Characterization of High-Tannin Fractions from Humus by Carbon-13 Cross-Polarization and Magic-Angle Spinning Nuclear Magnetic Resonance

Klaus Lorenz* and Caroline M. Preston

ABSTRACT
Condensed tannins can be found in various parts of many plants. Unlike lignin there has been little study of their fate as they enter the soil organic matter pool and their influence on nutrient cycling, especially through their protein-binding properties. We extracted and characterized tannin-rich fractions from humus collected in 1998 from a black spruce [Picea mariana (Mill.) Britton et al.] forest in Canada where a previous study (1995) showed high levels (3.8% by weight) of condensed tannins. A reference tannin purified from black spruce needles was characterized by solution 13C nuclear magnetic resonance (NMR) as a pure procyanidin with mainly cis stereochemistry and an average chain length of four to five units. The colorimetric proanthocyanidin (PA) assay, standardized against the black spruce tannin, showed that both extracted humus fractions had higher tannin contents than the original humus (2.84% and 11.17% vs. 0.08%), and accounted for 32% of humus tannin content. Consistent with the results from the chemical assay, the aqueous fraction showed higher tannin signals in the 13C cross-polarization and magic-angle spinning (CPMAS) NMR spectrum than the emulsified one. As both tannin-rich humus fractions were depleted in N and high in structures derived from lignin and cutin, they did not have properties consistent with recalcitrant tannin–protein complexes proposed as a mechanism for N sequestration in humus. Further studies are needed to establish if recalcitrant tannin–protein complexes and inhibition of soil enzymes. However, there is little chemically explicit information on the fate of tannins in humus, soil, or sediments (Bradley et al., 2000; Hernes and Hedges, 2000; Schofield et al., 1998). Preston (1999) found that tannin-rich fractions extracted from humus of a cutover forest site on northern Vancouver Island were depleted in N, and high in lignin and cutin structures.

In a previous study, high concentrations of condensed tannins were found by the proanthocyanidin assay in the organic layer of two Canadian forest sites dominated by black spruce (Lorenz et al., 2000). We investigated the nature of tannin in the humus by extracting and purifying tannin-rich fractions from the humus of one of these sites. In addition to changes in C and N, we characterized the fractions using colorimetric proanthocyanidin assay developed specifically for condensed tannins, and 13C NMR spectroscopy with CPMAS. To provide a standard for the chemical assay, condensed tannins were also extracted and purified from black spruce needles and characterized by solution 13C NMR.

MATERIALS AND METHODS
Isolation of Black Spruce Tannin
In the summer of 1998 a bulk sample of black spruce needles (ca. 200 g fresh weight) was collected at Lake Nipigon Forest, Ontario, Canada (for site details see Lorenz et al., 2000). Purified condensed tannin was prepared using standard methods (Preston, 1999): preextraction with hexane, extraction with 70% (v/v) aqueous acetone, followed by 70% (v/v) aqueous acetone, and cleanup of the aqueous phase by several washings with CHCl₃ and ethyl acetate. After solvents were removed by rotary evaporation, the crude extract was freeze-dried and then purified by chromatography on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) using 50% (v/v) aqueous methanol to remove sugars and low-molecular-weight phenolics, followed by 70% (v/v) aqueous acetone to elute the condensed tannin. Acetone was removed by rotary evaporation, and the aqueous phase was freeze-dried to give the purified tannin.

Abbreviations: CPMAS, cross-polarization and magic-angle spinning; DD, dipolar dephased; NMR, nuclear magnetic resonance; PA, proanthocyanidin; TOSS, total suppression of spinning sidebands.
Preparation of Tannin-Rich Humus Fractions

At Lake Nipigon Forest a bulk sample of humus (ca. 300 g fresh weight) was collected in the summer of 1998. After removal of debris and roots, the humus extraction was carried out following the conventional procedure for plant material. As found previously (Preston, 1999), washing the extract with ethyl acetate produced a high proportion of stable emulsion. This was processed separately including purification on Sephadex LH-20. Two tannin-rich fractions, designated emulsified and aqueous, were obtained.

Chemical Analysis

Carbon and nitrogen contents of dried humus samples (40°C) were detected by automatic combustion using a LECO (St. Joseph, MI) CR12 carbon analyzer and a LECO FP-228 N analyzer.

