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# Colorimetric Behavior and Seasonal Characteristic of Xylem Sap Obtained by Mechanical Compression from Silver Birch (*Betula pendula*)

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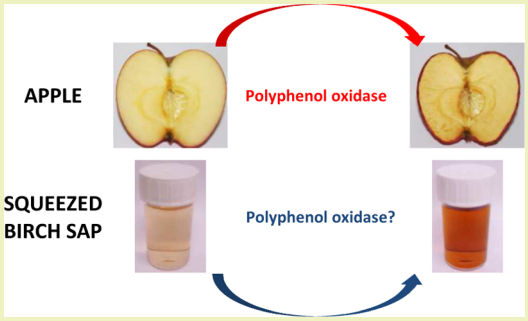
Supporting Information

**ABSTRACT:** This study investigated the color development in fresh birch xylem sap (*Betula pendula*) squeezed by mechanical compression, which was not seen in birch exudate and squeezed spruce sap. Altering the pH of the colored xylem sap demonstrated distinctive patterns of light absorption bands which suggested the formation of quinonoid intermediates by the enzymatic activity of polyphenol oxidases (PPO). Comparison with other PPOs by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) suggested the presence of the enzyme in the sap. The major phenol which acts as a substrate for PPO was identified as (−)-epicatechin. Inhibition of the color development was also attempted using $Na_2SO_3$, ethylenediaminetetraacetic acid (EDTA), and $N_2$ gas. The results showed that $Na_2SO_3$ was the most effective inhibitor. Knowledge on the differences between squeezed and exuded sap is important because squeezing can be envisaged as a pretreatment step for any process—such as a biorefinery—that utilizes biomass and has the advantage of utilizing trees that have recently been felled.




**KEYWORDS:** *Birch sap, Carbohydrates, Seasonal variation, Mechanical compression, UV−vis spectroscopy*

## INTRODUCTION

As one of the main constituents of biomass, wood is currently receiving a considerable amount of attention as the raw material for future biorefineries, aimed at the production of biofuels and commodity chemicals mainly through the efficient isolation and degradation of the polysaccharide components of wood fibres.[1−9] Xylem, the transportation system of the tree, however, hosts an aqueous substance termed xylem sap, which is rich in carbohydrates that are already in their monomeric form and easy to separate from the wood matrix by mere exudation.[10] The ascent of sap in the xylem tissue of plants is the upward movement of water from the root to the crown. Xylem is a complex tissue consisting of living and nonliving cells. The conducting cells in xylem are typically nonliving and include, in various groups of plants, vessels members and tracheids. Both of these cell types have thick, lignified secondary cell walls and are dead at maturity. Although several mechanisms have been proposed to explain the phenomenon, the cohesion−tension mechanism has the most evidence and support.[10] Reserve materials like sugars, on the other hand, are mainly stored in living cells. In the wood of silver birch soluble sugars and starch are mostly located in axial and ray parenchyma cells. The amount of total soluble sugars, sucrose, and glucose show significant variation within the stem. The amount is largest close to the cambium. Starch has been detected even close to pith in large stems.[11]

Maple and birch sap have been extensively studied because of their utilization in commercial foodstuffs.[13,14] Birch xylem sap, particularly, is used for the small-scale commercial production of birch syrup, and there are accounts of its pharmacological potential with detectable anti-inflammatory, antipyretic and phagocytosis-inhibiting effects.[15] Birch sap has been found to consist mainly of sugars, proteins, and free amino acids with significant seasonal variation affecting their concentrations.[12,16−18]

Sap has been traditionally collected from the living tree by exudation in springtime.[16,19] Xylem exudate is often considered to represent the true chemical composition of sap and it is useful in studies which investigate its physiological function.[20] In contrast, an alternative, efficient, and markedly faster method

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is to literally squeeze the sap out from a freshly felled tree by mechanical compression. Compression can be envisaged as a pretreatment step for any process that utilizes biomass and has the benefit of utilizing trees that have recently been felled. The chemical composition of sap obtained by compression is, however, most likely to be different from that of sap collected by exudation. This study constitutes a fundamental effort to elucidate the chemical composition of squeezed birch sap and to address the causes of a notable color change that spontaneously occurs straight after the compression. This discoloration may cause significant financial losses in highly valued wood products such as birch furniture, due to a deterioration of the appearance. Seasonal variations in the composition of squeezed sap and their effects on color change were also investigated. In addition, the chemical characteristics of birch sap (*Betula pendula*) were compared with sap from Norway spruce (*Picea abies* [L.] Karst.) where no comparable color change was detected. These aspects are important for the holistic, sustainable exploitation of woody biomass.

## EXPERIMENTAL SECTION

**Materials.** Ethylenediaminetetraacetic acid (EDTA), $Na_2SO_3$, NaOH, and HCl were all of analytical grade and purchased from Merck (Darmstadt, Germany). Milli-Q water (Direct-Q 3 UV, Millipore, USA) was used for all dilutions, samples, and chemical preparations in this study.

