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Comparison of Enzymatic, Alkaline, and UV/H_2O_2 Treatments for Extraction of Beetle-Infested Lodgepole Pine (BILP) and Aspen Bark Polyphenolic Extractives

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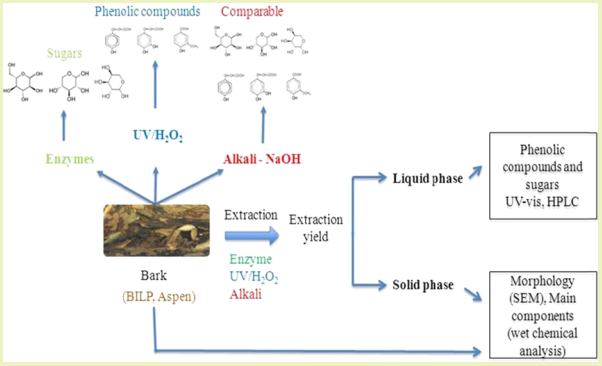
ABSTRACT: This paper describes the comparison of enzymatic, alkaline, and UV/H_2O_2 treatments for the extraction of beetle-infested lodgepole pine (BILP) and mixed aspen barks polyphenolic extractives. The concept of green polymers has become more appealing due to the presence of large volumes of processing residuals from the timber and pulp industries. This, in turn, supports the idea of developing new polymers based on bark extractives. Here, we used a chromatographic method to determine the chemical composition of some of the polyphenolic compounds in bark extractives and observed the effect of different extraction methods on extraction yield. Polyphenolic compounds separation was performed using HPLC in reverse-phase mode with an octadecylsilane (ODS), C18 column (3 μm particle size), and an UV detector. Detection wavelengths of 280, 310, and 370 nm were selected to allow better separation of each compound. The comparative studies and effects of enzymatic, alkaline, and UV/H_2O_2 treatments on extractives yield and component contents were investigated. UV/H_2O_2 treatment exhibited the highest yield with 54% of dry bark weight extracted and was found to degrade larger amounts of lignins/tannins than enzymatic and alkaline treatments. Conversely, enzymatic treatment was good for holocellulose.



KEYWORDS: *BILP, Mixed aspen bark, Polyphenolic extractives, HPLC, UV/H_2O_2, Enzymatic and alkaline treatments*

INTRODUCTION

Today, we are facing an environmental crisis, and its associated socio-ecological burden, mainly due to the extraction and processing of fossil fuels. Under these circumstances, it is urgent to reduce fossil resources consumption. As such, replacing petrochemical products with green biomaterials based on cost-effective renewable resources appears a viable solution. Petrochemical compounds such as phenol and its derivatives are produced in quantities over 10 million tons on an annual basis (essentialchemicalindustry.org/chemicals/phenol.html). Therefore, to alleviate environmental concerns, it is important to find new natural raw materials to produce polyphenolic compounds. One such raw material is woody biomass and bark, which is available in abundance as the byproduct from wood conversion industries.

Bark, similar to wood, is composed of cellulose, hemicellulose, lignin, extractives, and ash. Compared to wood, bark contains higher amounts of phenolic extractives, such as lignan, hydrolyzable and condensed tannins, which have been of great interest to scientists and green technologists during the last two decades. The main idea of this study to produce value-added chemicals that are rich in phenolic structures and thus suitable for wood adhesives,[1] PF resins,[2] polyols,[3] polyurethane foams,[4] and Novolak resins.[5] In addition, the lower toxicity of bark-based phenolics as compared to pure phenol would expedite resin production.[6]

In British Columbia, lodgepole pine (*Pinus contorta var. latifolia*) accounts for 24% of the total forest growing stock.[7] Large numbers of mature lodgepole pines have been damaged by the attack of the mountain pine beetle (*Dendroctonus ponderosae*) and its fungal associates. The scale of the mountain pine beetle attack in British Columbia is unprecedented. In 2010, the beetle attack had affected over 18 million hectares of forest and had killed 710 million m^3 of lodgepole pine.[8]

Aspen (*Populus tremuloides*) is considered as a source species and is important for maintaining biodiversity in the western and boreal regions in North America.[9] It is one of the main timber sources in North America and in some Scandinavian and Baltic nations.[10] Aspen accounts for 39.5% of the growing stock volume of the forested land base in these countries, which is almost equal to the 42.1% of the stock that is attributable to all coniferous species.[11] In order to better understand the bark extraction process and to provide better direction for the application of bark extractives, it is important to explore and compare the effect of

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fundamentally different treatments such as alkaline, enzymatic, and UV/H_2O_2 on bark.

