulosics such as barks [108].

Chemical treatment encompasses biomass deconstruction using any sort of chemical reagents through the dissolution of one or more of the major lignocellulosic macro components. Depending on the conditions, the treatment will be more prone to target polysaccharides or lignin.

- Acidic- The acid acts as a catalyst for polysaccharides hydrolysis. Depending on acid concentration and type, the attack on holocellulose can be partial, targeting solely the hemicelluloses fraction (dilute acid, resulting in a cellulose/lignin rich solid substrate) or total (concentrated acid, resulting in a mostly lignin substrate) by hydrolyzing both hemicelluloses and cellulose fractions. The concentrated acid treatment requires low temperatures and pressures, and avoids any need of enzymatic hydrolysis if the final end use is a sugar stream for fermentation, but on the other hand requires high chemical charges and consequent acid cost, with their environmental issues and high energy needed for acid recovery and re-use. Acid hydrolysis is one of the most employed processes in biomass treatment for ethanol production, being cheap and effective [109]. The downside for any acid treatment is the highly corrosive reaction environment that forces the use of resistant equipment and material (thereby increasing costs) and requires neutralization of the liquid stream to suitable pH before further processing and intensive washing of the solid stream. Additionally, if the sugar stream is to be used for fermentation, there are some degradation reactions of the monosaccharides, lignin and extractives that produce undesirable inhibitors that may require detoxification processes (furfural, 5-hydroxymethylfurfural, acetic acid, phenols, tannins, vanillin, ferulic acid and p-coumaric acid) [110,111].

  Mineral acids (sulfuric, phosphoric, nitric, hydrochloric) are the most commonly used although organic acids (formic, acetic, maleic, oxalic) have also been successively tested for polysaccharide hydrolysis. Although leading to lower inhibition products, the organic acids are more expensive making them less appealing for industrial purposes.

  Barks have been the subject of some studies regarding acidic pre-treatments, mostly for fermentation purposes [112,113].

- Alkaline- In this treatment, sodium, potassium, calcium and ammonium hydroxides are used as bases, mostly for lignin degradation and solubilization but also affecting the holocellulose fraction, mainly the hemicelluloses. Fractionation will depend on the alkali charge, temperature, time and additional reagents with some pre-treatments being carried at ambient temperatures, although the time in those cases can reach days. A rise in temperature will effectively reduce the reaction time to hours or minutes but at a cost of lowering selectivity.

  The most common alkaline use regarding lignocellulosic material is the kraft process to produce pulp, which besides soda also uses sodium sulfite to enhance delignification selectivity and reduce reaction time. Although removing up to 90% of lignin, at that point it starts to become less efficient, with residual lignin normally being removed through more selective reagents such as ozone, oxygen, chlorine dioxide to obtain a lignin free bleached pulp composed of cellulose fibers and some hemicelluloses. Alkaline hydrolysis leads to lower polymerization degree. Hemicelluloses are the most attacked and they depolymerize and solubilize as oligo or monosaccharides along with lignin, while cellulose suffers some degradation mostly through end-wise depolymerization (peeling). The lignin and most hemicelluloses are obtained in the liquid stream that can be further processed and separated to obtain a lignin rich residue (mostly monomeric and re-condensed moieties of lignin degradation products) and a hemicelluloses stream (oligo and monomeric sugars and their respective degradation products). The Kraft process has been adjusted to bark with some degree of success [114].

  The same can be done with sulfur-free process of soda, soda-anthraquinone (AQ) or alkaline-hydrogen peroxide, which can be more interesting if the lignin fraction is also to be targeted for commercialization. These processes tend to be used more with lignocellulosic biomasses other than wood.

- Organosolv- This designation refers to the delignification processes that use organic solvents such as alcohols, acids, ketones, ethers or amines (ethanol, ethanol, butanol, formic acid, acetic acid, acetone, γ-lactone, dioxane, ethylene diamine, etc.) with water as co-solvent at high temperatures and pressures to break the lignin-carbohydrate connections and degrade lignin, solubilizing its fragments. Several industrial processes have risen such as Alcell, Organocell and Formacell, although no large scale production took place. These processes can produce pulps with low residual lignin and its unsulfonated lignin is considered to be of higher value being suitable for a wide range of applications and better than kraft lignin [115,116]. Bark organosolv delignification can be achieved although with high chemical loads and resulting in pulps with high residual lignin [117,118].

- Ionic liquids (IL)- Salts normally composed of a large organic cation and organic/inorganic anion with melting temperature below 100°C and very low volatility can solubilize different

components of biomass depending on their cation/anion combination. The large pool of cations and anions that can be paired to produce ionic liquids and the subsequent product properties regarding thermophysical, biodegradation ability, toxicity and target selectivity makes them very interesting solvents with high tuning possibilities [119]. They can be used selectively to degrade and dissolve each of the major lignocellulosic components, from extractives [120] to the structural polymers [121,122]. An anti-solvent can be used to precipitate the dissolved component and the ionic liquid is supposed to be easily recovered to be recycled. If not produced from fossil resources, both ionic liquids and deep eutectic (DES) can be considered "green solvents", with IL major downside regarding conventional solvents being the high production and purification cost.

- Deep eutectic solvents (DES)- Similar to the ionic liquids in both very low volatility and high tuning possibility, the DES diverge from the IL by their low toxicity, biodegradability, easiness of synthesis and lower production cost. These low transition temperature mixtures are defined by the combination of two or more components (at least one hydrogen-bond donor and one hydrogen-bond acceptor) whose melting points in the specific mixture decrease significantly when compared to their individual components (DES are normally liquid at room or low temperatures) [123]. Their use is pretty much the same as with IL. Several studies addressed bark as a biomass deconstruction raw material using DES [124,125].

Thermochemical treatments encompass those where biomass physical and chemical changes occur mostly due to temperature degradation, envisaging energetically denser fuels (torrefaction) or biomass deconstruction (selective or non selective). Significant thermochemical changes start at temperatures just above 100 °C, affecting differently the major components of the lignocellulosic biomass with degradation products being mostly dependent on process time and temperature but also on the presence or absence of solvents and catalysts. Some treatments have low selectivity and degrade biomass to simple compounds/building blocks (H_2, CH_4, CO, etc.) or complex moieties derived from all biomass components (liquefaction, pyrolysis) with high number of compounds and low individual yield [126] while others, such as steam explosion or hydrothermal treatment can be tuned to target specific biomass components or act as pre-treatment to facilitate subsequent processes.

- Pyrolysis- Thermal degradation in the absence or near absence of oxygen (or any oxidizing agent) at temperatures between 300-800°C forming a gaseous, a liquid (tar or bio-oil) and a solid (char) stream [23,126]. This deconstruction method favors the liquid and solid products, which due to the lack of oxygen still maintain part of the structure and complexity of the feedstock material. The output depends on temperature and residence time, with the three main types being: slow pyrolysis (termed carbonization since it favors mostly char formation), working between 300-700 °C for long residence (up to days); fast pyrolysis favoring bio-oils (50-70%) with high heating rates

and low residence times (up to 10 s), and flash pyrolysis with very high heating rates (up to 10000 °C/s) and very short residence times (<0.5 s) promoting almost exclusively the bio-oil formation (75-80%) [127,128]. Bark has been the focus of intensive study in pyrolysis for energy, fuels and products regarding both the entire biomass [129–131] or fractions of it [132].

- Gasification- The thermal degradation in this process occurs in controlled deficient oxidizing atmospheres (20-50% below the stoichiometrically required for full combustion) at high temperatures (800-1000 °C) for the production of a fuel gas which is composed mostly by N_2, H_2, CO, CO_2, H_2O, CH_4, light hydrocarbons, tar and particulates. The gas can be enriched in one or more of the compounds depending on gasification design and conditions. The cleaned gaseous stream (usually called synthesis gas or syngas) can be used for energy production through burning or for chemicals and liquid fuels production through catalytic conversion using the Fischer-Tropsch process [126]. Barks have been used as raw material in several gasification tests, with positive results [133,134].

- Liquefaction- or solvolysis is another thermal degradation process whose work conditions fall in-between pyrolysis (similar thermochemical mechanisms) and hot water treatment (operates in liquid solvent) functioning at 200-400 °C under high pressures. It tries to depolymerize lignocellulosic and partially deoxygenate it through dehydration, decarboxylation or decarbonylation (oxygen removal through H_2O, CO_2 and CO elimination respectively), resulting in a biocrude with higher energetic value that can be refined to fuel through conventional technologies, while trying to prevent undesired reactions such as recondensation that form char and humins [135]. The role of the solvent is very important regarding operation cost, separation and recovery, reaction conditions, conversion yield and type of product obtained. Some of the solvents are water, hydrocarbons (e.g. naphthalene or toluene), anisoles, phenols (e.g. phenol, guaiacol) or alcohols (ethanol, glycerol) [136,137]. Several studies have addressed the use of bark for liquefaction to obtain bio-crudes for fuel or as phenol substitute in the synthesis of phenolic resins and polyurethane foams [138–140].

- Hydrothermal treatment- also called auto-hydrolysis or liquid hot water is the process that uses water at temperatures between 140-220 °C mostly for hemicelluloses degradation and decomposition to oligosaccharides and monosaccharides, reducing biomass recalcitrance toward further processing (e.g. enzymatic hydrolysis, pulping for hemicelluloses free pulps). Parts of the most vulnerable lignin polymeric sites are also degraded, releasing phenolic compounds, while most of cellulosic fibers remain intact. Since many extractives are water soluble, this process will also dissolve them into the liquid stream.

  Water self-ionization is the constant equilibrium that occurs in liquid water through which hydroxide (OH^-) and hydronium (H_3O^+) ions are formed from two water molecules. This

equilibrium is favored to the ionization products with higher temperatures increasing [OH^-] and [H_3O^+] which are responsible for hydrolysis reactions of polysaccharides. This means that water can act as reagent as well as solvent for the degradation products of lignocellulosic biomass. Since hemicelluloses are acetylated to some extent (depending on species), their cleavage also releases acetic acid, reducing the medium pH and auto-catalyzing the breakage of glycosidic bonds [141,142].

This method is industrially very enticing as it requires no chemicals addition, works at mild reaction conditions of temperature and pressure (below those of liquefaction, pyrolysis, gasification), separates partially or totally the hemicelluloses fraction from the solid residue (cellulose and lignin) leading to normally high enzymatic hydrolysis of this residue, avoids waste production and has low environmental impact. On the downside: high water volumes are usually required (increasing energetic cost to achieve the reaction temperature); diluted hydrolysate requires energy consumption for concentration; degradation products from monosaccharides, lignin and extractives (such as formic acid, acetic acid, levulinic acid, furfural, hydroxymethylfurfural, phenolic compounds, lignin degradation products) can prevent an ideal processing of both resulting streams, which usually occurs at harsher temperature conditions of autohydrolysis [142–144].

Both liquid and solid residue are interesting from the point of view of valorization, with hemicellulosic oligosaccharides and monosaccharides being enticing for the nutraceutical, pharmaceutical, polymers and food industries [145–147], and the cellulose/lignin rich residue to be further processed to a wide range of fuels, products and chemical compounds (depending on the subsequent processing and desired end-uses). Some studies focus on using autohydrolysis of bark as pre-treatment for recovery of hemicelluloses and extractives, or to prepare the remaining solid for saccharification and fermentation [148–150].

- Steam explosion- one of the most widely used lignocellulosic pre-treatment that combines the simultaneous effects of thermal, mechanical and chemical processes. High pressure saturated steam is imbued into the lignocellulosic matrix, partly condensing and acting as described previously in the hydrothermal treatment. After a short period of settling (seconds to minutes) the pressure is rapidly dropped by releasing the steam and condensed liquid. The pressure release leads to the rapid expansion of water embedded in biomass, “exploding” the material from within, and causing: mechanical fracture and particle downsizing; structural changes in cellulose (increasing its adsorption capacity and altering crystallinity); hemicelluloses hydrolysis and partial solubilization (as with autohydrolysis the acetyl groups released from hemicelluloses acidify the medium and promote further depolymerization); lignin degradation (mostly through cleavage of susceptible linkages such as β-O-4), partial solubilization in the liquor and recondensation in the lignocellulosic matrix (which might have negative impact in posterior treatments) [19,141]. A slurry with a cellulosic enriched solid and a liquid fraction rich in

hemicelluloses oligosaccharides/monosaccharides, soluble lignin and degradation products results from this process

Several methods have combined the steam explosion mechanism with addition of acid or alkaline chemical charge such as the ammonia fiber expansion (AFEX), CO_2 and SO_2 explosion pre-treatments that beside promoting the physical rupture of the lignocellulosic material, also chemically degrade fractions of it, making it more susceptible to further treatments [141].

Biochemical treatments encompass all the processes that use living agents, such as bacteria, fungi, yeasts and algae [151] or enzymes produced by them to deconstruct and convert lignocellulosic biomass in compounds and products through biochemical breakdown. Two distinct processes may be used: bioconversion by living agents or by enzymes (either free or immobilized) that do not need to consume the substrate to reproduce and maintain their existence, transforming specific molecules/polymers into desired compounds. Depending on the microorganism and the desired product and deconstruction pathways, several lignocellulosic bioprocessing strategies can be envisaged, including the production of biogas (mostly CH_4 and CO_2) through anaerobic treatment, composting, mushroom cultivation or fermentation [152,153].

Direct bioconversion from biomass without any prior treatment usually yields low conversions and requires very long periods.

- Enzymatic hydrolysis- enzymatic hydrolysis is mostly used for two purposes: 1) targeting the lignin polymer to degrade it, either to facilitate the subsequent enzymatic hydrolysis of the carbohydrate polymers or for other purposes (e.g. pulp delignification, lignin waste water treatment) [154,155]; 2) targeting the sugar polymers (hemicelluloses and cellulose) by hydrolyzing them into their respective monomeric constituents. In both cases, there is always the need of specific enzymes cocktails to fully deconstruct each polymer, being impossible to degrade any of these natural polymers in just one step [156,157].

  When lignin degradation is the focus, ligninases are the necessary enzymes, composed mainly by laccases and ligninolytic peroxidases (lignin- LiP, manganese- MnP, versatile- VP and dye-decolorizing- DyP-type peroxidases) obtained in most cases from fungi (more specifically white-rot fungi), although some bacteria also show lignin degradation enzymatic capacity [158]. Peroxidases also need to be assisted by oxidase enzymes to produce the peroxide or by several other enzymes (e.g. dehydrogenase).

  If the target is hemicelluloses, the enzymes are completely different with the main purpose to hydrolyze the glycosidic bonds between the monomeric moieties. Since hemicelluloses are diverse depending on raw material, the optimized combination of enzymes must account for

that: hardwood needs more xylanases, β-xylosidases, xyloglucanases and acetylxylan esterase while for softwood mannanases and beta-mannosidases are more relevant [159].

When hydrolyzing cellulose to glucose, cellulases require sets of enzymes to target different components of this homopolymer, being composed by endo-glucanases (randomly hydrolyze β-1,4-glycosidic bonds within the polymer, creating two end-chains), exo-glucanases or cellobiohydrolase (break the end-chains into cellobiose units) and β-glucosidase (hydrolyze cellobiose to two molecules of glucose) [157], as seen in Figure 5. Cellobiose is a strong inhibitor of endo and exo-glucanases which makes the β-glucosidase fundamentally necessary if the enzymatic hydrolysis of cellulose is to occur.

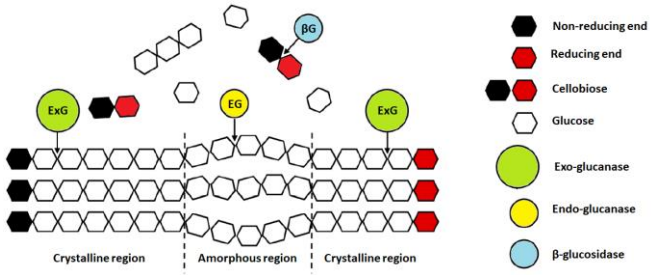


Figure 5. Scheme of cellulose enzymatic hydrolysis to glucose (adapted from [160]**)**

Variations in the composition of hemicelluloses such as those occurring between softwoods and hardwoods lead to different results if the same enzyme combo is used, and while cellulose is roughly the same regardless of species, its crystalline/amorphous ratio, degree of polymerization and accessibility can drastically change and affect the outcome of each specific biomass hydrolysis. Besides biomass variability, each enzyme optimal conditions also need to be taken in consideration (pH, stabilization agents, temperature) making the enzyme cocktail choice even more complex.

Additionally there is also the inhibitory effect of certain compounds derived from biomass degradation. Phenolic compounds derived from extractives and lignin are known to inactivate cellulases and xylanases [84,161], and degradation products from hemicelluloses and cellulose such as furfural, hydroxymethylfurfural, acetic acid, formic acid among others are also highly detrimental if above certain concentrations [162].

Although very interesting for biomass deconstruction, enzymatic hydrolysis still has to overcome some of its drawbacks (enzymes cost and lifetime, slow reactions, low solid loads) in order to be competitive at industrial scale.

Several studies have focused on the use of barks as feedstock for enzymatic hydrolysis after pre-treatments due to their availability and potential sugar source for bioethanol and other bioconversion molecules [84,163].

- Fermentation- is the biochemical breakdown of a compound or group of compounds by microorganisms like yeasts, bacteria, fungi and algae [151]. Although originally referring solely to anaerobic biotechnological processes for production of ethanol, acetone, isopropanol, butanol, ethanol or lactic acid, among others (by yeasts and bacteria), the term has spread to embark also aerobic processes such as acetic acid, citric acid, single cell proteins (yeast and bacteria), polyhydroxyalkanoate (PHA) which are a family of polyesters with more than 150 monomers that can be used for multiple purposes among which bioplastics, single cell oils from algae, fungi, bacteria or yeast to produce biodiesel or chemicals [164–166].

  Microorganisms feed on the sugar rich substrate (in the present case the sugar moieties obtained by hydrolysis of the lignocellulosic polysaccharides) and through specific biological pathways can convert them into a myriad of products. The number of compounds that can be synthetized by microbes is ever-growing especially due to genetically engineered strains that are modified to produce the desired compound at higher yields than normal while at the same time trying to eliminate, if possible, by-products formation (reducing separation and purification steps) and increasing the range of substrates to be used as feeding source [165]. From bioplastics to biofuels (ethanol, butanol, jet-fuel alka-(e)nes) [167], from pharmaceutical to flavoring compounds, from high volume-low value to high value-low volume compounds, the fermentation pathway (aided by genetic engineering, new reactors and processes) coupled to other biomass treatments is becoming more and more enticing, although there is still a long way to reach feasible and economically sound industrial production (except in a few cases such as ethanol and lactic acid) due to production costs and technical drawbacks.

  Although having lower polysaccharides content than wood or energy crop plantations, due to their low cost and availability barks have been forecast as potential feedstock for fuel and building blocks production through bioconversion methods, with abundant research focused on their use [122,166,168,169].

Each specific biomass characteristics and final products envisaged will determine the possible deconstruction pathways. More than one treatment can be combined to achieve the desired fractionation and end-product.

Case studies: *Picea abies* and *Eucalyptus globulus* barks

Within the bark biorefinery several species are considered enticing for their unique characteristics, whether due to specific physical characteristics, chemical composition or due to availability and expected low cost exploitability. Do to their large generation at sawmills and pulp mills, *Picea abies* (softwood) and *Eucalyptus globulus* (hardwood) barks show upgradable possibilities with no additional investment regarding handling, transportation and storage being required [72]. Of course the energetic input derived from the burning of these materials (their present use) would have to be met from other sources, while the main production line (timber or pulp) nor its products quality could be handicapped and the profitability of the new production hub would have to be financially enticing and technologically feasible.

Picea abies

Commonly known as Norway spruce, *Picea abies* is an evergreen coniferous tree from the Pinaceae family, and one among the 35 species of the genus, populating the temperate and boreal northern forests. It is one of Europe's most economic relevant species, with its wood being widely used for pulp and paper production, timber construction and furniture.

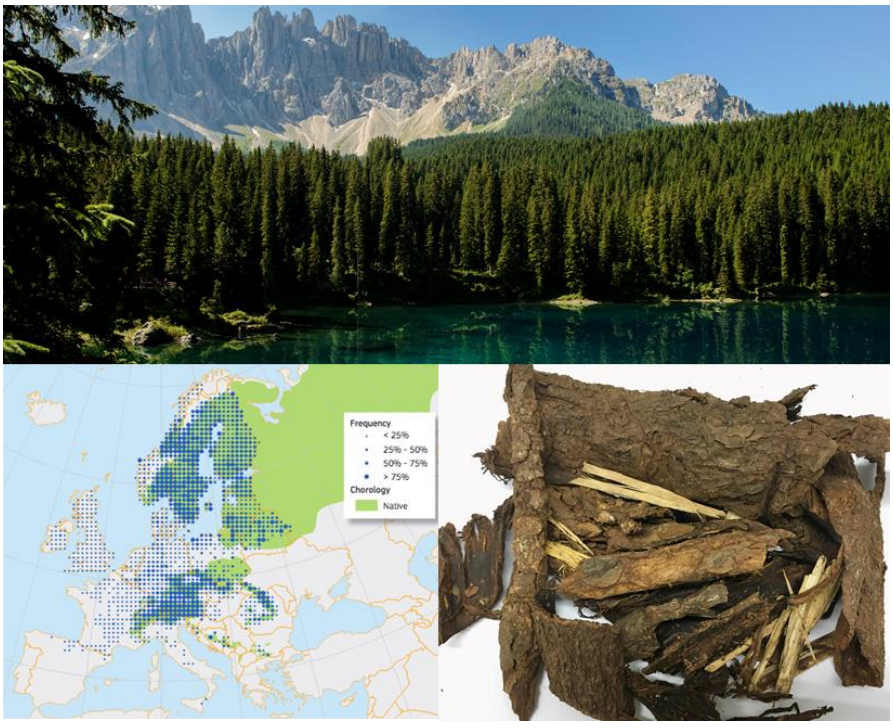


Figure 6. *Picea abies*: forest in Bolzano, Italy (top picture, copyright Son of Groucho, www.flickr.com: CC-BY); species distribution in Europe (bottom left) [170]**; industrial residual bark (bottom right)**

The bark (10-12% of the trunk diameter) [171] is removed from the bole upon debarking in the industrial sites and used as solid fuel for energy production through direct burning. According to the Swedish Forest

Agency [172] and the Natural Resource Institute of Finland [173] (2012 and 2018 respectively, and assuming a similar consumption for Sweden for the following years), the combined *Picea abies* debarked wood consumption by the industry was around 60 million m^3 yearly, meaning that (considering 12% bark average bole volume) around 8.2 million m^3 of bark was produced, or a theoretical weight annual value of 3.1 million tons (considering an average dry bark density of 380 $kg.m^{-3}$) [174]. The total *P. abies* bark production in Europe is surely higher since this species is used in wood industries in several other countries, as seen by the large distribution over Europe (Figure 6).

The chemical and physical characterization of *P. abies* bark has been extensively studied, considering the whole bark, focusing on inner and outer bark, or on the bark variability depending on the felling season. Table 3 shows some of the chemical characterizations of the different bark raw materials. The suberin content is below 2% [78] and was omitted in most of the studies. The values show a wide intra species variability, and although some can be explained by the use of different determination procedures, it is clear that this raw material is heterogeneous.

Overall this bark shows high apolar extractives (up to 8%), polar extractives (up to 30%) and lignin content (up to 35%) with low polysaccharide content (lower than 56%), rich in pectins (high galacturonic acid-GalA). Glucose content is low (below 41%) and highly dependent of bark fraction (much lower in inner than outer bark).

Table 3. Chemical composition of *Picea abies* bark in the literature.

	Ash	Apolar Ext.	Polar Ext.	Lignin	Polysacc	Glc	Man	Gal	Rha	Xyl	Ara	MeGlcA	GalA	Ref
F	5	7	19	26	41	-	-	-	-	-	-	-	-	[78]
M	3	4	17	24	41	27	4	2	0	4	4	-	-	
C	3	5	17	28	43	-	-	-	-	-	-	-	-	
winter	3	3	-	34	51	31	3	2	1	3	4	1	7	[175]
summer	3	2	-	37	48	28	2	2	1	4	5	1	6	
whole	3	28		17	51	-	-	-	-	-	-	-	-	[176]
inner	-	1	19	15	56	34	1	2	1	3	5	1	6	[42]
outer	-	2	6	35	36	17	2	2	1	5	4	1	5	
inner	-	2	30	3	56	41	2	3	1	3	7	-	-	[177]
outer	-	8	23	35	28	17	3	2	0	3	3	-	-	
inner	-	32		12	51	-	-	-	-	-	-	-	-	[178]
outer	-	16		33	49	-	-	-	-	-	-	-	-	

Particle size: F- fine (<0.180 mm), M- medium (0.250–0.450 mm), C- coarse (>2 mm)

This bark shows only 9 $MJ.kg^{-1}$ net calorific value at typical moisture content [174], even if the higher heating values are around 20 $MJ.kg^{-1}$ [176,179] meaning that the energy obtained is quite low unless the bark is oven-dried, thereby making its use for energetic purposes of low efficiency. This is probably one of the reasons for the intense research on alternative uses for this residue.

When used fresh, *P. abies* bark shows phytotoxic behavior toward seedlings, causing severe plant growth inhibition, which prevents its use as growing media [180]. Nevertheless, if composted, the bark appears

to lose its inhibition behavior, possibly becoming an interesting media for potted plants [181]. It has also been tested as sorbent for metal ion removal from aqueous solutions, proving to be an inexpensive source with potential for waste water treatment [182].

Miranda et al. [78] searched for a possible mechanical fractionation pathway that could lead to chemical different granulometric fractions as a first approach in the biomass deconstruction process, finding small decreases in extractives and ash content and small increment in lignin and polysaccharides between fine and coarse sized particles. Biogas after steam pretreatment and enzymatic hydrolysis was tested in *P. abies* forest residues with 8% bark with good results, although lower than if only wood was used [183]. Bio-oil production from pyrolysis of *P. abies* pulp and paper industrial residues was also tested (although it was not clear if including bark residues), showing high percentage of aromatic hydrocarbons and possible potential for fuel production [129]. Several other papers focus on bio-oil production for fuel oil from softwood barks, although none was found specifically for this species [184,185]. Regarding the polysaccharide fraction of this bark, several studies attempted glucose or ethanol production showing that, although possible, there are some drawbacks, mostly related to the recalcitrance and the somewhat lower polysaccharides content of *P. abies* bark [84,169,186,187]. Other studies focused on cellulosic and non-cellulosic components of *Picea* abies bark for cellulose fibers, nanocrystals and nanocomposites film formation [175,188–190]. Lignin has received little or no attention except as the recalcitrant fraction to remove or degrade for better performance applications regarding polysaccharides. Nevertheless its constitution, monomeric composition and possible structure were studied, pointing to a mostly G (guaiacyl) monomeric structure similar to respective wood [177,191].

The component that outsingles bark from other lignocellulosics is the extractive fraction, with most of the research focusing in it. Many studies dealt with different extraction methodologies by soxhlet, accelerated solvent extraction, acid assisted extraction, deep eutectic solvents or microwave assisted extraction [124,192–195]. Others studies targeted the composition of specific fractions (polar, apolar, tannin, phenolics, stilbenes) [175,196–199].

Since these extracts have biological activity [92,200], crude extracts, specific fractions or single compounds obtained from this bark have been tested against human pathogenic microbial [201–203], for plant fungal inhibition [204–206], as antioxidants [207,208], anti-leukemia [209], and for their immunomodulation activity [210] and antitumor activity [211].

The high content of apolar extracts of *P. abies* bark also led to its testing as raw material for production of tall oil through the recovery of its fatty and resin acids for fuel and chemicals production [212]. The recovery of polyphenols (mainly tannins) was also proposed for rigid and homogeneous tannin/furanic foams or in the formulation of adhesives for fiberboard [213–216].

Eucalyptus globulus

Eucalyptus globulus, also known as blue gum, is probably the industrially most important eucalypt species in the European Mediterranean zone, especially in Portugal, Spain, Italy, and to a lesser degree, in France (Figure 7). The easy pulping and the pulp and paper quality has promoted its cultivation as a pulpwood species.

Figure 7. *Eucalyptus globulus*: managed forest in Portugal (top picture, copyright J.R.Pinho: CC-BY); species distribution in Europe (bottom left) [217]**; industrial residual bark (bottom right)**

Bark represents 11-15% of the bole on a mass basis [54], and it is generated at industrial sites at a slightly higher proportion than a fifth of the bleached kraft pulp production [47], equivalent to roughly 124 000 tons annually for a large Portuguese pulpmill (500 000 pulp tons/year) [218]. According to CELPA [219], the annual industrial site production of bark in Portugal should be around 500 000 ton (considering a total pulp production of 2592 million tons, and taking into account that a fraction does not generate bark since it results from imported woodchips). The bark is used as solid fuel, burned in boilers on site, for electricity and heat generation. The energy produced from the bark accounts for roughly 13-14% of the total generated by biofuels (70% off all energy produced) within this industry, with black liquor representing almost the remaining 85% [219]. It is worth noting that the electric production within the pulp industrial complexes (obtained mostly from the burning of bark and black liquor) far exceeds its internal needs (3.44 TW.h produced vs 2.62 TW.h consumed), meaning that the main production process of pulp would not be hindered if part or all of the bark is processed for other purposes.

Being such an important residue, the physical, chemical and fuel characteristics of eucalypt bark have already received some attention, showing that bark is chemically similar to the respective wood, although

with a higher inorganic and extractives content and lower polysaccharide content. Table 4 presents some of the literature values obtained for the chemical composition of this bark.

Eucalyptus globulus bark can present very high inorganic content although most of it is probably due to exogenous contaminants that are easily embedded in its fibrous structure upon debarking, handling and storage. The extractives are higher than in the respective wood but compared to other species they are relatively low, especially the apolar extractives. The lignin content is similar and sometimes lower than in the respective wood, while polysaccharides content is quite high for a bark. Hemicelluloses are mainly glucuronoxylans.

Table 4. Chemical composition of *Eucalyptus globulus* bark in the literature (% as dry weight)

	Ash	Apolar Ext.	Polar Ext.	Lignin	Polysacc	Glc	Man	Gal	Rha	Xyl	Ara	MeGlcA	GalA	Ref
whole	4	1	-	22	69	-	-	-	-	-	-	-	-	[220]
whole	5	0	14	19	61	43	1	2	-	13	2	-	-	[221]
F	23	3	9	29	64	-	-	-	-	-	-	-	-	
M	16	1	6	29	53	37	1	2	-	12	1	-	-	[80]
C	4	1	5	22	70	-	-	-	-	-	-	-	-	
F	68	23		-	-	-	-	-	-	-	-	-	-	
M	24	21		12	79	-	-	-	-	-	-	-	-	[222]
C	20	17		13	84	-	-	-	-	-	-	-	-	
whole	2	1	5	19	72	50	0	2	0	12	2	2	3	[223]
whole	2	1	5	19	74	-	-	-	-	-	-	-	-	[224]

Particle size: F- fine (<0.180 mm), M- medium (0.250–0.450 mm), C- coarse (>2 mm)

The energetic value of bark is much lower than that of wood, with higher heating values (HHV) of 13 and 18 $MJ.kg^{-1}$ respectively [225], and lower basic density of 473 vs 567 $kg.m^{-3}$ [226]. The bulk density of milled bark is very low and almost independent on particle size (average of 169 $kg.m^{-3}$) [80]. These characteristics point to the inefficiency of eucalypt bark regarding its use for energy production.

Numerous studies addressed the viable upgrade of eucalypt bark in a biorefinery context. While most studies focused on the whole bark, a few considered the deciduous outer bark that could be collected through the natural shedding without felling the tree, or the inner and outer bark separately [227].

This bark may be used as substrate after composting with good results especially when mixed with pine bark [228] or as additive to growing media after hydrothermal treatment [229] aiming at substituting peat and avoiding its environmental negative impacts.

It was also tested as bio-sorbent for heavy metals such as uranium or chromium (for example to clean industrial waste waters) through adsorption and immobilization instead of more expensive activated carbon [230,231].

Torrefaction leads to better fuel properties by increasing the energetic density of the bark, its hydrophobicity and grindability [225], while pyrolysis of bark and residues including bark were also tested

to produce bio-oils similar to others produced with different biomasses [130,232]. Bio-oil production was also made via acid liquefaction (diethylene glycol, 2-ethylhexanol (2-EH) and p-toluene sulfonic acid) reaching up to 92% conversion yield at mild temperature conditions [233]. These bio-oils can be used for fuels, chemicals or energy production. Liquefied *E. globulus* bark was also applied as partial substitute (20%) of melamine-urea-formaldehyde resin for particleboard bonding [234]. Tannins recovered by extraction were used with softwood kraft lignin in "greener" adhesives formulations although other species seem to be more interesting than *E. globulus* [214]. The use of phenols extracted from eucalypt bark to partially substitute phenol derived from fossil fuels in phenol-formaldehyde adhesives also showed poorer result when compared to other species mostly due to low yields and tannin concentration [235].

Concentrated water extracts (obtained with or without catalyst) contained tannins and polyphenols that were tested as retanning agents, revealing good aptitude in leather retanning equivalent to commercial extracts of chestnut [236].

Due to its high polysaccharides content and fibrous nature, *E. globulus* bark was tested as raw material for pulp production to increase the raw-material fiber feedstock in a whole-tree pulping paradigm. The incorporation of bark with wood resulted in somewhat lower pulping performance regarding yield, delignification and pulp strength properties [237]. Neiva et al. [224] showed that optimized conditions for the delignification of bark and wood are different.

The lignin fraction of eucalypt bark has been studied with different methods and targets. Costa et al. [238] showed that organosolv or mild delignification processes are preferable if the resulting lignins are to be applied for production of functionalized aldehydes. Pre-treatment of bark with highly selective amine-sulfonate ionic liquids permitted the fractionation of a lignin rich liquid stream and a solid residue enriched in polysaccharides more prone to enzymatic hydrolysis degradation [122]. Matsushita et al. [222] reported that a hydrothermal pretreatment with carbon dioxide also increased the enzymatic hydrolysis to produce ethanol from the inner bark of *E. globulus*. Nevertheless, the studies on the possible utilization of both polysaccharides and lignin fractions seem to fall short on the potential of this species.

The extractives are the most studied component of eucalypt bark, even though their content is not very high, at least when compared to many other barks. Both lipophilic and polar extracts have been obtained from inner, outer and whole bark [38,239], at different heights and parts of the tree [45,240], through several techniques and solvents, and were chemically characterized [239,241–244], while the respective extractions were modeled and optimized towards specific fractions or compounds [245–247]. One of the most interesting class of compounds observed in this bark are the triterpenoids (e.g. β-sitosterol, β-amirine, oleanolic acid, betulinic acid, ursolic acid) contained in the apolar extract fractions, obtained either with dichloromethane, n-hexane or through supercritical extraction with CO_2/ethanol. These compounds show interesting biological activities such as antitumor, anti-HIV, antibacterial and anti-inflammatory possibly justifying their highly profitable recovery even if at a low yield [47,248–251].

Several polar and apolar crude extracts (obtained with several solvents) and compounds obtained from them showed antioxidant [45,221,252,253], antiproliferative potential of human breast cancer cells [218,223], and antimicrobial activity [45,254,255]. Jutakridsada et al. [256] propose the extraction of antioxidant compounds and the burning of the extracted bark, showing that the fuel properties were not significantly altered.

E. globulus bark extracts were tested for biosynthesis of gold nanoparticles, as "green synthesis" of specific nanostructures through reduction of soluble metal salts precursors, selectively producing nanoparticles with specific geometry and dimension and avoiding the use of detrimental chemicals [257].

Original research

The following section of the thesis regards the original research developed along the PhD program.

The objective of the work was to find alternative uses for the large amounts of bark residues produced in sawmills and pulp mills, focusing on two of the most intensively industrially used species. The first step was to collect the industrial barks from a Portuguese pulpmill and a Finnish sawmill (*E. globulus* and *P. abies* respectively) since the target was the raw material that exists in the industrial sites and not the virgin bark directly collected from the tree stems.

Bark is per se a complex material with highly differentiated tissues and cells, but it becomes more heterogeneous when collected at the industrial site mostly due to contamination from handling and debarking processes (e.g. wood and exogenous debris). Since the objective is to selectively deconstruct this biomass aiming at its full potential utilization, the original material characteristics must be very well known to properly choose the most adequate fractionation pathways.

The thesis is divided in four connected tasks.

The first task regards the characterization of each biomass (*E. globulus* and *P. abies*). The first and second papers focus on the industrial barks and test if it is possible to obtain chemically different fractions through particle size reduction by a simple mechanical process. Before fractionation, the bark was manually stripped from wood contaminants and all the fuel and chemical determinations were applied to each of the bark fractions and to wood (wood was used for comparison, being a much well known and studied material). The amount of information gathered and the extensive analysis lead to present the results of each bark in individual papers.

The third paper of the first task was focused on the fine characterization of the bark lignins. “Milled Wood Lignin” of both species was obtained through the classical Bjorkman procedure [258] and several analysis were made to determine lignin inter-unit linkage, end-groups and aromatic units: analytical pyrolysis (Py-GCMS), pyrolysis in the presence of tetramethylammonium hydroxide (Py-TMAH) , derivatization followed by reductive cleavage and modified methodology (DFRC and DFRC’), two-dimensional heteronuclear single-quantum coherence nuclear magnetic resonance (2D-HSQC-NMR). *Eucalyptus globulus* bark lignin presented characteristics very similar to those of the respective wood, but *Picea abies* bark presented data (from DFRC, Pyrolysis and 2D-HSQC) that showed some never before observed lignin monomers. The positive identification of those new lignin monomers was achieved through several different techniques and comparison to authentic standards proving that hydroxystilbene glucosides (mostly isorhapontin and at lower quantities astrigin and piceid) are true lignin monomers. The originality of this discovery merited the individual publication of the results in the fourth paper.

Having determined the chemical composition of both raw materials, the prospect of using the *E. globulus* industrial bark for bleached kraft pulp (BKP) production was tested in the second task, mostly due to its fibrous nature, relatively high cellulose and hemicelluloses contents and low lignin and extractives (when

compared to other barks). Based on previous work on bark pulping optimization [224], and knowing the detrimental effect that extractives and ash have on pulping and pulp characteristics, an alternative process was tested using hot water treatment under mild conditions prior to pulping to reduce these components in the biomass. Both untreated and treated barks were delignified with two different chemical charges and later bleached and refined up to 4500 rev. Morphological properties of unbleached and bleached (unbeaten and beaten) pulp fibers were measured and handsheets were produced from the pulps with their physical and mechanical properties determined to investigate the possible improvement (if any) of the hot water pre-treatment. The results were published in the fifth paper.

One of the characteristics that is commonly associated with barks is the high content in solvent extractable components, which normally is much higher than that of the respective wood. These easily obtainable crude extracts with composition depending on the solvent used contain compounds with diverse bioactivity, namely antioxidant and antimicrobial activities. In the third task, which lead to the fifth paper, four barks (among which are *E. globulus* and *P. abies*) were extracted with n-hexane, ethanol and water, and the crude extracts were evaluated for antioxidant, antimicrobial and anti-quorum sensing abilities. The extracted barks were used for further processing in task four. Several methodologies were applied to investigate the extracts antioxidant activity (ferric reducing antioxidant activity (FRAP), β-carotene bleaching test and free-radical scavenging assay) and regarding antimicrobial activity, disc diffusion essay and minimum inhibitory concentration (MIC) were tried against a large group of human pathogenic bacteria and yeasts. The objective of this third task was to determine the best solvents and most interesting crude extracts from several bark species, aiming at future recovery of antioxidant and antimicrobial compounds through simple extraction processes.

Since the extractives are known to be detrimental to several lignocellulosic biomass processes, among which to biological ones (e.g. enzymatic and fermentation), extractive-free *E. globulus* and *P. abies* barks were used as raw material (similar to those obtained from task three) in task four. The extractive-free barks were used as a source of fermentable sugars through enzymatic hydrolysis. Due to the recalcitrant nature of the lignocellulosic matrix, several auto-hydrolysis severity factors were tested to determine the most suitable conditions to separate the hemicellulosic sugars (in oligomeric or monomeric sugars) while enhancing biomass for subsequent enzymatic saccharification. The solid residues from the auto-hydrolysis were enzymatically hydrolyzed with two different commercial enzyme cocktails to determine their viability as substrates for production of monosaccharides (mainly glucose) through depolymerization of the cellulose and remaining hemicelluloses. Autohydrolysis followed by saccharification was tested to ascertain the possibility of selectively separating a rich stream in hemicellulosic oligosaccharides, a glucose solution derived from cellulose and a solid residue enriched in lignin.

The integrated scheme of the four tasks of this thesis (task one- orange; task two- yellow, task three- red, task fourth- purple) is presented in Figure 8. Following are the papers that compose the PhD thesis included in sequence, and formatted according to each journal.

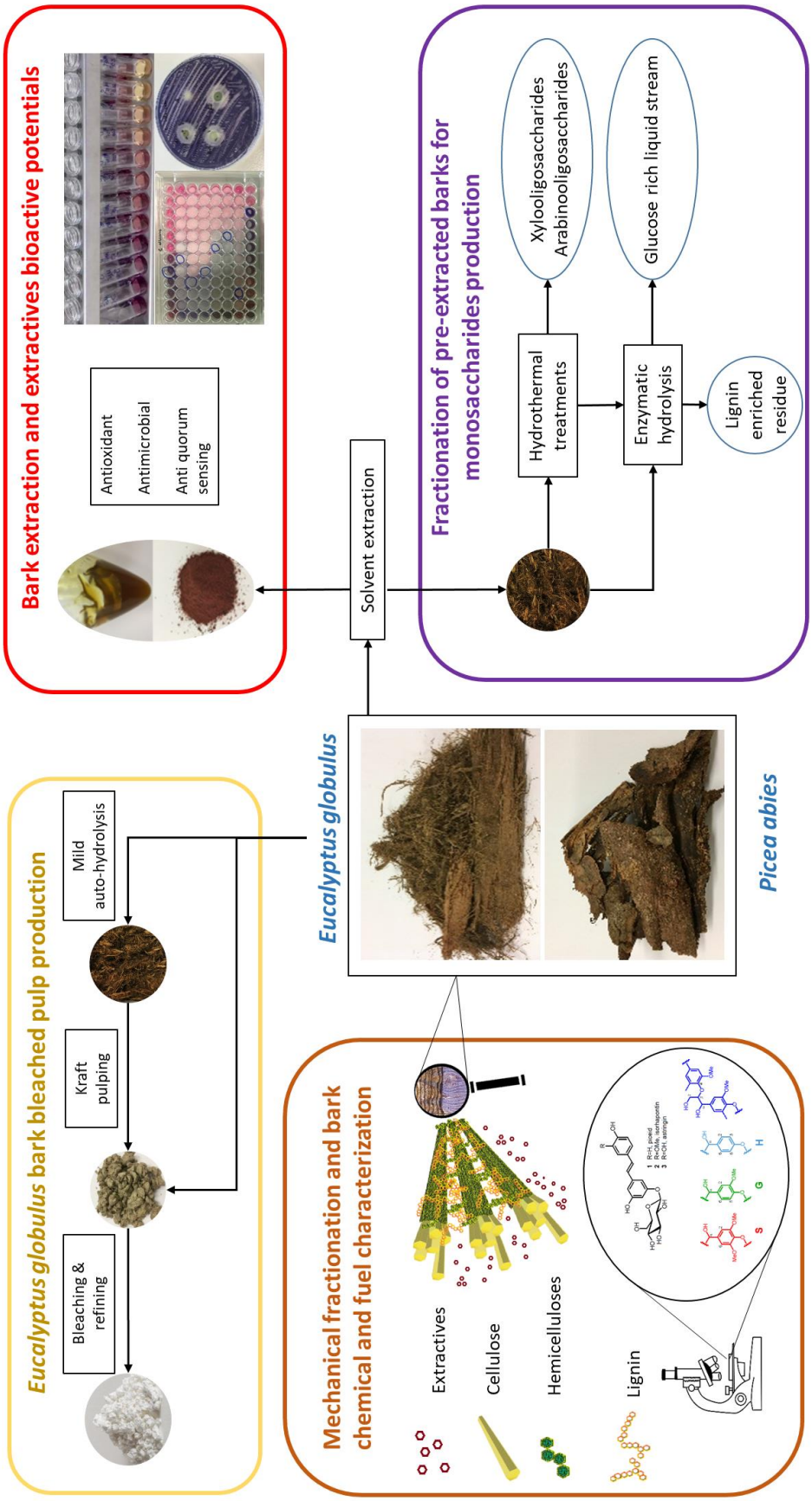
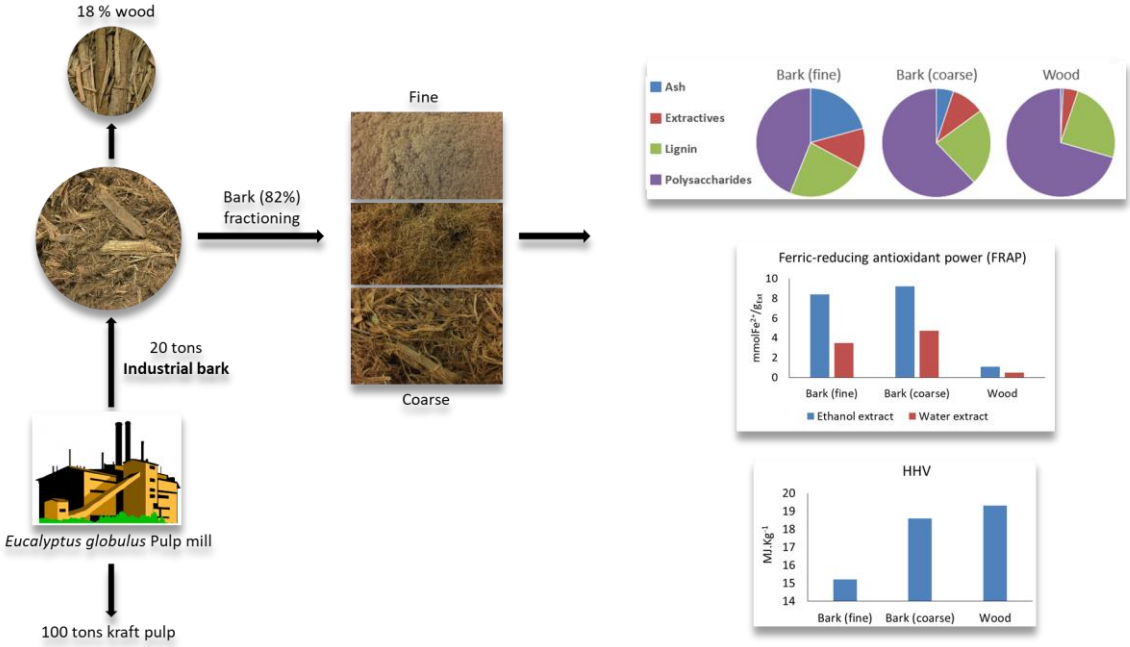


Figure 8. Thesis integrated scheme with four main tasks (task one- orange; task two- yellow, task three- red, task fourth- purple)

Paper 1:

Neiva, D.M., Araújo, S., Gominho, J., Carneiro, A.C., Pereira, H. 2018. Potential of *Eucalyptus globulus* industrial bark as a biorefinery feedstock: chemical and fuel characterization. *Industrial Crops and Products*, 123:262-270. https://doi.org/10.1016/j.indcrop.2018.06.070



Graphical abstract of the paper

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Potential of *Eucalyptus globulus* industrial bark as a biorefinery feedstock: Chemical and fuel characterization

Duarte M. Neiva[a,*], Solange Araújo[a], Jorge Gominho[a], Angélica de Cássia Carneiro[b], Helena Pereira[a]

[a] Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal
[b] Universidade Federal de Viçosa (UFV), Avenida Peter Henry Rolfs, 36571-000, Viçosa, Minas Gerais, Brazil

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ABSTRACT

Eucalyptus globulus bark is a residue from the pulp industry, traditionally used for energy production. This work aims at a more comprehensive knowledge of this industrial bark providing alternative possible uses based on its chemical and thermal characteristics. Bark and wood (18.3% of the total) were separated and bark was fractionated into fine (B_1, $\Phi < 0.180$ mm) medium (B_3, $0.450 < \Phi < 0.850$ mm) and coarse (B_6, $2 < \Phi < 10$ mm) fractions. B_1 showed a higher inorganic (21%), extractives (12.2%) and lignin (23.4%) contents than B_3/B_6 (3.7/5.1%, 8.9/9.8% and 21.6/22.8%, respectively) and much lower polysaccharide content (44% *vs* 63/62%). B_6 presented the highest contents of total phenolics (TFC, 271 mgGAE/g_{Ext}), flavonoids (FC, 106 mgCE/g_{Ext}) and condensed tannins (CTC, 65 mgCE/g_{Ext}) as well as antioxidant activities for Ferric Reducing Antioxidant Power (FRAP, 5.8 mmolFe^{2+}/g_{Ext}) and 2,2-diphenyl-1-picrylhydrazyl free radical scavenging assay (DPPH, 4.3 and 2.5 antioxidant activity index for ethanol and water extracts). B_3 and B_6 fractions showed similar proximate and ultimate analysis with higher High Heating Value (HHV close to 18 MJ/kg) and lower volatiles-to-fixed-carbon ratio (4.4) than B_1 (15.2 MJ/kg and 6.3 respectively). Bark has some detrimental thermal characteristics, such as high amounts of ash and chlorine (0.35%), presenting chemical features that point to their possible use in the food and pharmaceutical industries (high extractive content, phytochemical composition and antioxidant potential) or polysaccharides valorization (polyols, hemicellulose-derived oligomers, ethanol production).

1. Introduction

Barks are non-wood lignocellulosic forest products that are presently under increased scrutiny as a resource for value-added applications *e.g.* chemicals and bio-products, in addition to their already established use as solid fuel for energy and electricity production (Harkin and Rowe, 1971; Feng et al., 2013).

The underlying rationale for bark valorization is twofold. One relates to their large availability as byproducts or residues in wood-based industries where bark is removed from the tree logs before processing. The other derives from their intrinsic characteristics that include a large structural diversity and chemical richness, thereby allowing multiple product targeting and high potential value for biorefineries. However, this complexity requires a demanding and specific characterization at anatomical, chemical and physical levels to better determine the adequate routes to utilize their full potential. Bark differs chemically from wood with an overall higher proportion of ash and extractives. It varies between species and within each bark the composition depends also on the tissue *e.g.* inner and outer barks are distinct, especially in cork-rich barks (Sen et al., 2010; Leite and Pereira, 2017). The mechanical size reduction and screening yields fractions with different chemical and physical properties (Miranda et al., 2012a, 2013; Ferreira et al., 2015).

The industrial barks accumulated at mill yards have additional specific features that result from the harvesting, handling and debarking processes that may affect the feedstock composition *e.g.* presence of wood material, mineral and extraneous contaminations.

One important industrial bark is from the pulp mills processing eucalypt wood. Eucalyptus species are important raw-materials for pulp production and large scale plantations have been established with various species and hybrids. *Eucalyptus globulus* is the most widely cultivated in temperate regions, with a total global area estimated at around 2.3 million ha (Rejmánek and Richardson, 2011). It is also the most used species in the pulp and paper industries of the European southern countries, namely in Portugal, where the species is dominant covering 25.8% (8.12×10^5 ha) of the forest area (CELPA, 2016).

E. globulus wood has very good technological quality for producing

* Corresponding author.
E-mail address: duarteneiva@isa.ulisboa.pt (D.M. Neiva).

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high quality printing papers and it is well characterized (as reviewed in Pereira et al., 2010). The bark accounts for 11%–15% (oven-dry mass) of the bole (Quilhó and Pereira, 2001; Miranda et al., 2012b) which means that for each 100 tons of pulp produced, approximately 20 tons of bark can be generated in a pulp mill (Domingues et al., 2010). The bark residues are already incorporated in energy production by the pulp and paper industry *e.g.* in Portugal, they represented around 14% of the total energy produced from biofuels in 2015 (CELPA, 2016). However part of these bark residues can be directed to other uses if they prove to be more profitable.

The structure of *E. globulus* bark was described in detail (Quilhó et al., 1999, 2000) and several studies have determined its chemical composition whether in samples obtained from the industrial site debarking (Miranda et al., 2013; Neiva et al., 2014) or directly collected from the tree (Miranda et al., 2012b; Pereira, 1988). Overall *E. globulus* bark chemical composition show similarities with wood although with higher amounts of extractives and ash, and with lower carbohydrates contents and lignin (Miranda et al., 2012b, 2013; Neiva et al., 2014, 2016). The possibility of using the bark as a fiber source in pulping was already investigated (Miranda et al., 2012a,b; Neiva et al., 2016).

Eucalypt bark extractives also attracted attention with several studies focusing on the composition of apolar and polar fractions regarding the existence of bioactive compounds with pharmacological properties *e.g.* anti-inflammatory, antimicrobial, antibacterial, and probiotic properties as natural antioxidants (Kim et al., 2001; Santos et al., 2011; Domingues et al., 2011; Vázquez et al., 2008; Luís et al., 2014). Compounds included in the triterpenes family (*e.g.* oleanolic, betulinic and ursolic acids) show promising anti-tumoral activity among other properties (Li et al., 2002) while ellagic acid rhamnosides appear to be natural antioxidants (Kim et al., 2001).

Extraction with water and ethanol appears to be the preferable method to recover natural antioxidants due to their GRAS (Generally Recognized as Safe) status (Vázquez et al., 2012; Takeuchi et al., 2009).

The overall knowledge available for *E. globulus* bark shows its potential for various processing routes *e.g.* as a chemical source, fiber material or for thermochemical processing. However the studies available have focused on particular aspects of the bark and an integrative characterization is still lacking. This is particularly the case for the industrial bark stock as typically present in an operating pulp mill.

In this work we characterize the industrial *E. globulus* bark stock from a pulp mill yard in view of its use as a biorefinery raw-material. The wood content in the industrial bark was determined and mechanical fractionation of the bark into different sized particles was made. The fractions were evaluated in relation to chemical features including summative chemical composition and phytochemical profile of ethanol and water extracts (total phenolic compounds, tannins and flavonoids) and their antioxidant activity, as well as thermal properties including proximate and ultimate analysis, thermogravimetric analysis (TGA) and derivative thermogravimetric analysis (DTG). The valorization routes of this industrial residual stream are discussed under the concept of a full resource use (zero waste philosophy) and as a potential biomass source within a biorefinery concept, namely as a component unit within an integrative pulp biorefinery.

