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Tannin diagenesis in mangrove leaves from a tropical estuary: A novel molecular approach

PETER J. HERNES,[1,†] RONALD BENNER,[2] GREGORY L. COWIE,[3] MIGUEL A. GOÑI,[4]
BRIAN A. BERGAMASCHI,[5] and JOHN I. HEDGES[1]

[1]School of Oceanography, University of Washington, Box 357940, Seattle, WA 98195-7940, USA
[2]Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA
[3]Marine and Environmental Geosciences Group, Geology and Geophysics Department, University of Edinburgh, West Mains Road, Edinburgh EH9 3JW, UK
[4]Department of Geological Sciences, University of South Carolina, Columbia, SC 29208, USA
[5]U.S. Geological Survey, 2800 Cottage Way, California District Office, Sacramento, CA 95825, USA

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Abstract—Molecular-level condensed tannin analyses were conducted on a series of mangrove (*Rhizophora mangle*) leaves at various stages of decomposition in a tropical estuary. Total molecular tannin yields ranged from 0.5% ash-free dry weight (AFDW) in the most highly degraded black leaves (6–7 weeks in the water) up to >7% AFDW in fresh leaves (<1 week in the water). Total tannin exhibits an intermediate lability in these leaves relative to other measured biochemicals. Leaching is an important mechanism in tannin removal from leaves as indicated by the 30% loss of measurable tannin during a leaching experiment. Condensed tannin was >80% procyanidin (PC) with the remainder being prodelphinidin (PD). PD tannin, with its higher degree of hydroxylation, proved to be more labile than PC tannin. Average chain length of condensed tannin (degree of polymerization) exhibited an initial increase in response to leaching, but later decreased in the subsequent shift toward abiotic or microbially mediated chemical reactions. Several trends point toward a possible condensation reaction in which tannin plays a role in nitrogen immobilization. These include an apparent inverse correlation between molecular tannin and nitrogen, a positive correlation between molecular tannin and percent basic amino acids, ^{13}C-NMR data indicating transformation of tannin as opposed to remineralization, and ^{13}C-NMR data showing loss of condensed tannin B-ring phenolic carbons coupled with preservation of A-ring phenolic carbon. In addition to condensed tannin, the molecular method used also yielded several triterpenoids. Triterpenoids accounted for up to 3.5% AFDW of the leaf material and exhibited a threefold increase between yellow senescent leaves entering the estuary and black leaves. This trend is likely due to the weakening of protective cuticular membranes during leaf decomposition, which leads to increased yields in the acidic conditions used for tannin analyses.

1. INTRODUCTION

Tannin comprises as much as 20% of leaf (Benner et al., 1990a), needle (Hedges and Weliky, 1989), and bark tissues (Kelsey and Harmon, 1989), all major contributors to terrigenous organic matter cycling. Yet virtually nothing is known about tannin diagenesis at the molecular level. This is due in large part to the analytical challenges in measuring tannin. Although a large body of literature exists for molecular-level tannin studies in natural products, there has been little crossover to biogeochemistry and ecology. In this regard, the geochemistry of tannin is at much the same stage of development as that of its phenolic cousin, lignin, nearly three decades ago. The similarities are considerable: Each is phenolic, polymeric, unique to vascular plants, and has been well studied in natural products research. However, the contrasts are also considerable: Whereas lignin polymers are highly branched in a seemingly random manner, tannin macromolecules are often highly structured to the point whereby some have speculated a lock-and-key mechanism for how they function (Zucker, 1983). Whereas lignin predominates in woody tissue, tannin is often more abundant in leaves and needles. Finally, whereas lignin is relatively refractory, tannin is subject to numerous reactions. The latter point is important: Tannin offers the potential to study organic matter processing in a way unavailable for lignin or even structural polysaccharides.

Tannin in vascular plants occurs as two types, condensed and hydrolyzable (Fig. 1). A third type known as phlorotannin (because of the basic building block, phloroglucinol) is found in brown algae. Hydrolyzable tannin is made up primarily of gallic acid or its derivatives, which are often esterified to polyols such as glucose. The building blocks for condensed tannin are three-ring flavanols (note the terminology of extender units and terminal units in Figure 1). At least a dozen variations of these stereochemically active compounds are known to occur in condensed tannin. The most common linkages in condensed tannin are 4→8, whereas 4→6 linkages lead to branching (Fig. 1). Condensed tannin with these linkages is often referred to as proanthocyanidin (PA) due to the formation of cyanidins or related compounds on acid depolymerization. Although more rare, condensed tannin can also be found with A to A-ring and A to B-ring linkages, which strictly speaking, are not proanthocyanidins. More complete reviews of structure and chemistry of condensed tannin can be found in Hemingway (1988a,b), McGraw (1988), and Laks (1988). The structural

* Author to whom correspondence should be addressed (pjhernes@biol.sc.edu).
† *Present address:* Baruch Institute, University of South Carolina, Columbia, SC 29208, USA.