**Sap Collection.** The logs used for sap collection were obtained from Tuusula, Finland (60° 45′ 284″ N 24° 95′ 785″ E). Trees were felled and the logs were collected for the investigation on the 16th of February (winter), 28th of May (summer), and 23rd of September (autumn), 2010, for birch (*Betula pendula*) and on the 31st of August (autumn), 2010, for spruce (*Picea abies* [L.] Karst.). The logs were sawn into discs (30 cm in thickness each) at the laboratory by using a chain-saw and stored at −20 °C in a freezer until needed. As there is significant variation in, for example, sugar content and electrical conductivity,[12,16] along the length of the xylem in the living tree, the discs used in this study were carefully selected from the stem at a height of <2 m from the ground. The trees from which the discs were produced were mature and were over 70 years of age.

Each disc was gradually defrosted at 4 °C overnight, split, and debarked before squeezing (Figure 1). Sap was squeezed from the disc

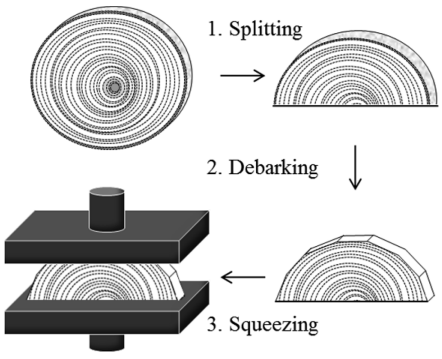



**Figure 1.** Squeezing process for the xylem sap collection from a wood disc.

using a Zwick Universal (Zwick Roell, Ulm, Germany) testing machine generally used for measuring the mechanical properties of wood (type 147570). The squeezing force applied was increased incrementally up to 100 kN (over a period of several seconds) and the maximum force maintained until the flow of sap ceased (ca. 10 s). Following collection, the sap was immediately prefiltered for further chemical analysis using Whatman Glass microfiber filters (Grade GF/C; 1.2 μm) (Whatman plc., UK). The amount of sap obtained from the discs was variable and although no precise measurements were conducted in this respect, it was clear that over 5% of the whole weight of the disc was easily squeezable as sap. The amount of squeezable sap was noted to depend upon the handling time after sampling and the storage time in the freezer.

**Control of Color Development.** Pure nitrogen gas was bubbled through the birch sap for 5 min to reduce the oxygen concentration and inhibit oxidation. In separate tests, sodium sulfite ($Na_2SO_3$), a reducing agent, and EDTA, a chelating agent, were immediately added to the sap following collection and before the commencement of color development.

**Color Development.** Photographing the color development of the xylem sap was conducted in a windowless room using a conventional digital camera (Ricoh GX200, Ricoh Company, Tokyo, Japan) with a tripod under uniform fluorescent lighting.

**Chemical Analysis of the Sap.** The carbohydrates in the sap were determined according to the NREL TP-510-42623 method using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) equipped with a CarboPac PA20 (ICS-3000; Dionex Corp., Sunnyvale, CA, USA).[21] Concentrations of total organic carbon (TOC), inorganic carbon (IC), and total carbon (TC) in the sap were determined using a Shimadzu TOC-5000A instrument (Shimadzu Corp., Kyoto, Japan). Formic acid was measured by ion chromatography (Dionex ICS-1500, Dionex Corp., Sunnyvale, CA, USA) following the procedure set out in ISO 10304-1 and a standard curve was constructed using sodium form. Acetic acid was analyzed by high performance liquid chromatography (HPLC) in a Dionex Ultimate 3000 column (Dionex Corp., Sunnyvale, CA, USA). Total Kjeldahl nitrogen (TKN) was determined according to the "modified SFS 5505" standard[22] to a precision of ±10%. The Kjeldahl-nitrogen instrument used in this study consisted of a combustion unit (Kjeldatherm, C. Gerhardt GmbH, Königswinter, Germany), a distillation unit (Vapodest 3S, C. Gerhardt GmbH, Königswinter, Germany), and a titration unit (716 DMS Titrino, Metrohm AG, Herisau, Switzerland). The UV-spectroscopy for this work was carried out on a Shimadzu double-beam spectrophotometer (model UV-2550; Shimadzu Corp., Kyoto, Japan). The concentration of soluble aromatic compounds was estimated by recording the absorbance at 280 nm. The concentration was calculated by employing an absorptivity of 20 $dm^3\ g^{-1}\ cm^{-1}$ in the Lambert−Beer equation.[23,24] Total phenolic content was also determined by a UV spectroscopic method which measures the absorbance at 300 and 350 nm in two sample solutions in different alkalinity.[25] For elemental analysis, an atomic absorption spectrophotometer (AAS) was used to determine the potassium content (Varian 220; Varian Medical Systems, Inc., California, USA) while inductively coupled plasma optical emission spectrometry (ICP-OES) was employed to determine the remaining elements (Perkin-Elmer 7100 DV; PerkinElmer, Massachusetts, USA).