2. Material and methods

2.1. Sampling and fractionation

Industrial bark (100 kg) from *Eucalyptus globulus* was collected after the debarking from a pulp mill from The Navigator Company, located in Setúbal, Portugal. The samples were air dried for several days followed by oven drying at 40 °C for 3 days. After manual homogenization, the material was bagged and stored in several lots, from which random samples were taken for further analysis.

The industrial bark was separated manually in wood (W) and bark (B) fractions by visual sorting. The bark fraction was knife-milled with a Retsch SM 2000 mill to pass a 10 × 10 mm screen (B) and sieved to six mesh sizes fractions: B_1 (mesh < 80, $\Phi < 0.180$ mm); B_2 (mesh 60/80, $0.180 < \Phi < 0.250$ mm); B_3 (mesh 40/60, $0.250 < \Phi < 0.450$ mm); B_4 (mesh 20/40, $0.450 < \Phi < 0.850$ mm); B_5 (mesh > 20, $0.850 < \Phi < 2$ mm); B_6 ($2 < \Phi < 10$ mm). Each fraction was weighed, humidity was determined and the yield calculated as oven-dryo.d. mass.

The samples B_1 (fine), B_3 (medium) and B_6 (coarse) were used for analysis, as well as the unsieved B sample that represents the bark as a whole, and the wood (W). To decrease the potential influence of particle size throughout the analyses, samples B_6 and B were milled to pass a 1 mm ouput sieve and the entire material obtained was used.

The wood sample was milled and the 40/60 mesh fraction ($0.250 < \Phi < 0.450$ mm) was used for analysis.

2.2. Chemical analysis

Ash content was determined by TAPPI standard method T15 os-58. Extractives content was obtained through Soxhlet extraction successively with dichloromethane, ethanol and water during 16 h for each solvent. The extraction thimbles were oven-dried and weighed after each extraction, and the extractives contents were determined through the dry weight variation after each extraction. Acid insoluble (klason) lignin and soluble lignin were determined in the extractive-free material according to TAPPI standard methods T222 om-88 and UM250 om-83 respectively. Ash content in the insoluble lignin was determined and deducted from the lignin content. The polysaccharides composition was determined in the hydrolysis liquor obtained from the lignin determination: the content of neutral monosaccharides, glucuronic acid and galacturonic acid was measured by separation through a Dionex ICS-3000 High Pressure Ion Chromatographer. For rhamnose, arabinose, galactose, glucose, glucuronic acid and galacturonic acid the column used was a Carbopac PA10 250 x 4 mm plus Aminotrap working at 25 °C with and eluent flow of 1 ml/min and a gradient flow as follow: 0–20 min 18 mM NaOH; 20–34 min 50 mM NaOH + 170 mM. For xylose and mannose the column used was Carbopac SA10 250 × 4 mm plus Aminotrap working at constant temperature and effluent flow (40 °C and 1.2 ml/min). The acetates were measured in a Waters 600 with a Biorad Aminex 87H column 300 × 7.8 mm working at 30 °C with a constant eluent flow of 0.6 ml/min of 10 nN H_2SO_4 with a UV/Vis detector at 210 nm. All the chemical analysis were made in triplicate.

The mineral content in ash was obtained as follows: Cl by EN 15289:2011 standard; B by spectrophotometry (420 nm) after oven burning at 600 °C; the remaining elements were determined after nitroperchloric acid digestion followed by atomic absorption spectroscopy (Ca, Mg, Fe, Zn, Cu, Mn, Ni, Pb, Cr), spectrophotometry (P and S at 725 and 420 nm respectively) and emission flame photometry (K). The determinations were made in duplicate samples.

2.3. Phytochemical profile and antioxidant activity of ethanol and water extractives

Phytochemical profile and antioxidant activity were determined for the ethanol and water extracts obtained in the chemical analysis (successive extraction). The methodology for determination of total phenols (TPC), flavonoids (FC) and condensed tannin (CTC) contents is described by Ferreira et al. (2015). The results obtained for each individual extract are reported to the mass of the respective extract, while the total content of TPC, FC and CTC in both extracts was calculated as a weighted average of the values for each extract taking into account the amount obtained of each extract.

TPC was determined by the Folin-Ciocalteu method and results reported as mg gallic acid equivalents (GAE)/$g_{Extract}$ through a calibration curve obtained using the same methodology. FC was estimated by the aluminium chloride colorimetric assay. The absorbances were measured at 510 nm and results reported as (+)-catechin equivalents (CE)/

$g_{Extract}$ through a calibration curve obtained using the same methodology. CTC were estimated by the vanillin-H_2SO_4 method with results and reported as (+)-catechin equivalents (CE)/$g_{Extract}$ through a calibration curve obtained using the same methodology.

The antioxidant activity of the extracts was estimated through the ferric-reducing antioxidant power (FRAP) and the free radical scavenging activity (DPPH) methods. The determination of the free radical scavenging activity (DDPH) followed the method described by Sánchez-Moreno et al. (1998).

The FRAP results were expressed in mmol Fe(II)/g extract and compared to those obtained with standards (ascorbic acid, catechin and gallic acid). The DPPH results were expressed as IC_{50} (extract concentration required for 50% DPPH inhibition) and as the antioxidant activity index, AAI (Scherer and Godoy, 2009) (AAI = final concentration of DPPH in the control sample/IC_{50}) which takes in consideration the mass of DPPH and the test sample used eliminating the concentration effect of the DPPH solutions. The antioxidant activity of the extracts is classified as poor if AAI < 0.5, moderate if 0.5 < AAI < 1, strong 1 < AAI < 2 and very strong when AAI > 2. The extracts were compared to a natural (catechin) and a synthetic (trolox) standard.

All the analyses were made in triplicate in the ethanol and water extracts.

2.4. Thermal properties

Proximate analysis was determined according to ASTM standard test method E870-82. Fixed carbon was calculated as the difference required to achieve 100% after summation of ash and volatile matter oven dry percentages.

Ultimate analysis followed ASTM D5373-08 test method using a Perkin-Elmer II 2400 elemental analyzer (Shelton, CT, USA). Oxygen content was determined by difference after determination of C, H, N, and ash content.

The determination of the higher heating value (HHV) followed ABNT NBR 8633 standard using an adiabatic bomb calorimeter IKA300 (Staufen, Germany).

Thermogravimetric analysis (TGA) and derivative thermogravimetry (DTG) were carried in a Shimadzu DTG-60H (Kyoto, Japan) with dynamic nitrogen atmosphere (gas flow of 50 mL min^{-1}) over a temperature interval of 10–900 °C with a 10 °C min^{-1} heating rate, using 2 mg ± 0.1 mg samples in a platinum container.

3. Results

3.1. Fractionation

The *Eucalyptus globulus* industrial bark collected from the pulp mill contained conspicuous wood chips and large wood pieces that were sorted out. The wood content was 18.3% (m/m) of the industrial bark. The milling of the bark-material (B) contained in the industrial bark yielded a heterogeneous distribution of the different granulometric fractions: B_1 (Φ < 0.18 mm) 17.4%, B_4 (0.45 < Φ < 0.85 mm) 19.9%, B_5 (0.85 < Φ < 2.00 mm) 20.2%, B_6 (2.00 < Φ < 10.00 mm) 27.7% with B_2 (0.180 < Φ < 0.250 mm) and B_3 (0.250 < Φ < 0.450 mm) fractions presenting lower yields of 5.0% and 9.8% respectively.

3.2. Chemical composition

Table 1 presents the chemical composition of the wood, bark and bark fractions, regarding content of ash, extractives and lignin as well as the monomeric composition of polysaccharides (neutral sugars, uronic acids and acetates).

Bark as a whole (B) is chemically different from wood (W). It has roughly seven times more ash (5.4% *vs.* 0.7%), and more than double the extractives content (9.9% *vs.* 4.5%). Both materials have a low content of apolar extractives (below 1%) and the main differences are found in the content of polar extractives (8.9% *vs.* 4.1%). Ethanol extractives have a lower proportion in the total extractives of bark than of wood (24% *vs.* 32%).

Lignin content was lower for bark than for wood (21.9% *vs.* 24.3%). The polysaccharides content, as determined by the sugars and acetic acid content after acid hydrolysis, shows that wood has a higher overall content than bark (70.8% and 61.1% respectively).The monomeric composition showed that bark, compared to wood, has an overall lower glucose (37.5% *vs.* 44.4%) and xylose (15.2% *vs.* 18.0%) contents although their relative percentage of total sugars is very similar to wood (approximately 62% glucose and 25% xylose).

When comparing the three granulometric fractions, the finest fraction B_1 has a much higher ash content (21.0%) than B_3 and B_6 fractions (6 and 4 times higher respectively) as well as the highest content of extractives (> 12%), mostly due to water soluble compounds.

The coarse fraction B_6 differentiates from the other two fractions in the relative proportion of ethanol and water extractives, with higher percentage of ethanol and much lower water extractives (23% and 66% of the total extractives, respectively, as compared to approximately 15% and 78% for B_1 and B_3). Klason and soluble lignin values are very similar for all fractions at 18.6–20.0% and 2.8–3.5% respectively. Although different in absolute value, the polysaccharides composition is similar for all fractions with predominance of glucose (57–62%) followed by xylose (24–26%). Overall the summative chemical composition was able to represent almost 100% of the material for all the samples.

Table 2 presents the ash mineral composition. Bark has a higher mineral content than wood, especially of Ca (30 times higher), Fe (21 times higher) and Mn (4.4 times). Calcium is the most abundant mineral of bark, corresponding to 75% of the total minerals, while in wood it only accounts for 19%. Cl in bark more than doubles the amount found in wood (0.35% *vs.* 0.16%).

Most of the mineral elements determined were quite similar between fractions with exception for Ca and Fe for which B_1 showed a considerable higher value than B_3 and B_6 (6.3% *vs.* 1.1% *vs.* 2.4% respectively for Ca and 846 *vs.* 383 *vs.* 296 ppm for Fe). Ca represents 85, 47 and 70% of the total mineral composition for B_1, B_3, and B_6 fractions.

3.3. Phenolic content of ethanol and water extractives

Table 3 presents the total phenolic content (TPC), flavonoids (FC) and condensed tannins (CTC) and also the antioxidant activity of ethanol and water extractives obtained by successive extraction with both solvents. The total values were calculated as a weighted average of the values for both extracts taking into account the extract mass obtained with ethanol and water (*i.e.* the water extractives are obtained in higher amounts than the ethanol extractives, Table 1).

Overall, the ethanol extracts always showed higher contents of TPC, FC and CTC than the water extracts.

Bark as a whole (B) has a phenolic content similar to that of wood with 238 and 233 mg GAE/g_{Ext} respectively. B presented 5% lower phenolic content than W for the ethanol extract and 29% higher for the water extract. Flavonoids were found in lower concentration in bark than in wood (94 *vs.* 112 mgCE/g_{Ext}), mostly due to a much lower content in the ethanol extract (25% lower than in wood) since the content is higher in the water extract than in the respective wood extract. The content of condensed tannins is similar in bark and wood (59 and 60 mg CE/g_{Ext} respectively), even if bark shows higher content in both ethanol and water extractives.

The antioxidant activity (AA) measured through the FRAP method showed that bark (both water and ethanol extracts) has over seven times more antioxidant power than wood. The ethanol extract AA value was more than twice that of the water extract. The tested standards

Table 1
Summative chemical composition (% o.d.) of the wood (W), bark (B) and bark granulometric fractions (B_1 ($\Phi < 0.180$ mm), B_3 ($0.250 < \Phi < 0.450$ mm) and B_6 ($\Phi > 2$ mm)) of *Eucalyptus globulus* industrial bark.

	W	B	B_1	B_3	B_6
Ash	**0.75 ± 0.12**	**5.37 ± 0.17**	**20.97 ± 0.49**	**3.69 ± 0.52**	**5.13 ± 0.24**
Extractives	**4.45 ± 0.18**	**9.86 ± 0.13**	**12.20 ± 0.05**	**8.92 ± 0.66**	**9.83 ± 0.17**
Dichloromethane	0.31 ± 0.02	0.92 ± 0.01	1.04 ± 0.03	0.51 ± 0.02	1.04 ± 0.07
Ethanol	1.42 ± 0.13	2.34 ± 0.06	1.67 ± 0.13	1.45 ± 0.15	2.30 ± 0.13
Water	2.72 ± 0.25	6.61 ± 0.19	9.49 ± 0.10	6.96 ± 0.80	6.49 ± 0.16
Lignin	**24.32 ± 0.23**	**21.86 ± 1.05**	**23.44 ± 0.48**	**21.64 ± 0.28**	**22.83 ± 0.92**
Klason	19.90 ± 0.21	18.92 ± 1.11	19.94 ± 0.64	18.59 ± 0.29	20.05 ± 0.95
Acid soluble	4.42 ± 0.04	2.95 ± 0.06	3.50 ± 0.16	3.05 ± 0.02	2.78 ± 0.11
Polysaccharides	**70.76 ± 0.60**	**61.14 ± 1.63**	**44.31 ± 2.56**	**63.37 ± 1.57**	**62.14 ± 1.96**
Rhamnose	0.30 ± 0.01	0.46 ± 0.02	0.46 ± 0.02	0.40 ± 0.02	0.48 ± 0.03
Arabinose	0.58 ± 0.01	1.55 ± 0.06	1.52 ± 0.04	1.32 ± 0.04	1.76 ± 0.09
Galactose	1.55 ± 0.02	1.60 ± 0.04	1.60 ± 0.02	1.51 ± 0.04	1.65 ± 0.05
Glucose	44.43 ± 0.40	37.47 ± 1.66	25.25 ± 2.03	39.38 ± 1.11	36.75 ± 1.35
Xylose	18.00 ± 0.50	15.21 ± 0.2	10.64 ± 1.07	16.04 ± 0.47	16.32 ± 0.82
Nannose	0.93 ± 0.01	0.37 ± 0.01	0.56 ± 0.03	0.31 ± 0.02	0.35 ± 0.02
Galacturonic acid	0.85 ± 0.03	1.66 ± 0.07	1.94 ± 0.11	1.43 ± 0.14	1.76 ± 0.12
Glucuronic acid	0.12 ± 0.01	0.12 ± 0.02	0.12 ± 0.00	0.12 ± 0.01	0.14 ± 0.01
Acetic acid	3.98 ± 0.09	2.71 ± 0.06	2.23 ± 0.01	2.85 ± 0.03	2.93 ± 0.09
Total	**100.3 ± 0.2**	**98.2 ± 0.7**	**100.9 ± 2.3**	**97.6 ± 1.5**	**99.9 ± 1.3**

The bold values signifies the main constituents of the lignocellulose material (ash, extractives, lignin and polysaccharides). Each of these main contituents is determined as the sum of smaller different parcels (unbolded).

Table 2
Mineral composition of the wood (W), bark (B) and bark granulometric fractions (B_1 ($\Phi < 0.180$ mm), B_3 ($0.250 < \Phi < 0.450$ mm) and B_6 ($\Phi > 2$ mm)) of *Eucalyptus globulus* industrial bark.

	W	B	B_1	B_3	B_6
Ca (%)	0.10	3.32	6.34	1.11	2.40
K (%)	0.15	0.30	0.36	0.45	0.28
Cl (%)	0.16	0.35	0.30	0.37	0.36
P (%)	0.01	0.05	0.06	0.03	0.03
Mg (%)	0.06	0.21	0.21	0.18	0.19
S (%)	0.03	0.05	0.06	0.11	0.04
Cu (ppm)	1	4	4	6	4
Fe (ppm)	26	546	846	383	296
Zn (ppm)	2	10	14	16	8
Mn (ppm)	168	731	740	655	733
B (ppm)	23	23	23	17	16
Ni (ppm)	2	4	5	1	3
Pb (ppm)	2	2	3	2	3
Cr (ppm)	2	5	5	3	3
Total (%)	**0.53**	**4.41**	**7.49**	**2.36**	**3.41**

presented a higher AA efficiency (17, 18 and 40 $mmolFe^{2+}/g_{standard}$ respectively for ascorbic acid, catechin and gallic acid) well above the AA values of bark and woood extracts; the closest value was given by the bark ethanol extract (7.8 $mmolFe^{2+}/g_{Ext}$).

In the free radical scavenging activity method, the concentration of extractives required for 50% DPPH inhibition (IC50) was 6.4 and 10.4 mg/L for bark and 5.9 and 39.8 for wood (ethanol and water extracts respectively). All extracts were less effective than trolox and catechin standards (IC50 = 3.5 and 2.7 mg/L respectively). Wood water extractives had very low antioxidant activity, requiring 40 mg/L to inhibit 50% of the DPPH. The antioxidant activity index (AAI) was above 2 for all extracts with exception of the water extract of wood (0.6).

The extracts from the bark coarse fraction (B_6) were richer than those of the B_1 and B_3 fractions in total phenolics, flavonoids and tannins for both ethanol (439 mg GAE/g_{Ext}, 169 mg CE/g_{Ext} and 136 mg CE/g_{Ext} respectively) and water extracts (211 GAE/g_{Ext}, 83 mg CE/g_{Ext}, 40 mg CE/g_{Ext} respectively). The extracts from B_1 and B_3 fractions showed similar results for total phenolics (196 *vs.* 199 $mgGAE/g_{ext}$), flavonoids (26 $mgCE/g_{ext}$ both) and condensed tannins (39 *vs.* 35 $mgCE/g_{ext}$).

The variation of the FRAP antioxidant activity among fractions was small with B6 presenting the best results (4.3 *vs.* 4.0 *vs.* 5.8 $mmolFe^{2+}/g_{Ext}$ for B_1, B_3 and B_6 respectively). The free radical scavenging activity (DDPH) was higher for the ethanol extract of B_3 (7.1 mg/L) in comparison with the results of B_6 and B_1 ethanol extract (IC50 ± 5.5 mg/L). For the water extracts, the coarser fraction presented significantly better IC50 results: 9.4 mg/L as opposing 15.8 and 16.8 g/L for fine and medium fraction. All fractions showed lower inhibition of DPPH than trolox and catechin standards.

3.4. Thermal properties

Table 4 presents the results of the proximate and ultimate analyses, and the higher heating value. Bark as a whole (B) had 7% less volatiles and 11% more fixed carbon leading to a V/FC ratio 16% lower than wood. The largest difference occurred for ash with 7 times higher values in bark than in wood.

The N, C and H bark contents were very similar to those of wood. The largest difference was for oxygen content with bark showing 42% as opposed to 46% of wood. Both H/C and O/C ratios were higher for wood (1.52 *vs.* 1.49 and 0.74 *vs.* 0.69 respectively). The energy density expressed as higher heating value (HHV) of wood was 19.3 MJ/kg and 5% higher than that of bark.

Regarding the different bark fractions, B_3 and B_6 showed very similar values for both proximate and ultimate analysis parameters with B_3 showing a slightly higher O content (44.6% *vs.* 42.4%) and a similar HHV. B_1 presented a very much higher ash content (20.7 *vs.* 4.4%) and V/FC ratio (6.3) when compared to the other fractions (around 4.5). The HHV was substantially lower for this fraction (15.2 MJ/kg).

The TGA and DTG analysis were made between 40 and 680 °C. Fig. 1 shows the results for the more relevant 250–600 °C interval.

Wood and bark present a similar behaviour regarding heat degradation with the first signs of mass loss starting near 300 °C and the highest rate of mass loss at 368 °C and 409° respectively. DTG shows that bark degradation is different from that of wood. The volatilization of bark increases between 300–350 °C and then the mass loss rate starts to slow between 380–410 °C, after which it slows to a constant variation of 0.001 mg $°C^{-1}$. For wood, the constant slope appears before the

Table 3
Phytochemical profile (total phenolic compounds, flavonoids and condensed tannins) and antioxidant activity (FRAP and DPPH methods) of ethanol and water extractives of wood (W), bark (B) and bark granulometric fractions (B_1 ($\Phi < 0.180$ mm), B_3 ($0.250 < \Phi < 0.450$ mm) and B_6 ($\Phi > 2$ mm)) of *Eucalyptus globulus* industrial bark.

	Standard	W	B	B_1	B_3	B_6
Phenolics (mg GAE/g_{Ext})						
Ethanol		403 ± 29	385 ± 7	423 ± 52	386 ± 10	439 ± 21
Water		144 ± 9	186 ± 6	156 ± 1	159 ± 2	211 ± 9
Total		233 ± 5	238 ± 6	196 ± 7	199 ± 7	271 ± 3
Flavonoids (mg CE/g_{Ext})						
Ethanol		208 ± 15	153 ± 4	138 ± 11	117 ± 3	169 ± 9
Water		63.1 ± 3.1	73.5 ± 0.9	6.8 ± 0.2	6.9 ± 0.4	83.3 ± 3.7
Total		112 ± 2.5	94 ± 1	26 ± 2	26 ± 3	106 ± 2
Condensed tannins (mg CE/g_{Ext})						
Ethanol		115 ± 6	125 ± 3	78 ± 11	63 ± 5	136 ± 4
Water		32 ± 1	36 ± 2	32 ± 2	29 ± 3	40 ± 2
Total		60 ± 2	59 ± 2	39 ± 3	35 ± 1	65 ± 4
FRAP antioxidant activity						
Ethanol (mmolFe^{2+}/g_{Ext})		1.1 ± 0.2	7.8 ± 0.2	8.4 ± 0.6	7.5 ± 0.2	9.2 ± 0.4
Water (mmolFe^{2+}/g_{Ext})		0.5 ± 0.0	3.6 ± 0.3	3.5 ± 0.1	3.3 ± 0.1	4.7 ± 0.2
Total (mmolFe^{2+}/g_{Ext})		0.7 ± 0.1	4.7 ± 0.2	4.3 ± 0.1	4.0 ± 0.1	5.8 ± 0.1
Ascorbic acid (mmolFe^{2+}/g)	17 ± 0.4					
Catechin (mmolFe^{2+}/g)	18 ± 0.5					
Gallic acid (mmolFe^{2+}/g)	40 ± 1.0					
DPPH antioxidant activity						
Ethanol IC50 (mg/L)		5.9 ± 0.3	6.4 ± 2	5.5 ± 0.2	7.1 ± 0.1	5.4 ± 0.2
Ethanol AAI		4.0 ± 0.2	3.7 ± 0.1	4.3 ± 0.2	3.3 ± 0.0	4.3 ± 0.2
Water IC50 (mg/L)		39.8 ± 2.8	10.4 ± 0.5	15.8 ± 1.0	16.8 ± 1.4	9.4 ± 0.4
Water AAI		0.6 ± 0.0	2.3 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	2.5 ± 0.1
Trolox IC50 (mg/L) AAI	3.5 ± 0.2 6.7 ± 0.3					
Catechin IC50 (mg/L) AAI	2.7 ± 0.4 8.6 ± 0.7					

highest degradation point is reached. After 450 °C, both wood and barks show a constant rate of degradation.

Regarding the different bark fractions (Fig. 1 bottom), the most susceptible to thermal degradation appears to be B_3 presenting the point at which the mass loss is more prominent at 327 °C while for the fraction B_1 and B_6 it happens at 355 and 362 °C respectively. B_1 residual mass is 2.1 and 1.6 times higher than that of B_3 and B_6 respectively, with B_6 being 35% higher than B_3.

4. Discussion

4.1. Fractionation

The industrial bark showed a substantial amount of wood. This high wood quantity results from the debarking operations and wood processing requirements, given the detrimental effect that bark may have in the subsequent wood processing. The inclusion of significant amounts of bark in pulping for either paper pulp or for dissolving pulp leads to lower product quality and higher processing problems. For instance, bark needs harsher pulping conditions than wood which leads to more rejects and higher chemical consumptions (Brannvall, 2009; Neiva et al., 2014, 2016). The high calcium content of bark (30 fold higher than wood) will cause higher precipitation of calcium-oxalate which can be problematic for both equipment and final product (Häärä et al., 2011).

However the wood loss to the process caused by the debarking should also be considered, since 18% of wood in the bark stream will account for roughly less 2% in the wood supply, which represents a high amount when taking the total processed feedstock. The debarking process may be selected and adjusted so that the bark residues contain

Table 4
Proximate and ultimate analyses and high heating value (HHV) of wood (W), bark (B) and bark granulometric fractions (B1 ($\Phi < 0.180$ mm), B3 ($0.250 < \Phi < 0.450$ mm) and B6 ($\Phi > 2$ mm)) of *Eucalyptus globulus* industrial bark.

	W	B	B_1	B_3	B_6
Proximate analysis					
Volatiles (%)	83.8 ± 0.7	77.9 ± 0.9	68.5 ± 0.2	78.1 ± 0.2	77.7 ± 0.3
Fixed carbon (%)	15.4 ± 0.6	17.1 ± 0.8	10.8 ± 0.4	17.5 ± 0.5	17.8 ± 0.2
Ash (%)	0.8 ± 0.1	5.0 ± 0.1	20.7 ± 0.5	4.4 ± 0.4	4.4 ± 0.2
V/FC ratio	5.4 ± 0.3	4.6 ± 0.3	6.3 ± 0.2	4.5 ± 0.1	4.4 ± 0.1
Ultimate analysis					
C (%)	46.6	45.8	39.0	45.2	46.0
H (%)	5.9	5.7	4.5	5.7	5.7
N (%)	0.7	0.8	1.0	0.8	0.8
O (%)	46.1	42.3	34.5	44.6	42.4
Atomic H/C ratio	1.52	1.49	1.38	1.51	1.49
Atomic O/C ratio	0.74	0.69	0.66	0.74	0.69
HHV (MJ/kg)	19.3	18.4	15.2	18.0	18.6

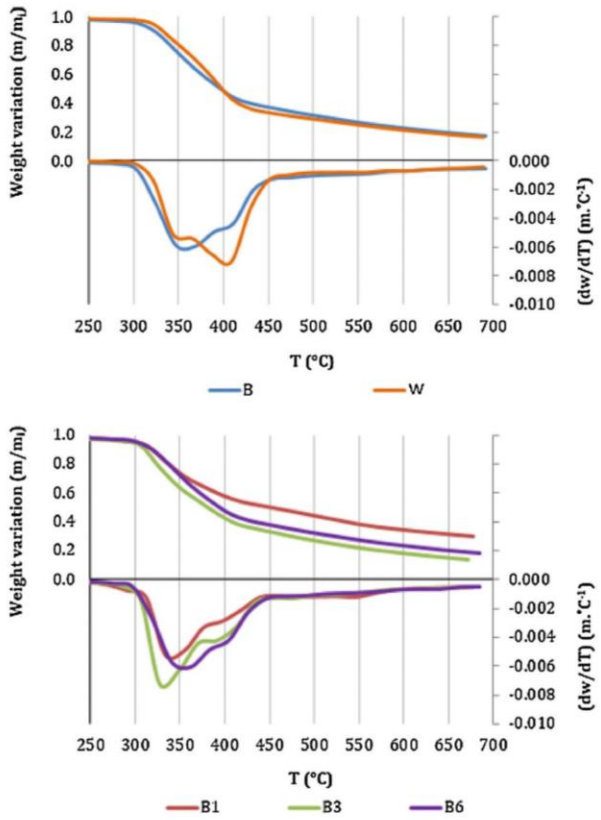

Fig. 1. Thermogravimetric analysis (TGA) and derivative thermogravimetric analysis (DTG) of wood (W) and bark (B) (top plot) and bark fractions B_1, B_3 and B_6 (bottom plot).

lower amounts of wood *e.g.* one Portuguese industrial site stated having as low as 3% of wood in the residual bark stream without decreasing the standards of the wood stream (pers. comm.).

The fractionation of the bark showed that this material produces a high amount of fines with no specific dominant fraction after sieving. Miranda et al. (2013) reported similar results with the fractions between 0.18–0.45 mm (B_2 and B_3) presenting a lower yield than the remaining fractions. The granulometric fractioning by milling depends on the bark structure and for other species the fines fraction is lower *e.g.* 2–3% oven dry mass for *Picea abies, Pinus sylvestris, Betula pendula* and *Pinus pinea* (Miranda et al., 2012a, 2013).

The bark of *E. globulus* shows higher friability leading to more fines production due to the high content in thin-walled parenchyma cells and brittle sclereids (respecting 50% *and* 7.3%) while long fiber bundles also form upon knife-milling due to a significant fiber content of 27.8% (Quilhó et al., 2000; Pereira et al., 2010). These features make bark more difficult to work with at industrial scale due to the entanglement of the long fibers in the machinery.

4.2. Chemical composition

The industrial bark has a much higher ash content than bark sampled directly from the tree due to mineral contamination by handling and storage at the mill yard. This has been shown previously *e.g.* by Miranda et al. (2013) who found 12.1% ash content in industrial *E. globulus* barks which is substantially higher than the value obtained in this study. Vázquez et al. (2008) and Neiva et al. (2016) reported similar values (4.7% and 3.5% respectively to the 5.4% ash content found here (Table 1). This suggests that the operations of log handling and subsequently of the residual bark transport and storage may be targeted towards a minimization of mineral contaminations taking into account that the bark collected directly from the tree stem has an ash content of 2.9% (Miranda et al., 2012b). Calcium is the major mineral element in the bark in part due to the calcium oxalate crystals present in the parenchyma cells of the bark and absent in wood (Pereira et al., 2010).

The chemical composition of bark is different from wood as known in general terms (Pereira et al., 2003; Fengel and Wegener, 1984) and specifically for *E. globulus* (Foelkel, 2011; Pereira et al., 2010).

Bark extractives content (9.9%, Table 1) more than doubles that of wood, in agreement with the reported range for bark extractives of 6.5–14% (Miranda et al., 2012b, 2013, Neiva et al., 2014, 2016, Vázquez et al., 2008; Pereira, 1988). Structural components of bark have lower contents in relation to wood when expressed in the summative chemical composition (Table 1) given the higher ash and extractives contents. However the polysaccharides/lignin, glucose/polysaccharides and xylose/polysaccharides ratios were identical in both materials (2.9, 0.62 and 0.25 respectively).

Bark showed a higher relative content in rhamnose, arabinose and galacturonic acid, which are major components of pectin, meaning that it probably has more pectin than wood. Similar results have been found before (Miranda et al., 2013; Neiva et al., 2016; Vázquez et al., 2008).

The fractions obtained by trituration and sieving showed chemical differences (Table 1). This is in relation with the structural features of the bark leading to specific grinding behaviour, as it has been shown for various species (Batista et al., 2013; Miranda et al., 2012a, 2017, Ferreira et al., 2015) including *E. globulus* (Miranda et al., 2013). The finest fraction had more than four times higher ash content than all others, corresponding to an increase in Ca and Fe in B_1 which seems to imply that at least part of these belong to exogenous materials, *e.g.* rocks and soil that contaminate the industrial bark.

The finest fraction also showed the highest content in extractives. This is a common occurrence reported for bark fractions of several species, including *E. globulus,* resulting from the accumulation in the fines of the more brittle cellular material of sclerified phloem and rhytidome (Freire et al., 2002; Miranda et al., 2013). This also explains the relative proportion of the structural components: the fines were enriched in lignin *e.g.* the polysaccharides/lignin ratio of B_1 was substantially lower than that of the other fractions (1.9 *vs.* 2.8). This tendency for the finest fraction of *E. globulus* bark to be enriched with lignin and depleted of polysaccharides was also previously reported (Miranda et al., 2013). Several other studies found that the fines are enriched in extractives and depleted in polysaccharides but the relation of lignin with particle size shows different variations for barks of different species (Miranda et al., 2012a, 2013, 2017; Bridgeman et al., 2007).

4.3. Chemical profile and antioxidant activity of extracts

Some phenolic molecules from plants act as natural antioxidants by preventing transition metal ions from initiating oxidation or by stopping oxidation chain reactions through donating the phenolic hydrogen atom to free radicals (Stasiuk and Kosubek, 2010). Besides their antioxidant capability, some of these compounds are also bioactive in many ways. The interest in these natural bioactive compounds is increasing given their possible end-uses for cosmetics, food additives and pharmaceutical products. Barks, with their high extractives content and compositional richness and diversity, are excellent raw materials for this purpose.

Bark and wood had similar contents of total phenols, flavonoids and condensed tannins (Table 3). The bark is however more interesting for extraction of these compounds because it has 2.2 times more polar extractives than wood.

Published results on the TPC of *E. globulus* bark in extracts obtained with different solvents (methanol, ethanol, water) and extraction methods (soxhlet, supercritical) show a wide range between 61 and 423

gallic acid equivalents per extract gram (Vázquez et al., 2008; Santos et al., 2011; Srivastava and Vankar, 2012; Lima et al., 2017). Luís et al. (2014) reported 263 GAE/g_{Ext} for wood and 253 mg GAE/g_{Ext} for stump bark ethanol extracts. Most of the values reported in the literature fall below those found here. Usually bark extracts have higher TPC than wood although for some species the opposite has been reported (Lamounier et al., 2012; Chang et al., 2001). Nevertheless, if reported to original material, bark has higher values than wood (21 *vs.* 10 mg GAE/g). Cadahia et al. (1997) found similar values of 14–23 mg GAE/g for bark extracts.

Wood extracts presented higher total flavonoid content (TFC) than bark. This also happened when comparing stump wood and stump bark of *E. globulus* (Luís et al., 2014). The flavonoid content of bark was lower than the 286.5 mgCE/g_{Ext} reported for ethanol/water extracts (Lima et al., 2017).

Comparing bark fractions, the coarse B_6 had higher total TFC, with 5 times more flavonoids and almost double condensed tannins than B_1 and B_3.

In spite of a similar phytochemical profile (Table 3), the ferric-reducing antioxidant power (FRAP) of bark is much higher than wood for both solvents (ethanol and water): with 2.2 times the polar extractives of wood, bark shows 14 times more antioxidant power (0.42 *vs.* 0.03 mmolFe^{2+}/g). This means that bark has compounds more prone to act as reducing agents of the transition metal ion from Fe^{3+} to Fe^{2+} or that the specific combination of compounds have a greater interaction effect.

Vázquez et al. (2008) reported lower FRAP antioxidant activity for *E. globulus* bark for ethanol and water extracts (85 and 161 mgAAE/g_{Ext}). Overall bark showed better FRAP results than most of 30 plant extracts of industrial interest (Dudonné et al., 2009).

In terms of free radical scavenging, all the extracts presented very good antioxidant activity (AAI > 2) except the wood water extract (AAI = 0.6). The ethanol extracts of both materials showed the equivalent antioxidant capacity of 43–60% of trolox and catechin standards. Luís et al. (2014) reported similar AAI results for *E. globulus*. Results on the antioxidant capacity of ethanol/water bark extracts of several eucalypt species were reported in the range 368–1042 mgTE/g_{Ext} (Miranda et al. 2016; Lima et al., 2017) which encompasses the value 590 mgTE/g_{Ext} reported here for the ethanol extract.

The coarse fraction B_6 was richer in ethanol extracts and phenolic compounds which also showed higher FRAP and DPPH antioxidant activity. The difference of flavonoids content in the water extracts between B_6 and B_1/B_3 apparently has lower influence in the FRAP results than in DPPH free-radical scavenging.

Although the wood and bark have fairly similar phytochemical profile, their extracts (both ethanol and water) are significantly different with bark presenting overall higher antioxidant properties.

4.4. Thermal properties

The chemical composition of a biomass influences its thermal properties being accepted that lignin and extractives increase the calorific values, while polysaccharides reduce the heat density due to their higher level of oxidation (Nordin, 1994). The gross calorific value of the main constituents of a lignocellulose biomass is the order of extractives > lignin > cellulose > hemicelluloses.

Eucalyptus barks tend to have lower HHV than the respective woods, as shown by Juizo et al. (2017) for nine *Eucalyptus* species. Although bark has higher extractive content, the lignin content is lower and it has higher ash leading to a lower high heating value (HHV).

The thermal properties obtained for the industrial *E. globulus* bark (Table 4) are similar to values reported by ECN (2017) for eucalyptus bark: 78.1% volatiles, 17.1% fixed carbon and 4.2% ash, ultimate analysis (47.4% C, 5.5% H, 0.3% N, 44.0% O) and 18.04 MJ/kg HHV. For *E. globulus* wood, Telmo et al. (2010) reported higher values of volatiles and lower fixed carbon contents (86.3% and 13.3% respectively) and similar CHNO percentages (46.2%, 5.8%, 0.2% and 47.2% respectively).

The extractives and hemicelluloses are more easily thermally degraded (Silva et al., 2016; Sen et al., 2014) which is why bark starts decomposing at slightly lower temperatures and at higher rates than wood (304 *vs.* 323 °C). Due to the higher lignin and cellulose contents (Table 1), wood shows a peak in DTG at higher temperatures than bark.

The proximate and ultimate analysis results of the fine fraction (B_1) are different from those of the other fractions B_3 and B_6 that were quite similar. The lower HHV of fraction B_1 is due to the 20% mineral content. The TGA and DTG curves reflect the chemical composition of the three fractions, with B_3 degrading faster than the others, mainly due to its higher polysaccharide and lower lignin contents and B_1 presenting a higher residual weight mostly due to its higher ash content.

4.5. Valorization potential

The bark from *E. globulus* benefits from being a side stream (or residue stream) of an already fully implemented indust0072y making it a perfect candidate to integrate within a biorefinery concept, aiming at a full resource use. The fact that the pulp industry has in place the industrial facilities and commodities required for chemical or thermal processing eases the economic feasibility of this valorization approach.

The characterization that was made in this work of the industrial bark collected at the pulp mill site allows two operational advices: i) the debarking equipment and process conditions should be tuned towards low wood losses; and ii) the handling and storage of bark should target a minimization of contamination from extraneous materials.

Residual bark is traditionally used for energy production and is a valuable contributor to the pulp mill energy balance. However, this bark has some detrimental thermal characteristics, such as high amounts of ashes and chlorine. Ashes are non-combustible materials that consume part of the energy produced to keep up the temperature of the system or to change state (fusion of certain ash components such as silica or calcium). The formation and deposit of slag, equipment corrosion, management, subsequent use and operation costs are also dependent on the composition and quantity of ash (Telmo et al., 2010; Gominho et al., 2012). The bark showed good calorific value and a high fixed carbon (Table 4), which is beneficial for carbonization processes. On the other hand its high Cl content (0.35%) hinders its use for solid biofuels such as pellets and briquettes, which can only have 0.03% of chlorine (EN 14961-2).

E. globulus bark is fibrous (Pirralho et al., 2014) and may be used for pulping even if the higher extractives content leads to lower kraft pulp yield in comparison with those of wood (41% *vs.* 48% (Neiva et al., 2016). The incorporation of some percentage of bark in the pulp feedstock allows production of pulps with adequate strength properties (Miranda et al., 2012b).

Fractionation by grinding is a required processing step and separation of the fines allows a differentiated valorization. In fact, the B_1 fine fraction was clearly different (Tables 1 and 2) and probably the fraction with less bioenergetic potential, mostly due to the high inorganic content. Fines contain a high content of polar extractives that should not be discarded if these are targeted. The high extractives content, their phytochemical profile and antioxidant activity points to their possible use in the food and pharmaceutical industries with several studies focusing on determining and isolating natural antioxidant molecules from both apolar and polar fractions (Vázquez et al., 2012; Kim et al., 2001; Santos et al., 2011; Freire et al., 2002; Domingues et al., 2010).

After removal of extractives, the bark can undergo valorization of polysaccharides that can be interesting for other applications, *e.g.* polyols for polyurethane foams (D'Souza et al., 2014), hemicellulose-derived oligomers (Moniz et al., 2013) or ethanol production (Lima et al., 2013).

Fig. 2 schematically summarizes possible valorization routes for *E.*

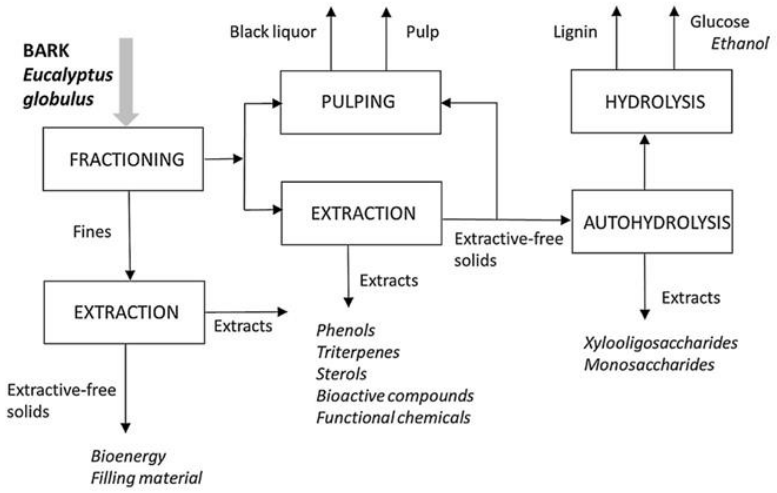

Fig. 2. Proposed flowsheet for *E. globulus* bark-based in biorefinery context.

globulus bark in accordance with the results obtained and the discussion made above. The schematic flowsheet includes a first selective mechanical fractionating with the fines targeted for the valorization of extractives and the other material directed to pulping with or without an extractives removal step.

5. Conclusions

A correct stem handling and debarking process as well as bark storage and transport are important to minimize wood losses for the pulping process and bark contamination from extraneous materials. The present industrial bark contained 18% of wood corresponding to a loss of roughly 2–3% of the wood stock in the debarking process.

Compared to wood, *E. globulus* bark presented higher extractives, similar lignin and lower polysaccharides contents. The phytochemical profile and antioxidant activity were higher than those of wood and although bark presents lower energetic value its fixed carbon content is higher than woods.

Size fractioning allowed obtaining differentiated fractions with different chemical and thermal properties. Fines showed lower bioenergetic potential, mostly due to the high inorganic content but are richer in extractives.

The *E. globulus* bark chemical composition, phytochemical profile and thermal properties seem to point to other potential uses apart combustion to generate energy, making it an interesting material to upgrade within the biorefinery concept. Extractive valorization due to their chemical functionalities and bioactivity indicates possible uses in the food and pharmaceutical industries. The remaining extractive-free bark can be considered as an additional fiber source for the pulping process, as well as for polysaccharides valorization, whether by deconstruction to monomers and subsequent fermentation or for end-use products such as polyols or hemicellulose-derived oligomers.

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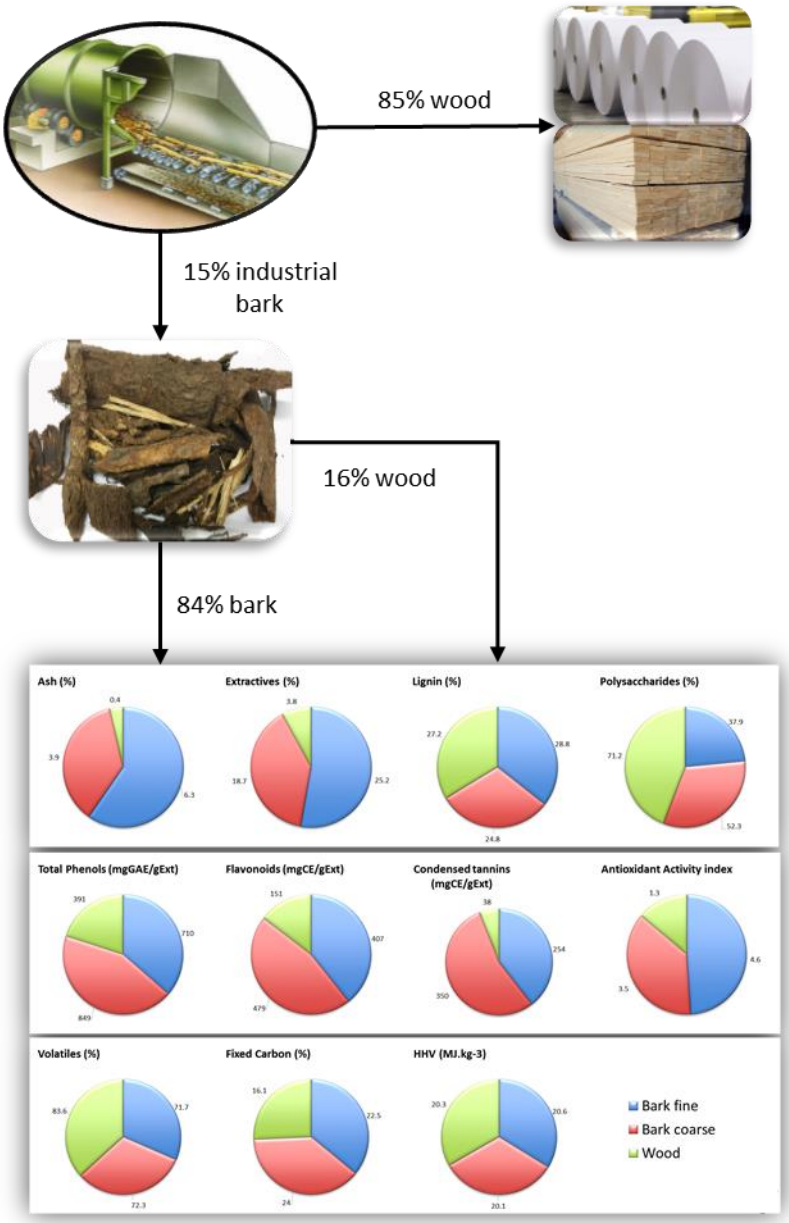

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RESEARCH ARTICLE

An integrated characterization of *Picea abies* industrial bark regarding chemical composition, thermal properties and polar extracts activity

Duarte M. Neiva[1]*, Solange Araújo[1], Jorge Gominho[1], Angélica de Cássia Carneiro[2], Helena Pereira[1]

1 Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Portugal,
2 Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil

* duarteneiva@isa.ulisboa.pt

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Abstract

The present work determines the chemical and thermal characteristics as well as the phytochemical and antioxidant potential of the polar extractives of the *Picea abies* bark from an industrial mill, their wood and bark components and also different bark fractions obtained by mechanical fractionation (fine B_1, Φ<0.180 mm, medium B_3, 0.450 < Φ<0.850 mm and coarse B_6, 2 < Φ<10 mm). The aim is to increase the knowledge on the *Picea abies* bark to better determine possible uses other than burning for energy production and to test an initial size reduction process to achieve fractions with different characteristics. Compared to wood, bark presented similar lignin (27%), higher mineral (3.9% vs 0.4%) and extractives (20.3% vs 3.8%) and lower polysaccharides (48% vs 71%) contents. Regarding bark fractions the fines showed higher ash (6.3%), extractives (25%) and lignin (29%) than the coarse fraction (3.9%, 19% and 25% respectively). Polysaccharide contents increased with particle size of the bark fractions (38% vs 52% for B_1 and B_6) but showed the same relative composition. The phytochemical profile of ethanol and water extracts presented higher contents for bark than wood of total phenols (2x higher), flavonoids (3x higher) and tannins (4-10x higher) with an increasing tendency with particle size. Bark antioxidant activity was higher than that of wood for ferric-reducing antioxidant power (FRAP, 10 vs 6 mmolFe^{2+}/g_{Ext} for the ethanol extract) and free radical scavenging activity (DPPH, 6 vs 18 mg/L IC50 for the ethanol extract) methods. The different bark fractions antioxidant activity was very similar. Bark thermal properties showed a much lower volatiles to fixed carbon ratio (V/FC) than wood (3.1 vs 5.2) although the same higher heating value (20.3 MJ/kg). The fractions were quite similar. Bark presented chemical features that point to their possible upgrade, whether by taking advantage of the high extractives with bioactive compounds or the production potential for hemicellulose-derived oligomers with possible use in nutraceutical and pharmaceutical industries.

study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Introduction

Norway spruce (*Picea abies* (L.) Karst.) is a major softwood species in Europe used extensively in pulp mills for mechanical and kraft pulp production, and in sawmills to produce wood components. The industrial processing originates large quantities of biomass as residual materials that accumulate at mill site and may be considered as side-streams available for valorization under a full resource utilization concept.

Barks are non-wood forest products that are receiving increased attention as a potential feedstock for an integrated valorization—the so-called bark-based biorefinery—given their availability and concentration at industrial processing sites, as well as their potential for a combination of value-added applications *e.g.* chemicals and bio-products, that add to their current use as solid fuel [1,2]. Barks of the different industrial tree species show great diversity in structural and chemical composition, as well as in physical and mechanical fractionation properties, thereby leading to specific valorization routes. Numerous bark characterization studies have been recently carried out in view of their potential use in biorefineries [3–9].

Norway spruce bark is one of the barks that has been researched quite intensively, since it is an abundant material i.e. it represents 10–15% volumetric content in logs, and is still an undervalued feedstock because it is used mostly for fuel in combustion, although its rich chemical composition allows considering a better valorization as a source of high-value chemicals e.g. directed to production of adhesives, resins and plastics as well as to bioactive extracts [10].

The chemical composition of Norway spruce bark has been reported for the bark as a whole [11, 12] as well as for the inner and outer bark fractions [13]. Norway spruce bark is characterized by a large proportion of extractives e.g. 21.6% [14] or 28.3% [15]. The hydrophylic extractives have attracted attention, namely tannins [16, 17] and stilbenes [18] as well as lipophilic extractives [19].

Polysaccharides are also major constituents of Norway spruce e.g. together, hemicelluloses, pectins and cellulose constitute about 50% of the inner bark, 33% of the outer bark [13] and 40% of the whole bark collected after debarking in a pulp mill [20, 14].

In this sense, a bark biorefinery that first extracts valuable compounds such as extractives [21, 22], tannins [10, 23], non-cellulosic polysaccharides [20, 24] and cellulose [25], and thereafter converts the residue into biofuels and energy could be interesting [26].

The industrial barks accumulated at mill yards have additional specific features that result from the harvesting, handling and debarking processes that may affect the feedstock composition *e.g.* presence of wood material, mineral and extraneous contaminations. For instance, Ngueho Yemele [27] observed a high wood content of 19.9% in the industrial spruce bark from a sawmill. Since composition of wood and bark differ greatly, experimental results obtained with pure bark may not translate exactly into the industrial scale, therefore advising the characterization of the specific industrial bark.

The industrial bark of *Picea abies* collected at the pulp mill yard was studied here in view of its potential use as a source of chemicals within a bark-based biorefinery. The wood content in the industrial bark was determined and mechanical fractionation of the bark into different sized particles was made. The fractions were evaluated in relation to chemical features including summative chemical composition and the phenolic profile of ethanol and water extracts (total phenolic compounds, tannins and flavonoids) and their antioxidant activity, as well as thermal properties including proximate and ultimate analysis, thermogravimetric analysis (TGA) and derivative thermogravimetric analysis (DTG). The valorization routes of this industrial residual stream are discussed under the concept of a full resource use (zero waste philosophy) and as a potential biomass source within a biorefinery concept.

Material and methods

Sampling and fractionation

A 100 kg sample of large chips from Norway spruce (*Picea abies* (L.) Karst.) bark was collected after the debarking from a sawmill located in Jyvaskyla, Finland. The chips were air dried for several days under well ventilated conditions and frequent mixing, followed by oven drying at 40˚C for 3 days.

An aliquot of the industrial bark was visually observed and the wood (W) chips were separated from the bark (B) fractions and quantified. The bark fraction was knife-milled with a Retsch SM 2000 mill to pass a 10x10 mm screen (B) and sieved to six mesh sizes fractions: B_1 (mesh <80, Φ <0.180 mm); B_2 (mesh 60/80, 0.180<Φ<0.250 mm); B_3 (mesh 40/60, 0.250<Φ<0.450 mm); B_4 (mesh 20/40, 0.450<Φ<0.850 mm); B_5 (mesh >20, 0.850<Φ<2 mm);B_6 (2<Φ<10 mm). Each fraction was weighed and the yield calculated as o.d. mass.

The chemical analysis were performed on the samples B_1 (fine), B_3 (medium) and B_6 (coarse), as well as on the unsieved B sample that represents the bark as a whole, and the wood (W). The combination of the wood and the bark fractions in their respective proportions represent the whole industrial bark as obtained.

Before analysis, the samples B_6 and B were milled to pass a 1 mm ouput sieve and the entire material obtained was used. The wood sample was milled and the 40/60 mesh fraction (0.250<Φ<0.450mm) used for analysis.

Chemical analysis

Ash content was determined by TAPPI standard T15 os-58 and extractives by successive Soxhlet extraction with dichloromethane, ethanol and water overnight for each solvent. Total lignin was determined in the extractive-free material as acid insoluble lignin (klason) and soluble lignin according to TAPPI standards T222 om-88 and UM250 om-83 respectively. Klason lignin was adjusted taking in consideration its ash content. Hydrolysis liquor resulting from T222 om-88 was used to ascertain polysaccharides composition regarding neutral monosaccharides, glucuronic acid, galacturonic acid and acetates by separation through a Dionex ICS-3000 High Pressure Ion Chromatographer, using an Aminotrap plus Carbopac SA10 column. All the chemical analysis were made in triplicate.

The ash composition was determined as follows: Cl by EN 15289:2011 standard; B by spectrophotometry (420 nm) after oven burning at 600˚C; the remaining elements were determined after nitro-perchloric acid digestion followed by atomic absorption spectroscopy (Ca, Mg, Fe, Zn, Cu, Mn, Ni, Pb, Cr), spectrophotometry (P and S at 725 and 420 nm respectively) and emission flame photometry (K). Two duplicates were tested and result expressed as average.

Ethanol and water extractives

The composition and antioxidant activity of the ethanol and water extracts obtained by successive extraction were analyzed in relation total phenols (TPC), flavonoids (FC) and condensed tannins (CTC) contents as previously described [28]. TPC results were reported as mg gallic acid equivalents (GAE)/$g_{Extract}$ and FC and CTC as (+)-catechin equivalents (CE)/$g_{Extract}$ through a calibration curves.

The antioxidant activity of the extracts was estimated by two methods: ferric-reducing antioxidant power (FRAP), expressed in mmol Fe(II)/g extract and the results compared to standards (ascorbic acid, catechin and gallic acid); free radical scavenging activity (DPPH) as described by Sánchez-Moreno [29]. The DPPH results were expressed as IC_{50} (extract

concentration required for 50% DPPH inhibition) and as antioxidant activity index (AAI = final concentration of DPPH in the control sample/IC_{50}) which takes in consideration the mass of DPPH and test sample decreasing the concentration influence of the DPPH solution used [30]. The antioxidant activity is classified as poor if AAI<0.5, moderate if 0.5<AAI<1, strong 1<AAI<2 and very strong when AAI>2. DPPH results were compared to a natural and a synthetic standards ((+)-catechin and trolox, respectively.

