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Grafting of aminated oligogalacturonans onto Douglas fir barks. A new route for the enhancement of their lead (II) binding capacities

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ABSTRACT

Chemical modification of Douglas fir bark and its subsequent utilization in adsorption of PbII from aqueous solutions was investigated. A new approach to enhance the natural properties of bark by covalent grafting of oligogalacturonans was developed. The polysaccharidic moiety of barks was functionalized by periodate oxidation and derivatized after reductive amination in presence of aminated oligogalacturonic acid. PbII adsorption isotherms of derivatized barks were then determined and compared with the capabilities of crude barks using the Langmuir adsorption model in terms of affinity (b) and maximum binding capacities (q_{max}). Derivatization resulted in significant enhancements of the q_{max} values (up to $\times 8$), along with little change of the affinity parameter.

1. Introduction

The ability of barks to bind heavy metal ions from polluted waters and the impact of various operating factors on this property have been largely described [1–4]. One of our previous works [5] has demonstrated heavy metal binding capabilities of Douglas fir barks. Not only is biosorption a cost-effective tool for wastewater treatment, but it could also be the starting point of new ways of utilization and/or valorization of forest or agricultural by-products [6]. It has been expected that advantage could be taken from the strong adsorption capacities displayed by barks, a low-cost forest by-product, to remove heavy metal ions from aqueous solutions [7]. Moreover, barks are available in large amounts from sawmills and paper plants.

Heavy metal ion binding capabilities are partly due to carboxylic acid moieties of polysaccharides such as pectin [8,9] as well as phenolic functions present in lignin [10] or condensed tannins [11]. Moreover, many authors assumed that these capabilities could be improved by selective chemical modification [12,13]. Several modifications [14] consist in acid treatment using HCl, alkali treatment using NaOH, Fenton oxidation with H_2O_2/Fe^{2+} or formaldehyde stabilization. The aim of this study was to enhance the metal adsorption capacities of barks by introduction of covalently bound carboxyl groups. Our specific goal was to convert a common forest by-product into valuable biosorbent able of high metal ion loading using an aqueous-based functionalization and derivatization method. Chemically modified barks can be used to selectively remove heavy metals ions from industrial or mine wastewater. Such methodology, originally designed at laboratory scale, was actually industrialized [15]. The main objectives of the present study were first to establish the efficiency of derivatization through reductive amination with aminated oligogalacturonan following periodic oxidation of bark polysaccharidic moieties. In a second part, Langmuir isotherm parameters characterizing adsorption of lead by crude and derivatized barks were deduced from equilibrium binding studies. Lastly we studied the influence of the oligogalacturonan degree of polymerization on the heavy metal adsorption capabilities as well as the steric hindrance of the amine on the percentage of aldehyde function substitution.

2. Experimental

2.1. Materials

Douglas (*Pseudotsuga menziesii*) barks were obtained from a local sawmill in Limousin region (France). They were dried in a ventilated oven (40 °C), ground (particle size <200 μm) and stored in desiccators at room temperature.

2.2. Chemicals

All solvents and reagents (pectin, 1,3-diaminopropane, ammonia, Boc_2O, sodium periodate, sodium cyanoborohydride and

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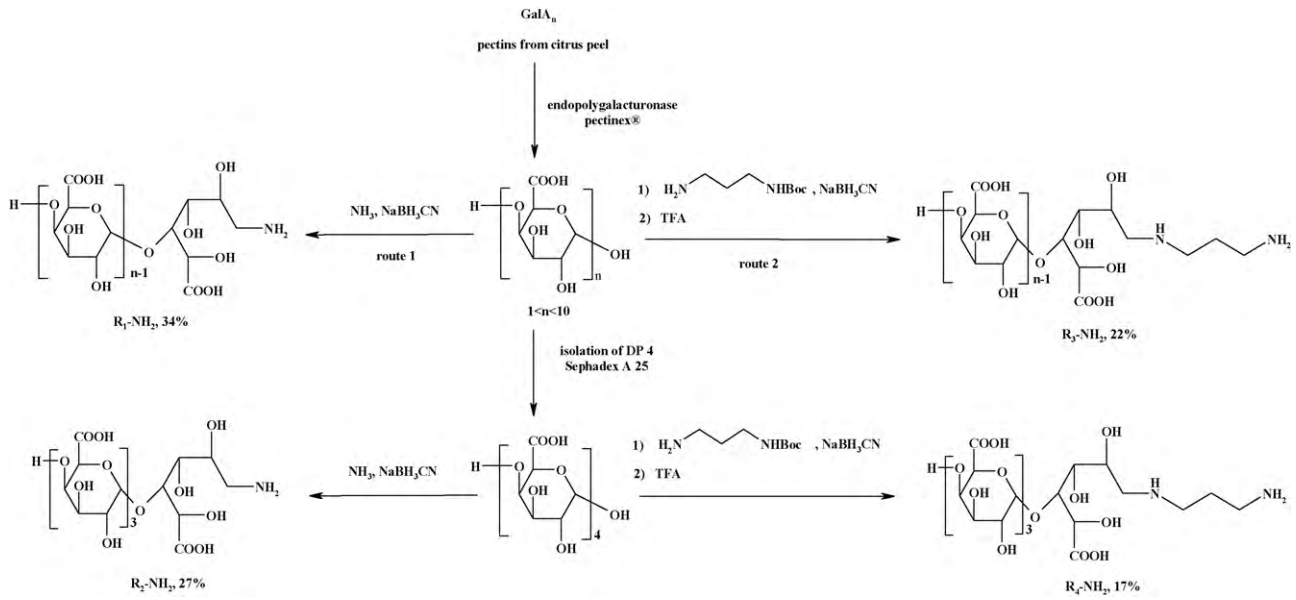

