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FULL PAPER

Enhancing the antioxidant activity of technical lignins by combining solvent fractionation and ionic liquid treatment

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Abstract: A grass soda technical lignin (PB1000) underwent a process combining solvent-fractionation and treatment with an ionic liquid (IL), and a comprehensive investigation of the structural modifications was performed using HPSEC, ^{31}P NMR, thioacidolysis and GC-MS. Three fractions with distinct reactivity were recovered from successive ethyl acetate (EA), butanone (MEK) and methanol (MeOH) extractions. In parallel, a fraction deprived of EA extractives was obtained. The samples were treated with methyl imidazolium bromide [HMIM]Br using either conventional heating (CH) or microwave irradiation (MW). The treatment allowed to solubilise 28% of the EA insoluble fraction and yielded additional free phenols in all the fractions, as a consequence of depolymerisation and demethylation. The gain of the combined process in terms of antioxidant properties was demonstrated through DPPH$^{\bullet}$ radical scavenging test. Integrating further IL safety-related data and environmental considerations, this study paves the way for the sustainable production of phenolic oligomers competing with commercial antioxidants.

Introduction

The industry is more and more demanding of biobased phenolic compounds, to be used as building blocks for polymer synthesis or valued for their antiradical activity, especially in the field of polymers, materials and cosmetics. Phenolics derived from plant biomass are in particular potential alternatives to synthetic commercial antioxidants like Bisphenol A [1] and *t*-butylated hydroxytoluene (BHT)[2]. Among them ferulic acid is already known for its potential regarding various applications [3] but its low availability from plant sources might hinder industrial developments. As polymer of phenyl propanoids representing up to 30% of the plant biomass, lignins represent one of the major potential sources of biobased phenolics. [4] Moreover, the valorisation of technical lignins generated as industrial by-products would increase the sustainability of lignocellulose-based biorefineries.[5]

Various strategies driven by the green chemistry principles have been designed to convert technical lignins into functional molecules or assemblies while mitigating their variability processing from botanical origin and transformations during industrial treatments.[6] These strategies include fractionation and depolymerisation. Fractionation can be performed by ultrafiltration, potentially directly integrated into the pulping process,[7] or by solvent extraction, which is advantageously performed at ambient temperature and pressure and does not require specific equipment.[8] On the other hand, among the depolymerisation strategies (thermochemical, biological or chemo-catalytic)[9], the recently reported implementation of methyl imidazolium bromide ionic liquid (IL) [HMIM]Br provides a way to produce lignin oligomers with increased free phenol content (PhOH) and decreased polymerisation degree, as a consequence of the selective cleavage of aryl-alkyl ether bonds (Figure 1).[10] In particular, it was shown to generate new phenol groups from the methoxy groups of lignin syringyl and guaiacyl units. Such phenolic oligomers have a great potential as antioxidant additives for the formulation of materials, since they are likely to combine miscibility in the polymer matrix and limited migration towards the environment due to their oligomeric structure.[11,12] Despite this potential, the process has, until now, only been applied to lignin models and the antioxidant properties of the oligomers had not been assessed yet. Advantages of using [HMIM]Br for the transformation of lignins are foreseen to potentially ensure the homogeneity of the reaction medium, to avoid the use of additional chemical reagents and to perform the reaction in mild conditions (T<110°C, time<40min) compared to most lignin catalytic conversion processes. Moreover, in this context of use, this ionic liquid can be recycled. All these advantages also *a priori* render the process attractive from an environmental point of view. However, the sustainability assessment of the process requires further health and safety information on [HMIM]Br which had not been provided so far. The present paper aims at assessing the

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possibility to transfer the IL newly-developed process[10] to technical lignin fractions. The objective was to recover antioxidant extracts soluble in ethyl acetate (EA), one of the conventional solvents recommended for their relatively limited health hazard and environmental footprint as assessed from HSE criteria compiled from various sources.[13] EA was selected here in order to separate extractives from the IL/water layer. Since alkali grass lignin Protobind 1000 (PB1000) from GreenValue LLC is available at industrial scale and is already known for its antioxidant properties,[14] it was selected as starting material for this study. This lignin was subjected to a three-step semi-continuous solvent fractionation process in order to recover three structurally distinct soluble fractions (F1-F3). In parallel, the same lignin sample was submitted to an extensive EA washing to recover a EA insoluble residue (F4) and validate the possibility of recovering soluble compounds through chemical treatment of this fraction. PB1000 and the four fractions were submitted to the previously optimized IL-treatment using microwave-irradiation (MW) or conventional heating (CH) as heating process. The advantage of microwave-irradiation was the short reaction time (10 s), whereas the conventional heating was used to generate more severe modifications relevant to investigate structure-properties relationships. The phenolic monomers and oligomers were extracted from the reaction media with EA and the ethyl acetate extracts (EAE) were analysed by chromatographic and spectroscopic methods in order to assess the efficiency of the conversion, elucidate mechanisms and identify molecules of interest for further developments. The antioxidant properties of PB1000 and the EAE as well as those of the insoluble residues recovered from the most drastic process (CH, 40 min) were assessed through DPPH• radical scavenging test. The half-max-effective concentration EC_{50} was used as criteria to compare the performance of the different samples. The study demonstrates the technical advantage of implementing the [HMIM]Br-based treatment in a cascading approach combining fractionation and depolymerisation. In addition, sustainability considerations of the process are further discussed according to safety data obtained on the IL.

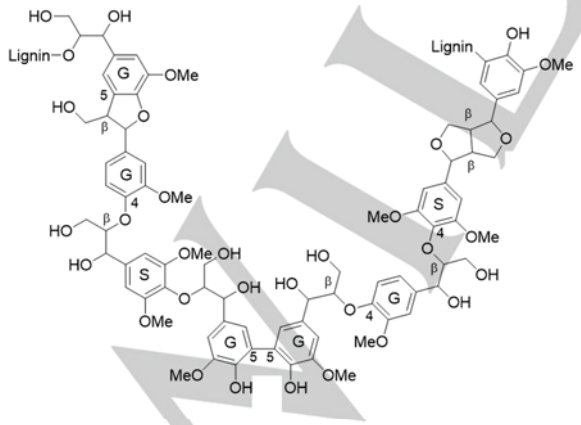

Figure 1. Model for the chemical structure of lignin showing the G and S phenylpropane units linked together by interunit labile aryl-alkyl ether bonds (β-*O*-4) and resistant bonds (5-5, β–5, β– β).[10]

Results and Discussion

1. Recovery of different PB1000 fractions and their contrasted reactivity towards [HMIM]Br-treatments

PB1000 fractionation

PB1000 was fractionated at a semi pilot scale (1 kg dry powder) through a semi-continuous process intended for future industrial development.[8] This process consisted in a 3-step sequential extraction with solvents of increasing polarity (Figure 2) and yielded 31% wt. for the EA-soluble fraction F1, 19% wt. for the MEK-soluble fraction F2, and 23% for the MeOH-soluble fraction F3. Since F2 and F3 contained residual EA-soluble compounds (37 and 7% respectively), PB1000 was also submitted, at lab scale (g), to a drastic extensive washing with EA yielding an EA-insoluble fraction F4 (50% of the weight of the starting PB1000) subsequently used only to validate the depolymerisation process.