All the analyses were made in triplicate and results expressed as average with standard deviation.

Thermal properties

Proximate analysis was determined according to ASTM E870-82 standard for ash and volatile matter, with fixed carbon calculated by difference to 100%. Ultimate analysis followed ASTM D5373-08 standard using a Perkin-Elmer II 2400 elemental analyzer (Shelton, CT, USA), with oxygen content determined by difference after C, H, N, and ash content determination. The higher heating value (HHV) was determined following ABNT NBR 8633 standard using an adiabatic bomb calorimeter IKA300 (Staufen, Germany).

Thermogravimetric analysis (TGA) and derivative thermogravimetry (DTG) were carried out in a Shimadzu DTG-60H (Kyoto, Japan) with dynamic nitrogen atmosphere (gas flow of 50 mL min^{-1}) between 10–680˚C with a 10˚C min^{-1} heating rate, using 2 mg ± 0.1 mg samples in a platinum container.

Results

Fractionation

The industrial bark included wood pieces (W) that could be clearly out singled and corresponded to 15.9% (m/m) of the total.

Mechanical grinding and granulometric sieving of the bark-only material (B) contained in the industrial bark yielded the following fraction distribution: B_1 (< 0.180 mm) 3.5%, B_2 (0.18<Φ<0.25 mm) 1.9%, B_3 (0.25<Φ<0.45 mm) 4.0%, B_4 (0.45<Φ<0.85 mm) 9.6%, B_5 (0.85<Φ<2.00 mm) 27.6% and B_6 (2.00<Φ<10.00 mm) 53.4%. The material was brittle and fractionated easily into coarse granules (B_5 and B_6) that together constituted 81.0% of the total.

Chemical composition

Table 1 summarizes the results obtained for the chemical composition of the industrial bark and of its wood and bark fractions, regarding content of ash, extractives and lignin as well as the monomeric composition of polysaccharides (neutral sugars, uronic acids and acetates).

The bark (B) has a high content of extractives (20.3% of o.d. material) mainly constituted by polar compounds soluble in ethanol and water, that represent together 73% of the total extractives. Lignin content is 26.9% and polysaccharides represent 47.9% of the bark. Polysaccharides are mainly constituted by glucose (55.9% of the total units) while the composition of hemicelluloses is dominated by a high presence of arabinose and xylose (10.8% and 9.6% of the total units, respectively) and of galacturonic acid (11.7%); galactomannans are present in lower amounts with galactose and mannose representing respectively 4.1% and 5.3% of the total units.

The composition of wood (W) is different mainly regarding the much lower content in extractives that represent only 3.8% of the material and the also much lower ash content i.e. 0.38% vs. 3.88% in bark (Table 1). The polysaccharides, that represent 71.2% of the wood, have a composition that differs from that of bark by the dominance of galactomannans (mannose and galactose correspond to 20.2% and 2.9% of the total units), with less proportion of uronic

Table 1. Chemical composition (in % o.d. mass) of the industrial bark (IB) of *Picea abies*, the wood (W) and bark-only (B) fractions, as well as of three granulometric fractions obtained after grinding and sieving of bark (B_1, < 0.180 mm, B_3 (0.25<Φ<0.45 mm) and B_6 (2.00<Φ<10.00 mm).

	IB	W	B	B1	B3	B6
Ash	**3.32 ± 0.07**	**0.38 ± 0.01**	**3.88 ± 0.08**	**6.28 ± 0.11**	**4.21 ± 0.10**	**3.87 ± 0.02**
Extractives	**17.64 ± 1.30**	**3.78 ± 0.21**	**20.25 ± 1.5**	**25.18 ± 0.19**	**21.69 ± 0.63**	**18.74 ± 0.18**
Dichloromethane	4.68 ± 0.05	0.81 ± 0.03	5.41 ± 0.05	8.17 ± 0.24	6.44 ± 0.06	4.81 ± 0.06
Ethanol	3.93 ± 0.24	0.87 ± 0.05	4.51 ± 0.27	6.62 ± 0.37	5.37 ± 0.16	4.13 ± 0.23
Water	9.03 ± 1.16	2.11 ± 0.20	10.34 ± 1.34	10.39 ± 0.56	9.88 ± 0.63	9.80 ± 0.12
Lignin	**26.92 ± 0.74**	**27.22 ± 0.29**	**26.86 ± 0.82**	**28.75 ± 0.92**	**29.92 ± 1.59**	**24.81 ± 0.30**
Klason	26.08 ± 0.70	26.9 ± 0.30	25.93 ± 0.77	27.74 ± 0.96	29.03 ± 1.63	23.84 ± 0.31
Acid soluble	0.83 ± 0.06	0.32 ± 0.01	0.93 ± 0.07	1.01 ± 0.04	0.89 ± 0.05	0.98 ± 0.04
Polysaccharides	**51.56 ± 0.92**	**71.18 ± 1.51**	**47.87 ± 0.81**	**37.86 ± 0.32**	**42.61 ± 1.49**	**52.27 ± 0.26**
Rhamnose	0.48 ± 0.02	0.07 ± 0.00	0.55 ± 0.03	0.56 ± 0.01	0.49 ± 0.05	0.73 ± 0.04
Arabinose	4.56 ± 0.34	1.32 ± 0.02	5.17 ± 0.4	4.05 ± 0.20	4.15 ± 0.35	6.31 ± 0.13
Galactose	1.96 ± 0.09	2.03 ± 0.03	1.95 ± 0.1	1.74 ± 0.03	17.7 ± 0.07	2.17 ± 0.09
Glucose	29.49 ± 0.21	43.89 ± 0.73	26.78 ± 0.11	20.71 ± 0.23	23.97 ± 1.31	29.39 ± 0.2
Xylose	4.97 ± 0.31	7.11 ± 0.94	4.57 ± 0.19	3.18 ± 0.05	4.22 ± 0.49	4.21 ± 0.08
Mannose	4.42 ± 0.35	14.34 ± 1.80	2.55 ± 0.08	2.54 ± 0.01	2.95 ± 0.06	2.27 ± 0.03
Galacturonic acid	4.48 ± 0.12	0.74 ± 0.01	5.62 ± 0.14	4.42 ± 0.07	4.36 ± 0.17	6.54 ± 0.10
Glucuronic acid	0.22 ± 0.01	0.09 ± 0.00	0.25 ± 0.01	0.28 ± 0.01	0.25 ± 0.02	0.24 ± 0.01
Acetic acid	0.62 ± 0.01	1.60 ± 0.06	0.44 ± 0.00	0.38 ± 0.02	0.45 ± 0.02	0.42 ± 0.01
Total	**99.4 ± 0.4**	**102.5 ± 1.2**	**98.9 ± 0.3**	**98.1 ± 0.6**	**98.4 ± 1.1**	**99.7 ± 0.7**

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acids and by a higher acetylation degree (acetic acid corresponds to 2.5% of the units vs. 0.9% in bark).

The composition of the industrial bark as a whole is similar to the bark composition with differences in direct relation to the proportion of the wood it contains (Table 1).

The three granulometric fractions show some chemical compositional differences. The fine fraction B_1 has a higher ash content (6.3%) that decreases in the B_3 and B_6 fractions (4.2% and 3.9% respectively). The fine fraction also shows the highest content of extractives (25.2%), that decrease with the increase of particle size to 18.7% in the B_6 fraction, mostly due to a decrease in the dichloromethane soluble compounds. The coarse fraction B_6 differentiates from the other two by the lower proportion of Klason lignin (23.8% and 27.7% for B_6 and B_1 respectively) and the highest polysaccharide content (52.3% and 37.9% for B_6 and B_1 respectively) The monomeric composition of the polysaccharides was similar between fractions with a slight enrichment in glucose in the coarse fraction (56.2% and 54.7% of all units for B_6 and B_1 respectively).

Ash mineral composition

Table 2 presents the results obtained for the ash mineral composition. Calcium is the most important mineral of bark, corresponding to 1.5% of the material, followed by potassium (0.2%); the content of manganese is comparatively high (0.05%). Cl and S are present corresponding to 0.04 and 0.05% respectively.

In wood, the content of the different minerals is much lower given the overall low ash content. It is noteworthy that the potassium content has a much higher relative proportion than in bark.

The different granulometric fractions of bark show a quite similar composition with exception for the iron content which is four to five times lower in the coarse fraction regarding the medium and fine fractions (Table 2).

Table 2. Mineral composition (in % o.d. mass or ppm) of the industrial bark (IB) of *Picea abies*, the wood (W) and bark-only (B) fractions, as well as of three granulometric fractions obtained after grinding and sieving of bark (B_1, < 0.180 mm, B_3 (0.25<Φ<0.45 mm) and B_6 (2.00<Φ<10.00 mm).

	IB	W	B	B1	B3	B6
Ca (%)	1.25	0.14	1.46	1.74	1.55	1.52
K (%)	0.21	0.14	0.22	0.25	0.24	0.24
P (%)	0.05	0.02	0.06	0.08	0.07	0.05
Mg (%)	0.07	0.02	0.08	0.09	0.07	0.08
S (%)	0.05	0.04	0.05	0.05	0.05	0.04
Cl (%)	0.04	0.03	0.04	0.04	0.04	0.02
Cu (ppm)	9	34	4	4	8	4
Fe (ppm)	96	53	104	253	228	54
Zn (ppm)	137	31	158	158	146	170
Mn (ppm)	397	73	458	436	394	486
B (ppm)	16	25	14	21	20	23
Ni (ppm)	1.6	0.9	1.8	2.2	3.1	1.0
Pb (ppm)	3.8	3.5	3.9	3.5	7.0	3.3
Cr (ppm)	2.5	1.6	2.7	4.3	6.0	2.5

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Ethanol and water extractives

The phenolic composition of the ethanol and water extracts obtained by the successive extraction with both solvents is shown in Table 3 regarding contents in total phenolics, flavonoids and condensed tannins. The antioxidant activity of the extracts is also included as measured by the FRAP and DPPH methods.

The ethanol extracts contain much higher values of phenolics, flavonoids and tannins than the subsequent water extracts. The bark ethanol extracts have a high proportion of phenolic compounds (851 mg GAE/g_{Ext}) in which flavonoids and condensed tannins constitute the major classes (476 mg CE/g_{Ext} and 360 mg CE/g_{Ext} respectively). The bark water extracts contain a much lower amount of phenolic compounds i.e. 187 mg GAE/g_{Ext} total phenolics, 98 mg CE/g_{Ext} flavonoids and 40 mg CE/g_{Ext} condensed tannins.

Compared to bark, wood has a lower phenolic content especially of tannins e.g. 391 mg GAE/g_{Ext} total phenolics, 151 mg CE/g_{Ext} flavonoids and 38 mg CE/g_{Ext} condensed tannins in the ethanol extracts (Table 3).

The extracts from the different bark granulometric fractions did not show considerable differences apart from the total phenolics in B_6 that had a higher content in the ethanol extracts and a lower content in the water extracts.

The FRAP antioxidant activity of bark extracts (Table 3) showed that ethanol extracts have more antioxidant power than water extracts (10.0 mmolFe^{2+}/g_{Ext} vs. 2.6 mmolFe^{2+}/g_{Ext}). The values are lower than those obtained for usual antioxidant compounds e.g. ascorbic acid, catechin or gallic acid (17, 18 and 40 mmolFe^{2+}/g_{Ext} respectively). The wood had a lower antioxidant activity compared to bark.

As regards the free radical scavenging activity, the concentration of extracts required for 50% DPPH inhibition (IC50) was 6.3 and 20.7 mg/L for bark ethanol and water extracts respectively. Both extracts are less effective than trolox and catechin standards (IC50 = 3.5 and 2.7 mg/L respectively). The wood ethanol and water extractives have very low antioxidant activity (IC50 = 17.8 and 40.6 mg/L respectively). The bark ethanol extracts have an antioxidant activity index (AAI) above 2 (AAI = 3.3) that corresponds to a strong antioxidant classification.

Table 3. Chemical composition (total phenolics, flavonoids, condensed tannins) and antioxidant properties (FRAP and DPPH) of the ethanol and water extracts of the industrial bark (IB) of *Picea abies*, the wood (W) and bark-only (B) fractions, as well as of three granulometric fractions obtained after grinding and sieving of bark (B_1, < 0.180 mm, B_3 (0.25<Φ<0.45 mm) and B_6 (2.00<Φ<10.00 mm). Determinations on standards are included for FRAP (ascorbic acid, catechin and gallic acid) and for DPHH (trolox and catechin) antioxidant properties.

	IB	W	B	B1	B3	B6	Standard
Phenolics (mg GAE/gExt)							
Ethanol	778 ± 33	391 ± 41	851 ± 32	710 ± 15	772 ± 22	849 ± 31	
Water	176 ± 9	119 ± 9	187 ± 9	311 ± 25	288 ± 14	193 ± 8	
Flavonoids (mg CE/gExt)							
Ethanol	424 ± 10	151 ± 16	476 ± 9	407 ± 1	407 ± 12	479 ± 11	
Water	88 ± 7	37 ± 4	98 ± 7	101 ± 4	109 ± 1	106 ± 7	
Condensed tannins (mg CE/gExt)							
Ethanol	309 ± 5	38 ± 4	360 ± 6	254 ± 7	271 ± 7	350 ± 18	
Water	35 ± 2	10 ± 1	40 ± 2	61 ± 1	56 ± 1	40 ± 2	
FRAP antioxidant activity							
Ethanol (mmolFe^{2+}/gExt)	9.4 ± 0.2	6.0 ± 0.1	10.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	10.0 ± 0.0	
Water (mmolFe^{2+}/gExt)	2.4 ± 0.1	1.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.8 ± 0.0	2.5 ± 0.1	
Ascorbic acid (mmolFe^{2+}/g)							17 ± 0.4
Catechin (mmolFe^{2+}/g)							18 ± 0.5
Gallic acid (mmolFe^{2+}/g)							40 ± 1.0
DPPH antioxidant activity							
Ethanol IC50 (mg/L)	8.1 ± 0.4	17.8 ± 1.3	6.3 ± 0.2	5.1 ± 0.4	4. 7 ± 0.2	6.8 ± 0.3	
Ethanol AAI	3.3 ± 0.1	1.3 ± 0.1	3.7 ± 0.1	4.6 ± 0.3	5.0 ± 0.2	3.5 ± 0.2	
Water IC50 (mg/L)	23.9 ± 0.9	40.6 ± 2.5	20.7 ± 0.6	15.8 ± 0.6	13.7 ± 0.2	20.4 ± 1.2	
Water AAI	1.0 ± 0.0	0.6 ± 0.0	1.1 ± 0.0	1.5 ± 0.1	1.7 ± 0.0	1.1 ± 0.1	
Trolox IC50							3.5 ± 0.2
AAI							6.7 ± 0.3
Catechin IC50							2.7 ± 0.4
AAI							8.6 ± 0.7

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The FRAP antioxidant activity of the different bark granulometric fractions is similar. The DDPH free radical scavenging activity of both ethanol and water extracts was similar for the three granulometric fractions with the coarser fraction always presenting a lower antioxidant activity than the other fraction.

Thermal properties

Table 4 presents the results of the proximate and ultimate composition as well as the higher heating value. In comparison to wood, bark has more ash, less volatiles and more fixed carbon leading to a lower V/FC ratio (3.1 vs. 5.2). The elemental composition of bark and wood is similar and the differences of the H/C and O/C ratios are of small magnitude (1.39 *vs.* 1.49 and 0.63 *vs.* 0.67, respectively for bark and wood). The higher heating value (HHV) of bark and wood is the same at 20.3 MJ/kg. The bark fractions show small differences in thermal properties with the fixed carbon increasing from smaller to bigger fraction (22.5 vs 24 for B_1 and B_6 respectively). The coarser fraction showed a lower higher heating value than the other two fractions.

Fig 1 shows the results for the TGA and DTG analysis made between 250 and 690˚C. Mass loss starts near 300˚C but the thermal degradation at higher temperatures differs somewhat between bark and wood. For bark the highest rate of mass loss spreads in the temperature range of 360–403˚ and for wood the highest degradation rate is reached at 385˚C. After 450˚C, both wood and barks show a constant rate of degradation of 0.001 mg˚C^{-1}.

Table 4. Proximate and ultimate analysis (in % o.d. mass) and high heating value (MJ/kg) of the industrial bark (IB) of *Picea abies*, the wood (W) and bark-only (B) fractions, as well as of three granulometric fractions obtained after grinding and sieving of bark (B_1, < 0.180 mm, B_3 (0.25<Φ<0.45 mm) and B_6 (2.00<Φ<10.00 mm).

	IB	W	B	B1	B3	B6
Proximate analysis						
Volatiles (%)	74.7 ± 0.6	83.6 ± 0.4	73.0 ± 0.7	71.7 ± 0.3	72.1 ± 0.9	72.3 ± 0.5
Fixed carbon (%)	22.2 ± 0.6	16.1 ± 0.4	23.4 ± 0.6	22.5 ± 0.6	23.6 ± 0.9	24.0 ± 0.4
Ashes (%)	3.1 ± 0.1	0.3 ± 0.0	3.6 ± 0.1	5.8 ± 0.2	4.3 ± 0.0	3.7 ± 0.0
V/FC ratio	3.4 ± 0.0	5.2 ± 0.1	3.1 ± 0.1	3.2 ± 0.1	3.1 ± 0.1	3.0 ± 0.1
Ultimate analysis						
C (%)	48.6	49.1	48.5	49.0	50.0	48.6
H (%)	5.7	6.1	5.6	5.7	5.8	5.8
N (%)	1.0	0.7	1.0	1.2	1.0	1.0
S (%)	0	0	0	0	0	0
O (%)	41.4	43.7	41.0	37.8	39.0	40.7
Atomic H/C ratio	1.40	1.49	1.39	1.40	1.39	1.43
Atomic O/C ratio	0.64	0.67	0.63	0.58	0.58	0.63
HHV (MJ/kg)	20.3	20.3	20.3	20.6	20.7	20.1

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The bark fractions do not show differences in thermal degradation and the TGA and DTG curves are largely superposed.

Discussion

The industrial bark of *Picea abies* collected at mill site included a significant proportion of wood that derived from the debarking process. A high wood content in a sawmill residual bark was previously reported at up to 21% [10, 27]. Although this wood proportion represents a loss in the wood supply to the subsequent main process, it is important to notice that Norway spruce bark has structural features that are incompatible with some wood applications, namely those of pulp and veneer production. The technical relevance of such contamination to the process depends on the structural features of the specific bark; in some cases, the impact may not be so important e.g. for fibrous barks such as those of *Eucalyptus globulus* that may even be

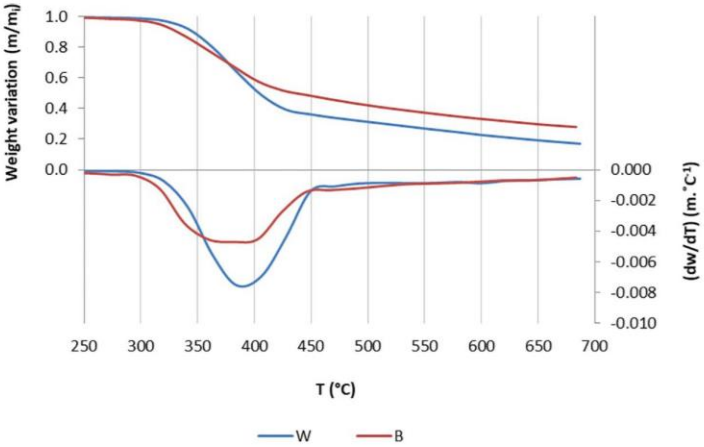

Fig 1. TGA and DTG of the wood (W) and bark-only (B) fractions of *Picea abies* industrial bark.

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considered as a pulping feedstock [6, 31, 32]. However, this is not the case of *Picea abies* bark and a conservative option is taken by the mills to avoid bark contamination in the feedstock. Therefore, if the industrial *Picea abies* bark stream is directed for specific targeted applications, the presence of wood in amounts similar to those found in this work–ca. 16%—has to be taken into consideration. This means that the composition of the industrial bark will somewhat differ from that of the bark itself (Table 1).

The mechanical fractionation of *Picea abies* bark showed that the material is easily comminuted and fines are not produced in significant amounts e.g. particles < 0.25 mm represented only 5.4% of the material. This is of practical importance for raw-material pre-processing and shows that sieving operations may not be required if they are not detrimental to the following unit operations. Similar results on the fractionation behavior of Norway spruce bark were also reported by Miranda [14].

The *Picea abies* bark contains a substantial amount of extractives, mainly polar compounds (14.8% of the bark, Table 1). Such high content of extractives has been reported in many studies with values similar to those found here [10, 13, 33, 34]. The high extractives content (20%), and substantial apolar fraction (5%) follows the same behavior than other softwood species, with Douglas-fir, loblolly pine, scots pine and stone pine showing values in the range of 19–30% total extractives and 2–7% apolar ones.[3, 14, 35, 36]

When targeting a specific industrial bark stream, the extractives content will depend on its wood proportion since wood has a much lower extractive content (3.0% of polar extractives, Table 1). When considering the extraction of polar compounds successively with ethanol and water, the results showed that most of the phenolics were extracted by ethanol (Table 3). In fact, the ethanol extracts contained a very high proportion of phenolic compounds, mostly constituted by flavonoids (476 mg CE/g_{Ext}) and condensed tannins (360 mg CE/g_{Ext}). The subsequent water extraction solubilizes the remaining phenolic compounds which correspond to much lower proportions of the extract (Table 3); in the water extract an important proportion of solubilized sugars should be present [10]. However, to fully account for the potential production of phenolic extracts from the *Picea abies* bark, the fraction extracted by water should also be taken into consideration given their yield in terms of the initial bark material e.g. ethanol only extracts about 65% of the total phenolics (Table 3). However, from a practical point of view, it should be considered that the ethanol and water extracts differ in relation to their concentration in phenolics (high concentration in the ethanol extract and lower in the subsequent water extract) and to their antioxidant activity.

The bark ethanol extracts are classified as being very strong antioxidants with an AAI above 3.5, although their free radical scavenging activity is lower than that of usual antioxidants, either synthetic or natural, e.g. trolox and catechin (6.7 and 8.6 respectively). The same applies to the FRAP antioxidant power which is half of that of usual antioxidant compounds e.g. ascorbic acid (10 mmolFe^{2+}/g_{Ext} vs. 17 mmolFe^{2+}/g_{Ext} respectively). Being a sequential extraction it is normal that the water extracts have lower antioxidant properties but nevertheless they still have compounds with antioxidant capability that are not solubilized in ethanol; since the water extractives yield is much higher than that of ethanol (Table 1), this fraction cannot be ignored if the aim is obtain compounds with antioxidant activity. When compared to wood, bark has a much higher extraction yield (five times more) and the compounds extracted also have higher antioxidant activity.

The high content of extractives in Norway spruce has attracted the attention of research and several studies showed their potential [37, 21]. The practical use of the bark for production of tannins was proposed by Kempainnen [10] who could obtain water extracts with up to 50% tannin content at pilot-scale. Lacoste [38] showed that purified tannins from *Picea abies* bark could be used to produce foams.

The composition of *Picea abies* bark regarding structural components (Table 1) is in line with published reports for this species [10, 14]. In comparison to other softwoods barks such as Douglas-fir, loblolly or Scots pines It shows lower lignin content (27% vs 30–44%) but higher polysaccharides (48% vs 24–38%) [3, 14, 35]. It is noteworthy that the bark hemicelluloses contain a high proportion of arabinose, xylose and galacturonic acid, together representing 32.1% of the polysaccharides while the composition of wood polysaccharides is quite different, with glucose representing 61.7% of the total, and mannose and galactose 23.1% (Table 1). The high hemicellulosic content of *Picea abies* bark allows to consider it as a potential source of oligosaccharides by mild treatments such as hydrothermal processes after the removal of polar extractives. Hydrothermal treatments have been applied to various types of biomass and proposed for production of xylooligosaccharides using e.g. rice straw [39], corn cobs [40] or even wood [41]. A further material valorization along chemical fractionation routes may consider delignification of the cellulose and lignin enriched solids to yield a cellulose-rich solid fraction and a soluble lignin [4, 25].

Regarding the compositional profile of the different granulometric fractions (Table 1), the fines showed a higher extractive content (approximately one third more extractives than the coarse fraction) as well as more mineral content. This enrichment in extractives and minerals in the smallest particles obtained after grinding was already found for *Picea abies* bark [14] as well as for barks of other species [7, 8] and for other biomass types [42,43]. As regards structural components, fines were enriched in lignin when reported on an extractive- and ash-free basis (42.0% vs. 32.0% in the coarse fraction), but the polysaccharide profile was similar, as also previously reported [14].

When compared to wood, bark had higher fixed carbon content which is also visible in the behavior of the TGA plot, leading to a higher weight variation after the 450˚C. Bark starts to decompose at lower temperatures, probably due to the higher extractives content that are easily degraded. On the other hand, the DTG curve is less pronounced than for wood since bark has a lower volatiles content. The TGA and DTG plots of the three fractions were almost identical, which means that the chemical variations found (Table 1) did not alter significantly their thermal degradation in the absence of oxygen.

The results obtained point out that *Picea abies* industrial bark is suitable as a biomass source for valorization within a biorefinery concept, allowing fractionation into chemicals or an energy use. It should also be referred that the bark may be used for energy production either directly after collection (HHV of bark and wood is the same at 20.3 MJ/kg, Table 4) or at any point along the fractionation. Francezon and Stevanovic presented small decreases (up to 5%) on higher heating values of black spruce (*Picea mariana*) bark after several extraction processes have been reported [44]. The energetic content of the P*icea abies* bark is on the lower end of the range published for several softwood species that go from 19.6 MJ/kg up to 25 MJ/kg [45].

An example of a possible fractionation sequence is shown in Fig 2. Alongside the conventional use of bark as a solid biofuel, the *Picea abies* bark granulated material may be extracted by a solvent sequence and the obtained raw extracts purified to obtain bioactive compounds or chemicals, while the extracted solids may be either used for energy e.g. as biooils through liquefaction or pyrolysis, or used for carbohydrate oligomer production by e.g. hydrothermal treatments, and further delignified to yield lignin fractions and cellulose that can be directed to cellulose-based materials or bioethanol.

Conclusions

The industrial bark has a substantial amount of wood and extraneous material derived from harvesting, handling and debarking processes leading to specific features that have to be accounted for if this material is to be used in a biorefinery context.

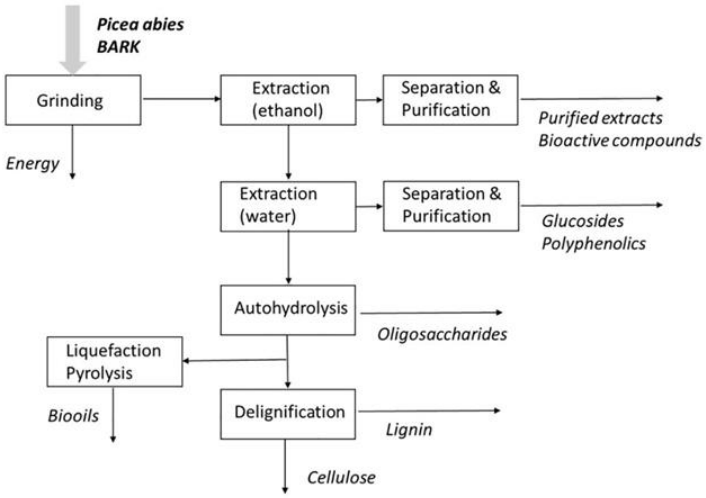

Fig 2. Example of possible fractionation sequence for Picea abies industrial bark biorefinery.

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Chemically, bark is very different from wood, presenting a much higher extractive content, with the crude polar fraction showing very good antioxidant activity, and has similar lignin and lower polysaccharides content. The polysaccharides are richer in hemicelluloses, especially in arabinose and galacturonic acid as opposing the mannose in the wood. Regarding energy properties, bark presented a lower volatiles to fixed carbon ratio and a more controlled thermal degradation than wood but the same calorific value.

As for the fractionation of the bark in different sized particles, the fractions had some chemical differences (the fine fraction was richer in ash, extractives and lignin, and lower in polysaccharides contents) but were very similar in thermal characteristics. The mechanical fractionation as a first processing step in a biorefinery seems therefore unnecessary due to its operational costs.

Overall, the chemical and thermal characteristics of *Picea abies* bark show a possible upgrade potential, whether by taking advantage of the high content in extractives with bioactive compounds or the high amount of hemicelluloses allowing production of oligomers for possible use in nutraceutical and pharmaceutical applications or production of biomaterials such as biofilms or adhesives, while the remaining solid residue may be used for biofuels, chemicals or direct energy production.

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Author Contributions

Conceptualization: Duarte M. Neiva, Jorge Gominho, Helena Pereira.

Data curation: Duarte M. Neiva, Solange Araújo, Jorge Gominho, Angélica de Cássia Carneiro.

Formal analysis: Duarte M. Neiva, Solange Araújo, Angélica de Cássia Carneiro, Helena Pereira.

Funding acquisition: Helena Pereira.

Investigation: Duarte M. Neiva, Solange Araújo, Jorge Gominho, Angélica de Cássia Carneiro.

Methodology: Duarte M. Neiva.

Project administration: Jorge Gominho, Helena Pereira.

Resources: Duarte M. Neiva, Solange Araújo, Jorge Gominho, Angélica de Cássia Carneiro, Helena Pereira.

Supervision: Jorge Gominho, Helena Pereira.

Validation: Duarte M. Neiva.

Visualization: Duarte M. Neiva, Helena Pereira.

Writing – original draft: Duarte M. Neiva, Helena Pereira.

Writing – review & editing: Duarte M. Neiva, Jorge Gominho, Helena Pereira.

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Paper 3:

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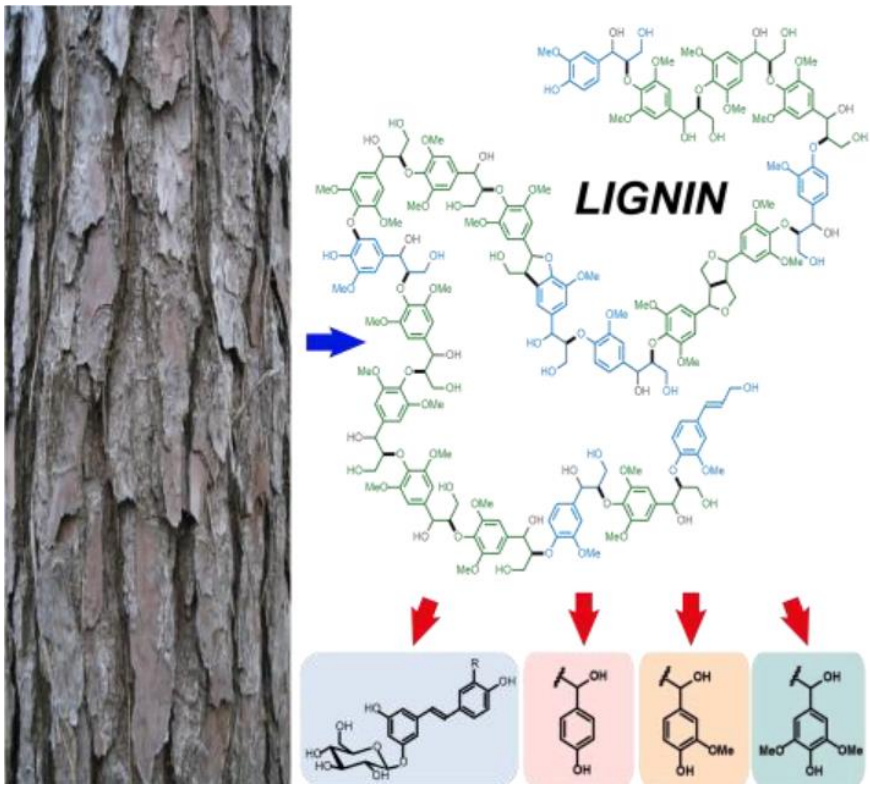

Graphical abstract of the paper

Lignin from Tree Barks: Chemical Structure and Valorization

Duarte M. Neiva[+,[a, b]] Jorge Rencoret[+,[a]] Gisela Marques,[a] Ana Gutiérrez,[a] Jorge Gominho,[b] Helena Pereira,[b] and José C. del Río*[a]

Lignins from different tree barks, including Norway spruce (*Picea abies*), eucalyptus (*Eucalyptus globulus*), mimosa (*Acacia dealbata*) and blackwood acacia (*A. melanoxylon*), are thoroughly characterized. The lignin from *E. globulus* bark is found to be enriched in syringyl (S) units, with lower amounts of guaiacyl (G) and *p*-hydroxyphenyl (H) units (H/G/S ratio of 1:26:73), which produces a lignin that is highly enriched in β-ether linkages (83%), whereas those from the two *Acacia* barks have similar compositions (H/G/S ratio of ≈5:50:45), with a predominance of β-ethers (73–75%) and lower amounts of condensed carbon–carbon linkages; the lignin from *A. dealbata* bark also includes some resorcinol-related compounds, that appear to be incorporated or intimately associated to the polymer. The lignin from *P. abies* bark is enriched in G units, with lower amounts of H units (H/G ratio of 14:86); this lignin is thus depleted in β-O-4′ alkyl–aryl ether linkages (44%) and enriched in condensed linkages. Interestingly, this lignin contains large amounts of hydroxystilbene glucosides that seem to be integrally incorporated into the lignin structure. This study indicates that lignins from tree barks can be seen as an interesting source of valuable phenolic compounds. Moreover, this study is useful for tailoring conversion technologies for bark deconstruction and valorization.

Introduction

The search for an alternative to replace fossil fuels for the production of chemicals, products, and energy has found in lignocellulosic biomass the most widespread and available source of renewable raw materials. With agricultural crops being mostly intended for food production, forest biomass and residues from both agricultural and forestry industries are seen as the most reliable sources of biomass for the production of biofuels, bioproducts, and value-added chemicals, especially if inserted in a biorefinery context with full resource valorization and a zero-waste philosophy.[1,2]

In this regard, tree barks, which are generated in significant amounts as waste from the wood industries (e.g., timber or pulp and paper industries) or as residues resulting from forest management, are considered potential upgradable side streams for value-added applications.[3,4] These widely available and low-cost residues are mostly used as solid fuel for the production of energy and heat or for horticultural use, despite the high chemical potential that this lignocellulosic biomass might offer. The rationale for valorization of bark is its high availability, chemical richness, and structural diversity, allowing for the targeting of multiple products. However, its higher complexity requires better knowledge and understanding of its composition and structure, and perhaps more demanding and adequate processing routes. Moreover, as tree barks contain significant amounts of lignin (sometimes with higher lignin content than their respective woods), bark deconstruction routes should target this abundant aromatic polymer for the production of fuels, chemicals and materials that are nowadays produced from fossil resources.[5,6] Many studies have focused on the deconstruction and uses of the different bark fractions,[3,4,7,8] but only a few have addressed in detail the characterization of the lignin fraction,[9–11] even though lignin is a major component in these forest wastes that can be valorized as a source of platform chemicals, biofuels, and biobased materials.

Lignin is a complex phenylpropanoid polymer that has a structural role in plant cell walls while also providing hydrophobicity and protection against pathogens. Lignin is synthesized by the oxidative radical polymerization of three main hydroxycinnamyl alcohols—*p*-coumaryl, coniferyl, and sinapyl al-

[a] D. M. Neiva,[+] Dr. J. Rencoret,[+] Dr. G. Marques, Prof. A. Gutiérrez, Prof. J. C. del Río
Department of Plant Biotechnology
Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS)
CSIC, Av. Reina Mercedes, 10, 41012-Seville (Spain)
E-mail: delrio@irnase.csic.es

[b] D. M. Neiva,[+] Prof. J. Gominho, Prof. H. Pereira
Centro de Estudos Florestais
Instituto Superior de Agronomia, Universidade de Lisboa
Tapada da Ajuda, 1349-017 Lisboa (Portugal)

[+] These authors contributed equally to this work.

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cohols (so-called monolignols)—to produce a branched backbone of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units.[12] Besides the three canonical monolignols, a growing number of other phenolic compounds have also been documented as behaving as true lignin monomers. These include phenolic compounds, such as monolignol ester conjugates, catechols, and methoxycatechols, arising from the truncated biosynthesis of monolignols or ferulate esters, among others. In addition, phenolic compounds derived from other biosynthetic pathways have also been found incorporated into the lignin of several plants. Such compounds include the flavone tricin, which is present in the lignins of grasses and other monocots,[13–15] the hydroxystilbenes piceatannol, isorhapontigenin, and resveratrol, which are incorporated into the lignin of palm fruit endocarps,[16,17] and their corresponding *O*-glucosylated counterparts (astringin, isorhapontin and piceid), which have recently been reported to be incorporated into the lignin of Norway spruce bark,[18] and the hydroxycinnamic amides tyramine ferulate, which is found in the lignins of some Solanaceae,[19] and diferuloylputrescine, which is found in the lignin of maize kernels.[20] These new additions to the family of lignin precursors show that this biopolymer is far more complex than previously thought, providing further evidence that any phenolic compound present in the lignifying zone of the cell wall can be incorporated into the lignin polymer through similar oxidative reactions.[12,21] More importantly, these discoveries greatly expand the range of valuable phenolic compounds that can be obtained from lignins, thus enhancing the value of what is considered a waste product of forestry and agricultural activities.

The overall lignin content and the composition and relative abundance of the different monomeric units, especially the S/G ratio and the distribution of the different linkage types and functional groups, are important parameters to understand the lignin structure, as well as its chemical properties and reactivities. Detailed knowledge of the lignin structure is a prerequisite to optimize and tailor the conditions for processing, aiming at lignocellulosic deconstruction for subsequent valorization of their components. In this sense, the present study focuses on the comprehensive structural characterization of lignins from the barks of a series of trees, including the softwood Norway spruce (*Picea abies*) and the hardwoods eucalyptus (*Eucalyptus globulus*), mimosa (*Acacia dealbata*), and blackwood acacia (*A. melanoxylon*). Eucalyptus and Norway spruce are representative of the major hardwood and softwood species used by the timber and pulp and paper industries in Europe, with bark accounting for roughly 10–15% of the bole mass,[7,8,22] which is generated as waste in large quantities at industrial sites. For the *Acacia* species, the continuous fight against these invasive species also generates large amounts of bark residues, since a common method to mitigate their proliferation is removing the bark without felling the tree, thus preventing sprouting from the stump. The detailed characterization of the lignin structure of these barks will be highly relevant for the further valorization of these abundant lignocellulosic wastes.

Results and Discussion

Composition of the main constituents of the barks

The abundances of the main constituents (namely, the contents of dichloromethane, ethanol and water extractives, Klason lignin, acid-soluble lignin, polysaccharides, and ash) of the different barks selected for this study are shown in Table 1. A wide diversity in the content of the different constituents was observed among all barks. In general terms, all barks had a high content of extractives, which were particularly prominent for *A. dealbata* bark, accounting for about 46% of the total bark, most of which were due to polar compounds. A high content of polar extractives has also been reported for other *Acacia* species, such as *A. mangium*, accounting for 38% of the total bark.[3] Great differences were also observed in the content of structural polysaccharides, with the barks of the two *Acacia* species showing the lowest amounts of structural polysaccharides (≈21–29%) when compared to *E. globulus* (≈61%) and *P. abies* (≈48%). One interesting feature regarding the composition of polysaccharides of *P. abies* bark was the higher content of xylose (≈5%) with respect to mannose (≈3%), which is in contrast to that found in its respective wood (≈7% xylose vs. ≈14% mannose) and what is commonly found in the woods of other conifers.[8] Regarding the lignin content, the bark from *A. melanoxylon* presented a very high value (≈55%), which might be the result of polyphenolics condensation during the Klason lignin determination procedure, implying that these compounds are intimately associated to the lignocellulosic matrix, even after successive extractions with dichloromethane, ethanol, and water. *A. dealbata* bark presented the lowest lignin content, which accounted for

Table 1. Abundance of the main constituents (wt% dry basis) of the different barks (average of three replicates). PA: *P. abies*; EG: *E. globulus*; AD: *A. dealbata*; AM: *A. melanoxylon*.

Components	PA[a]	EG[b]	AD	AM
Extractives				
dichloromethane	5.4±0.1	0.9±0.1	1.0±0.0	2.2±0.1
ethanol	4.5±0.3	2.3±0.1	37.5±0.3	5.9±0.2
water	10.3±1.3	6.6±0.2	7.8±0.8	6.1±0.7
Lignin				
Klason lignin	25.9±0.8	18.9±1.1	16.7±0.3	54.3±0.4
acid-soluble	0.9±0.1	3.0±0.1	1.9±0.1	1.0±0.1
Polysaccharides				
rhamnose	0.5±0.1	0.5±0.1	0.2±0.1	0.3±0.1
arabinose	5.2±0.4	1.6±0.1	1.9±0.1	2.2±0.1
galactose	2.0±0.1	1.6±0.1	1.2±0.1	1.5±0.1
glucose	26.8±0.1	37.5±1.6	19.0±0.6	12.4±0.1
xylose	4.6±0.2	15.2±0.2	3.7±0.7	2.2±0.1
mannose	2.6±0.1	0.4±0.1	0.6±0.2	0.5±0.1
galacturonic acid	5.6±0.1	1.7±0.1	0.5±0.1	0.8±0.2
glucuronic acid	0.3±0.1	0.1±0.1	0.6±0.1	0.8±0.1
acetic acid	0.4±0.1	2.7±0.1	0.9±0.1	0.6±0.1
Ash	3.9±0.1	5.4±0.2	3.3±0.1	5.6±0.1

[a] From reference [9]. [b] From reference [7].

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roughly 19%. The lignin contents in *P. abies* and *E. globulus* barks accounted for 26.8% and 21.9%, respectively. In general terms, the lignin content in barks was found to be higher than in their respective woods, as was also observed for other tree species, such as willow and cork oak.[11,23]

In this work, the structural characteristics of the lignins from the different barks were thoroughly addressed. For this, the "milled-bark" lignin (MBL) preparations were isolated according to the classical protocol,[24] and were subsequently analyzed by various techniques, including analytical pyrolysis, in the absence and in the presence of tetramethylammonium hydroxide (TMAH), derivatization followed by reductive cleavage (DFRC), and 2D NMR spectroscopy.

Lignin composition as determined by Py-GC/MS

The composition of the lignins isolated from the different barks was first addressed by pyrolysis–gas chromatography–mass spectrometry (Py-GC/MS; Figure 1). The identities and relative molar abundances of the released lignin-derived phenolic compounds are listed in Table 2. Significant differences were observed among the different lignins. The lignin from *P. abies* bark exhibited a composition typical of softwoods (Figure 1A), with the release of phenolic compounds derived mostly from G lignin units (≈78% of all phenolic compounds), including guaiacol (peak 2), 4-methylguaiacol (peak 4), 4-ethylguaiacol (peak 6), and 4-vinylguaiacol (peak 7), together with lower amounts of compounds derived from H lignin units (≈22% of all phenolic compounds), including phenol (peak 1), 4-methylphenol (peak 3), and 4-ethylphenol (peak 5). The lignin from *E. globulus* bark released phenolic compounds derived mostly from S lignin units (≈70%; Figure 1B), including syringol (peak 10), 4-methylsyringol (peak 13), 4-ethylsyringol (peak 16), 4-vinylsyringol (peak 18), syringaldehyde (peak 25), and acetosyringone (peak 26), together with lower amounts of compounds derived from G lignin units (≈28% of all phenolics) and from H lignin units (≈2% of all phenolics), with a S/G ratio of approximately 2.5. In the case of the barks from the two *Acacia* species, the pyrograms showed a similar distribution of lignin-derived phenolic compounds (Figure 1C,D), with a predominance of those derived from G lignin units (≈53–57%), alongside lower amounts of compounds derived from S lignin units (≈33–36%) and H lignin units (≈10–12%), and with similar S/G ratios of around 0.6–0.7. However, and surprisingly, the lignin from *A. dealbata* bark also released high amounts of resorcinol (Figure 1C, peak 15, accounting for ≈35% of all phenolic compounds), which is absent in the pyrograms of the other bark lignins. Resorcinol is a phenolic compound that is not derived from lignin units and its peculiar structure, with two hydroxy groups in *meta* position, suggests that it may derive from moieties with flavonoid/hydroxystilbene skeletons that might be incorporated or intimately associated to the lignin polymer. It is well known that some flavonoids, such as the flavone tricin, are incorporated into the lignins in grasses and other monocotyledons.[13–15] Likewise, hydroxystilbenes, particularly piceatannol, have been found incorporated into the lignins of palm fruit endocarps.[16,17] Resorcinol was released from the lignin of *A. dealbata* bark even after exhaustive extraction with different solvent systems, reinforcing the idea that it belongs to phenolic moieties that are strongly associated to the lignin polymer.

It has been reported that the lignins from other barks, such as that from cork oak (*Quercus suber*) bark, also include ferulates in their structure.[23,25] However, ferulates (and *p*-hydroxycinnamates in general) cannot be analyzed by Py-GC/MS, owing to decarboxylation during pyrolysis.[26,27] The occurrence of ferulates, and other *p*-hydroxycinnamates, in these lignins can, however, be evaluated by performing pyrolysis in the presence of tetramethylammonium (TMAH), a methylating reagent that prevents decarboxylation during pyrolysis and releases intact *p*-hydroxycinnamates (as their permethylated derivatives).[26,27] Figure 2 shows the chromatograms of the compounds released during Py-TMAH of the different lignins. The identities and relative molar abundances of the released compounds are listed in Table 3. The distribution of the lignin-derived compounds follows the same trend as those released by conventional pyrolysis. The lignin from *P. abies* bark released predominantly G lignin units with minor amounts of H lignin units, whereas the lignin from *E. globulus* bark released mostly S lignin units with lower amounts of G and H lignin units and the lignins from the two *Acacia* species released similar amounts of G and S lignin units, with minor amounts of H lignin units. More importantly, the Py-TMAH chromatograms (Figure 2) also showed the release of *p*-hydroxycinnamates (as their methyl derivatives), which are incorporated into these lignins, including the methyl derivatives of *p*-coumarates (peak 29, *p*CA), ferulates

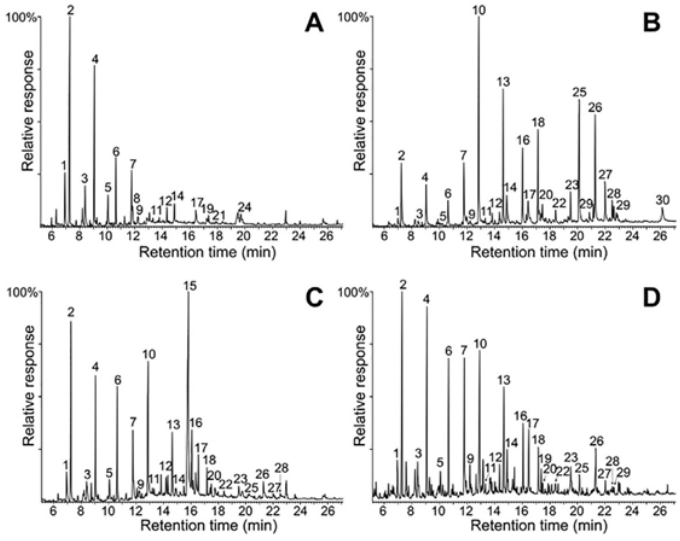

Figure 1. Py-GC/MS chromatograms of the MBLs isolated from the barks of *P. abies* (A), *E. globulus* (B), *A. dealbata* (C), and *A. melanoxylon* (D). The identities and relative abundances of the lignin-derived phenolic compounds released are listed in Table 2.

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Table 2. Identities and relative molar abundances of the lignin-derived phenolic compounds released after Py-GC/MS of the MBLs isolated from the different barks. PA: *P. abies*; EG: *E. globulus*; AD: *A. dealbata*; AM: *A. melanoxylon*.

Entry	Compound	PA	EG	AD	AM
1	phenol	10.2	1.0	3.7	5.0
2	guaiacol	32.8	6.9	11.2	15.2
3	4-methylphenol	6.3	0.9	2.2	3.1
4	4-methylguaiacol	19.9	4.4	6.6	12.5
5	4-ethylphenol	3.5	0.1	1.6	2.0
6	4-ethylguaiacol	6.6	2.5	6.1	8.4
7	4-vinylguaiacol	6.1	5.4	5.1	9.2
8	4-vinylphenol	2.0	0.0	0.0	0.0
9	eugenol	0.6	0.2	0.5	1.3
10	syringol	0.0	17.6	10.1	10.8
11	*cis*-isoeugenol	0.5	0.5	0.3	0.6
12	*trans*-isoeugenol	1.5	1.1	1.0	1.7
13	4-methylsyringol	0.0	9.5	4.1	7.0
14	vanillin	3.0	3.5	0.7	3.1
15	resorcinol	0.0	0.0	35.3	0.0
16	4-ethylsyringol	0.0	5.2	3.7	3.8
17	acetoguaiacone	3.5	2.2	1.7	3.8
18	4-vinylsyringol	0.0	6.6	1.3	2.8
19	guaiacylacetone	0.9	0.8	0.5	1.2
20	4-allylsyringol	0.0	1.0	0.5	0.5
21	propiovanillone	0.3	0.6	0.3	0.5
22	*cis*-4-propenylsyringol	0.0	1.0	0.4	0.7
23	*trans*-4-propenylsyringol	0.0	2.1	1.2	1.5
24	dihydroconiferyl alcohol	2.3	0.0	0.0	0.0
25	syringaldehyde	0.0	10.9	0.3	1.3
26	acetosyringone	0.0	8.1	1.1	2.7
27	syringylacetone	0.0	3.4	0.3	0.7
28	propiosyringone	0.0	1.2	0.3	0.3
29	syringyl vinyl ketone	0.0	0.7	0.1	0.3
30	*trans*-sinapaldehyde	0.0	2.6	0.0	0.0
	H [%]	22.0	2.0	11.7[a]	10.1
	G [%]	78.0	28.1	52.5[a]	57.4
	S [%]	0.0	69.9	35.9[a]	32.5
	S/G ratio	0.0	2.5	0.7	0.6

[a] Relative abundances calculated without resorcinol (Figure 1, peak 15).

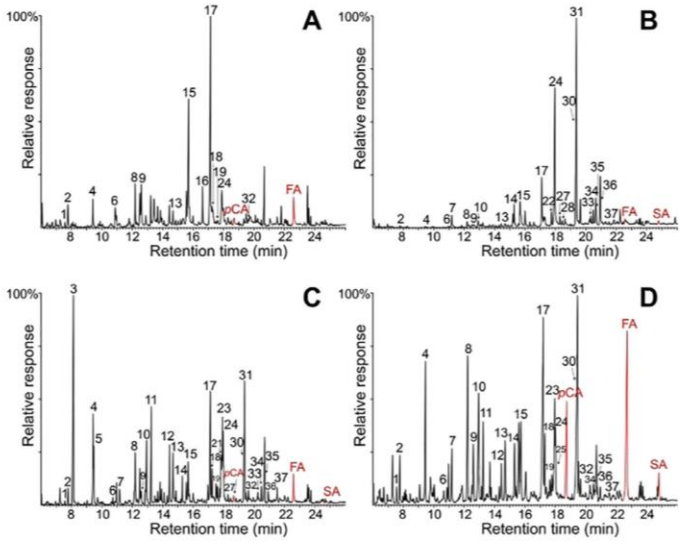

Figure 2. Py-TMAH-GC/MS chromatograms of the MBLs isolated from the barks of *P. abies* (A), *E. globulus* (B), *A. dealbata* (C), and *A. melanoxylon* (D). The identities and relative abundances of the lignin-derived phenolic compounds released are listed in Table 3.

(peak 38, FA), and sinapates (peak 39, SA), that were particularly abundant in the lignin from *A. melanoxylon* bark (accounting for ≈5% *p*CA, ≈10% FA, and ≈1% SA among all released compounds). The lignin from *E. globulus* bark released only trace amounts of ferulates and sinapates. It is important to remark the occurrence of *p*-hydroxycinnamates in the lignins of these tree barks, as they have not been reported in the lignins of their respective woods.

Py-TMAH of the lignin from *A. dealbata* bark (Figure 2C) also released significant amounts of other phenolic compounds (as their methyl derivatives) that derived neither from lignin nor from *p*-hydroxycinnamate moieties, such as 1,3-dimethoxybenzene (peak 3), 2,4-dimethoxytoluene (peak 5), and methyl 2,4-dimethoxybenzoate (peak 21), and which are absent from the rest of the lignins studied here. These compounds are structurally related to the resorcinol released by Py-GC/MS from the lignin of *A. dealbata* bark (Figure 1C and Table 2) and may arise from moieties with flavonoid/hydroxystilbenoid skeletons incorporated or intimately associated to the lignin polymer. The lignin from *P. abies* bark also released significant amounts of a compound that was not derived from lignin or *p*-hydroxycinnamates, namely methyl 3,5-dimethoxybenzoate (peak 16), which was absent from the rest of the lignins and that arose from hydroxystilbene moieties that are integrally incorporated into the lignin polymer (see below).

Analysis by derivatization followed by reductive cleavage

Additional information regarding the lignin monomeric units, as well as other phenolic units potentially incorporated into the lignin polymer, was obtained through chemical degradation by a method called derivatization followed by reductive cleavage (DFRC), a chemical degradative method that cleaves β-ether bonds in lignin and releases the corresponding lignin monomers involved in these linkages.[28] The chromatograms of the compounds released from these lignins are shown in Figure 3. The lignin from *P. abies* bark released the *cis* and *trans* isomers of the guaiacyl (*c*G and *t*G) lignin monomers (as their acetate derivatives), as corresponds to a conifer lignin, whereas the lignins from the barks of *E. globulus*, *A. dealbata*, and *A. melanoxylon* also released the *cis* and *trans* isomers of the syringyl (*c*S and *t*S) lignin monomers (as their acetate derivatives). Interestingly, the chromatogram of the DFRC degradation products released from *P. abies* bark also showed a series of peaks that were identified by comparison with authentic standards as the hydroxystilbenes resveratrol, isorhapontigenin, and piceatannol (peaks 1–3 in Figure 3A). The release of these compounds during DFRC indicates that at least a part of the hydroxystilbenes are incorporated into the lignin polymer of *P. abies* bark as β-ether-linked structures (those cleaved by the DFRC degradation method). Hydroxystilbenes have also been found incorporated into the lignins of other plant tissues, such as palm fruit endocarps, where they have been shown to behave as authentic lignin mono-

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Table 3. Identities and relative molar abundances of the compounds released after pyrolysis-TMAH of the MBLs isolated from the different barks. PA: *P. abies*; EG: *E. globulus*; AD: *A. dealbata*; AM: *A. melanoxylon*.