Because of the versatile chemical composition of bark, researchers have expended tremendous efforts in developing extraction procedures that allow the isolation of specific phenolic compounds. Among these procedures, extraction using organic solvents such as methanol and ethanol has emerged as one of the most promising techniques for extracting phenolic compounds, especially condensed tannins, with high purity and intact structural properties.[12,13]

However, bark extraction with organic solvents has severe limitations, with notably low extractives yield, generally ranging from 10% to 20% in bark weight.[14] In this sense, the use of alkaline solvents, such as 1% NaOH, significantly increases the extractives yield and releases large amounts of phenolic compounds.[15,16]

Nevertheless, extraction with alkaline solvents is a complex process, which involves, as with polar solvents, the swelling and dissolution of polymers into cell walls, and also hydrolysis reactions. Hence, the extractives obtained using alkaline solvents not only contains phenolic compounds but also fractions of aliphatic acids and hemicelluloses.[17] The low selectivity of alkaline extraction, combined with the large diversity in composition of tree barks, requires a systematic and thorough investigation of extraction process effects on each species of tree bark.

Enzymatic treatment is environmentally friendly. Tree bark mainly contains cellulose, hemicellulose, and lignin. Because of the very selective nature of enzymes, to proceed to extraction, it is necessary to use a cocktail of enzymes that include exoglucanase, endogluconase, β-glucosidase, and ligninases. These enzymes are already widely used for process integration to reduce the number of process steps and increase yield.[18] Using a combination of cellulases, hemicellulases and ligninases initiates hydrolytic degradation of the plant cell wall, which retains phenolics in the polysaccharide−lignin network by hydrogen or hydrophobic bonding. Direct enzyme catalysis is another approach to cleave ether and ester linkages between phenol and plant cell wall polymers, as explained by Pinelo et al.[19]

The mechanism of photocatalysis by UV/TiO_2 has been described by several authors[20−24] who have studied the degradation of various wastes, in particular lignin. As of the multifunctionality of lignin compared to phenol, wavelength ranges of lower energy are sufficient to initiate the degradation.[23] The main reactive species produced in the photocatalytic process by UV/H_2O_2 are hydroxyl and superoxide radicals,[25] which are proposed to allow the degradation of bark components.

The aim of the work was to determine and compare the effectiveness of alkaline, a cocktail of enzymes, and UV/H_2O_2 treatment of BILP and mixed aspen barks to improve the extraction efficiency and polyphenolic extractive yields. The raw materials considered for this study were beetle-infested lodgepole pine (BILP) bark and mixed aspen bark; both species have a worldwide distribution but are most abundant in North America. In addition, we sought to select the most appropriate wavelengths for each phenolic compound in bark polyphenolic extractives. Biotechnological processes enable the discerning separation of main fractions (cellulose, hemicellulose, and lignin) from bark constituents.

EXPERIMENTAL SECTION

Chemicals and Standards. Sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), and acetic acid (CH_3COOH) were purchased from Caledon Laboratory Chemicals. Catechol, tannin, Folin−Denis reagent, and sodium chlorite ($NaClO_2$) were purchased from Sigma-Aldrich. Cellulose extraction thimbles and grade 42 filters were purchased from Whatman. Mountain pine beetle-infested lodgepole pine and mixed aspen barks were provided by FP Innovations and ground to particle size below 0.212 mm (US70 mesh size). All the chemicals used in this study were reagent grade and were used without further purification.

Chemical Composition of Bark before and after Extraction. *Ash Contents in Bark.* Ash contents in the bark were determined by oxidizing dry bark at 580 °C with a TGA instrument. About 10 mg of oven-dried sample was weighed in a platinum pan and heated from room temperature to 580 °C, at a heating rate of 10 °C/min using a thermal gravimetric analyzer (TGA-Q500, TA Instruments, U.S.A.). The final ignition temperature was 575 °C according to the ASTM D1102-84 method-suggested temperature for the determination of ash contents in wood. Bark ash contents were reported as the percentage of remaining residues after treatment. Five samples of each bark were tested to generate standard deviation.

Preparation of Extractive-Free Bark. Extractive-free bark was prepared according to ASTM D1105-96. Ethanol−toluene extractives and hot water extractives contents in bark were then reported. The ethanol−toluene extractive values listed in this paper refer to the bark extraction with successive ethanol−toluene mixture and 95% ethanol.

Lignin Content of Bark. The lignin contents of BILP and aspen bark was determined according to a modified procedure for the evaluation of acid-insoluble lignin content in wood and pulp.[26] Briefly, 0.2 g of oven-dried extractive-free bark was combined with 2 mL of 72% sulfuric acid and incubated at 30 °C for 1 h. Fifty mL of distilled water was then added, and the mixture was autoclaved at 120 °C. The solution was then filtered into glass crucibles, grade 40−60, and lignin content was determined using eq 1.

$$\text{Lignin (\%)} = \left(\frac{m_{be}}{m_b}\right) \times \left(\frac{m_{ic} - m_c}{m_{be}}\right) \quad (1)$$

where m_{be} is the oven-dry weight of extractive-free bark, m_b is the oven-dry weight of bark, m_{lc} is the oven-dry weight of crucible and of lignin, and m_c the oven-dry weight of crucible.