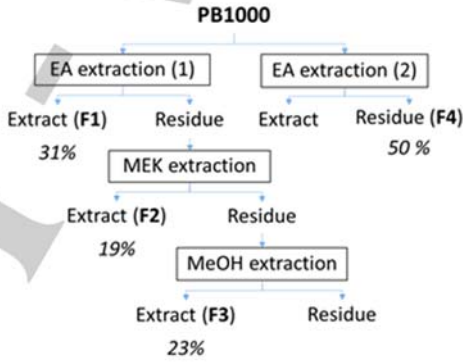

Figure 2. Fractionation scheme of PB1000 by sequential extraction with (EA) ethyl acetate, (MEK) butanone, and (MeOH) methanol, and recovery yields (% wt./PB1000) of the fractions (F1-F4).

One main characteristic of these set of four fractions was the increasing proportion of lignin inter unit aryl-alkyl β-*O*-4 linkages content from F1 to F4, as reflected by the increasing thioacidolysis yields (Table 1).[15] Since our previous study carried out on dioxan-isolated model lignins showed the β-*O*-4 linkages to be the most privileged target of the IL treatment allowing partial depolymerisation,[10] these fractions were thus expected to show increasing reactivity towards the IL-treatment.

Table 1. Thioacidolysis yield of PB1000 and its fractions.

Samples	Thioacidolysis yield (µmol g^{-1})
PB1000	83
F1	32
F2	96
F3	166
F4	197

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In order to determine the influence of structural variations on the efficiency of the depolymerisation process, PB1000 and its four derived fractions were subjected to heating treatments in [HMIM]Br, either under microwave irradiation (MW, 10 s, 110°C) or under conventional heating (CH, 110°C, 20 or 40 min). In line with the objective of the treatment to recover EA-soluble functional extracts, the efficiency of the treatments was assessed on the base of the recovery yields of the EAE after treatment and the characteristics of the extracts, selecting average molar masses and PhOH as major criteria for further applications (Table 2). Moreover, to estimate the solubility gain, the proportion of EAE soluble compounds with respect to the total products recovered was calculated and compared to the EA solubility of the initial sample (Figure 3).

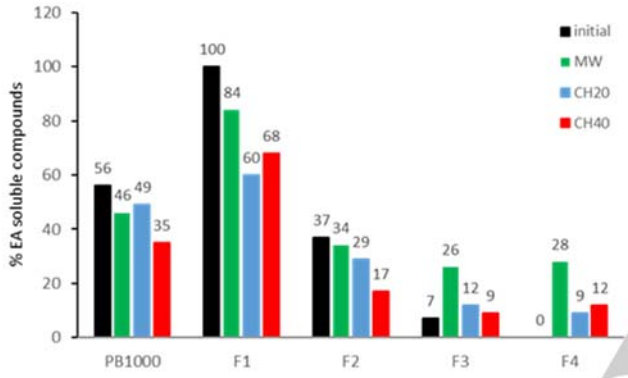

Figure 3. EA soluble compounds proportion (% wt.) of the initial samples (PB1000 and its fractions F1-F4) and the reaction products recovered upon [HMIM]Br treatments with (MW) microwave, (CH20) conventional heating 20 min, and (CH40) conventional heating 40 min. Values after treatments are calculated based on the EA extract (EAE) and EA extraction residues (EAR) recovery yields.

Effect of the [HMIM]Br treatments on the recovery of EAE

As a consequence of the fractionation process, the four fractions exhibited initial contrasted solubility in EA: F1 was 100% EA soluble, whereas F4 was poorly soluble and F2 and F3 only partially (37 and 7%, respectively). The [HMIM]Br treatments induced a decrease of the proportion of EA soluble compounds of all the samples, except for F3 and F4 which exhibited on the contrary an increase. The highest effect of the [HMIM]Br treatment was observed for F4 in the MW conditions. Indeed, 15% of this insoluble fraction could be solubilized and 28% of the products formed was soluble. The EAE represented 8% of PB1000. Three-fold- and twice lower solubility were observed in CH20 and CH40 conditions, respectively.

Table 2. Yields and characteristics of the EAE recovered after treatment by [HMIM]Br ((-MW) microwave irradiation, (-CH20) conventional heating 20 min, (-CH40) conventional heating 40 min) compared to the initial sample PB1000 and its fractions F1-F4.

Samples	EAE yields[a] (%)		PhOH[b] (mmol g^{-1})	Molar mass averages[c] (M_n, M_w, $g.mol^{-1}$) and polydispersity (PD)		
	/starting fraction	/PB1000		M_n	M_w	PD
PB1000						
Initial	56	56	2.68	1015	1260	1.2
MW	36	36	4.55	580	814	1.4
CH20	45	45	3.95	606	843	1.4
CH40	29	29	4.22	802	936	1.2
F1						
Initial	100	31	3.88	896	1089	1.2
MW	72	22	4.43	805	1190	1.5
CH20	59	18	5.45	664	882	1.3
CH40	59	18	6.62	680	876	1.3
F2						
Initial	37	7	2.76	914	1085	1.4
MW	28	5	4.14	978	1407	1.4
CH20	26	5	5.74	700	935	1.3
CH40	16	3	8.27	676	872	1.3
F3						
Initial	7	2	1.18	961	1212	1.3
MW	21	5	2.78	851	1140	1.3
CH20	9	2	7.26	752	945	1.3
CH40	7	2	11.94	709	937	1.3
F4						
Initial	0	0	1.59	1165	1856	1.6
MW	15	8	6.25	865	1144	1.3
CH20	5	2	3.96	867	1153	1.3
CH40	7	3	4.64	971	1282	1.3

[a] EAE recovery yields expressed with respect to the mass of sample treated by [HMIM]Br (/ starting fraction) and to the mass of PB1000 submitted to fractionation before treatment (/ PB1000); [b] determination by ^{31}P NMR after phosphorylation in pyridine/deuterated chloroform of the whole initial samples and of the EAE recovered after IL treatment; [c] HPSEC determination after dissolution-filtration in THF of the EAE recovered before and after IL treatment (values given for PB1000 and F4 before treatment correspond to the whole samples).

In contrast, the treatment of the totally soluble fraction F1 led to the decrease of the solubility with production of insoluble material (16 to 40% depending on the treatment). The maximum solubility decrease was observed with the CH conditions, indicating that these conditions potentially favored recondensation reactions of the EA soluble compounds due to a longer reaction time. In the case of the only partially EA soluble fractions F2 and F3 two

contrasted behavior was observed: F2 behavior similar to F1 with a solubility decrease enhanced in CH conditions, and F3 behavior similar to F4 with solubility increase (up to 19% solubility increase) in MW. The EAE recovered from F3 accounted for 21% of the fraction and 5% of PB1000. The treatment of PB1000 without any previous fractionation led to an intermediate behavior with a solubility decrease observed only in the most severe conditions CH40. In conclusion, the [HMIM]Br treatments could produce EA-soluble compounds from fractions showing poor initial EA-solubility, and the presence of EA-soluble compounds in the initial sample led to an apparent decrease in EA-solubility most probably due to recondensation reactions favored by CH conditions.