**Chemical Analysis of Sap by Solvent Extraction.** Ethyl acetate (Sigma Aldrich, Missouri, USA) was used as solvent. A 25 mL portion of the filtered sap and 50 mL of ethyl acetate were added in a separatory funnel and shaken for 2 min. The lower phase was drained and reloaded to another separatory funnel with 50 mL of pure ethyl acetate. The solvent extraction was performed three times with pure ethyl acetate and the ethyl acetate supernatant from each extraction was collected separately. The solvent was removed from the supernatant by a rotavapor at 40 °C. The extract was dissolved in 1 mL of pyridine (Sigma Aldrich, Missouri, USA). (−)-Epicatechin (Sigma Aldrich, Missouri, USA) was used as an internal standard for GC-MS.

A 250 μL portin of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 5% trimethylchlorosilane (TMCS) (Sigma Aldrich, Missouri, USA) was added to the vials and sonicated for 5 min. The sample was then subjected to gas chromatography coupled with mass spectrometry (GC-MS). The GC-MS analyses were performed on a Thermo Fisher Scientific Trace 1300 equipped with an ISQ Single Quadrupole Mass Spectrometer and a TR-5MS capillary column (30 m × 250 μm × 0.25 μm) (Thermo Fisher Scientific, Massachusetts, USA), using helium as the carrier gas (1 mL/min). The injector temperature was set to 270 °C. The temperature program

proceeded as follows: 2 min (100 °C), 15 °C/min (100−260 °C), 10 min (260 °C). The MS monitored a mass range of 30−600 $m/z$ to collect identifying fragments from the electron impact (EI) source. A preliminary identification of the compounds was carried out by using MassBank and our own mass spectra library which is based on the mass spectra of model compounds.[26]

**Molecular Weight Determination of the Proteins in Sap.** The molecular weight of proteins in the sap was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% Citerion Stain Free gel, BioRad. The proteins were visualized using the Bio-Rad's Criterion Stain Free gel imaging system (BioRad Laboratories In., Hercules, CA, USA) or by staining the gel with Coomassie brilliant blue. A part of the sample was concentrated 10 times by using a Vivaspin 20 centrifugal concentrator (MWCO 10 000 Da, Sartorius AG, Goettingen, Germany). The birch sap was centrifuged (14 000*g*, 10 min), and the clear supernatant and the pellet were run in the gel. A polyphenol oxidase (*p*-diphenol oxidase, EC 1.10.3.2) was used as a model substance in the protein determination.[27] *Melanocarpus albomyces* laccase produced in *T. reesei* and *Trametes hirsuta* laccase were provided by VTT (Technical Research Centre of Finland, Espoo, Finland) and were run in SDS-PAGE with the sap samples and the protein maker.

## RESULTS AND DISCUSSION

**Chemical Characteristics of the Colored Xylem Sap.** Table 1 shows the results from chemical analyses of the birch xylem sap and the spruce sap obtained from sample trees felled at different times of the year. The results agreed well with published data which report that sap contains monomeric and oligomeric sugars, starch and other polysaccharides, phenolic compounds, organic acids, and enzymes as well as various metal ions (see Table 2 for a detailed analysis of the metals).[16−18,28−30] Starch, which can be degraded releasing hexoses into the xylem vessel is, in birch, actively stored from autumn until January.[12] The results show that the autumn felled materials had the highest total sugar content followed by summer and then winter felled wood (Table 1). This suggests that the increase in the concentrations of sugars and starch in the defoliation season is probably due to a reduction in the consumption of carbon for growth. Although only formic acid and acetic acid concentrations were determined, the acid content appeared to be the highest in autumn, which no doubt affects the pH of the sap (Table 1).

**Table 1. Chemical Composition of Colored Birch Sap from Three Different Seasons and Spruce Sap in Autumn**

| | birch | | | spruce |
|---|---|---|---|---|
| | winter | summer | autumn | autumn |
| pH | 6.45 | 6.00 | 5.50 | 6.00 |
| carbon and nitrogen (mg/L) | | | | |
| TOC | 1402 | 2916 | 3312 | 1168 |
| TC | 1404 | 2920 | 3320 | 1169 |
| IC | 2.03 | 3.95 | 7.88 | 0.66 |
| TKN | 13 | 47 | 32 | 76 |
| acids (mg/L) | | | | |
| formic acid | 0.75 | 8.96 | 9.88 | 0.81 |
| acetic acid | 22.6 | 0.00 | 132 | 0.00 |
| total | 23.4 | 8.96 | 142 | 0.81 |
| free sugar contents (mg/L) | | | | |
| arabinose | 0.18 | 1.30 | 0.00 | 0.70 |
| rhamnose | 0.20 | 0.51 | 0.00 | 0.25 |
| galactose | 10.1 | 86.3 | 51.6 | 3.52 |
| glucose | 1043 | 964 | 1302 | 730 |
| xylose | 1.55 | 22.1 | 14.9 | 1.12 |
| fructose | 1027 | 1434 | 1645 | 640 |
| total | 2082 | 2509 | 3014 | 1375 |