Holocellulose and α-Cellulose Content of Bark. Holocellulose and α-cellulose contents in extracted bark were determined following a procedure developed by Browning et al.[27] A total of 0.5 g of oven-dried extractive-free bark was weighed, and 16 mL of buffer solution was added. The mixture was placed in a water bath at 70 °C and, 1 mL of 27% (w/v) $NaClO_2$ was added every 30 min for the following 4 h. The filtered holocellulose was separated in two sets of experiments. The first set was oven-dried at 65 °C and weighed, while the second set of filtered holocellulose was kept in the desiccator for 24 h and then transferred into beakers for the α-cellulose determination. Three mL of 17.5% NaOH solution was added to the prepared holocellulose and incubated at 20 °C, followed by another 6 mL after 5 min. The total treatment lasted 45 min. Then distilled water was added, and the mixture was allowed to stand for 1 h. Once the caustic treatment was completed, the solution was filtered under vacuum into 40−60 grade crucibles and washed with 30 mL of distilled water. Crucibles and samples were then oven-dried overnight at 65 °C. Holocellulose and α-cellulose contents were determined according to eqs 2 and 3.

$$\text{Holocellulose (\%)} = \left(\frac{m_{be}}{m_b}\right) \times \left(\frac{(m_{hc} - m_c)}{m_{be}}\right) \quad (2)$$

$$\alpha\text{-cellulose (\%)} = \left(\frac{m_{be}}{m_b}\right) \times \left(\frac{(m_{\alpha c} - m_c)}{m_{be}}\right) \quad (3)$$

where m_{be} is the oven-dry weight of extractive-free bark, m_b is the oven-dry weight of bark, m_{hc} is the oven-dry weight of holocellulose and crucibles, $m_{\alpha c}$ is the oven-dry weight of α-cellulose and of crucible, and m_c is the oven-dry weight of the crucible.

Enzymatic Treatment. Enzymatic hydrolysis was performed using a cocktail of three enzymes, cellulase (E.C. 3.2.1.4) from *T. reesei* (6.3 U/mg), β-glucosidase (E.C. 3.2.1.21) from almonds (7.55U/mg) (both

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enzymes were purchased from Biochemika-Fluka), and Novozym51003 laccase (E.C.1.10.3.2) from genetically modified *Aspergillus sp.* (1000 LAMU/g = 3.57 IU/mL/min) (obtained from Novozymes, Franklinton, NC, U.S.A.). Enzyme reactions were performed at solid:liquor ratio of 1:10 by inserting ground bark (BILP and aspen bark) in 0.2 M sodium acetate/acetic acid buffer (pH 5.6), containing 10 Ug^{1-} of each enzyme at 45 °C for 48 h. The enzymatic-treated flasks were agitated in a rotary shaker at 150 rpm. After hydrolysis, 600 μL of extractives were removed from the treated barks, passed through a 0.22 μm filter, and stored for subsequent analysis of phenolic compounds.

Alkaline Treatment. Bark powders were extracted using 1% NaOH solution with a solid:liquor ratio of 1:10 at 100 °C for 120 min. Bark soluble fractions were filtered, and the liquid fractions were collected and stored at −10 °C. Bark residues were washed with excessive hot water and oven-dried at 65 °C to constant weight. The extraction yield was calculated based on eq 4.

$$\text{Yield } (\%) = \frac{m_b - (m_{bef} - m_f)}{(m_b)} \quad (4)$$

where m_b is the oven-dry bark weight, m_{bef} is the weight of the oven-dried bark after extraction, and m_f is the oven-dry filter weight.

UV/H_2O_2 Treatment. Beetle-infested lodgepole pine (BILP) and aspen bark samples were treated with UV/H_2O_2 in 300 mL Erlenmeyer flasks under room temperature conditions. The ground barks were combined with 1% NaOH at a solid:liquor ratio of 1:10 and then exposed with conventional UV lamps (Philips, 40Watt) for 3 h with 100 mM of H_2O_2.[28] H_2O_2 concentration of 100 mM was adjusted due to acting as a free-radical eliminator at higher concentrations, which decreases the amount of hydroxyl radicals in the solution.

Sample Preparation for HPLC. After three consecutive treatments, the total phenolic contents from bark extract were assessed by dissolving the extractives in hot water, according to the method reported by Yu et al.[29] Bark extractive samples (0.1 g) were mixed with 2 mL of hot water in test tubes and heated for 1 h in a boiling water bath. The samples were cooled at room temperature and centrifuged at 10000*g* for 10 min (model Avanti J-E Centrifuge, Beckman Coulter, U.S.A.). Samples were filtered through a 0.45 μm filter and analyzed by HPLC. The treatments selected in this study allowed extracting both polar and nonpolar fractions from bark, and thus, a tiny fraction of the extractives were not completely dissolved as the HPLC sample preparation stage was completed. Consequently, the following study on bark polyphenolic extractives only considered the hot water-soluble fractions.