Besides initial EA-solubility, the fraction dependent effect of [HMIM]Br treatments on the amount of EAE recovered could be related to the fraction's structural differences in terms of β-*O*-4 bonds content. Indeed, in the case of F1 and F2, exhibiting the lowest amount of β-*O*-4 bonds according to thioacidolysis yields, the proportion of EA soluble compounds in the reaction mixtures after treatment decreased, whereas in the case of F3 and mainly F4, this proportion significantly increased: the results are in agreement with our previously reported statements and suggest that β-*O*-4 linkages are effectively the most privileged targets of the IL treatment. This also demonstrates that the efficiency of the depolymerisation process is able to counterbalance the effect of recondensation reactions, occurring during the treatment and that may be particularly important in the case of long reaction times (CH compared to MW).

In all cases, and whatever the treatment, the yield of the reaction was good but never reached 100% due to losses of compounds, mainly volatiles (not identified nor quantified) during the reaction and soluble and insoluble compounds during the separation process (precipitation of the products and removal of the water/IL layer).

Molar mass distribution of the EAE

Whatever the sample, the weight-average molar mass (M_w) of the EAE, before or after treatment, did not exceed 2000 g mol^{-1}, indicating that the EAE were essentially composed of oligomers (less than 10 phenylpropane units). Nevertheless, some variations in molar distribution appeared between the samples upon treatments. Increasing EAE average molar masses was observed from F1 to F3, in agreement with the use of solvent with increasing polarities for PB1000 fractionation process.[16,17] The effect of the [HMIM]Br treatment on the molar masses depended on the fraction and conditions used. In the case of F3, all the treatments induced a decrease in average molar masses with an increasing effect from MW to CH20 and CH40. For F1 and F2 a decrease in the molar mass was only observed in CH conditions and the use of MW by contrast led to a slight increase. In the case of F4, the EAE produced by the treatments exhibited molar masses 1.4- to 1.6-fold lower than that of the initial F4 insoluble fraction, and of the same range as the non-modified EAE of the other fractions. A slight increase was observed in the CH conditions. This result supported the hypothesis that recondensation reactions were favored by the CH conditions.

Evidences of demethylation and depolymerisation upon [HMIM]Br treatment

GC-MS analysis was performed to investigate the phenolic monomers present in the EAE (Supplementary information S1). It revealed the presence of a mixture of compounds (phenolic acids, ketones and aldehydes accounting in total for 1.2% of PB1000) in F1, the absence of monomeric compounds in F2 and F3 before treatment, and the formation of new compounds for all the fractions after treatment. The chromatograms of the EAE recovered after treatment revealed the presence of phenolic monomers diagnostic of demethylation (all fractions) and/or depolymerisation (F2 and F3). In F1 the treatment led to the total conversion of acetosyringone, the major EAE phenolic monomer before treatment, into its once- and twice-demethylated counterparts (1-(3,4-dihydroxy-5-methoxyphenyl)-ethan-1-one **C** and 1-(3,4,5-trihydroxyphenyl)-ethan1-one **D**) and to the disappearance of all the other phenolic extractives, most probably involved in recondensation reactions. In F2 and F3 the treatment led to the production of two ketones **A** and **B** previously identified as acidolysis ketones from experiments on lignin models.[10] In F2 only the demethylated form **B** was detected whereas in F3 both were formed. The analysis of the thioacidolysis products also indicated that demethylation took place within lignin units linked through β-*0*-4 bonds (Supplementary information S2). Indeed, catechol, 5-hydroxyguaiacyl, and 3,4,5-trihydroxyphenyl thioethylated derivatives were detected after thioacidolysis of all the treated samples. The proportion of these demethylated units was higher in the CH-treated samples, with an increased effect for 40 min. An important feature arising from these results is that demethylation and depolymerisation through the cleavage of β-*O*-4 bonds seem to be concomitant but independent processes. Both the demethylation and depolymerisation reactions diagnosed herein allow the appearance of new phenolic groups initially involved in ether bonds and could thus account for the increase of PhOH evidenced further.

PhOH functionality gained by IL-treatment combined with EA extraction

In order to assess the functionality gained through IL treatment combined with subsequent extraction, the PhOH of the EAE after treatment were compared to the initial PhOH of the samples. As shown by the higher PhOH of F1 (the PB1000 EA soluble fraction) compared to PB1000, EA extraction alone provided a way to recover compounds with higher functionality. However, this functionality was even higher when the IL treatment was applied before recovery of the EAE (3.95-11.94 versus 3.88 mmol g^{-1}), except for F3-MW (2.78 mmol g^{-1}). Moreover, whatever the sample and the conditions, the [HMIM]Br treatment led to an increase of PhOH compared to the starting sample. This effect increased from F1 to F3, for which a maximum 10-fold increase was obtained (Table 2). In the case of F3, the increase in PhOH was concomitant to the decrease in average molar masses, suggesting that the treatment induced the cleavage of ether

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bonds with subsequent release of phenol groups, as previously demonstrated on lignin models.[10]

The PhOH enrichment (ΔPhOH, mmol g^{-1}) through IL treatment and subsequent EA extraction increased with the thioacidolysis yield of the initial fraction, whatever the treatment (Figure 4). This was consistent with the major contribution of depolymerisation reactions through β-*O*-4 bonds cleavage to the formation of new phenol groups. However, PB1000 and its residual F4 fraction did not followed the same tendency as the other three fractions. In particular, their ΔPhOH upon CH treatments were lower than expected from the behavior of these fractions. This suggested that a higher proportion of phenols formed by depolymerisation were present in the EA insoluble residues compared to the other fractions. Moreover, the weak differences in terms of mass distribution, and in fact the unclear correlation between thioacidolysis yield of the starting fraction and the M_w of the obtained EAE, once more indicated that in the case of fractions for which the depolymerisation process could be highly efficient (F3 or F4), recondensation reactions also occur. Interestingly, these recondensations reactions seemed not to impact the global PhOH. This could be also a proof of synergy between both demethylation and depolymerisation processes. In all cases, the efficiency of the treatment conditions in terms of phenols production was increased from MW to CH20 and CH40. As a major feature, the [HMIM]Br treatments was shown to induce an increase of PhOH, that may be the consequence of both depolymerisation and transformation of methoxy groups into phenol ones.