In conclusion, the basic chemical analyses suggested that there were little differences between the main chemical components of squeezed (Table 1) and exuded birch saps.[14,16,17] However, squeezing brought about a temporal color change in the sap which is described in detail in the following sections. Such color change has not been reported for exuded birch sap. It is evident that the color change must originate from chemical differences, and we speculated that polyphenols may be responsible for the color development, either by complex formation with metal ions or by enzymatic oxidation. Hence, a more refined chemical analysis on, e.g., different oligosaccharides or various organic acids, was not within the scope of this study.

**Color Development in the Sap.** Figure 2 shows the color change in the xylem sap squeezed from a defrosted birch wood disc. Color development in the squeezed xylem sap was recorded by photographing it over a 1000 min period at room temperature (Figure 2). The results from the UV−vis measurements confirmed an increase in absorbance in the 300−600 nm wavelength range (Figure 3), accounting for the color development (typically the human eye's response to

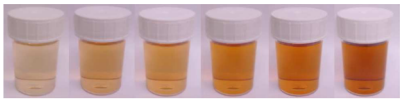



**Figure 2.** Color development of the fresh xylem sap (winter) after the squeeze.

**Table 2. Concentration of 12 Elements in the Squeezed Sap and Wood in Birch and Spruce[a]**

| | Element Contents (ppm) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Si | Al | Fe | Ca | Mg | Mn | Cu | Na | K | P | S | Cl |
| birch | | | | | | | | | | | | |
| winter sap | – | – | <1 | 10.0 | 2.4 | – | 1.91 | – | 49 | 3.5 | – | – |
| summer sap | – | – | <1 | 37.6 | 21.8 | – | 1.89 | – | 156 | 26.5 | – | – |
| autumn sap | – | – | <1 | 25.7 | 12.0 | – | 1.86 | – | 114 | 21.3 | – | – |
| wood (summer)[b] | 90 | 3 | 7 | 500 | 90 | 90 | – | 7 | 400 | 40 | 100 | 70 |
| spruce | | | | | | | | | | | | |
| autumn sap | – | – | <1 | 60.9 | 7.5 | – | 1.94 | – | 143 | 24.7 | – | – |
| wood (summer)[b] | 0 | 0 | 0 | 700 | 90 | 90 | – | 0 | 300 | 20 | 0 | 0 |

[a]Chemical elements that were not determined are expressed as –. [b]Reference 45.

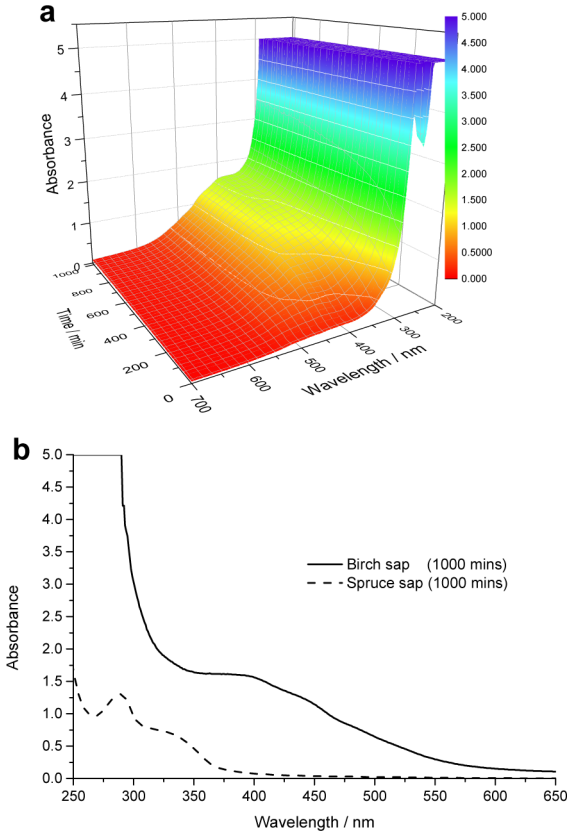



**Figure 3.** UV–vis absorbance spectra of (a) the color development in winter birch xylem sap as a function of time and (b) absorbance spectra of birch and spruce sap over 1000 min after squeeze.