HPLC Analysis. The HPLC system included a Dionex BioLC 20 Series HPLC instrument equipped with a GP50 gradient pump, AS40 auto sampler, AD25 absorbance detector, and Chromeleon for data collection and analysis (CMS); all of them from Dionex Technologies (USA). Ten μL of sample was injected by the autosampler. A Hypersil ODS C18 column (100 mm × 4.6 mm, particle size 3 μ) from Alltech (U.S.A.) was used for chromatographic separation. For elution, the two mobile phase gradients were used with a flow rate of 1.3 mL/min: 0.1% formic acid in aqueous solution as (eluent A) and ACN as organic mobile phase (eluent B). The initial isocratic range started from 0 to 4 min at 5% ACN pursued a linear increase until reaching 23% after 10 min. To obtain better separation of polyphenols, an isocratic range from 10 to 15 min at 23% ACN was applied. ACN concentration was further increased, linearly, to 50% within 4 min to elute the most preserved analytes directly, followed by reverting back to the initial conditions from 50% to 95% for 1 min. Chromatograms were recorded at three different wavelengths, 280, 310, and 370 nm. The bark polyphenolic extractives were separated and characterized based on their reported retention time (t_r) values.[30]

Folin−Denis Method. Folin−Denis is a common spectrophotometric method to assess the total phenolic contents in the samples. An intense blue color was produced after 30 min in the reaction mixture due to the reduction of phenol.[31] The Folin−Denis reagent is a combination of phosphomolybdic−phosphotungstic acid in alkaline solution. In this study, catechol was used as a reference standard in place of tannic acid or gallic acid. Thus, the total phenolic contents were calculated as catechol equivalents from the calibration curve (mg cat equiv/g of treated dry extract).

Scanning Electron Microscopy (SEM). Samples of beetle-infested lodgepole pine (BILP) and aspen bark after enzyme, alkaline, and UV/H_2O_2 treatments were oven-dried at 50 °C for 1 h, and a dense layer of samples were kept in the sample holder mended on a carbon ribbon. Until analysis, the sample assembly was maintained in plasma for 60 s in a SC7620 mini sputter coater (Polaron) purged with argon to eliminate air from the samples. SEM with a JEOL model JSM-6610LV microscope supplemented by an Oxford/SDD EDS detector was used for observing the bark samples before and after treatments.[32]

RESULTS AND DISCUSSION

Bark Composition. The major components of bark from infested lodgepole pine and aspen are summarized in Table 1.

Table 1. Chemical Composition of Beetle-Infested Lodgepole Pine and Aspen Bark

composition (%)	BILP bark	aspen bark
ethanol−toluene extractives	19.25 ± 0.54	18.39 ± 0.71
water extractives[a]	9.39 ± 0.38	17.13 ± 1.21
total extractives[b]	28.64	35.52
Klason lignin[c]	33.12 ± 0.60	34.25 ± 0.59
holocellulose[c]	39.15 ± 1.72	31.55 ± 0.75
α-cellulose[c]	24.79 ± 1.02	19.16 ± 1.43
ash contents	4.13 ± 0.76	5.69 ± 0.49
total extracts[d]	100.91	101.32

[a]Yield obtained after extraction using ethanol−toluene. [b]Addition of ethanol−toluene and water extractives. [c]Calculated as the percentage of initial dry bark weight. [d]Addition of total extracts, Klason lignin, holocellulose, and ash contents.

Bark major components analysis was reported in terms of extractives, which correspond to the soluble fractions in the ethanol/toluene mixture and 95% ethanol, water, and the bark main components, which refer to lignin, holocellulose, and α-cellulose. The addition of bark fractions from infested lodgepole pine and aspen resulted in 100.91% and 101.32% of total bark weight, respectively. The slight deviation observed may be due to some experimental errors. Infested lodgepole pine bark and aspen bark contained 4.13% and 5.69% of ash, respectively, which falls between the values obtained for other softwood and hardwood species such as *Tsuga heterophylla* and *Quercus mongolica*.[33,34]

Infested lodgepole pine and aspen bark total extractives contents were 28.64% and 35.52% of dry bark weight, which is generally high compared to the 16.60% obtained for *Pinus pinaster* after dichloromethane, ethanol, and water extraction and the 12.10% reported for the bark belly of *Quercus cork Linnaeus*, after successive extraction with ethanol and water, yet within the range of the 43.40% reported for *Populus* hybrid.[35−37] The extractives content in barks was assessed by subtracting the weight before and after extraction and not by direct evaporation of the concentrated extractives. As a result, the values could be slightly overestimated due to possible weight losses during manipulations. In addition, the high extractives values may be due to other effects, such as bark particles size, which drastically affect the fractionation and extraction yield, resulting in general difficulties in precisely comparing the extractives content obtained in the literature.[38] It is interesting to note that our results revealed higher extractives content in the mixed aspen bark compared to lodgepole pine, which is unusual although such high values are reported for *Populus* hybrid.[37]