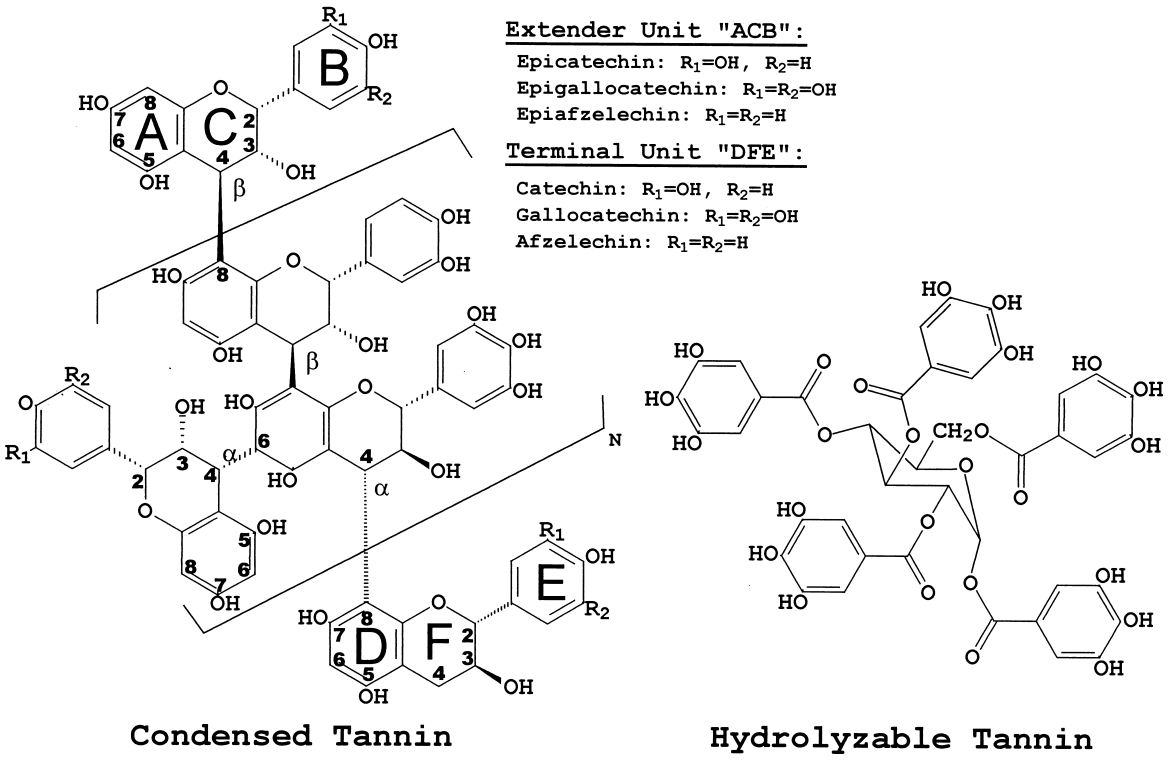

Fig. 1. Structures of typical condensed and hydrolyzable tannins.

contrast between condensed and hydrolyzable tannin has led some to attribute much different functionality of the two polyphenols within plants (Zucker, 1983).

Unlike carbohydrates, lipids, amino acids, and pigments, which are ubiquitous in organic matter and have both marine and terrestrial sources, tannin (along with lignin and cutin) is uniquely terrestrial. Thus, tannin has the potential to provide source information that is complementary to lignin and cutin. For instance, monocotyledons cannot be distinguished by lignin composition and only weakly by cutin (Goñi and Hedges, 1990). However, one condensed tannin monomer, *ent*-epicatechin, is unique to monocotyledons, and propelargonidin-containing polymers are much more common in monocotyledons than dicotyledons (Ellis et al., 1983). Conversely, hydrolyzable tannin is only found in dicotyledons (Okuda et al., 1995). Tannin-related compounds can also be used to distinguish between angiosperms and gymnosperms, as flavones are found primarily in angiosperms. In addition, condensed tannin dimers and trimers in angiosperms appear to contain more species-dependent taxonomic information that may be useful in certain environments where potential sources are more constrained (Haslam, 1989).

Tannin is the fourth most abundant component of vascular plant tissue, following cellulose, hemicellulose, and lignin. However, as mentioned above, in rapidly cycling leaf and needle tissue, tannin content can be as high as 20%. Thus in addition to biomarker potential, tannin greatly contributes to the characteristics of bulk organic matter, including color, astringency, and reactivity.

Historically, interest in tannin stems from its ability to bind with protein nitrogen during the tanning process. Geochemically, potential nitrogen binding and nitrogen immobilization by tannin is also of great interest. Nitrogen immobilization (the incorporation of exogenous nitrogen into organic matter) in sediments and submerged leaves is a poorly understood process, and the study of tannin may shed some light on this key process.