Proanthocyanidin Assay

Condensed tannins in the humus and tannin-rich humus fractions were analyzed by the colorimetric proanthocyanidin (butanol–HCl) assay, standardized against the purified condensed black spruce tannin (Lorenz et al., 2000; Preston, 1999).

Nuclear Magnetic Resonance Analysis

General structural information on the black spruce tannin was obtained by solution 13C NMR (Ayres et al., 1997; Czochanska et al., 1980; Newman et al., 1987) of 100 mg of the purified black spruce tannin dissolved in 3 mL of 1:1 D2O and acetone. The spectrum was obtained at 75.47 MHz on a Bruker MSL 300 spectrometer (Bruker Instruments, Karlsruhe, Germany) using a 10-mm sample tube, inverse-gated decoupling, 45° pulse, 0.2-s acquisition time, and 2-s relaxation delay. Spectra were processed with line broadening, and chemical shifts reported relative to tetramethylsilane, using the acetone peak at 30.7 ppm as a secondary standard.

To obtain solid-state 13C CPMAS NMR spectra, approximately 100 mg of sample material was packed into a 7-mm-o.d. rotor and spun at 4.7 kHz on the same instrument operating at 75.47 MHz for 13C. Spectra were acquired with 1 ms contact time, 2 s recycle time, and 6000 scans, and were processed using 30 to 40 Hz line-broadening. Dipolar dephased (DD) spectra were generated by inserting a delay period of 40 to 50 μs without 1H decoupling between the cross-polarization and acquisition portions of the CPMAS pulse sequence. The DD spectra were obtained in combination with the sequence for total suppression of spinning sidebands (TOSS). Chemical shifts are reported relative to tetramethylsilane at 0 ppm (secondary reference adamantane).

![Fig. 1. Solution 13C nuclear magnetic resonance (NMR) spectrum of (a) purified black spruce tannin and (b) expansion of C3–C2 region.](image-url)
RESULTS AND DISCUSSION

Nuclear Magnetic Resonance of Black Spruce Tannin

The solution $^{13}$C NMR spectrum of the black spruce tannin was interpreted according to previous studies (Ayres et al., 1997; Czochanska et al., 1980; Newman et al., 1987). As shown in Fig. 1a, the sharp, clean peak at 144.7 ppm and large signals at 116 and 119 ppm are characteristic features of a pure procyandin (two OH groups on the B ring). The C2–C3 region is shown in more detail in Fig. 1b. Signals for C3 in a chain terminating position [C3(term)] of condensed tannins are at 65 to 69 ppm, while signals for C3 in the chain-interior and upper chain-ending positions [C3(int)] are at 69 to 75 ppm. The C2 region is 75 to 85 ppm, divided into C2\text{cis} (75–80 ppm) and C2\text{trans} at 80 to 85 ppm. The C2\text{trans} region may be further divided into terminal and interior units at approximately 80 to 81.5 ppm and 81.5 to 85 ppm, respectively. Chemical shift cutoffs are reported for this tannin and will vary slightly for different structures and NMR conditions. For this tannin preparation, the stereochemistry at C2–C3 was calculated as 78% \text{cis} and 22% \text{trans}, and the average chain length as 4.6. A higher proportion (approximately two-thirds) of the \text{trans} units are terminal. This purified black spruce tannin was used as a standard in the colorimetric proanthocyanidin assay.

Tannin Analysis and Nuclear Magnetic Resonance of Humus

The NMR spectrum of the humus sample before extraction is shown in Fig. 2a. Features in both the normal CPMAS and DD spectra are similar to those previously observed for humus (Kögel-Knabner et al., 1992; Lorenz et al., 2000; Preston, 1999). The alkyl C region shows a peak at 33 ppm with a shoulder at 30 ppm for more rigid and more mobile CH$_2$, respectively. The latter comes from components with greater molecular motion in the solid state as indicated by higher persistance of the signal at 30 ppm in the DD spectrum. Less accumulation of long-chain CH$_3$ from cutin, suberin, plant waxes, and microbial biomass in the organic layer compared with the humus sample previously collected in 1995 at the same site is indicated by the lower alkyl-C intensities (cf. Lorenz et al., 2000). Also, the peak at 56 ppm for methoxyl C, the phenolic peak with maximum at 145 ppm and a shoulder at 153 ppm, and the weak and broad peak at 130 ppm indicates a higher proportion of guaiacyl lignin structures compared with tannin than in 1995. In the humus sample from 1995, with much higher tannin content, peaks at 130, 145, and 155 ppm were sharp and well resolved, and the methoxyl signal less so.

