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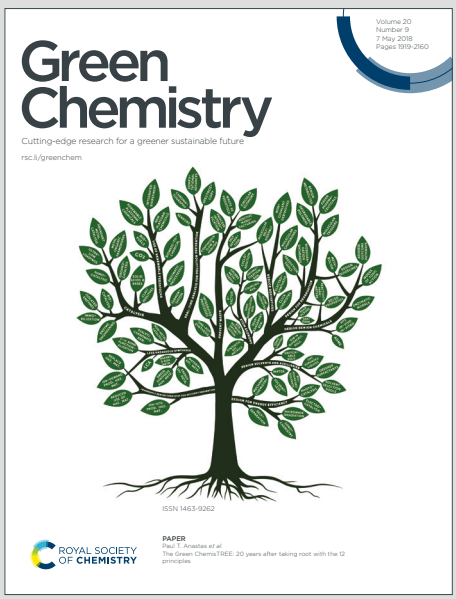

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ARTICLE

Accessing suberin from cork via ultrafast supercritical hydrolysis

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Natural biopolyesters in the form of suberin could be a promising resource for the synthesis of a wide array of chemicals, fuels and materials. However, access to this molecule is limited by a chemical-intensive and laborious work-up procedure based on alkaline hydrolysis or methanolysis. Herein, we propose the ultrafast supercritical hydrolysis as a novel and scalable approach for the facile access to suberin from cork. Supercritical water (SCW) with high or low ionic properties (pKw of 13 and 15) would allow the liquefaction of lignocellulosic materials while avoiding the hydrolysis of ester linkages. Therefore, extractives and carbohydrates partition in aqueous products while suberin and lignin are enriched in the solid products. Suberin yield reached as high as 66.6% of the dry cork at 386℃ and 1.27 s (pKw = 13), owing to the removal of up to 94.7% of polysaccharides content of the original cork biomass. The occurrence of repolymerization reaction, however, was observed under this condition, including an increase in Klason lignin presumably due to pseudo-lignin formation. Meanwhile, lower repolymerization products accompanied by a 77.1% polysaccharides and 14% lignin removal was achieved when working at 395℃ and 0.25 s (pKw = 15), at the expense of lower suberin yield of 59.8%. It appears that even a longer reaction time of 1.27 s would not lead to hydrolysis of ester linkages in suberin but influences the extent of liquefaction of the lignocellulosic matrix and the formation of repolymerization products. A lower ion product and a reaction time of less than 1 s would be preferable to strike a balance between maximum polysaccharides removal and minimum repolymerization products generation. This study has contributed towards the development of a new sustainable process to access valuable molecules from cork and has highlighted the importance of obtaining high quality and highly functionalized biopolymers as a source of new building blocks.

I. Introduction

Polymers of biological origin have attracted significant attention in recent years as a sustainable resource for the production of fuels, chemicals and materials of industrial relevance. Lignocellulosic materials, which are found abundantly in agro-industrial wastes and residues, can be an effective source of important molecules and building blocks that can be processed into bioenergy, biofuels, biochemicals and other value added products. The under-appreciated tree bark, for example, is of particular interest as a resource for biologically active compounds, such as cellulose, hemicellulose and lignin. One of the major issues that needs to be addressed in utilizing lignocellulosics is the dire need for an effective biomass fragmentation that would enable industrial application. An effective biomass fragmentation strategy is characterized to comprise of the least possible number of steps; to minimize, if not completely avoid the use of organic chemicals and to anchor on well-accepted process fundamentals practiced in the chemical industry.[1]

Cork oak forests, spanning about 2 million hectares worldwide can be found in Portugal (34%) Spain (27%), Morocco (18%) and Algeria (11%).[1] Approximately 300,000 tons of external cork bark are harvested annually, primarily to produce wine bottle stoppers and cork thermal flooring. During processing, up to 25% of cork is transformed into powder as a by-product.[2] Having no direct industrial application, it is mainly used by the industry to produce energy by burning. Cork is comprised of extractives (8-20%), carbohydrates (6-25%), suberin (30-50%) and lignin (15-30%), all of which have distinct structures and promising applications.[3,4] Therefore, cork by-products have a huge potential to be exploited for other applications other than its current uses.

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Electronic Supplementary Information (ESI) available: SF1. Photos of the original cork biomass and solid hydrolysis products. *SF2 Summary of suberin compounds identified via GC-MS; SF3. Formation of repolymerization products. ST1. Chemical summative analysis data; ST2. Typical GC-MS chromatograph for suberinic acid monomers; SL. List of chemicals See DOI: 10.1039/x0xx00000x*

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There is huge body of work related to cork fractionation achieved by chemical methods, intended for the recovery of suberin. Suberin is a cell wall macromolecule that plays a vital role in controlling water and solute diffusion in tissue layers and as a protective barrier against pathogenic invasion and thermal fluctuations.[5] It is located in the secondary cell wall, together with some lignin and is sandwiched in between the primary and tertiary walls whose major constituents include polysaccharides and lignin.[6] It has been widely accepted to consist of polyaliphatic and polyphenolic domains linked together in a three-dimensional polyester network. The polyaliphatic part is composed of α,ω-diacids and ω-hydroxy fatty acids (also called suberinic fatty acids) and glycerol, with chain lengths typically from C_{16} to C_{26}.[7] Meanwhile, the polyphenolic part consists largely of hydroxycinnamic acids such as ferulic, p-coumaric and caffeic acids and minor quantities of p-coumaryl, coniferyl and sinapyl monolignol alcohols.[8] Due to its interesting structure, suberin and its fatty acid constituents has found application as an additive in offset lithographic printing, as binders in particle boards, polyurethane foams and plywood and pharmaceuticals and cosmetics. [9–11]

Despite the abundance and promising applications, suberin and its fatty acids are not commercially available in the market because they are difficult to produce synthetically. [10,12] Thus, interest in naturally sourced suberinic fatty acids has been attracting attention to obtain novel highly-functional polymers that cannot be currently synthesized. The main concern as to why it is not as widely valorised as cellulose and lignin is due to the limited access to it. The conventional approach is intuitively carried out due to the positioning of the biopolymers within the cork cell wall. Moreover, there exists an inter-monomer ester linkage between the hydroxyacids and diacids (polyaliphatic constituents), an ester or ether crosslink between the polyaliphatics and polyphenolic part and a C-C, amide and ether bonds connecting the polyphenolic part to the rest of the cell wall components.[13] Thus, the isolation of suberinic fatty acids from cork follows a sequential and laborious train of removing extractives, followed by suberin recovery through alkali methanolysis[14,15] or alkali hydrolysis[16], then acid hydrolysis for cellulosic fractions, leaving lignin behind. Alkaline hydrolysis is a harsh process, where ω-hydroxyfatty acids with epoxy and hydroxyl containing derivatives are obtained. Methanolysis was reportedly a milder approach, where alkanedioic and hydroxyalkanoic acids are obtained. Both processes require the use of a huge amount of chemicals, long reaction times (several hours and involved at least nine steps in succession) and laborious work up procedure, only to generate small yields (few grams) that would only be suitable for characterization. Furthermore, these processes require neutralization of reactants.

Recent developments for fractionation include the application of ionic liquids (IL)[17,18], and reductive catalytic fractionation (RCF)[19,20]. Choline-based ILs in so far was the most suitable system to dissolve suberinic fractions, leaving the lignocellulosic domains behind. However, the effective separation of ILs from suberin as well as the regeneration of the ILs remains to be a major hurdle for this approach. RCF is more attuned to lignin solubilisation, therefore, RCF products are a cocktail of mono-, di- and oligomeric fractions of lignin alongside suberin monomers that need to be effectively separated. Clearly, there is a need for a more sustainable approach towards accessing cork fractions, something that would facilitate industrial scale up.