Finally, tannin offers the potential to reconstruct reaction and processing history. Tannin shows an intermediate degradability unlike the other major biochemicals, e.g., 1 to 5 yr in pine litter samples (Tiarks et al., 1992). As such, tannin may be an important tracer of intermediate rate processes. The electron transfer sensitivity of tannin might record the redox history of the organic matter as a whole. For instance, vicinol diols are prone to quinone formation in alkaline conditions. As shown in Figure 1, the B-ring of condensed tannin can be mono-, di-, or trihydroxylated. Thus, a monohydroxylated B-ring should be more stable toward oxidation than a dihydroxylated, and both should be more stable than a trihydroxylated B-ring. Ratios of the three ring types should provide redox or other diagenetic information. Another parameter sensitive to reaction history is the degree of polymerization, or the ratio of total condensed tannin (extender plus terminal units; see Fig. 1) to terminal units. Smaller polymers and oligomers are more water-soluble than larger ones, and therefore more subject to leaching. In addition, studies have shown that larger polymers are more resistant than smaller polymers to microbial degradation (Grant, 1976; Field and Lettinga, 1991). Therefore, degree of polymerization overall should provide an integration of such processes.

This study involves early diagenesis of mangrove leaves (*R. mangle*) submerged in a tropical estuary. Mangrove swamps represent an important link between terrestrial and marine ecosystems. Senescent mangrove leaf material is an important source of carbon, nitrogen, and other nutrients for estuarine food webs (Odum and Heald, 1975). In addition, utilizing solid-state ^{13}C-NMR and Folin-Denis analysis, Benner et al. (1990a) showed that much of mangrove leaf carbon is in the form of tannin. However, without molecular-level tannin information, little could be determined about the diagenetic pathway of tannin. Because of the quantitative importance of tannin but lack of sensitivity in the methods used, Benner et al. (1990a) concluded "Clearly, molecular-level methods for the characterization and quantification of tannins . . . in nonwoody vascular plant tissues need to be developed. Parallel application of such methods to soil and sediment samples may explain, in part, why a comparably large fraction of these organic materials remain uncharacterized." In large part, this observation became the impetus for the development of such a molecular-level method from existing techniques in the natural products literature (Hernes and Hedges, 2000). The application of this new method to the mangrove leaf samples of Benner et al. (1990a) represents the first study of the geochemistry of tannin at the molecular level.

2. MATERIAL AND METHODS

All sample collection and workup procedures are described in detail by Benner et al. (1990b). Briefly, green and yellow mangrove leaves were collected from trees and yellow, orange, brown, and black mangrove leaves collected from the water and surficial sediment of a tropical estuary in the Bahamas. Collected were 50 to 100 leaves in each of eleven color stages to account for tree-to-tree variability in composition. All leaves were oven-dried at temperatures below 60°C and ground up to pass through a 40-mesh screen. Benner et al. (1990b) calculated diagenetic mass loss from the leaves using relative mass per unit leaf surface area. A leaching experiment was conducted using the ground yellow leaves from trees in distilled water on a shaker table for 20 h at 4°C. Both dissolved and particulate fractions were freeze dried and ground. Elemental, solid-state ^{13}C-NMR and Folin-Denis analyses were conducted by Benner et al. (1990a).

As indicated in Benner et al. (1990b), lignin phenols were analyzed as trimethyl silyl (TMS) derivatives by gas chromatography (GC) following CuO oxidation (Hedges and Ertel, 1982). Benner et al. (1990b) also reported neutral aldose plus cyclitol yields, as determined by acid hydrolysis and also quantified by GC as TMS derivatives (Cowie and Hedges, 1984). Cutin acids can be measured simultaneously with lignin using CuO oxidation (Goñi and Hedges, 1990), and previously unpublished yields quantified from the lignin GC chromatograms are presented here. Previously unpublished uronic acid yields were measured by the hydrolysis and GC method of Walters and Hedges (1988). Finally, amino acid compositions were measured in acid hydrolyzed samples (70 min at 150°C in 6 N HCl under N_2) by HPLC. Chromatography was performed after OPA derivatization using reverse-phase C_8 or C_{18} columns and charge-matched recovery standards as outlined by Cowie and Hedges (1992).

Molecular-level condensed tannin analyses were done in duplicate by acid depolymerization in the presence of excess phloroglucinol to capture the released carbocations. Details can be found in Hernes and Hedges (2000). Briefly, ~50 mg of bulk organic matter was depolymerized in 3 mL acetone:water (70:30 vol.%) with an acid strength of 1.0 mol/L HCl and ~0.25 mol/L phloroglucinol. Interflavan bonds are protonated and broken, leaving the lower structural unit intact (can be either a terminal or extender unit) and the upper extender unit as a carbocation (Fig. 2). The carbocation is then captured by phloroglucinol either alpha or beta to the C-ring at C-4, producing a monomer-phloroglucinol adduct (Fig. 2). A side reaction in this system is the quantitative conversion of PD tannin (and to a much lesser extent, PC tannin) to a four-membered ring via the addition of acetone (Fig. 2) (Hernes and Hedges, 2000).

Depolymerization was carried out at 30°C for 24 h in culture tubes purged with Ar. All solvents were sparged with Ar. After 24 h, 10 mL water was added along with ~150 μg hematoxylin as an internal standard. Samples were extracted three times with ~5 mL ethyl acetate. The ethyl acetate was passed through a sodium sulfate drying column and evaporated under a stream of nitrogen. The samples were then placed in a vacuum dessicator overnight to remove any residual water or acid.