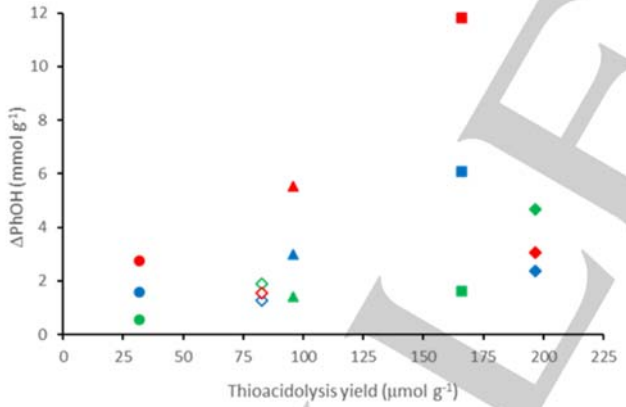

Figure 4. Enrichment in PhOH upon (green) MW-, (blue) CH20- , and (red) CH40- [HMIM]Br treatments (difference between the EAE PhOH content after treatment and the initial PhOH content of the sample) as a function of the thioacidolysis yield of the initial samples (◇) PB1000, and its fractions (•) F1, (▲) F2, (■) F3 and (◆) F4.

In order to assess the effect of the PhOH increase on the antioxidant properties, the EAE showing the highest PhOH (CH40 samples) were selected for the further radical scavenging tests. The corresponding EA insoluble residues were also tested in view of a potential use of all the fractions of PB1000.

2. Interest of the products of fractionation and [HMIM]Br treatment as potential antioxidants

Antioxidant properties (AOP) of the phenolic monomers compared to reference antioxidants

The main phenolic monomers detected in the EAE after treatment, namely acidolysis ketones (compounds **A** and **B**, Figure 5) and acetosyringone demethylated once or twice (compounds **C** and **D**, Figure 5) were tested for their DPPH• radical scavenging capacity according to a test previously used to compare the AOP of lignin models and technical lignins.[14] EC_{50} (concentration of tested sample necessary to reduce 50% of the radicals) was used for this comparison.

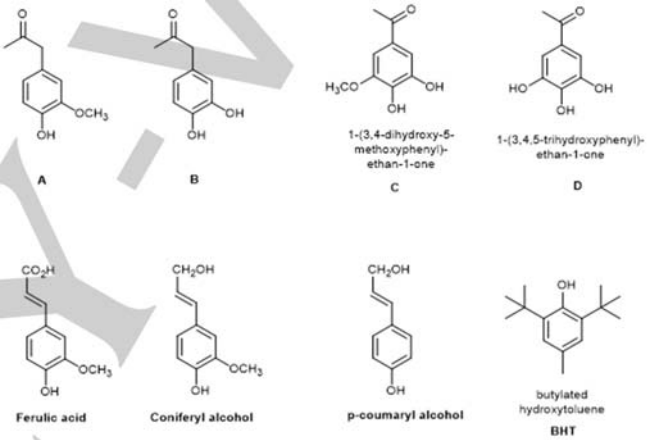

Figure 5. Structures of phenolic compounds compared for their radical scavenging activity: (**A**-**D**) compounds detected by GC-MS in the EAE after IL treatment of PB1000 fractions, (ferulic acid, coniferyl alcohol, *p*-coumaryl alcohol) lignin model compounds referred as monolignols, and (BHT), a commercial antioxidant.

A, **B**, **C** and **D** showed higher AOP (EC_{50} lower than 0.2 g L^{-1}) than the other lignin model compounds tested (ferulic acid, coniferyl alcohol and *p*-coumaryl alcohol) (Figure 6). The lowest EC_{50} was reached with the twice-demethylated acetosyringone (compound **D**) four times lower than that of ferulic acid (0.21 g L^{-1}), which is a reference for natural antioxidants. The radical scavenging capacity of lignins, and phenolics in general, relies on the ability of phenols to trap free radicals in the medium after the loss of a proton[18] followed by the stabilization of this radical by mesomerism. The best performance of compounds **C** and **D** is consistent with the presence of highly electron withdrawing group conjugated to the aromatic ring and to the presence several phenols carried by adjacent carbons.[19]

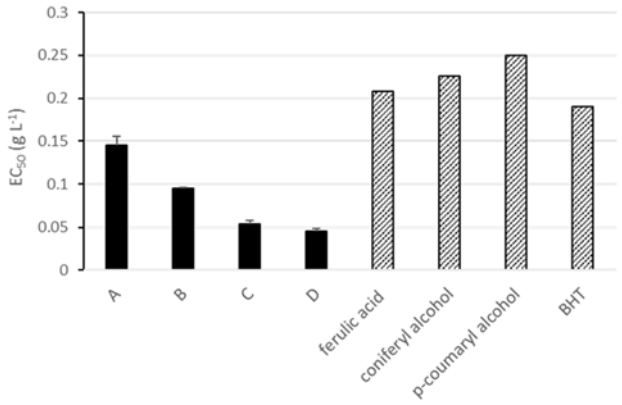

Figure 6. DPPH• radical scavenging capacity of phenolic compounds produced by IL treatment of PB1000 fractions (compounds **A**-**D**) compared to monolignols and a commercial synthetic antioxidant (BHT). error < 1%.

Interestingly, all the compounds tested were competitive with a commercial antioxidant like BHT (EC_{50}=0.19 g L^{-1}).Thus, these compounds might be advantageously purified or synthetised and used as antioxidant for commercial applications. However, in order to avoid costly purification steps and take profit of possible synergies between phenolic compounds, the AOP of the mixtures of products recovered from the treatments (EAE and EA extraction residues) was considered.

AOP of the EAE and EA extraction residues compared to PB1000 and its fractions

All the fractions (F1-F3) and the products recovered from the CH40 IL treatment of these fractions exhibited DPPH• radical scavenging capacity similar or higher than that of the PB1000 reference sample, according to EC_{50} (Table 3). Concerning the untreated fractions, the results are in accordance with previous studies on the fractionation of other technical lignins (organosolv lignin BIOLIGNIN™ and LignoBoost kraft lignins), showing the general higher radical scavenging activity of the EA soluble fraction compared to the other ones. [20] The IL treatment of the fractions led to the production of EAE with enhanced radical scavenging activity compared to PB1000 and the fraction considered. This enhancement could be explained by the increased of PhOH after treatment and the presence of the highly antioxidant phenolic monomers in EAE. In addition, the EA extraction residues (EAR) also exhibited interesting higher activity than their corresponding starting fraction, except for F1. In the case of F3, the AOP of the insoluble residue was even twice better than the EAE, which confirmed that some neo-formed phenol groups were carried by some compounds of higher molar mass insoluble in EA after treatment. According to the EC_{50}, F2 appeared as the most interesting fraction with respect to the production of antioxidant through IL treatment, since the radical scavenging activity of both the EA- soluble and insoluble fraction after treatment were the best.

Table 3. DPPH• radical scavenging capacity (EC_{50}) of PB1000, its fractions (F1-F3) and their reaction products ((EAE) EA extracts and (EAR) EA extraction residues) recovered upon [HMIM]Br IL treatment with conventional heating during 40 min.