Fig. 1. Hydrolysis and derivatization steps of pectin from citrus peel.

trifluoroacetic acid) were purchased from Aldrich, Acros, or VWR. Milli-Q deionized water (Millipore system) was used throughout all experiments. Chloroform was distilled over CaH_2. Other solvents were used without further purification.

2.3. Chemical modification of Douglas fir barks

2.3.1. Oligogalacturonan isolation

Oligogalacturonans were obtained by enzymatic hydrolysis of commercial pectin from citrus (Sigma). 25 μL of endopolygalacturonase (Pectinex®) was added to 100 mL of a 10 g L^{-1} pectin solution. The mixture was stirred at 55 °C for 35 min. Oligomers of high degree of polymerization were removed by precipitation with 3 volumes of ethanol. Galacturonic acid was removed by size exclusion chromatography (Biogel P2). Oligogalacturonans solubilized in water were then demethylesterified; this solution was alkalinized by addition of 0.1 N aqueous NaOH and pH was maintained at 10 throughout incubation at 4 °C until no pH drop was observed (~72 h); after neutralization with Amberlite IR 120 (H+) resin, this solution was concentrated under vacuum and freeze-dried. The resulting oligogalacturonan fraction was then purified by ion exchange chromatography [16]. A column (Ø 2 cm, 38 cm) of DEAE Sephadex A-25 was equilibrated with a solution of 10 mM NH_4HCO_3 at pH 8.2. Demethylesterified oligogalacturonic acids (250 mg) were slowly loaded onto the column at a flow rate of 20 mL h^{-1}. Oligogalacturonic acids were eluted with 1 L linear gradient from 10 mM to 0.4 M NH_4HCO_3. Oligogalacturonic acid content was monitored by the orcinol reaction [17]. The purified fractions were concentrated, lyophilized and analyzed by mass spectrometry.

2.3.2. tert-Butyl 3-aminopropylcarbamate

According Dardonville et al. [18], a 0.5 M solution of Boc_2O (1 eq.) in $CHCl_3$ was added drop wise over a 2 h period to a 0.25 M solution of 1,3-diaminopropane (5 eq.) in $CHCl_3$ cooled in an ice-bath. The reaction mixture was stirred overnight at room temperature and filtered. The filtrate was concentrated under vacuum and the resulting oil, dissolved in EtOAc (400 mL), was washed with half-saturated brine (3 × 150 mL), dried ($MgSO_4$) and concentrated under vacuum to afford pure mono-Boc-protected 1,3-diaminopropane. Rf = 0.3 ($CHCl_3$/MeOH, 7/3, v/v), ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.39 (s, 9H), 1.57 (quint, 2H, J = 6.7 Hz), 2.74 (t, 2H, J = 6.7 Hz), 2.87 (s, 2H), 3.16 (q, 2H, J = 6.4 Hz), 5.1 (s, 1H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 28.4 (3C), 33.5, 38.4, 39.7; 79.0, 156.2.

2.3.3. Oligogalacturonic acids derivatization

The general synthesis of aminated oligogalacturonans is presented in Fig. 1.

2.3.3.1. Derivatization without spacer. Oligosaccharides (460 mg) were dissolved in 180 mL of water. Ten equivalents of ammonia (0.470 mL) and 2 eq. (96 mg) of sodium cyanoborohydride were added to the solution. The mixture was kept at room temperature under magnetic stirring for 30 min. Amine formation was monitored by TLC (butanol/acetic acid/water) (2/1/1) (v/v/v) and revealed by ninhydrin.

2.3.3.2. Derivatization with spacer. Tetrasaccharides (100 mg) were dissolved in 1 mL of water, then were added 3 mL of a methanolic solution containing 10 eq. of *tert*-butyl 3-aminopropylcarbamate (244 mg) and 16 eq. of $NaBH_3CN$ (136 mg). The mixture was stirred at 80 °C for 1 h. The mixture was evaporated to dryness and the solid was taken up with 10 mL of water. Excess amine was removed by 8 washes with chloroform (8 × 20 mL); the reducing agent was removed by size exclusion chromatography (Biogel P2, eluent:water). Deprotection was carried out by TFA at room temperature. Oligosaccharide mixture was treated the same way.