The following day, samples were redissolved in ~200 μL pyridine and small aliquots derivatized with equal amounts of Regisil, i.e., bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane, by heating for 10 min at 60°C. A Shimadzu AOC-14 autoinjector introduced samples (2 μL) onto an HP 5890 gas chromatograph equipped with a 0.25 mm × 30 m capillary column coated with DB35ms liquid-phase (J&W Scientific) and a flame ionization detector. Splitless injection was carried out using a column-loading time of two minutes. The injection port and detector were maintained at 310°C. Helium was used as the carrier gas. The oven temperature was maintained at 70°C during column loading, then increased to 200°C at 25°C/min. A second ramp of 4°C/min increased the oven temperature to 330°C, which was then maintained for 30 min. Electronic pressure control was also used, with an initial column head pressure of 13 psi held for two minutes and then increased to 30 psi at 1 psi/min. A typical chromatographic trace is shown in Figure 3.

Selected samples were injected onto a gas chromatograph/mass spectrometer (HP 5890 gas chromatograph interfaced to an HP 5970 mass selective detector) for peak identification. Although standards exist for many monomeric compounds, only very recently have phloroglucinol adducts become commercially available. For this study, catechin adducts were identified by the depolymerization of a catechin-catechin dimer (Leuven Bioproducts) in the presence of phloroglucinol. Remaining adduct identities are based on mass spectral fragmentation patterns and elution orders as determined by the catechin and epicatechin adducts. Triterpenoid identities were determined from commercially available standards as well as a library of trimethylsilylized naturally occurring compounds generated by W. Greenaway (University of Oxford, Oxford, UK).

Quantification was done using the hematoxylin internal standard and relative responses to available standards. Where stan-

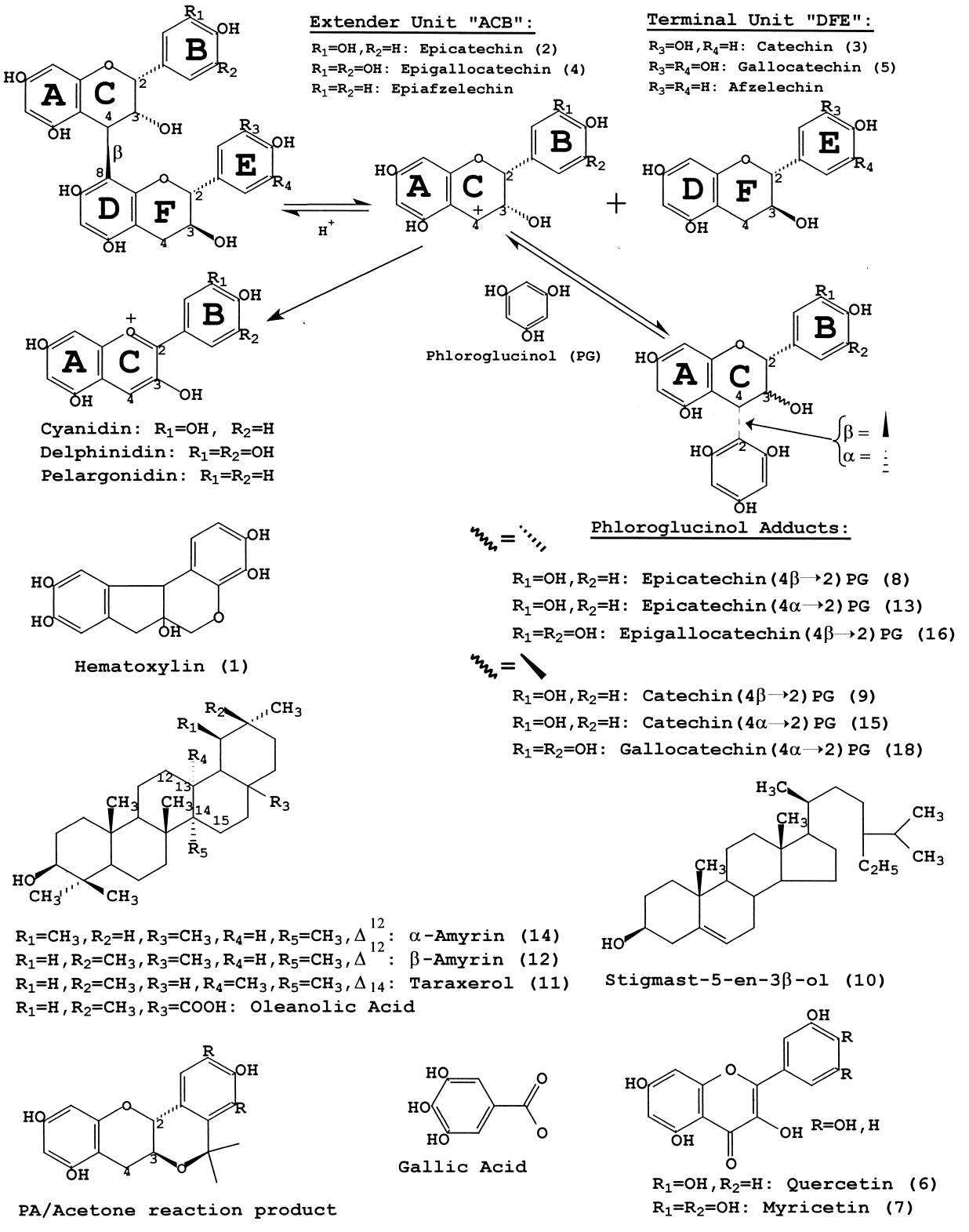

Fig. 2. Acid depolymerization of a condensed tannin dimer with and without phloroglucinol. Structures of all compounds quantified are shown. Numbers in parentheses correspond to elution order as shown in Figure 3.