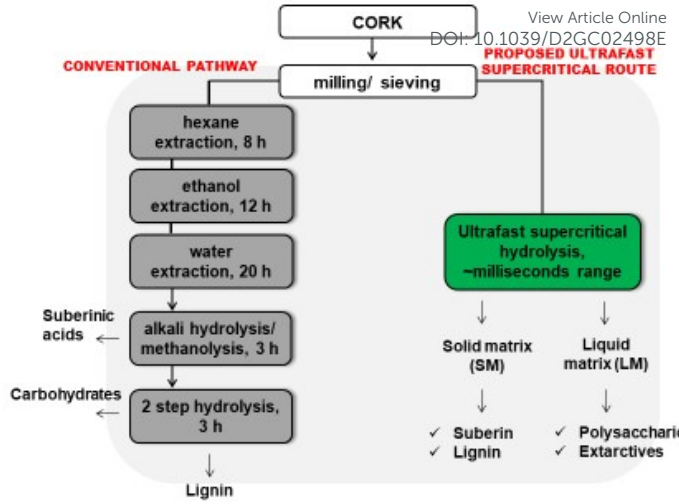

Figure 1. Comparison of conventional approaches to cork fractionation and the proposed ultrafast supercritical route

We therefore envisioned a novel process that would allow the removal of the lignocellulosic matrix instead, for facile recovery of suberin, as illustrated in Fig. 1. In such scenario, it is necessary to utilize a solvent that has high diffusing ability to penetrate through the tertiary and secondary walls and dissolve the primary cell wall components. Amongst solvents, water is generally considered as safe, nontoxic, environmentally benign and non-contaminating. When water is in its supercritical state, it exhibits properties that considerably differs than ambient conditions. It was revealed that the important factor in the control of hydrolysis reaction is the ions concentration.[21,22] Control over the reaction rates can be achieved such that side reactions faster than depolymerization reactions can be avoided, facilitating high selectivity. Thus, Cocero and co-workers[23] have designed a Sudden Expansion Micro-Reactor (SEMR) that allows for an instantaneous heating and cooling achieved by sudden decompression due to the Joule–Thompson effect with residence times effectively reaching as short as 40 ms. The extent of reaction can be effectively controlled by manipulating water properties (by varying temperature and pressure) and residence time inside the reactor. The SEMR has been previously demonstrated as an effective approach in valorizing lignocellulosic biomass, particularly cellulose, hemicellulose and lignin, in model compounds and actual biomass.[23–26]

In this study, we have leveraged ultrafast supercritical water as the reaction media for the fractionation of cork. We demonstrated that hydrolysis with the SEMR led to the simplification of the process consequently reducing processing time and chemical utilization for accessing cork fractions as compared with conventional approaches. We also showed that supercritical water (SCW) either as a low-ionic or high-ionic

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reaction media will allow the hydrolysis of the lignocellulosic matrix and will avoid the hydrolysis of both linear aliphatic ester links and acylglycerol ester links, keeping suberin intact in a lignin-suberin complex. This paves way to accessing suberin selectively and obtaining new building blocks that could not be achieved from any other biopolymers. As suberin is a new addition to the SEMR portfolio, characterization of recovered suberin fatty acids was also reported herein. Finally, we provided insights into the formation and avoidance of unwanted substances such as repolymerization products during processing.

II. Results and Discussion

1. Characterization of as-received cork

The chemical composition of cork is highly variable and is affected by diverse factors including age of tree, geographical origin and climate.[27–29] Thus, the as-received cork was subjected to an extensive component analysis, details of which are provided in the methods section. The major components of cork can be categorized into four, namely extractives, suberin, polysaccharides and lignin. Table ST1 summarizes the results of the chemical summative analysis carried out on the cork sample, in comparison with published data in the literatures. Due to some differences in the experimental procedures used by other researchers, only the major components were compared. Overall, it can be seen that the properties of the as-received cork were comparable with published data. The components in the as-received cork can be ranked as follows: suberin > lignin > polysaccharides > extractives. This ranking order is common for corks from Q. suber of Portugal. Q. suber cork from Bulgaria and Turkey were reported to have more polysaccharides than lignin.[30]

The extractives are low-to-medium molecular weight compounds that improves cork impermeability and that would impart organoleptic properties in wine when cork is used as a bottle stopper.[31] These compounds typically consist of waxes, triterpenes, n-alkanes, sterols, phenols and polyphenols, which, when removed, will not affect the structural integrity or mechanical properties of the cork. The extractives are further classified into two: the aliphatics or waxes that can be extracted by low polarity solvent such as hexane and the phenolics, which are extracted by ethanol and water.[32] The cork sample was found to contain more phenolics (7.6 g / 100 g cork) than aliphatic extractives (3.6 g / 100 g). GC-MS analysis revealed that the hexane extractives contain major triterpenes, such as fredelan-3-one, γ-sitostenone, Lup-20(29)-en-28-al and betulinic acid. Glycerol and long chain aliphatic alcohols such as 1-nonadecanol, docosanol and tricosanol were also present. Fatty acids such as palmitic and octadecanoic acid were also found. Compared with those reported by Sen et al.[31], more fatty acids were present in the hexane extracts in the current sample. Meanwhile, phenolics sequentially extracted by ethanol and water, consist of carbohydrates and sugar derivatives, some of which could have originated from free sugars that surfaced upon size reduction. Ethanol extractives were found to contain levolglucosan, arabitol, xylose, myo-inositol, lactose, arabinopyranose, ethylene and propylene glycol, lactic acid, oxalic acid, succinic acid and mallic acid L-rhamnose and galactopyranose. Vanillin and vanillic acid which are lignin derivatives were also detected. Fatty acids such as palmitic acid and hexadecanoic acid were also present as well as major triterpenes, such as fredelan-3-one and betulinic acid. Meanwhile, glycerol, arabinose, hexadecanoic acid and octadecanoic acid were found in water extractives.

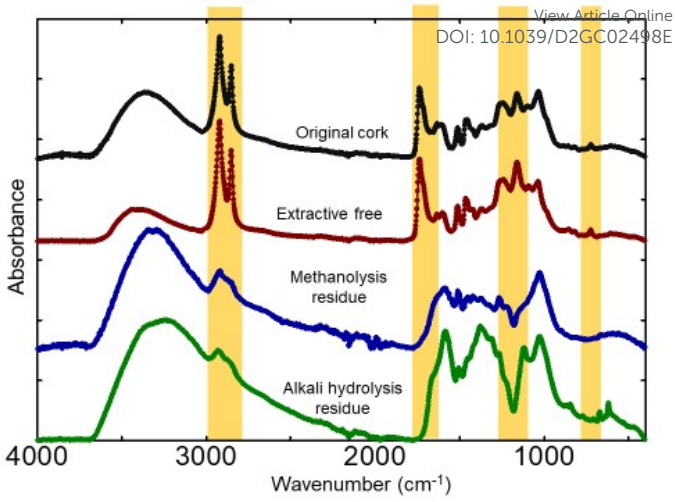

Figure 2. Fourier transform infrared spectra of the as-received and extractive free cork biomass compared with residues obtained after alkaline methanolysis and alkali hydrolysis

Table 1. Suberin FTIR intensities (adapted from Graca and Pereira, 2000) [ref. 36]

Wavenumber, cm-1	Assignment
2921	Asymmetric –CH2- alkyl chains
2852	Symmetric –CH2- alkyl chain
1737	C=O ester bonds
1242	Epoxide ring
1148	C-O-C ester bonds
724	C-H bending

Alkali hydrolysis and alkaline methanolysis are the most commonly adopted approach for suberin determination. Both approaches entail gravimetric analyses of suberin in terms of hydrophobic long-chain lipids released from the cleaving of ester bonds in cork. Alkali hydrolysis takes shorter time (1.5 hr) and allows for a smaller amount of cork sample (as small as 100 mg). In contrast, alkali methanolysis takes 3 hr and 1.5 g sample, but is more desirable as it permits a separate quantification (using HPLC) of the glycerol fraction that solubilized in water after solvent partitioning. It is worth noting that in most of older literatures, the aqueous glycerol was unaccounted for, which underestimated the total suberin reported. Nevertheless, in the context of this manuscript, total suberin refers to the mass of suberin gravimetrically obtained from post-alkaline methanolysis organic fraction plus aqueous glycerol. Therefore, total suberin quantity reported in this study could be generally higher than those previously reported. The total suberin recovered from the cork sample via alkali hydrolysis and alkaline

methanolysis was found to be 46.5 g/100 g cork and 47.8 g/ 100 g cork, respectively. Despite the difference in the technique, the values do not differ significantly, similar to the observations by Pinto et al.[16] .