2.3.4. Bark grafting

2.3.4.1. Periodic oxidation and reductive amination of bark. The first chemical step (Fig. 2) consisted in aldehyde group generation. To this end, the cellulosic and hemicellulosic moieties of Douglas fir bark were oxidized by periodic acid. Douglas fir bark (6 g) was suspended in a 0.157 M $NaIO_4$ aqueous solution. In order to avoid radical-induced depolymerization reactions, the reaction was conducted in the dark in the presence of propanol-1 as radical scavenger with a 9:1 (v/v) $NaIO_4$/propanol-l ratio. The reaction mixture was stirred at room temperature for 7 days and then reaction was stopped by destruction of excess periodate with ethylene glycol. The oxidized product was rinsed with milli-Q water and air-dried at 40 °C. In a second step, 200 mg of dialdehyde bark (DAB) were derivatized by reductive amination with 0.026 M aminated

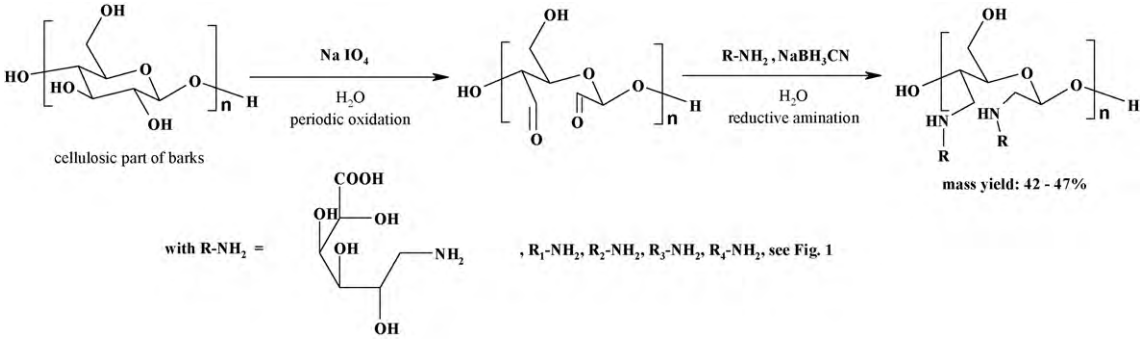

Fig. 2. Functionalization and derivatization steps of the cellulosic and hemicellulosic moieties of Douglas fir bark.

oligosaccharide solutions (DP4 oligogalacturonan, oligogalacturonans mixture or galacturonic acid, with or without spacer arm). $NaBH_3CN$ at a concentration of 0.064 M was used as reducing agent and pH was adjusted to 6.5; the resulting reaction mixture was stirred at room temperature for 5 days. Derivatized barks were rinsed with milli-Q water, air-dried at 40 °C and stored in desiccators.

2.3.4.2. Determination of substitution degrees. Acidity measurement was conducted according to Boehm [19]. 50 mg of crude or modified barks were suspended in 10 mL of 0.1 M $NaHCO_3$ solution and stirred during 72 h under nitrogen. The suspensions were filtered on sintered glass (porosity 3); excess alkali was back titrated by 0.1 N HCl. The substitution degree of bark (Table 1) was then estimated by the following formula:

$$\% = \frac{(n_{COOH}(\text{aminated bark}) - n_{COOH}(\text{NaBH}_3\text{CN bark}))/DP}{n_{CHO}(\text{dialdehyde bark})} \times 100$$

2.4. Chemical characterizations

2.4.1. Mass spectrometry

Mass spectrometry analyses were conducted by the platform "Biopolymers-Interactions-Structural Biology" located at the INRA Center of Nantes (INRA Research Unit 1268) (http://www.angers-nantes.inra.fr/plates_formes_et_plateaux_techniques/plate_forme_bibs). Electrospray ionization (ESI-MS data acquisition were performed on a ESI ion trap (IT) instrument (LCQ Advantage, Thermo Electron, USA). Samples were dissolved in 1:1 MeOH–water at concentrations of 0.1 or 0.05 mg mL^{-1}. Introduction was performed at a flow rate of 2.5 μL min^{-1} in negative or in positive ionization mode. Matrix-assisted laser desorption/ionization (MALDI)-MS data acquisition were performed on a M@LDI LR (Waters, UK) and MALDI Autoflex III Smartbeam (Brüker Daltonics, Deutschland). Samples were dissolved at a concentration of 0.1 mg mL^{-1}. One microliter of sample was spotted on a stainless steel MALDI target plate and one microliter of matrix, consisting of 2,5-dihydroxybenzoic acid (DHB) dissolved at 10 mg mL^{-1} was then added and was allowed to dry.