Samples	EC_{50} (g L^{-1})		
	Untreated	EAE	EAR
PB1000	0.40 ± 0.01	-	-
F1	0.27 ± 0.03	0.11 ± 0.00	0.42 ± 0.08
F2	0.27 ± 0.01	0.11 ± 0.00	0.15 ± 0.01
F3	0.38 ± 0.03	0.33 ± 0.01	0.17 ± 0.01

Benefit of combining fractionation and IL treatment

Thanks to PB1000 fractionation and the subsequent IL treatment of the fractions a set of functional antioxidant products with distinct characteristics was generated. The mapping of the different samples according to their antioxidant property (EC_{50}), molar mass distribution (Mn, Mw), and phenolic content (PhOH) (Figure 7) highlights that the EAE recovered after IL treatment of the fractions combined several advantages: lower molar masses (Mn ~700 g mol- 1), higher PhOH (6-12 mmol g-1) and higher radical scavenging activity (EC_{50}=0.10-0.17 g L-1).

Moreover, it shows that the molar mass differences between the fractions were leveled by the IL treatment. These characteristics make them good candidates as antioxidant ingredients for the formulation of plastics or cosmetics or for the synthesis of new bio-based polymers and multi-functional molecules. Based on these results, an integrated process could be proposed and its sustainability was discussed.

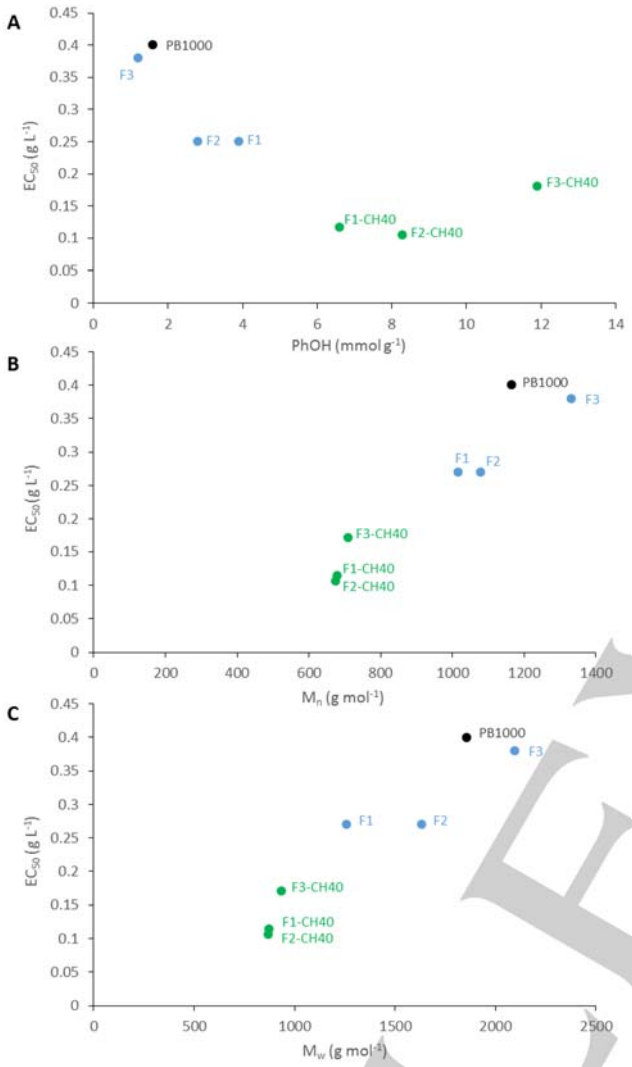

Figure 7. Mapping of PB1000 and its products according to (A) EC_{50} and PhOH content, (B) EC_{50} and M_n, and (C) EC_{50} and M_w: (black) starting PB1000 sample, (blue) PB1000 fractions, (green) ethyl acetate extracts (EAE) recovered after [HMIM]Br treatment with conventional heating during 40 min (CH40).

3. Towards a sustainable cascade integrated process

Taken together, the results showed that IL treatment could be advantageously applied to lignin fractions for different purposes: production of competitive antioxidant extracts, increase of lignin free phenol content, partial dissolution of insoluble residues. In all cases, the demethylation and depolymerisation induced by the treatment is beneficial. To design a cascade approach and preliminary assess its sustainability, it is necessary to consider the technical performances of the products but also the recovery yields and safety aspects.

Recovery yields and technical gain

The process proposed in Figure 8 allows the production of a total of 67.7% of products with enhanced antioxidant activity compared to PB1000, including 35.5% EA-soluble oligomers enriched in PhOH. In order to optimize the gain in AOP and PhOH, the IL treatment conditions selected are the most drastic ones (conventional heating, 40 min), which on contrary do not favor the formation of soluble material. However, since the insoluble products exhibit high antioxidant properties, they could be advantageously incorporated directly as filler in plastics or functionalized by grafting of the PhOH. In the first step of the process, a functional fraction is directly obtained from PB1000 by EA extraction. In the second step, the residue is submitted to a MEK extraction combined with IL treatment of the extract to recover both EA-soluble and –insoluble highly antioxidant products. In the last stage, the MEK extraction residue undergoes a MeOH extraction combined with IL treatment to recover EA soluble oligomers highly enriched in phenol groups together with antioxidant insoluble compounds. Due to its lowest content in lower-molar-mass phenolic extractives, the final residue of the process might find applications for instance as filler in materials.[21]

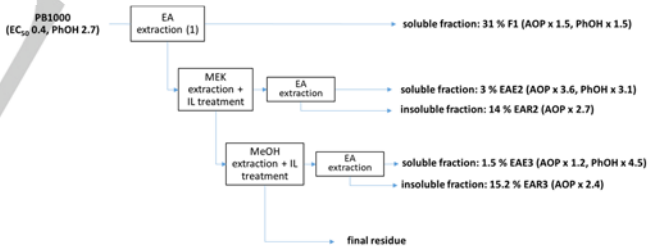

Figure 8. Scheme of a process proposal yielding 35.5% of EA soluble products (F1, EAE2, EA3) and 29.2% EA insoluble products (EAR2, EAR3) showing a gain in terms of antioxidant property ($AOP=1/EC_{50}$) and free PhOH content compared to the starting material.

Safety and environment related aspects and further sustainability considerations

Sustainability assessment of the developed value chain was further examined for physico-chemical hazards and environmental impacts potentially arising from the key chemicals involved. With regard to the fire hazard, the two main critical points of the process in terms of safety are the use of organic solvents (EA, MEK, and MeOH) for the extraction steps and the

use of a new ionic liquid ([HMIM]Br). Of course, the well-established flammability of mentioned solvents has to be handled from supply to process end-use, which leads in this particular context to limited issues according to mild operations conditions. Despite their flammability properties, such materials remain still recommendable as extraction solvents, with regard to HSE criteria taken into consideration with some prioritising rules.[22] In particular, these substances are not mentioned in any list of the EU REACH regulation designating substances of particular concern due to their adverse health or environmental impacts. A summary of results pertaining to physical hazards potentially associated to the use of [HMIMBr] is provided in Table 4.

Moreover, EA and MeOH could potentially be substituted by biobased recommended alternatives, bio ethyl lactate and bio ethanol, respectively (this latter one being nowadays totally bio-sourced). MEK, if not so easily substituted, and although having some toxicity hazard, might at least be produced from biomass in the future, in a cost-effective way as an intermediate to biobutanol.[23]

Table 4. Data summary pertaining to fire and corrosivity to metal hazards.