Table 2. Extractives Yield and Chemical Composition of Bark Residues of Beetle-Infested Lodgepole Pine (BILP) and Aspen Bark after Extraction with Different Methods

	extraction method	extractives yield (%)	Klason lignin (%)	holocellulose (%)	α-cellulose (%)
BILP	alkaline	52.25 ± 1.44	19.61 ± 0.31	26.77 ± 2.40	22.16 ± 1.56
	enzymatic	49.53 ± 2.15	25.13 ± 0.53	16.73 ± 2.61	8.86 ± 2.14
	UV/H_2O_2	54.10 ± 2.10	15.08 ± 1.69	22.42 ± 0.29	13.22 ± 0.67
Aspen	alkaline	48.29 ± 0.55	22.76 ± 0.21	26.35 ± 1.31	18.64 ± 1.92
	enzymatic	48.83 ± 2.67	21.70 ± 1.19	20.38 ± 2.56	10.81 ± 0.78
	UV/H_2O_2	53.66 ± 2.67	16.06 ± 0.87	27.08 ± 2.14	15.85 ± 2.34

Aspen bark exhibited considerably higher amounts of water extractives compared to infested lodgepole pine bark, indicating a larger presence of polar extractives such as lignans, neolignans, and hydrolyzable tannins.[14] Infested lodgepole pine and aspen bark's main components exhibited similar content of Klason lignin, but the aspen holocellulose content was lower.[39]

Treated Bark Composition. Infested lodgepole pine and aspen barks were extracted using alkaline, enzymatic, and UV/H_2O_2 treatment, and the influence of these treatments on bark chemical composition is listed in Table 2 as well as shown in Figure 1. Infested lodgepole pine bark extractives yield ranged

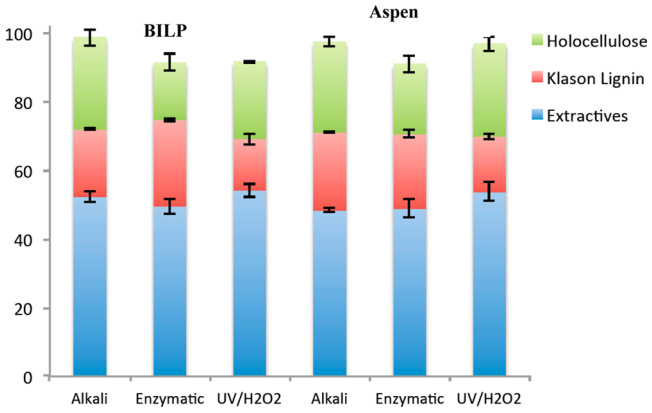

Figure 1. Bark residue composition of beetle-infested lodgepole pine (BILP) and aspen after three different treatments.

from 49.00% to 55.00% depending on the treatment used, whereas aspen bark extractives yield was slightly lower, ranging from 48.29 to 53.00%.

UV/H_2O_2 was found to be the most efficient treatment, as suggested by the higher extractives yield obtained compared to alkaline and enzymatic treatments, but the difference in extractives yield between the different treatments did not exceed 6% in weight. Although only slight differences in extractives yield were observed depending on the treatments used, we found different effects on the main component constituents.

As indicated in the results, UV/H_2O_2 treatment resulted in the extraction of a greater content of Klason lignin in both bark species, whereas the enzymatic treatment resulted in the extraction of greater contents of holocellulose and α-cellulose. The lower holocellulose and α-cellulose content observed in enzymatic-treated bark residues is possibly due to the presence of cellulase and β-glucosidase in the enzyme cocktail, which resulted in the removal of large amounts of polysaccharides from the bark. Matsushita et al.[40] previously observed the swelling of the phloem parenchyma's primary cell wall and the presence of many nanoclefts in the phloem fiber's secondary wall after hydrothermal pretreatment, which may allow greater access of enzymes to the secondary cell wall cellulose, supporting enzymatic hydrolysis.

In addition, the lower Klason lignin content observed after UV/H_2O_2 treatment supports its strong oxidative nature, which would preferentially degrade phenolic structures in bark such as lignin and condensed tannins.[41] In general, alkaline treatments resulted in the extraction of Klason lignin and holocellulose with yields very similar to that of enzymatic treatment, although the α-cellulose content did not vary, which suggests that the decrease in holocellulose is mainly due to the degradation of hemicellulose.

SEM images at different magnifications were obtained for control, alkaline, enzymatically, and UV/H_2O_2-treated BILP and aspen barks reported in this work. Figure 2 illustrates the surface morphology; the treated bark revealed the surface roughness while the untreated surface appears smooth.