Table 1
Substitution degrees of barks. GalA: galacturonic acid; DP4: oligogalacturonans with a degree of substitution of 4; oligo: oligogalacturonans with an average degree of polymerization of 3.

Bark	Without spacer		With spacer	
	GalA	Oligo	DP4	Oligo
n_{COOH} (mmol g^{-1})	1.755	2.361	1.963	3.017
DP	1	4	4	2.7
%	4.96	3.13	1.89	7.68

2.4.2. FT-IR spectroscopy

Crude, dialdehyde, and modified barks were characterized by Infrared (IR) spectroscopy with a 1000 FT-IR PerkinElmer Spectrum spectrometer in the 400–4000 cm^{-1} frequency range.

2.4.3. NMR spectroscopy

^1H and ^{13}C Nuclear magnetic resonance (NMR) spectroscopies were performed with a Brücker DPX-400 spectrometer. Chemical shifts are reported as δ in parts per million.

2.5. Biosorption experiments

2.5.1. Study of lead adsorption isotherms

Biosorption experiments were carried out in batch conditions by adding 10 mg of barks to 10 mL of a single metal aqueous solution ($PbNO_3$) at concentrations ranging from 0 to 2000 ppm (19.31 meq. L^{-1}). The initial pH of each metal solution was adjusted to 5 by adding drop wise HNO_3 (0.1 N). Suspensions were shaken at room temperature during 2 h to ensure equilibrium. Adsorbent was finally separated from the solution by vacuum filtration through a sintered glass filter (porosity 3). The maximum lead adsorption capacity q_{max} and the Langmuir constant b were then graphically obtained from the Langmuir adsorption isotherm methodology as previously described [5].

2.5.2. Metal ion analytical determinations

Concentrations of residual PbII in the filtrate were determined by flame atomic absorption spectrometry at 283.6 nm with an Analyst 400 PerkinElmer spectrophotometer, in the concentration range 0–10 ppm (0–96.5 μeq. L^{-1}). The amount of adsorbent-bound lead was calculated from the difference between initial and final concentrations of the metal ion in the solution.

3. Results and discussion

3.1. Chemical modifications of barks

3.1.1. Oligogalacturonans and their derivatization

The general synthesis of aminated oligogalacturonans is presented in Fig. 1. Oligoglacturonans were obtained from commercial pectin after endopolygalacturonase hydrolysis using Pectinex® [20]. After elimination of oligogalacturonic acid with high DP (>10) by ethanol precipitation and removal of galacturonic acid by size exclusion chromatography on Biogel P2, a mixture of oligogalacturonic acids (33% mass yield) with DP ranging from 2 to 10 was obtained. The average DP determined through MALDI mass spectrometry was estimated to 4. Pure oligomers were then purified by anion exchange chromatography on a Q-Sepharose column using NH_4HCO_3 solution as eluent according to Suzuki et al. [16]. Each oligogalacturonan was then unambiguously characterized by the

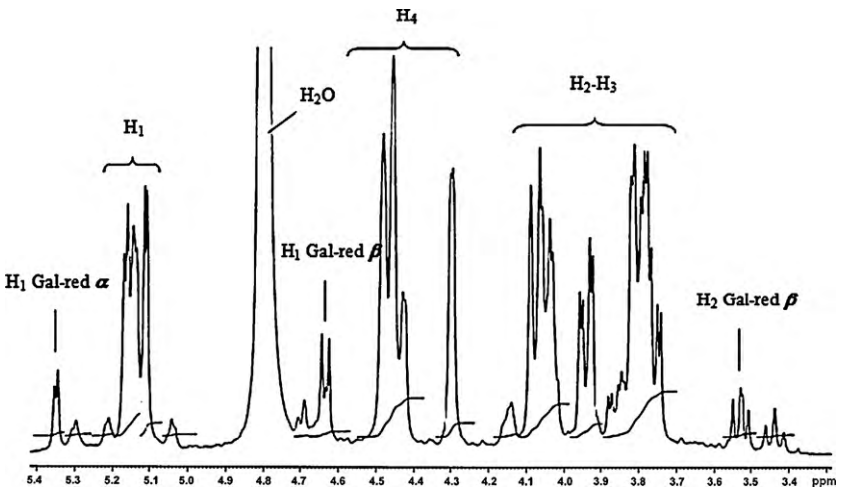

Fig. 3. ^{1}H NMR spectrum of oligogalacturonic acid DP4.

Table 2
^{1}H NMR data (δ in ppm, J in Hz) of DP4 oligogalacturonic acid in D_2O at 25 °C.