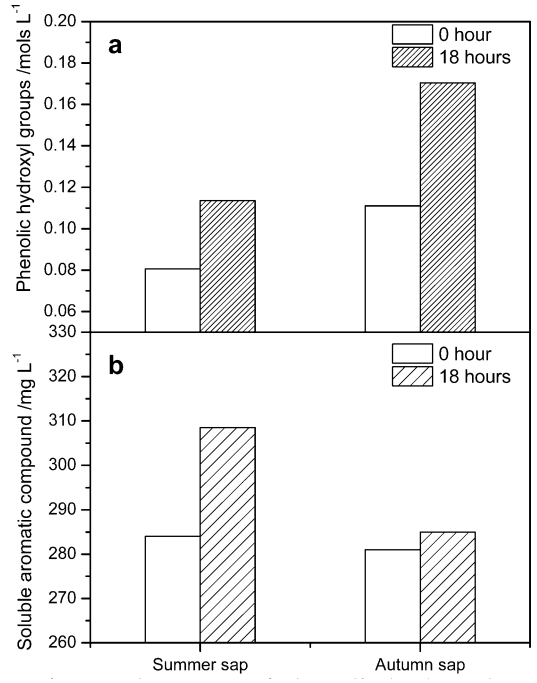



**Figure 4.** Accumulation of (a) total amount of phenolic hydroxyl groups and (b) soluble aromatic compounds in sap after its squeezing.

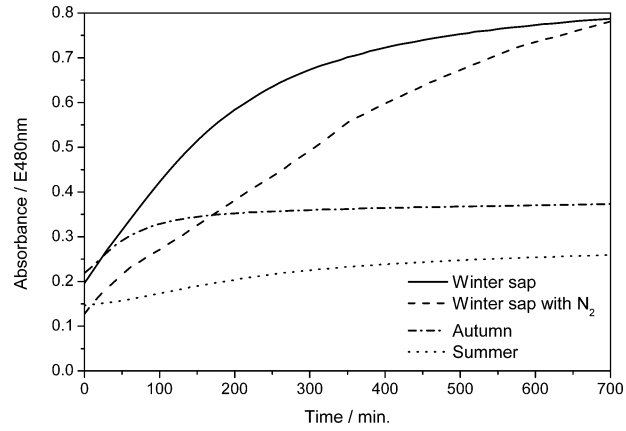



**Figure 5.** Temporal curves of the absorbance of birch xylem sap obtained in summer, autumn, and winter as well as and winter sap bubbled with $N_2$ gas immediately after the sap collection (E480 nm).

electromagnetic radiation is in the 400–760 nm range).[31] This color development has not been reported in previous research on birch sap obtained by exudation.[30,32] Interestingly, this color development was not observed in squeezed spruce xylem sap (Figure 3). It has been reported that a browning chemical reaction occurs in heated birch sap, in so-called Maillard reactions.[18] In contrast, our finding differs from the reported browning reaction since it was observed at room temperature.

Figure 4 shows that soluble aromatic compounds and phenolic substances are present in the xylem sap as determined by spectrophotometric determination. It is apparent that their concentrations increase as the color develops. There was also significant seasonal variation in the accumulation of soluble aromatic compounds and the total amount of phenolic hydroxyl groups, even though there was no clear correlation between the concentrations and season (Figure 4). In general, birch sap has an abundance of phenolics as secondary metabolites like catechin.[29] These phenolic substances are so-called leucochromophores and are believed to act as color precursors and chromophores under certain conditions.[33,34] Authentic catechols and phenols are also known as leucochromophores and when they react with metal ions such as iron (Fe) and copper (Cu), they form strong colored complexes which possess a maximum light absorption at 600 nm.[35] The results showed that autumn sap had a higher UV–vis absorbance at the end of the color development, compared to summer sap (Figure 5) and the magnitude of the phenolic accumulation correlated well with the color development in the xylem saps (Figure 4). In addition, sugar concentration does not seem to be involved in the color development as added glucose and fructose did not result in any significant change (Figure S1 in the Supporting Information).

As shown in Figure 5, a marked decrease in the color development reaction rate was observed after $N_2$ had been bubbled through the sap. It was apparent that oxygen is clearly involved in the color development due to the fact that not only is the reaction slower, but also it occurs to a similar extent without oxygen.

In order to investigate the pH sensitivity of xylem sap that had already acquired coloration, the ambient pH of the colored sap was adjusted to a range from 2.06 to 12.32 with HCl and NaOH. The UV–vis spectra of the sap at each pH were measured (Figures 6 and 7). Summarizing the results, the colored sap was found to be sensitive to a change in pH and exhibited higher absorptions under alkaline conditions and lower absorptions in neutral–acidic conditions (Figure 7). This finding suggests that the compression process could squeeze

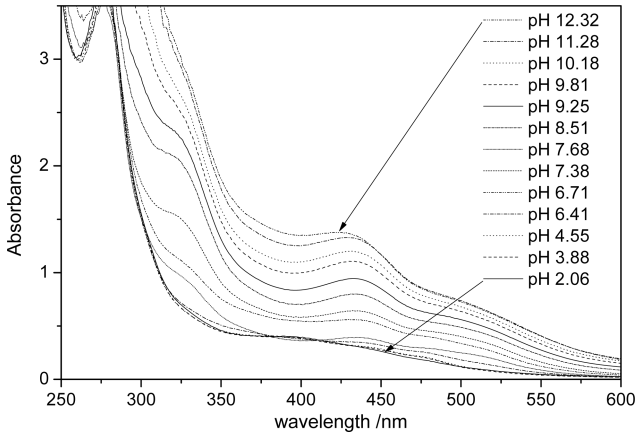



**Figure 6.** UV−vis spectra of xylem sap at different pH (autumn birch sap). The pH of completely discolored sap was altered.