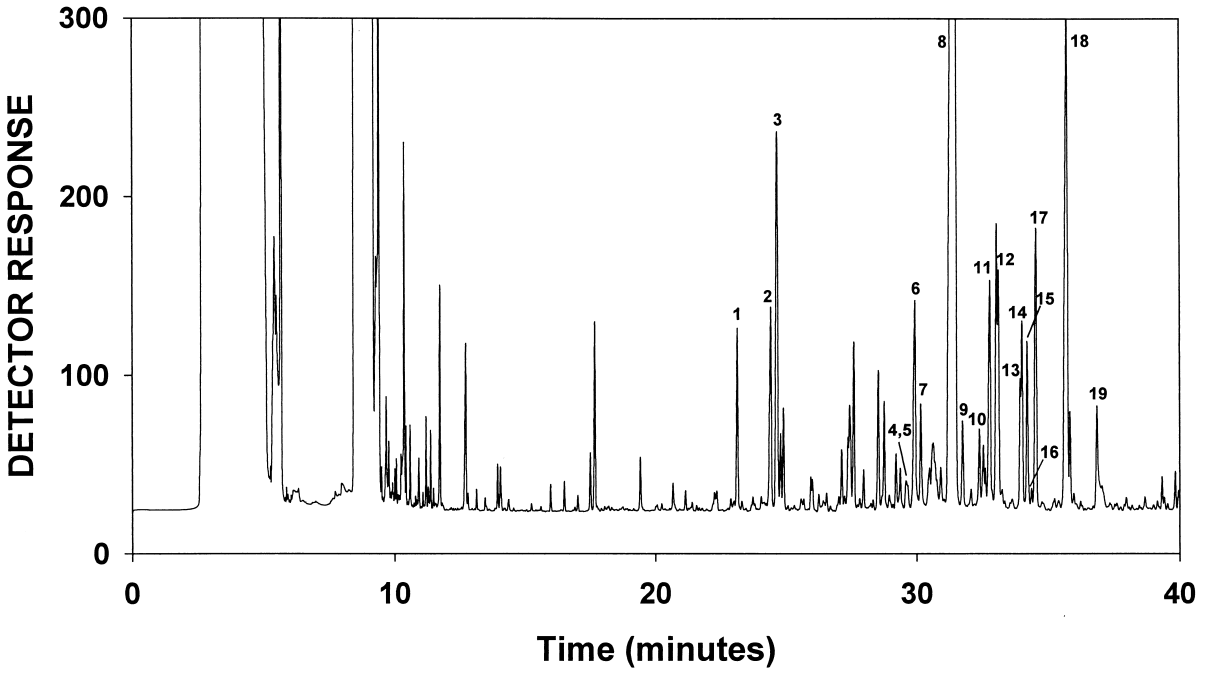

Fig. 3. GC trace of yellow mangrove leaves. 1 = Hematoxylin, 2 = Epicatechin, 3 = Catechin, 4 = Epigallocatechin, 5 = Gallocatechin, 6 = Quercetin, 7 = Myricetin, 8 = Epicatechin(4β→2)phloroglucinol, 9 = Catechin(4β→2) phloroglucinol, 10 = Stigmast-5-en-3β-ol, 11 = Taraxerol, 12 = β-Amyrin, 13 = Epicatechin(4α→2)phloroglucinol, 14 = α-Amyrin, 15 = Catechin(4α→2)phloroglucinol, 16 = Epigallocatechin(4β→2)phloroglucinol, 17 = PC/acetone(4→2) phloroglucinol, 18 = Gallocatechin(4α→2)phloroglucinol, 19 = PC/acetone(4→2)phloroglucinol.

dards were unavailable, the hematoxylin response was used as a default. The reproducibility of all the major compounds was ±5 to 10% mean deviation, whereas trace compounds were within ±10 to 30%.

3. RESULTS AND DISCUSSION

3.1. Bulk Decomposition Indices

In the early diagenesis of leaves, color is often a useful marker for the various stages of decomposition. During sample collection, Benner et al. (1990b) sorted submerged mangrove leaves into eleven different groups based on gradations of color, but ultimately opted to combine them into yellow, orange, brown, and black categories. A relationship established between average leaf mass and area indicated 2% mass loss in yellow submerged leaves as compared to yellow senescent leaves still attached to trees. Corresponding 6, 27, and 36% mass losses were determined for orange, brown, and black leaves, respectively. Benner et al. (1990b) estimated that these losses corresponded to approximately 1 week submerged in water for orange leaves, 4 weeks for brown leaves, and 6 to 7 weeks for black leaves. Relative mass-to-area ratios proved useful for normalizing molecular data between color types when discussing diagenetic trends. Much of our discussion also adopts this format in which [Compound X] × [Percent mass remaining] (i.e., 100, 98, 94, 73, and 64% for the yellow senescent leaves and four leaf colors present in the water) is normalized to the content of Compound X in yellow senescent leaves. In this formulation, percentage yields above 100% represent increases in the "absolute" yields of Compound X, as opposed to conservative behavior. An example of such an increase was shown by Benner et al. (1990a) for total nitrogen, in which nitrogen, after an initial drop in the yellow submerged leaves, steadily increased in the orange, brown, and black leaves to absolute recoveries of 150% (50% more than the original level). Increasing nitrogen in submerged plant material has been documented in several different environments with several different types of plant tissue and is commonly referred to as nitrogen immobilization (Rice and Tenore, 1981; Rice, 1982; Qualls, 1984; Bowden, 1986; Melillo et al., 1984; Benner et al., 1991). Given the potential relationship between tannin and nitrogen, this becomes an important trend to keep in mind when interpreting the tannin molecular data.