Entry	Compound	Origin	PA	EG	AD	AM
1	4-methoxystyrene	H	1.0	0.0	0.3	0.8
2	1,2-dimethoxybenzene	G	4.0	0.5	1.3	2.3
3	1,3-dimethoxybenzene	FL/ST	0.0	0.0	20.1	0.0
4	3,4-dimethoxytoluene	G	3.7	0.3	6.0	6.7
5	2,4-dimethoxytoluene	FL/ST	0.0	0.0	3.4	0.0
6	4-methoxybenzaldehyde	H	3.7	0.7	0.2	1.0
7	1,2,3-trimethoxybenzene	S	0.0	2.5	1.0	2.2
8	3,4-dimethoxystyrene	G	5.3	1.2	4.2	7.4
9	methyl 4-methoxybenzoate	H	6.2	0.9	1.1	2.8
10	3,4,5-trimethoxytoluene	S	0.0	1.4	4.0	5.8
11	1,3,5-trimethoxybenzene	FL/ST	0.0	0.0	6.4	3.1
12	2,4,6-trimethoxytoluene	FL/ST	0.0	0.0	3.4	1.7
13	3,4-dimethoxypropenylbenzene	G	1.9	0.8	3.3	3.3
14	3,4,5-trimethoxystyrene	S	0.0	3.3	1.3	2.6
15	3,4-dimethoxybenzaldehyde	G	22.1	8.8	3.2	5.0
16	methyl 3,5-dimethoxybenzoate	FL/ST	3.7	0.0	0.0	0.0
17	methyl 3,4-dimethoxybenzoate	G	28.5	8.6	7.3	11.6
18	3,4-dimethoxyacetophenone	G	2.2	1.7	2.0	2.4
19	1-(3,4-dimethoxyphenyl)-2-propanone	G	0.8	0.1	1.1	0.7
20	1-(3,4,5-trimethoxyphenyl)-1-propene	S	0.0	1.5	0.6	0.9
21	methyl 2,4-dimethoxybenzoate	FL/ST	0.0	0.0	3.9	0.0
22	*cis*-1-(3,4-dimethoxyphenyl)-2-methoxyethylene	G	2.5	1.2	0.9	1.0
23	methyl 3,4-dimethoxy-benzeneacetate	G	3.8	0.8	4.3	2.0
24	3,4,5-trimethoxy-benzaldehyde	S	0.0	23.9	3.4	3.8
25	*trans*-1-(3,4-dimethoxy-phenyl)-2-methoxyethylene	G	2.3	1.3	0.9	0.8
26	*cis*-1-(3,4-dimethoxyphenyl)-3-methoxyprop-1-ene	G	0.8	0.0	0.4	0.3
27	*cis*-1-(3,4-dimethoxy-phenyl)-1-methoxyprop-1-ene	G	0.9	1.5	0.5	0.2
28	*trans*-1-(3,4-dimethoxy-phenyl)-1-methoxyprop-1-ene	G	0.3	0.9	0.3	0.2
29	methyl *trans*-4-*O*-methyl-*p*-coumarate	*p*CA	1.0	0.0	0.7	5.0
30	3,4,5-trimethoxy-acetophenone	S	0.0	10.1	2.8	2.5
31	methyl 3,4,5-trimethoxy-benzoate	S	0.5	19.1	4.9	8.9
32	*trans*-1-(3,4-dimethoxy-phenyl)-3-methoxyprop-1-ene	G	0.7	0.0	0.7	0.7
33	1-(3,4,5-trimethoxyphenyl)-2-propanone	S	0.0	1.2	0.7	0.7
34	1-(3,4,5-trimethoxyphenyl)-2-methoxypropane	S	0.0	1.7	0.7	0.6
35	*cis*-1-(3,4,5-trimethoxy-phenyl)-2-methoxyethylene	S	0.0	2.1	1.1	0.7
36	*trans*-1-(3,4,5-trimethoxy-phenyl)-2-methoxyethylene	S	0.0	2.1	0.8	0.7
37	methyl 3,4,5-trimethoxy-benzeneacetate	S	0.0	0.8	1.0	0.5
38	methyl *trans*-4-*O*-methyl-ferulate	FA	4.0	0.5	1.7	10.3
39	methyl *trans*-4-*O*-methyl-sinapate	SA	0.0	0.6	0.1	1.0

H: *p*-hydroxyphenyl units; G: guaiacyl units; S: syringyl units; *p*CA: *p*-coumarates; FA: ferulates; SA: sinapates; FL/ST: flavonoids/stilbenoids.

mers.[16,17] Moreover, the chromatogram also showed the release of significant amounts of glucose (as its peracetate), which indicates that the hydroxystilbenes are incorporated into this lignin as their corresponding *O*-glucosides (Figure 3E), namely resveratrol-*O*-glucoside (piceid), isorhapontigenin-*O*-glucoside (isorhapontin), and piceatannol-*O*-glucoside (astringin).

The lignin from *A. dealbata* bark also released some phenolic compounds as their acetate derivatives (peaks 4–7 in Figure 3C), but their structures could not be fully established. The mass spectra of these compounds showed a molecular ion peak at *m/z* 440 and four consecutive losses of 42 mass units (fragments at *m/z* 398, 356, 314, and 272) that indicate the presence of four hydroxy groups (as acetates) in the structure (Figure 3F). The spectra are similar to those of acetylated tetrahydroxychalcones, but comparison with authentic standards of the common chalcones 2,3′,4,4′-tetrahydroxychalcone, 2′,3,4,4′-tetrahydroxychalcone (butein), and 2′,4,4′,6′-tetrahydroxychalcone (naringenin chalcone) ruled out this type of structure. Additional work is still in progress to fully identify the structure of these compounds, which might correspond to polyphenolic moieties incorporated or closely associated to the lignin from *A. dealbata* bark and that could be at the origin of the resorcinol released during Py-GC/MS and the related compounds released during Py-TMAH.

An interesting feature of the DFRC degradation method is that it cleaves β-ether linkages but leaves γ-esters intact, and therefore is also a powerful tool to identify monolignol ester conjugates with different acyl groups attached to the γ-OH of the lignin side chain, such as acetates and *p*-coumarates, which are common components in the lignins of many plants.[29–34] However, no traces of *p*-coumaroyl monolignol ester conjugates could be detected among the DFRC degradation products, most probably because they are below the detection limit, even in the case of the lignin from *A. melanoxylon* bark, which has the highest *p*-coumarate content, as indicated

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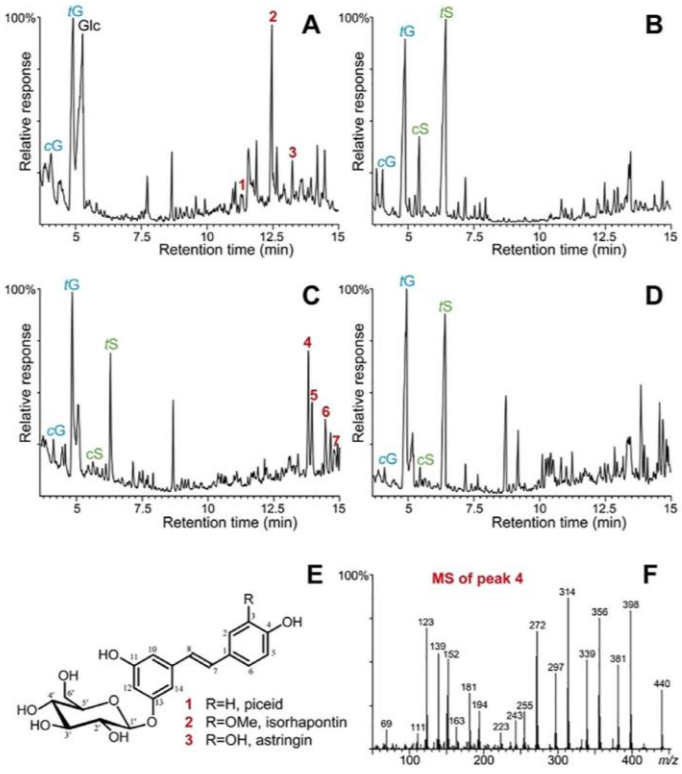

Figure 3. A–D) Chromatograms of the DFRC degradation products released from the lignins isolated from the barks of *P. abies* (A), *E. globulus* (B), *A. dealbata* (C), and *A. melanoxylon* (D). *c*G, *t*G, *c*S and *t*S are the normal *cis*- and *trans*-coniferyl (G) and sinapyl (S) alcohol monomers (as their acetate derivatives). Peaks in red color correspond to the different hydroxystilbene compounds: **1**: resveratrol; **2**: isorhapontigenin; **3**: piceatannol, as their acetyl derivatives. Glc: glucose (as the acetate derivative). Peaks 4–7 correspond to unidentified isomeric compounds with a molecular ion at *m/z* 440. E) Structures of the hydroxystilbene glucosides. F) mass spectrum of peak **4** released from the lignin of *A. dealbata* bark.

by Py-TMAH. Moreover, the lignin from some barks, such as that from cork oak bark, also showed significant levels of native acetates acylating the γ-OH.[23] To analyze the occurrence of native acetate groups attached to the γ-OH of the lignin side chain, the original DFRC protocol was slightly modified (so-called DFRC′) by replacing acetylating reagents for propionylating ones.[29,31] The chromatograms of the DFRC′ degradation products released from the lignins isolated from each of the barks are shown in Figure 4. The chromatograms show the release of originally γ-acetylated guaiacyl (cG_{ac} and tG_{ac}) and syringyl (cS_{ac} and tS_{ac}) lignin units, confirming that naturally occurring acetates acylate the γ-OH groups of these lignins, particularly in the lignin from *P. abies* bark, with up to 7% of acetylated G units. This finding was somewhat unexpected, as the lignins from conifer woods are not acetylated at the γ-OH,[31] and this is the first report of a lignin from a conifer tissue that shows significant levels of acetylation at the γ-OH. The lignin from *E. globulus* bark also showed some levels of acetylation of the lignin side chain, which occurred predominantly over the S lignin units (8% of the total S units are acetylated) whereas G units were barely acetylated (2% of the G units). This is the opposite to what occurred in the corresponding *E. globulus* wood, where G lignin units were preferentially acetylated.[31] In contrast, the lignins from the two *Acacia* barks were scarcely acetylated at the γ-OH.

Lignin structural units and interunit linkages as elucidated by 2D NMR

The lignins isolated from the different barks were also analyzed by 2D HSQC-NMR spectroscopy, which provided useful information regarding the lignin composition and the proportion of the different interunit linkages. The side-chain (δ_C = 50–98 ppm; δ_H = 2.5–6.8 ppm) and the aromatic/unsaturated (δ_C = 98–155 ppm; δ_H = 5.8–7.8 ppm) regions of the spectra are shown in Figure 5. The main lignin substructures found are displayed at the bottom.

The aromatic/unsaturated regions of the spectra showed signals from the aromatic rings and unsaturated side chains of the different H, G, and S lignin units, as well as from *p*-hydroxycinnamates (ferulates, FA, and *p*-coumarates, *p*CA) and cinnamaldehyde end groups (J). The lignin from *P. abies* bark showed signals from G and H lignin units, whereas the spectrum of the lignin from *E. globulus* bark showed signals from S and G lignin units, whereas signals from H units were barely detected. The lignins from the barks of the two *Acacia* species presented signals from all three S, G, and H lignin units. Signals from *p*-coumarates (*p*CA) and ferulates (FA) were only detected in the spectrum of the lignin from *A. melanoxylon* bark, corroborating the Py-TMAH data that indicated the occurrence of significant amounts of *p*-hydroxycinnamates in this lignin. Strong signals from cinnamaldehyde end groups (J) were also observed in the spectra of *E. globulus* and *A. melanoxylon* barks, and with lower intensity, in the lignin from *A. dealbata* bark.

However, the most remarkable feature in this region of the spectra was the presence of strong signals at around δ_C = 100–110 ppm/δ_H = 6.0–6.5 ppm in the spectra of the lignins from *P. abies* and *A. dealbata* barks, which are related to the atypical phenolic compounds released from these lignins by Py-GC/MS, Py-TMAH, and DFRC. In the case of *A. dealbata* bark, these signals (Figure 5, gray) are related to the resorcinol released during Py-GC/MS, the similar compounds released during Py-TMAH, and the still unknown phenolic compounds released during DFRC. However, extensive NMR analysis using different techniques (HSQC, HMBC, HSQC-TOCSY) failed to fully establish their structure. These signals seem to derive from polyphenolic moieties with flavonoid/hydroxystilbenoid skeletons, including condensed tannins, that are apparently incorporated or closely associated to the lignin polymer but whose structure remains elusive to us. However, in the case of the lignin from *P. abies* bark, the new signals (Figure 5, pink) could be unambiguously assigned (with the aid of authentic standards) to the hydroxystilbenes (principally isorhapontigenin, but also piceatannol and

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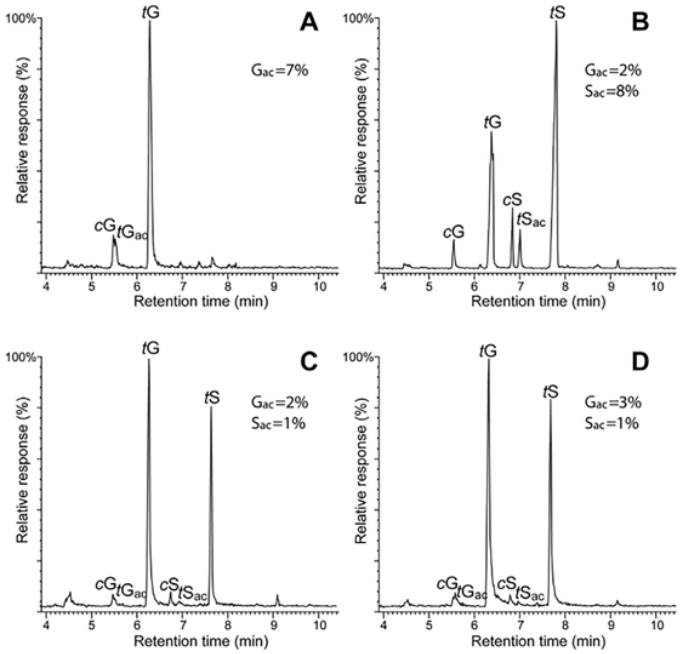

Figure 4. Reconstructed ion chromatograms (sum of the ions at *m/z* 222, 236, 252, and 266) of the DFRC′ degradation products released from the lignins isolated from the barks of *P. abies* (A), *E. globulus* (B), *A. dealbata* (C), and *A. melanoxylon* (D). *c*G and *t*G are the normal *cis*- and *trans*-coniferyl (G) alcohol monomers (as their propionylated derivatives; *m/z* 236); *c*S and *t*S are the normal *cis*- and *trans*-sinapyl (S) alcohol monomers (as their propionylated derivatives; *m/z* 266); tG_{ac} is the γ-acetylated *trans*-coniferyl (G) alcohol monomer (as the propionylated derivative; *m/z* 222); tS_{ac} is the γ-acetylated *trans*-sinapyl (S) alcohol monomer (as the propionylated derivative; *m/z* 252).

resveratrol) that were released during DFRC. These signals are similar to those previously observed in the spectra of the lignins from palm fruit endocarps, which were assigned to the hydroxystilbene piceatannol incorporated into the lignin structure.[16,17] In addition, the occurrence in the aliphatic–oxygenated region of the spectrum of strong signals from glucose at $\delta_C = 72.9$ ppm/$\delta_H = 3.17$ ppm (Glc_2), $\delta_C = 76.5$ ppm/$\delta_H = 3.24$ ppm (Glc_3 and Glc_5), $\delta_C = 69.3$ ppm/$\delta_H = 3.17$ ppm (Glc_4), and $\delta_C = 60.2$ ppm/$\delta_H = 3.79$–3.40 ppm (Glc_6), together with the occurrence of a signal for the linkage between the hydroxystilbene and the glucose moieties in the HMBC spectrum at $\delta_C \approx 158$ ppm/$\delta_H \approx 4.7$ ppm,[18] conclusively confirmed that these hydroxystilbenes are glucosylated and that the phenolic compounds that are incorporated into the lignin polymer are the corresponding *O*-glucosides, namely isorhapontin (isorhapontigenin-*O*-glucoside), astringin (piceatannol-*O*-glucoside), and piceid (resveratrol-*O*-glucoside). Piceid, astringin, and isorhapontin are known compounds occurring among the extractives from *P. abies* bark.[35–37] However, these compounds are highly soluble in water and other solvents and, as the bark was subjected to exhaustive extraction with different solvents (dichloromethane, ethanol and water) aimed at removing all the extractives prior to lignin isolation (and the MBL preparation was additionally exhaustively washed with different organic solvents), it is possible to assume that the hydroxystilbene glucosides observed are linked to the lignin by covalent bonds and do not correspond to residual free molecules. This assumption is also supported by the absence from the HSQC spectrum of signals from the unsaturated bonds that evidenced their participation in radical coupling reactions. In addition, exhaustive analysis by diffusion-ordered spectroscopy (DOSY) also confirmed that hydroxystilbene glucosides were integrally incorporated into the lignin polymer of *P. abies* bark.[19] Interestingly, the occurrence of significant amounts of hydroxystilbene glucosides in a 'milled bark tannin-lignin" fraction isolated from Norway spruce bark was previously reported, although the authors suggested without any experimental evidence that they were linked to the condensed tannin moiety instead of the lignin polymer.[9]

The aliphatic–oxygenated regions of the spectra gave information on the different interunit linkages present in lignin. The most prominent signals in this region of the spectra corresponded to typical lignin substructures (Figure 5), including signals from β-O-4′ alkyl–aryl ethers (**A**), β–5′ phenylcoumarans (**B**), β–β′ resinols (**C**), 5–5′ dibenzodioxocins (**D**), spirodienones (**F**), and cinnamyl alcohol end groups (**I**). In addition, the HSQC spectrum of the lignin from *P. abies* bark also showed other signals that were assigned to substructures involving hydroxystilbenes glucosides, including signals for a benzodioxane ($\mathbf{P_b}$) structure arising from 8-O-4′ coupling of two hydroxystilbene glucosides, signals for a phenylcoumaran structure ($\mathbf{P_c}$) involving 8–10′ coupling of two hydroxystilbene glucosides, as well as signals for a benzodioxane structure (**V**) formed by β-O-4′ cross-coupling of coniferyl alcohol and astringin, and that presented similar correlations to those signals observed for the incorporation of the hydroxystilbene piceatannol in the lignins of palm fruit endocarps.[16,17] The definitive assignments of the structures involving the incorporation of hydroxystilbene glucosides into the lignin polymer were attained by detailed HSQC-TOCSY and HMBC experiments, as already described.[18] The occurrence of these structures ($\mathbf{P_b}$, $\mathbf{P_c}$, **V**) conclusively demonstrates that hydroxystilbene glucosides behave as true lignin monomers in *P. abies* bark, participating in radical coupling reactions during lignification and being integrally incorporated into the lignin structure.

The relative abundances of the main lignin interunit linkages and end groups, as well as the abundances of the different lignin units (H, G, and S), *p*-hydroxycinnamates (*p*CA and FA), and hydroxystilbene glucosides (P) of the lignins from the different barks, estimated from volume integration in the HSQC spectra, are shown in Table 4. Important differences were found among the lignins from the different barks. The lignin from *P. abies* bark presented mostly G lignin units, with lower amounts of H lignin units (H/G ratio of 14:86), in agreement with the data obtained from Py-GC/MS. In addition, this lignin contained large amounts of hydroxystilbene glucosides (36 units per 100 aromatic lignin units), mostly isorhapontin, incorporated into its structure. This composition makes this

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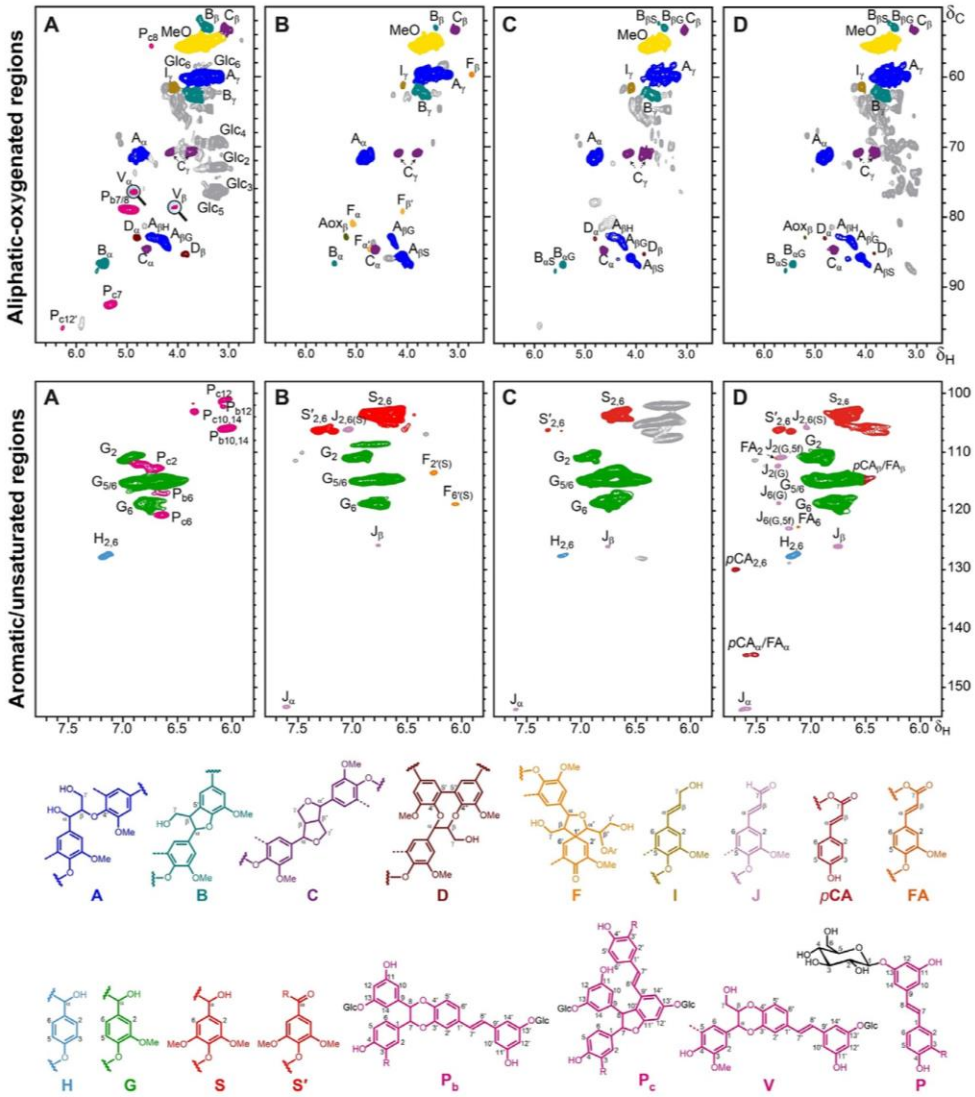

Figure 5. Side-chain (δ_C = 50–98 ppm; δ_H = 2.5–6.8 ppm) and aromatic (δ_C = 98–155 ppm; δ_H = 5.8–7.8 ppm) regions of the 2D HSQC-NMR spectra of the MBLs isolated from the barks of *P. abies* (A), *E. globulus* (B), *A. dealbata* (C), and *A. melanoxylon* (D). The main lignin structures identified are depicted at the bottom. **A**: β-O-4′ alkyl-aryl ethers; **B**: β-5′ phenylcoumarans; **C**: β-β′ resinols; **D**: 5-5′ dibenzodioxocins; **F**: β-1′ spirodienones; **I**: cinnamyl alcohol end groups; **J**: cinnamaldehyde end groups; ***p*CA**: *p*-coumarates; **FA**: ferulates; **H**: *p*-hydroxyphenyl units; **G**: guaiacyl units; **S**: syringyl units; **S′**: Cα-oxidized syringyl units; **P**: hydroxystilbene glucosides (isorhapontin, R = OCH_3; astringin, R = OH; piceid, R = H); $\mathbf{P_b}$: 8-O-4′/3′-O-7 benzodioxane structures involving isorhapontin (R = OCH_3), astringin (R = OH) or piceid (R = H) units; $\mathbf{P_c}$: 8–10′/11′-7 phenylcoumaran structures involving isorhapontin (R = OCH_3), astringin (R = OH) or piceid (R = H) units; **V**: β-O-4′/3′-O-α benzodioxane structure formed by cross-coupling of astringin (R = OH) and coniferyl alcohol.

lignin highly condensed, with a low abundance of β-O-4′ ether linkages (44% of all interunit linkages) and a high abundance of condensed linkages, mostly phenylcoumarans (20%), dibenzodioxocins (5%), resinols (4%), and other condensed linkages involving coupling of hydroxystilbene glucosides (benzodioxanes $\mathbf{P_b}$, 13%; phenylcoumarans $\mathbf{P_c}$, 12%; benzodioxanes **V**, 2%). By contrast, the lignin from *E. globulus* bark contained mostly S lignin units and lower amounts of G and H lignin units (H/G/S ratio of 1:26:73; S/G ratio of 2.8), in agreement with the Py-GC/MS data. This composition makes this lignin highly enriched in β-O-4′ ether linkages (83% of all interunit linkages) and depleted in condensed linkages, consisting mostly of resinols (8%), spirodienones (5%), and phenylcoumarans (4%). The lignins from *A. dealbata* and *A. melanoxylon* barks presented a rather similar composition, with a slight predominance of G over S lignin units (S/G ratio of 0.9) and with lower amounts of H units (≈4–5%). This composition produced a lignin with a high content of β-O-4′ linkages (≈73–75%), and with a lower abundance of condensed linkages, such as phenylcoumarans (12%), resinols (≈7–8%), dibenzo-

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Table 4. Structural characteristics (lignin interunit linkage types, end groups, aromatic units, and S/G ratio, *p*-hydroxycinnamate and hydroxystilbene contents) from volume integration of $^{1}H/^{13}C$ correlation signals in the HSQC spectra of the MBLs isolated from the different barks. PA: *P. abies*; EG: *E. globulus*; AD: *A. dealbata*; AM: *A. melanoxylon*.

Components	PA	EG	AD	AM
Lignin interunit linkages [%]				
β-O-4′ aryl ethers (**A**)	44	83	73	75
phenylcoumarans (**B**)	20	4	12	12
resinols (**C**)	4	8	8	7
dibenzodioxocins (**D**)	5	0	4	4
spirodienones (**F**)	0	5	3	2
benzodioxanes ($\mathbf{P_b}$)	13	0	0	0
phenylcoumarans ($\mathbf{P_c}$)	12	0	0	0
benzodioxanes (**V**)	2	0	0	0
Lignin end-groups[a][%]				
cinnamyl alcohol end-groups (**I**)	2	2	3	3
cinnamaldehyde end-groups (**J**)	0	4	2	6
Lignin aromatic units				
H [%]	14	1	5	4
G [%]	86	26	50	51
S [%]	0	73	45	45
S/G ratio	0	2.8	0.9	0.9
***p*-Hydroxycinnamates[b]**				
p-coumarates *p*CA [%]	0	0	0	4
ferulates FA [%]	0	0	0	4
Hydroxystilbene units P [%][b]	36	0	0	0

[a] Expressed as a fraction of the total lignin interunit linkage types **A**–**V**. [b] *p*-Coumarate, ferulate and hydroxystilbene contents are expressed as percentages of total lignin content (H + G + S = 100).

dioxocins (4%), and spirodienones (≈2–3%). The main differences among the lignins from the two *Acacia* barks were the occurrence of small amounts of *p*-coumarates and ferulates in the lignin from *A. melanoxylon* bark, that could not be detected in the spectrum of the lignin from *A. dealbata* bark (although *p*-coumarates and ferulates occurred at lower levels in this lignin, as indicated by Py-TMAH), as well as the occurrence of large amounts of still unknown polyphenolic compounds that are presumably incorporated or closely associated to the lignin of *A. dealbata* bark and are absent in the lignin from *A. melanoxylon* bark.

Bark lignin valorization potential

Lignin, the most abundant natural polymer with an aromatic skeleton, has long been considered a waste product of the pulping industry, although its combustion is important for the internal energy supply to the process. Nowadays, lignin is increasingly seen as an attractive renewable feedstock for producing chemicals, materials, and fuels that are currently obtained from fossil resources,[5,6] with a potential market value estimated at about 12 billion € by 2020–2025 for new lignin-based products.[38] Barks have the advantage of often presenting higher lignin contents than their respective woods, making them interesting raw materials for obtaining lignin for different uses. The best example is the bark of *A. melanoxylon*, with 55% lignin content, which represents 72% of the structural cell-wall components.

Although the variability and complexity of the lignin structure in barks can be seen as a limitation for their industrial extraction and subsequent transformation and utilization, it can also provide a wide range of possibilities and specific end uses for each of the lignin polymers for the production of different phenolic compounds for diverse chemical and pharmaceutical industries. The high S/G ratio of the lignin from *E. globulus* bark, together with its enrichment in β-ether units indicates a more reactive and easier to depolymerize lignin that will result in a higher yield of monomers; on the other hand, the enrichment of G lignin units in the lignin from *P. abies* bark, despite being a more condensed lignin and, therefore, more difficult to depolymerize, could be advantageous for example for the production of high added-value compounds such as vanillin for use in the flavor and fragrance industry or for the synthesis of epoxy resins.[39,40]

The significant content of *p*-hydroxycinnamates in the lignin from *A. melanoxylon* bark, which have not been described in lignins from woods, could make this bark an unconventional source of *p*-hydroxycinnamates, which are mostly produced from grass lignins. More important is the occurrence of significant amounts of hydroxystilbene glucosides that are integrally incorporated into the lignin of *P. abies* bark, which makes this an interesting feedstock for obtaining highly valuable hydroxystilbenes. Bark lignins enriched in resorcinol moieties, such as that from *A. dealbata* could serve as raw materials for the production of resorcinol–formaldehyde resin, which is mainly used as thermosetting binders for wood. Resorcinol is considerably more reactive than phenol, but it is less used in resins preparation, owing to its higher cost. In this sense, *A. dealbata* bark lignin, after undergoing a thermochemical depolymerization process, could be considered a valuable raw material for the industrial preparation of resorcinol-containing resins for wood adhesives or other commercial uses, such as semiconductor photocatalysts[41] and organic aerogels.[42] Finally, as occurs with lignins from other sources, the aromatic nature of bark lignins makes them suitable materials to mimic and replace phenol in polyurethane (PU) formulation and in phenol–formaldehyde (PF) resins for adhesives formulation in medium density fiberboards (MDF) manufacture.[43,44]

Conclusions

This study provided a comprehensive characterization of the lignins from the barks of several species, including the softwood *P. abies*, and the hardwoods *E. globulus*, *A. dealbata*, and *A. melanoxylon*. A wide diversity in the content, composition, and structure of the lignin polymers was observed among the different barks. This knowledge will be of great help for the development of efficient conversion technologies of these lignocellulosic materials, that have been considered as waste and will aid in their full valorization. The occurrence in some of these lignins of phenolic compounds that are different from the traditional monolignols, such as the hydroxystilbene glucosides present in the lignin of *P. abies* or the still unknown poly-

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phenolic compounds apparently incorporated into the lignin of *A. dealbata*, expands the range of products that can be obtained from these lignins, thus enhancing the value of these waste materials that are produced in high abundance at low cost by forestry operations and by the timber and pulp and paper industries.

Experimental Section

Samples

Barks from *P. abies* and *E. globulus* were collected after debarking in industrial sites at a sawmill near Jyvaskyla, Finland, and at The Navigator Company pulp mill located in Setúbal, Portugal, respectively. Both samples were manually sorted to remove wood contamination from the debarking process. The barks from the two Acacia trees were collected directly from trees at Sintra (*A. melanoxylon*, ≈40 year-old specimens) and Buçaco (*A. dealbata*, ≈6 year-old specimens), Portugal. The barks were air-dried, knife milled and successively Soxhlet extracted with dichloromethane (2 L, 24 h), ethanol (2 mL, 24 h) and water (2×2 mL, 24 h). The Klason lignin content was determined in the extractive-free material following the TAPPI standards T222 om-88 (and corrected for ash and protein contents), whereas the acid-soluble lignin was determined spectrophotometrically following the TAPPI method UM250 om-83. The composition of polysaccharides was determined in the Klason lignin hydrolysates as neutral monosaccharides, glucuronic acid, galacturonic acid, and acetates through separation by a Dionex ICS-3000 High Pressure Ion Chromatographer, using an Aminotrap plus Carbopac SA10 column. All chemical analyses were made in triplicate.

Lignin isolation

The "milled-bark" lignin (MBL) preparations were obtained from extractive-free samples using ball-milling conditions, as previously described.[33] The ball-milled materials (≈80 g) were extracted with 90:10 *v/v* dioxane/water mixture (2 L) under continuous stirring in the dark for 12 h. The solution was centrifuged and the supernatant, which contained the lignin, was then collected by decantation. This extraction process was repeated three times, using fresh dioxane/water mixture each time, and the supernatants combined. Crude lignins were obtained after removal of the solvent on a rotary evaporator at 40 °C and the isolated lignins were subsequently purified as described previously.[33] The yields of the crude MBLs (calculated as the percentage of the Klason lignin content) were 37% (*P. abies*), 21% (*E. globulus*), 49% (*A. dealbata*), and 11% (*A. melanoxylon*).

Analytical pyrolysis

Pyrolysis of the lignins was performed at 500 °C (1 min) in a 3030 micro-furnace pyrolyzer (Frontier Laboratories Ltd., Fukushima, Japan) connected to a GC 7820A (Agilent Technologies, Inc., Santa Clara, CA) and an Agilent 5975 mass-selective detector. The column used was a 30 m×0.25 mm i.d., 0.25 μm film thickness, DB-1701 (J&W Scientific, Folsom, CA). The GC oven was heated from 50 °C to 100 °C at 20 °C min^{-1} and then ramped to 280 °C at 6 °C min^{-1} and held for 5 min. Helium (1 mL min^{-1}) was used as the carrier gas. For the pyrolysis in the presence of tetramethylammonium hydroxide (Py-TMAH), the lignins were mixed with a droplet of TMAH (25 wt% in methanol) prior the pyrolysis. The released compounds were identified by comparison of their mass spectra with those present in the NIST and Wiley mass spectral libraries and by comparison with reported data.[45]

Derivatization followed by reductive cleavage (DFRC)

DFRC degradation was performed according to the classical procedure,[28] and the details have been described previously.[33] Briefly, the lignin (≈10 mg) was first treated with 8:92 *v/v* acetyl bromide/acetic acid mixture (2.5 mL) under stirring (2 h, 50 °C), after which it was dried. Powdered Zn (50 mg) and a 5:4:1 *v/v/v* dioxane/acetic acid/water mixture (2.5 mL) were added and allowed to react for 40 min at room temperature. The liquid phase was removed, treated with saturated ammonium chloride solution (3 mL) and then extracted with dichloromethane (10 mL, then 2×5 mL). After evaporating the organic phase to dryness, the lignin degradation products were acetylated with acetic anhydride/pyridine prior to analysis by GC-MS. To evaluate the occurrence of native acetate groups attached to the lignin, the original DFRC method was slightly modified by using propionylating reagents (denoted as DFRC′) instead of acetylating ones, as previously described.[29,31] The DFRC and DFRC′ lignin degradation products were analyzed by on a Saturn 4000 GC-MS apparatus (Varian, Walnut Creek, CA). The column used was a 12 m×0.25 mm i.d., 0.1 μm film thickness, DB5-HT (J&W Scientific, Folsom, CA). Helium (2 mL min^{-1}) was used as the carrier gas. The samples were injected directly onto the column by using a septum-equipped programmable injector (Varian 8200 autosampler, Varian, Folsom, CA) that was heated from 120 °C (0.1 min) to 330 °C at a rate of 200 °C min^{-1} and held until the end of the analysis. The GC oven was heated from 120 °C (1 min) to 380 °C (10 min) at a rate of 10 °C min^{-1}. The GC-MS transfer line was set to 300 °C.

NMR spectroscopy

2D NMR spectra were recorded on an AVANCE III 500 MHz instrument (Bruker, Karlsruhe, Germany) fitted with a cryogenically cooled 5 mm TCI gradient probe with inverse geometry, at the NMR facilities of the General Research Services of the University of Seville (SGI-CITIUS). The MBL sample (≈40 mg) was dissolved in [D_6]DMSO (0.75 mL). The residual DMSO signal (δ_C = 39.5 ppm; δ_H = 2.49 ppm) was used as the internal reference. The HSQC experiments used the Bruker standard pulse programs "hsqcetgpsisp2.2". The detailed NMR experimental conditions were described previously,[46] and the signals were assigned according to reported values.[13,18,33,46,47] Quantifications of lignin units and interunit linkages were performed as described previously.[10,33] Briefly, the signals used to quantify the relative abundances of the aromatic units were $H_{2,6}$, G_2, $S_{2,6}$, $pCA_{2,6}$, FA_2, and P_{c12}/P_{b12}—as signals $H_{2,6}$, $S_{2,6}$, and $pCA_{2,6}$ involve two proton–carbon pairs, their volume integrals were halved. The various interunit linkages were quantified via the volume integrals of the $\mathbf{A}_\alpha$, $\mathbf{B}_\alpha$, $\mathbf{C}_\alpha$, $\mathbf{D}_\alpha$, $\mathbf{F}_\alpha$, $\mathbf{P}_{b7}$, $\mathbf{P}_{c7}$, and $\mathbf{V}_\alpha$ correlation signals. The relative abundances of cinnamyl alcohol end groups (**I**) were estimated by integration of the signal $\mathbf{I}_\gamma$, which was also halved as it involves two proton–carbon pairs, whereas the abundances of cinnamaldehyde end groups (**J**) was determined by integration of the signal $\mathbf{J}_\alpha$ and comparing that with $\mathbf{I}_\alpha$.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biomass · NMR spectroscopy · phenol · pyrolysis · renewable resources

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Paper 4

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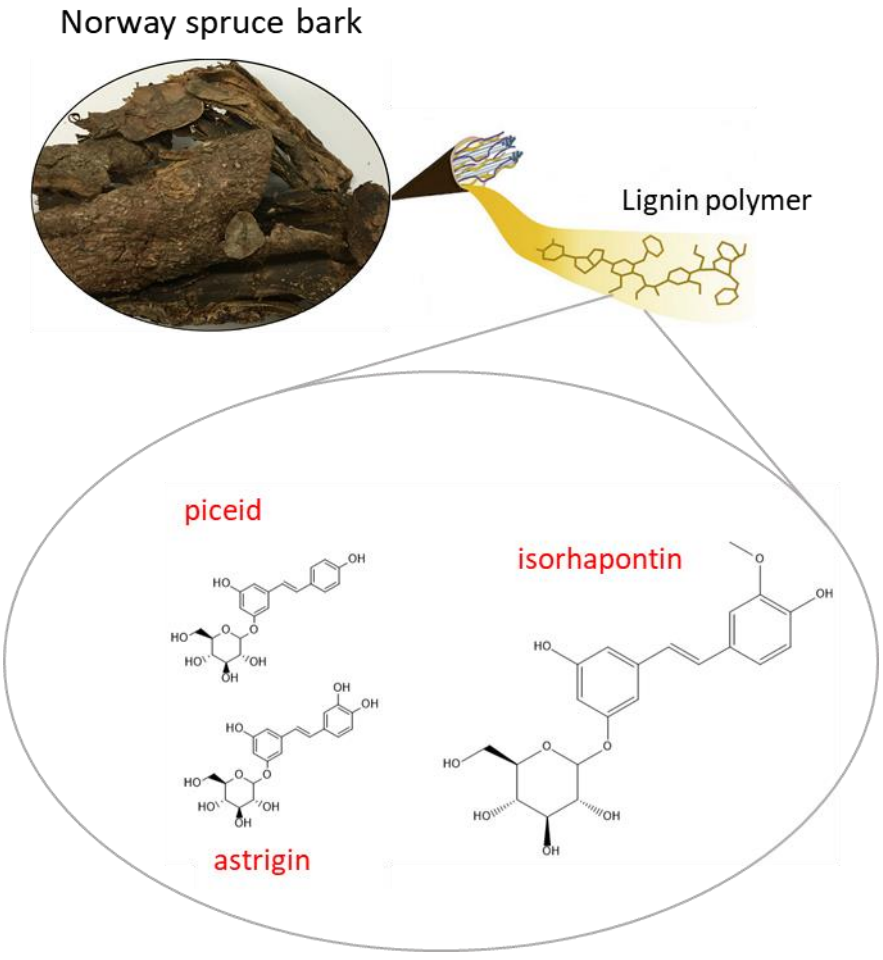

Graphical abstract of the paper

Hydroxystilbene Glucosides Are Incorporated into Norway Spruce Bark Lignin[1][OPEN]

Jorge Rencoret,[a] Duarte Neiva,[b] Gisela Marques,[a] Ana Gutiérrez,[a] Hoon Kim,[c,d] Jorge Gominho,[b] Helena Pereira,[b] John Ralph,[c,d] and José C. del Río[a,2,3]

[a]Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS), CSIC, Avda. Reina Mercedes, 10, 41012-Seville, Spain

[b]Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal

[c]Department of Energy Great Lakes Bioenergy Research Center, the Wisconsin Energy Institute, University of Wisconsin-Madison, Madison, Wisconsin 53726

[d]Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706

ORCID IDs: 0000-0003-2728-7331 (J.Re.); 0000-0002-5913-5976 (D.N.); 0000-0002-6431-8267 (G.M.); 0000-0002-8823-9029 (A.G.); 0000-0001-7425-7464 (H.K.); 0000-0003-3419-6075 (J.G.); 0000-0002-5393-4443 (H.P.); 0000-0002-6093-4521 (J.Ra.); 0000-0002-3040-6787 (J.C.d.R.).

Recent investigations have revealed that, in addition to monolignols, some phenolic compounds derived from the flavonoid and hydroxystilbene biosynthetic pathways can also function as true lignin monomers in some plants. In this study, we found that the hydroxystilbene glucosides isorhapontin (isorhapontigenin-*O*-glucoside) and, at lower levels, astringin (piceatannol-*O*-glucoside) and piceid (resveratrol-*O*-glucoside) are incorporated into the lignin polymer in Norway spruce (*Picea abies*) bark. The corresponding aglycones isorhapontigenin, piceatannol, and resveratrol, along with glucose, were released by derivatization followed by reductive cleavage, a chemical degradative method that cleaves β-ether bonds in lignin, indicating that the hydroxystilbene glucosides are (partially) incorporated into the lignin structure through β-ether bonds. Two-dimensional NMR analysis confirmed the occurrence of hydroxystilbene glucosides in this lignin, and provided additional information regarding their modes of incorporation into the polymer. The hydroxystilbene glucosides, particularly isorhapontin and astringin, can therefore be considered genuine lignin monomers that participate in coupling and cross-coupling reactions during lignification in Norway spruce bark.

Lignin is a complex aromatic polymer derived essentially from the oxidative coupling of three monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols (Boerjan et al., 2003; Ralph et al., 2004). Other phenolic compounds, including monolignol ester conjugates (with acetates, *p*-hydroxybenzoates, *p*-coumarates, or ferulates) or compounds derived from the truncated biosynthesis of monolignols (such as caffeyl alcohol or 5-hydroxyconiferyl alcohol and the hydroxycinnamaldehydes), have also been widely found to act as true lignin monomers in the lignins of many plants (Ralph et al., 1994, 2019; del Río et al., 2007, 2008, 2015; Chen et al., 2012, 2013; Rencoret et al., 2013; Tobimatsu et al., 2013; Lu et al., 2015; Karlen et al., 2016).

Recent investigations have shown that phenolic compounds derived from other biosynthetic pathways can also behave as true lignin monomers participating in radical coupling reactions and becoming integrally incorporated into the lignin polymer. The flavone tricin was the first phenolic derived from outside the monolignol biosynthetic pathway to be implicated in lignification (del Río et al., 2012; Lan et al., 2015). Tricin was first discovered in lignin preparations from wheat (*Triticum durum*) straw (del Río et al., 2012), and further studies indicated that it is widely present in the lignins

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[2]Author for contact: delrio@irnase.csic.es.

[3]Senior author.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: José C. del Río (delrio@irnase.csic.es).

J.C.d.R. conceived the study; D.N. and H.P. obtained the initial samples; J.Re., D.N., and G.M. isolated the lignins and performed the experiments; J.C.d.R. and J.Re. discovered the hydroxystilbene glucosides incorporated into the lignin; A.G., H.K., J.G., H.P., and J.Ra. contributed to the experimental design and the discussion of the results; J.C.d.R. wrote the paper; all authors critically reviewed the manuscript.

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of all grasses as well as in other monocots (Rencoret et al., 2013; del Río et al., 2015; Lan et al., 2016a, 2016b) and can be manipulated biogenetically (Eloy et al., 2017; Lam et al., 2017). More recently, we also reported the occurrence in macaúba (*Acrocomia aculeata*), carnauba (*Copernicia prunifera*), and coconut (*Cocos nucifera*) palm fruit endocarps of a second class of polyphenolic compounds, hydroxystilbenes (piceatannol, resveratrol, and isorhapontigenin), that behave as authentic lignin monomers participating in coupling and cross-coupling reactions during lignification and becoming integrally incorporated into the lignin structure (del Río et al., 2017; Rencoret et al., 2018). Flavonoids and hydroxystilbenes, unlike the monolignols that derive from the shikimate biosynthetic pathway, are metabolic hybrids deriving from a combination of the shikimate and acetate/malonate-derived polyketide pathways. These phenolic metabolites are known to participate in oxidative radical cross-coupling reactions with monolignols to produce 'nonconventional' lignans (termed flavonolignans and stilbenolignans) that have two phenylpropanoid units linked together through a diversity of linkages (Begum et al., 2010; Chambers et al., 2015). The wide natural occurrence in plants of these types of hybrid compounds arising from the cross-coupling with monolignols is an indication that at least some members of the flavonoids and hydroxystilbenes are also compatible with lignification.

In this paper, we provide evidence that hydroxystilbene glucosides are present in the lignin of Norway spruce (*Picea abies*) bark, participate in radical coupling reactions, and are integrally incorporated into the lignin structure. Part of the importance of this finding lies in their being the first examples of the authenticated incorporation of phenolic glycosides into lignins. Glucosides have been purported to exist in certain lignins, but it was not clear how they could be present as such. After all, if a phenol is protected with a glucoside, it simply cannot be oxidized to a radical and therefore cannot participate in lignification. In principle, it is possible for phenolic endgroups in lignin to be glucosylated after polymerization. However, this possibility is regarded with skepticism, as it is not easy for a large enzyme to penetrate the hydrophobic lignin polymer domain, and it is unclear what the purpose of phenolic glucosylation of the polymer would be. In this study, we propose an elegant solution to this conundrum by demonstrating that the glucosylated phenolic group is not one that would normally participate in radical coupling, and that another phenolic-OH participates in radical coupling in these novel monomers.

RESULTS AND DISCUSSION

Release of Hydroxystilbenes by Derivatization Followed by Reductive Cleavage

The 'milled wood lignin' (MWL) preparation obtained from Norway spruce bark was first analyzed by derivatization followed by reductive cleavage (DFRC), a chemical degradative method that selectively cleaves β-ether linkages in lignin, releasing the corresponding lignin monomers involved in these linkages (Fig. 1). The lignin released the cis- and trans-isomers of the guaiacyl (*c*G and *t*G) lignin monomers (as their acetylated derivatives), as is typical from a conifer lignin. More importantly, the chromatogram also indicated the occurrence of substantial amounts of a peak that was identified as isorhapontigenin 2 by comparison with the retention time and mass spectrum of an authentic standard. Minor amounts of the related resveratrol 1 and piceatannol 3 were also identified among the DFRC degradation

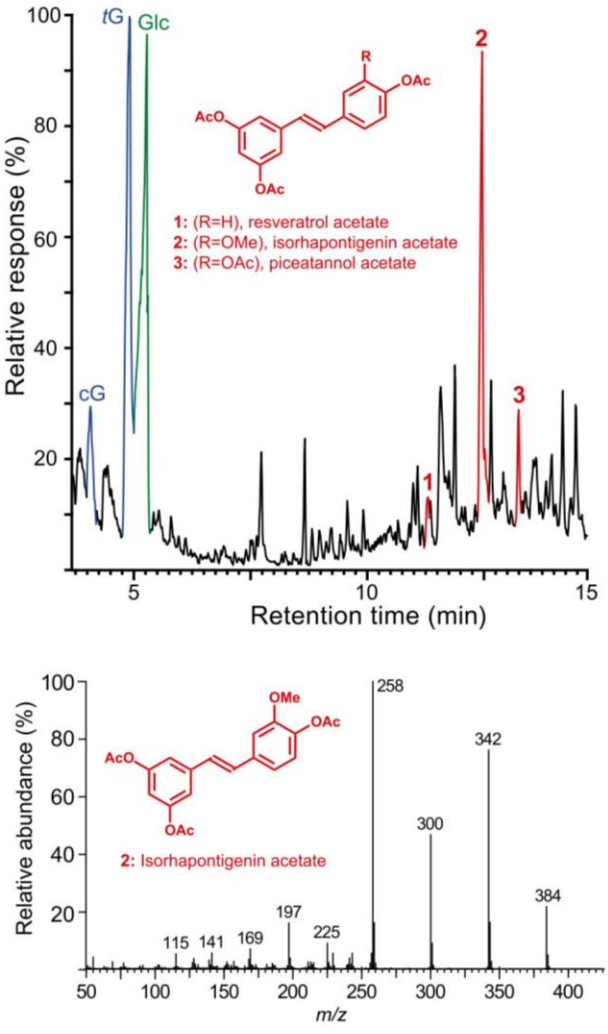

Figure 1. Release of hydroxystilbenes from the lignin of Norway spruce bark by reductive cleavage. Top: Total-ion chromatogram of the DFRC degradation products released from the MWL lignin isolated from Norway spruce bark, showing the presence of the hydroxystilbenes resveratrol 1, isorhapontigenin 2, and piceatannol 3, as their acetate derivatives. *c*G and *t*G are the cis- and transconiferyl alcohol monomers (as their acetate derivatives). Note the occurrence of a peak from glucose Glc (as its peracetylated derivative). Bottom: Electron-impact mass spectrum of isorhapontigenin acetate 2.

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products. The release of these compounds indicates that hydroxystilbenes are present in the lignin of Norway spruce bark. Hydroxystilbenes, particularly piceatannol, have been recently found incorporated into the lignins of other plant tissues, such as palm fruit endocarps, where they behave as true lignin monomers (del Río et al., 2017; Rencoret et al., 2018). Interestingly, the chromatogram also showed the release of important amounts of a compound that was identified as glucose (as its acetate), which seemed to indicate that the hydroxystilbenes were incorporated into the lignin as the corresponding hydroxystilbene glucosides, the so-called isorhapontin (isorhapontigenin-*O*-glucoside), astringin (piceatannol-*O*-glucoside), and piceid (resveratrol-*O*-glucoside; Fig. 2); it is evident that the phenolic glucosides were cleaved under the acidic conditions of DFRC releasing glucose and the corresponding hydroxystilbene aglycones. The DFRC data, therefore, indicated that at least a fraction of the hydroxystilbene glucosides, particularly isorhapontin, were incorporated into the lignin of Norway spruce bark as β-ether linked structures, the ones cleaved by the DFRC degradation method.

The hydroxystilbene glucosides piceid, astringin, and isorhapontin are known to occur in the extracts of Norway spruce bark (Hammerbacher et al., 2011; Latva-Mäenpää et al., 2013; Mulat et al., 2014). However, these compounds are highly soluble in water, and because the bark was subjected to exhaustive extraction with various solvents (dichloromethane, ethanol, and water) aimed at removing all of these hydroxystilbene glucosides and other extractives before lignin isolation (and the MWL preparation was additionally exhaustively washed with different organic solvents), it is reasonable to contend that the hydroxystilbene glucosides observed are linked to the lignin by covalent bonds and do not contaminate the lignin as free compounds.

Mode of Incorporation of Hydroxystilbene Glucosides into the Lignin as Determined by Two-dimensional NMR

Additional information regarding the composition and structure of the lignin isolated from Norway spruce bark, including the mode of incorporation of the hydroxystilbene glucosides into the lignin polymer, was obtained from two-dimensional heteronuclear single-quantum coherence NMR (2D-HSQC-NMR; Fig. 3). The aromatic region of the spectrum (Fig. 3B) gave information regarding the different lignin and hydroxystilbene units present in the lignin preparation. The signals for the C_2/H_2, C_5/H_5, and C_6/H_6 correlations from G-lignin units were observed in this region of the spectrum, clearly indicating the occurrence of a G-rich lignin in Norway spruce bark, as corresponds to a lignin from a conifer. Minor amounts of signals from $C_{2,6}/H_{2,6}$ and $C_{3,5}/H_{3,5}$ correlations of H-lignin units were also observed. Signals from hydroxystilbenes were also clearly present in this part of the spectrum in the region at $\delta_C/\delta_H \sim 100\text{-}108/5.9\text{-}6.5$, and were similar to those from the hydroxystilbenes observed in the 2D-HSQC-NMR spectra of lignins from palm fruit endocarps (del Río et al., 2017; Rencoret et al., 2018), including signals from isorhapontigenin and piceatannol, and confirming the results observed by DFRC.