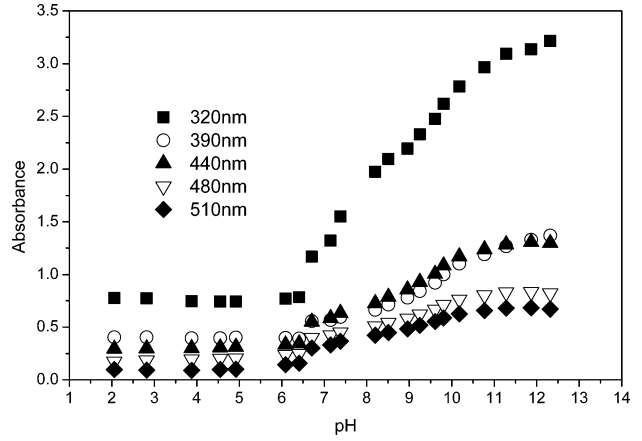
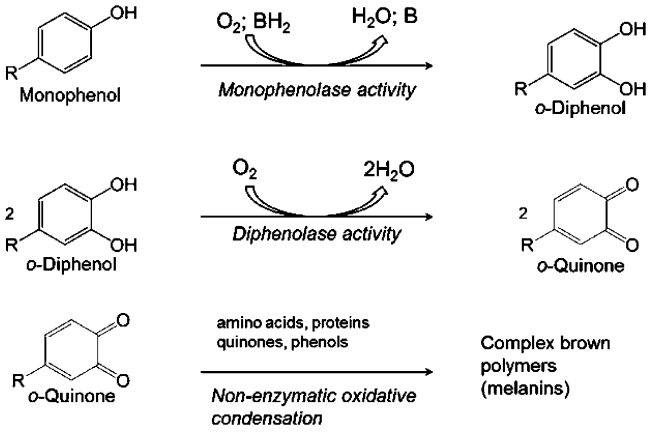



**Figure 7.** pH dependency of color formation in the colored xylem sap.

out leucochromophores, for example, polyphenols consisting of catechols, hydroquinones, and pyrogallol from the parenchyma cells into the sap. They may be partially responsible for the color development because the color formation of the leucochromophores in the sap is bound to become more activated under alkaline conditions, particularly in the presence of metal ions as they act and form brown color during mechanical pulp production.[36]

As an additional or alternative mechanism to the activation of leucochromophores, we hypothesize that an enzymatic reaction, namely polyphenol oxidase (PPO)-catalyzed browning could be a major cause of the color development. The occurrence of the color development in the sap is similar to the browning reaction by PPO on account of the following: (i) the color development is only seen in mechanically squeezed birch xylem sap and not in exudation from the intact birch xylem,[14] and (ii) the reaction takes hours and occurs at room temperature.[37] Enzymatic reactions have been considered to be one of the major causes of discoloration in freshly cut wood surfaces.[38] A similar enzymatic oxidation process to that occurring during the browning of freshly cut apple peel dominates the darkening of fresh wood veneer surfaces produced from hardwood.[39] This phenomenon might affect the visual quality of birch wood products, for example, veneer in the plywood industry. Veneer is produced from a green log by rotary peeling which continuously destroys wood cells due to the harsh physical cutting processes employed during plywood manufacture. It has been observed that discoloration occurs on freshly peeled birch veneer surfaces within half an hour of peeling.[40] Bauch mentioned that discoloration in living wood and in felled trees is caused by multiple biological factors including reactions with oxygen and PPO.[38] PPO is normally present in most plant cells and the browning color reaction occurs as a result of oxidation by PPO.[41,42] The compression process in this study could probably provide enough force to destroy the wood cell complex and the collected xylem sap may include PPO which was originally located in the plant cell structure and would never be released if the plant cell remained intact.[43] Thus the compression process could trigger the release of PPO from the cell wall structure, thereby causing enzymatic reactions that result in the browning of the birch sap (Scheme 1).