Polysaccharides as monomeric sugars were obtained via two-step acid hydrolysis. Cellulosic carbohydrates are expressed in terms of glucose while non-cellulosic carbohydrates, mainly hemicellulose, consist of xylose and arabinose. The cork sample has a total polysaccharide content of 14.6 g/100 g cork, which is within the range observed in previous works.[30,33] This value is lower than the cellulose content in typical woody trees and materials. Also, cellulose (as glucose) and hemicellulose (as xylose + arabinose) was almost of the same quantity. While cellulose is a major contributor in the rigidity of the plant cell wall, it is not the case in corks. Lignin is the second structural component in cork. It imparts rigidity to the cell walls, as well as mechanical strength, stress resistance and durability. Lignin in the cork sample amounts to 21.1 g / 100 g cork, also relatively lower than most woody trees.[34] Other minor constituents such as moisture and protein were found as 3.7 ± 0.2 g/ 100g and less than 0.001 g/ 100g of cork, respectively. It is worth noting that polysaccharides and lignin depolymerisation favours acidic to neutral conditions whereas suberin depolymerisation is favoured in alkaline conditions.[35]

The as-received cork, extractive free cork and residues after alkali hydrolysis and alkali methanolysis were analysed by FTIR, as shown in Fig. 2. This provided information regarding the presence of ester bonds that signify the presence of suberin. There are six important peak intensities assigned to suberin, the corresponding assignments of which are shown in Table 1. The dominant peaks of 2921 and 2852 cm^{-1} are attributed to the long-chain aliphatics in suberin. Meanwhile, the ester bonds are represented by high-intensity 1737 and 1148 cm^{-1} vibrations.[36] These were clearly identified in the as-received and extractive-free cork. After the alkali hydrolysis and alkaline methanolysis, these six peaks were significantly diminished in the residues, which confirmed the removal of the suberin from the cork.

2. Ultrafast hydrolysis conditions

Cork has low permeability to liquid water and water vapour ($280.5x10^{-3}$ $mol{\cdot}m^{-1}s^{-1}Pa^{-1}$ and 110.1×10^{-13} $mol{\cdot}m^{-1}s^{-1}Pa^{-1}$)[37], hence, it is difficult for ambient water to simply penetrate the cell walls. However, by controlling the properties of water at the proximity of supercritical regions, water properties significantly change which enable water to function as a reaction media. The properties of water at the proximity of supercritical regions has been previously discussed by Cantero et al.[38] More recent updates for calculations were incorporated in this current work benchmarking from the values reviewed by Brunner, 2014[39], particularly on the determination of the ionization constant of water. The experimental conditions are summarized on Table 2, and water properties at the stipulated conditions were calculated accordingly. Considering that the cork powder has higher percentage of lignin than polysaccharides, reaction conditions used in this study were benchmarked from Abad et al.[24] to begin with (386°C, 0.3 s).

The stipulated experimental conditions resulted to low densities and both high [UFC-1, UFC-2] and low [UFC-3] ion product values. With low density, water molecules have better diffusibility into the biomass substrate. UFC-1 and UFC-2 have close density values and a pK_w of 13 which is one magnitude higher than pK_w of water at room temperature with reaction time as the distinguishing element. Meanwhile, UFC-3 resulted to the lowest density value, the shortest reaction time and a pK_w = 15, translating to a lower molal concentration of H^+/OH^- than water at ambient conditions.

Ultrafast hydrolysis experiments were carried out in a continuous microreactor that permits control of reaction time in the milliseconds range. The specifications, characteristics and configuration of the continuous microreactor has been reported extensively elsewhere.[23,24] The occurrence of supercritical hydrolysis in the ultrafast microreactor is first evidenced by the liquefaction of some cork components. It was observed that the pH of the exiting product stream after hydrolysis reduced from pH = 7 to a pH = 5, suggesting the presence of small acid monomers in the product. The supercritical hydrolysis products in all experiments consist of two major fractions: namely the solid matrix (SM) and liquid matrix (LM). LM and SM were separated by centrifugation.

Since operating at supercritical conditions demands energy consumption, Cantero et al.[40] has already reported the energetic studies for the continuous microreactor utilized in this study. The assumptions previously reported can be deemed as valid on two conditions (1) the continuous microreactor has the same configuration and (2) lignocelullosics, particularly sugars, are the main products hydrolyzed in the system. They have reported that biomass hydrolysis under supercritical conditions, when integrated with commercial combined heat and power (CHP) schemes, such as that of power generation by gas turbines and steam injection, can be carried out without extra heat requirements. It has been estimated that about 2.2 kg natural gas per kg product and 19.5 $kW{\cdot}kg^{-1}$ of work requirement per kg product is would be needed per 0.39 $w{\cdot}w^{-1}$ sugars concentration.

Table 2: Summary of experimental conditions for ultrafast supercritical hydrolysis

Experimental code	Reaction temperature, ^oC	Pressure, bar	Reaction time, s	Density, $kg{\cdot}m^{-3}$	pK_w	$[H+]/[OH-]$, $mol{\cdot}L^{-1}$
UFC-1	389 ± 4	272 ± 4	0.43	398	13.1	7.7×10^{-8}
UFC-2	386 ± 4	264 ± 4	1.27	411	13.0	9.3×10^{-8}
UFC-3	395 ± 12	265 ± 5	0.25	238	15.1	2.2×10^{-9}

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Table 3: TDS, TSS and TCC yields for the experimental conditions

Experimental code	*TDS, %	*TSS, %	Total carbohydrate content, g·L^{-1} glucose equivalent
UFC-1	19.1	80.9	2.5
UFC-2	25.5	74.5	5.9
UFC-3	22.9	77.1	3.2

*normalized to 100%

3. Characterization of liquid hydrolysis products

The liquid matrix for UFC-3 was extracted with dichloromethane after partial drying and the aqueous fraction was analyzed using HPLC and the organic fraction was analyzed via GC-MS after derivatization. The aqueous fraction, after a two-step acid hydrolysis, was found to contain 30 µg glucans and 33 µg xylans per mL of aqueous fraction, confirming the liquefaction of the cellulosic fractions from cork. On the other hand, a mg of organic fraction was revealed to contain docosanol (0.8 µg) and glycerol (4.3 µg) presumably from hexane extractives; palmitic acid (1.4 µg), vanillin (0.5 µg) and vanillic acid (1.2 µg), attributed to ethanol extractives and stearic acid (0.7 µg) associated with water extractives. This result highlighted the low dielectric constant of SCW, similar to that of non-polar organic compounds, thus allowing dissolution of organic compounds.

4. Characterization of solid hydrolysis products

SM was observed to be pasty, which signals the collapse of the stacked structure of cork during the hydrolysis process due to the dissolution of some portions of the primary cell wall. Upon oven-drying, the SM converted to an agglomerated mass that could be as hard as stone. Hence, lyophilization was performed to facilitate the analysis of the SM. Fig. SF1 shows the images of the original cork biomass in comparison with the UFC-2 and UFC-3 solid hydrolysis products and the carbohydrate-lignin complex after suberin extraction.