The aliphatic-oxygenated region of the spectra (Fig. 3A) gave information about the different substructures, characterized by their diagnostic interunit linkages, present in the lignin. In this region, typical signals from lignin substructures, including the correlation signals from β–*O*–4′ alkyl-aryl ethers A, β–5′ phenylcoumarans B, β–β′ resinols C, 5–5′ dibenzodioxocins D, and cinnamyl alcohol end-groups I, were clearly observed. Moreover, the signals for the C_2/H_2 (Glc_2), C_3/H_3(Glc_3), C_4/H_4(Glc_4), C_5/H_5 (Glc_5), and C_6/H_6 (Glc_6) correlations of glucose units were also clearly visible in this region of the spectrum, confirming the occurrence of the hydroxystilbene glucosides, already advanced by DFRC. The linkage between the hydroxystilbenes and the glucose moiety was definitively proved by long-range correlation experiments; the heteronuclear multiple-bond correlation (HMBC) spectrum (Fig. 4) clearly demonstrated that the glucose moiety is linked to a phenolic unit of the resorcinol part of the hydroxystilbenes, indicating the occurrence of isorhapontin, astringin, and piceid, the respective glucosides of isorhapontigenin, piceatannol, and resveratrol. As noted above, we can discard the occurrence of free hydroxystilbene glucosides in the lignin preparation as these compounds are highly soluble in water and other solvents, and have been removed during the exhaustive solvent extraction before the MWL isolation protocol. This contention is also compellingly supported by the absence of signals from double bonds between 7- and 8-position (or 7′- and 8′-position) in the 2D-HSQC-NMR spectrum, a feature evidencing their participation in radical coupling reactions.

Most importantly, signals from structures involving the coupling of hydroxystilbene glucosides were found in the HSQC spectrum (Fig. 3). Signals for benzodioxane (P_b) and phenylcoumaran structures (P_c) involving coupling of two hydroxystilbene glucosides, and signals for benzodioxane structures (V) formed by cross-coupling of astringin and coniferyl alcohol, presented

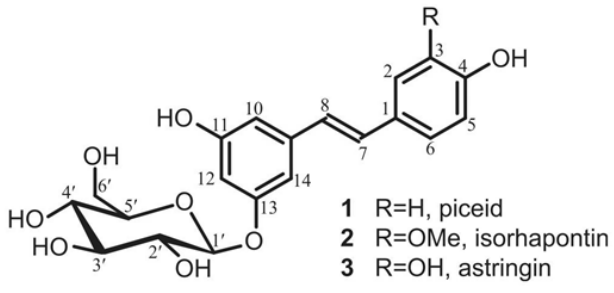

Figure 2. Structure of the hydroxystilbene glucosides piceid 1, isorhapontin 2, and astringin 3; for convenience, glucose has been drawn linked to C-13 of the hydroxystilbene instead of C-11, as the latter OH participates in some coupling structures.

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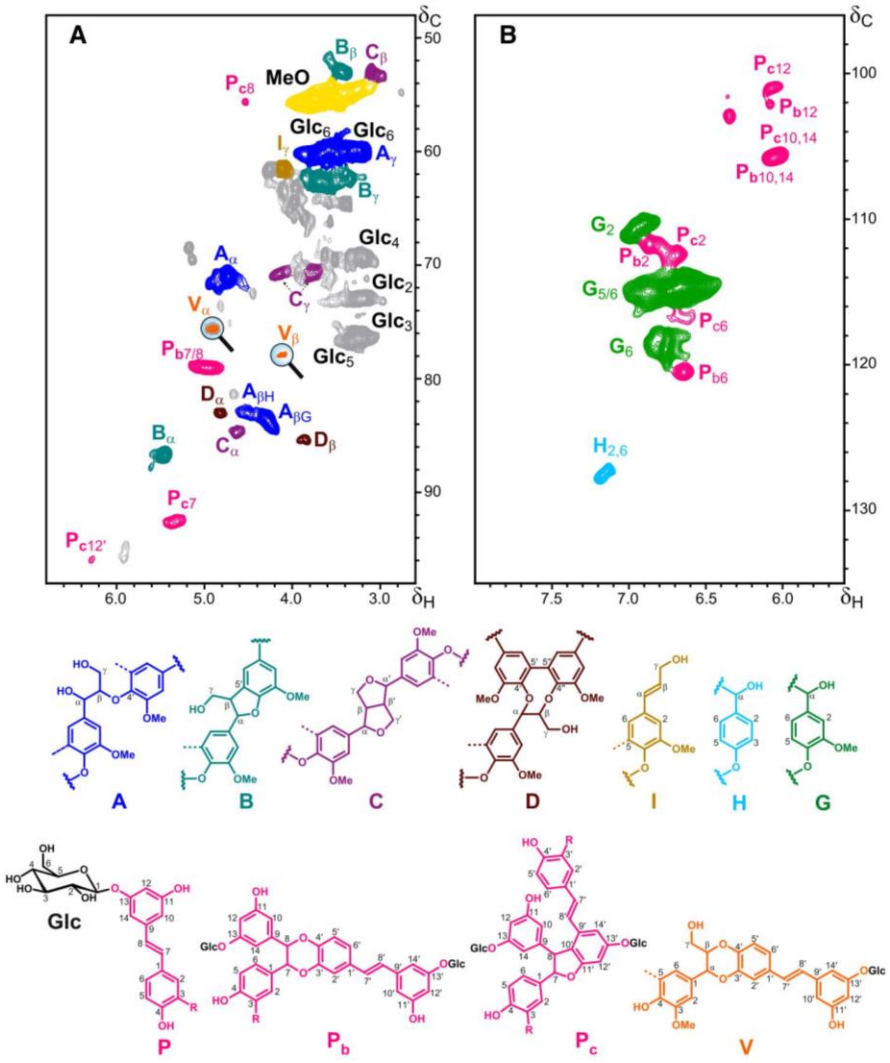

Figure 3. 2D-HSQC-NMR spectrum (in dimethyl sulfoxide-d_6) of the MWL preparation isolated from Norway spruce bark. A, Aliphatic-oxygenated (δ_C/δ_H 48–98/2.6–6.8) and aromatic [δ_C/δ_H 96–135/5.6–8.0; (B)] regions. The main structures found are as follows: A: β–O–4′ alkyl-aryl ethers; B: β–5′ phenylcoumarans; C: β–β′ resinols; D: 5–5′ dibenzodioxocins; I: cinnamyl alcohol end-groups; H: p-hydroxyphenyl units; G: guaiacyl units; P: hydroxystilbene glucosides (isorhapontin, R = OCH_3; astringin, R = OH; piceid, R = H); Glc: glucose units; P_b: 8–O–4′/3′–O–7 benzodioxane structures involving isorhapontin (R = OCH_3), astringin (R = OH), or piceid (R = H) units; P_c: 8–10′/11′–7 phenylcoumaran structures involving isorhapontin (R = OCH_3), astringin (R = OH), or piceid (R = H) units (please note that other phenylcoumaran structures arising from other linkages, such as 8–5′ and 8–12′, may also occur, but only the 8–10′ linkage has been authenticated due to the occurrence of signal $P_{c12'}$); V: β–O–4′/3′–O–α benzodioxane structure formed by cross-coupling of astringin and coniferyl alcohol.

similar correlations to those observed for the incorporation of the hydroxystilbene piceatannol into the lignins of palm fruit endocarps (del Río et al., 2017). Thus, the strong signal present at δ_C/δ_H 79.0/5.00 is similar to the signal for the C_7/H_7 and C_8/H_8 correlations of the benzodioxane structure ($P_{b7/8}$) formed by 8–O–4′

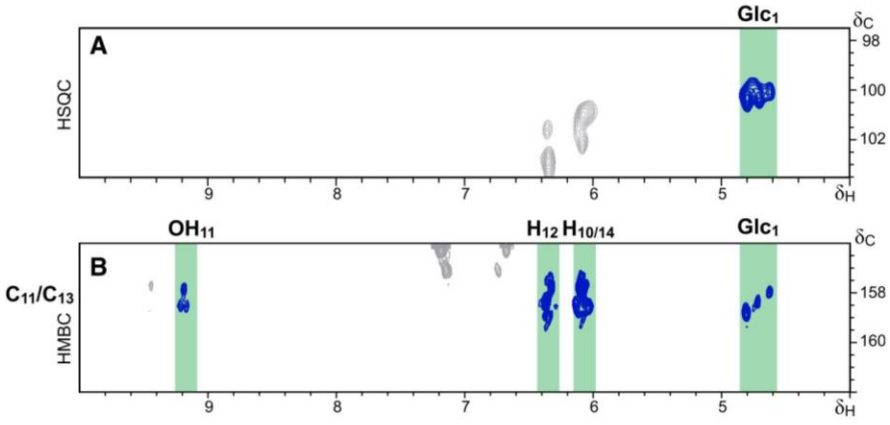

Figure 4. Proof of the linkage between Glc and the C-13 of the hydroxystilbene unit. A, Section of the HSQC spectrum of the lignin of Norway spruce bark showing the correlation of the anomeric carbon of glucose (Glc_1); B, Section of the long range ^1H–^{13}C correlation HMBC spectrum of the lignin from Norway spruce bark, showing the correlations within 2–3 atoms of C-13, and demonstrating that glucose is linked to the C-13 of hydroxystilbenes. Note that the correlations of C-11 overlap with those of C-13.

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coupling of two piceatannol units observed in the HSQC spectrum of the lignin of palm fruit endocarps; in the case of the lignin from Norway spruce bark, with isorhapontin being the major hydroxystilbene glucoside incorporated into the lignin structure according to the DFRC results, this benzodioxane structure would be formed by the coupling of an isorhapontin (or an astringin) unit at its 8-position to the 4–*O*-position of an astringin unit. Therefore, two different benzodioxane substructures would be expected to be produced, one comprising two astringin units and another comprising isorhapontin and astringin units. These types of structures are similar to the hydroxystilbene glucoside dimers, piceasides G and H (the *RR* and *SS* isomers, respectively, formed by 8–*O*–4′ coupling of two astringin units), and piceasides E and F (the *RR* and *SS* isomers, respectively, formed by 8–*O*–4′ coupling of an isorhapontin and an astringin unit), that have been identified among the ethanol-water and acetone-water extractives of Norway spruce bark (Li et al., 2008; Gabaston et al., 2017). Because isorhapontin is the major hydroxystilbene glucoside present in the lignin of Norway spruce bark, and the benzodioxane structure requires an astringin unit to form the benzodioxane skeleton, the predominant benzodioxane structure would be that formed by 8–*O*–4′ coupling of an isorhapontin unit at its 8-position with the 4′–*O*- of an astringin unit, in structures analogous to those of piceasides E and F.

The HSQC spectrum of the lignin from Norway spruce bark also showed two signals at δ_C/δ_H 92.6/5.36 (P_{c7}) and at δ_C/δ_H 55.6/4.52 (P_{c8}) that are similar to the signals for the C_7/H_7 and C_8/H_8 correlations of the phenylcoumaran structure formed by 8–10′ coupling of two piceatannol units, similar to the stilbene dimer scirpusin B, observed in the HSQC spectra of the lignins from palm fruit endocarps (del Río et al., 2017; Rencoret et al., 2018). In the case of Norway spruce bark, this phenylcoumaran structure would predominantly be formed by coupling of two isorhapontin units, with a skeleton similar to stilbene dimer bisisorhapontigenin A, and with smaller amounts of isorhapontin and astringin cross-coupled structures. However, it is important to note that, besides the 8–10′ coupling, other phenylcoumaran structures can also be formed by other types of coupling between two hydroxystilbenes, including the 8–5′ and the 8–12′ coupling structures that have been found in other plants, including Norway spruce (Iliya et al., 2002; Yao and Lin, 2005; Li et al., 2008; Francezon et al., 2017; Gabaston et al., 2017). Differentiating among the different phenylcoumaran structures, however, is a difficult task as the C_7/H_7 and C_8/H_8 correlation signals are very similar for all types of phenylcoumaran structures involving two hydroxystilbenes, regardless the type of coupling (8–5′, 8–10′, or 8–12′). In the case of the lignins from palm fruit endocarps, the differentiation among the different types of linkages and phenylcoumaran structures involving two hydroxystilbenes was possible via the occurrence of a characteristic and intense signal at δ_C/δ_H 95.9/6.28 for the $C_{12'}/H_{12'}$ correlations of the phenylcoumaran structure formed by 8–10′ coupling of two piceatannol units (as in the dimeric stilbene scirpusin B), and that was clearly apparent in the HSQC spectra. In the case of the lignin from Norway spruce bark, this signal ($P_{c12'}$) was also present in the HSQC spectrum, indicating the occurrence of a phenylcoumaran structure involving 8–10′ coupling of two hydroxystilbene glucosides (principally two isorhapontin units, having the skeleton of the dimeric stilbene bisisorhapontigenin A); however, the intensity of this signal was somewhat weak in comparison with the signals of P_{c7} and P_{c8}, suggesting that other phenylcoumaran structures arising from other coupling structures (possibly 8–5′ or 8–12′) might also be present in this lignin. In fact, different phenylcoumaran dehydrodimeric structures arising from 8–5′ homocoupling and cross-coupling of isorhapontin and astringin, such as the diastereomer pairs piceasides A and B (astringin dimers), piceasides C and D (astringin-isorhapontin dimers), and piceasides O and P (isorhapontin dimers), have been found among the extractives of Norway spruce and black spruce (*Picea mariana*) barks (Li et al., 2008; Francezon et al., 2017; Gabaston et al., 2017). In addition, the dimeric phenylcoumaran structure formed by 8–12′ coupling of two isorhapontigenin units, termed bisisorhapontigenin B, has been identified in species of the *Gnetum* genus (Iliya et al., 2002; Yao and Lin, 2005). However, it is important to note that these are coupled substructures occurring into the lignin polymer, and that we have to discard the occurrence of simple dimeric stilbenes in the lignin isolated from Norway spruce bark due to the absence of the characteristic signals for the double bonds in the HSQC, a feature that indicates the stilbene's participation in radical coupling reactions; apparently radical coupling at the 8-position occurs first, as we hope to examine using biomimetic coupling reactions, and possible molecular modeling studies, in due course. Moreover, and as already noted above, actual dimeric structures would have been removed during the exhaustive extraction with dichloromethane, ethanol, and water before the lignin isolation process.

A key finding in the HSQC spectrum (Fig. 3) was two signals at δ_C/δ_H 75.8/4.90 (V_α) and δ_C/δ_H 78.0/4.17 (V_β) that are similar to the C_α/H_α and C_β/H_β correlation signals of the benzodioxane structure formed by cross-coupling of piceatannol and monolignols observed in the spectra of the lignins from palm fruit endocarps (del Río et al., 2017). In the lignin from Norway spruce bark, the benzodioxane structure (V) would uniquely be formed by cross-coupling of coniferyl alcohol and the catechol moiety of astringin; isorhapontin, with a guaiacyl ring, cannot form this type of benzodioxane structure. The occurrence of all of these coupled and cross-coupled structures involving hydroxystilbene glucosides indicates that these phenolic compounds behave as authentic lignin monomers participating in radical coupling reactions during lignification to become

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integrally incorporated into the lignin polymer of Norway spruce bark. Zhang and Gellerstedt (2008) also found hydroxystilbene glucosides in a 'milled bark tannin-lignin' fraction that was isolated from Norway spruce bark after exhaustive removal of extractives with different solvents, and proposed that they might be linked to the condensed tannins. However, we can rule out this type of linkage as condensed tannins are not present in the MWL preparation studied here; in addition, the occurrence of the cross-coupled benzodioxane structure V provides evidence of the direct linkage between hydroxystilbene glucosides and the lignin polymer.

Another approach for demonstrating that hydroxystilbene glucosides are incorporated into the lignin polymer and are not present as free molecules or dimeric structures is by diffusion-edited ^{1}H-NMR, also termed Diffusion-ordered Spectroscopy. Diffusion NMR experiments can resolve compounds of different molecular size spectroscopically in a mixture based on their differing diffusion coefficients. Analysis of the MWL from Norway spruce bark by diffusion-edited ^{1}H-NMR (Fig. 5) indicated that the aromatic protons at δ_H ~6.10 assigned to the H_{10}, and $H_{12,14}$ of isorhapontin, as well as the protons at δ_H ~3.23 of the $H_{2/3/4/5}$ of the glucose moiety, must have similar translational diffusion coefficients as the aromatic protons of G-lignin units (δ_H ~6.50–7.00), also providing additional evidence that hydroxystilbene glucosides are incorporated into the lignin polymer. It can be clearly noted that the sharp signals from contaminants present in the MWL that are seen in the normal ^{1}H-NMR are completely absent in the diffusion-edited spectrum (Fig. 5).

Radical Coupling of Hydroxystilbene Glucosides and Cross-Coupling with Monolignols and the Growing Lignin Polymer

Hydroxystilbene glucosides, as for the hydroxystilbenes, and because the crucial phenolic-OH (4-position) remains free (Fig. 6), are compatible with the radical coupling reactions that typify lignification, and are therefore expected to participate in radical coupling reactions with other hydroxystilbenes glucosides as well as with monolignols and become integrally incorporated into the lignin polymer. As occurs with monolignols and the corresponding hydroxystilbenes isorhapontigenin and piceatannol, the hydroxystilbene glucosides isorhapontin and astringin can be oxidized by peroxidases and/or laccases to form radicals that are stabilized by resonance (Fig. 6A). These radicals can eventually couple and cross-couple with another hydroxystilbene glucoside molecule forming a variety of dehydrodimers and higher dehydro-oligomers (Fig. 6, B–E), similar to the structures with benzodioxane and phenylcoumaran skeletons observed by 2D-NMR in the lignin of Norway spruce bark. In addition, the hydroxystilbene glucosides (and their dimers and higher oligomers) can also cross-couple with monolignols and the growing lignin polymer via radical coupling reactions to become integrally incorporated into the lignin structure, as shown in Figure 7. As indicated above, the hydroxystilbene glucoside astringin, with its catechol ring, can cross-couple with coniferyl alcohol forming the benzodioxane structure V, that is formed via *β–O–4′* radical coupling of coniferyl alcohol (at its *β*-position) and astringin (at its 4′–*O* position) followed by internal trapping of the quinone methide intermediate by the 3′-OH and forming the

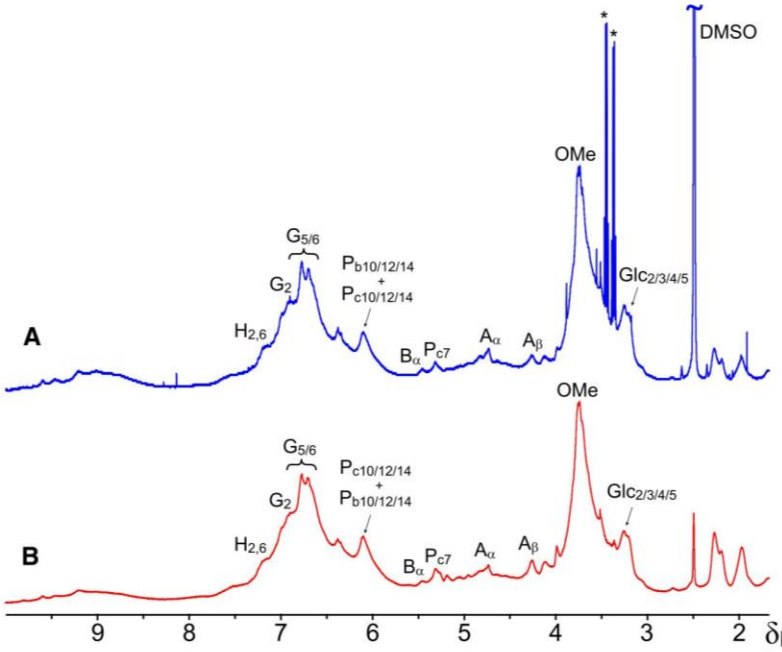

Figure 5. Analysis of the MWL isolated from Norway spruce bark by diffusion-edited ^{1}H-NMR. Comparison of standard ^{1}H-NMR (A) and diffusion-edited ^{1}H-NMR (B) of the MWL preparation isolated from Norway spruce bark indicated that the hydroxystilbene glucosides have translational diffusion coefficients similar to those of the lignin, and providing further evidence of its incorporation into the polymer. *Major contaminants. DMSO, dimethyl sulfoxide.

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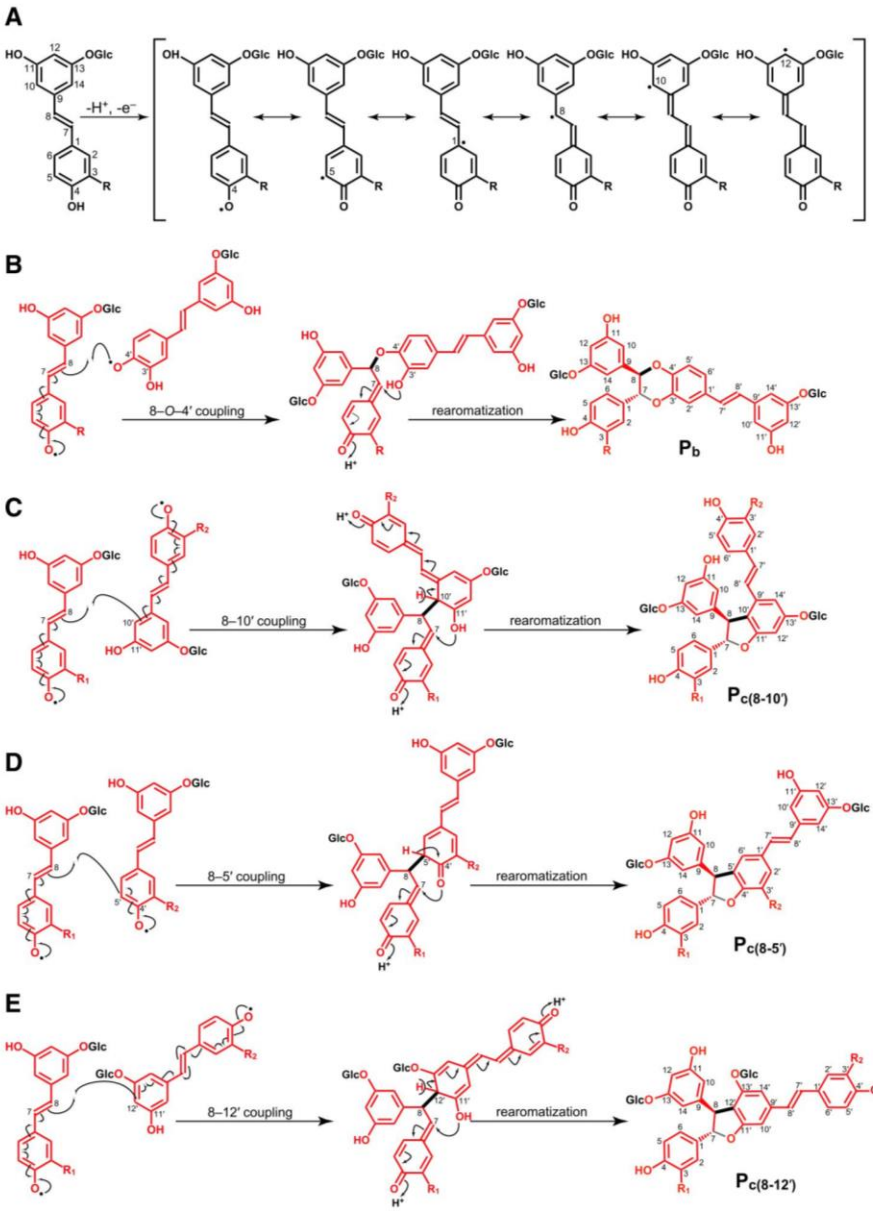

Figure 6. Hydroxystilbene glucoside radicals and dehydrodimeric radical coupling reactions. A, Oxidative radicalization resulting from one-electron oxidation of isorhapontin (R = OCH_3, isorhapontigenin-*O*-glucoside), astringin (R = OH, piceatannol-*O*-glucoside), and piceid (R = H, resveratrol-*O*-glucoside) stabilized by delocalization; resonance forms are displayed, in which the single-electron density is shown to localize at the 4–*O*-, 5-, 1-, 8-, 10-, and 12-positions. B, Dehydrodimerization products arising from 8–*O*–4′ coupling of isorhapontin (R = OCH_3) or astringin (R = OH) with another astringin unit producing a benzodioxane structure; this structure is similar to the stilbene glucoside dimers piceasides E and F (R = OCH_3), and piceasides G and H (R = OH) referred to in the text. C, Phenylcoumaran structures arising from the 8–10′ coupling of isorhapontin (R_1,R_2 = OCH_3) and astringin (R_1,R_2 = OH) units; this structure has the same skeleton as the stilbene dimers bisisorhapontigenin A (R_1 = R_2 = OCH_3) and scirpusin B (R_1 = R_2 = OH), referred to in the text. D, Phenylcoumaran structures arising from the 8–5′ coupling of isorhapontin (R_1,R_2 = OCH_3) and astringin (R_1,R_2 = OH) units; this structure is similar to that of the stilbene glucoside dimers piceasides A and B (R_1 = R_2 = OH), piceasides C and D (R_1 = OCH_3, R_2 = OH), and piceasides O and P (R_1 = R_2 = OCH_3), referred to in the text. E, Phenylcoumaran structures arising from the 8–12′ coupling of isorhapontin (R_1,R_2 = OCH_3) and astringin (R_1,R_2 = OH) units; this structure presents the same skeleton as that of the stilbene dimer bisisorhapontigenin B (R_1 = R_2 = OCH_3).

benzodioxane bridge (Fig. 7A). On the other hand, the hydroxystilbene glucoside isorhapontin, with its guaiacyl ring, can easily cross-couple with coniferyl alcohol in different ways, including via 4–*O*–*β* or 5–*β* forming the *β*–*O*–4′ alkyl-aryl ether or *β*–5′ phenylcoumaran structures (Fig. 7, B–C); it can also couple with lignin oligomers (or the polymer) to form the 5–5′/4–*O*–*β*′′-linked dibenzodioxocin structures shown in Figure 7D. In fact, several stilbenolignans formed by cross-coupling of isorhapontigenin with monolignols through different linkage types (Fig. 8) have been found in other gymnosperms, such as the gnetumonins B and C, the *threo* and *erythro* forms of the aryl-alkyl ethers arising from 4′–*O*–*β* cross-coupling of isorhapontigenin and coniferyl alcohol, or the phenylcoumarans gnetofuran A and gnetucleistol F arising from the 5′–*β* cross-coupling of isorhapontigenin and coniferyl or sinapyl alcohol, respectively (Ma et al., 2017). The occurrence of all of these stilbenolignans, not to be confused with stilbenolignins, reveal the compatibility of hydroxystilbenes, and more particularly of isorhapontigenin, with lignification. Although the occurrence of stilbenolignans involving hydroxystilbene glucosides have not been reported so far, the cross-coupling of hydroxystilbene glucosides, and more particularly isorhapontin that presents a guaiacyl unit, with monolignols is feasible and can be anticipated. However, unambiguous identification of these cross-coupled

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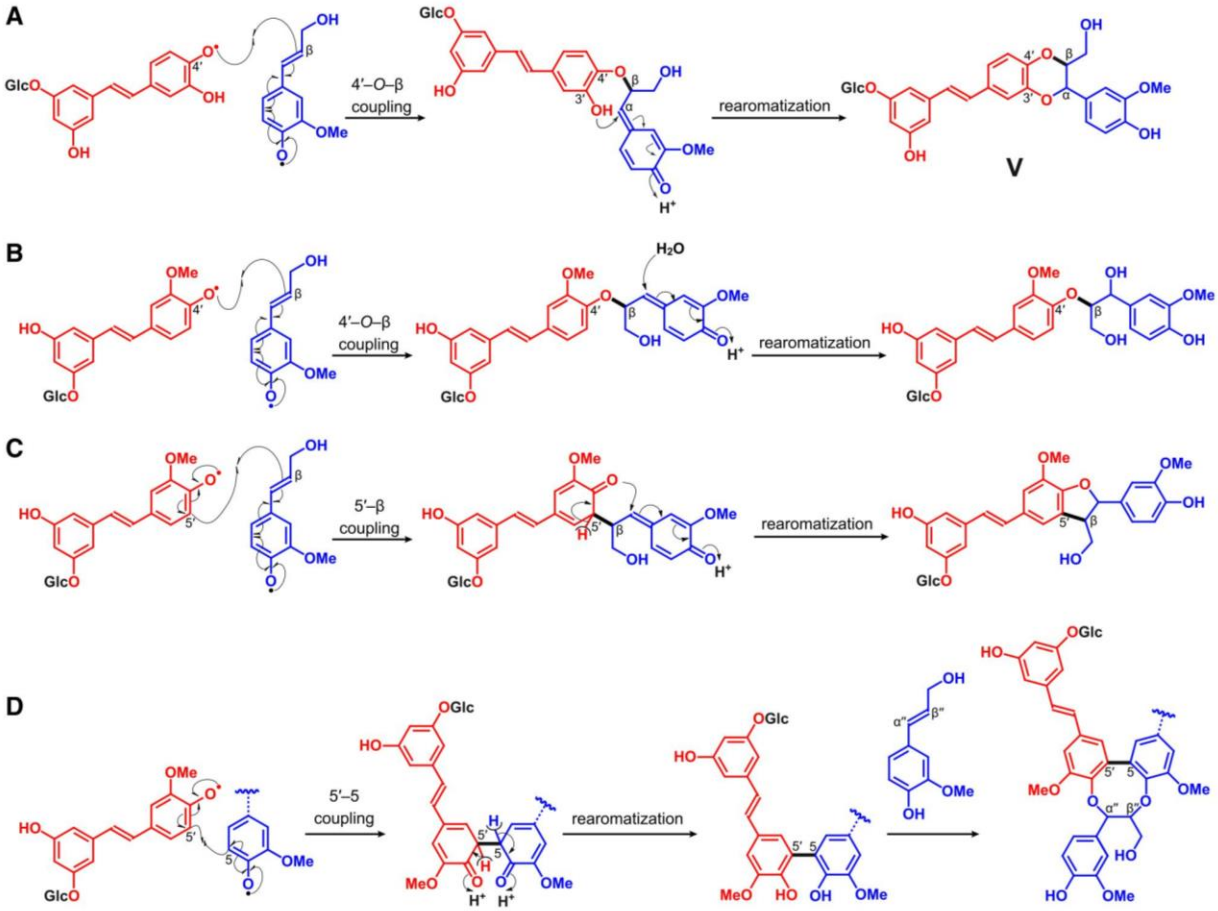
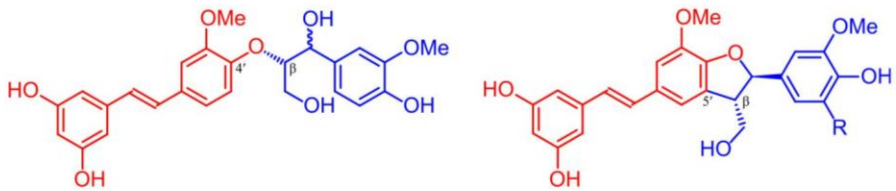


Figure 7. Dehydrodimerization products arising from oxidative cross-coupling of hydroxystilbene glucosides with monolignols or the growing lignin polymer. A, *β–O–*4′ coupling of astringin and coniferyl alcohol, producing the benzodioxane structure V; B, *β–O–*4′ coupling of isorhapontin and coniferyl alcohol, producing *β–O–*4′ alkyl-aryl ether structures; C, *β–*5′ Coupling of isorhapontin and coniferyl alcohol, producing phenylcoumaran structures; D, 5–5′ Coupling of isorhapontin and guaiacyl phenolic polymer end-units, producing dibenzodioxocin structures.

structures of isorhapontin with monolignols is not a trivial task as the NMR signals overlap with those of the typical *β–O–*4′ alkyl aryl ether A, *β–*5′ phenylcoumaran B, and the 5–5′ dibenzodioxocin D lignin structures. In any case, the release of hydroxystilbenes upon DFRC and the inherent polymeric nature of the lignin seem to suggest the existence of such linkages between the hydroxystilbene glucosides and the lignin



gnetumonin B (*threo*)
gnetumonin C (*erythro*)

gnetofuran A (R=H)
gnetucleistol F (R=OMe)

Figure 8. Examples of stilbenolignans involving cross-coupling of isorhapontigenin and monolignols. Gnetumonins B and C are the *threo* and *erythro* aryl-alkyl ether structures arising from 4′*–O–β* cross-coupling of isorhapontigenin and coniferyl alcohol; gnetofuran A and gnetucleistol F are the phenylcoumaran structures arising from the 5′*–β* cross-coupling of isorhapontigenin and coniferyl or sinapyl alcohol, respectively.

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polymer. Additional work with authentic models and detailed NMR studies are needed to unambiguously confirm this contention.

If the hydroxystilbene glucosides are fully integrated into the lignin polymer in Norway spruce bark, then they should also be considered true lignin monomers participating in radical coupling reactions during lignification in this bark tissue. Thus, the hydroxystilbene glucosides, particularly isorhapontin and astringin, can also be added to the list of 'nonconventional' phenolic lignin monomers recently discovered in plants, including phenolics from different biosynthetic pathways such as the corresponding hydroxystilbene aglycones, particularly piceatannol, discovered in the lignins of palm fruit endocarps (del Río et al., 2017; Rencoret et al., 2018), the flavone tricin present in the lignins of all grasses and other monocots (del Río et al., 2012; Lan et al., 2015, 2016a, 2016b), or the hydroxycinnamic amides, such as tyramine ferulate in tobacco and other Solanaceae (Negrel et al., 1996; Ralph et al., 1998), and diferuloylputrescine, recently identified in the lignin of maize grain kernels (del Río et al., 2018). This would be the first report of a phenolic glucoside acting as authentic lignin monomer. All of these discoveries continue to provide further evidence of the plasticity of the lignification process and indicate that, as has been noted early on, "any phenolic transported to the lignifying zone of the cell wall can, subject to simple chemical concerns, be incorporated into the polymer" (Ralph et al., 2008).

Implications for Prior Claims of Lignin Glucosides

Lignin-(poly)saccharide binding has long fascinated plant cell wall researchers (Terrett and Dupree, 2019). Apart from the well-authenticated example in which ferulates on arabinoxylans in commelinid monocots provide a powerful mechanism for extensive cell wall cross-linking (Ralph, 2010), securing definitive evidence for other types of lignin-polysaccharide cross-linking, particularly in noncommelinids, has been a challenge essentially unmet. The proposed benzylic-polysaccharide ethers, for example, have been only weakly 'observed' in NMR spectra (Balakshin et al., 2007), but there is one validated account of evidence from degradation and isolation of a lignin-sugar unit from *Pinus densiflora* (Japanese red pine; Nishimura et al., 2018). Glucosides (or, more generally, glycosides) also have weak evidence from NMR spectra (Balakshin et al., 2007), but the assignments are again unauthenticated and there is the additional conceptual problem as to how they result. Logically, they cannot arise from the phenol-glucosylated monolignols, coniferin and syringin, that are known to be derivatives stored in the vacuole (Dima et al., 2015) and which, in the case of coniferin and in softwoods, may be deglucosylated and the usual monomer used for lignification (Terashima et al., 2016); this is simply because a free-phenol is an absolute requirement to enable the generation of the phenolic radical that is used in the radical coupling reactions that typify lignification. If there truly are glucosides in 'normal' lignins, the glucoside must be introduced post-polymerization, and it is difficult to imagine how or why this might happen. For these reasons, the claims of glucosides in lignins, and the weak and tenuous NMR evidence for them, have never been compelling. Here, however, we have a very simple way for explaining how such glucosides might in fact appear in the polymer. If a lignin precursor has more than one phenolic-OH, and at least a single phenolic-OH capable of undergoing radicalization and enabling the radical coupling reactions, then there is a sound mechanism by which phenolic glucosides can be accommodated. The stilbene glycosides here provide one such example (and flavonoid glucosides might also provide another example, although they have not been authenticated as lignin monomers yet). Another in 'normal lignification' must be via, for example, a resinol unit in which only one of the phenols is glucosylated. Interestingly, this type of half-glucosylated pinoresinol metabolites has been identified in Arabidopsis (*Arabidopsis thaliana*; Morreel et al., 2014). The important point here is that there is finally a plausible pathway for creating lignins bearing phenolic glucosides.

Biosynthesis, Role of Hydroxystilbene Glucosides in the Lignin of Norway Spruce Bark, and Prospects for Metabolic Engineering

The biosynthesis of hydroxystilbenes is controlled by the stilbene synthase that catalyzes the formation of the stilbene backbone from three malonyl-CoA units and one CoA-ester of a cinnamate derivative. In the case of Norway spruce, the biosynthesis of the major tetrahydroxystilbene glucosides, astringin and isorhapontin, goes through the formation of resveratrol from *p*-coumaroyl-CoA by the corresponding stilbene synthase, which is further modified through hydroxylation, *O*-methylation, and *O*-glucosidation reactions to produce isorhapontin and astringin (Hammerbacher et al., 2011). The production of isorhapontin and astringin was found to be enhanced by fungal infection, suggesting that these phenolic metabolites have a role in antifungal defense (Hammerbacher et al., 2011). It is therefore evident that the incorporation of hydroxystilbene glucosides into the lignin polymer in bark, which is the outermost layer that covers the wood and gives protection to the tree against external agents, can provide antifungal and antimicrobial properties, contributing to resistance to disease and to pathogenic attack. In addition, and as also occurs in the palm fruit endocarps, the incorporation of hydroxystilbene glucosides into the lignin may allow for the production of higher amounts of lignin by incorporating other phenolic compounds present in the cell wall into the lignin polymer, which indeed will produce more condensed

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structures, as the phenylcoumarans and benzodioxane structures shown above illustrate, thus reinforcing and strengthening the cell wall.

The identification in recent years of nonconventional lignin monomers, not usually present in the lignins of other plants, as is the case of the hydroxystilbene glucosides described here, can open up new ways to design and engineer the lignin structure to produce polymers with new or improved properties, as already considered with other phenolic compounds (Vanholme et al., 2008, 2012; Grabber et al., 2010; Wilkerson et al., 2014; Mottiar et al., 2016). Metabolic engineering to introduce hydroxystilbene glucosides into the lignin polymer could provide a means to increase disease resistance in plants by adding antifungal and antimicrobial properties, and may provide a way of producing lignins with special properties, including enhanced hydrophilicity, a trait potentially proposed to improve enzymatic wall digestibility (Grabber et al., 2010). The particular structure of the hydroxystilbene glucosides, with a pendant glucose moiety that obviously does not participate in the radical coupling reactions, makes these phenolic metabolites potentially interesting for introducing specific functionalities into the lignin via functionalization of the glucose moiety.

CONCLUSION

Hydroxystilbene glucosides (isorhapontin, astringin, and piceid) have been found incorporated into the lignin of Norway spruce bark, participating in radical coupling reactions during lignification, and should therefore be considered as authentic lignin monomers. The occurrence of hydroxystilbene glucosides in the lignin of Norway spruce bark seems to have a role in plant protection, providing antifungal and antimicrobial properties to the bark; in addition, the incorporation of phenolic metabolites beyond the traditional monolignols allows for the production of higher amounts of lignin, which may be more condensed, thus providing additional protection to the bark.

MATERIALS AND METHODS

Samples

The Norway spruce (*Picea abies*) bark used for this study was obtained from a sawmill located in Jyvaskyla, Finland, after the debarking process, and the chemical composition has been published elsewhere (Neiva et al., 2018). The air-dried samples were milled using a knife-mill (1 mm screen) and successively extracted with dichloromethane (2000 mL) in a Soxhlet apparatus for 24 h, ethanol (2000 mL, 24 h), and hot water (two times 2000 mL, 24 h at 100°C). Klason lignin content was estimated as the residue after sulfuric acid hydrolysis of the pre-extracted material, corrected for ash and protein content, according to the TAPPI method T222 om-88, and presented a value of 30.7% ± 0.8 (three replicate samples were used).

Lignin Isolation and Purification

The MWL preparation was obtained from extractive-free bark according to the classical procedure (Björkman, 1956). Around 40 g of extractive-free material were finely ball-milled in a Retsch PM100 planetary ball mill (Retsch) for 5 h at 400 rpm using a 500 mL agate jar and agate ball-bearings (20 × 20 mm). The ball-milled material was then extracted three times with dioxane-water, 90:10 (v/v; 25 mL of solvent/g of milled bark), and the isolated lignin was exhaustively purified as described elsewhere (del Río et al., 2012). The MWL yield was 37% of the Klason lignin content.

DFRC

DFRC degradation was performed according to the original protocol (Lu and Ralph, 1997), and the detailed explanation can be found elsewhere (del Río et al., 2012). Briefly, around 10 mg of MWL were treated with 2.5 mL acetyl bromide in acetic acid (8:92, v/v) at 50°C for 2 h. The solvents and excess of bromide were removed by rotary evaporation, then dissolved in dioxane/acetic acid/water (5:4:1, v/v/v), and 50 mg of powdered zinc was added. After 40 min stirring at room temperature the mixture was transferred into a separatory funnel with dichloromethane and saturated ammonium chloride. The pH of the aqueous phase was adjusted to less than 3 by adding 3% (v/v) HCl, the mixture vigorously mixed, and the organic layer separated. The water phase was extracted twice more with dichloromethane. The combined dichloromethane fractions were dried over anhydrous $NaSO_4$, and the filtrate was evaporated in a rotary evaporator. The lignin degradation products were acetylated with an acetic anhydride: pyridine solution (1:1 v/v) for 1 h at room temperature and dissolved in dichloromethane for a subsequence gas chromatography/mass spectrometric analysis. A Saturn 4000 (Varian) equipment fitted with a capillary column (DB5-HT, 15 m × 0.25 mm i.d., 0.1 μm film thickness; from J&W Scientific) was used to analyze the lignin degradation products obtained upon DFRC. The samples were injected with an autoinjector (Varian 8200), which was programmed from 120°C (0.1 min) to 330°C (until the end of the analysis) at a rate of 200°C min^{-1}. The oven was programmed from 120°C (1 min) to 380°C (5 min) at a rate of 10°C min^{-1}. The temperature of the transfer line was set at 300°C during the analysis. Helium was used as carrier gas at a rate of 2 mL min^{-1}. The different hydroxystilbenes (resveratrol, isorhapontigenin, piceatannol) were identified by comparison with authentic standards.

NMR Spectroscopy

Multidimensional NMR spectra (2D HSQC, 2D HMBC, 2D HSQC-TOCSY) experiments were recorded on an AVANCE III 500 MHz instrument (Bruker) fitted with a cryogenically cooled 5 mm triple resonance gradient probe with inverse geometry. Around 40 mg of lignin sample were dissolved in 0.75 mL of dimethyl sulfoxide-d_6. The central solvent peaks were used as internal references (δ_C/δ_H 39.5/2.49). The HSQC experiments used Bruker's standard "hsqcetgpsisp2.2" pulse program (adiabatic-pulsed version), the HMBC experiments used Bruker's standard "hmbcgplpndqf" pulse program with long-range *J*-coupling evolution times of 62.5 ms [J_{LR} = 8 Hz, and/or 80 ms (J_{LR} = 6.25 Hz) when required], and the diffusion-edited ^{1}H-NMR used the pulse program "ledbpgp2s1d". The detailed NMR experimental conditions have been described elsewhere (del Río et al., 2012).

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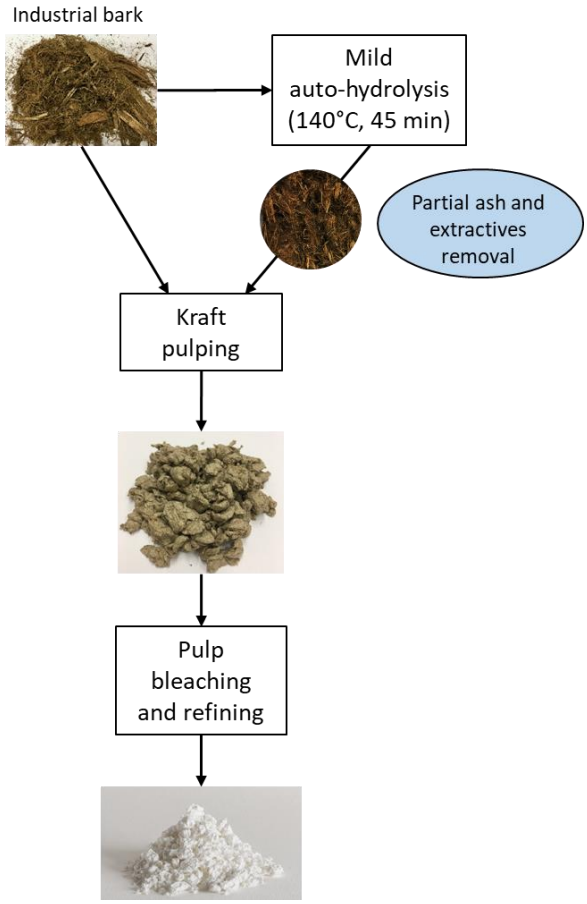

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THE POTENTIAL OF HYDROTHERMALLY PRETREATED INDUSTRIAL BARKS FROM *E. globulus* AS A FEEDSTOCK FOR PULP PRODUCTION

Duarte M. Neiva,[1] Jorge Gominho,[1] Luís Fernandes,[2] Ana Lourenço,[1] Catarina Chemetova,[1] Rogério M. S. Simões,[2] and Helena Pereira[1]

[1]Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Lisboa, Portugal
[2]Unidade de Materiais Têxteis e Papeleiros, Universidade da Beira Interior, Covilhã, Portugal

This study focused on the use of industrial *eucalyptus globulus* bark as an alternative fiber source for bleached pulp and paper production. Bark has high extractives and ash contents (7.7% and 3.5%, respectively) but a mild hydrothermal pretreatment was tested, decreasing its values to 2.8% and 2.4%, respectively. Untreated and pretreated bark were kraft pulped at 15% and 20% (as Na_2O) active alkali conditions. The pretreatment improved delignification when using low active alkali; kappa number 25.4 vs 17.5, and shives 3.1% vs 0%, respectively, with untreated and pretreated bark. The pretreatment resulted in a lower chemical demand to obtain pulps with similar yield and kappa number. It was possible to produce bleached pulps with good handsheet optical, physical, and mechanical properties with slightly lower values than those of industrial eucalypt wood pulps; e.g., brightness > 85% vs 87%, tear index > 4.2 vs 5.6 mn.m^2.g^{-1}, tensile index > 62 vs 69 n.m.g^{-1} for bark and wood pulps, respectively.

KEYWORDS. Kraft pulp, pretreatment, chemical analysis, handsheet properties, fiber morphology

INTRODUCTION

Eucalyptus globulus is one of the most important species cultivated in intensive short-rotation plantations for short fiber pulp and paper production in the temperate and Mediterranean regions.[1] Bark, tree tops, leaves, and stumps are considered biomass residues, either left on the plantation ground for soil nutrition (usually branches and leaves) or burned at the industrial site for energy and heat production (bark and stumps). However, several studies have shown their potential value, either by incorporating them in the pulping process[2–5] or for extraction of valuable compounds such as sugars and bioactive polyphenols from extractives.[6–10]

E. globulus bark can represent as much as 20% of the o.d. mass of the bole, with an average of 11%.[1,11] This value is in good agreement with the reference that one ton of bleached pulp generates around 20% of that in bark, making it widely available at the mill.[12] Presently, this low-value byproduct is used as solid fuel in steam boilers to generate heat/electricity, and even though it is important for this energy-intensive industry, other end uses might prove economically more interesting.

The bark of *E. globulus* has higher extractives and ash contents than wood, and these are detrimental to the pulping process and paper quality.[13,14] The anatomical features are quite

Address correspondence to Duarte M. Neiva, Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017, Lisboa, Portugal. E-mail: duarteneiva@isa.ulisboa.pt

different, with wood presenting on average 66% fibers, as opposed to bark, with 35% of fibers and almost 50% of parenchyma cells.[15,16]

The raw material shortage in some regions of the world and the increasing consumption of pulp and board products (402 million tons in 2011 and expected to reach 521 million by 2021[17]) have triggered the search for alternative fiber sources to meet this demand, with new wood and non-wood species being studied as well as residual materials.[3,18–21] In this context, *E. globulus* bark may be an interesting candidate.

This article aims at studying the feasibility of using the *E. globulus* bark that is obtained as a biomass residue from the pulp industry as a fiber source for production of kraft pulps with characteristics similar to those of wood pulps. The novelty of this work is the authors' proposal and testing of a pretreatment aimed at removing part of the extractives and contaminants from bark that are known to have detrimental effects on the process and quality of pulp and paper. The chemical composition of the untreated and pretreated industrial bark was determined, and the kraft pulping, pulp morphological and handsheet characteristics of several pulps, produced under different conditions, were tested and compared to a pulp produced with industrial *E. globulus* wood chips.

EXPERIMENTAL

Sampling

Eucalyptus globulus Labill. bark residues were obtained from a Portuguese pulp and paper mill (Portucel Soporcel Group). This industrial bark, which also contained some different-sized wood chunks, was fractionated using a knife mill (Retsch SM 2000) with an output sieve of 6 × 6 mm, and screened with a 2 mm sieve to remove small particles and fines.

Bark Pretreatment

Bark pretreatment was conducted using distilled water, with a liquid-to-solid ratio of 10/1, in a stainless-steel batch reactor (ca. 4L) with fluid recirculation. The heating time to temperature was 45 min, and the time at maximum temperature (140°C) was set at 45 min. At the end of the pretreatment, a sample was recovered from the remaining 3 L of liquor, the solid residue was thoroughly washed with hot and cold water, and the excess water removed. The pretreated bark was pulped without drying, taking into account its dry matter content, previously determined.

Chemical Characterization

Both initial and pretreated bark samples were analyzed regarding ash, extractives (dichloromethane, ethanol and water), lignin (Klason and soluble), and holocellulose contents as well as neutral monosaccharides and acetic acid. The methodology and equipment used was previously described.[22] The results are presented as percentage of the oven-dry material.

The liquor samples from the pretreatment were filtered through 0.45 μm membranes and analyzed by HPLC. The HPLC system (Waters, Milfort, USA) was equipped with an Aminex HPX-87H column with a cation H^+-guard column (Bio-Rad). Elution took place at 50°C with 5 mM H_2SO_4. A refractive index detector was used to detect glucose, xylose, arabinose, and acetic acid. Furfural and hydroxymethyl-furfural (HMF) were detected with an UV/VIS detector at 280 nm. Oligosaccharides (OS) were determined by an indirect method based on quantitative acid hydrolysis of the liquors with 4% (w/w) H_2SO_4 at 121°C for 60 min. The variation of sugar monomers, before and after liquor hydrolysis, was due to OS depolymerization. Total phenolic compounds were determined by the Folin-Ciocalteu colorimetric method.[23]

Kraft Pulping and Bleaching

Industrial bark was cooked in a forced circulation reactor (ca. 6L) with a West N4400 Single Loop Temperature Controller. The bark was submitted to kraft pulping with and without pretreatment and the conditions used were as follows: active alkali 15 and 20% (as Na_2O); sulfidity 30% (as Na_2O); liquor-to-wood ratio 10:1; time to temperature (165°C) 100 min;

and total reaction time 214 min. The solid residue (pulp) was thoroughly washed with hot and cold water until achieving neutral pH, defibrated, and screened to remove shives.

Unbleached pulps were submitted to an elemental chlorine-free sequence, at medium consistency (10%), D0ED1D2, where E stands for alkaline extraction, performed at 60°C for 60 min, and D for chlorine dioxide stages. Chlorine dioxide loads (as active chlorine) in D0 stage (45°C, 30 min) were determined using a kappa factor of 0.2 (ClO_2 charge,% = 0.2 x kappa number). In subsequent stages, fixed loads of 1.2% in D1 stage (70°C, 120 min) and 0.5% in D2 stage (70°C, 180 min) were applied, aiming to achieve a pulp brightness of 88–90% ISO. Bleached pulps were beaten in a PFI mill (Norway) at 500, 1500, 2500, and 4500 revolutions under a refining intensity of 1.66 N/mm (ISO 5264-2). For comparison, data from pulping of industrial chips in the same reactor, with 22% active alkali, 30% sulfidity, 4:1 liquor to wood ratio, time to temperature (160°C) 100 min, and total reaction time of 160 min, were used.[24]

Pulp Characterization

Bark pulps **Bt_{15}** and **Bt_{20}** correspond to the pretreated bark pulped under low and high active alkalinity charges (15% and 20% as Na_2O, respectively), while **B_{15}** and **B_{20}** correspond to bark with no previous treatment under low and high active alkalinity.

Pulp and reject yields were obtained gravimetrically and all were reported as percentage of the original material (untreated bark). Kappa number was determined using a TIM865 titration manager from TitraLab® following TAPPI Useful Test Method UM 246 and pulp viscosity through SCAN-CM 15:88, respectively, and hexenuronic acids (HexAc) by spectrophotometry (Shimadzu, UV-160A) according to Li.[25] Kappa number was corrected ($Kappa_{corr}$) to account solely for the residual lignin using the following expression.

$$Kappa_{corr} = Kappanumber - 0.086 \times (Hex\,Ac)$$

Fiber morphological properties (fiber population, length, width, coarseness, and fines) of unbleached pulps and bleached pulps before and after beating were determined using MORFI LB01 Fiber Size Analyzer (Techpap, France).

Schopper-Riegler (pulp freeness) was determined by ISO 5267-1 and water retention value (WRV) according to Silvy.[26]

To evaluate paper properties, handsheets with basic weight of 60 g/m^2 were produced and conditioned following ISO 5269-1 and ISO 187 standards. Bulk, tensile, and tear indexes were examined according to ISO 5270 standard. Scott-Bond with a Tester Model B (Precision Scientific Petroleum Instruments, IL, USA) was used. Pulp optical properties of brightness, opacity, and light scattering coefficient were determined through ISO 2470-1, ISO 2471, and ISO 9416, respectively.

RESULTS AND DISCUSSION

Chemical Analysis

The chemical composition of the industrial bark and pretreated bark are reported in Table 1, expressed as mass percentage of original bark. The industrial bark has high amounts of extractives and contaminants that are substantially above those of wood: extractives 7.7% vs., e.g., 4.4%[18] or 3.0%,[3] and ash 3.5% vs. 0.4-1%.[1,3,18,22]

A hydrothermal pretreatment was conducted, removing 7.2% (w/w) of the initial

TABLE 1. Summative chemical analysis of raw and pretreated barks reported to initial bark

(% o.d.)	Bark	Pretreated Bark
Ash	3.5	2.4
Total extractives	7.7	2.7
Dichloromethane	0.5	0.1
Ethanol	2.7	0.8
Water	4.5	1.7
Total lignin	22.5	21.5
Klason lignin	19.4	18
Soluble lignin	3.1	3.5
Holocellulose	70.3	69.9

TABLE 2. Monosaccharides and acetic acid in raw and pretreated barks

(%)	Bark	Pretreated Bark
Glucose	34.6	34.5
Xylose	11.2	11.1
Galactose	1.3	1.2
Mannose	0.9	0.6
Arabinose	0.7	0.5
Acetic acid	4.1	4.4

oven-dry material. It substantially decreased the ash (3.5% vs. 2.4%) and extractive contents (7.7% vs. 2.8%), while lignin decreased slightly and holocellulose remained almost unaltered (22.5% vs. 21.5% and 70.3% vs. 69.9%, respectively) by the process.