**Scheme 1. Simplified Scheme of PPO Reaction[44]**




**Characterization of the Elemental Content, Molecular Weight of Proteins, and the Possible Substrate for PPO Reaction in Sap.** The results from the determination of the element content (Table 2) showed that Fe was not found in the birch sap despite the fact that birch xylem has a certain amount of Fe (ca. 7 ppm).[45] This suggests that Fe resides inside the cell wall from which some chemical components are hard to squeeze out. It is also evident that small quantities of Cu were present in the xylem sap in both birch and spruce (Table 2), which may imply the possible presence of PPO in the xylem birch sap. (A defining feature of PPOs is that they have a coppers located in their active site.[46]) However, this finding still does not explain why the color development occurs in squeezed birch sap, but not in spruce.

To get a further idea about the protein content within the sap sample, the high molecular weight components were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). These high $M_w$ components are, to a large extent, proteins because the main wood polymers (cellulose, hemicellulose, and lignin) are largely insoluble in water. In general, xylem sap from wood collected by root pressure contains a large number of proteins (e.g., poplar contains more than 100 different proteins in the sap).[47] As shown by the SDS-PAGE analysis in Figure 8, the sap samples (lanes 3−7) contain proteins with different molecular weights except for the clear supernatant of the centrifuged sap (lane 6). The molecular weight of PPO depends on the species of the plant, such as cabbage (40 kDa) and butter lettuce (60 kDa).[48,49] The analysis with two model PPOs showed that they

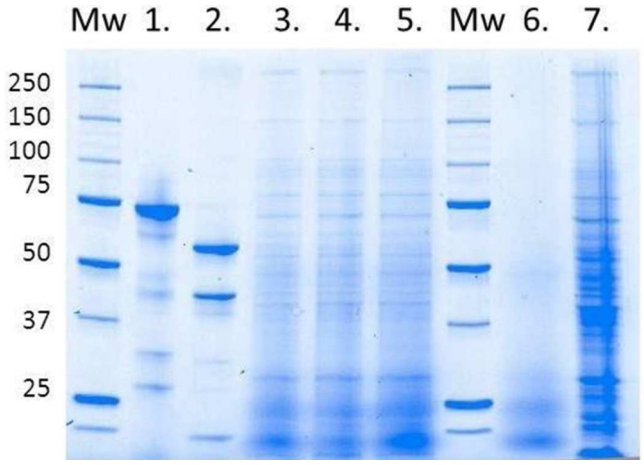



**Figure 8.** SDS-PAGE of the birch sap. MW, molecular weight (kDa) and marker protein: (1) 5 $\mu$g of purified Melanocarpus albomyces laccase produced in *T. reesei*, (2) 5 $\mu$g of purified Trametes hirsuta laccase, (3) 35 $\mu$L of birch sap, (4) 15 $\mu$L of 10 times concentrated birch sap, (5) 25 $\mu$L of 10 times concentrated birch sap, (6) 35 $\mu$L of clear supernatant of the centrifuged sap, and (7) pellet from the centrifuged sap.

produced bands between 37 and 75 kDa on SDS-PAGE (lanes 1 and 2 in Figure 8). Within this molecular weight range, several protein bands can be found in the sap (lane 3, 4, 5, and 7), suggesting that an enzyme with similar molecular weight to PPOs could exist in the sap. We emphasize, however, that the SDS-PAGE analysis does not provide any hard evidence on the structure of the proteins. To elucidate the structural details would require the use of, e.g., nuclear magnetic resonance spectroscopy, but such extensive analytical efforts would be outside the scope of this manuscript.

The results from GC-MS analysis (Figures S2 and S3 in the Supporting Information) indicated that the predominant phenol in sap is (−)-epicatechin (Figure 9). The concentration

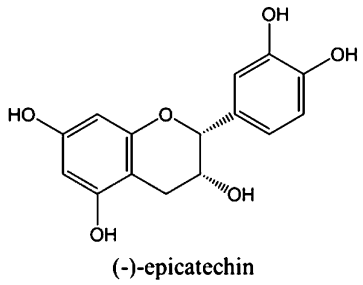



**Figure 9.** Structure of (−)-epicatechin which was the major phenol found in sap.

of (−)-epicatechin in the sap was 262.73 mg/L, quantified from the chromatogram, which exceeded the concentration of other phenols by a 10-fold margin, making it the only significant phenolic compound in sap (Figure S2 in the Supporting Information). (−)-Epicatechin has often been found to function as a substrate for PPO oxidation, for example, in apples, and it is considered a major contributor to browning in PPO reactions.[43] A significant amount of (−)-epicatechin has also been found in the exudate from silver birch.[29] It is believed that the concentration of the polyphenol like (−)-epicatechin is directly correlated with the degree of browning in PPO reaction. Furthermore, the oxidation of (−)-epicatechin by PPO can be reversed by ascorbic acid which converts a portion of *o*-quinones (Scheme 1) to the original polyphenols.[50]