The PA assay of the composite humus sample showed a very low tannin content (0.08%; Table 1). Three years ago, 3.8% was found in the organic layer at the same site (Lorenz et al., 2000). These differences are also supported by the DD spectrum. In the DD spectrum of the low-tannin humus in this study, a broad peak characteristic of tannins can be observed at 105 ppm but the broad phenolic region has no resolved signals for lignin or tannin, and there is a small but distinct methoxyl signal at 57 ppm. By contrast, the DD spectrum of the 1995 humus sample had almost completely resolved phenolic peaks at 145 and 155 ppm, and almost no methoxyl signal, although the broad peak around 105 ppm was similar in intensity. This DD signal, which has been identified as a characteristic marker for condensed tannins (Wilson and Hatcher, 1988), seems to persist even when the tannin chemically identifiable by the proanthocyanidin assay has become very low (Preston, 1999).

Condensed tannins from black spruce needles are a
Table 1. Analysis of mass, tannins, C, and N in the humus extraction.

<table>
<thead>
<tr>
<th></th>
<th>Mass</th>
<th>Tannin†</th>
<th>Carbon</th>
<th>Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>mg g⁻¹</td>
<td>%</td>
<td>g</td>
</tr>
<tr>
<td>Humus</td>
<td>290.74</td>
<td>0.8</td>
<td>100.0</td>
<td>476</td>
</tr>
<tr>
<td>Emulsified fraction</td>
<td>0.78</td>
<td>28.4</td>
<td>9.2</td>
<td>34.2</td>
</tr>
<tr>
<td>Aqueous fraction</td>
<td>0.50</td>
<td>111.7</td>
<td>23.2</td>
<td>134.6</td>
</tr>
</tbody>
</table>

† Proanthocyanidin assay based on black spruce tannin.
‡ Enrichment factor.

significant input to humus in Lake Nipigon Forest as shown in a previous litter decomposition study (Lorenz et al., 2000). Traces of tannins could be observed in the NMR spectrum even of the low-tannin humus sampled in 1998, but it is not known if this is an indicator of restricted decomposition because high contents of tannins inhibit the decomposers (Nicolai, 1988; Preston, 1999). More complex, insoluble, or modified tannins may be responsible for the persistence of tannin features in the spectra (Handley, 1961; Francois et al., 1987; Toutain, 1987). We do not know the reason for the difference in humus tannin contents measured at this site in 1995 and 1998.

Tannin-Rich Fractions of Humus

Extraction and Tannin Analysis

Applying the extraction procedure for plant material to a bulk humus sample was successful. As found previously (Preston, 1999), the acetone–water extract separated into a stable emulsion and an aqueous fraction, which were purified separately on the Sephadex LH-20 column. Table 1 shows that the aqueous fraction was higher in tannins than the emulsion (111.7 and 28.4 mg g⁻¹) and accounted for 23.2 and 9.2%, respectively, of humus tannin. Both fractions were enriched in C but depleted in N relative to the original humus. Very similar results were found after extraction of humus from old-growth clearcuts dominated by the ericaceous shrub salal (Gaultheria shallon Pursh) on northern Vancouver Island (Preston, 1999). Compared with the original humus (2.5 mg g⁻¹ tannin) the aqueous and emulsified fractions obtained in the previous study were also depleted in N and enriched in C, and accounted for 27% of humus tannin content.

Nuclear Magnetic Resonance of Humus Fractions

Both the normal CPMAS and DD spectra of the residue are very similar to those of the starting humus (Fig. 2b). Minor differences can be observed in the alkyl C region with lower contribution at 33 ppm for more rigid long-chain CH₃ compared with the starting humus. Consistent with this, the DD spectrum of the residue has a slightly higher intensity for the mobile component of long-chain CH₃. The DD spectrum also shows loss of intensity of the 146 ppm shoulder, consistent with loss of tannins, although there are no differences in the aromatic region of the normal CPMAS spectrum.

Spectra from the emulsified and the aqueous humus fractions are shown in Fig. 3a and 3b. Both tannin-rich fractions have a high proportion of alkyl C, with slightly higher intensity at 30 than 33 ppm. Most of this alkyl C is rigid, as shown by the low intensity in the DD spectra, and probably originates from plant cutin and suberin, or microbial biomass.