	EA	MeOH	MEK	[HMIM] Br
Ignitability Heat Release Rate	easy medium	easy moderate	easy large	Very difficult Very low
Fire retardancy	none	none	none	Very significant
Fire induced toxicity	COx emissions in fire	COx emissions in fire	COx emissions in fire	COx, HCN from unexpected fire conditions
Corrosivity to metals (C and stainless steel	nd	nd	nd	Significant for neat IL and IL with 10% added water

Also often considered as green solvents *per se*, ionic liquids remain controversial for safety issues[24] and chiefly require a case-by-case analysis in the context of use, since safety assessments are not based only on intrinsic properties, but essentially on apparatus and testing procedure dependent properties (flash point, thermal stability, metal corrosivity).[25] A preliminary assessment of [HMIM]Br safety profile has been performed and is provided herein (with technical details in Supplementary information S3), integrating both physico-chemical hazard (fire, corrosivity to metals) and eco-toxicological properties of the IL, in line with REACH regulation safety data needed for future registration. This study also provides new insights regarding [HMIM]Br thermal stability and fire behavior, showing in particular a remarkable resistance to ignition and a flame retardancy property. A first order evaluation of the corrosivity potential of the neat IL has indicated that further investigation on the matter will be required for the appropriate selection of the reactor material owing to practical conditions of used of the IL.

Regarding the environmental properties of the IL,[26] the selected test battery includes the tests required by annex VII of the REACH regulation (substance manufactured or imported into European Union between 1 and 10 tons per year) and immunotoxicity tests on the three-spined stickleback (*Gasterosteus aculeatus*). The results (Table 5) showed that [HMIM]Br has a low the toxicity for *D. magna* and *P. subcapitata* (EC_{50}>100 mg L^{-1}). No biodegradation was observed in the manometric respirometry test, leading to conclude that [HMIM]Br is not-rapidly biodegradable. These results are consistent with those already obtained on compounds of the imidazolium family which demonstrate that the toxicity increases, and the biodegradability is enhanced with the length of side alkyl chain. The fish immunotoxicity tests indicated that [HMIM]Br has low toxic effects on the stickleback splenocytes by inducing necrosis, likely due to the bromine anion associated to the imidazolium-based cation of [HMIM]Br. Recyclability of [HMIM]Br was already examined in our previous study. It was concluded that 75% of clean IL can be recovered at the end of the treatment and could be used again for similar reactions for several cycles.[10] All these results bring a contribution to safe-by-design biorefinery involving the studied value chain.

Table 5. Overview of the results obtained for the methyl-imidazolium bromide [HMIM]Br.

Test	Results	Classification for aquatic environmental hazards
Daphnia magna immobilization test (OECD 202)	EC_{50} 48h: 414 mg L^{-1} (352 – 477 mg L^{-1})[a]	Not classified for acute aquatic hazard
Algal growth inhibition test *Pseudokirchneriella subcapitata*, (OECD 201)	NOEC[b] 72h: 62.5 mg L^{-1} EC_{10} 72h: 227 mg L^{-1} (209 – 242 mg L^{-1}) EC_{50} 72h: 346 mg L^{-1} (327 – 361 mg L^{-1})	
Manometric respirometry test (OECD 301F)	No biodegradation observed	Not readily biodegradable. Further investigation is needed to conclude on the classification for the long-term aquatic hazard
Fish innate immune responses (*Gasterosteus aculeatus*)	Stimulate immune responses Cytotoxic effect	-[c]

[a] 95% confidence interval;[b] No Observed Effect Concentration;[c] not relevant

Conclusions

The possibility to transfer the [HMIM]Br treatment to technical lignins was demonstrated, using PB1000 as commercial reference sample. Safety assessment of [HMIM]Br highlighted the effective flame retardant property of this IL and provided data on its eco-toxicological footprint, not departing from other ILs of the imidazolium family useful for future REACH registration. The treatment induced both depolymerisation and demethylation of lignin, leading to the formation of additional free phenols. Based on these results and after checking that similar effects were obtained with other commercial technical lignins including Kraft

lignin (data not shown), an integrated cascade process combining IL treatment and solvent extractions was designed so as to optimize the recovery yield of EA soluble extracts with enhanced performance compared to the technical lignin. The first step directly provides a functional EA extract, while the second and third steps combine extractions with IL treatment to further improve functionalities for applications as antioxidant or building blocks. Indeed, the extracts consisted of free-phenol rich oligomers with antioxidant properties favorably competing with ferulic acid and BHT. Besides their high antiradical activity, these extracts have the advantage to show standardized average molar masses (872-937 g mol^{-1}), low polydispersity (1.2-1.3) and high free phenol content (up to 11.9 mmol g^{-1}), which provides opportunities of green bio-based innovation in plastics and cosmetics formulation.

Experimental Section

General materials and methods

Technical grass (mixed wheat straw/Sarkanda grass) alkali lignin PROTOBIND 1000 (PB1000) was purchased from GreenValue LLC (USA).[27] Ionic liquid [HMIM]Br was synthesised following a previously reported procedure.[28] Ethyl acetate was purchased from Carlo Erba Reagents (France) and used as received. All other reagents, compounds **A** (ref 410659) and **B** (ref 796883), were purchased from Sigma-Aldrich Chemical Co. (USA) and were used as received.

TLC experiments were performed on an aluminium strip coated with Silica Gel 60 F_{254} from Macherey-Nagel, revealed under UV-light (254 nm), then in the presence of a 5% w/w ethanolic solution of phosphomolybdic acid. Evaporations were conducted under reduced pressure at temperatures below 35°C unless otherwise stated. Column chromatography (CC) was carried out with an automated flash chromatography PuriFlash system and pre-packed INTERCHIM PF-30SI-HP (30 µm silica gel) columns. ^{1}H, ^{13}C spectra were recorded in CD_3OD at 400, 100 MHz respectively on a Bruker Ascend 400 MHz instrument. Chemical shifts are reported in parts per million relative to internal references (solvent signal).

Lignin fractionation and EA solubility test

EA extraction (1). PB1000 (1 kg) was fractionated by a three-step sequential solvent extraction process, according to a previously published approach.[8] The following solvents were used sequentially in a semi-continuous process: ethyl acetate (EA); 2-butanone (MEK); methanol. Lignin was loaded in the first solvent in the glass column. After settlement, EA was pumped by the HPLC pump at a flow rate of 2-4 mL.min^{-1} in the column. The solubilized fraction was collected at different heights in the column. The solvent was removed via vacuum evaporation and the final solvent was removed by vacuum drying. The recovered solvent was re-used in the process. When the concentration of solubilized lignin was very low, the second solvent was added by the pump in the column. For each solvent the procedure was repeated till after 3 solvent extractions, the residual lignin fraction was collected from the column. This fraction was dried at maximum temperature of 40°C.