	H_1	H_2	H_3[a]	H_4[a]	H_5[a]
Gal-red α	5.35 d, $J_{1,2}$ = 3.5	3.88 dd, $J_{2,1}$ = 3.5, $J_{2,3}$ = 10.5	4.03	4.42	4.80
Gal-red β	4.63 d, $J_{1,2}$ = 7.7	3.55 dd, $J_{2,1}$ = 7.9, $J_{2,3}$ = 9.9	3.79	4.45	4.80
Gal-2	5.17 d, $J_{1,2}$ = 3.6	3.79 dd, $J_{2,1}$ = 3.7, $J_{2,3}$ = 10.7	4.09	4.48	4.82
Gal-3	5.14 d, $J_{1,2}$ = 3.3	3.81 dd, $J_{2,1}$ = 3.7, $J_{2,3}$ = 10.7	4.05	4.45	4.82
Gal-ter	5.11 d, $J_{1,2}$ = 3.7	3.74 dd, $J_{2,1}$ = 3.7, $J_{2,3}$ = 10.7	3.94	4.29	4.80

[a] $J_{3,4}$ and $J_{4,5}$ coupling constants could not been resolved.

typical m/z value that corresponded to its molecular ion peak [M−H]. The predominant DP4 oligomer was fully characterized by the means of NMR spectroscopic analysis. ^{1}H spectrum is given in Fig. 3. The complete assignment of the proton (Table 2) and carbon spectra (Table 3) was achieved by performing 2D COSY and 2D HMBC experiments. All data are in agreement with the literature related to oligogalacturonic acid [21]. In the anomeric region, five doublets were observed. The anomeric protons of non-reducing galacturonic residues exhibited three doublets with a coupling constant of 3 Hz corresponding to α-glycosidic bonds. The reducing galacturonic residue exhibited two doublets at 5.35 and 4.58 ppm corresponding to the α ($J_{1,2}$ = 3 Hz) and β ($J_{1,2}$ = 7 Hz) configurations respectively. Based on the integrated intensities of the H1-α and H1-β signals, α/β-ratio was estimated to 40/60. DP of oligogalacturonic acid was then easily determined by comparison of the integration of anomeric protons and was confirmed to be 4. These results were in agreement with mass spectrometry.

Table 3
^{13}C NMR data (δ in ppm) of DP4 oligogalacturonic acid in D_2O at 25 °C.

	C_1	C_2	C_3	C_4	C_5	C_6
Gal-red α	92.63	68.43	69.01	78.56	70.99	175.58
Gal-red β	96.6	70.85	72.89	77.64	72.89	175.67
Gal-2	99.67[a]	68.59	69.01	78.45	72.04	175.28
Gal-3	99.66[a]	68.59	69.02	78.39	72.04	175.28
Gal-ter	99.66[a]	68.54	69.10	79.14	72.04	175.28

[a] Chemical shifts may be reversed.

Reducing end of DP4 oligogalacturonic acid was then converted into amine prior to reaction with dialdehyde barks. This derivatization method is frequently used for oligosaccharide detection [22,23]. To this end, two strategies were adopted:

- On the one hand, amino group was introduced by reductive amination reaction between purified DP4 oligomer with ammonia in presence of $NaBH_3CN$ (Fig. 1, route 1) as reducing agent in aqueous media. The reactions were monitored by TLC and the amino group at the reducing end was clearly shown after ninhydrine spray. After purification by size exclusion chromatography, structure of modified product was unambiguously characterized by ESI mass spectrometry which showed the characteristic value of m/z = 721.27 that corresponded to the molecular ion peaks $[M-H]^-$ of the expected products and m/z = 704.33 corresponding to the molecular ion peaks $[M-H_2O]^-$ (Fig. 4a).
- On the other hand, incorporation of amino group was performed by reaction with aminated spacer arm (Fig. 1, route 2). The general route adopted for this conversion consisted in condensation of N-Boc-1,3-diaminopropane by reductive-amination with the reducing end of DP4 oligosaccharide followed by deprotection of the terminal N-Boc residue. ESI mass spectrometry of N-Boc protected and unprotected aminopropyl-DP4 oligogalacturonic acids showed the expected signals at m/z = 881.3 ($[M+H]^+$) and m/z = 763.3 ($[(M-H_2O)+H]^+$) respectively (Fig. 4b). Structural modification of pure DP4 oligogalacturonic acid was also observed on ^{1}H NMR spectra. The fixation of N-Boc aminopropyl chain was confirmed ^{1}H NMR analysis by appearance of a signal at 1.47 ppm with an integrated intensity of 9 protons and disappearance of ^{1}H signal of the reducing galacturonic residue. Deprotection of Boc-protected amino group by diluted TFA was then confirmed by the disappearance of the signal at 1.47 ppm and no significant change in the osidic region of spectrum.