3.2. Tannin Yields and Composition

Four terminal units and four extender units of condensed tannin were identified overall in the acid depolymerization mixture from the mangrove leaf samples analyzed (Table 1). Included in our survey were the six basic leaf categories reported by Benner et al. (1990a,b), along with Orange #5, which represents the most degraded of three orange leaf subgroups, and Brown #6, which represents the least degraded of the three brown leaf subgroups. The rationale for including the latter two samples is discussed below. The structural precursors of all analyzed compounds (before trimethylsilyl derivatization) are shown in Figure 2. Because alpha and beta adducts from the extender units are a result of carbocation chemistry and not

Table 1. Tannin and triterpenol compositions and yields (mg g^{-1} tissue AFDW) from decaying mangrove leaves[a].

Leaf color	Condensed tannin										Triterpenols				
	Terminal Units				Extender Units				Tannin-related						
	C	EC	GC	EGC	C	EC	GC	EGC	GA	FON	αAM	βAM	TRX	STG	OLA
Collected from trees															
Green	5.49	2.03	0.21	0.31	3.5	39.8	7.96	0.13	0.02	3.63	0.24	0.47	1.82	0.66	nd
Yellow	6.05	2.74	0.22	0.59	3.1	36.0	10.4	0.21	0.04	6.58	2.64	3.58	4.32	1.28	nd
Collected from water															
Yellow	5.76	3.51	0.26	0.40	3.6	43.0	11.6	0.29	0.21	6.30	2.21	4.25	4.39	1.33	nd
Orange	6.22	3.55	0.16	0.29	4.2	48.3	9.28	0.37	0.30	6.32	3.85	5.60	5.61	1.99	nd
Orange #5	4.21	1.97	0.05	0.11	1.9	38.3	6.65	0.17	0.14	2.00	3.10	4.66	3.77	1.32	nd
Brown #6	2.09	0.86	nd	nd	1.4	20.7	1.18	0.11	0.09	0.49	3.65	6.45	3.34	1.39	0.21
Brown	1.67	1.15	nd	nd	1.1	10.8	0.52	0.13	0.07	0.49	6.06	10.3	6.72	1.86	0.66
Black	1.09	0.59	nd	nd	0.3	3.31	0.21	0.00	0.02	nd	6.90	13.7	6.80	2.73	4.05
Laboratory leached yellow tree leaves															
Leaves	4.02	1.82	0.17	0.19	2.4	36.9	11.4	0.13	0.01	2.85	2.53	4.04	3.97	1.08	nd
Leachate	13.01	4.30	1.45	1.43	6.2	52.6	3.84	0.12	0.13	11.2	<0.01	0.15	nd	nd	nd
Mass balance	5.87	2.33	0.43	0.45	3.2	40.11	9.82	0.13	0.03	4.58	2.01	3.24	3.15	0.86	nd

[a] Abbreviations: AFDW = ash-free dry weight, C = (+) catechin, EC = (−) epicatechin, GC = (+) gallocatechin, EGC = (−) epigallocatechin, GA = gallic acid, FON = total flavones, αAM = α-amyrin, βAM = β-amyrin, TRX = taraxerol, STG = stigmastenol, OLA = oleanolic acid, nd = not detected.

indicative of interflavan stereochemistry (interflavan bonds are all thought to be *trans* to the hydroxyl group at C-3), alpha and beta adducts of the same monomer type (i.e., catechin(4α→2) phloroglucinol and catechin(4β→2)phloroglucinol) are summed. Total yields of the condensed tannin ranged from 5.5 mg g^{-1} ash-free dry weight (AFDW) in the black leaves to 72.3 mg g^{-1} AFDW in the orange leaves. In addition to condensed tannin, other tannin-related peaks were identified, including gallic acid, which is potentially derived from hydrolyzable tannin, and flavones (6 and 7), which are structurally related to condensed tannin (Fig. 2). Gallic acid was a minor constituent, ranging from 0.02 to 0.30 mg g^{-1} AFDW, whereas total flavones ranged from undetected to 6.58 mg g^{-1} AFDW.