In order to confirm whether suberin was kept intact in the SM, FTIR analyses was carried out, as shown in Fig. 3. Overall, the six important peak intensities attributed to suberin; 2921, 2852, 1737, 1242, 1148, 724 cm^{-1} as discussed earlier were preserved, denoting that the main suberin fractions have remained intact. It was also observed that a shoulder at 1737 cm^{-1} was starting to develop. This observation was more prominent for UFC-1 and UFC-2. This can be attributed to the endpoints of suberin linkages, possibly indicating more accessible or exposed suberin chains arising from their dissociation from the cell wall matrix. Further characterization via TGA and DSC were carried out to confirm whether the ultrafast supercritical hydrolysis has not affected the properties of recovered suberin. TGA and DSC are powerful approaches to demonstrate the changes in the properties of materials, by virtue of their thermal behavior. Fig. 4 shows the TGA curves for the original cork biomass, the three hydrolysis solid products and that of the suberin free residue. It can be seen that all

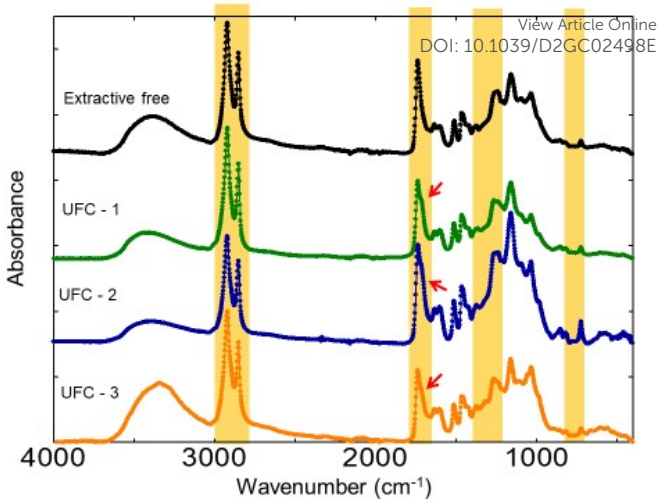

Figure 3. Fourier transform infrared spectra of extractive free cork biomass compared with the solid matrix recovered after ultrafast hydrolysis reaction

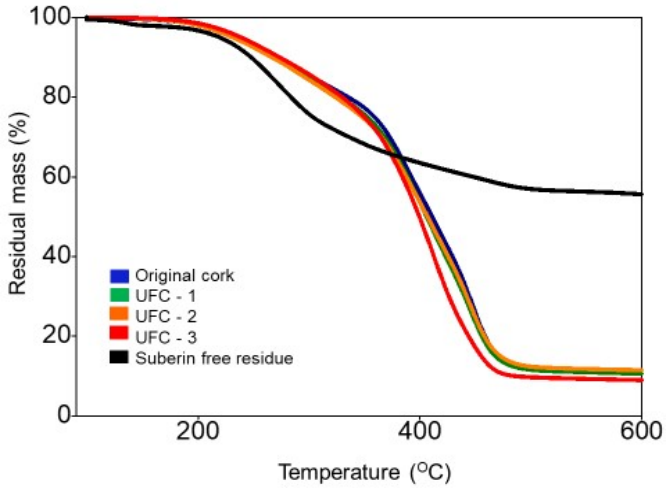

Figure 4. Thermogravimetric analysis curves of supercritical hydrolysis solid products in comparison to original cork biomass and suberin free residue

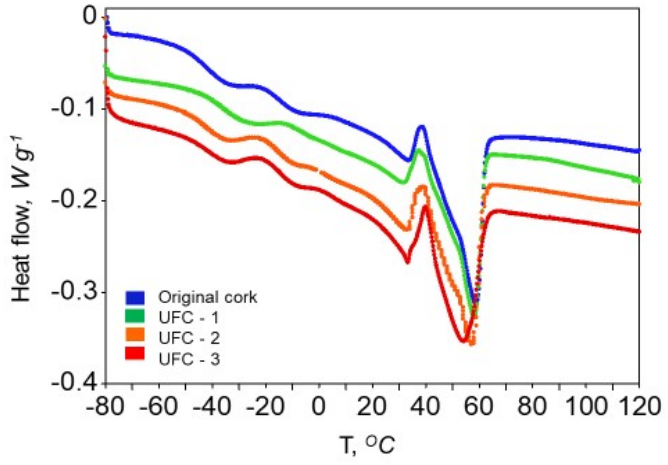

Figure 5. Differential scanning calorimetry thermograms supercritical hydrolysis solid products in comparison to original cork biomass [curves were slightly shifted vertically for clarity]

curves exhibited a single stage weight loss or decomposition. All samples were found to be thermally stable at around 200°C. Table 4 summarizes the degradation and onset temperatures derived from the TGA. The $T_{5\%}$ of the original cork at 233.7°C is close to values reported Ferreira et al., (231.4°C). [17] The suberin free residue had the lowest $T_{5\%}$ largely attributed to its lignocellulosic components, with the carbohydrates easily degrading at temperatures below 200°C. UFC-2 registered slightly lower $T_{5\%}$ than UFC-1 and UFC-3, presumably due to the presence of more volatile compounds arising from liquefaction of cork components. The residue recorded at $T_{600\ C}$ did not change that much, except for the suberin free residue, owing to its high content of lignin and ash. Nonetheless, their values were not significantly different from one another, indicating that the suberin material has been preserved in the solid hydrolysis products and remains to be the major constituent of the recovered SM. The DSC thermograms of the original cork material and the recovered SM are compared in Fig. 5. The second endothermic peak values and melting enthalpies are found in Table 5. In general, all samples elicited one weak and one strong endothermic peaks, approximating at 33°C and 59°C, respectively, indicating that the cork material and SM consists of at least two types of biopolymers. In general, the second melting peaks for SM slightly shifted to a slightly lower end with UFC-3 having 54°C. Meanwhile, melting enthalpies generally increased, with UFC-1 registering 39.4 $J{\cdot}g^{-1}$. These findings indicate that there was change in the composition of the SM, arising from the ultrafast hydrolysis reaction. The lower