Chemical modifications of oligogalacturonan mixture and galacturonic acid were successfully carried out according to the same procedures and led to the formation of the expected products without apparent hydrolysis of oligomers (Fig. 5). Unfortunately, we failed to attach a spacer arm to galacturonic acid probably because of the concomitant C5–C3 lactonization, leading to many by-products and low yields.

3.1.2. Bark grafting

3.1.2.1. Bark derivatization. Functionalization of bark involved the formation of dialdehyde functions in the polysaccharidic moieties

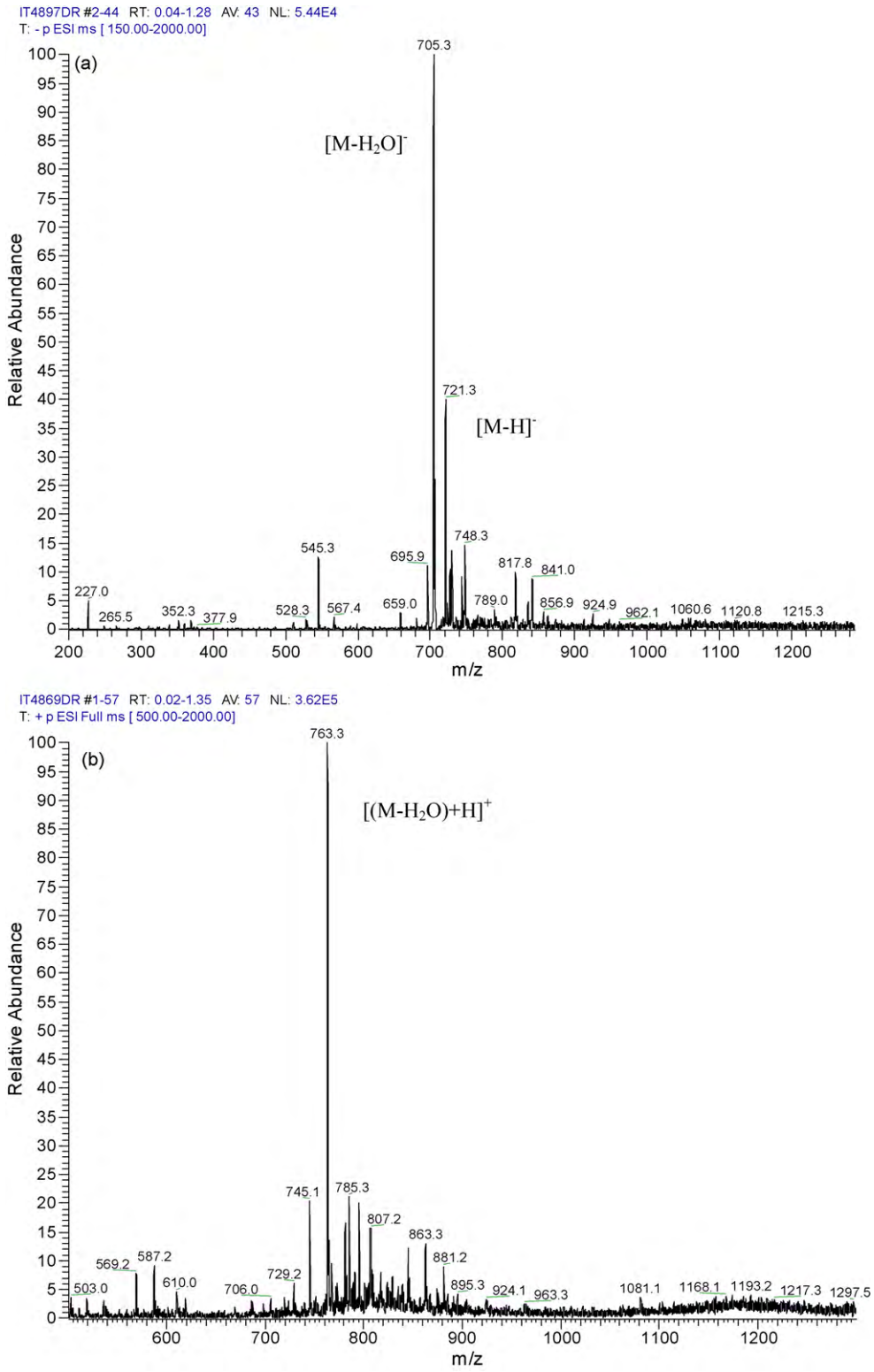

Fig. 4. Negative full ESI-IT-MS spectra of the amino-oligogalacturonic acid DP4 (a) and positive full ESI-IT-MS spectra of the aminopropyl-oligogalacturonic acid DP4 (b).

by periodate ($NaIO_4$) oxidation. The methodology has to produce high aldehyde functional group content and while preventing the degradation of bark. Typical aldehyde concentrations could be estimated through the Canizzaro reaction [24,25]. In our case, it was evaluated to 8.00 mmol g^{-1} for dialdehyde barks, indicating that the operating conditions are well adapted to Douglas fir barks. Periodic oxidation led to the formation of aldehydic functions that constitute grafting sites for aminated oligogalacturonans. The amount of amine that can be grafted is a function of the concentration of the aldehyde groups generated onto the barks surface.