Compositionally, condensed tannin consisted of predominantly catechin (3) and epicatechin (2) (often referred to collectively as procyanidin or PC tannin), which are the most common types of condensed tannin. Catechin and epicatechin terminal units ranged from 8 to 20% and 3 to 11%, respectively, of the total condensed tannin, whereas catechin and epicatechin extender units represented 5 to 7% and 61 to 79%. The remainder of the condensed tannin consisted of gallocatechin (5) and epigallocatechin (4). Together, these prodelphinidin (or PD) tannins ranged from undetected to <1% for (5) and undetected to 1% for (4) in the terminal units, and 4 to 18% for (5) and undetected to <1% for (4) in the extender units. Generally, tannin compositions remained stable through to the orange stage (approximately 1 week in the water), before undergoing noticeable change in the brown and black stages.

3.3. Triterpenols

In addition to tannins, four triterpenols and a sterol were recovered from the acid depolymerization mixture: α-amyrin (14) and β-amyrin (12) (0.24–6.90 mg g^{-1} AFDW and 0.47–13.7 mg g^{-1} AFDW, respectively), taraxerol (11) (1.82–6.80 mg g^{-1} AFDW), oleanolic acid (10) (undetected to 4.05 mg g^{-1} AFDW), and the sterol stigmast-5-en-3β-ol (10) (0.66–2.73 mg g^{-1} AFDW). All compounds were found in higher concentrations in the brown and black leaves than in the yellow leaves, indicating preferential preservation or chemical release from their matrices. Total yields ranged from 3.2 to 34.2 mg g^{-1} AFDW. Our results can be qualitatively compared to the work of Killops and Frewin (1994). Using GC-MS, these authors detected taraxerol and β-amyrin as the primary triterpenols in *R. mangle* leaves, along with stigmast-5-en-3β-ol and α-amyrin (tentatively identified as lupeol in their study, but confirmed with an authentic standard as α-amyrin in this study) in lesser amounts.

The triterpenols and sterol present in these mangrove leaves are very unreactive and, in fact, show absolute increased recoveries relative to yellow senescent leaves. Killops and Frewin (1994) reported that β-amyrin in *R. mangle* leaves was largely confined to the epicuticular wax, whereas taraxerol appeared to be a cutin component. They further suggest that the resistance of cuticular membranes to degradation allows such triterpenols to persist into the sediments where they were able to detect them. Thus, the absolute increase in triterpenol recoveries in this study may be indicative of a weakening cuticular membrane structure that, while still capable of providing in situ protection, is more susceptible to the acid conditions in our tannin analyses. Oleanolic acid, which is only measurable in the brown and black leaves, could also result from weakening of cuticular membranes or may, in fact, be produced within the leaves by oxidation of β-amyrin (see Fig. 2 for structural relationships. β-amyrin and oleanolic acid differ only in the functional group R_3). The latter, however, does not seem likely given that α-amyrin and β-amyrin yields increase in parallel

Table 2. Tannin compositional parameters from decaying mangrove leaves[a].

Leaf Color		Percent PC:PD tannin			Percent 2,3-*cis*:2,3-*trans*		
	X_n	Ext	Term	Total	Ext	Term	Total
Collected from trees							
Green	7.4	84:16	93:7	86:14	78:22	29:71	71:29
Yellow	6.2	79:21	92:8	81:19	73:27	35:65	67:33
Collected from water							
Yellow	6.9	80:20	93:7	82:18	74:26	39:61	69:31
Orange	7.1	84:16	96:4	86:14	78:22	38:62	73:27
Orange #5	8.4	85:15	98:2	87:13	82:18	33:67	76:24
Brown #6	8.9	94:6	100:0	95:5	89:11	29:71	82:18
Brown	5.4	95:5	100:0	96:4	87:13	41:59	79:21
Black	3.3	94:6	100:0	96:4	88:12	35:65	71:29
Laboratory leached yellow tree leaves							
Leaves	9.2	77:23	94:6	79:21	73:27	32:68	69:31
Leachate	4.1	94:6	86:14	92:8	84:16	28:72	70:30

[a] Abbreviations: X_n = degree of polymerization, PC = procyanidin (catechin and epicatechin), PD = prodelphinidin (gallocatechin and epigallocatechin), 2,3-*cis* = epicatechin and epigallocatechin, 2,3-*trans* = catechin and gallocatechin, Ext = extender units, Term = terminal units.

(Table 1). If oxidation were an important mechanism, then the oxidative counterpart of α-amyrin (ursolic acid) would also be expected in the brown and black leaves, but it was not detected. In addition, it is not apparent why only one of the methyl groups would be selectively oxidized to a carboxylic group. Stigmast-5-en-3β-ol is a common sterol in vascular plant tissues and shows a weak increasing trend.

3.4. Source Signature

Overall, the green and senescent yellow mangrove leaves from trees exhibit a composition consistent with that expected for fresh angiosperm dicotyledon leaves, with a PD tannin content of $<$ 20% (Table 2), as compared to gymnosperm needles which typically have PD contents $>$ 50%. The taxonomic generalizations presented here are based solely on the analyses of $\sim$120 plant tissues using the identical tannin molecular method (Hernes and Hedges, 1999). This avoids the pitfalls of comparing tannin data obtained from the widely ranging analytical conditions used in previous taxonomic studies, although in general, the trends obtained with this technique compare well with the broader literature (see Hernes and Hedges, 1999, 2000).