Following the different treatments, the total phenolic compounds in aspen and BILP bark extractives were assessed. Enzymatic treatment resulted in 6.95 ± 0.40 mg/g and 8.82 ± 0.51 mg/g, alkali treatment provided 4.3 ± 0.66 mg/g and 4.64 ± 0.44 mg/g, and UV/H_2O_2 treatment offered 5.62 ± 0.30 mg/g and 8.0 ± 0.52 mg/g, respectively. Generally, extractives from BILP exhibited higher amounts of phenolic compounds compared to aspen. The extractive levels in barks are higher than in wood, and similarly, the total phenols level in bark has been found to be higher,[42] although some studies have shown higher polyphenols levels in wood than in bark.[43,44]

The values found in the literature for the total phenols content of methanol:water (80:20, v/v) extracts of Eucalyptus bark (*E. camaldulensis, E. globulus, and E. rudis*) were in the range of 2.5 to 91.6 mg/g.[45] Vázquez et al.[46] reported a value of 1.48 mg/g for *Eucalyptus globulus* bark extracted with this solvent. Subsequently, the estimation of total phenolic compounds should be reflected as a polyphenol index, depending upon the applied method. In addition, the lower value possible effects of other inhibiting species present in the solution mixture, such as ascorbic acid.[47] The values obtained after the different treatments of BILP and mixed aspen bark were generally low compared to the values found in the literature for total phenols. However, the extraction with organic solvents generally resulted in low extractives yield in bark (20.8%)[48] obtained with a cyclohexane:ethanol (1:2, v/v) solvent mixture compared to the treatments reported in this paper.

Most coelutions occurred between the peaks of ferulic acid and quercitrin, so the gradient was modified in this part of the chromatogram to allow a progressive elution of components while minimizing overlapping. The chromatograms corresponding to the better separation of polyphenolic extractives treated with a cocktail of enzymes are shown in Figure 3 from (A−C) for BILP and (D−F) for aspen bark. This demonstrates that all analytes can be successfully separated at three different wavelengths. Polyphenols constituents of the extractives obtained after enzymatic cocktail treatment of BILP and aspen