Grafting of each aminated oligogalacturonans was performed by reductive amination according to the procedure described in the experimental section. With values ranging from 1.89 to 7.68% (Table 1), the degrees of substitution are small but con-

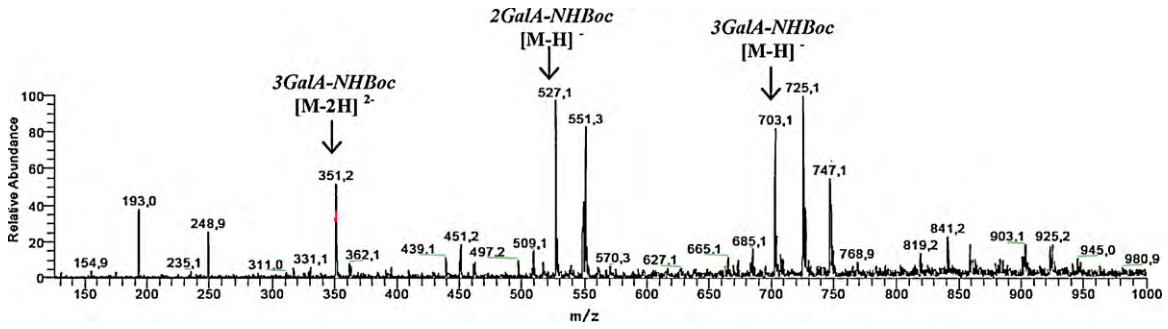

Fig. 5. Negative full ESI-IT-MS spectra of N-Boc aminated oligogalacturonic acid mixture. With: GalA: galacturonic acid, 2GalA: aminopropyl-di-galacturonic acid (DP2), 3GalA: aminopropyl-tri-galacturonic (DP3).

sistent with our previous data [12]. Moreover, Table 1 suggests that steric hindrance of oligogalacturonans limits their grafting onto barks, therefore decreasing their degree of substitution. Oligogalacturonans, more voluminous than GalA, present in that case a lower reactivity towards aldehydic sites which then become less accessible. Lastly, the addition of a spacer arm between the oligogalacturonan amine and the aldehydic grafting site increased the resulting degree of substitution.

3.1.2.2. Bark characterization. FT-IR spectra of crude, dialdehyde, and functionalized barks are presented in Fig. 6. The aldehyde functionalization is clearly identified through the changes of absorption bands at 1735 cm^{-1} (C=O stretching of aldehydes). The derivatization was then confirmed by the decrease of the absorption band at 1735 cm^{-1} and by the apparition of characteristic bands originating from the grafting of the ligands: 1629 cm^{-1} (COO^- stretching) and 1591 cm^{-1} (N–H deformation). The characteristic NH_2 absorption bands at 3300–3500 cm^{-1} did not show up because of the overlap with the large OH band at 3200–3600 cm^{-1}.

3.2. Lead binding capacities of crude and grafted barks

The adsorption of lead onto crude or grafted Douglas fir barks was studied through the Langmuir isotherm methodology. The Langmuir parameters, q_{max} (maximum lead adsorption capacity) and b (Langmuir constant, *e.g.* affinity), graphically deduced from Fig. 7 are presented in Table 4. The results show that adsorption behavior of Pb^{2+} ion on both crude and grafted barks can be satisfactorily described by Langmuir isotherms. Quantitatively, and in

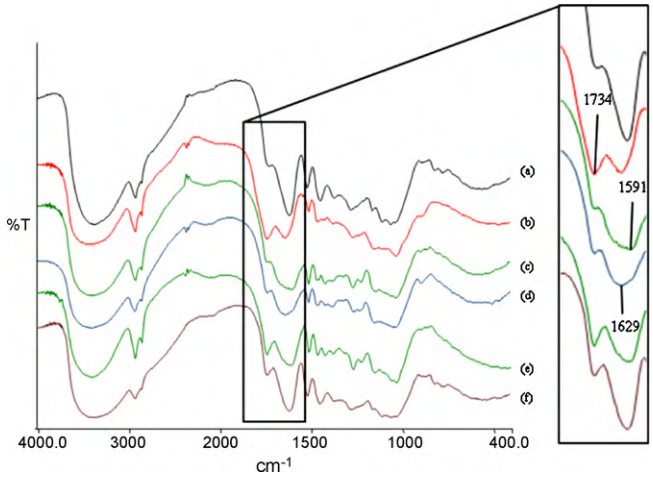

Fig. 6. FT-IR spectra of (a) crude, (b) dialdehyde barks, and barks grafted with (c) oligogalacturonic acid -with spacer, (d) DP4 with spacer, (e) oligogalacturonic acid, and (f) galacturonic acid.