The NMR spectra also indicate a high proportion of lignin-derived structural components in both fractions. The methoxyl peak of lignin is present in both the normal CPMAS and DD spectra, with higher intensity for
the emulsified fraction. For this fraction, the phenolic region has three barely resolved maxima, at 145, 148, and 152 ppm, characteristic of lignin–tannin mixtures. For the aqueous fraction, however, the split phenolic peak (145 and 155 ppm) in both spectra is more characteristic of tannin, consistent with its higher tannin content (111.7 vs. 28.4 mg g\(^{-1}\)). The higher intensity of the peak at 116 ppm is also consistent with incorporation of proanthocyanidin subunits. In the di-O-alkyl region of both fractions no distinct peak at 104 ppm can be observed, and the broad O-alkyl signal at 73 to 74 ppm most likely originates from the side-chain of lignin and the C2 and C3 of tannins rather than carbohydrate, especially for the aqueous fraction. However, both fractions have a broad signal characteristic of tannins at 106 ppm in the DD spectra. By comparison with NMR of black spruce needle litter from the Lake Nipigon site (Lorenz et al., 2000), both fractions appear like black spruce needles that have undergone loss of most extractives, carbohydrates, and protein. If these tannin-rich fractions are the rigid remains of plant structures, the alkyl intensity is more likely to be from plant than from microbial input. However, further insights would require molecular-level analyses.

The NMR spectra of these fractions are also consistent with those found for the fractions from northern Vancouver Island (Preston, 1999). These had similar features, although the proportion of lignin structures was higher, probably resulting from the higher influence of very large, persistent coarse woody debris in coastal forests (Preston et al., 1998).

**CONCLUSIONS**

In a previous litter decomposition study, tannins were rapidly lost from black spruce needles at the Lake Nipigon site (Lorenz et al., 2000), although an accumulation of condensed tannins was observed in the organic layer. This study found no unusually high content of condensed tannins by the PA assay in the humus collected three years later at the same site. However, it was still possible to extract tannin-rich fractions with characteristic signatures in the NMR spectra from this low-tannin humus, while comparison of NMR spectra and the PA assay indicate that humus tannins are at least partially invisible to the latter. Spectra of both fractions suggested a structural complex of lignin, tannin, and cutin but both were depleted in N (cf. Preston, 1999).

Many questions remain regarding the chemical nature, and pathways of chemical or microbial transformation of tannins in humus and organic matter. It is especially important to elucidate whether recalcitrant tannin–protein complexes are an important mechanism for N sequestration in black spruce and other N-limited ecosystems. Probably more soluble litter fractions high in N are leached out, decomposed very quickly, oxidized upon contact with minerals, or incorporated in humic structures (Bradley et al., 2000; Francois et al., 1987; Hernes and Hedges, 2000; Schofield et al., 1998; Toutain, 1987). Our extraction procedure, based upon that used for fresh plant material, extracted fractions high in plant structural polymers and low in N. In this and a previous study (Preston, 1999), the fractions accounted for around 30% of the humus tannin that was visible to the chemical assay, but may have failed to extract tannins associated with proteins or other N-containing structures if these were either inherently insoluble, or further protected due to cross-linking or other structural transformations. While complexes of condensed tannins and proteins are largely insoluble, they are only held by physical forces, and can be disrupted by solvents such as acetone that break hydrogen bonds (Hagerman et al., 1998; Spencer et al., 1988). It is therefore also possible that tannin–protein complexes may have been disrupted by acetone-water.

While we do not fully understand the fate or function of tannins in forest humus, and no one has shown direct, unequivocal evidence for tannin–protein complexes in humus, tannins probably contribute to associated forest management problems observed in black spruce ecosystems like the development of thick mor horizons, nutrient limitation, and increased sequestration of N in the forest floor with increasing stand age (Titus et al., 1995; Pastor et al., 1987; Smith et al., 1998). For example, an incubation study of black spruce humus with added condensed tannins and nitrogen showed little recovery of added tannins after ten weeks, and a reduction of N mineralization (Bradley et al., 2000). However, this was counteracted by N fertilization, indicating that tannins may have more effect on nutrient-limited sites, consistent with studies of Northup et al. (1998, 1999). One possibility is that litter decomposition might be slowed down if the protein-binding properties of tannins affect functioning of extracellular fungal enzymes. Further studies should include monitoring annual variation of humus tannin content, determining the effects of tannins on soil enzyme activity, and developing methods to isolate or otherwise demonstrate the presence and function of tannin–protein complexes in forest humus.

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**REFERENCES**