**Inhibition of Color Development.** As shown in Figure 10, a decline in UV−vis absorbance was detected in the

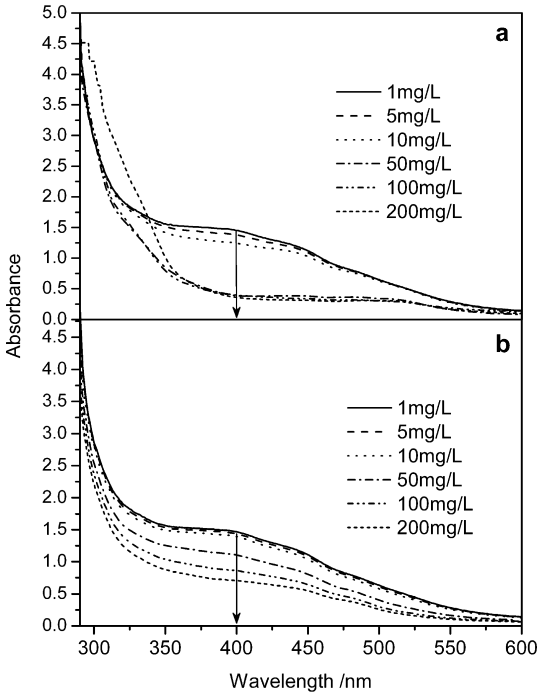



**Figure 10.** UV−vis spectra of the color formation of birch xylem sap inhibited with different concentrations of (a) $Na_2SO_3$ and (b) EDTA.

experiments with inhibitors added to the xylem sap. The results indicate that there is an apparent effect of the inhibitors on color development. Two conventional PPO inhibitors were separately applied to the xylem sap before the color change occurred in order to control the color development and to further explore the possible influence of PPO as its cause. Quantification of PPO activities can be made by determination of quinone formation with a spectroscopic technique that measures the absorption at 480 nm.[51] A decrease in UV−vis absorbance clearly correlated with the concentrations of the inhibitors (Figure 11). $Na_2SO_3$ has a higher inhibition performance than EDTA in terms of the color development

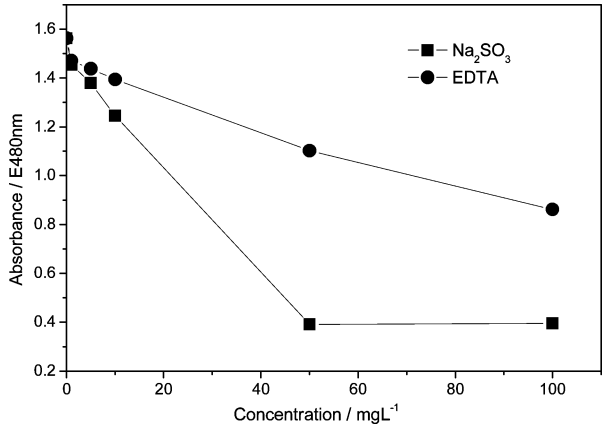



**Figure 11.** Inhibitory effect of $Na_2SO_3$ and EDTA for the color development in the winter xylem sap.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in the sap. This finding is in accordance with previous reports on PPO inhibitors, with regard to the function of each inhibitor.[44] Several inhibitors for PPO activities are in use in the food industry such as the reducing agent, $Na_2SO_3$, and the chelating agent, EDTA.[44] It is known that EDTA inhibits PPO activity by its chelating function and removes the active copper site from the enzyme before its activation.[52] On the other hand, sulfites are known to be one of the most powerful enzymatic browning inhibitors because they act as reducing agents and have the ability to directly inhibit PPO.[44] Inhibition of the browning reaction by sulfites is actually irreversible inactivation of PPO.[53] Sulfites impede the polymerization of quinones by preventing the accumulation of *o*-quinones; they may also form stable achromatic compounds.[54] Two detailed mechanisms have been suggested to explain the PPO inhibition by sulfites: (i) sulfites react with intermediate quinones in the melanosis reaction (complex brown polymer) forming sulfoquinones; (ii) they irreversibly react with PPO causing complete inactivation.[55]

## CONCLUSIONS

We observed a spontaneous discoloration in squeezed birch xylem sap which was a phenomenon that has not been reported for birch sap obtained by exudation. The results suggested that discoloration was due to enzymatic oxidation caused by PPO. (−)-Epicatechin was considered to be the major phenol substrates for enzymatic oxidation. The inhibition experiments suggested that $Na_2SO_3$ was the most effective inhibitor which is believed to prevent enzymatic reaction by reducing oxygen in the sap and directly inactivating enzyme, supporting the idea that the discoloration of the squeezed sap is an oxidation process with enzymatic browning.

More research is required to understand the details of squeezable chemical compounds in birch xylem sap. Specifically, a detailed verification of PPO activities is also needed. Our work has demonstrated that sap—albeit with chemical properties distinct from those of exuded sap—can be efficiently isolated from the fresh wood matrix by mechanical squeezing. This is a procedure that can be envisaged in any biorefinery processes that use fresh wood material.

## ASSOCIATED CONTENT

### Supporting Information

Sugar effects, chromatogram, and mass spectra from GCMS. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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