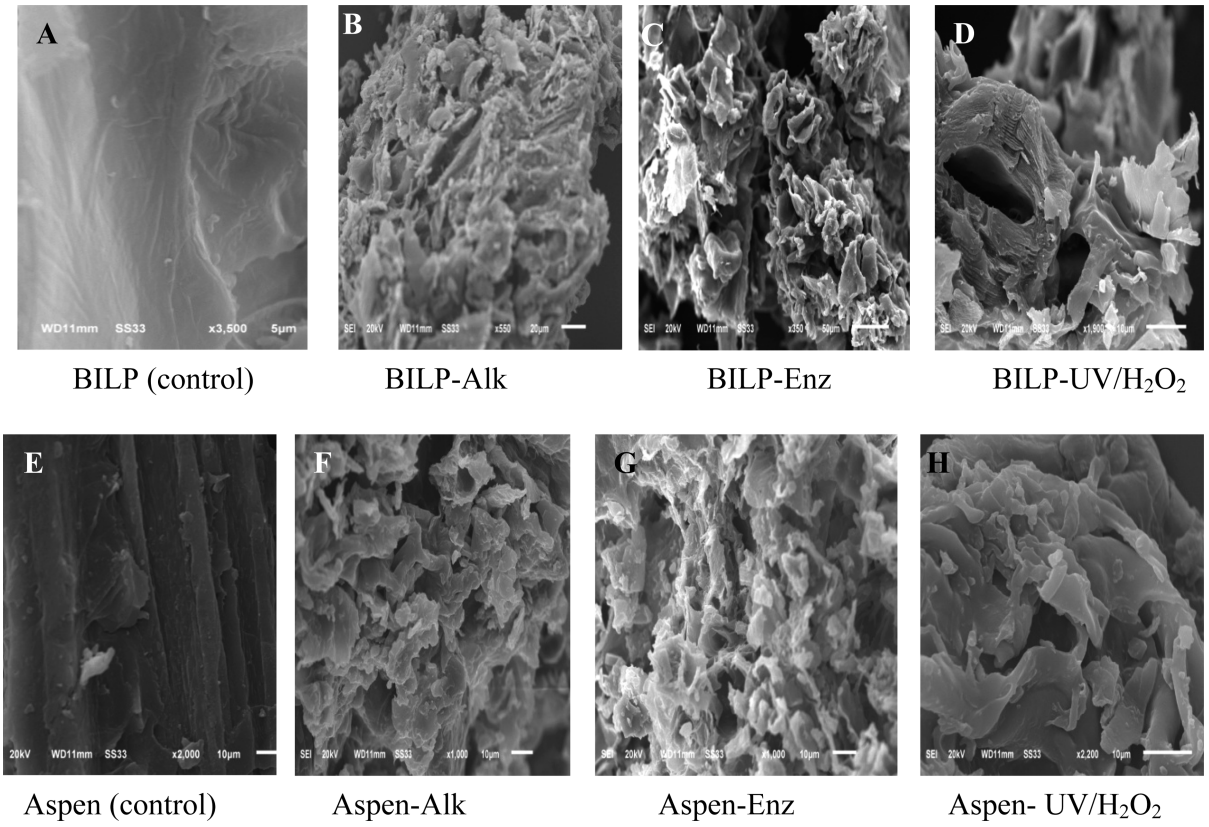

Figure 2. Scanning electron microscopy of beetle-infested lodgepole pine (BILP) and aspen barks. (A) Control BILP bark, (B) treated with alkaline, (C) treated with cocktail of enzymes, and (D) treated with UV/H_2O_2. (E) Control aspen bark, (F) treated with alkaline, (G) treated with cocktail of enzymes, and (H) treated with UV/H_2O_2 (used different magnifications).

bark exhibited better separation at three different wavelengths as compared to the extractives from other treatments.

In all cases, the yields from enzymatic cocktails were comparable to the other two treatments, but the enzymatic treatment resulted in better separations of polyphenolics, while the chromatograms of the other treatments showed overlapping separations. The separation of polyphenolic complex molecules in bark under acidic mobile phase with the combination of acetonitrile gradient is a common method in reverse-phase liquid chromatography.

It was reported that under alkaline conditions, the caffeic and syringic acids may overlap, as can *p*-coumaric acid and vanillic acid. Under alkaline conditions, phenolic acid tends to complex with sodium and prevents an efficient separation due to alteration of the hydrophilicity of analytes, thereby reducing its interaction with the stationary phase.[49] Furthermore, under alkaline conditions, syringic acid and ferulic acid were not completely separated; moreover, *p*-hydroxybenzoic acid shifted as an unsymmetrical peak. As a result, syringic acid and ferulic acid, as well as caffeic acid and hydroxycinnamic acid, nearly overlapped.[50]

Although the difficulties in separating analytes may be due to the samples basicity, samples dilution during preparation, as well as the use of 0.1% formic acid as eluent, may prevent these phenomena. The very low selectivity of alkaline and UV/H_2O_2 compared to enzymatic treatment lead to obtaining a very diverse range of molecules with close structural features, which could also complicate analytes separation.

Degradation of various products treated by AOPs using UV light and H_2O_2, linked to specific pH dependent mechanisms, produces very reactive hydroxyl radicals (•OH) in the reaction mixture, thus making it more susceptible to oxidation.[51] Nevertheless, the chromatogram and the chromatographic peak patterns were comparable to those reported in a previously published article.[30]

As a result, these two treatments have low selectivity embedded in very diverse structures that might be difficult to separate during chromatography. Moreover, UV/H_2O_2 and alkaline treatment lead to highly basic extractives, which could prevent an effective separation due to possible interactions between analytes and column. However, our results indicate that enzymatic treatment due to its highly specificity, low pH, and cleavage of selective bonds among the molecules will eventually lead us to observe better separation of polyphenolic compounds during HPLC.

CONCLUSIONS

The effect of three different treatments on the surface morphology and chemical composition of bark residues and polyphenolic extractives from BILP and aspen barks were studied. The total chemical composition of BILP bark was ash (ca. 4%), extractives (ca. 28%), Klason lignin (ca. 33%), holocellulose (ca. 39%), while mixed aspen bark residues consisted of ash (ca. 5%), extractives (ca. 35%), Klason lignin (ca. 34%), and holocellulose (ca. 31%), respectively. The HPLC analysis shows fast, selective, sensitive, and reliable determination of the most common polyphenolics in bark extractives. UV/H_2O_2 treatment was able to preferentially remove phenolic compounds, with limited effect on the sugar concentration of the bark hydrolysates. Under these conditions, the greater removal of