Table 2 presents the monosaccharides and acetic acid resulting from the total hydrolysis of the carbohydrate polymers, reported as mass percentage of the original industrial bark. There is almost no variation with the pretreatment, although there is a slight decrease of mannose and arabinose contents (0.9, 0.7 vs. 0.6, 0.5, respectively). The acetic acid, mainly derived from the acetyl group in xylan, exhibits slightly higher values, which was not expected since a portion was removed in the pretreatment, as shown in Table 3. We could not find an explanation for this slight increase.

Table 3 shows the chemical characteristics of the liquor resulting from the hydrothermal pretreatment. The sugars and phenolics that were solubilized are mainly derived from the bark extractives and, to a small extent, from lignin. The mild conditions used in this pretreatment favored the removal of sugars as oligosaccharides,[27,28] with monomers of glucose, xylose, and arabinose comprising solely 17%, 14%, and 28% of each, respectively. The phenolics showed the highest concentration in the liquor (1.33 g/L).

TABLE 3. Pretreatment liquor chemical characteristics

	C (g/L)	% as Oligosaccharides
Glucan	1.28	83
Xylan	1.07	86
Arabinan	0.84	72
Acetic acid	0.19	–
HMF	0.04	–
Furfural	0.10	–
Phenolics (as GAE)	1.33	–

The conditions of the pretreatment were such as to try to prevent extensive degradation of the carbohydrate polymers (cellulose and hemicelluloses), leading to only 2% loss, while at the same time reducing the extractives and soluble phenolic components of the bark, which can have a detrimental effect in the kraft process, increasing its chemical demand.[13,14]

The washing of the bark prior to the delignification process might not be possible at an industrial scale. Skipping this phase would probably lead to a slight decrease in delignification, since part of the pretreatment liquor (with a lower pH) would remain absorbed in the pulp, decreasing the alkalinity of the kraft liquor.

Kraft Pulping

The pulping results are presented in Table 4. Bark pulps were obtained with a lower screened yield (41–42.4%) and higher residual lignin than wood chip pulps. Miranda[3] reported higher values of pulp yield (47.2%) for bark pulps, although the delignification degree was smaller (36.1 kappa number).

The pulping conditions that were used (165°C and 114 min at maximum temperature) correspond to previously optimized conditions to produce pretreated industrial bark pulp with kappa number 17.[18] Due to the high hydration capacity of bark,[5] the liquor-to-wood ratio used was 10:1, which is higher than the industrial standards for wood delignification (3–4:1) and may have a negative impact on operational conditions and equipment capacity, including black liquor evaporators.

The screened pulp yield and kappa number were relatively similar for the pulps $\mathbf{Bt_{15}}$ and $\mathbf{Bt_{20}}$, showing that, for pretreated barks, the active alkalinity has little impact on these parameters, at least within the range studied. On the other hand, for bark without pretreatment, the impact of using low active alkali charges results in some rejects (3.1%) and pulp with both

TABLE 4. Kraft pulping conditions and pulps characterization for wood and different barks

		Untreated Bark		Pretreated Bark	
	Wood* (W)	(B_{15})	(B_{20})	(Bt_{15})	(Bt_{20})
Total yield (%)	49.1	45.5	41.1	41.0	41.3
Screened yield (%)	48.4	42.4	41.1	41.0	41.3
Kappa	13.3	25.4	17.3	17.5	17.6
HexA (μmol/kg)	39.0	41.0	59.2	34.8	39.4
$Kappa_{corr}$	9.9	21.9	12.3	14.5	14.2
Viscosity (cm^3/g)	780	1012	855	992	824

*Data from Gominho.[22]

higher screened yield and kappa number. This decrease in the delignification degree is probably due to the depletion of reaction chemicals, given by reactant consumption by the extractives.

The pretreatment of the industrial bark results therefore in a lower chemical demand to obtain pulps with similar yield and kappa number.

The comparison of the pretreated industrial bark with wood chips[18] shows that chemical composition cannot explain the differences in pulping yield. Anatomical characteristics certainly influence bark yield due to higher loss of small-sized parenchyma cells and phloem vessels during pulping and washing.[5] For instance, the parenchyma tissues of *E. globulus* bark phloem that represent 50% in volume[16] are easily attacked and degraded during kraft pulping.

Although having similar kappa number, pulps **Bt_{15}** and **Bt_{20}** differ in the amount of hexenuronic acids. This means that the use of higher active alkalinity results in a stronger attack on polysaccharides, namely on hemicelluloses.[29]

Pulp viscosity decreased with increasing active alkali charge, with pulps **Bt_{15}** and **B_{15}** showing higher viscosity values (992 cm^3/g vs. 1012 cm^3/g) with respect to **Bt_{20}** and **B_{20}** (824 cm^3/g vs. 855 cm^3/g). The pulp viscosity of both pretreated bark pulps was somewhat lower than the corresponding untreated pulps, indicating that the pretreatment may somehow affect the lignocellulosic structure with negative impact to cellulose upon pulping.

Pulp Morphological Properties

Table 5 shows the fiber morphological properties of unbleached unbeaten pulps and bleached unbeaten and beaten (4500 PFI revolutions) pulps for wood and bark.

Overall, wood fibers showed a higher length and fiber population/g and lower fiber width, coarseness, and fines than bark. Wood fiber length and width were similar to those found for bleached *E. globulus* pulps.[30,31] No data could be found in the literature for bark to allow comparison. Wood and bark fibers showed little variation of length, width, and coarseness with bleaching.

The coarseness of bark pulp fibers was higher than that of wood pulp fibers, indicating a higher fiber wall thickness. In fact, fiber wall thickness for *E. globulus* wood and bark of around 5 μm and 7 μm, respectively, were reported.[3] The use of pretreatment and of different active alkali charges in the bark pulping seems to produce little variation in the pulp fibers' morphological properties.

Handsheet Optical Properties

Optical properties of wood and bark bleached handsheets are presented in Table 6. Brightness attained for all unrefined pulps was almost identical (88–90%), showing some decline with beating. Pulps **Bt_{15}** and **Bt_{20}** (pretreated barks) showed a similar behavior to wood pulp toward refining, achieving, at highest beating level, 87% brightness, with **B_{15}** and **B_{20}** showing 85% brightness.

Regarding opacity, all bark pulps proved to be more susceptible to beating, decreasing

TABLE 5. Morphological properties of unbleached, bleached unbeaten, and bleached beaten pulp fibers of wood and bark pulps

	Pulp	Beating (PFI)	Fibers (millions/g)	Length* (μm),	Width (μm)	Coarseness (mg/100m)	Fines (% in area)
W**	Unbleached	0	19	826	17.7	6.0	6.2
	Bleached	0	23	808	17.2	6.2	7.5
		4500	23	801	17.7	6.2	7.6
B_{15}	Unbleached	0	20	768	18.7	7.9	10.6
	Bleached	0	20	768	18.9	7.8	10.4
		4500	21	770	18.3	7.5	8
B_{20}	Unbleached	0	21	759	18.5	7.3	7.9
	Bleached	0	21	754	18.6	7.4	7.6
		4500	23	801	17.7	6.2	7.6
Bt_{15}	Unbleached	0	21	766	18.7	7.7	8.9
	Bleached	0	21	770	18.5	7.7	9.2
		4500	23	753	18.4	7.1	9.3
Bt_{20}	Unbleached	0	22	757	18.2	7.3	10
	Bleached	0	22	747	18.2	7.5	10.7
		4500	20	774	18.7	7.8	10.6

* weighted in length; ** data from Gominho.[22]

7–9% for 4500 rev as opposed to the 4% drop for wood. Pulps **Bt_{15}** and **Bt_{20}** achieved higher opacity values than the respective **B_{15}** and **B_{20}**, which appears to indicate a positive influence of the pretreatment in this parameter.

Regarding light scattering coefficient, the pulps produced with pretreated bark showed higher values than those from untreated bark. Higher active alkalinity also led to higher light scattering coefficients. The use of pretreatment and/or higher alkalinity has an impact in the carbohydrate polymers, leading to lower degree of polymerization and fiber-fiber bonding, consequently increasing fiber-air interfaces and therefore leading to higher light scattering coefficient. According to Carvalho,[32] there is an inversely proportional relation between degree of polymerization and light scattering coefficient.

The decrease in all three parameters with beating was expected, since a positive variation in fiber bonding is known to have a detrimental effect on optical properties.[33] Nonetheless, the effect of beating was more pronounced for bark than for wood.

Handsheet Physical/Mechanical Properties

Figure 1 shows the physical and mechanical properties of the four bleached eucalypt bark pulps and, for comparison, a pulp produced with eucalypt industrial chips under the same bleaching process. Unbeaten pulps produced with eucalypt wood should have a °SR and WRV in the range of 16–24 and 100–130%, respectively,[34] which was met by all the bark

TABLE 6. Optical properties of wood and bark bleached handsheets (unbeaten and beaten)

	Brightness (%) PFI (Rev)		Opacity % PFI (Rev)		Light Scatt. Coef. ($m^2.kg^{-1}$) PFI (Rev)	
	0	4500	0	4500	0	4500
W*	89	87	85	81	52	39
B_{15}	88	85	82	73	44	30
B_{20}	88	85	83	76	48	33
Bt_{15}	88	87	83	76	47	34
Bt_{20}	90	87	85	78	51	36

* Data from Gominho.[22]

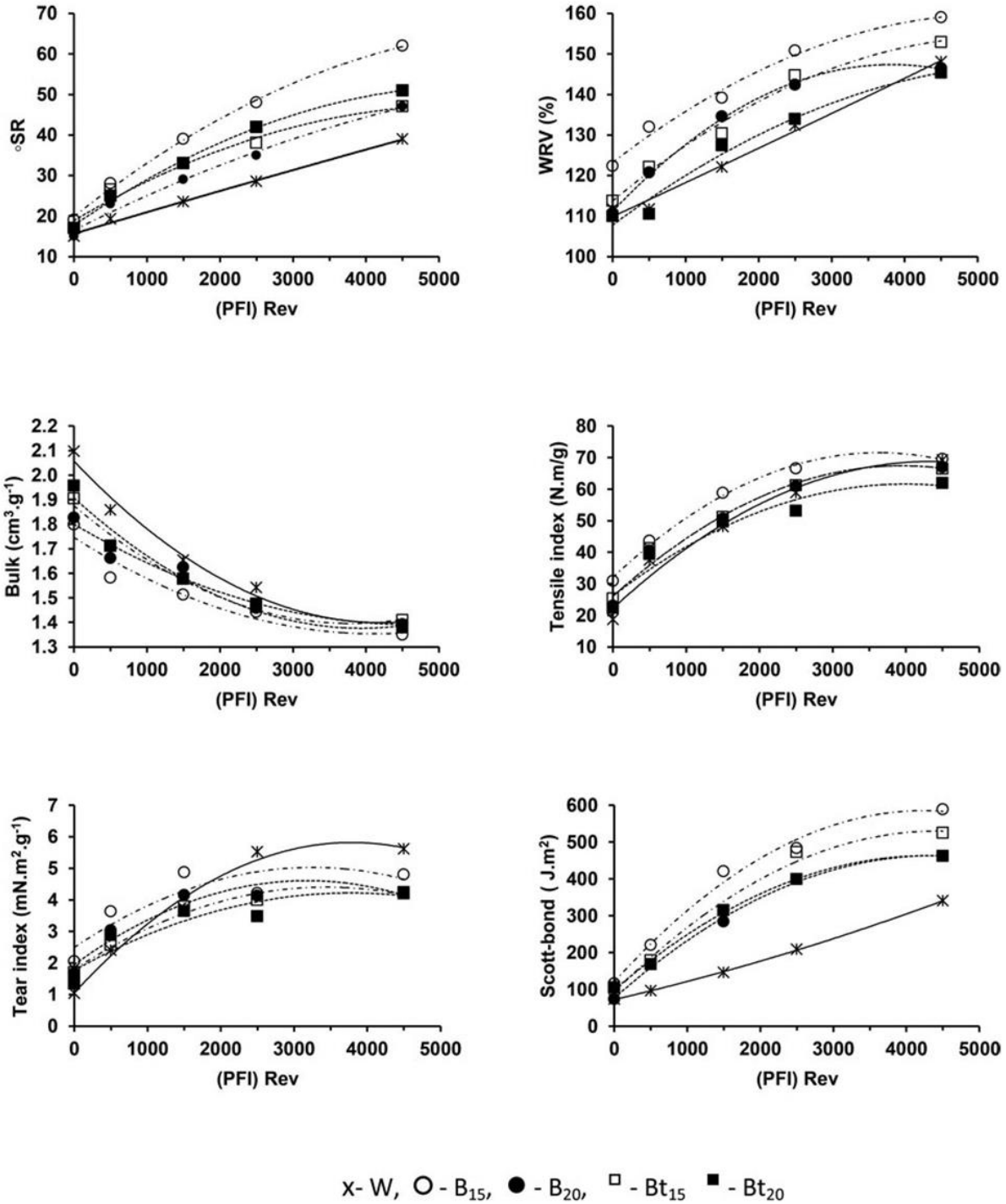

FIGURE 1. Wood and bark pulps' handsheet properties.

pulps. Upon beating, the bark pulps showed a higher increase in drainability than the wood pulp, with pulp $\mathbf{B_{15}}$ always showing the highest increase along the beating, reaching way above 50°SR at 4500 rev. This behavior of bark pulps under beating has been previously shown by Miranda,[3] and is certainly a consequence of their anatomical structure and fiber morphology. The higher fines content of these pulps (Table 5) also plays a role in the observed behavior.

The bleached pulps from pretreated bark always showed lower WRV values than those from untreated bark, regardless of the active

alkalinity (**Bt_{15}** and **Bt_{20}** lower than **B_{15}** and **B_{20}**, respectively), and high alkalinity also led to lower WRV, regardless of the use of pretreatment (Bt_{20} and Bt_{20} lower than **Bt_{15}** and **B_{15}**, respectively). This should be due to the effect of removal of hemicelluloses in the pretreatment and with higher active alkalinity upon pulping.

One of the most important properties when determining handsheet quality is the bulk, since it is an indirect measure of the fibers' conformity and structural organization.[35] This property also determines the handsheet strength, with high bulk normally indicating low paper strength. Refining will increase the paper strength at the cost of lowering bulk (i.e., structure densification). Pulp **B_{15}** presented the lowest bulk and highest handsheet strength values relative to the other bark pulps. This is probably due to the lower degradation of the carbohydrate polymers, namely the hemicelluloses that contribute to increase fiber bonding and conformity.

Pulps **Bt_{15}**, **Bt_{20}**, and **B_{20}** showed similar behavior regarding bulk, tensile, and tear indexes, and pulps **Bt_{15}** and **B_{15}** showed a higher Scott-bond than the other two. It appears that the lower active alkalinity improved the Scott-bond ability, especially at higher beating degrees.

Compared to wood, it is clear that the bark pulps have a lower tear index at higher beating, with similar results being reported for *Europhyla* barks pulps for which the paper tear strength was much lower than that of the wood pulp.[5] On the other hand, both wood and bark show comparable tensile strength with bark, presenting a much higher paper surface strength (Scott-bond).

CONCLUSIONS

E. globulus bark available at the pulp mill may be used to produce pulps with a kappa number similar to that of wood pulps (kappa number 17) and can be bleached using a standard bleaching sequence to a brightness degree similar to that of wood pulps. Refining of bark pulps is easier than refining wood pulps. The handsheet optical, physical, and mechanical properties of the bleached bark pulps studied in this work are comparable to those obtained for *E. globulus* wood.

However, bark delignification is more difficult to achieve and lower yields are obtained in comparison with wood. The use of a mild hydrothermal pretreatment proved beneficial in decreasing the ashes (31%) and extractives content (67%), while at the same time preventing polysaccharides degradation (2% loss). At low active alkali, the use of this hydrothermal pretreatment led to pulps with much lower kappa number than for untreated bark.

Process and economic impacts from withdrawing bark from its current uses, as well as the operation costs, should be evaluated in the future, to determine the viability of the process.

Overall, industrial bark of *E. globulus* appears to produce pulps with approximate characteristics of wood pulp, at least in terms of the parameters studied in this work. Other parameters, such as extractive content, pitch count, dirt count, bleachability, and brightness stability, might be needed to determine if bark is a good raw material for pulp production.

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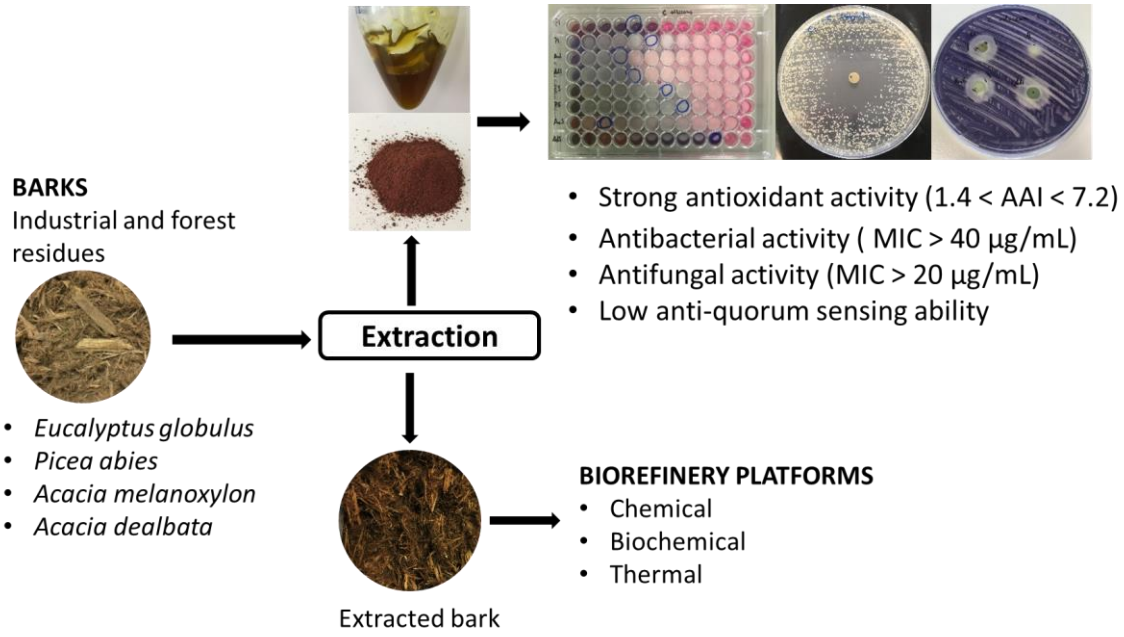

Graphical abstract of the paper

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ORIGINAL

Bark residues valorization potential regarding antioxidant and antimicrobial extracts

Duarte M. Neiva[1] · Ângelo Luís[2] · Jorge Gominho[1] · Fernanda Domingues[2] · Ana P. Duarte[2] · Helena Pereira[1]

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Abstract

Bark residual side streams from industries (*Eucalyptus globulus*—Eg and *Picea abies*—Pa) or from control of invasive species in Mediterranean countries (*Acacia melanoxylon*—Am and *Acacia dealbata*—Ad) are burned for energy production, although their high content of extractable compounds points to a possible valorization as sources of phytochemicals with antioxidant and antimicrobial activities. Non-polar and polar extracts were obtained, and their phenolic contents, antioxidant activity, antiquorum sensing and antimicrobial potential against several human pathogenic microbes (nine bacteria and two yeasts) were determined. Extraction yield ranged from 0.5 to 37% of barks dry weight varying with species and solvent used, and both water and ethanol extracts presented strong or very strong scavenging antioxidant ability. Eg and Pa non-polar extracts showed the lowest minimum inhibitory concentration for gram-positive bacteria (0.04–1.25 mg/mL), while Ad presented the best results among polar extracts regarding bacteria (0.16 mg/mL for *K. pneumoniae*) and yeast strains (0.02–0.04 mg/mL). Non-polar extracts showed great response against both *Candida* species (MIC = 0.04–0.63 mg/mL). Each extract had different antimicrobial activity showing that species and solvents can be used to tailor compounds to target specific pathogens. Information regarding these bioactive extracts from residual forest side streams can provide possible utilization routes for natural compounds recovery prior to combustion.

Abbreviations

EgH	*Eucalyptus globulus n*-hexane extract
PaH	*Picea abies n*-hexane extract
AmH	*Acacia melanoxylon n*-hexane extract

✉ Duarte M. Neiva
duarteneiva@isa.ulisboa.pt

[1] Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisbon, Portugal

[2] CICS-UBI, Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, Avenida Infante D. Henrique, 6200-506 Covilhã, Portugal

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AdH	*Acacia dealbata n*-hexane extract
EgET	*Eucalyptus globulus* ethanol extract
PaET	*Picea abies* ethanol extract
AmET	*Acacia melanoxylon* ethanol extract
AdET	*Acacia dealbata* ethanol extract
EgW	*Eucalyptus globulus* water extract
PaW	*Picea abies* water extract
AmW	*Acacia melanoxylon* water extract
AdW	*Acacia dealbata* water extract

Introduction

The research on new antioxidant and antimicrobial compounds derived from natural resources has become an important quest in the food, pharmaceutical, cosmetic and polymer industries because of public perception and interest in naturally derived products, better use of resources, lower production cost, pathogens developing resistance to conventional antibiotics or substituting synthetic compounds that might have a detrimental public health effect (Co et al. 2012; Royer et al. 2013; Wikaningtyas and Sukandar 2016).

Plants are very interesting resources to be studied and exploited due to their wide diversity and availability; some compounds present in the extractives (terpenoids, resin acids, flavonoids, stilbenes, tannins, lignans) are produced by the plant as secondary metabolites, for protection against external and internal stresses such as insects or pathogen attacks, as well as to act as antioxidants to decrease biological decay from harsh oxidative conditions from natural physiological processes within the cells (Angelis et al. 2016). Several plant parts have been studied as potential sources of bioactive compounds (fruits, seeds, leaves, stumps, wood) (Luís et al. 2012, 2014, 2016; Dezsi et al. 2015; Laboukhi-Khorsi et al. 2017) with barks receiving substantial attention and proving to be a potentially interesting source of lipophilic and polar compounds (Chang et al. 2001; Angelis et al. 2016; Rajan et al. 2017; Lima et al. 2018; Burčová et al. 2018).

The usually high extractives content of barks, especially when comparing to the respective woods, makes them particularly interesting raw materials for obtaining these bioactive compounds after easy or mild extraction conditions and preferably using GRAS solvents (generally recognized as safe). The solvent choice is paramount if the extracts are to be used in the food, pharmaceutical or nutraceutical industries, with *n*-hexane, ethanol and water being industrially applied to such end uses (Takeuchi et al. 2009). One of the main problems of barks as raw material derives from the large variation in extractives content and composition with species, age, edaphoclimatic conditions, season and tree health (Krogell et al. 2012; Kemppainen et al. 2014; Rajan et al. 2017). Nevertheless, the chemical variability of barks collected at industrial sites tends to decrease due to homogenization and diversity of collection sites.

The bark species studied here were chosen due to their relevance in the timber and pulp and paper industries (*Eucalyptus globulus* and *Picea abies*) or due

to their availability from control of invasive species (*Acacia melanoxylon* and *Acacia dealbata*). Several studies have focused on alternative uses of *E. globulus* and *P. abies* barks rather than direct burning for energy and electricity production, describing their chemical composition and possible uses, whether for pulp production, organic growing media formulation or ethanol and biogas production (Kemppainen et al. 2012, 2014; Chemetova et al. 2018; Neiva et al. 2016, 2018a, b), with some of them specifically focusing on the use of the extractives component (Valimaa et al. 2007; Pinto et al. 2013; Hubert et al. 2016; Angelis et al. 2016; Jablonsky et al. 2017; Tanase et al. 2018b). *Acacia* species are considered invasive in several European countries (mostly Mediterranean), and the fight to reduce or prevent their proliferation is constant and costly. In Portugal, one of the methods to mitigate proliferation of *A. dealbata* is to remove the bark without felling the tree, thereby reducing or preventing sprouting. For *A. melanoxylon*, the tree is felled and the wood is used due to its quality. In both cases, the bark is collected in large quantities or left in the field, therefore being a potential source for valorization. They can have potential applications with economic gains that could and should be valued to partially ease the economic resources associated with these invasive species combat and management or be used within the concept of a full resource use valorization, zero waste philosophy, where these species are cultivated for profitable resources (Souza-Alonso et al. 2017). These species have also been the subject of some studies regarding their composition and possible uses (Seigler 2003; Pizzi 2006; Luís et al. 2012).

Oxidation is necessary for normal metabolic processes in living organisms (energy production, cell growth regulation), but it is also responsible for cell and tissue damages (cell membrane lipid peroxidation, DNA mutation) as well as for food decay or deterioration of natural and synthetic materials (metals and polymers). The effort to delay or prevent auto-oxidation and prolong the shelf life of food and nutraceutical products leads to the addition of synthetic antioxidant compounds (e.g., butylated hydroxytoluene or butylated hydroxyanisole) whose long-term consumption may have potentially toxicological detrimental effects on human and animal health (Rodríguez-Rojo et al. 2012; Luís et al. 2014). Natural antioxidants are highly desirable for the nutraceutical and cosmetic industries working as scavengers of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide which can be originated from exogenous factors or be generated in biological functions within the cells (Chang et al. 2001; Co et al. 2012; Hubert et al. 2016). The mechanism for antioxidant protection regarding food product shelf live, polymeric and other materials is mostly through scavenging of ROS species, but when it comes to protection associated with polyphenols under physiological (in vivo) conditions, the antioxidative mechanism appears to be more complex. Within the cell, the antioxidant mechanism is mostly achieved through activation of transcription factors that regulate the activity of cytoprotective proteins and endogenous antioxidants (Forman et al. 2014; Ávila et al. 2017). Nevertheless, several natural polyphenols or crude plant extracts have been tested and determined to have a positive influence on activating transcription factors (such as Nrf2) acting as indirect antioxidants (Tanigawa et al. 2007; Eggler et al. 2008; Forman et al. 2014; Kumar et al. 2014).

Regardless of their end use, new, harmless natural antioxidants are therefore a very enticing product for several industries.

Naturally derived compounds have also been investigated for their pharmacological effects, with several studies finding interesting antiallergic, antiinflammatory, anticancer, antiquorum sensing and antimicrobial properties (Valimaa et al. 2007; Alfredsen et al. 2008; Ignat et al. 2013; Kalia 2013; Luís et al. 2014; Mocan et al. 2015; Tanase et al. 2018a). Although the use of antibiotics has significantly reduced infectious diseases and increased life spans of humans and livestock, the side effect is the increase in pathogenic strains that are stronger and more adapted to external aggression. This pathogenic resistance to conventional antibiotics increases the potential threat to public health with several bacteria and fungi being documented as gaining resistance to specific antibiotics or growing coordinated population responses to counteract external aggressions. Additionally, there is also the increase in negative side effects derived from the use of higher dosages or stronger antibiotics on the human organism. Alternative approaches are needed to combat the more resilient pathogenic strains, whether by searching for new biocidal or biostatic molecules or, in the case of some bacteria, by reducing the virulence through impeding their quorum sensing mechanisms that lead to virulence factors such as exopolysaccharide synthesis, biofilm formation or swarming motility (Kalia 2013; Vasavi et al. 2013). The search for natural compounds with antimicrobial and antiquorum sensing potential has led to extensive research on secondary metabolites from plants either in the crude extracts or by isolating specific compounds and testing them against different pathogens (Cowan 1999; Silva and Fernandes Júnior 2010; Savoia 2012).

It is possible to find some information regarding the antimicrobial activity of extracts from *P. abies* and *E. globulus* leaves, stumps, wood or knotwood (Valimaa et al. 2007; Khan et al. 2009b; Luís et al. 2014, 2016) and *P. abies* bark (Ignat et al. 2013; Salem et al. 2016; Burčová et al. 2018). No information was found for the bark of *E. globulus* and both acacia species.

This work is a first approach to the possible use of *E. globulus*, *P. abies*, *A. melanoxylon* and *A. dealbata* barks as sources of extractable compounds with a significant bioactivity (antioxidant, antimicrobial, antifungal and antiquorum sensing). Several crude extracts were studied to ascertain the best solvent extraction route and yields and estimate phytochemical content of extracts. These crude extracts were further studied regarding their antioxidant activity through different methods, since no single assay can accurately measure all antioxidant mechanisms in a complex system (Prior et al. 2005), their antimicrobial activity tested against several human pathogenic microbes (bacteria and yeasts) through disc diffusion and minimum inhibitory concentration determination, as well as their potential to inhibit quorum sensing. Although some information exists on *P. abies* and *E. globulus* antioxidant and antimicrobial activity of crude extracts or of some of their compounds, no information was found in the literature for the *Acacia* species studied here.

Materials and methods

Sampling and extraction

Eucalyptus globulus bark was collected after industrial debarking operation, at The Navigator Company pulp mill located in Setúbal, Portugal. *Picea abies* bark was collected at an industrial sawmill near Jyvaskyla, Finland. *Acacia melanoxylon* and *Acacia dealbata* barks were collected directly from trees at Sintra and Buçaco, Portugal, respectively. The industrial debarking process left substantial amounts of wood in the *E. globulus* and *P. abies* barks that were manually sorted and removed.

All the barks were air-dried, using an oven with air circulation at 25 °C until constant humidity (between 9 and 13% depending on species) and knife-milled to particle sizes below 1 mm. Extracts were obtained through solvent extraction using a Soxhlet apparatus for 16–24 h with roughly 10 g of oven-dry bark and a solvent to bark ratio of 20. The *n*-hexane extracts were obtained from the original bark samples, while the ethanol and water extracts were obtained from the *n*-hexane-extracted material. The extracts were concentrated in a rotary evaporator and further dried in a vacuum oven at 35 °C and 150 mbar. They were kept in the refrigerator afterwards until dissolution for analysis. The yields were determined by weight variation of the barks after each extraction and reported as percentage of oven-dry (o.d.) initial bark.

Monosaccharide composition

Monosaccharide composition of solubilized carbohydrates was determined after acid hydrolysis of each extract by HPIC (Dionex ICS-3000, using an Aminotrap plus Carbopac SA10 column). Samples (0.35 g) were first hydrolysed under strong acid concentration with 3 mL H_2SO_4 (72%) at 30 °C for one hour after which they were diluted with 84 mL of distilled water and further reacted at 120 °C in an autoclave for another hour. After filtration, the sample was injected in the HPIC.

Bark extracts phenolic profile

Total phenolic content (TPC) was determined by the Folin–Ciocalteu assay and reported as gallic acid equivalents (mg GAE/g_{ext}) using a calibration curve ($Abs = 1.493\ [GA] + 0.0254$, $R^2 = 0.99$) obtained through six gallic acid solutions ranging from 0.06 to 0.6 g/L.

Flavonoids content (FC) was estimated according to the aluminium chloride colorimetric method, where absorbances were measured at 415 nm, and reported as quercetin equivalents (mg QE/g_{ext}) using a calibration curve ($Abs = 0.0078\ [Q] - 0.0089$, $R^2 = 1.00$) obtained with eight quercetin solutions ranging from 0 to 200 mg/L.

Condensed tannins (CT) were estimated by the vanillin-H2SO4 method, where absorbances were measured at 500 nm, and reported as (+)-catechin equivalents

(CE) using a calibration curve (Abs = 2.0147 [C] − 0.0032, $R^2 = 1.00$) obtained with six (+)-catechin solutions ranging from 0.01 to 0.1 g/L.

The detailed methodologies can be found elsewhere (Luís et al. 2012; Ferreira et al. 2015).

Bark extracts antioxidant activity

The antioxidant activity of the crude extracts was determined by three methods: FRAP (ferric reducing antioxidant power), β-carotene bleaching (BCB) and DPPH scavenging assays.

FRAP (ferric reducing antioxidant power) assay shows the extract potential to reduce Fe(III) to Fe(II). The antioxidant activity can be measured spectrophotometrically by the change in the solution colour to blue resulting from the reduction in Fe(III)-2,4,6-tripyridyl-s-triazine (TPTZ) to Fe(II)-2,4,6-tripyridyl-s-triazine (TPTZ). Briefly, 180 µL of the extract solution was mixed with 540 µL of distilled water and 5.4 mL of FRAP solution (83.3% 0.3 M acetate buffer, 8.3% 10 mM TPTZ and 8.3% 20 mM ferric chloride v/v) and allowed to react for 30 min at 37 °C, after which absorbance was measured at 595 nm. The results were expressed as trolox equivalents (TE) through a calibration curve (Abs = 5.66 [TE] − 0.0038, $R^2 = 1.00$) obtained for trolox solutions with concentrations between 0.02 and 0.2 mg/mL.

The β-carotene bleaching test (BCB) measures the ability of an antioxidant to inhibit lipid peroxidation. Linoleic acid radical will attack the β-carotene, which will lose its characteristic orange colour. The antioxidant activity of an extract can be measured by the attack inhibition of linoleic acid radicals towards β-carotene by spectrophotometry at 470 nm. Briefly, 0.4 mg of β-carotene in chloroform, 20 µL linoleic acid, 400 mg Tween 40 and 1 mL of chloroform were mixed and then dried in the rotary evaporator. In total, 100 mL of oxygenated distilled water was added slowly and under agitation to the residue to form an emulsion. Five mL of this emulsion was added to 300 µL of different extract methanolic solutions (5, 50, 100, 250, 500, 750, 1000 mg/L) and left to react at 50 °C for one hour. The control's (300 µL methanol) absorbance was read (470 nm) at the beginning of the reaction (t = 0), and after one hour, all samples and control absorbances were read and the inhibition percentage calculated as $I\,\% = [(Abs^{t=1}\text{sample} - A^{t=1}\text{control})/(A^{t=0}\text{control} - A^{t=1}\text{control})]$. All absorbances were obtained against a blank consisting of the emulsion without the β-carotene. Butylated hydroxytoluene (BHT) standard was used as reference for comparison purposes.

The DPPH scavenging assay determines the capacity of a compound or group of compounds to stabilize the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) radical. When in radical form, this compound has a purple colouration with a characteristic absorption peak at 517 nm that turns to yellow when in DPPH2 form. Therefore, the antioxidant activity towards DPPH radicals could be determined spectrophotometrically by the decrease in absorbance at 517 nm. The procedure follows Scherer and Godoy (2009). Briefly, aliquots of 0.1 mL of methanolic extract solutions (25, 50, 100, 150, 200 and 250 mg/L) were added to 3.9 mL of different methanolic solutions of DPPH (0.2, 0.124 and 0.08 mM) and

incubated for 90 min at room temperature in the dark, after which the absorbance was measured at 517 nm. The inhibition of the DPPH was calculated as $I\% = [(Abs0–Abs1)/Abs0] \times 100$, where Abs0 is the control absorbance (0.1 mL of methanol) and Abs1 is the absorbance of each test sample after 90-min incubation. Three curves were obtained (sample concentration vs I%), and the IC50 was calculated as the sample concentration required to inhibit 50% of the DPPH. The antioxidant activity was determined as the antioxidant activity index (AAI = final concentration of DPPH in the control sample/IC50). The scavenging antioxidant ability was classified as: weak $AAI \leq 0.5$; moderate $0.5 < AAI \leq 1$; strong $1 < AAI < 2$; and very strong when $AAI \geq 2$. Two antioxidant standards, gallic acid and quercetin, were used for comparison. All assays were carried out in duplicate. DPPH solutions were prepared daily.

Antimicrobial activity and antiquorum sensing properties

Microorganism strains and culture media

The crude extracts antimicrobial activity was tested against 11 human pathogenic microbes: two yeasts (*Candida albicans* ATCC 90028 and *Candida tropicalis* ATCC 750) and nine bacteria: four gram-positive strains (*Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* LMG 16779 and *Staphylococcus aureus* ATCC 25923) and five gram-negative strains (*Acinetobacter baumannii* LMG 1025, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella enterica serovars* Typhimurium ATCC 13311). For the antiquorum sensing activity, the bacterium used was *Chromobacterium violaceum* ATCC 12472. These reference strains were acquired from the American Type Culture Collection (ATCC) or the Belgium Co-Ordinated Collections of Micro-Organisms/Laboratory of Microbiology (BCCM/LMG).

The reference strain cultures were stored at −80 °C in 20% glycerol, from which a sample was collected, subcultured and maintained in the fridge for future use in all the assays. From the fridge sample, subcultures were prepared in brain–heart infusion agar (BHI) for bacteria and Sabouraud dextrose agar (SDA) for yeasts 24 h prior to any antimicrobial test. All repetitions were achieved in different days and using different subcultures. For the disc diffusion assay, Müller–Hinton agar (MHA) was used for bacteria and MHA with 2% glucose and 0.5 µg/mL blue methylene was used for yeasts. The broth medium used for the minimum inhibition concentration (MIC) was Müller–Hinton broth (MHB) for the bacteria, and for the yeasts, a broth solution was prepared as follows: 3-(*N*-morpholino)propanesulfonic acid (MOPS, 35.53 g) and RPMI-1640 medium (5.215 g, supplemented with glutamine and phenol red and without bicarbonate) were dissolved in 400 mL of distilled water, pH adjusted to 7.0 ± 0.1 at 25 °C (with 1 M NaOH), water added to a final volume of 0.5 L, filtered sterilized and finally stored at 4 °C until required.

Disc diffusion assay (DD)

The disc diffusion assay was performed according to the M2-A8 and M44-A2 methods from the Clinical and Laboratory Standard Institute (CLSI) for bacteria and yeasts, respectively. From each subcultured plate, a sample was collected and suspended in saline solution to achieve 0.5 McFarland (inoculum density of $1–2\times10^8$ colony-forming units/mL for bacteria and $1–5\times10^6$ for yeasts) and subsequently used to inoculate the MHA petri dishes. Sterile filter paper discs (6 mm diameter) were impregnated with 20 µL of each extract diluted in dimethyl sulfoxide (DMSO) (200 mg/mL) such as to achieve 4 mg/disc. The discs were placed in the previously inoculated MHA petri dish and incubated at 37 °C for 24 h (bacteria) or 48 h (yeasts) before the diameters of inhibition zones were measured. Negative control consisted of a disc impregnated with 20 µL of DMSO and positive controls of discs with tetracycline (30 µg/disc) as bacteriostatic agent and amphotericin B (25 µg/disc) as antifungal.

Antiquorum sensing activity

The antiquorum sensing activity of the extracts was determined through the biomonitor strain *C. violaceum* ATCC 12472. The bacterial suspension was obtained by aerobic growth (30 °C, 250 rpm and overnight) in Luria–Bertani (LB) broth. From this suspension, a sample adjusted to an optical density of 1, measured spectrophotometrically at 620 nm, was inoculated in a plate with LB agar. The antiquorum sensing activity was measured through a disc diffusion assay similar to the one expressed previously with the plates being incubated at 30 °C for 24 h after which the inhibition of the pigment (violacein) produced around the disc ring of colourless, but viable cells were measured. The quorum sensing inhibition was determined by QSI = d2–d1 with d2 representing the diameter (mm) where the violet colouration starts to manifest and d1 the diameter where the bacteria growth inhibition ends. Resveratrol (5 µg/disc) was employed as positive control.

Minimum inhibition concentration (MIC) determination

The second method to assess the influence of the extracts on microorganisms' growth was the resazurin microtiter assay. Sterile 96-well plates were used to determine the minimum inhibitory concentration (MIC). For bacteria, the procedure was as follows: 90 µL of MBH broth solution was added to the first column and 50 µl for the remaining columns; 10 µL of each extract (200 mg/mL in DMSO) was added to the first well of each line; and after homogenization, 50 µL of the first well was passed to the second well and from this to the adjacent and so on as to successively dilute the extract concentration remaining 50 µL in each well in serially halved concentrations. In all wells, it was further added: resazurin (10 µL, 0.1% in MHB), 30 µL of MHB and finally 10 µL of bacterial suspension (0.5 McFarland). A set of controls was added: a line with tetracycline instead of sample as positive control, a line without extracts and a line without bacterial suspension (MHB solution instead). For the yeasts, the procedure was similar with the following variations: the final volume

was 200 µL instead of the 100 µL for the bacteria; the resazurin-sterilized solution (50 µL, 20 mg/mL in water) was added directly to the suspension of yeast (10 µL of 0.5 McFarland inoculum diluted in 10 mL of broth culture media); 100 µL of this solution was added to each well already containing 100 µL of decreasing extract concentration. In the case of yeasts, the positive control was obtained with amphotericin B. The plates were prepared in triplicate and placed in an incubator set at 37 °C for 24 h after which the colour change was assessed visually with the MIC being the well (respective concentration in mg/mL) that still exhibits purple colouration adjacent to the first one that turned pink or colourless.

Statistical analysis

Experiments were made at least in triplicate and results expressed as mean with respective standard deviation, as an average of two experiments for the BCB assay or modal values in the case of MIC. The STATISTICA (version 6.1 from StatSoft) software was used to test the significant differences among means by applying one-way ANOVA (analysis of variance) with post hoc Tukey HSD (honestly significant difference) test with an alpha equal to 0.05.

Results

Extraction

Table 1 shows the extraction yields for *n*-hexane (EgH, PaH, AmH and AdH) followed by ethanol (EgEt, PaEt, AmEt and AdEt) or water (EgW, PaW, AmW and AdW) extractions for *Eucalyptus globulus* (Eg), *Picea abies* (Pa), *Acacia melanoxylon* (Am) and *Acacia dealbata* (Ad) barks. *Picea abies* presented a significantly higher non-polar extractives content (4% o.d) than the remaining barks, and *A. dealbata* showed the highest polar extract content regardless of the solvent used (37%) with 2.5–12.3 times the content in the other barks. Water proved to be a better polar extraction solvent for *E. globulus* (three times higher yield) and *P. abies* (1.6 times higher yield) than ethanol while the opposite occurred for *A. melanolylon* (1.5 higher for ethanol).

Table 1 Bark extraction yields (% o.d mass) for *n*-hexane, ethanol and water solvents

Solvent	Eg	Pa	Am	Ad
n-hexane	**0.50** ± 0.05 a	**4.06** ± 0.16 d	**1.41** ± 0.09 c	**0.78** ± 0.06 b
Ethanol	**2.98** ± 0.23 a	**9.35** ± 0.10 c	**6.83** ± 0.06 b	**36.54** ± 0.48 d
Water	**9.28** ± 0.12 ab	**14.88** ± 1.67 b	**4.46** ± 0.77 a	**36.77** ± 2.90 c

Eg (*E. globulus*), Pa (*P. abies*), Am (*A. melanoxylon*) and Ad (*A. dealbata*)

Mean values (bold) in a row with different letters are significantly different according to one-way ANOVA with post hoc Tukey HSD test with $\alpha = 0.05$

Extracts monosaccharides

After an acid hydrolysis, the resulting solutions were analysed by HPIC (high-pressure ionic chromatography) and the main monosaccharide monomers determined. Table 2 shows the results for ethanol and water extracts. The same procedure was applied to the *n*-hexane extracts, but only *A. dealbata* extract showed a slight content of glucose (3.1 mg/g_{ext}—not shown in Table 2), with the remaining extracts presenting only trace amounts of monosaccharides.

The total monosaccharides content ranged from 3 to 25% for AdEt and PaW extracts, respectively. Overall, water extracts presented a higher total sugar content than the respective ethanol extract (1.5–2.9 times higher), with the exception of *A. melanoxylon* bark, which contained the same amount of both polar extracts (213 and 219 mg/g_{ext}). Glucose was the most abundant sugar in all the extracts (41–99% relative percentage) with the exception of the *E. globulus* water extract (27%), where the most relevant monosaccharide was arabinose (36%). The amount and content of all monosaccharides with the exception of glucose were higher in water extracts than in ethanol extracts.

Bark extracts phenolic, flavonoid and tannin contents estimation

Table 3 shows the results for the phenolic, flavonoids and tannin contents as well as the antioxidant activity of the *n*-hexane, ethanol and water extracts of the four barks.

The total phenolic content (TPC) of the *n*-hexane extracts was very low, with *E. globulus* showing the highest content (36 mg GAE/g_{ext}). The determinations of the flavonoid (FC) and condensed tannins (CT) contents for the non-polar extracts were impossible to conduct due to turbidity formation during the experimental procedures that impaired spectrophotometry readings. The TPC for ethanol and water extracts showed significant differences between species and solvent used ranging from 138 mg GAE/g_{ext} (AmW) to 704 mg GAE/g_{ext} (AdEt). The ethanol TCP was always higher than the corresponding water TPC. While *A. dealbata* bark presented the highest phenol content for both polar extracts (704 and 586 mg GAE/g_{ext}) when expressed in terms of raw material, the values are even higher in relation to the other barks, for example six times higher (215.5 vs 36.1 mg GAE/g_{bark} for AdW and PaW) up to 42 times higher content (257 vs 6.2 mg GAE/g_{bark} for AdEt and EgEt).

Regarding flavonoids (FC), water was a better extracting medium (ranging between 15.9 and 30.9 mg QE/g_{ext}) than ethanol (3.9–19.4 mg QE/g_{ext}). *Eucalyptus globulus* bark extract presented the highest amount of flavonoids (EgW—30.9 mg QE/g_{ext}), although, if the amount obtained from the bark is reported, *A. dealbata* (AdW) yielded the highest value (5.8 mg QE/g_{bark}).

Regarding condensed tannins (CT), Ad presented a substantially higher quantity (440.3 and 377.3 mg CE/g_{ext} for ethanol and water, respectively) than all the other extracts (2.2–377.3 mg CE/g_{ext}). Water was less efficient than ethanol to remove and dissolve tannins from barks showing much lower values (e.g., 83.2 vs 11.2 for AmEt and AmW, respectively).

Table 2 Monosaccharide composition of ethanol and water extracts after hydrolysis from *E. globulus*, *P. abies*, *A. melanoxylon* and *A. dealbata* barks expressed as mg/g_{ext}

	Ethanol				Water			
	EgEt	PaEt	AmEt	AdEt	EgW	PaW	AmW	AdW
Rhamnose	**9.0** ± 0.3 b	**0.9** ± 0.0 a	**35.0** ± 0.3 e	0 a	**14.0** ± 0.2 c	**10.2** ± 0.4 b	**32.1** ± 0.7 d	**0.9** ± 0.0 a
Arabinose	**6.7** ± 0.2 c	**4.4** ± 0.0 bc	**65.4** ± 1.3 e	**0.3** ± 0.0 a	**60.9** ± 0.8 d	**81.2** ± 0.6 f	**59.9** ± 0.4 d	**3.2** ± 0.1 b
Galactose	**4.2** ± 0.2 d	**0.6** ± 0.0 b	**1.3** ± 0.0 c	**0** a	**27.0** ± 0.2 g	**21.5** ± 0.0 f	**13.6** ± 0.2 e	**4.1** ± 0.1 d
Glucose	**39.3** ± 0.6 b	**76.6** ± 0.7 d	**108.7** ± 0.8 g	**27.6** ± 0.3 a	**45.1** ± 0.8 c	**101.7** ± 0.7 f	**88.9** ± 3.0 e	**31.7** ± 0.3 a
Mannose	**2.5** ± 0.1 c	**2.4** ± 0.1 bc	**0.5** ± 0.0 a	**0** a	**12.8** ± 0.1 e	**29.3** ± 0.9 f	**7.8** ± 0.1 d	**1.2** ± 0.0 ab
Xylose	**1.6** ± 0.1 b	**0.4** ± 0.1 a	**8.4** ± 0.2 d	**0** a	**8.4** ± 0.1 d	**4.4** ± 0.4 c	**11.1** ± 0.3 e	**0.4** ± 0.0 a
Total	**63.2** ± 1.5 c	**85.1** ± 0.7 d	**219.3** ± 2.6 f	**27.9** ± 0.0 a	**168.2** ± 1.8 e	**248.3** ± 0.9 g	**213.4** ± 4.4 f	**41.5** ± 0.5 b

Mean values (bold) in a row with different letters are significantly different according to one-way ANOVA with post hoc Tukey HSD test with $\alpha = 0.05$

Table 3 Total phenolic content, flavonoids content, condensed tannin content and antioxidant activity of *n*-hexane, ethanol and water extracts from *E. globulus*, *P. abies*, *A. melanoxylon* and *A. dealbata* barks

	n-hexane				Ethanol				Water			
	EgH	PaH	AmH	AdH	EgEt	PaEt	AmEt	AdEt	EgW	PaW	AmW	AdW
Phytochemical composition												
TPC (mg GAE/g_{ext})	**36** ± 3 a	**27** ± 3 a	**17** ± 1 a	**17** ± 1 a	**208** ± 8 d	**375** ± 8 f	**149** ± 8 bc	**704** ± 16 h	**172** ± 11 c	**242** ± 17 e	**138** ± 1 b	**586** ± 11 g
TPC (mg GAE/g_{bark})	**0.2** ± 0.01 ab	**1.1** ± 0.1 a	**0.2** ± 0.02 a	**0.1** ± 0.01 a	**6.2** ± 0.2 ab	**35.0** ± 0.7 c	**10.1** ± 0.6 ab	**257** ± 5.9 e	**15.9** ± 1.0 b	**36.1** ± 2.6 c	**6** ± 0.0 ab	**215.5** ± 4.0 d
FC (mg QE/g_{ext})	–	–	–	–	**18.1** ± 1.4 cd	**19.4** ± 0.4 de	**14.5** ± 0.4 b	**3.9** ± 0.1 a	**30.9** ± 1.8 g	**20.7** ± 0.5 e	**25.6** ± 0.9 f	**15.9** ± 0.6 bc
FC (mg QE/g_{bark})	–	–	–	–	**0.5** ± 0.04 a	**1.8** ± 0.04 d	**1.0** ± 0.02 b	**1.4** ± 0.05 c	**2.9** ± 0.16 d	**3.1** ± 0.08 e	**1.1** ± 0.04 bc	**5.8** ± 0.21 f
CT (mg CE/g_{ext})	–	–	–	–	**12.5** ± 0.7 a	**61.1** ± 1.5 b	**83.2** ± 5.5 c	**440.3** ± 9.4 e	**2.2** ± 0.3 a	**13.1** ± 0.8 a	**11.2** ± 1.2 a	**377.1** ± 8.3 d
CT (mg CE/g_{bark})	–	–	–	–	**0.4** ± 0.02a	**5.7** ± 0.14 b	**5.7** ± 0.37 b	**160.9** ± 3.43 d	**0.2** ± 0.03 a	**1.9** ± 0.11 ab	**0.5** ± 0.06 a	**138.6** ± 2.9 c
Antioxidant activity												
FRAP (mg TE/g_{ext})	**15** ± 3 a	**29** ± 1 a	**33** ± 1 a	**26** ± 1 a	**489** ± 10 e	**530** ± 3 f	**323** ± 9 c	**1295** ± 32 h	**402** ± 4 d	**346** ± 3 c	**292** ± 6 b	**1132** ± 10 g
FRAP (mg TE/g_{bark})	**0.18** ± 0.01 a	**1.11** ± 0.1 a	**0.24** ± 0.02 a	**0.13** ± 0.01 a	**15** ± 0.3 ab	**50** ± 0.2 b	**22** ± 0.6 ab	**473** ± 11.6 c	**37** ± 0.3 ab	**51** ± 0.4 b	**13** ± 0.3 ab	**416** ± 3.5 c
BCB—IC_{50} (mg/L) (1)	> 1000	> 1000	> 1000	> 1000	427	406	> 1000	197	465	563	634	188
BCB—inhib % at 1 g/L (2)	40	21	17	0	71	76	49	83	76	66	62	95

Table 3 (continued)

	n-hexane				Ethanol				Water			
	EgH	PaH	AmH	AdH	EgEt	PaEt	AmEt	AdEt	EgW	PaW	AmW	AdW
DPPH—IC_{50} (mg/L) (3)	–	–	–	–	**14.6** ± 5.6 ab	**12.5** ± 4.9 ab	**16.3** ± 5.9 ab	**5.3** ± 1.7 a	**22.1** ± 7.0 ab	**17.8** ± 7.2 ab	**28.3** ± 10.4 b	**5.6** ± 2.1 a
DPPH—AAI (4)	–	–	–	–	**2.7** ± 0.2 bc	**3.1** ± 0.2 c	**2.4** ± 0.2 abc	**7.2** ± 0.9 d	**1.7** ± 0.2 ab	**2.2** ± 0.2 abc	**1.4** ± 0.1 a	**6.9** ± 0.5 d

Mean values (bold) in a row with different letters are significantly different according to one-way ANOVA with post hoc Tukey HSD test with $\alpha = 0.05$

TPC total phenolic content, *TF* total flavonoids, *CT* condensed tannins, *GAE* gallic acid equivalent, *QE* quercetin equivalents, *CE* catechin equivalents, *TE* trolox equivalents

(1) BHT IC50 = 29, (2) BHT inhibition % at 1 g/L = 100, (3) gallic acid IC50 = 2.1 ± 0.8 mg/L and quercetin IC50 = 4.32 ± 0.39 mg/L, (4) gallic acid AAI = 23.6 ± 1.7 and quercetin AAI = 12.2 ± 1.71

Bark extracts antioxidant activity

The results for the three antioxidant activity assays performed on the bark extracts are presented in Table 3.

The ferric reducing antioxidant power (FRAP) of all the non-polar extracts was very low (15–33 mg TE/g_{ext}). Ethanol was more efficient than water to extract compounds with antioxidant activity, with FRAP values between 11 and 53% higher for ethanol when compared with the equivalent water extracts (e.g., 346 vs 530 for PaW and PaEt). *Acacia dealbata* bark extract showed the highest FRAP values with more than double those obtained by the other barks and was a better antioxidant than trolox (above 1000 mg TE/g).