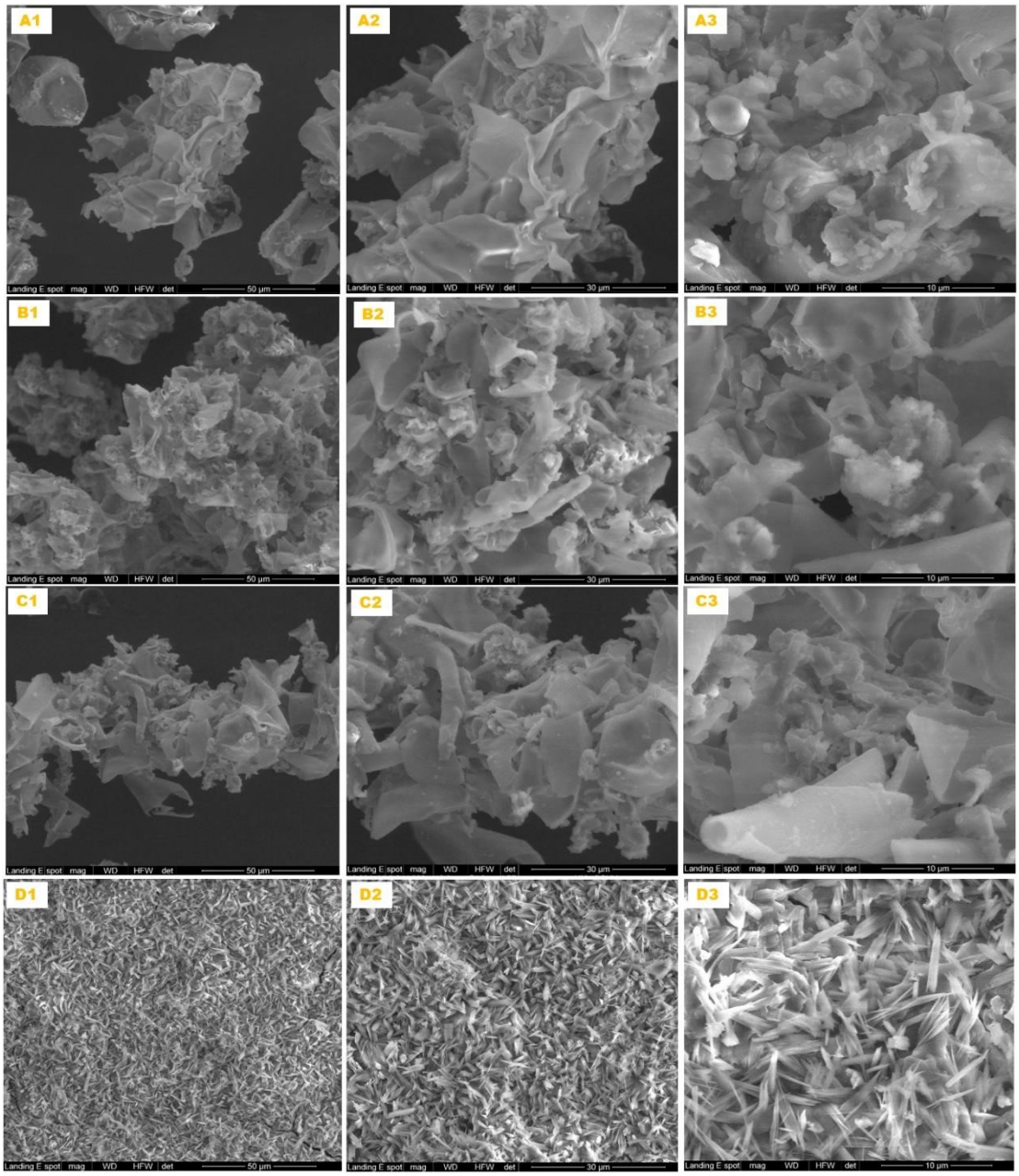

Figure 6. Scanning electron micrographs (SEMs) of the original cork biomass [A series] in comparison with solid hydrolysis products [UFC-2 for B and UFC-3 for C series] and carbohydrate-lignin complex after suberin extraction [D series]. A1, B1, C1 and D1 taken at 2000x magnification, A2, B2, C2 and D2 at 4000x magnification and A3, B3, C3 and D3 at 10,000x magnification.

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heat flow was attributed to a lower cellulose content[41], although ash formation could also trigger higher melting enthalpies.

Scanning electron micrographs (SEMs) of the original cork biomass in comparison with the solid hydrolysis products and the carbohydrate lignin complex after suberin extraction is presented in Fig. 6. The original cork biomass presented the structure with several layers of folds that suggests the stability of the cell walls. The solid hydrolysis products structurally resembled that of the original cork biomass, indicating that the removal of the polysaccharides do not affect the micromorphology, but may affect the fold thickness[42], which is not necessarily apparent in this SEM results. This observation further highlighted that lignin is a major stuctural component of the cork cell wall and that ultrafast supercritical hydrolysis can diffused through the cork biomass to liquefy the polysaccharides without hydrolyzing suberin. Meanwhile, the carbohydrate-lignin complex showed flaky appearance of smaller particles that indicated the absence of suberin that originally interconnected those particles.

5. Further separation of suberin and lignin

While we propose ultrafast supercritical hydrolysis to replace the exhaustive chemical procedures involving alkaline methanolysis, it was still utilized in confirming the effectiveness of the alternative approach, in the absence of a direct protocol that would enable quantitative determination of suberin. Henceforth, similar to the chemical summative analysis, the lyophilized SM were subjected to sequential alkaline methanolysis for suberin analysis and two-step hydrolysis for carbohydrate and lignin analysis. Fig. 7 illustrates the individual compositions of the SM in comparison with the original biomass.

The total suberin is defined as aliphatics + glycerol, total polysaccharides is the sum of cellulose (as glucose) and hemicellulose (as arabinose and xylose) and total lignin refers to the sum of Klason lignin + acid insoluble lignin. It is apparent that polysaccharides decreased and suberin significantly increased in all the conditions tested than the conventional method. UFC-1 and UFC-3 have almost the same level of total polysaccharides remaining, at 2.9 g and 3 g per 100 g of cork, respectively, corresponding to 77.9% and 77.1% reduction, respectively than the conventional method. Meanwhile, UFC-2 only had 0.7 g/ 100 g cork polysaccharides remaining, which means almost 94.7% of total polysaccharides were removed. Polysaccharides are labile biomass fractions, therefore they quickly hydrolyze.[43] High pK_W has favoured their hydrolysis. Table 5 breaks down the individual polysaccharide molecule. Glucose, xylose and arabinose were substantially decreased in all experimental conditions. It was found that xylose and arabinose were easily removed by the ultrafast supercritical treatment, such that for UFC-2, only glucose was left detected. The total suberin for UFC-1 and UFC-3 was also marginally the same, at 60.3 g and 59.8 g/100 g cork respectively, corresponding to a 26% and 25% increase in recovery. This indicates that a lower ion product content can afford the same polysaccharides removal as the high ion product at a shorter reaction time. On the other hand, UFC-2 contains 66.6 g/ 100 g cork total suberin which corresponds to a 39% increase in suberin recovery.

Table 4. TGA and DSC-derived data: Degradation temperature and onset temperature derived from TGA curves and Second endothermic peak and melting enthalpies from DSC

	Original cork	UFC-1	UFC-2	UFC-3	Suberin free residue
$T_{5\%}$,℃	233.7	233.9	230.9	238.4	219.2
$T_{10\%}$, ℃	269.3	270.0	266.2	273.3	247.8
wt $T_{600}{}^{o}C$, %	10.5	10.5	11.3	8.9	55.5
Second endothermic peak, ℃	58.7	58.2	57.0	54.1	-
Melting enthalpies, $J{\cdot}g^{-1}$	33.9	39.4	39.3	38.0	-

Table 5. Residual monosaccharide composition of detected in solid hydrolysis products in comparison with the original cork biomass (g / g oven dry weight of cork).

(per g/g of dry cork)	Glucose	Xylose	Arabinose
Conventional method	308.0	63.0	5.7
UFC - 1	79.6	7.4	0.6
UFC - 2	22.7	0.0	0.0
UFC - 3	90.0	5.0	0.6

Table 6: Indices for the ratio between suberin and lignin ($r_{S/L}$) and suberin and aqueous glycerol ($r_{S/Gly}$)

Experimental code	$r_{S/L}$	$r_{S/Gly}$
Conventional method	2.3	11.9
UFC-1	3.2	13.2
UFC-2	2.8	13.8
UFC-3	3.0	12.8

Meanwhile, lignin was not removed as effectively as polysccharides. This is attributed to the complexity of the lignin structure as well as its tight association with suberin. UFC-1 and UFC-3 had 18.4% and 14% removal of the original lignin initially present in cork. Surprisingly, measured lignin for UFC-2 slighly increased. This occurrence maybe attributed to the formation of pseudo-lignin material bearing polyaromatic phenolic nature arising from the aromatic compounds released by lignin depolymerization.[44] Cunha et al.[45] carried out experiments at 200°C, wherein they were able to remove 29% and 35% of total lignin and polysaccharides present in their cork. Their operating conditions could lead to higher ion product than UFC-1. Thus, It can be inferred that high ion product would be beneficial for the removal of lignin. The observed removal of polysaccharides and lignin can now be related to the FTIR findings as discussed earlier. As the shoulder that started to appear at 1737 cm^{-1} (C=O ester bonds) was more apparent with UFC-1 and UFC-2, it

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can be inferred as indication of more polysaccharides and lignin removal.

Two useful indices can be used to describe the suberin macromolecule, the ratio of suberin to lignin ($r_{S/L}$) and the ratio of suberin to aqueous glycerol ($r_{S/Gly}$). The calculated values are summarized in Table 6. The relative proportion of suberin and lignin in cork, denoted by $r_{S/L}$ is an important indicator of the stiffness and rigidity the cork structure. The $r_{S/L}$ value typically ranges from 1.0 – 3.2. [46] The calculated $r_{S/L}$ for the conventional treatment is 2.2. The $r_{S/L}$ values after ultrafast supercritical treatment were found generally higher than that of the conventional method, further supporting the removal of lignin from SM relative to the increase in suberin recovered. The $r_{S/L}$ value could be an important index which can be used in exploring other methods for the separation of suberin from lignin.

Due to the repolymerization reaction, UFC-2 had the lowest improvement in $r_{S/L}$ value. Meanwhile, $r_{S/Gly}$ indicates the suberin macromolecular structure.[46] The calculated $r_{S/Gly}$ for the conventional method is 11.9. Similar to the $r_{S/L}$, the $r_{S/Gly}$ values also increased after ultrafast supercritical treatment, with values that lie within the typical range of 8.2-14.5.[46] The highest $r_{S/Gly}$ of 13.8 for UFC-2 meant that there is relatively more suberin monomers recovered relative to glycerol. This could mean that some glycerol possibly degraded due to the longer reaction time.