EA extraction (2). PB1000 (6.5 g) was dissolved in 250 mL EA, the mixture was stirred at room temperature during 30 minutes. The resulting solid residue was filtered and the filtrate was recovered. The procedure was repeated 9 times to obtain an exhaustive extraction. The combined EA extracts were concentrated under reduced pressure and the final solvent was removed by vacuum drying. The residual lignin fraction was collected and dried at maximum temperature of 40°C.

EA solubility test. Lignin solubility was determined gravimetrically by dispersing 100 mg of lignin in 10 mL of EA (room temperature, 30 min), then centrifuging the suspension (20°C, 20 min, 4000g) and drying the solid residue at 40 °C for 48 h. Solubility was determined in duplicate, based on the amount of solid residue.

[HMIM]Br treatments

Whatever the treatment, the IL was vacuum-dried at RT before use.

Treatment with microwave irradiation. MW irradiation experiments were conducted in an Anton Paar Monowave 300 instrument. The sample (200 mg) and [HMIM]Br (2 g) were placed in an Anton Paar 30 mL reaction tube equipped with a magnetic stirrer. The mixture was irradiated with P=300 W, T_{max}=110 °C, ramp 30 s, hold 10 s, with full air cooling and stirring. At the end of the reaction, the solid residue was filtered, washed with water (20 mL) and EA (20 mL). The filtrate was recovered, the layers were separated and the aqueous layer was extracted with EA (2x20 mL). The combined EA extracts were dried over $MgSO_4$ and concentrated under reduced pressure bellow 35°C. The crude soluble mixture was analyzed by ^{31}P NMR, HPSEC, and thioacidolysis.

Treatment of lignin upon conventional heating. The sample (200 mg) and [HMIM]Br (2 g) were placed into an Ace pressure tube equipped with a magnetic stirrer under an inert atmosphere, flushed with Ar. The tube was closed and the mixture was stirred in an oil bath at 110 °C. After 20 or 40 min, the solid residue was filtered and the same downstream procedure as for MW irradiation was applied to the solid residue and the filtrate.

Synthetic procedure for compounds C and D

Acetosyringone (500 mg, 2.5 mmol) and [HMIM]Br (2.5 g, 6 equivalents) were placed in an Anton Paar 30 mL reaction tube equipped with a magnetic stirrer. The mixture was irradiated with P=300 W, T_{max}=110 °C, ramp 30 s, hold 10 s, with full air cooling and stirring. At the end of the reaction, water (20 mL) and EA (20 mL) were added. The layers were separated and the aqueous layer was extracted with EA (2x20 mL). The combined EA extracts were dried over $MgSO_4$ and concentrated under reduced pressure bellow 35°C. The crude mixture was purified by flash column chromatography (eluted with 50 to 100% ethyl acetate in cyclohexane) to yield 63 % of product **C** (1-(3,4-dihydroxy-5-methoxyphenyl)-ethan-1-one) and 26 % of product **D** (1-(3,4,5-trihydroxyphenyl)-ethan-1-one). The products were characterized by NMR (Supplementary information S4).

*Compound **C**: R_f* = 0.34 (cyclohexane/ethyl acetate 1:1). ^{1}H NMR (400 MHz, CD_3OD, 25°C): δ_H 2.53 (s, 3H, CH_3), 3.91 (s, 3H, CH_3O), 7.19 (m, 2H, H_2 and H_6). ^{13}C NMR (100 MHz, CD_3OD, 25°C): δ_C 24.9 (CH_3), 55.3 (CH_3O), 103.6, 110.2 (C_2, C_6), 127.9, 139.9, 144.9, 147.9 (4*C_q), 198.3 (CO).

*Compound **D**: R_f* = 0.23 (cyclohexane/ethyl acetate 1:1). ^{1}H NMR (400 MHz, CD_3OD, 25°C): δ_H 2.48 (s, 3H, CH_3), 7.05 (s, 2H, H_2 and H_6). ^{13}C NMR (100 MHz, CD_3OD, 25°C): δ_C 24.8 (CH_3), 107.89 (C_2, C_6), 128.0, 139.1, 145.2 (4*C_q), 198.4 (CO).

Chemical analysis of lignin and lignin-derived products

GC-MS analysis. 20 μL of EA solutions (1 mg mL^{-1}) previously dried on sodium sulfate were silylated with 100 μL of bistrimethylsilyl-trifluoroacetamide (BSTFA) and 10 μL of GC-grade pyridine. The silylation was completed within a few minutes at room temperature. GC-MS analyses were performed in splitless mode on an Agilent 7890A gas chromatograph coupled to an Agilent 5977B mass spectrometer, with a poly(dimethylsiloxane) column (30 m × 0.25 mm; Rxi-5Sil, RESTEK), working in the temperature program mode from 70 to 330 °C at +30°C min^{-1}, during 20 min, with helium as carrier gas. The chromatographic system was combined to a quadrupole mass spectrometer operating with electron impact ionisation (70 eV) and positive mode detection, with a source at 230°C and an interface at 300°C, and with a 50 to 800 m/z scanning range.[29]

Quantitative ^{31}P NMR and samples preparation. Derivatisation of the samples with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane (TMDP, Sigma-Aldrich, France) was performed according to a reported procedure.[30] Lignin samples (20 mg) were dissolved in 400 μL of a mixture of anhydrous pyridine and deuterated chloroform (1.6:1 v/v). Then was added 150 μL of a solution containing cyclohexanol (6 mg mL^{-1}) and chromium(III)acetylacetonate (3.6 mg mL^{-1}), which served as internal standard and relaxation reagent respectively and 75 μL of TMDP. NMR spectra were acquired without proton decoupling in $CDCl_3$ at 162 MHz, on a Bruker Ascend 400 MHz spectrometer. A total of 128 scans were acquired with a delay time of 6 s between two successive pulses. The spectra were processed using Topspin 3.1. All chemical shifts were reported in parts per million relative to the product of phosphorylated cyclohexanol (internal standard), which has been observed to give a doublet at 145.1 ppm. The content in hydroxyl groups (in mmol g^{-1}) was calculated on the basis of the integration of the phosphorylated cyclohexanol signal and by integration of the following spectral regions: aliphatic hydroxyls (150.8–146.4 ppm), condensed phenolic units (145.8-143.8 ppm; 142.2-140.2 ppm), syringyl phenolic hydroxyls (143.8-142.2 ppm), guaiacyl phenolic hydroxyls (140.2–138.2), *p*-hydroxyphenyl phenolic hydroxyls (138.2–137.0 ppm), and carboxylic acids (136.6-133.6 ppm).