The results for the β-carotene bleaching test (BCB) were reported as the quantity necessary to inhibit 50% of the linoleic acid radical, IC50, and as the highest inhibition possible for an extract concentration of 1 g/L. All the non-polar extracts and AmEt needed higher concentrations than 1 g/mL to reach a 50% inhibition. EgH was the most efficient non-polar extract obtaining 40% inhibition at 1 g/L with all the other three species obtaining between 0 and 21%. Water extracts were better in stabilizing the radical than the respective ethanol extracts, with the exception of the *P. abies* bark for which the ethanol extract showed a higher inhibition (76% vs 66%) and lower IC50 (406 vs 563 mg/L). *Acacia dealbata* presented the best results with AdW inhibition of 95% at 1 g/L and with IC50 of 188 mg/L. All the results were well below those of the standard butylated hydroxytoluene (BHT), with IC50 of 29 mg/L and 100% inhibition at 250 mg/L.

As with FRAP, the DPPH antioxidant activity followed the same trend of Ad > Pa > Eg > Am, with ethanol extracts having a higher antioxidant activity ($2.4 < AAI < 7.2$) than the respective water extracts ($1.4 < AAI < 6.9$). The coefficient of variation (standard deviation divided by mean) of IC50 (between 31.6 and 40.4%) was much higher than that of AAI (between 7.1 and 12.9). All the extracts are classified as having strong (AmW, EgW) or very strong (all remaining polar extracts) antioxidant activities.

Antimicrobial activity and antiquorum sensing properties

Disc diffusion and antiquorum sensing activities

The disc diffusion assay measures the influence of the crude extracts on the normal growth of bacteria and yeasts on agar plates. The larger the diameter without growing bacteria or yeast around the paper disc soaked with extract, the higher the growth inhibition properties of that extract. These inhibition zones are presented in Table 4 for all 12 extracts paired with the 9 bacteria (gram-positive: *B. cereus*, *E. faecalis, L. monocytogenes, S. aureus*, and gram-negative: *A. baumannii, E. coli, K. pneumoniae, P. aeruginosa, S. Typhimurium*) and 2 yeasts (*C. albicans* and *C. tropicalis*) along with the positive controls (30 µg tetracycline for

Table 4 Antimicrobial and quorum sensing inhibition zones (mean diameters in mm ± standard deviation) of *n*-hexane, ethanol and water extracts from *E. globulus*, *P. abies*, *A. melanoxylon* and *A. dealbata* barks (4 mg per disc)

	n-hexane				Ethanol				Water				Control
	EgH	PaH	AmH	AdH	EgEt	PaEt	AmEt	AdEt	EgW	PaW	AmW	AdW	
Gram-positive													Tetracycline
B. cereus	**13.3** ± 0.5 ef	**9.3** ± 1.0 bcd	– a	**7.9** ± 1.9 ab	**11.7** ± 0.4 cdef	**14.1** ± 0.5 fg	**12.2** ± 0.1 ef	**16.9** ± 0.1 g	**8.9** ± 0.3 bc	**11.9** ± 1.6 def	**11.0** ± 1.6 cde	**16.4** ± 1.0 g	**30.0** ± 0.8
E. faecalis	– a	– a	– a	– a	– a	**13.7** ± 0.5 c	**10.7** ± 1.2 b	**17.8** ± 1.1 d	– a	**9.9** ± 0.5 b	– a	**16.3** ± 0.4 d	**25.2** ± 0.6
L. monocytogenes	– a	– a	– a	– a	**18.2** ± 2.1 d	**15.5** ± 0.5 bcd	**12.8** ± 2.8 b	**29.0** ± 0.8 e	**17.4** ± 1.2 cd	**14.4** ± 0.1 bc	**14.9** ± 0.4 bc	**30.4** ± 0.3 e	**18.3** ± 0.6
S. aureus	**11.1** ± 0.5 bc	**8.1** ± 0.7 a	– a	**8.8** ± 0.2 ab	**16.7** ± 0.8 e	**15.5** ± 0.4 e	**14.2** ± 1.4 de	**20.7** ± 1.3 f	**15.6** ± 0.7 e	**14.3** ± 0.8 de	**11.7** ± 1.4 dc	**21.8** ± 0.4 f	**30.3** ± 0.5
Gram-negative													Tetracycline
A. baumannii	**17.6** ± 0.9 c	**8.5** ± 2.8 a	**9.8** ± 2.4 a	**16.6** ± 2.4 c	**11.1** ± 0.3 ab	**9.5** ± 0.4 a	**9.4** ± 1.0 a	**14.6** ± 0.8 bc	**11.2** ± 1.6 ab	**10.4** ± 2.3 ab	**9.9** ± 0.6 ab	**16.7** ± 1.1 c	**25.6** ± 0.3
E. coli	– a	– a	– a	– a	**8.1** ± 2.0 ab	**8.6** ± 0.8 ab	**8.7** ± 0.6 ab	**13.0** ± 1.0 c	**7.5** ± 1.3 ab	**9.9** ± 1.8 bc	**8.6** ± 1.2 ab	**13.1** ± 2.6 c	**23.3** ± 0.5
K. pneumoniae	– a	– a	– a	– a	**15.9** ± 0.2 e	**12.3** ± 0.8 b	**14.2** ± 0.2 cd	**20.5** ± 0.9 f	**15.7** ± 0.4 de	**13.5** ± 0.7 bc	**13.4** ± 0.7 bc	**21.1** ± 0.7 f	**22.3** ± 0.5
P. aeruginosa	– a	– a	– a	– a	**10.4** ± 0.8 b	**10.1** ± 0.9 b	**10.2** ± 0.4 b	**15.0** ± 1.1 c	**11.6** ± 1.1 b	**11.0** ± 0.8 b	**10.9** ± 0.8 b	**16.8** ± 0.9 c	**11.5** ± 0.6
S. typhimurium	– a	– a	– a	– a	**8.8** ± 0.7 bc	– a	**7.9** ± 1.7 abc	**14.3** ± 0.4 d	**8.7** ± 1.2 bc	**9.1** ± 1.2 bc	**9.2** ± 0.8 c	**15.6** ± 0.5 d	**28.5** ± 0.5
Yeast													Amphotericin B
C. albicans	**18.2** ± 2.4 c	– a	**11.1** ± 1.6 b	**16.8** ± 2.5 c	– a	– a	– a	**10.2** ± 1.3 b	– a	– a	– a	**12.9** ± 1.0 b	**20.3** ± 0.6
C. tropicalis	**25.2** ± 3.1 f	– a	**12.2** ± 2.2 c	**23.6** ± 2.5 ef	**10.7** ± 0.2 bc	**10.2** ± 0.7 bc	**9.1** ± 0.7 abc	**20.3** ± 0.6 de	**10.0** ± 0.4 abc	**8.4** ± 1.1 abc	**7.5** ± 0.5 abc	**18.4** ± 0.5 d	**21.5** ± 0.6
Quorum sensing inhibition													Resveratrol

Table 4 (continued)

	n-hexane				Ethanol				Water				Control
	EgH	PaH	AmH	AdH	EgEt	PaEt	AmEt	AdEt	EgW	PaW	AmW	AdW	
C. violaceum	**6.9** ± 1.8 e	**2.1** ± 0.9 abc	**6.1** ± 0.5 de	**7.2** ± 2.0 e	**3.5** ± 1.3 cd	– a	– a	– a	**3.1** ± 0.4 bc	– a	– a	– a	**8.5** ± 0.7

Mean values (bold) in a row with different letters are significantly different according to one-way ANOVA with post hoc Tukey HSD test with $\alpha = 0.05$

bacteria, 25 µg amphotericin B for yeasts and 5 µg resveratrol for quorum sensing per disc). Negative control (DMSO) showed no inhibition for all species.

The *n*-hexane extracts had no limiting effect on the growth of most of the gram-negative bacteria, with the exception of *A. baumannii* where EgH and AdH presented the best results along with *A. dealbata* water extract. Of the four gram-positive bacteria studied, only half (*B. cereus and S aureus*) were growth-inhibited by the *n*-hexane extract, although only mildly, with results being worse than for most of the polar extracts. *Acacia melanoxylon n*-hexane extracts presented the worst results towards bacteria, being slightly antibacterial only for *A. baumannii* (d = 9.8 mm). EgH presented the best results of the non-polar extracts.

Regarding polar extracts influence on bacteria, *A. dealbata* always presented the best growth inhibition with water extracts being slightly better than ethanol extracts. Overall, the inhibitory effect was less effective for the gram-negative bacteria than for the gram-positive ones, showing lower inhibition diameters (11.96 vs 15.68 mm for gram-negative and gram-positive diameter averages, excluding the totally ineffective results). The higher inhibition diameters were obtained by *A. dealbata* extracts for the *L. monocytogenes* (30.4 mm). The control tetracycline obtained inhibition diameters between 11.5 mm (*P. aeruginosa*) and 30.3 mm (*S. aureus*).

For the yeasts, the results were more pronounced for *n*-hexane extracts than for polar ones. Among the polar extracts, only *A. dealbata* presented any influence on *C. albicans* with all other extracts being completely ineffective. *C. albicans* suffered less growth impairment from the extracts than *C. tropicalis*, with the latter being more inhibited by the EgH and AdH extracts (25.2 mm and 23.6 mm, respectively), with AdEt also showing good results (20.3 mm).

Regarding the extracts' quorum sensing inhibition properties, the non-polar extracts were, with the exception of the polar *E. globulus*, the only ones that showed any capability to inhibit the quorum sensing. The best results were obtained for AdH and EgH extracts (7.2 and 6.9 mm, respectively). As for the polar extracts, ethanol extracts were slightly better than water extracts, for example 3.5 mm and 3.1 mm, respectively, for *E. globulus*.

Minimum inhibition concentration (MIC)

The resazurin microtiter assay was used to assess the minimal concentration required to inhibit the growth of the microbes studied. The concentrations in mg/mL of all crude extracts as well as the positive controls (tetracycline and amphotericin B for bacteria and yeasts, respectively) in µg/mL and negative controls (only DMSO without extract/standard) are shown in Table 5.

The concentrations required to inhibit the gram-negative species were very high with bacteria like *E. coli*, *P. aeruginosa* or *S. Typhimurium* being virtually indifferent to all the extracts, with the exception of AdEt. Regarding *K. pneumonia*, both ethanol and water extracts from each bark produced almost similar inhibition. For *A. baumannii*, the lowest concentration was obtained for AdW (0.313 mg/mL) followed by EgH and AdEt (both 1.25 mg/mL). For the gram-negative, the closest crude extract to the control was AdW regarding *K. pneumoniae* but still being 2600 times less efficient than tetracycline.

Table 5 MIC values (mg/mL) of *n*-hexane, ethanol and water extracts from *E. globulus*, *P. abies*, *A. melanoxylon* and *A. dealbata* barks and positive and negative controls

	n-hexane				Ethanol				Water				Controls (µg/mL)	Negative control
	EgH	PaH	AmH	AdH	EgEt	PaEt	AmEt	AdEt	EgW	PaW	AmW	AdW		
Gram-positive													Tetracycline	DMSO
B. cereus	0.04	0.04	0.63	0.63	0.31	0.16	0.63	0.31	2.5	5	0.63	0.63	0.06	> 20
E. faecalis	0.16	1.25	> 10	5	10	2.5	10	5	5	5	5	2.5	0.06	> 20
L. monocytogenes	0.16	1.25	> 10	5	2.5	2.5	5	2.5	2.5	10	5	2.5	0.06	> 20
S. aureus	0.08	0.31	5	5	1.25	0.63	> 10	> 10	> 10	5	2.5	> 10	0.06	> 20
Gram-negative													Tetracycline	DMSO
A. baumannii	1.25	2.5	5	5	2.5	2.5	5	1.25	2.5	5	5	0.31	0.06	> 20
E. coli	> 10	> 10	> 10	> 10	10	10	10	1.25	10	10	10	2.5	0.06	> 20
K. pneumoniae	> 10	5	> 10	> 10	0.63	1.25	1.25	0.31	0.63	1.25	1.25	0.16	0.06	> 20
P. aeruginosa	> 10	5	> 10	> 10	> 10	10	> 10	1.25	10	> 10	10	> 10	0.24	> 20
S. typhimurium	> 10	5	> 10	> 10	> 10	> 10	> 10	2.5	> 10	>10	> 10	2.5	0.24	> 20
Yeast													Amphotericin B	DMSO
C. albicans	0.31	0.16	0.63	0.31	0.16	0.16	5	0.02	0.63	10	10	0.04	0.25	> 20
C. tropicalis	0.04	0.04	0.04	0.04	0.08	0.08	0.16	0.04	0.31	1.25	5	0.08	0.5	> 20

Regarding gram-positive bacteria, *E. globulus* *n*-hexane bark extract was clearly the most efficient to inhibit the growth of these bacteria with MIC values ranging from 0.04 to 0.16 mg/mL. Most of the polar extracts showed poor antibacterial activity with PaEt being the most efficient of them all, reaching from 0.16 to 2.5 mg/mL. *Bacillus cereus* was clearly the most susceptible of the gram-positive species with EgH crude extract being 667 times less efficient than the positive control.

As for the yeasts, it is clear that *C. albicans* was less affected by the extracts than *C. tropicalis* with the only exception of *A. melanoxylon* polar extracts for which the MIC for *C. albicans* is half of the *C. tropicalis*. For *C. albicans*, the polar extracts of *A. melanoxylon* were almost ineffective and the PaW presented a much higher MIC than PaEt (10 vs 0.16 mg/mL, respectively). The most efficient crude extract for *C. albicans* was AdEt (0.02 mg/mL) with only 80 times lower MIC than the control amphotericin B. As for *C. tropicalis*, almost all extracts showed good antifungal activity (except AmW) with the best results occurring for all polar extracts and the *A. dealbata* ethanol extract (0.04 mg/mL) having only 80 times less efficiency than amphotericin B (0.5 µg/mL).

Discussion

The four barks were chosen due to their availability as industrial residues in pulping mills (*E. globulus* and *P. abies*) and sawmills (*P. abies*) and as biomass residues from the eradication of invasive species (*A. melanoxylon* and *A. dealbata*) in southern Europe. In fact, the availability of the barks is required to decrease the cost associated with their collection, transportation and management, in view of their integration into biorefineries leading to a valorization in line with each bark-specific characteristic.

In this kind of study, the solvents choice is paramount since the compounds extracted are to be used in the food, pharmaceutical or nutraceutical industries, with *n*-hexane, ethanol and water already being industrially applied to such end uses. Therefore, these three solvents were tested here regarding yield, chemical profile and activities of the extracts. In this work, *n*-hexane was used to decrease the possible detrimental influence that the residual solvent in the extracts could have on the bacteria and yeast growth during the antimicrobial assays.

P. abies bark has a relatively high content in lipophilic extractives, but this content was low in the other barks (Table 1). The yields obtained are in accordance with published data (Santos et al. 2011; Miranda et al. 2012, 2013; Neiva et al. 2018a). The amount of polar extractives was higher than that of lipophilic extractives regardless of the polar solvent used (Table 1). According to Neiva et al. (2018a, b) the total extractives obtained through a sequential extraction of dichloromethane, ethanol and water of *E. globulus* and *P. abies* barks were 10% and 20%, respectively. This means that by using *n*-hexane and water it is possible to extract almost the totality of extractives present in the *E. globulus* (9.8%) and *P. abies* (19%) barks. The variation in extraction yield between the ethanol and water extractives is a combination of the sugars (here calculated as monosaccharides after acid hydrolysis of the extract, Table 2), total phenolic compounds (TPC, Table 3) and other compounds, such as

alkaloids or saponins that could not be accounted for within these assays. The *E. globulus* and *P. abies* water extracts showed much higher monosaccharides content than their ethanol extracts, which probably explains the higher water extract yield. On the other hand, for *A. melanoxylon*, ethanol was able to extract higher quantities of sugars and phenols increasing the extract yield for this solvent when compared to water. As for the *A. dealbata*, the higher sugar content in the water extracts made up for the lower phenolic content, and therefore, both polar solvents presented very similar extraction yields.

The possible drawback from using water instead of ethanol as a solvent is that the increase in yield leads to a lower phenolic concentration with the extract getting richer in sugars as seen from the monosaccharides content in the EgEt (6%) and PaEt (9%), which is much lower than the EgW (17%) and PaW (25%). The phenolic composition also is not identical between solvents: ethanol solubilized much better the tannins in the barks and showed worst response in solubilizing the flavonoid components (Table 3). The higher phenolic content in alcoholic solvents when compared to water was shown in several studies (Zhao et al. 2006; Co et al. 2012; Luís et al. 2014). The higher phenolic content in the ethanol extract as well as its lower monosaccharides content is probably the reason for its better overall results regarding antioxidant and antimicrobial properties than water extracts.

The monosaccharide composition after acid hydrolysis of the polar extracts (Table 2) shows that ethanol extracts are richer in glucose, and in some cases in rhamnose, with both representing 50–99% of all monosaccharides, while in the water extracts this relative abundance drops to 35–79% due to the sharp increase in the other monomers. Besides existing as free monosaccharides and oligosaccharides, part of these sugars derives from the degradation of flavonoids or stilbenes that normally occur in plants as glycosides (glucoside, galactoside, arabinoside, rutinoside) (Kemppainen et al. 2014; Xiao 2015; Angelis et al. 2016). For example, the stilbenes glycosides in *P. abies* bark are estimated to be 5–10% in weight (Kemppainen et al. 2014). The low monosaccharide content in the AdEt may be connected to the fact that it also has very low flavonoid content. Overall, the total monosaccharide content of the extracts appears to have a negative correlation with the antioxidant activity ($R=-0.81$, $R=-0.78$, $R=-0.78$ for FRAP, BCB and DPPH methods, respectively).

Few studies account for the monosaccharide content of the polar extractive fractions. Hafizoglu and Holmbom (1995) reported on the acetone extract of *Abies nord manniana* bark monosaccharides plus sucrose content of 14–25% which is not far from those seen here (except for *A. dealbata* that shows lower values). Kemppainen et al. (2014) present total sugars of the water extracts of *P. abies* bark collected in the winter of 24% (similar to the 25% presented here, Table 2) with glucose also accounting for the largest share (75% of the monosaccharides).

The total phenolic content (TPC) of the *n*-hexane extracts was very low compared to the polar extracts, i.e. most of these compounds have higher affinity to more polar solvents. Between the two polar solvents used, ethanol appears to be a better solvent for extraction of phenolic components than water.

Flavonoids and tannins could not be measured in the non-polar extracts due to formation of turbidity that impeded the spectrophotometric measurements.

According to Co et al. (2012), the methodology might not be compatible with non-polar extracts.

Both FRAP and DPPH methods were strongly positively correlated with TFC ($R=0.97$ and 0.97) and CT ($R=0.97$ and $R=0.98$), while BCB test correlated positively with TFC ($R=0.79$) while showing a poorer correlation to CT ($R=0.66$). As for TF, the correlation was negative regarding antioxidant activity methods ($R=-0.72$, $R=-0.21$, $R=-0.77$ for FRAP, BCB and DPPH, respectively). Several studies found positive correlation between total phenolic and tannin contents with antioxidant activity (Luís et al. 2014). Flavonoids were also considered to have antioxidant activity, although, in the present case, higher contents lead to lower FRAP and AAI. One possible explanation might reside in the fact that for each polar solvent used (ethanol and water) the flavonoids tended to be more easily extracted by water and tannins by ethanol, with ethanol always showing higher antioxidant activity (FRAP and DPPH) implying that condensed tannins might have a higher influence on the antioxidant activity than the flavonoids. As for the capacity to inhibit lipid peroxidation (BCB test), water extracts appear to show higher potential than the ethanol.

Of all the extracts studied, *A. dealbata* bark presented the best results if the main goal of the extraction is to obtain compounds with antioxidant properties, showing even higher potential than the standard trolox in the FRAP method. When combined with the extremely high extraction yield (37% for either polar extract), it makes this bark very suitable for phenolic extraction with antioxidant activity. Furthermore, the easily extractable tannins with ethanol (160 mg CE/g_{bark}) might also be an interesting route to valorize this residue. This has already been done commercially for some acacia species (Pizzi 2006). Nevertheless, all polar extracts showed strong (EgW and AmW) or very strong (with $AAI \geq 2$) antioxidant activity with ethanol being significantly better solvent than water for this purpose.

In vitro antibacterial activity of the extracts was tested with two methods, one for an overall screening (disc diffusion method, DD) and the second to determine their quantitative potential (minimum inhibitory concentration, MIC). When comparing both methods, some discrepancies were found with DD method presenting inhibition halos for most of the bacteria (even gram-negative) when exposed to the polar extracts, while showing limited or no effect for the non-polar extracts (with the exception of *A. baumannii*). The high concentration of extracts in the DD method (200 mg/mL in DMSO) will probably result in some microbe growth inhibition that will not be registered in the MIC method where the highest concentration tested is 10 mg/mL. On the other hand, in the MIC test, the *n*-hexane extracts presented very interesting results regarding both gram-positive bacteria (especially the EgH and PaH) and yeasts (all non-polar extracts), which was not expected when looking at the preliminary results from DD. This is probably due to the greatest limitation of the DD method that depends on the diffusion capacity that each compound has in the agar medium, leading to poorer results than expected for compounds with low mobility.

Nevertheless, both assays indicate that gram-negative bacteria are less susceptible to the extracts than gram-positive bacteria and yeasts with similar results being reported in the literature (Nostro et al. 2000; Ignat et al. 2013). Besides the

peptidoglycan cell wall, common to both gram-positive and gram-negative bacteria, the latter also have an outer lipopolysaccharide membrane making them less permeable to lipophilic compounds. Furthermore, gram-negative bacteria also have the capacity to actively efflux detrimental compounds (trans-envelope multidrug efflux pumps) through the membrane, drastically reducing the accumulation of many antibiotics in the cell (Zgurskaya et al. 2018).

Overall, the results indicate that *A. melanoxylon* bark presents the most limited effect on the microbes tested, with the other three barks showing interesting biostatic potential regarding different microbes. The non-polar extract fraction of *E. globulus* bark showed very interesting potential regarding all gram-positive bacteria and both *Candida* strains. As for the ethanol extracts, *P. abies* presented the best results for *B. cereus* and *S. aureus*, which might be in part explained by the known presence of several stilbene glycosides (e.g., isohapontin, piceid, astringin) and their aglycones that have demonstrated antifungal and antibacterial activity (Co et al. 2012). *Acacia dealbata* water and ethanol extracts presented the lowest MIC values regarding some of the gram-negative bacteria (*A. baumannii, K. pneumoniae* and *P. aeruginosa*). Regarding both *Candida* strains, results show that both non-polar and polar extracts have very interesting biostatic effect, with water extracts showing a substantial reduction in action when compared to ethanol.

Several studies have positively related total phenolic content and respective antibacterial activity. In the present case, this was only valid for gram-negative bacteria, where the *A. dealbata* bark polar extracts showed the highest TPC as well as the lowest MIC when compared to the other barks. For the gram-positive ones, that relation was not observed.

It is possible to find in the literature some information regarding the antimicrobial activity of extracts from *P. abies* and *E. globulus* leaves, stumps, wood or knotwood (Valimaa et al. 2007; Khan et al. 2009b; Luís et al. 2014, 2016) and *P. abies* bark (Ignat et al. 2013; Salem et al. 2016; Burčová et al. 2018). No information could be obtained on the bark of *E. globulus*, *A. dealbata* and *A. melanoxylon*.

Ignat et al. (2013) found for ethanolic extracts of *P. abies* bark similar DD inhibition halos (10–15 mm) for *S. aureus*, *P. aeruginosa* and *E. coli*, while on the other hand presenting no growth inhibition effect for the water extracts regarding the same strains, which is contradictory to the current results, where water extracts presented equal or better inhibition zones than ethanol extracts. Burčová et al. (2018) found for ethanol extracts of *P. abies* almost no growth inhibition effect against *B. cereus, S. aureus, L. monocytogenes* and *E. coli* presenting a 14-mm inhibition zone against *P. aeruginosa*. The *n*-hexane extracts showed the highest effects against *B. cereus* and *P. aeruginosa*, while no effects were registered against *C. albicans* for both ethanol and *n*-hexane extracts. Salem et al. (2016) presented much lower MIC values for methanolic *P. abies* bark extracts (0.08 to 0.43 mg/mL) for several bacteria (*P. aeruginosa, E. coli, L. monocytogenes*, *S. aureus* and *B. cereus*) than the ones found here (0.16 to > 10 mg/mL) for the same species. Luís et al. (2014) presented slightly lower MIC values of ethanol crude extracts from stump bark of *E. globulus* for several of the same bacteria and yeasts with the gram-negative ones also presenting the worst results.

As alternative for antibiotics, or to be used simultaneously, the inhibition of the quorum sensing ability of some bacteria might be another interesting approach to reduce their virulence and group coordinated action (Kalia 2013; Defoirdt et al. 2013). The mediation of the quorum sensing is done through the interchange of small signal molecules that regulate the gene expression to produce pathogenicity and symbiosis phenotypes when the bacteria population reaches a certain density. The objective is to interfere with this mechanism to prevent virulent factors as exopolysaccharide synthesis or biofilm formation that can increase up to 1000 times the bacteria population resistance to antibiotics (Kalia 2013). The results show that only the non-polar extracts and the *E. globulus* polar showed some interference in the purple pigment (violacein) production of the *C. violaceum* bacterium, that is controlled by quorum sensing, meaning that in the non-polar extracts (especially the EgH, AmH and AdH), some compounds are able to inhibit this phenomenon. The halos registered were smaller than the ones obtained by *E. globulus* essential oil (10 mm) (Luís et al. 2016) and 4 out of 21 other essential oils from different plants (Khan et al. 2009a).

The overall results of this work show that different barks and solvents can be used to obtain extracts with distinct composition and characteristics, whether to obtain antioxidant or antimicrobial compounds. Each extract presented different antimicrobial activity showing that both species and solvents can be used to tailor extractable compounds to target specific pathogenic bacteria or yeasts. Nevertheless, additional work is needed to better understand which compounds or synergies of compounds, present in the crude extracts, are responsible for the antioxidant and antimicrobial effects.

Conclusion

Barks from industrial side stream flows, for example *E. globulus* or *P. abies*, or from biomass collected from forest operations to control invasive species, for example *A. melanoxylon* and *A. dealbata*, have valorization potential due to their high extractives content. Extraction may therefore integrate a bark-based biorefinery as a first step prior to further processing in chemical, biochemical or thermal platforms.

Extraction with different non-polar and polar solvents yields crude extracts with different characteristics that have very interesting antioxidant (mostly those from polar extracts) and antimicrobial activities. All the polar extracts from the four barks demonstrated strong or very strong antioxidant activity as inhibitors of free radicals or lipid peroxidation with ethanol proving to be a more efficient solvent than water to obtain those compounds.

As for the overall antimicrobial activity, *E. globulus* and *P. abies* barks presented the best non-polar extracts regarding the minimum inhibitory concentration for most of the gram-positive bacteria, while *A. dealbata* bark presented the best results among the polar extracts regarding the effect on both bacteria and yeasts pathogenic strains. All the *n*-hexane extracts showed great response against both *Candida* species. The gram-negative bacteria presented high resistance to almost all extracts with *K. pneumoniae* being the most susceptible.

Regarding the antiquorum sensing, the only extracts that showed any activity were the non-polar extracts from all barks and the polar ones from *E. globulus*.

Crude bark extracts present within their composition compounds that might be interesting for the cosmetic, pharmaceutical, food and polymer industries as cheap and natural antioxidants and bioactive systems.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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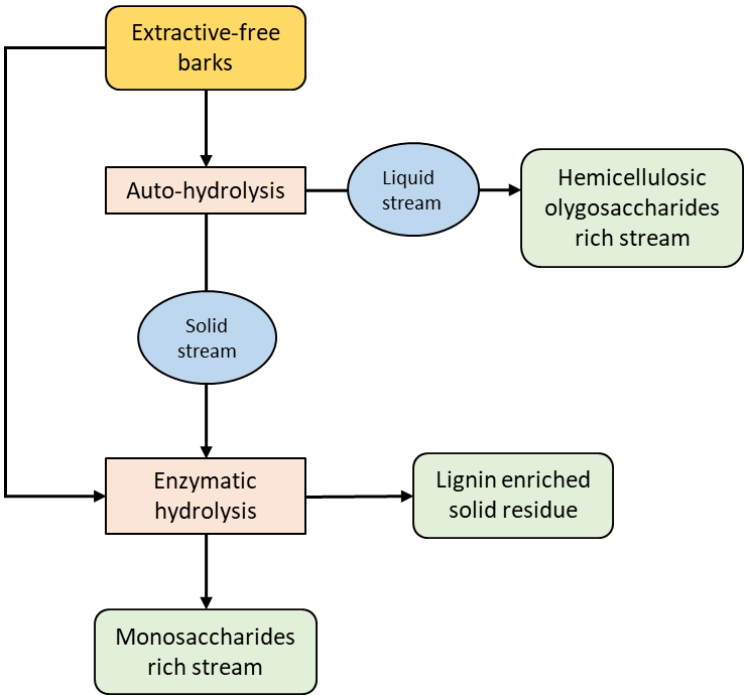

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Fractionation and valorization of industrial bark residues by autohydrolysis and enzymatic saccharification

Duarte M. Neiva[a,*], Ricardo A. Costa[a], Jorge Gominho[a], Suzana Ferreira-Dias[b], Helena Pereira[a]

[a] Universidade de Lisboa, Instituto Superior de Agronomia, Centro de Estudos Florestais, Lisboa, Portugal
[b] Universidade de Lisboa, Instituto Superior de Agronomia, Centro de Investigação em Agronomia, Alimentos, Ambiente e Paisagem, Lisboa, Portugal

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ABSTRACT

Eucalyptus globulus (Eg) and *Picea abies* (Pa) residual barks from pulp and solid wood industries were studied regarding their fractionation. Extractive-free barks were treated through autohydrolysis (severity factors 3.3–4.7) to obtain non-cellulosic rich oligosaccharides/monosaccharides moieties, followed by enzymatic saccharification of the (mostly unaltered) lignin/cellulose enriched residues. The maximum sugar yields obtained from autohydrolysis were 11 and 14 g/100 g extractive-free bark for Eg and Pa with typical fermentation inhibitors reaching 8 g/100 g. Two commercial enzymes (Saczyme Yield and Ultimase BWL40) were tested to extractive-free bark and residues from autohydrolysis. Ultimase allowed 73% and 51% global sugar yield for Eg and Pa, respectively, while Saczyme showed poorer results especially when applied to Pa. The solids obtained after saccharification were highly enriched in lignin. Autohydrolysis followed by enzymatic saccharification enabled bark fractionation into streams rich in xylooligosaccharides or arabinooligosaccharides (hemicellulosic polymers), glucose from cellulose and lignin remaining in the solid residue.

1. Introduction

The shift from a petrol based to a biobased industrial production of chemicals, products and fuels requires an adequate and extensive use of available biomass. In that sense, conversion of underused materials, side-streams or residues of already implemented wood-based industries is key to increase sustainability in the generation of biomaterials and biofuels (Van Heiningen, 2006).

One significant residue in the solid wood and pulp&paper industries is bark that is stripped from the logs before processing. Bark represents nearly 12% of the stem's mass, and is mainly used for energy production by direct burning in central heating and power (CHP) plants, regardless of the valorization potential given by its rich and diverse chemical composition (Hejnowicz, 2007; Quilhó and Pereira, 2001).

Two of the most important industrial tree species in Europe are *Eucalyptus globulus*, extensively planted and used in the south Mediterranean region, especially in Portugal and Spain, for pulp&paper industries (Cerasoli et al., 2016; Neiva et al., 2018a) and *Picea abies*, a Northern European endemic species that spreads throughout central Europe used for timber and pulp (Caudullo et al., 2016; Neiva et al., 2018b). Bark residues generated from these species are difficult to calculate but institutional estimates refer 0.5 Mton for *E. globulus* in Portugal (2017) and 1.4 Mton for *P. abies* in Finland (2018) (CELPA, 2017; Luke, 2018).

Already available at industrial sites, these residues are very enticing upgradable raw materials for fuel and chemical production since handling, transportation and storage costs are already supported (Lundmark et al., 2018). Accordingly, a substantial amount of research has focused on their possible use for multiple purposes and end-uses, and although no industrial application has yet been devised, future commercial implementation might be not only technically but economically viable.

The chemical fraction of barks that attracts most attention is the extractable non-structural components, viewed as possible source of a myriad of compounds and biopolymers (*e.g.* fatty and resin acids or alcohols, terpenes, steroids, flavonoids, stilbenes, tannins) that can be used by important industries (Burčová et al., 2019; Domingues et al., 2010; Jutakridsada et al., 2017; Kreps et al., 2017; Lacoste et al., 2015). Nevertheless, the integrated processing to use the full chemical potential means that the extractive-free solid residues will have to be dealt with. Although bark can be burned after extraction without major consequences for its fuel properties (Jutakridsada et al., 2017), other more interesting end uses might be envisaged. The extractive-free bark residues are composed by the three structural polymers (cellulose, hemicelluloses and lignin) and their fractionation into monomeric sugars and lignin moieties may be a very interesting pathway for

* Corresponding author.
E-mail address: duarteneiva@isa.ulisboa.pt (D.M. Neiva).

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valorizing the overall bark stream. In the case of cork-rich barks a fourth structural component specific to cork is also present, suberin, and may open an additional valorization pathway (Leite and Pereira, 2017). Since extractives are considered detrimental for many lignocellulosic deconstruction processes (*e.g.* delignification, enzymatic hydrolysis, fermentation), the extractive-free barks should be less recalcitrant than the original material (Frankó et al., 2018; Neiva et al., 2016).

Fractionation of the lignocellulosic cell wall structure is not an easy task, with no "one step process" able to deliver the three polymers as pure fractions. Since the production of biofuels or chemicals based on the biological conversion of monomeric sugars through fermentation is one of the most looked upon large scale use of lignocellulosic biomass, the fractionation of both cellulose and hemicelluloses should be achieved without extensive degradation. Hemicelluloses derived oligosaccharides are also seen as interesting products for the pharmaceutical and food industries, showing prebiotic and antioxidant activities, among other uses (Gullón et al., 2012; Moure et al., 2006).

One of the most eco-friendly and simple pre-treatment processes is the hydrothermal treatment, also called autohydrolysis, that uses liquid water at high temperatures to break glycosidic bonds and solubilize mono, di and oligosaccharides (mainly from hemicelluloses and partially from amorphous cellulose) also with degradation and removal of the more accessible and fragile lignin portions. The hydrolysis of acetyl groups from hemicelluloses creates *in situ* acidic conditions that improve the hydrolytic deconstruction of the polymer backbone, avoiding the use of either acidic or caustic conditions and their associated costs (Healey et al., 2015). Autohydrolysis allows to obtain a hemicellulose derived sugar rich liquid stream and to increase the enzymatic accessibility of the resulting lignin and cellulose enriched solid for saccharification, enabling better yields and higher productivity (Moniz et al., 2013, 2015). Additionally, hemicelluloses, and more specifically oligosaccharides, are known to inhibit cellulases impairing enzyme-catalyzed saccharification (Silva-Fernandes et al., 2015; Yang et al., 2011).

Although hydrolysis of the lignocellulose polysaccharides to sugar monomers can be achieved by chemical processes, the enzymatic processes are seen as industrially more viable since they require lower energy consumption, operate at milder conditions and are more selective. Nowadays, new and more efficient enzymatic cocktails are being developed to overcome the recalcitrant nature of cellulose. However, the production and cost of enzymes still impairs their economic viability for most of the applications.

Pre-treatments followed by enzymatic hydrolysis and/or fermentation targeted to contribute to the lignocellulosic fractionation goal have been tested in different raw materials such as industrial wood and non-woody residues, including barks (David and Atarhouch, 1987; Frankó et al., 2018; Kemppainen et al., 2012; Lima et al., 2013; Matsushita et al., 2010; Miranda et al., 2019; Santos et al., 2012).

The objective of this study was to fractionate previously extracted barks of *Eucalyptus globulus* and *Picea abies* using different autohydrolysis pretreatment conditions and test their influence on the saccharification of the solid residues catalyzed by two enzyme cocktails (Saczyme Yield and Ultimase BWL40). These sequential treatments aimed at a controlled deconstruction of the lignocellulosic matrix to obtain streams enriched in xyloolygosaccharides/arabinoolygosaccharides from hemicelluloses, glucose from cellulose and also a lignin residue. Through such environmentally friendly processes, an alternative end-use to these industrial residues aside from direct burning for energy purposes is proposed.

2. Material and methods

2.1. Sampling

Extractive-free barks of *Eucalyptus globulus* (Eg) and *Picea abies* (Pa) were used in this work. The first was collected from a pulp mill of The Navigator Company located in Setúbal, Portugal, and the second from a sawmill located in Jyvaskyla, Finland. A full characterization of these barks was already reported (Neiva et al., 2018a, 2018b). The barks were milled through a 2 × 2 mm sieve and fully extracted with a sequence of dichloromethane (16 h), ethanol (24 h) and water (24 h).

2.2. Enzyme preparations

The commercial enzymes used were: Saczyme Yield (henceforth Saczyme), a cocktail of cellulases, β-glucosidases and amyloglucosidases with a cellulase activity of 195 Filter Paper Units(FPU)/mL and β-glucosidase activity of 25 International Unit(IU)/mL; and Ultimase BWL 40 (henceforth Ultimase), a concoction of β-glucanases and xylanases from *Trichoderma reesei* with a cellulase and β-glucosidase activity of 127 FPU/mL and 28 IU/mL, respectively (Miranda et al., 2019). Enzymes were kindly donated by Novozymes A/S. Cellulase and β-glucosidase activities were measured two weeks before enzymatic hydrolysis. At the end of the experiments, the activities were once again measured and no significant changes were detected.

2.3. Chemical composition analysis of extractive-free and autohydrolysed barks

The summative chemical analyses of untreated (extractive-free) and pretreated barks were determined according to TAPPI standard methods. Ash content was obtained according to TAPPI 211 om-02. Total lignin was obtained as the sum of Klason and soluble lignin with determination of Klason lignin by TAPPI 222 om-02 (corrected to ash content) and soluble lignin by TAPPI UM 205 om-93 measuring the absorbance at 205 nm. The polysaccharides composition was determined in the hydrolysate of Klason lignin. The neutral monosaccharides, glucuronic and galacturonic acid were determined by high pressure ion-exchange chromatography (HPIC) in a Dionex ICS3000 equipped with a PAD detector with a CarboPac PA10 (4 × 250 mm) column plus Aminotrap and a NaOH + CH_3COONa eluent with a 1 mL/min flow at 25 °C; acetic acid was determined in a Waters 600 and measured with a UV/Vis detector at 210 nm, with a Biorad Aminex 87H HPX (300 × 7.8 mm) column and a 10 mN H_2SO_4 eluent with a 0.6 mL/min flow at 30 °C. All analyses were made in duplicate.

2.4. Hydrothermal pretreatments

The *E. globulus* and *P. abies* extractive-free barks were subjected to five different hydrothermal pretreatments with water (autohydrolysis) under temperature and time conditions between 150 and 190 °C and 60–120 min. The severity factor (SF) of each pretreatment was calculated according to:

$$SF = \log\left(t.\ e^{\frac{T-100}{14.75}}\right)$$

where T is the temperature (°C) and t is the time of autohydrolysis (min).

The SF of the five pretreatments was as follows: 3.3 (150 °C and 60 min), 3.6 (150 °C and 120 min), 4.0 (170 °C and 90 min), 4.4 (190 °C and 60 min) and 4.7 (190 °C and 120 min). The temperature and time values of these pretreatments correspond to the factorial points of a factorial design (experiments with SF of 3.3, 3.6, 4.4 and 4.7) with a center point (SF = 4.0; 170 °C and 90 min) (Lundstedt et al., 1998). These conditions were chosen to have a wide range of severity factors from mild to harsh conditions (SF between 3.3 and 4.7).

The autohydrolysis were carried in 100 mL steel micro digesters rotating in an oil bath with a liquid-to-solid ratio of 10:1. The pretreated barks were separated from the autohydrolysis liquor, thoroughly washed with water and the mass loss calculated based on oven dried material (o.d.). The hydrolysate liquor was subjected to an acid hydrolysis with sulfuric acid (the same conditions for lignin

Table 1
Autohydrolysis conditions, yield and chemical composition (g/100 g extractive-free bark) of the original material (OM) and the solid residues obtained after the autohydrolysis of *Eucalyptus globulus* (Eg) and *Picea abies* (Pa) and lignin monomeric composition (hydroxyphenyl-H, guaiacyl-G and syringyl-S units) determined by pyrolysis.

		E. globulus						*P. abies*					
Temperature (°C)		OM	150		170	190		OM	150		170	190	
Time (min)			60	120	90	60	120		60	120	90	60	120
Severity factor			3.3	3.6	4.0	4.4	4.7		3.3	3.6	4.0	4.4	4.7
Yield (%)		100	84	76	71	69	68	100	71	74	66	65	66
Ash		7.2	6.3	4.7	5.8	5.8	5.6	4.0	3.0	2.8	3.0	1.8	1.9
Lignin	Klason	21.9	21.7	20.1	21.7	22.8	24.4	31.8	28.8	31.5	32.3	34.9	35.9
	Soluble	3.0	2.3	1.7	1.3	1.2	1.1	1.0	0.6	0.5	0.4	0.6	0.6
	Total	24.9	23.9	21.8	23.0	24.0	25.6	32.8	29.4	32.0	32.7	35.5	36.5
Sugars	Arabinose	1.7	0.3	0.1	0.2	0.1	0.1	6.9	0.6	0.4	0.1	0.1	0.1
	Galactose	1.7	0.8	0.4	0.2	0.0	0.0	2.6	1.2	1.1	0.7	0.3	0.0
	Glucose	38.6	39.5	42.7	39.0	37.2	36.1	32.2	26.4	29.2	26.9	27.9	28.2
	Xylose	15.6	12.4	7.7	3.4	1.8	1.2	5.2	4.8	4.7	3.7	2.9	1.9
	Mannose	0.8	0.9	0.7	0.7	0.5	0.4	4.4	3.3	3.2	1.8	0.8	0.6
	Galacturonic acid	1.4	0.4	0.2	0.1	0.0	0.0	4.6	2.0	1.4	0.2	0.1	0.0
	Glucuronic acid	0.0	0.1	0.0	0.0	0.0	0.3	0.0	0.0	0.1	0.0	0.0	0.0
	Acetic acid	0.8	2.7	1.6	0.7	0.3	0.2	3.7	0.4	0.6	0.3	0.2	0.1
	Total	60.6	57.0	53.4	44.3	40.0	38.4	59.6	38.6	40.8	33.7	32.2	30.9
Glucose/other sugars		1.8	2.3	4	7.5	13.4	15.9	1.2	2.1	2.5	3.9	6.5	10.3
Sugars/lignin		2.4	2.4	2.5	1.9	1.7	1.5	1.8	1.3	1.3	1	0.9	0.8
Glucose/lignin		1.6	1.7	2	1.7	1.6	1.4	1	0.9	0.9	0.8	0.8	0.8
H		5	9	4	4	5	4	21	21	22	21	21	23
G		24	32	31	33	33	33	79	79	78	79	79	77
S		70	60	65	63	61	63	–	–	–	–	–	–
S/G		2.9	1.9	2.1	1.9	1.8	1.9	–	–	–	–	–	–

determination) and the polysaccharides (neutral monosaccharides and acetates) determined before and after the acid hydrolysis as mentioned previously. Furfural (F) and 5-hydroxymethylfurfural (HMF) contents were also ascertained through the same method used for acetic acid determination in both autohydrolysis liquors, and soluble lignin in the post acid autohydrolysis liquor. All analyses were made in duplicate and reported as the average value.

2.5. Enzymatic saccharification

The reactions were carried out in jacketed cylindrical glass reactors in 50 mM citrate buffer solution at 4.8 pH and 50 °C with magnetic stirring during 48 h. A buffer-to-bark ratio of 30:1 was used (60 mL to 2 g of bark, 3.3% solid load-SL), and the amount of enzyme added was 45 FPU/g of bark.

Two additional saccharification tests were performed for autohydrolysed bark at severity factor 4.0 (central point): the first, increasing the enzymatic hydrolysis solid load to 5% (henceforth SL = 5%); and the second by hydrolyzing enzymatically the slurry system obtained in autohydrolysis, without separation of the liquor and solid streams (henceforth Slurry).

In total, eight enzymatic saccharifications were performed for each bark species and enzymes: saccharification of the extractive-free original material (OM), of the autohydrolysed barks with different SF (3.3, 3.6, 4.0, 4.4, 4.7) (Frankó et al., 2015), SL = 5% and Slurry. All enzymatic hydrolysis were performed in duplicate and reported as average values.

To follow the saccharification time-course, 0.5 mL aliquots were extracted through syringe filters (0.45 µm) at 1, 3, 5, 7, 24, 30 and 48 h reaction time, and polysaccharide conversion as reducing sugars (glucose equivalents per total monosaccharides in the solid loaded to the reactor) determined using the phenol-sulfuric acid assay (Zhang et al., 2009). The 48 h aliquot was also analyzed by HPIC (the same methodology as explained before) for determination of the neutral monosaccharides produced at the end of the enzymatic saccharification. The solid residues were thoroughly washed and separated by centrifugation at 5000 rpm for 10 min for pyrolysis analysis. All analyses were made in duplicate.

Final saccharification results (48 h) were expressed as: total monosaccharides concentration (sugar concentration, g/L); glucose yield (as % of the extractive-free bark glucose) and non-glucose yield (% of the amount of non-glucose monosaccharides in the extractive-free bark); reactor sugar yield (monosaccharides obtained after enzymatic hydrolysis/total monosaccharides of the solid added to the reactor); global sugar yield (monosaccharides obtained after enzymatic hydrolysis/monosaccharides of original extractive-free bark).

2.6. Analytical pyrolysis

The extractive-free barks, autohydrolysis and enzymatic hydrolysis solid residues were milled to a fine powder on a Retsch MM200 mixer mill and dried under vacuum over phosphorus pentoxide. Approximately 100 µg of each sample were weighted and pyrolysed at 550 °C for 1 min in a CDS Pyroprobe 5150 Pyrolyzer connected to an Agilent GC 7890B coupled to a mass detector system 5977B, and the fused-silica capillary column used was a ZB-1701 (60 m × 0.25 mm i.d. × 0.25 µm film thickness). The oven heating program started at 40 °C (held for 4 min), ramp 1 to 70 °C at a rate of 10 °C/min, ramp 2 to 100 °C at 5 °C/min, ramp 3 to 265 °C at 3 °C/min (held for 3 min), and ramp 4 to 270 °C at 5 °C/min (held for 9 min); the temperature of the injector and the GC/MS were kept at 270 °C and 280 °C, respectively, and the carrier gas was helium with a total flow of 1 mL/min. The compounds were identified using the Wiley, NIST libraries and literature (Faix et al., 1990; Ralph and Hatfield, 1991). Lignin derived products (hydroxyphenyl-H, guaiacyl-G and syringyl-S units) were determined and expressed as molar percentage of all lignin units and the S/G ratio determined for *E. globulus* bark. Tables A1, A2 and A3 in Appendix A show the full pyrolysis results.

3. Results and discussion

3.1. Chemical composition

The chemical composition of the extractive-free bark for both species used in the present study is reported in Table 1, along with the composition of the solid fractions resulting from autohydrolysis

conducted with severity factors (SF) between 3.3 and 4.7 and respective lignin monomeric composition determined by analytical pyrolysis. Both barks presented high mineral content reaching above 7% in mass in Eg, as previously reported (Neiva et al., 2018a, 2018b), that was not removed during the extraction of the original material.

Eg showed a lower lignin content of 24.9% (Table 1), enriched in syringyl units (S/G/H = 70/24/5), with 3.0% corresponding to soluble lignin, while Pa presented a more condensed structure without S units with a G/H = 79/21 and a lower percentage of soluble lignin (1.0%). These values are characteristic of hardwoods and softwoods and very similar to the respective woods (Lourenço and Pereira, 2018; Rencoret et al., 2009). These different lignin characteristics point to a much higher recalcitrant nature of the Pa bark when compared to Eg bark, in accordance with the knowledge that softwood biomass is harder to delignify, hydrolyze or in a broader sense fractionate into the three main polymers that compose the cell wall material (Frankó et al., 2018; Kemppainen et al., 2012; Santos et al., 2011). Eg and Pa extractive-free barks presented similar contents of total sugars (61% and 60% respectively) but with considerable differences regarding their composition. While Eg showed higher glucose and xylose content, corresponding to almost 90% of all sugars, meaning a xylan enriched hemicelluloses backbone, in Pa hemicelluloses are more heterogeneous with the predominance of arabinose (7% of the bark), xylose (5%), galacturonic acids (5%) and mannose (4%), typical of glucomannans and arabinoxylans presence.

The glucose/other monomers ratio was higher for Eg (1.8) than for Pa (1.2), pointing to a more interesting material for saccharification aiming at ethanol production, since glucose is the most easily consumed sugar for most of the current bioprocesses while pentose sugars (arabinose and xylose) show lower fermentability, at least when using yeasts (Lima et al., 2013). Furthermore, the sugar/lignin and glucose/lignin ratios are higher for Eg than for Pa (Table 1), suggesting an easier fractionation of the Eg bark since lignin is considered the highest recalcitrant factor in biomass deconstruction.

3.2. Autohydrolysis

This pre-treatment was chosen to reduce bark recalcitrance, promote cellulose accessibility to enzymes and obtain hemicellulosic sugars from the matrix without substantial decomposition to undesirable compounds. A relatively high liquid/solid ratio (L/S) was needed (10 mL/$g_{ext\text{-}free\ bark}$) mostly due to the highly fibrous nature of the Eg bark (Pereira et al., 2010), leading to a very high water absorption and consequent low free liquid availability. For the denser and less fibrous Pa bark, the L/S ratio could be reduced, although in the current work both barks were tested under the same L/S ratio. Autohydrolysis was performed under relatively high severity factors between 3.3 and 4.7 mostly due to the expected recalcitrance of the barks. Similar SF have been applied to other lignocellulosic residual materials to solubilize hemicelluloses and obtain oligosaccharides (Moniz et al., 2013, 2015).

The chemical compositions of the solid residues after autohydrolysis are presented in Table 1, while the composition of the hydrolysates pre and post acid hydrolysis is shown in Table 2.

E. globulus bark was less susceptible to autohydrolysis at low SF with only 16% mass solubilization as compared to the 29% for *P. abies*. Nevertheless, with increments in SF, the degradation pattern regarding mass loss became quite similar for both species (32 and 35% respectively at the highest SF of 4.7). Slightly lower yields of 20–30% and 15–25% have been reported for Eg and Pa wood autohydrolysis, respectively, under similar conditions (Romaní et al., 2011; Song et al., 2008).

Glucose solubilization with hydrothermal treatments of Eg bark was almost negligible regardless of autohydrolysis conditions (lower than 2.5 g/100 $g_{ext\text{-}free\text{-}bark}$, SF = 4.7) while for Pa bark a significant portion was lost with treatment corresponding to as high as 5.8 g/100 $g_{ext\text{-}free\text{-}bark}$ which corresponds to roughly 18% of all glucose (in polymeric form). This is probably related to the existence of easily degraded glucomannans in Pa bark hemicelluloses while in Eg glucose is almost exclusively related to cellulose. Xylose and mannose were harder to hydrolyze than arabinose (requiring higher SF), in accordance with previous reports (Le Normand et al., 2012) showing that most arabinose and galacturonic acid are hydrolyzed and solubilized below 140 °C and mannose and xylose depolymerization occurs preferentially at or above 160 °C.

Polymeric glucose content in the autohydrolysis solid residues changed substantially with SF in relation to the other polymeric sugars with the glucose/other sugars ratio increasing from 1.8 to 15.9 and from 1.2 to 10.3 for Eg and Pa barks respectively (Table 1). This evidences the preferential hydrolysis of the hemicellulosic monomers mainly of xylose, arabinose, mannose and galacturonic acid. On the other hand, the ratio glucose/lignin in the solids remained mostly unaltered apparently pointing to a solid residues enriched in both lignin and cellulose even though some small proportion of lignin was degraded and dissolved, as seen by its presence in the liquors ranging from 0.6 to 2.5 g/100 $g_{ext\text{-}free\text{-}bark}$ (Table 2).

Klason lignin content was higher in the autohydrolysis residues at higher severity factors than in the extractive-free barks (Table 1, *e.g.* 21.9 *vs* 24.4 g/100 g for Eg original bark and autohydrolysed solids at SF 4.7, respectively). This has been previously reported for hot water and steam pre-treatments, and associated to extractives deposition or to sugars and their degradation products condensation reactions with lignin, remaining in the solid residue as pseudo-lignin (Carvalheiro et al., 2009; Robinson et al., 2002). Since the bark was previously extracted, this increment in Klason lignin should only derive from sugar condensation reactions, which can also partially explain the sharp decline of monosaccharides in the corresponding hydrolysate, even after accounting for their loss due to further degradation to furfural and 5-hydroxymethylfurfural.

An interesting observed variation regarding the *E. globulus* bark is the decline in the syringyl/guaiacyl ratio (S/G) with pre-treatment independent of the autohydrolysis severity factor, dropping from 2.9 (extractive-free bark) to 1.8–2.1 in the autohydrolysis residues. The syringol, methyl-syringol and vinyl syringol, which account to 42% of all S unit in the pyrograms of the original material, increase up to 73% with increasing autohydrolysis severity factor, while trans-propenylsyringol, syringilacetone and trans-sinapaldehyde decrease their percentage from 33% to 9%.(data in Annex Table A2) These results seems to show that there is a higher loss of S units compared to G and H, which is plausible since the β-O-4 alkyl-aryl bonds, typical of the S units are easier to break than the more condensed structures usually occurring with G and H.

The results from Table 2 show that the hydrolyzed and solubilized sugars during autohydrolysis are mostly in oligomeric form at SF below 4.4 (*e.g.* monomeric xylose in hydrolysis liquor is 1.6 g/100 g of extractive-free bark, which after acid hydrolysis of the liquor increases to 7.7 g/100 g, corresponding to 80% of the xylose being in oligosaccharide form).