6. Characterization of the Recovered Suberinic acids

Suberin is a major distinguishing factor for the complexity of cork biomass. Hence, characteristics of suberinic acids were also investigated. The chain lengths of the isolated suberinic acids was characterized using gel permeation or size exclusion chromatography (GPC/SEC). Bento et al.[47] have reported the use of SEC to assess the effect of increasing catalyst concentration (sodium methoxide) on the methanolysis process. Meanwhile, about 1 mg of suberin from UFC-2 was subjected to GPC analysis, together with suberin from chemical summative analysis and suberin obtained from alkali hydrolysis. The chromatographs obtained from the analysis are shown in Fig. 8. The chromatographs of suberin via sodium methoxide are represented by (1) - UFC-2 and (2) -conventional method, while (3) represents the suberin obtained from alkali hydrolysis. Chromatographs (1) and (2) consists of about five prominent but overlapping peaks as follows: a broad band of peaking at around 14.2 min, 2nd peak at about 16.29 min, a 3rd around 17.5 min, 4th at the 19.5 min and 5th at 22 min. The trend of the chromatograph follows similarly that of Bento et al.[43] Following their characterization, the broad band is assigned to long chain suberinic fragments with glycerol and the 4th band and 5th band is assigned to the suberinic acids in monomeric units. This again illustrates that the long chain suberinic acids were preserved even after the ultrafast hydrolysis treatment. Meanwhile, chromatogram (3) revealed that alkali hydrolysis led to much smaller units than the alkaline methanolysis products. This confirms that alkali hydrolysis is harsher than the

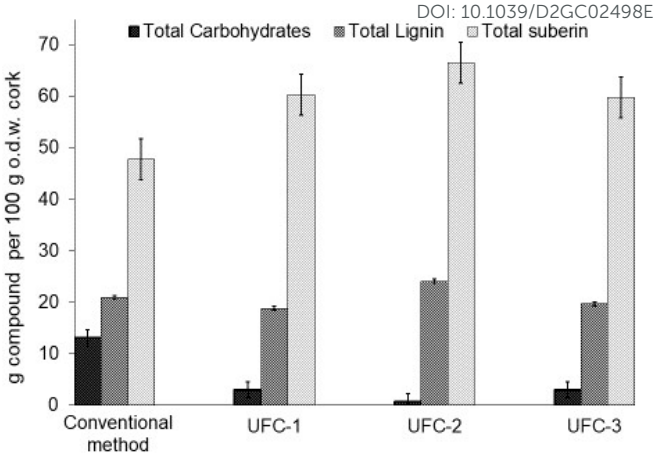

Figure 7. Changes in product distribution of the solid matrix recovered after ultrafast hydrolysis reaction

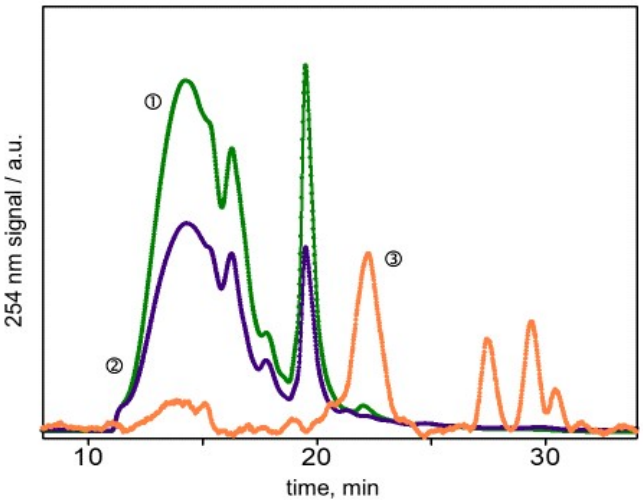

Figure 8. GPC analysis of suberin samples using Phenogel 5µ 100 Å and 1000 Å, corresponding to molecular. 1 – suberin sample obtained from UFC -2, 2 suberin sample obtained from chemical summative analysis (conventional method) and 3 – suberin sample obtained from alkali hydrolysis

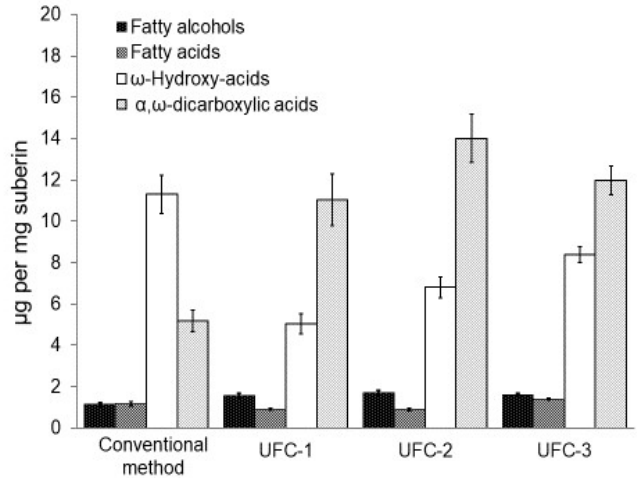

Figure 9. Distribution of suberinic acid families detected in suberin samples from conventional and ultrafast hydrolysis methods

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alkali methanolysis and could lead to the destruction of novel long polymeric chains in suberin.

Suberinic acids can be identified individually using GC-MS. A typical GC-MS chromatograph for suberinic acid monomers are shown in Fig. S3, while Table S4 summarized the identities and quantities of the major suberin acids determined from suberin samples via conventional method and ultrafast supercritical treatment. Only the major peaks are presented herein, following NIST and related literatures[15,18] and does not involve full evaluation of other molecules detected that cannot be assigned. The compounds were detected as their corresponding methyl ester/ trimethylsilyl derivatives. Suberin monomeric families are categorized into four, which are the fatty alcohols, fatty acids, ω-hydroxy-acids and α,ω-dicarboxylic acids. Fig. 9 illustrates the distribution of suberin families via conventional approach in comparison with ultrafast supercritical hydrolysis. All the suberin families are present in the suberin samples. Ultrafast treatment generally resulted to higher suberin monomer yields. The fatty alcohols detected include: eicosan-1-ol (C20) and docosan-1-ol (C22). The fatty acids detected include octadecanoic acid (C18), eicosanoic acid (C20) and docosanoic acid (C22). The major ω-hydroxy-acids detected are 16-hydroxyhexadecanoic acid (C16), 18-hydroxyoctadec-9-enoic acid (C18), 22-hydroxydocosanoic acid (C22), 24-hydroxytetracosanoic acid (C24), 9,10,18-trihydroxyoctadecanoic acid (C18) and 9,10-epoxy-18-hydroxyoctadecanoic acid (C18). Meanwhile, the major α,ω-dicarboxylic acids present in the sample include hexadecanedioic acid (C16), octan-1,8-dioic acid (C8), nonan-1,9-dioic acid (C9), 9,10-dihydroxyoctadecanedioic acid (C18), docosanedioic acid (C22), decan-1,10-dioic acid (C10) and undecanedioic acid (C11). This further supported that the suberin macromolecule was protected from hydrolysis reaction under supercritical conditions.