Thioacidolysis. Thioacidolysis of lignins (5 mg) was carried out according to literature published protocol,[31] using heneicosane ($C_{21}H_{44}$, Fluka) as internal standard (IS). Lignin-derived p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) thioacidolysis monomers were analyzed as their trimethylsilyl derivatives by a gas chromatography−mass spectrometry (GC-MS) instrument (Saturn 2100, Varian) equipped with a poly(dimethylsiloxane) column (30 m × 0.25 mm; SPB-1, Supelco) and using the following heating program: 40 to 180 °C at 30 °C min^{-1}, then 180 to 260 °C at 2 °C min^{-1}. The mass spectrometer was an ion trap with an ionization energy of 70 eV and positive mode detection. The determination of the thioethylated H, G, and S monomers was performed from ion chromatograms reconstructed at m/z 239, 269, and 299, respectively, as compared to the IS signal measured from the ion chromatogram reconstructed at m/z (57 + 71 + 85). The molar yield of the detected thioethylated monomers was calculated on the basis of the Klason lignin content of the sample, determined according to a published procedure.[32]

Molar Mass distribution. The apparent number-average- (M_n) and the weight-average- (M_w) molar mass of the samples were estimated by high-performance size-exclusion chromatography (HPSEC) using a styrene - divinylbenzene PL-gel column (Polymer Laboratories, 5 μm, 100 Å, 600 mm x 7.5 mm I.D.) with photodiode array detector (Dionex Ultimate 3000 UV/vis detector) set at 280 nm, and using BHT-stabilized THF (1 mL min^{-1}) as eluent. The samples were solubilized in THF and filtered on PTFE membrane (0.45 μm) before injection. The molar mass averages were assessed from the apparent molar masses determined by a calibration curve based on polyethylene oxide standards (Igepal, Aldrich) and lignin model dimers.[33]

Assessment of antioxidant properties

Preparation of the solutions. A lignin sample was weighed in a 2 mL microfuge tube and the solvent (90/10 v/v dioxane/water mixture) was added in order to obtain concentrations between 0.1 and 0.5 mg mL^{-1}. The dispersion was homogenized using a vortex (Heidolph TOP-MIX 94323, Fisher Scientific Bioblock, Vaulx Milieu, France) for 30 s at 20 000 Hz. The resulting solutions were tested for their radical scavenging activity.

Measurement of the free radical scavenging activity by DPPH• test.

The free radical scavenging activity of the samples was evaluated by measuring their reactivity toward the stable free radical DPPH• according to a published method.[14] In a quartz cuvette, 77 μL of the sample dioxane/water solution was added to 3 mL of a 6 × 10^{-5} mol L^{-1} DPPH• solution, prepared daily in absolute ethanol. The absorbance at 515 nm of each sample was monitored using an UV− visible double-beam spectrophotometer (Shigematsu Scientific Instrument, USA), until reaching a plateau. A blank was prepared under the same conditions, using 77 μL of solvent instead of the sample solution. All kinetics were obtained from at least six solutions, prepared from three different lignin preparations. The kinetics of disappearance of DPPH• was obtained by calculating at each time the difference between the absorbance of the blank solution and the absorbance of the sample. When the absorbance reached a plateau, the percentage of residual DPPH• was calculated and plotted vs the concentration of soluble lignin in the sample tested. The concentration of antioxidant extract needed to reduce 50% of the initial DPPH• (noted EC_{50}, with EC standing for efficient concentration) was determined from this linear curve.

Safety assessment of [HMIM]Br

Overall multicriteria safety analysis has been inspired by the global strategy developed by some of the authors of this manuscript for greener use of ionic liquids in general, as exemplified by Eshetu *et al*.[34] in the case of energy storage in electrochemical devices.

Fire hazard: examined by fire calorimetry testing based on the use of the most polyvalent fire calorimeter designated as the Fire Propagation Apparatus, following ISO 12136. See also ESI for further details on procedures and complementary details on achieved experimental data.

Corrosivity screening tests: carbon and stainless-steel specimen, partially immerged in plastic cells containing neat IL and IL added with 10% water, following home-made procedure (exposure in an oven regulated at 100°C during 8 days): mass loss determined before and after exposure with calibrated balance, inspired from procedure developed for IL corrosivity assessment by German ILs producer IO-LI-TEC.

Ecotoxicity tests. Ecotoxicity tests required by annex VII of the REACH regulation (substance manufactured or imported into European Union between 1 and 10 tons per year) have been performed. They are presented briefly in Table 6. In addition to these regulatory tests, fish immunomarker tests were conducted to study possible long-term effects on aquatic ecosystems. Their protocol is detailed in a previous paper.[26]

Table 6. Summary of the ecotoxicity tests performed on [HMIM]Br.

Organisms	Test method	Effect	Endpoints	Expression of results	Test duration
Micro-algae *Pseudokirchneriella subcapitata*	OECD 201, 2011	Chronic	Growth	NOEC EC_{10} EC_{50}	72 hours
Micro-crustaceans *Daphnia magna*	OECD 202, 2004	Acute	Mobility	EC_{50}	48 hours
Activated sludge receiving predominantly domestic sewage	OECD 301F, 1992	Ready Biodegradability	Oxygen consumption	% biodegradation	28 days

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Authors contributions

A. Majira synthetised the model compounds, performed the ^{31}PNMR analyses of the raw and derivated lignin samples and provided technical support for the experiments with [HMIM]Br.

L. Cézard performed the thioacidolysis analysis of the raw and derivated lignin samples.

M. Thierry contributed to the optimisation of the IL-treatment and investigated the effect of the treatment on PB1000, in the framework of her MSc thesis.

F. Pion prepared PB1000 fraction F4 at lab scale and contributed to the design of the integrated cascade process.

A. Bado-Nilles, P. Pandard, T. Jayabalan and G. Marlair performed the safety related analysis of [HMIM]Br and contributed to the selection of process conditions.

P.-H. Ducrot contributed to the design of the IL route for lignin conversion and to the elucidation of reaction mechanisms.

C. Lapierre performed the analysis of lignin extractives and contributed to the identification of lignin demethylation and depolymerisation products by mass spectrometry.

B. Godon, L. Foulon and V. Aguié-Béghin conceived, performed and analysed the measure of antioxidant activity of the raw and derivated lignin samples.

J.C. van der Putten performed the semi-continuous fractionation processes and characterised lignin fraction F1-F3.

R.J.A. Gosselink coordinated the semi-continuous fractionation process implemented on PB1000 and provided the characterised lignin fractions F1-F3.

S. Baumberger coordinated the study within the framework of Zelcor project and designed the cascade process.

B. Cottyn designed the [HMIM]Br process, implemented it to PB1000 and its fractions and coordinated the analysis of the lignin raw material and derivatives.

All the authors participated in the scientific discussions, red and approved the final manuscript.

Keywords: lignins • biorefinery • green chemistry • antioxidant • ionic liquid

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FULL PAPER

Entry for the Table of Contents

FULL PAPER

A cascade biorefinery process is presented. Upgrading of technical lignins and sustainable production of phenolic oligomers of interest as substitutes for commercial antioxidants.

A. Majira, B. Godon, L. Foulon, J.C. van der Putten, L. Cézard, M. Thierry, F. Pion, A. Bado-Nilles, P. Pandard, T. Jayabalan, V. Aguié-Béghin, P.H. Ducrot, C. Lapierre, G. Marlair, R.J.A. Gosselink, S. Baumberger, B. Cottyn**

Page No. – Page No.

Enhancing the antioxidant activity of technical lignins by combining solvent fractionation and ionic liquid treatment

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