Fig. 1 shows known possible inhibitors in subsequent fermentation and enzymatic processes (furfural, 5-hydroxymethylfurfural (HMF), acetic acid and soluble lignin) and total monosaccharides resulting from each autohydrolysis. Furfural and HMF concentrations tend to increase with autohydrolysis severity conditions through degradation of pentoses and hexoses respectively. Nevertheless, a significant reduction of these compounds can also occur as they can further degrade into other aldehyde products and formic acid if harsh enough conditions are used (Palmqvist and Hahn-Hägerdal, 2000; Yu et al., 2010) (*e.g.* 3.5 *vs* 2.2 g/$g_{ext\text{-}free\ bark}$ for furfural in Eg autohydrolysis liquor before and after acid hydrolysis).

The sharp monosaccharides reduction in the hydrolysate (right plot in Fig. 1) above SF = 3.6 without a correspondent increase in degradation products (left plot in Fig. 1) also points to the possible occurrence of condensation reactions between the sugars and lignin,

Table 2
Autohydrolysis liquor composition and composition after mild acid hydrolysis (values separated by //) as g per 100 g of extractive-free barks of *Eucalyptus globulus* and *Picea abies*, including monosaccharides, sugar degradation products and soluble lignin.

	E. globulus					*P. abies*				
T (°C)	150		170	190		150		170	190	
t (min)	60	120	90	60	120	60	120	90	60	120
Severity factor	3.3	3.6	4.0	4.4	4.7	3.3	3.6	4	4.4	4.7
Soluble lignin	-//0.7	-//1.0	-//1.5	-//2.2	-//2.5	-//0.6	-//0.9	-//1.4	-//1.8	-//1.6
Monosaccharides										
Rhamnose	0.1//0.3	0.1//0.3	0.1//0.3	0.1//0.1	0.0//0.0	0.1//0.8	0.1//0.7	0.1//0.3	0.0//0.0	0.0//0.0
Arabinose	0.4//1.2	0.7//1.2	0.7//0.7	0.2//0.2	0.0//0.0	1.0//6.4	1.9//6.5	1.6//2.6	0.2//0.2	0.0//0.0
Galactose	0.1//0.8	0.2//1.1	0.5//1.0	0.4//0.5	0.1//0.1	0.1//1.3	0.3//1.5	0.5//1.4	0.3//0.6	0.1//0.1
Glucose	0.1//0.5	0.1//0.6	0.1//0.6	0.3//0.7	0.3//0.5	0.2//2.6	0.4//3.0	0.6//3.2	0.5//2.4	0.2//0.8
Xylose	0.1//2.9	0.3//5.8	1.6//7.7	1.7//2.4	0.1//0.2	0.1//0.7	0.2//1.0	0.2//0.9	0.1//0.5	0.1//0.1
Mannose	0.0//0.2	0.0//0.2	0.0//0.4	0.1//0.2	0.2//0.1	0.0//1.1	0.0//1.5	0.0//1.6	0.3//1.5	0.2//0.4
Galacturonic acid	-//0.0	-//0.2	-//0.0	-//0.0	-//0.0	-//0.8	-//0.6	-//0.2	-//0.1	-//0.0
Glucuronic acid	-//0.1	-//0.0	-//0.0	-//0.0	-//0.0	-//0.1	-//0.1	-//0.0	-//0.0	-//0.0
Acetic acid	0.4//1.0	0.8//2.0	1.9//2.8	3.6//3.2	3.9//3.4	0.1//0.6	0.4//0.7	0.7//1.0	1.1//1.3	1.2//1.4
Degradation products										
Furfural	0.0//0.2	0.1//0.4	0.8//1.2	3.2//2.6	3.5//2.2	0.0//0.2	0.1//0.4	0.6//0.8	0.9//0.8	0.7//0.6
Hydroxymethylfurfural	0.0//0.0	0.0//0.0	0.1//0.0	0.4//0.1	0.7//0.2	0.0//0.0	0.0//0.1	0.1//0.2	0.5//0.3	0.8//0.3

leading to an increase of Klason lignin as mentioned before.

The non-cellulosic sugars recovery in the hydrolysate presented the best results for *E. globulus* and *P. abies* (within the range studied) when applying the SF of 4 (90 min at 170 °C) and 3.6 (120 min at 150 °C) respectively. This means that for Pa, 14 $g/g_{ext\text{-}free\ bark}$ total monosaccharides can be recovered in an hydrolysate mostly composed by arabinose, glucose, galactose and mannose (almost 80% in oligomeric form) with 2.1 $g/g_{ext\text{-}free\ bark}$ of inhibitors (mostly soluble lignin and acetic acid) and for Eg, 11 $g/g_{ext\text{-}free\ bark}$ total monosaccharides in an hydrolysate composed mostly of xylose (80% of which in oligomeric form) with 5.5 $g/g_{ext\text{-}free\ bark}$ of inhibitors (acetic acid, soluble lignin and furfural).

By using an autohydrolysis treatment under the right conditions, a substantial part of the hemicelluloses could be recovered in oligomeric/monomeric fractions. Both Pa and Eg, at their best SF showed a near 50% recovery of hemicellulose sugars of almost exclusively xylose for Eg and of a mixture rich in arabinose for Pa. These hydrolysates could potentially be used for obtaining xylooligosaccharides (XOS) and arabinooligosaccharides (AOS) with application in the food and pharmaceutical industries (Belorkar and Gupta, 2016). These hydrolysates must contain high content of XOS and AOS, and very low level of free monomers. However, the separation of the simple monomers is very expensive meaning that not all hydrolysates presented in Table 2 are attractive, particularly those obtained from *Picea abies*. Alternatively, these hydrolysates can be used for ethanol production by fermentation or for the production of monosaccharides after hydrolysis under diluted acid conditions for further biotechnological transformations to valuable compounds (*e.g.* xylitol, arabitol).

3.3. Enzymatic saccharification

The enzymatic hydrolysis of the extractive-free barks and solid

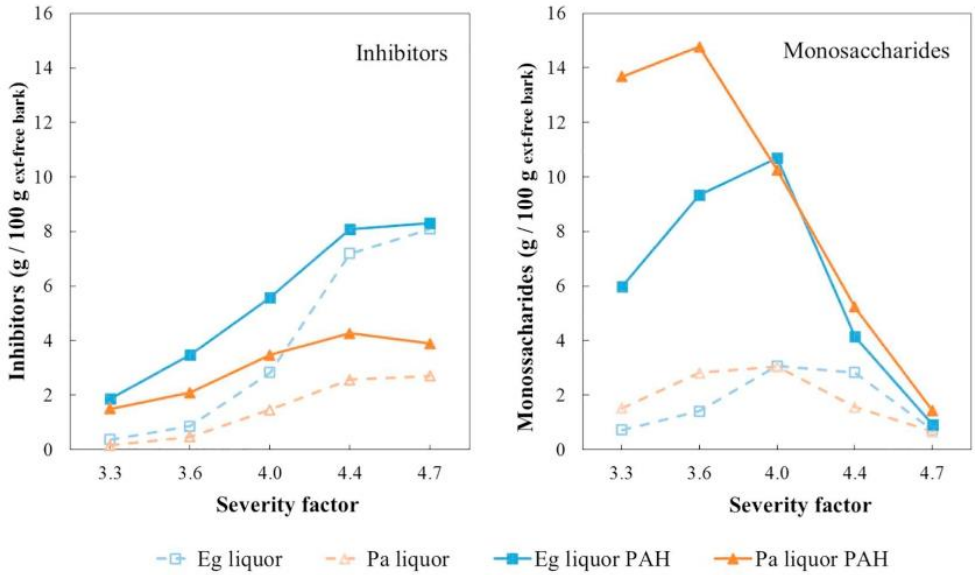

Fig. 1. Possible inhibitors (acetic acid, lignin, furfural and 5-hydroxymethylfurfural) (left) and total monosaccharides (right) present in the autohydrolysis liquors obtained at different severity factors (SF) as g per 100 g of extractive-free bark before (open symbols) and after acid hydrolysis (PAH; closed symbols) for *Eucalyptus globulus* (Eg) and *Picea abies* (Pa).

residues after autohydrolysis were achieved at a low solid load (SL = 3.3%) mainly due to the Eg bark capacity to absorb water and thus reducing the free liquid availability and preventing a proper agitation of the medium. An additional test was performed at 5% SL (for the severity factor SF = 4 only). Above this value, the reaction medium was so viscous that agitation was totally impeded. Another test was made using both liquid stream and solid residue from the autohydrolysis to determine the feasibility of direct saccharification of the slurry. This test could indicate if both cellulosic and hemicellulosic sugars used together could promote a higher global sugar yield and evaluate the possible inhibitory effect of the autohydrolysis liquor in saccharification. These experiments were made only for the autohydrolysed fractions at SF = 4 for both barks and were designated as "slurry".

Saczyme Yield and Ultimase BWL-40 were mainly designed to be used in combination with amylases to improve grain starch saccharification yields. These enzyme blends are not specifically developed for the hydrolysis of lignocellulosic biomass but they have been used for cell wall degradation or to hydrolyze β glucans, xylans and other non-starch polysaccharides present in starch grains to reduce viscosity and improve filterability of the worth. Since they showed interesting cellulase and β-glucosidase activities, they were used for the saccharification of autohydrolysed extractive-free barks. It was expected that the cell wall could have been sufficiently degraded so that these cocktails could produce good saccharification yields. Also, Saczyme Yield and Ultimase BWL 40 were previously tested by our group for the saccharification of olive pomace and olive stones, showing 80 and 90% of glucan conversion in pomace, and 40 and 55% in stones, respectively, after 5 h reaction (Miranda et al., 2019).

Fig. 2 presents the polysaccharides conversion (as glucose equivalents per total sugars of each solid residue obtained under different severity factors) along the time-course of the enzymatic hydrolysis (48 h), for each autohydrolysis solid residues and untreated extractive-free barks, catalyzed by Saczyme or Ultimase. Fig. 3 presents the comparative results from the experiments using different solid load and slurry performed for the autohydrolysis severity factor SF = 4. The enzymatic dosage in each hydrolysis assay was 45FPU/g of bark.

The autohydrolysis pre-treatment seems to be essential for saccharification of the Eg bark (only 3 to 10% of sugars were produced after 48 h for the untreated bark) independently of the enzyme formulation, while for Pa the untreated bark showed significant sugar conversion reaching as high as 38% (Fig. 2). The initial sugar conversion rates of Pa barks were higher than those of Eg barks with half the maximum conversion occurring in the first three hours while for Eg barks the time required was between 5 and 24 h.

The effect of the pre-treatments severity factor (SF) seems to be much more important on the saccharification of Eg bark than on Pa bark with sugar conversion increments of 67% (Saczyme) and 51% (Ultimase) between SF = 3.3 and SF = 4.7 in comparison with 10–13% increments for Pa bark. Similar results were reported for non-extracted untreated Eg barks with negligible sugar conversion (Matsushita et al., 2010), while Kemppainen et al. (2012) showed that untreated Pa bark could reach near 30% hydrolysis yield after 48 h (as DNS reducing sugars).

The two barks showed divergent results regarding the "slurry" experiment. While for Pa the sugar conversion was lower than if autohydrolysis liquor was removed prior to solid residue saccharification, for Eg the conversion was improved by doing the enzymatic hydrolysis with the slurry. This seems that the use of the autohydrolysis liquor has a negative impact in the saccharification of the Pa bark and not in the Eg bark, even though the amount of possible inhibitors (lignin, acetic acid and sugar degradation products) is higher for Eg than for Pa (Fig. 1, left plot).

Some of the results show higher than 100% conversion, which implies that the phenol/sulfuric acid methodology not only responds to reducing sugars but also to other components dissolved in the solution. This has been previously detected in other studies for similar reducing sugar measurement methods like the DNS assay (Kemppainen et al., 2012).

Table 3 (*E. globulus*) and Table 4 (*P. abies*) show the neutral sugars present in the hydrolysate at the end of the enzymatic hydrolysis (48 h) by HPIC, the enzymatic hydrolysis yield, global sugar yields and the lignin main type units present in the remaining solid residue.

The Ultimase enzymatic cocktail presented better results than Saczyme for both species, in some cases more than doubling the saccharification yields for the same substrates.

For Eg bark, Ultimase appears to be less dependent on the autohydrolysis severity factor, presenting total sugar contents between 29 $g/g_{ext\text{-}free\ bark}$ (SF = 4.7) and 37 $g/g_{ext\text{-}free\ bark}$ (SF = 4.0) when comparing to the sharper variation seen with Saczyme, with 14 $g/g_{ext\text{-}free\ bark}$ (SF = 3.3) and 33.5 $g/g_{ext\text{-}free\ bark}$ (SF = 4.0). As for the Pa bark, the variation with SF was less pronounced for both enzymes (12–17 $g/g_{ext\text{-}free\ bark}$ for Saczyme and 23–30 $g/g_{ext\text{-}free\ bark}$ for Ultimase).

Contrary to Eg bark, which showed almost no saccharification without pre-treatment, the Pa extractive-free bark presented a substantial sugar content after enzymatic hydrolysis with 36% and 47% glucose yields for Saczyme and Ultimase respectively.

Comparing the reactor sugar yields, it is possible to see that autohydrolysis severity conditions have a positive effect on the sugar conversion although, when reported as the global sugar yield process, the gains for Saczyme are marginal regardless of using or not any pre-treatment. When using Ultimase, the global sugar yield shows increments from 29% (untreated) to a maximum of 51% (SF = 3.6).

Although Saczyme presents higher cellulase activity than Ultimase (195 *vs* 127 FPU/mL) and similar β-glucosidase activity (25 *vs* 28 IU/mL), the results show that Ultimase is a better enzyme cocktail than Saczyme for this kind of biomass saccharification, presenting both higher glucose and global sugar yields. The presence of unknown enzyme activities on these enzyme cocktails may be responsible for their different behaviour.

Although only Ultimase is supposed to have xylanases, the "SL = 4.0, Slurry" experiment shows that both enzyme blends can degrade oligomeric xylose from the hydrolysates, presenting for Eg 9.4 and 11.4 $g/g_{ext\text{-}free\ bark}$ of Xylose + Mannose in the reaction medium for Saczyme and Ultimase respectively. The Slurry experiment also shows that the Eg autohydrolysis liquors only slightly inhibit the enzymatic hydrolysis with global glucose yield being reduced from 89% (34.3 $g/g_{ext\text{-}free\ bark}$) to 79% (30.4 $g/g_{ext\text{-}free\ bark}$) for Ultimase and even less for Saczyme (82% to 78%). As for the Pa, the autohydrolysis liquor appears to inhibit considerably more the enzymatic hydrolysis with a variation from 48% (15.5 $g/g_{ext\text{-}free\ bark}$) to 33% (10.6 $g/g_{ext\text{-}free\ bark}$) for Saczyme and a 69% to 57% for Ultimase. Matsushita et al. (2010) tested the saccharification of *E. globulus* bark slurry after steam explosion hydrothermal pre-treatment obtaining approximately 36% monosaccharides yield after 24 h incubation, which is lower than the value obtained here (73%, Table 3). Frankó et al. (2015) presented 51% glucose yields from a slurry of steam-treated *Picea abies* bark (after hot water extraction to remove a significant part of the hydrophilic extractives) which is slightly lower than the obtained for the slurry experiment in this work (57%).

The solid load increment to 5% proved to be very detrimental towards Saczyme saccharification, with both barks showing much lower total monosaccharides when compared to the 3.3% solid load experiment, affecting mostly the glucose yield and consequently the global sugar yield (55% *vs* 38% and 29% *vs* 19% for Eg and Pa global sugar yields respectively). When Ultimase was applied, almost no variation was noticed for Pa bark but a highly positive effect was observed for Eg bark with a sugar yield increase from 61% to 72%. Lima et al. (2013)

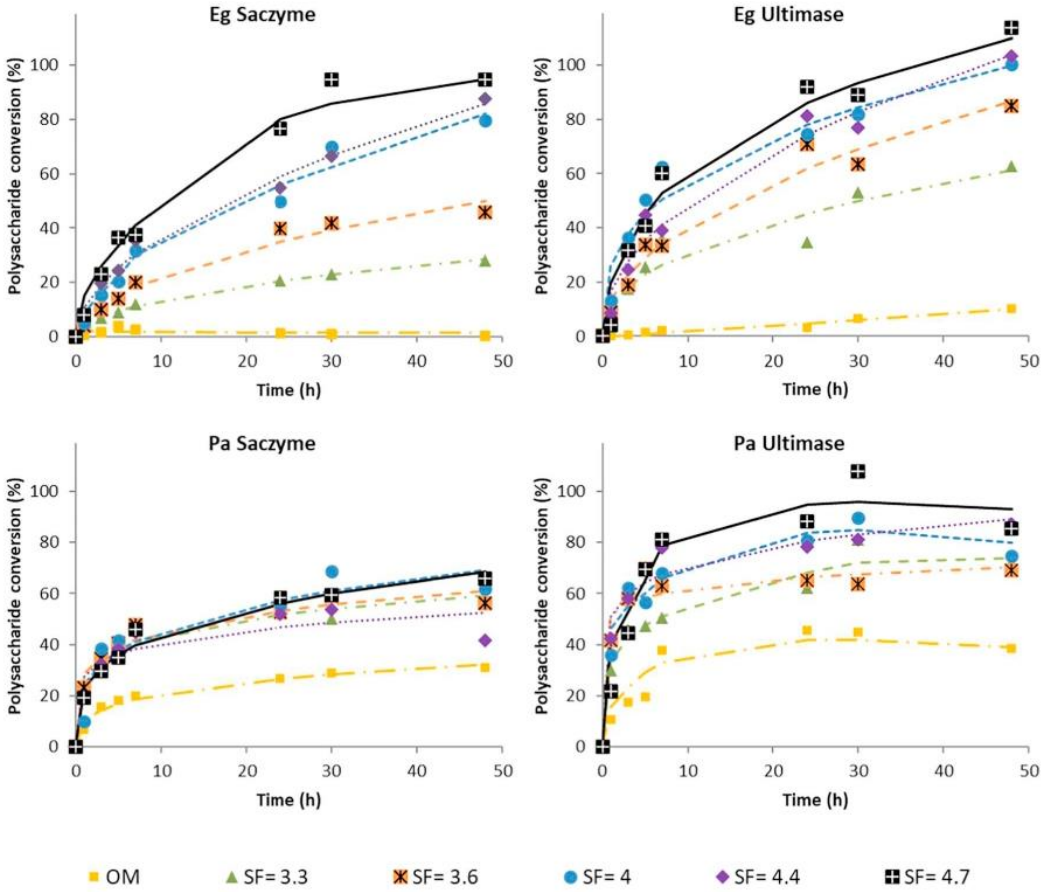

Fig. 2. Polysaccharide conversion time-course by enzymatic hydrolysis at 3.3% solid load (SL) of *Eucalyptus globulus* and *Picea abies* barks using Saczyme and Ultimase for the untreated (OM) and autohydrolysed bark residues under different severity factors (SF).

presented up to 65% glucose conversion after acidic and alkaline pre-treatments of *E. grandis* and *E. grandis* × *E. urophylla* which is far below the values obtained here with Ultimase at the same 5% SL for Eg, but above the 55% obtained with Saczyme. For *E. globulus* residues (bark, branches and leaves) after autohydrolysis (210 and 230 °C), 60% glucose conversion and a hydrolysate with 17.1 g/L of glucose was obtained (Silva-Fernandes et al., 2015), below the 29 g/L obtained here with Ultimase.

The best final sugar concentration of the liquid stream was obtained as expected for the experiments with higher solid load (SF = 4, SL = 5%), reaching 31 g/L for Eg and 18 g/L.

Since neither autohydrolysis nor enzymatic saccharification target specifically the lignin polymer, it is expected that the chemical changes within this polymer are lower than in many of the current delignification processes and that the final recovered lignin will maintain some resemblance to the original lignin. This might be a process to obtain aromatic compounds or lignin polymers without significant chemical alterations and free of specific contaminants such as sulfur typical from kraft or sulfite lignins. The potential market of lignin-based phenol and carbon fiber, among others, that can reach over 13 billion dollars (Smith et al., 2016) makes this residual stream highly desirable from an integrated process for multiple end-products.

Looking at the overall sugar recovery process that combines autohydrolysis liquor sugars and enzymatic saccharification of *E. globulus* and *P. abies,* the maximal recovery amounted to 540 kg and 440 kg of monomeric sugar moieties per ton of pre-extracted Eg and Pa barks respectively (Ultimase with SF = 4, SL = 5% and SF = 3.6 respectively for Eg and Pa). If the aim is solely glucose, the yields will be 406 kg and 314 kg per ton, respectively. Assuming a theoretical 0.51 g/g of ethanol from hexoses conversion, the highest ethanol production from glucose would be 207 and 160 kg of ethanol per extractive-free ton of *E. globulus* and *P. abies* barks.

The global mass balance of the best conditions (excluding solid load increase and slurry experiments) for each extractive-free bark is presented in Fig. 4.

4. Conclusions

Autohydrolysis followed by saccharification allowed fractionation of *Eucalyptus globulus* (Eg) and *Picea abies* (Pa) barks into three fractions: one liquid stream rich in hemicellulose oligosaccharides, one glucose solution and a lignin enriched solid residue.

Optimal autohydrolysis conditions of Eg and Pa extractive-free barks produced xylooligosaccharides/arabinooligosaccharides rich streams with low inhibitors formation. Efficient enzymatic saccharification of the solid required autohydrolysis with Eg presenting better results reaching almost total cellulose saccharification. Ultimase proved to be more efficient than Saczyme in all cases. Direct saccharification of autohydrolysis slurry was possible for Eg, while solid load increments to 5% improved saccharification for both species.

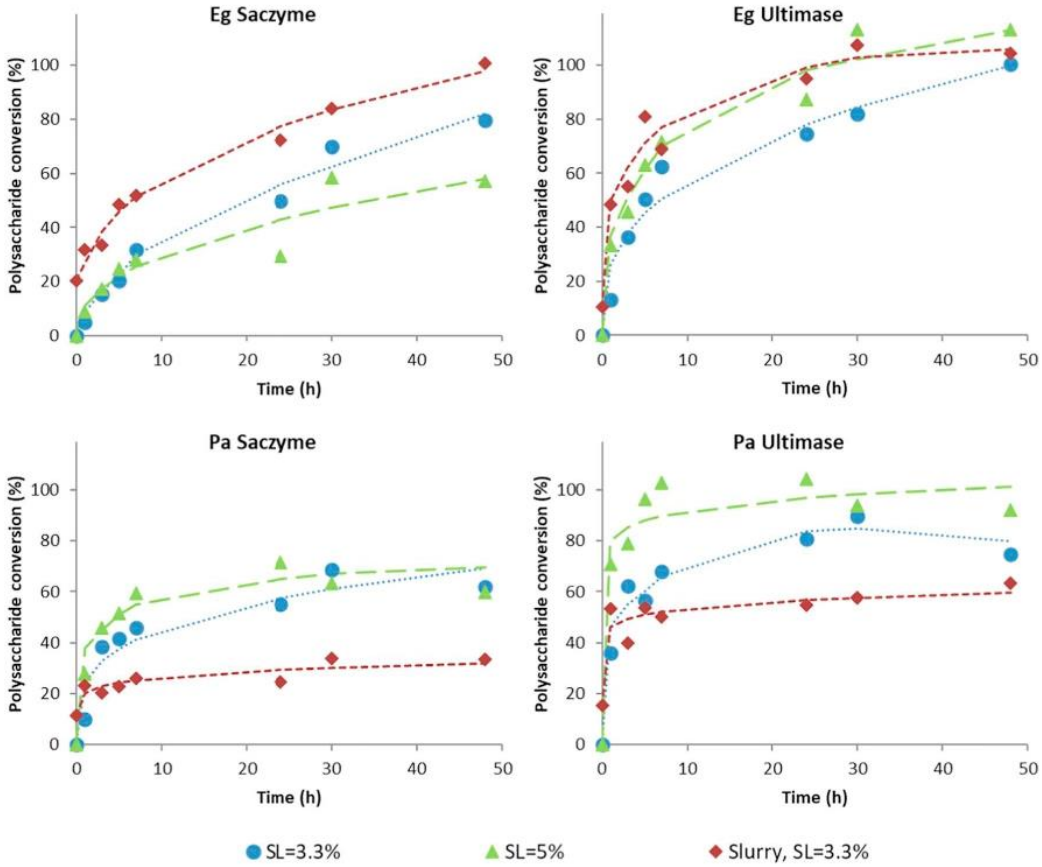

Fig. 3. Polysaccharide conversion time-course by enzymatic hydrolysis (Saczyme and Ultimase) of *Eucalyptus globulus* and *Picea abies* autohydrolysed bark residues at severity factor (SF = 4), with solid load 3.3% and 5% and slurry (SL = 3.3%).

Table 3
Monosaccharide content (g/100 $g_{ext\text{-}free\ bark}$) obtained in the hydrolysate after 48 h-enzymatic hydrolysis (Saczyme and Ultimase) for *Eucalyptus globulus* bark without (SF: 0) and with previous autohydrolysis under different Severity Factors (SF: 3.3–4.7) and lignin monomeric composition (hydroxyphenyl-H, guaiacyl-G and syringyl-S units) determined by pyrolysis.

	Saczyme						Ultimase									
Severity factor	0	3.3	3.6	4.0	4.0 SL = 5%	4.0 Slurry	4.4	4.7	0	3.3	3.6	4.0	4.0 SL = 5%	4.0 Slurry	4.4	4.7
Arabinose	0.1	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.7	0.0	0.0
Galactose	0.1	0.1	0.0	0.0	0.0	0.9	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0
Glucose	0.5	10.2	20.7	31.6	21.2	30.3	18.5	24.3	2.2	26.5	31.9	34.3	40.4	30.4	30.3	28.3
Xylose + mannose	0.5	2.5	1.2	1.0	1.6	9.4	3.0	1.8	0.8	4.7	4.2	2.4	2.6	11.4	1.3	0.8
Fructose	0.0	0.7	0.5	0.9	0.4	0.7	0.5	0.6	0.1	0.0	0.5	0.0	0.4	0.8	0.5	0.0
Total monosaccharides	1.2	13.5	22.4	33.5	23.2	42.0	22.1	26.8	3.3	31.2	36.6	36.7	43.4	44.0	32.1	29.1
Sugars concentration (g/L)	0	5	10	16	16	14	11	13	1	12	16	17	31	15	16	14
Reactor sugar yield (%)	2	24	42	76	52	–	55	70	5	55	69	83	98	–	80	76
Global sugar yield (%)	2	22	37	55	38	69	37	44	5	51	60	61	72	73	53	48
Glucose yield (%)	1	26	54	82	55	78	48	63	6	69	82	89	105	79	79	73
Non-glucose yield (%)	3	15	8	9	9	53	16	12	5	21	22	11	14	62	8	4
H	4	4	4	4	5	5	7	6	7	4	5	5	5	4	6	6
G	24	24	28	25	28	26	29	28	25	26	27	28	27	28	29	26
S	72	72	67	70	67	69	64	66	68	69	68	67	68	68	65	69
S/G	3.0	3.0	2.4	2.7	2.3	2.7	2.2	2.3	2.7	2.6	2.6	2.4	2.5	2.5	2.2	2.6

SL = solid load; reactor sugar yield = (total monosaccharides obtained after enzymatic hydrolysis) / (total monosaccharides of the solid residue added to the reactor) × 100; Global sugar yield = (total monosaccharides obtained after enzymatic hydrolysis) / (total monosaccharides of in the extractive-free bark) × 100.

Table 4
Monosaccharide content (g/100 $g_{ext\text{-}free\ bark}$) obtained in the hydrolysate after 48 h-enzymatic hydrolysis (with Saczyme or Ultimase) for *Picea abies* bark without (SF: 0) and with previous autohydrolysis under different Severity Factors (SF: 3.3–4.7) and lignin monomeric composition (hydroxyphenyl-H and guaiacyl-G) determined by pyrolysis.

	Saczyme						Ultimase									
Severity factor	0	3.3	3.6	4.0	4.0 SL = 5%	4.0 Slurry	4.4	4.7	0	3.3	3.6	4.0	4.0 SL = 5%	4.0 Slurry	4.4	4.7
Arabinose	0.4	0.0	0.0	0.0	0.0	1.9	0.0	0.0	1.2	0.1	0.0	0.0	0.0	2.0	0.0	0.0
Galactose	0.2	0.3	0.1	0.3	0.3	1.0	0.3	0.3	0.2	0.1	0.1	0.1	0.1	0.6	0.0	0.0
Glucose	11.7	12.6	10.8	15.5	9.8	10.6	10.6	13.1	15.1	23.4	28.4	22.1	22.5	18.2	24.0	22.0
Xylose + mannose	0.8	0.9	0.8	1.0	0.8	1.8	0.9	0.8	1.1	1.2	1.4	1.1	1.2	2.1	0.9	0.9
Fructose	0.1	0.5	0.5	0.4	0.2	0.9	0.5	0.4	0.0	0.1	0.6	0.0	0.4	0.9	0.5	0.3
Total monosaccharides	13.3	14.4	12.1	17.2	11.1	16.1	12.3	14.7	17.5	24.9	30.4	23.4	24.2	24.0	25.5	23.2
Sugars concentration (g/L)	4	7	5	9	8	5	6	7	6	12	14	12	18	8	13	12
Reactor sugar yield (%)	22	37	30	51	33	–	38	47	29	65	75	70	72	–	79	75
Global sugar yield (%)	22	24	20	29	19	27	21	25	29	42	51	39	41	40	43	39
Glucose yield (%)	36	39	33	48	30	33	33	41	47	73	88	69	70	57	75	68
Non-glucose yield (%)	6	7	5	6	5	20	6	6	9	6	8	5	6	21	5	4
H	20	17	20	18	14	22	17	18	19	14	26	25	17	23	30	27
G	80	83	80	82	86	78	83	82	81	86	74	75	83	77	70	73

SL = solid load; reactor sugar yield = (total monosaccharides obtained after enzymatic hydrolysis) / (total monosaccharides of the solid residue added to the reactor) × 100; Global sugar yield = (total monosaccharides obtained after enzymatic hydrolysis) / (total monosaccharides of in the extractive-free bark) × 100.

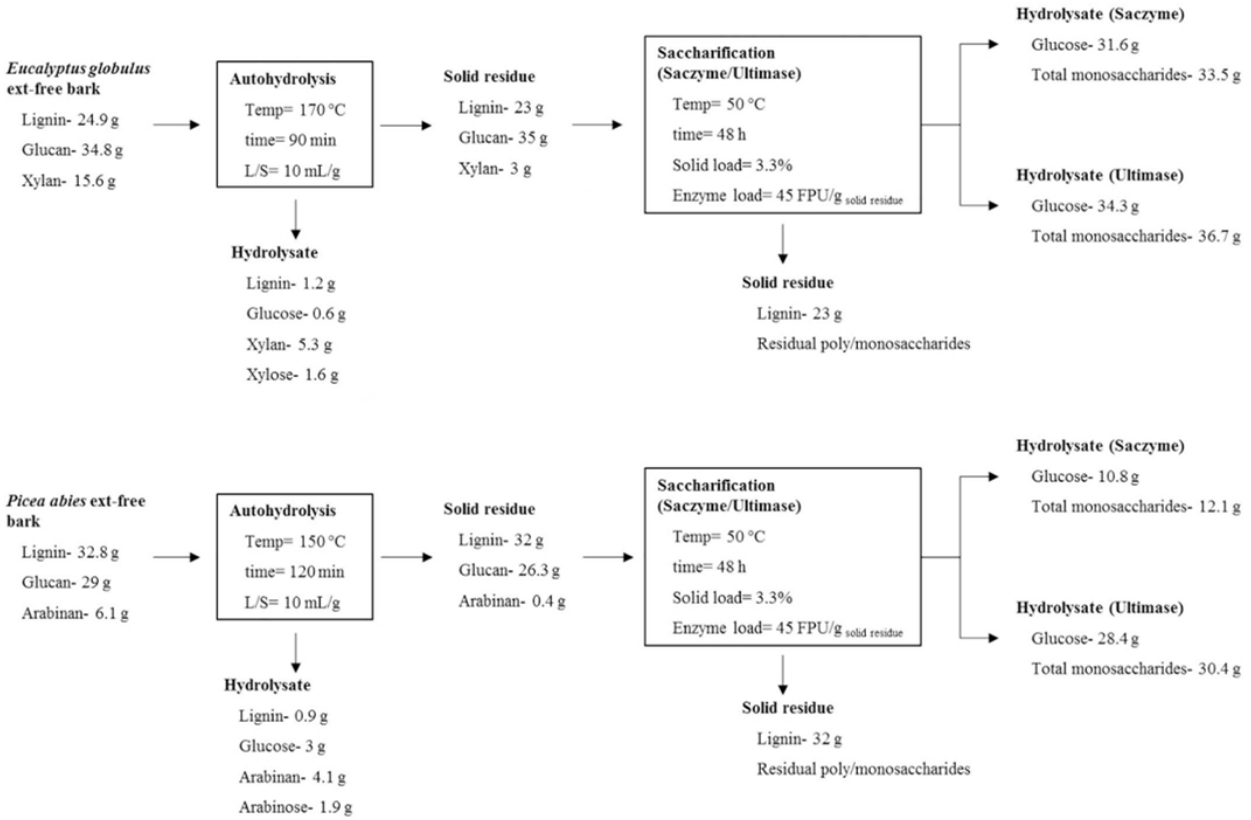

Fig. 4. Global mass balance for the valorization route of *Eucalyptus globulus* and *Picea abies* extractive-free barks. Glucan was calculated from glucose content multiplying by a factor of 0.9 and xylan and arabinan from xylose and arabinose contents multiplying by a factor of 0.88.

CRediT authorship contribution statement

Duarte M. Neiva: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing.**Ricardo A. Costa:**Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing.**Jorge Gominho:**Writing - review & editing, Resources, Funding acquisition, Supervision.**Suzana Ferreira-Dias:**Conceptualization, Methodology, Writing - review & editing, Resources, Supervision.**Helena Pereira:**Writing - review & editing, Resources, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare no competing financial interests or personal relationships that could have any influence in this work.

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Appendix A. Annex

Table A1
Identified compounds and relative abundances of the lignin-derived phenolic compounds released after Py-GC/MS of *Eucalyptus globulus* extractive-free bark (OM) and autohydrolysis solid residues at specified severity factors.

Severity factor	OM	3.3	3.6	4	4.4	4.7
Compound						
Phenol	4	6	3	3	4	3
Guaiacol	7	8	7	9	12	10
4-methylphenol	2	2	1	1	2	1
4-methyl guaiacol	3	3	4	4	6	5
4-ethyl guaiacol	1	1	0	1	1	1
4-vinyl guaicol	6	9	10	13	11	12
eugenol	1	1	1	0	0	0
Propylguaicol	0	0	0	0	0	0
Syringol	8	7	3	13	15	15
cis-isoeugenol	0	0	0	0	0	0
trans-isoeugenol	3	3	3	2	1	2
4- methyl syringol	8	9	8	10	13	11
Vanilin	1	2	2	1	1	1
Homovanillin	1	2	1	1	1	1
4-ethyl syringol	1	2	1	1	2	2
acetoguaiacone	1	2	1	1	1	1
4-vinyl syringol	13	13	18	20	16	20
guaiacylacetone	1	1	1	0	0	0
4-allylsyringol	2	2	2	1	1	1
Propioguaiacone	0	0	0	0	0	0
trans-coniferyl alcohol	1	1	1	1	0	0
cis-4-propenylsyringol	2	2	1	2	2	2
Propinylsyringol	2	2	2	1	1	1
trans-4-propenylsyringol	13	5	6	4	3	3
Syringaldehyde	4	4	6	2	1	2
Homosyringaldehyde	1	2	3	1	1	1
Acetosyringone	4	3	3	3	3	2
trans-coniferyl alcohol	0	0	0	0	0	0
Coniferyl aldehyde	0	0	1	0	0	0
Syringylacetone	7	4	6	2	3	2
Propiosyringone	0	1	1	1	0	1
cis-sinapyl alcohol	0	1	1	0	0	0
Dihydrosinapyl alcohol	0	0	0	0	0	0
trans-sinapyl alcohol	0	0	0	0	0	0
trans-sinapaldehyde	3	3	4	1	0	1

Table A2
Identified compounds and relative abundances of the S-lignin derived phenolic compounds released after Py-GC/MS of *Eucalyptus globulus* extractive-free bark (OM) and autohydrolysis solid residues at specified severity factors.

Severity factor	OM	3.3	3.6	4	4.4	4.7
Compound						
Syringol	12	12	5	21	24	24
4- methyl syringol	11	15	13	16	22	17
4-ethyl syringol	1	3	2	2	3	3
4-vinyl syringol	19	22	28	32	27	32

(*continued on next page*)

Table A2 (*continued*)

Severity factor	OM	3.3	3.6	4	4.4	4.7
Compound						
4-allylsyringol	3	3	4	2	2	2
cis-4-propenylsyringol	3	4	1	3	3	3
Propinylsyringol	3	3	3	1	1	1
trans-4-propenylsyringol	19	9	10	6	4	5
Syringaldehyde	5	7	9	3	2	4
Homosyringaldehyde	2	3	5	2	2	1
Acetosyringone	5	5	5	5	4	3
Syringylacetone	10	7	9	3	6	3
Propiosyringone	1	1	1	1	1	1
cis-sinapyl alcohol	1	1	1	1	0	0
Dihydrosinapyl alcohol	0	0	0	0	0	0
trans-sinapyl alcohol	0	0	0	0	0	0
trans-sinapaldehyde	5	4	5	1	0	1

Table A3
Identified compounds and relative abundances of the lignin-derived phenolic compounds released after Py-GC/MS of *Picea abies* extractive-free bark (OM) and autohydrolysis solid residues at specified severity factors.

Severity factor	OM	3.3	3.6	4	4.4	4.7
Compound						
Phenol	13	13	13	12	12	13
Guaiacol	20	20	22	21	22	24
4-methylphenol	8	7	7	7	8	9
4-methyl guaiacol	11	13	13	14	16	18
4-Ethyl phenol	0	1	1	1	1	1
4-ethyl guaiacol	2	2	2	2	2	2
4-vinyl guaicol	17	20	19	17	21	20
4-Propenyl guaicol	2	2	2	10	1	1
cis-isoeugenol	2	2	2	1	1	1
trans-isoeugenol	6	6	6	5	5	4
Vanilin	4	4	4	2	3	3
Homovanillin	2	1	1	0	1	0
Acetoguaiacone	2	2	2	2	2	2
Guaiacylacetone	1	1	1	1	1	1
Dihydroconiferyl alcohol	2	3	3	3	4	0
cis-coniferyl alcohol	0	0	0	0	0	0
trans-coniferyl alcohol	7	2	1	1	1	0

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Integrative results and discussion

The bark of two of the most industrially used species in Europe, *Eucalyptus globulus* (Eg) and *Picea abies* (Pa), were the focus of this thesis in regard to their possible deconstruction and upgradable approaches in a biorefinery context. These highly abundant residues are now mostly burned for energy and heat production in pulp and wood mills, and their chemical richness is thereby wasted. In this thesis, a thorough chemical characterization was achieved for each species, underlining their most interesting features and therefore pointing to possible deconstruction pathways and end uses. Although these barks have been extensively studied, it was possible to gather new knowledge and to propose alternative end uses.

The barks studied here were wittingly collected from industrial sites and not directly from trees. This will undoubtedly have some influence on the results when compared with previous research data obtained from barks collected from the tree but, in our opinion, it will provide better and more accurate information if the aim is to give a "new life" to these residues. The bark residues will vary from mill to mill, depending on processing, but as it happens when one works with biomass, such variability is unavoidable.

The studied barks of the two species are quite distinct and it was therefore expected that they had significant chemical differences and therefore deconstruction pathways and possible end uses.

The first thing that was obvious from the visual analysis of the industrial barks was that they were highly contaminated with wood (18% and 16% for Eg and Pa respectively), probably due to the strict requirements of the main production process that sacrifices some wood in favor of the final product quality. This not only leads to a 1.5-2% waste of the total wood supply for the main process but also increases the physical and chemical complexity of the industrial bark stream, making it harder to work with. Another thing that was obvious from the mechanical fractionation and analysis of the chemical composition was that the handling, transportation and storage of the bark (especially for Eg) at the industrial site need to be improved since a great deal of mineral extraneous materials was found to contaminate this residue. After mechanical downsizing, the fine fraction was enriched in inorganic matter (up to 21% vs 4-5% for the coarser fractions, Paper 1). Therefore, if the bark stream is to be used effectively, the handling, transportation and debarking process should be adjusted to reduce these contaminants.

Eucalyptus globulus bark showed similar chemical composition to that of the respective wood (Paper 1) although with higher ash and extractives content (mainly the polar extractives) and with lower lignin and polysaccharides (with quite similar monomeric sugar moieties). On the other hand, *P. abies* bark showed a more pronounced distinction from its wood (Paper 2) with higher ash content and much higher extractives (up to 20%) regarding both apolar and polar fractions. The lignin content was almost the same and the structural polysaccharides were much lower, presenting distinct relative monosaccharides abundances with lower mannose content in the bark than in the wood, and significantly higher arabinose and galacturonic acid content. *E. globulus* bark showed a higher acetylation of the hemicelluloses than *P. abies* bark but for both it was less than that of the respective woods. The proposed mechanical

fractionation was aiming at a possible retrieval of particle sized fractions with distinct compositions given that many studies reported highly differentiated compositions of inner and outer fractions of the barks. Although different, the variation that was found is, in our opinion, not high enough to dispend the energy and time to accomplish this task, except if needed to remove the mineral contaminated fraction of the fines (mostly for *E. globulus* bark).

As for the thermal properties, both barks showed a lower volatiles to fixed carbon ratio than the respective woods with *E. globulus* bark presenting lower calorific power than its wood (due to lower lignin and higher inorganic content). Although chemically quite different, the wood and bark of *P. abies* showed remarkable CHNO and HHV similarities, which implies that the increase in ash is probably compensated by the HHV of the extractives since the apolar extracts are known to be highly energetic in regard to polysaccharides.

The chemical composition and thermal properties of the barks indicates that better end-uses besides burning should be envisaged. The low energetic density and high ash content makes these biomasses less efficient. Besides, *E. globulus* also has high chlorine content which is detrimental to the boilers as well as the environment. The extractives are the first, and in many cases, the most interesting fraction to be explored. Nevertheless, once the extractives are removed, the extractive-free bark will be enriched in the structural components that should be further explored in an integrative concept.

The bark lignin and extractives were more finely scrutinized to further the knowledge on these specific fractions. The extractives fraction will be later discussed in the text.

Regarding the lignin polymer and its potential valorization (Paper 3), the “milled wood lignin” or better in this case “milled bark lignin” showed for *E. globulus* a highly enriched β-O-4’ aryl ethers linkages (83% of all inter-unit linkage types, higher than the 76% of the respective wood [259]) with very high S/G ratio (2.8, similar to respective wood) and some degree of acetylation at the γ-OH (S_{ac}= 8%, G_{ac}=2%). *P. abies* bark presented a predominantly G-lignin type (86%) and absence of S units, characteristic of softwood lignin, showing a very condensed structure with only 44% β-O-4’ aryl ethers linkages and 29% from condensed linkages (phenylcoumarans, resinols and dibenzodioxocins) as well as others related to the hydroxystilbenes. The *P. abies* bark showed higher lignin condensation in relation to its wood [259] and significant levels of acetylation at the γ-OH (G_{ac}=7%), which had never been reported in any conifer wood lignin. These characteristics point to a more reactive and theoretically easier to depolymerize *E. globulus* lignin and a more condensed *P. abies* lignin, with the first requiring less intense conditions and resulting in higher yields of S units and the later, although more recalcitrant, yielding high-valuable hydroxystilbenes and G units that can be used for production of compounds such as vanillin [260].

Intriguing and promising results were found for the lignin composition of the *P. abies* bark. Through DFRC, 2D-HSQC-NMR, HMBC, normal and diffusion edited ^{1}H-NMR, the conclusive presence of hydroxystilbenes glucosides (mainly isorhapontin, but also in lower amounts astrigin and piceid) in the lignin structure was obtained (Paper 4). The number of true lignin monomers has grown in the past decades with new

compounds being assumed of taking part in the lignification. With this work, the number increases with hydroxystilbene glucosides compounds here reported for the first time as building block units of *P. abies* bark lignin. This discovery may seem merely academic but in fact it has interesting possible ramifications. The first one, as reported for other compounds, is that the lignin polymer is much more complex than previously thought of, with monomers derived from other biosynthetic pathways (e.g. acetate/malonate-derived polyketide pathway) apart from the monolignol-derived shikimate pathway being incorporated in the lignin structure [34,36]. The second and most interesting one is the authenticated finding, for the first time, of glucosides in the lignin structure (Paper 3 and 4). The presence of glucosides in lignin has been proposed previously as occurring in some lignins, but the assimilation mechanism into the polymeric structure was unclear, with direct glucosylation of phenolic groups being viewed with skepticism at best for its supposed biosynthesis impediments (enzyme penetration in hydrophobic lignin structure very unlikely) [261]. In this case, the already glucosylated stilbenes have a second phenolic group that will participate in the radical coupling with lignin. This elegant solution is a much more plausible explanation for the occurrence of glucosides in the lignin structure. The third reason regards the possibility to biologically modify and engineer the lignin polymer aiming at specific traits (e.g. antifungal, enhanced hydrophilicity) or by functionalization of the glucose moiety. This is, in our opinion, one of the most interesting discoveries in this thesis.

The bark of *E. globulus* presented significant polysaccharides content and lower lignin than the respective wood (Paper 1). The chemical features combined to its fibrous nature, lead us to believe that it could be an interesting raw material to increment the fiber supply for pulping purposes. The physical and chemical traits of the *P. abies* bark, as determined here and throughout the literature, showed very poor perspectives of pulpability leading to its exclusion as raw material for this purpose. The idea of cooking bark and wood together had previously been tested with poor results [237], since both materials clearly show different optimal pulping conditions [224].The use of hot water pre-treatment to partially wash and decrease the extractives and ash content was tested here and allowed significantly better reaction yields, although the global yield between treated and untreated was quite similar once reported to original material (taking into account the pre-treatment mass loss) (Paper 5). Nevertheless the pre-treatment allowed higher delignification especially when using low chemical charges, reaching kappa number of 17 with only 15% active alkali. The resulting pulps were subjected to bleaching and refining, producing handsheets with similar physical characteristics than those from the respective wood. Although the tear index was lower for the bark pulps, the tensile index was similar and the paper surface strength was even higher than that of the wood. The main drawback on using bark for pulping lies on the global pulping yield, which is around 41%, much lower than the 50% achieved with wood at industrial production. Nevertheless, since bark has 10% lower polysaccharides content, it is fair to assume that it delignifies pretty well, which is in agreement with the determined high S/G ratio and β-O-4' aryl ethers linkages (paper 3). Another drawback for bark pulping is that the higher ash content might hinder its use for finer applications such as dissolving pulps produced from bleached pulp.

As stated before, one of the most interesting component of the barks and that most differentiates them from wood, is the extractive fraction. Both species had much higher content of extractives in bark than in wood (9.9% vs 4.4% for *E. globulus* and 20.2% vs 3.8% for *P. abies*), with more polar extracts than apolar ones (Paper 1 and 2). The characteristics and composition of the extracts is highly dependent on the solvent used and their antioxidant and antimicrobial activity clearly reflects it. In the work developed in task 3 (Paper 6), in addition to *E. globulus* and *P. abies* barks we included barks from two other species (*Acacia dealbata* and *A. melanoxylon*). Ethanol extracts showed higher phenolic content than water extracts, more specifically regarding condensed tannins (water was more efficient in extracting flavonoids) and the antioxidant activity results, independent of the methodology, proved that ethanol extracted better compounds that prevent oxidation (whether lipid and metal oxidation or free-radical scavenging and neutralization). Although highly antioxidant extracts could be produced from both barks, *P. abies* bark showed higher extraction yields, total phenolic content and antioxidant activity, making it a more interesting raw material for the procurement of biobased antioxidant compounds. Regarding antimicrobial activity, the extracts from both species proved mostly ineffective against Gram-negative bacteria, with *E. globulus* bark extracts showing higher growth inhibitory characteristics against Gram-positive bacteria than *P. abies* bark extracts, especially for the n-hexane extracts. The most impressive results were nevertheless obtained against both *Candida* strains where inhibition occurred at concentrations as low as 40 $\mu g.mL^{-1}$. The increased virulence behavior of some bacteria strains (e.g. biofilm formation, swarming motility) is one of the reasons for the increased microbial resistance to antibiotics. This behavior occurs through molecular communication between bacterium (quorum sensing) when in adverse environment conditions and the disruption of this communication mechanism could prove highly productive in preventing antidrug resistant strains [88]. The crude extracts anti-quorum sensing ability was tested, but alas, it proved to be mostly ineffective. The overall results of Paper 6 show that *Acacia dealbata* bark was clearly more interesting for obtaining antimicrobial extracts, although *E. globulus* and *P. abies* barks could also be looked upon as raw materials to extract bioactive compounds.

The extractives bioactivity and the expected improvement in the subsequent processing of the extracted barks indicate that this step in the deconstruction pathway should be very enticing if a bark based biorefinery is to take place.

Thinking on the sequential integrative deconstruction potential and the full valorization of barks, one fractionation pathway was applied to the extractive-free barks, including an autohydrolysis step and an enzymatic saccharification, aiming at exemplifying one of the possible conversion routes. The extracted barks, and in consequence enriched in the main cell wall structural components (hemicelluloses, cellulose and lignin), were subjected to a sequential fractionation obtaining a liquid stream rich in xylooligosaccharides or arabinooligosaccharides (depending on the species used), after autohydrolysis, and to a glucose enriched liquid stream and a lignin enriched solid residue after the enzymatic saccharification of the autohydrolysis non-solubilized material.

By testing a range of autohydrolysis conditions, it was possible to determine the best conditions for hemicelluloses degradation and solubilization as oligosaccharides. *P. abies* hemicelluloses required lower severity factors to partially depolymerize and solubilize than those of *E. globulus,* producing 14 and 12 g of monosaccharides/oligosaccharides per 100 g of extractive-free bark, respectively. These oligomeric streams are of interest for the food and pharmaceutical industries (e.g. prebiotic potential), although they can also be totally depolymerized to monosaccharides for ethanol fermentation or biotechnological transformation to valuable compounds such as xylitol and arabitol. The autohydrolysis solid residue (enriched in cellulose and lignin) was then enzymatically saccharified with two commercial enzymes (Saczyme Yield and Ultimase BWL40), and the results showed that higher glucose and total sugar yields could be obtained with *E. globulus* bark than with *P. abies* bark. Alternatively, saccharification was also performed directly in the autohydrolysis slurry (liquid and solid residue) increasing the global sugar yield up to 73%.

Papers 6 and 7 provide a possible deconstruction pathway to fully use the chemical potential of these barks, with extractives, hemicelluloses, cellulose and lignin being obtained sequentially through simple and environmentally clean processes, while paper 5 shows a technically viable alternative for the *E. globulus* bark more related to the pulp and paper industry, where it is mainly produced.

Conclusions

Industrial barks are highly interesting and upgradable residual streams with potential to increase the biomass available for bio-based products, building blocks, fine chemicals and fuels. Already available and at low cost, these non-wood lignocellulosic materials can be deconstructed using already established processes (normally associated to wood processing) with small adjustments to the specific characteristics of bark and taking into consideration the species at hand.

An integrated pathway to make use of each lignocellulosic major chemical component can be achieved. Barks are characterized by a rich extractive fraction with high value low volume bioactive components, and highly desirable for several important industries. Crude extracts could be obtained through solvent extraction that showed high antioxidant activity and low minimum inhibitory concentration against some human pathogenic microbial.

Since extractives can be detrimental to other transformation processes, the removal of extractives was designed as a first step in the fractionation process. The extractive-free barks, enriched in the structural components can be further fractionated through green-processes such as autohydrolysis and enzymatic hydrolysis producing a hemicellulosic rich stream of oligosaccharides, a glucose stream and a lignin residue. The process might be adjusted to obtain liquid streams targeted for production of fuel such as ethanol, building blocks such as xylitol, arabitol, lactic acid and succinic acid, and to obtain phenolic components from the solid lignin fraction.

The use of kraft pulping after autohydrolysis documented here for *Eucalyptus globulus*, gives a possible fibre end-use for this residue increasing the feedstock for pulp and paper production.

Barks are non-wood lignocellulosics that are much less explored and known than wood, with possible unique chemical characteristics. The detailed chemical characterization made in this thesis showed for the first time that hydroxistilbenes glucosides are present in *Picea abies* lignin, as a new lignin monomer, thereby providing a plausible biochemical solution for the occurrence of glucosides in this phenolic polymer and opening the possibility of bioengineering the addition of functionality groups for production of a lignin with special attributes (hydrophilicity, bioactivity).

This thesis presents only a few of the possible deconstruction pathways that can be followed and of the products that can be obtained from the barks of *Eucalyptus globulus* and *Picea abies.*

Future work

Several studies can be envisaged to further deepen the knowledge already acquired on the barks and to advance on the utilisation of their main components.

Regarding the extractives:

- Testing of different extraction techniques and conditions, studying their effects on extraction yield and extract chemical composition.
- Separation and purification of the crude extracts to obtain, if not pure compounds, at least families of compounds
- Testing single compounds or families of compounds for antioxidant activity or human pathogenic microorganisms' growth inhibition to determine those responsible for each phenomena, either alone or in synergies.

Regarding the lignin fraction:

- The solid residues from the enzymatic saccharification from paper 7 (mostly lignin under the best enzymatic conditions) need to be further studied. A comparison to the already available MBL (milled bark lignin) in paper 3 will show us the impact that the sequential treatment has on the final solid (lignin).
- After purification, this "lignin" could be tested as additive in several products such as a phenol substitute in adhesives.
- The hydroxystilbenes glucosides determined in the milled bark lignin could occur in the bark of other Picea species or even in other softwoods, and so a new study could be designed to evaluate fine lignin composition of other barks.

Regarding the polysaccharides fraction:

- The autohydrolysis and enzymatic hydrolysis combo should be optimized regarding either total monosaccharides obtained or type of oligosaccharides obtained (from hemicelluloses) in the autohydrolysis liquid stream.
- Other enzymatic cocktails could be tested on the same substrates (extractive-free barks) to improve saccharification.
- The autohydrolysis liquid stream rich in oligosaccharides should be analysed in more detail to ascertain their characteristics and from there envisage possible end uses.

Other deconstruction pathways could also be studied on these barks e.g. pyrolysis, fractionation using deep eutectic solvents or ionic liquids.

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