Overall, UFC-1 had almost the same quantities of suberin families as with the conventional method (18.6 and 18.8 µg per mg suberin), while UFC-2 and UFC-3 have substantially higher compounds detected, both roughly having about 23.4 µg per mg suberin. It was observed that ω-hydroxyacids were higher in the conventional method which almost halved in UFC-1 and UFC-2 and two-thirds for UFC-3. However, α,ω-dicarboxylic acids in UFC-1 and UFC-3 almost doubled and tripled in UFC-2 versus the values obtained for the conventional method. These data suggested that the cleaving and fragmentation of the suberin macromolecule can be affected by the pretreatment. The formation in α,ω-diacids can be a result of the cleavage of α-links on both sides due to the removal of polysaccharides and lignin on either side of the monomer.[7] This trend was also observed by Karnaouri et al.[13] after chemoenzymatic pretereatment of birch outer bark. Aside from the major suberin monomers, glycerol, phenolics and extractives are also detected. Glycerol was found to be smaller in UFC-1 and UFC-2, further corroborating that longer reaction time could result into the occurrence of some parallel degradation reactions. UFC-3 had almost same glycerol quantity as in the conventional method. The major phenolics detected include cis- and trans-ferulic acid and vanillin. All suberin samples from ultrafast supercritical hydrolysis generally led to an increase in phenolics amount than the conventional method. As more lignocellulosics detached from SM, more suberin is obtained and this accompanies higher amount of phenolics. Major extractives include lupenone and betulinic acid.

7. Formation of repolymerization by-products

Under ultrafast conditions, lignin repolymerization could potentially take place.[24] The formation of lignin repolymerization products is an important topic that needs to be discussed since suberin remains in the solid matrix, and the introduction of repolymerization products may induce additional complexities for subsequent processing. Initially, it was assumed that the repolymerization products formation would manifest in the Klason lignin characterization. However, there was no visible irregularity in Klason lignin weight until UFC-2 products were analysed. The formation of solid precipitates in the aqueous fraction collected after solvent partitioning post-alkaline methanolysis (the same aqueous fraction that contains glycerol), was observed (Fig. S5). These precipitates settled out and stabilized after several hours and was not evident in the sample collected from the chemical summative analysis. Herein after, these precipitates were denoted as “char”. In the beginning, char was assumed to be lignin fragments not filtered properly. However, the amount of char formed increased as the reaction duration increased. Hence, char was collected and GPC analysis was carried out in order to investigate its properties. Klason lignin was also included in this analysis as a benchmark and any change in the behavior will be illustrated in the GPC curve. UFC-1, UFC-2 and UFC-3 yielded 7.8%, 11.9% and 7.3% of repolymerization product (% per o.d.w. of solid hydrolysis product), respectively. The GPC chromatograms of the analyzed samples are shown in Fig. 9. First, the curve of the Klason lignin for all solid hydrolysis products follows a single peak, eluting between 8-10 min (peak at about 8.9 min). However, the “char” samples eluting between 6.8 – 10 min possessed bimodal peaks at about 7.2 and 8.3 min, respectively. This means that these “char” precipitates do not possess Klason lignin structure. Since it has a shorter elution time, it indicates that the molecular weight of “char” is lower than that of their Klason lignin counterpart. Moreover, UFC-2 appears to have more lighter fractions than sample from UFC-1 or UFC-3. Since UFC-2 generated the most repolymerization product (11.9%), this indicates that the formation of repolymerization products takes place at longer reaction times. Therefore, there occurs competing reaction between the cleaving of polysaccharide and lignin from cork (and their liquefaction) and the condensation reactions (formation of pseudo-lignin and char). Since more precipitates are formed at longer reaction time, it is therefore not advisable to work under these conditions to avoid repolymerization products, despite having higher suberin yields.

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IV. Summary and Conclusions

Ultrafast supercritical hydrolysis was demonstrated as a promising approach towards the fractionation of cork. SCW having low density and high diffusibility could have effectively penetrated the cork, to liquefy the lignocellulosic components in the primary cell wall. SCW having either low-ion (pKw = 15) or high-ion products (pKw = 13) facilitated the removal of polysaccharides and lignin from cork while hydrolysis of both linear aliphatic ester links and acylglycerol ester links in suberin were avoided, as confirmed by FTIR. TGA and DSC analysis also supported that the presence of preserved suberin in the solid matrix maintains a similar behaviour as in the original cork materials, with minor differences due to the removal of other cork components. Suberin recovery of as high as 66.6 % of dry cork could be obtained at 386°C and 1.27 s (pKw = 13) due to the removal of up to 94.7% polysaccharides content of cork. However, Klason lignin was found to increase, owing to the possible formation of pseudo-lignin materials from resulting from lignin degradation. Moreover, under this conditions, repolymerization products up to 11.9% of the solid hydrolysis products would form. Suberin recovery of 59.8% could be obtained 395°C and 0.25 s (pKw = 15), accompanied by 77.1% polysaccharides and 14% lignin removal. Only 7.3% of repolymerization products has formed. It appears that the ion product concentration does not lead to the hydrolysis of ester linkages in suberin due to the very short reaction time. High ion concentrations at short time would enhance both polysaccharides and lignin removal. Low ion concentrations at short time would result to relatively lower removal of both polysaccharides and lignin. Although, reaction time has more pronounced effect on the liquefaction of other components and the occurrence of repolymerization products. As the reaction time is prolonged, in an attempt to also remove more lignin, pseudo-lignin and repolymerization products start to surface, affecting product purity. The present results have contributed towards the goal of simplifying cork fractionation to recover valuable macromolecules while avoiding a chemical-intensive and laborious isolation procedure.

V. Methods

A. Cork preparation

Cork granules were kindly supplied by Ainia Centro Tecnologico Valencia. The average particle size of the as-received cork granules was determined via screening with a vibratory sieving apparatus for 15 min using sieves with the following mesh sizes (2000 µm, 1000 µm, 500 µm and 250 µm). The cork granules were found to have an average size of 1000 mm. Cork granules were then milled using a Retsch SM 100 cutting mill with an output sieve of 250 µm then further homogenized to 100µm powders using ball mill Retsch PM100 to avoid clogging inside the tubular reactor. The particle size was confirmed via DLS (dynamic light scattering), with a d(0.9) at about 108 µm. The ballmilled particles are denoted as "cork powder".

B. Characterization of Cork

Chemical summative analysis of the cork granules included quantification of ash and moisture content, extractives, suberin, carbohydrates and lignin and was performed in a sequential order according to the Laboratory Analytical Procedure (LAP) from NREL and published literatures. [45,46] This list of chemicals used used are listed in the ESI (S6).

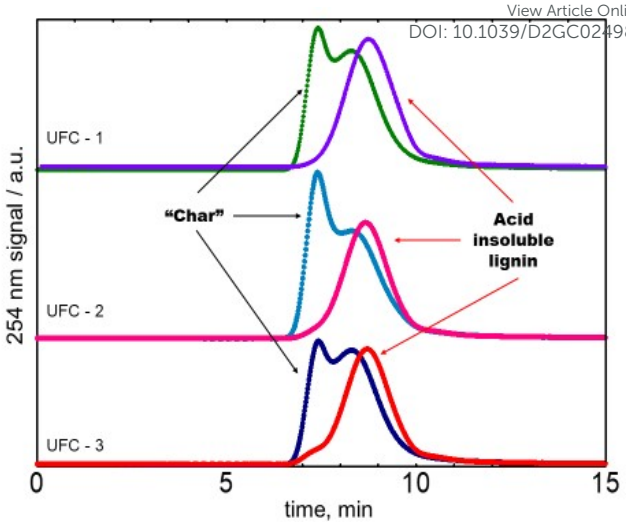

Figure 10. GPC analysis, Jordi Gel Sulphonated Plus 10000 Å 250 x 10 mm column with UV detector, 254 nm. The column is operated 35°C, with a mobile phase solution of water:methanol 90:10 vol, adjusted to pH=12 at a flow rate of 1 ml/min

B.1 Determination of ash and moisture content. The moisture content of the cork granules was determined by measuring the weight difference before and after drying in an oven at 105°C overnight. The weight of the oven dried samples were obtained after cooling down in a dessicator. For ash determination, about 0.3 g samples of milled cork granules were placed in a porcelain crucible and oxidized in a muffle furnace at 550°C for 6 h. Samples were cooled down in an oven at 105°C, then placed in a desiccator to cool down further prior to weighing. Moisture and ash content was reported as fraction of the initial milled cork granule sample

B.2 Determination of the extractives content. Extractives present in cork powder have been removed via successive solvent extraction using 190 mL of n-hexane [8 h], ethanol [10 h] and water [20 h]. The extractions were carried out in a Sohxlet apparatus. Solvents were separated and recovered using a rotary evaporator while the extractives were dried in an oven at 50°C overnight. The mass of the solid residues was obtained and reported as a percentage of the oven dry milled cork. Total extractives of the cork were taken as the sum of the n-hexane, ethanol and water extractives. About 1 mg of the extractives were analysed via GC-MS for qualitative determination of its compounds, refer to C.4 below for the details of the procedure.