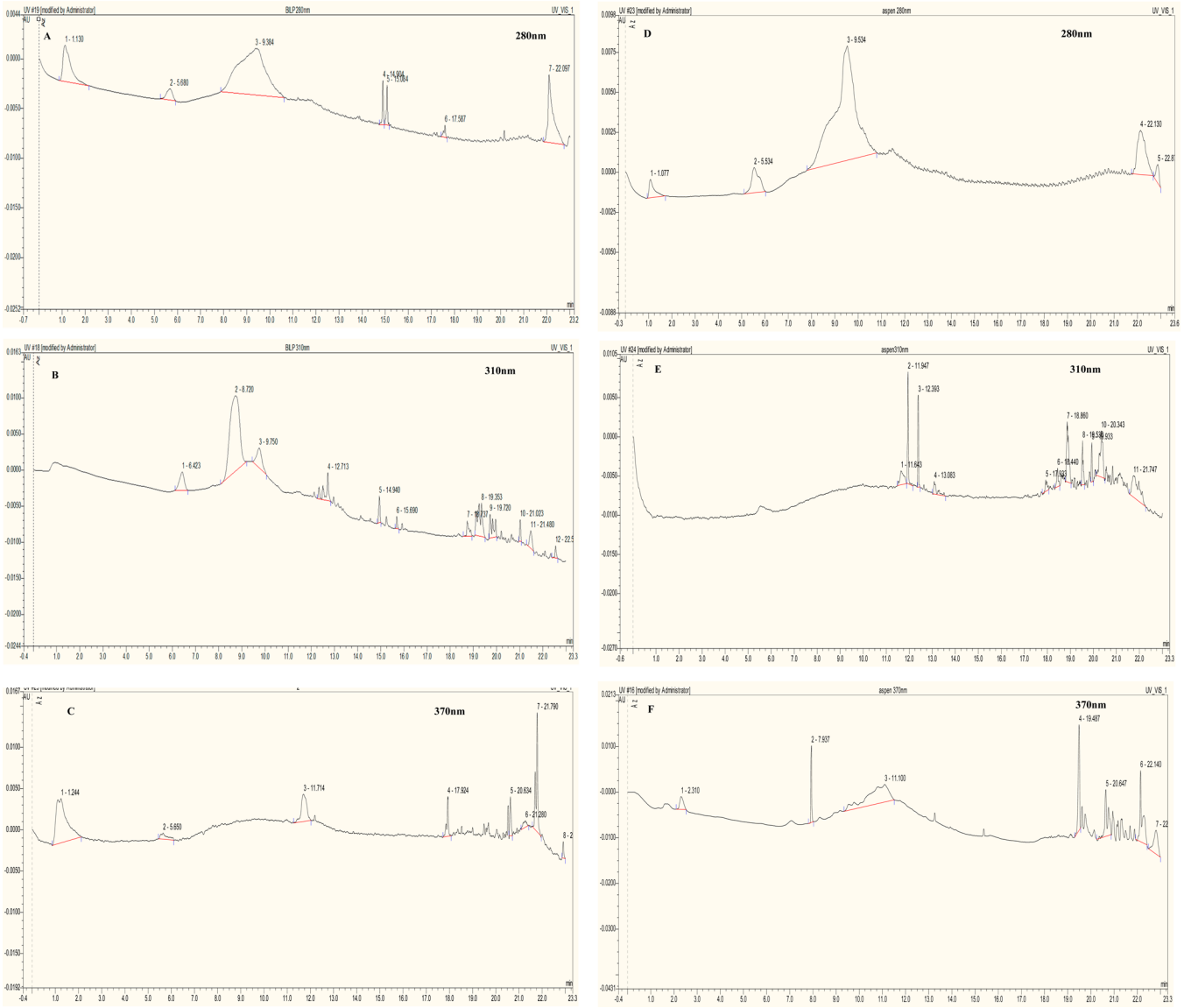

Figure 3. Chromatograms of BILP bark (A, B, C) and aspen bark (D, E, F) polyphenolic extractives. The BILP bark polyphenolic extractives at $\lambda_{280\ nm}$ are gallic acid (1.13), *p*-hydroxybenzoic acid (5.68), syringic acid (9.38), rutin (14.90), taxifolin (15.08), myrecetin (17.5) and kaempferol (22.0); at $\lambda_{310\ nm}$ are (+)-catechin (6.42), caffeic acid (8.72), syringic acid (9.75), *p*-coumaric acid (12.71), rutin (14.9), taxifolin (15.69), fisetin (18.73), trans-resveratrol (19.35, 19.72), apigenin (21.02, 21.48), and kaempferol (22.5); and at $\lambda_{370\ nm}$ are gallic acid (1.24), *p*-hydroxybenzoic acid (5.65), ethyl gallate (11.71), myrecetin (17.92), quercetin (20.63), and apigenin (21.8). Aspen bark polyphenolic extractives at $\lambda_{280\ nm}$ are gallic acid (1.07), *p*-hydroxybenzoic acid (5.53), syringic acid (9.53), and kaempferol (22.1); at $\lambda_{310\ nm}$ are ethyl gallate (11.64, 11.94), *p*-coumaric acid (12.39), ferulic acid (13.08), myricetin (17.93), fisetin (18.44, 18.86), trans-resveratrol (19.53, 19.93), quercetin (20.34), and apigenin (21.74); and at $\lambda_{370\ nm}$ are protocatechuic acid (2.31), vanillic acid (7.93), ethyl gallate (11.1), trans-resveratrol (19.48), quercetin (20.64), and kaempferol (22.1). All peak assignments were characterized based on their (t_r) values of polyphenolic standards as reported by Aznar et al.[30]

phenolic compounds, expressed in terms of catechol equivalent (8.0 ± 0.52 mg/g in BILP bark extractives and 5.62 ± 0.30 mg/g in aspen bark extractives) was observed. Furthermore, these results elucidate the effects of different treatments on bark-derived polyphenols, which can be considered as promising methods for applications in bioconversion and polymer industrial processes.

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Notes

The authors declare no competing financial interest.

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