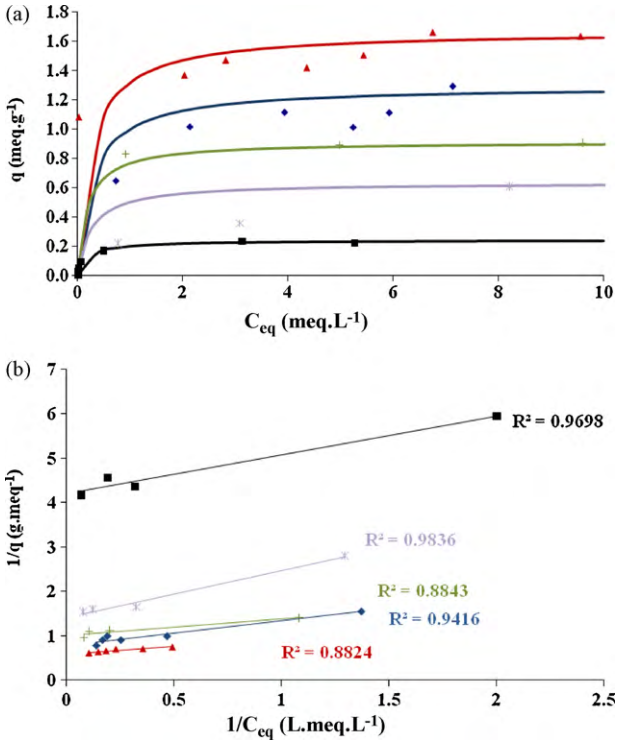

Fig. 7. Lead (II) adsorption on barks: (a) isotherms, (b) Langmuir linearization. (■) Crude bark; modified barks: (✳) galacturonic acid-grafted without spacer, (+) oligogalacturonic acid-grafted without spacer, (♦) DP4-grafted with spacer, (▲) oligogalacturonic acid-grafted with spacer.

the case of lead, the grafting of oligogalacturonans onto barks could increase up to 8 times the value of q_{max}. This property could be justified by the chemical modification of barks that introduces on their surface a larger number of ion exchange sites. The results show therefore that the maximal capacity of adsorption of lead (q_{max}) increases in function of the degree of polymerization of the grafted oligogalacturonans. Such an observation was also correlated with an increase of the carboxylic sites of grafted barks (Table 1). For a

Table 4
Langmuir parameters of crude and modified barks. GalA: galacturonic acid; DP4: oligogalacturonans with a degree of substitution of 4; oligo: oligogalacturonans with an average degree of polymerization of 3.

Bark	Crude	Without spacer		With spacer	
		GalA	Oligo	DP4	Oligo
q_{max} (meq. g^{-1})	0.24	0.63	0.91	1.29	1.78
b (L meq.$^{-1}$)	4.79	3.78	5.25	3.37	3.71

same grafting site used, the mole number of carboxylic functions is higher for oligogalacturonans presenting highest value of degree of polymerization. In this case, and as discussed previously, the degree of substitution of barks is lowered because of a lower reactivity towards the aldehydic functions which become less accessible probably because of oligogalacturonan bulkiness. That is the reason why our study was directed to the grafting of oligogalacturonans with a spacer arm. In the case of oligogalacturonans, such a strategy favored the reductive amination reaction and then, the grafting of barks. The value of the degree of substitution of barks is doubled, this is also the case of the q_{max} value. Not only does amine spacer contribute to increase the substitution degree of bark but oligogalacturonans could adopt typical "egg box" conformation in presence of lead [26] as it naturally occurs in presence of calcium within the cell walls. Lastly, and from a qualitative point of view, it is of interest to observe that the *b* value or Langmuir constant is similar whatever the nature of the grafting. The increase of lead adsorption capacities from crude to modified barks is only explained by the grafting of the carboxylic functions, which means a same kind of ion exchange groups presenting the same affinity for lead. Then, the resulting *b* values remains unchanged or at least, similar.

4. Conclusion

An original method for the enhancement of lead (II) binding capacities of Douglas fir barks is proposed. This is achieved through a chemical modification of their polysaccharidic moiety in aqueous medium. The grafting of oligogalacturonans (R–COO^- ligand) induces an increase of the barks heavy metal binding capacities while the Langmuir constant remains unchanged. The addition of a spacer arm onto oligogalacturonans, favored the reductive amination reaction and then, the grafting of barks. The values of their degree of substitutions, as well as their maximal heavy metal adsorption capacities increase significantly.

Our work demonstrated that barks, a common by-product of the forest industry, could be easily converted, for specific utilizations, into ion exchange resins with high specificity and/or affinity towards defined metals ions.

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