B.3 Determination of the suberin content. Two methods for suberin isolation was considered for this study, namely: methanolysis and alkali hydrolysis. The protocol for suberin extraction by alkali methanolysis was adapted from a procedure from Pereira. 1.5 g of extractive-free milled cork was refluxed in a 100 mL solution of 3 %(w/v) of $NaOCH_3$ in CH_3OH for about 3 h. After cooling to room temperature, the mixture was filtered and the solid residue was refluxed again with 100 mL CH_3OH for 15 min. The collected filtrates were combined and acidified with 2 M H_2SO_4 until a pH of 6-7 was reached. The solvent was evaporated using a rotary evaporator. The residue was suspended in 50 mL water and the

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methanolysis products were recovered by extracting with 50 mL of CH_2Cl_2 three times (solvent partitioning). The water fraction was set aside for glycerol determination. The recovered organic fraction combined and dried over anhydrous Na_2SO_4 and the solvent was evaporated using a rotary evaporator. The dried extracts were determined gravimetrically and is referred to as "suberin". Total suberin is calculated as the sum of suberin monomers and glycerol. Alkali hydrolysis was carried out according to Pinto et al.[16] Briefly, 100 mg of extract free cork was reacted with 10 ml of 0.5 M KOH in ethanol/ water solution (with a 9:1 v/v ratio) at 70℃ for 1.5 h. The mixture was cooled to room temp, acidified to pH = 3-3.5, then extracted 3 times with methyl tert-butyl ether (MTBE).

B.4 Determination of aqueous glycerol. The water fraction collected from the solvent partitioning step with CH_2Cl_2 from B.3. contained the aqueous glycerol. The column used for the separation of the compounds was Shodex SH-1011 at 50℃, using sulphuric acid (0.01 N) as the mobile phase with a flow of 0.8 ml min−1. A Waters IR detector 2414 was used and the concentration was determined using a 5-point calibration curve with a glycerol standard.

B.5 Determination of the carbohydrates and lignin content. Suberin-free residue was subjected to a two-step hydrolysis process. Firstly, 3 mL of 72% sulfuric acid was added to 300 mg of suberin-free residue and was incubated at 30℃ for 30 min. Afterwards, 84 mL of deionized water were added and samples were heated at 120℃ for 60 min. The final product was vacuum filtered and a 50 mL liquid aliquot was used to determine the soluble lignin as well as carbohydrates. The remaining solid was collected to analyse the insoluble lignin and ash content. The liquid aliquot was analysed with a UV-Visible spectrophotometer to determine the soluble lignin. The wavelength was set at 205 nm and the used extinction coefficient had a value of 18.675 L $g^{-1}cm^{-1}$.[48] A similar liquid aliquot was neutralized with calcium carbonate to a pH between 5 and 6 and then analysed with HPLC to identify and quantify the structural carbohydrates. The HPLC conditions were the same as section C.4. The solid was dried at 105℃ for 24 h, cooled in a desiccator, and weighed, and denoted as Klason lignin. After that, the sample was placed in a muffle furnace at 550℃ for 24 hr and the remaining residue is denoted as the ash content. The ratio of the mass of suberin to the mass of lignin recovered is calculated as follows:

$$r_{S/L} = \frac{mass\ of\ total\ suberin,g}{mass\ ot\ total\ lignin,g} \qquad \text{Eq. 1}$$

Meanwhile, the ratio of the mass of suberin to the mass of aqueous glycerol recovered is calculated as follows:

$$r_{S/\mathrm{Gly}} = \frac{mass\ of\ suberin\ monomers\ determined\ gravimetrically,g}{mass\ ot\ aqueous\ glycerol,g} \qquad \text{Eq. 2}$$

C. Ultrafast reaction

The cork suspension at an initial concentration of 40 g/L was prepare in water and was maintained under vigorous mixing until and during pumping onto the reactor. Product samples collected consist of two major fractions, the aqueous matrix (AM) and the solid matrix (SM). Separation of the 2 fractions was carried out via centrifugation. The AM was subjected to partial drying and liquid-liquid extraction with CH_2Cl_2. The aqueous phase was subjected to two-step acid hydrolysis and HPLC analysis while the organic phase was submitted to GC-MS analysis. Meanwhile, the SM was freeze dried and subjected to FTIR analysis, elemental analysis, DSC/TGA. The freeze dried fraction was subjected to methanolysis and lignocellulosic fractionation following the methods outlined in section B.3, B.4 and B.5.

C1. FTIR analysis. The FTIR experiments were carried out using a Bruker Tensor 27. Samples were analysed in the wavelength range of 4000 cm^{-1} to 600 cm^{-1} with a resolution of 4cm^{-1}.

C2. DSC/TGA. Differential Scanning Calorimetry (DSC) was used to determine glass transition temperature of lignin in biomass and solid samples. The used device was a Mettler Toledo DSC 3+.

C3. Suberin molecular weight determination. About 1 mg of each sample was dissolved in 0.1% w/v LiBr in DMSO. The column used was Phenogel 5µ 1000 Å with corresponding a molecular weight range 1k – 75kDa, respectively.

C4. GC-MS analysis. Suberin samples between 5-10 mg were derivatized prior to analysis. The samples were dissolved in 250 µl of pyridine, and the hydroxyl and carboxyl groups in the compounds are converted into trimethylsilyl (TMS) ethers and esters with the addition of 250 µl of bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and 50 µl of chloromethylsilane (hexadecane was added as internal standard). The mixture was heated at 70℃ for 30 min in an oven. The derivatized extracts were immediately analyzed by GC–MS by injection in a GC–MS (Agilent 7820 GC and 5977B quadrupole MS and HP-5MS column, 30 m, 0.25 mm x 0.25 µm) operated as follows: 80℃, 4℃·min-1 until 310℃ then held at 310℃ hold for 15 min. [18] Data were acquired using Mass Hunter Work Station Software B.07.00 and compounds were identified based on EI-MS fragmentation patterns using the Wiley-NIST reference library as well as other published literatures[18]. Quantification was carried out using calibration curves constructed for external standards of the major suberin monomer families [1-pentadecanol, R^2 = 0.9999; 16-hydroxyhexadecanoic acid, R^2 = 0.9975; hexadecanedioic acid, R^2=0.9923; cinnamic acid, R^2=0.9997; glycerol, R^2 = 0.9885 and ferulic acid, R^2=0.9975]. Samples were analysed in duplicates and triplicates.

C5. Char analysis [repolymerization products]. The molecular weight distribution of the char samples was determined by gel permeation chromatography (GPC) with a Jordi Gel Sulphonated Plus 10000 Å 250 x 10 mm column and using a Waters IR detector 2414 (210 nm) and a Waters dual λ absorbance detector 2487 (254 nm). The column was operated at 35℃. The mobile phase was a solution water:methanol 90:10 vol, adjusted to pH = 12 at a flow rate of 1 mL/min. Samples were dissolved directly in the eluent with the concentration of about 1-2 mg/mL The injection volume was 25 µL.

C6. Scanning electron microscopy. The morphologic features of the samples were observed by a QUANTA 200 FEG (FEI) scanning electron microscope, operated in low vacuum mode. No pre-processing was carried out. The images were obtained at various magnifications, 2000x, 4000x and 10,000x, respectively.

Author Contributions

Elaine Mission: Main author, Conceptualization, Methodology, Experimentation, Investigation, Validation, Formal analysis, Visualization, Writing- Original draft preparation, and Funding acquisition

Maria Jose Cocero: Correpsonding author, Conceptualization, Resources, Supervision, Writing- Review and Editing and Funding acquisition

Conflicts of interest

The authors do not have any conflicts of interest to declare.

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