A second commonly measured parameter is the relative proportion of 2,3-*cis* forms (epicatechin and epigallocatechin) to 2,3-*trans* forms (catechin and gallocatechin) (see Fig. 2). The 2,3-*cis* forms in the green and yellow senescent leaves constitute 78 and 73%, respectively, of all extender units, but only 29 and 35% in the terminal units (Table 2). This pattern also is typical of dicotyledon leaves, as compared to gymnosperm needles in which 2,3-*cis* forms make up $<$ 50% of all extender units (Hernes and Hedges, 1999).

A third diagnostic source indicator is the degree of polymerization. This parameter corresponds to the total yield of condensed tannin divided by the corresponding yield of terminal units and gives an indication of the average chain length. Values for green and senescent yellow leaves were 7.4 and 6.2, which are higher by a factor of three than most other dicotyledon leaves analyzed with this technique (Hernes and Hedges, 1999).

Other source indicators include flavones, which are more common in dicotyledon leaves than gymnosperm needles (Hernes and Hedges, 1999), and the flavone content of the green and senescent yellow mangrove leaves are representative. The triterpenols α-amyrin, β-amyrin, and taraxerol measured in these samples appear to be exclusive to angiosperms and rare in monocotyledons (Gershenzon and Croteau, 1991). Finally, in comparison to other dicotyledon leaves, the tannin content of these pendant mangrove leaves was the highest of 16 temperate and 24 tropical species analyzed by this method (Hernes and Hedges, 1999).

A primary concern when using any compounds as source markers is the effect of diagenesis on the overall source signature. This study provides an excellent opportunity to look at some of these effects for molecular tannin and triterpenoids. In comparing the black submerged leaves to the senescent yellow leaves from trees, several relevant changes in composition are evident: (1) PD content drops from 8% to undetected in terminal units and from 19 to 4% overall, (2) flavones are no longer detected in the black leaves, (3) the 2,3-*cis* content of extender units decreases from 27 to 12% but only from 33 to 29% overall, (4) the calculated degree of polymerization decreases from 6.2 to 3.3, (5) measured total triterpenoids increase by a factor of three, and (6) overall tannin content decreases from 59 mg g^{-1} to 5.5 mg g^{-1} (Tables 1 and 2). Thus, while the composition of the black leaves is still recognizable as dicotyledon in origin, it would be difficult to attribute that composition specifically to mangrove leaves if the source was unknown.

3.5. Comparison of Total Molecular Tannin to Bulk Estimates

Previously, Benner et al. (1990a) established the quantitative importance of bulk tannin in senescent and decaying mangrove (*R. mangle*) leaves using ^{13}C-NMR and Folin-Denis analyses.

Although ^{13}C-NMR estimates of tannin were relatively constant at ~20 wt.% during leaf senescence and decomposition, Folin-Denis estimates were much more variable, ranging from ~14% tannic acid equivalent (TAE) in yellow submerged leaves to < 1% TAE in brown and black submerged leaves. Because hydrolyzable tannin had been previously thought to be more soluble in water than condensed tannin (e.g., Zucker, 1983), Benner et al. (1990a) hypothesized that the extractable tannin measured using the Folin-Denis reagent was primarily hydrolyzable. They also suggested that the residual tannin (i.e., the component of bulk tannin as estimated by ^{13}C-NMR that is unaccounted for by Folin-Denis results) in the leaf complex was condensed. Although this may be partly true, it is worth noting that on a mass-balance basis, the residual tannin actually increased by 15 to 25% in the latter stages of decomposition, suggesting that at least that much of the residual tannin in these latter stages may in fact be chemically altered tannin that was previously extractable.

A primary concern when using either ^{13}C-NMR or Folin-Denis to estimate tannin is that both are subject to false positives. Since both measurements rely on functional groups instead of the whole molecule, any non-tannin compound with similar functionalities will register as tannin. Such spurious contributors include lignin, lignan, and aromatic amino acids in the case of ^{13}C-NMR, and ascorbic acid and peptides in the case of Folin-Denis. Lignin has been measured in these mangrove leaves (Benner et al., 1990b) at ~2% and thus is a minor contributor to total phenolics as measured by ^{13}C-NMR (confirmed by dipolar dephasing experiments). Similarly, the contribution of aromatic amino acids has been measured at < 0.5% (Cowie, unpubl. data).

Our molecular-level tannin data (Table 1) do not definitively confirm or refute the hypothesis of Benner et al. (1990a) for selective loss of hydrolyzable tannin. On one hand, the total condensed tannin measured molecularly is somewhat less than the residual tannin (0.5 to 7.2% AFDW) estimated from the difference between the ^{13}C-NMR and colorimetric analyses (~8 to ~20%). The solvent system (i.e., 70:30 v/v acetone: water) used in our depolymerization, however, is identical to that used by Benner et al. (1990a) for extraction before Folin-Denis analysis. In addition, the pattern we measured along the color series is similar to the pattern Benner et al. (1990a) determined using Folin-Denis reagent (Fig. 4) with an initial increase in the yellow and orange leaves followed by a rapid drop in the brown and black leaves. Although we detected gallic acid (potentially derived from hydrolyzable tannin) only in trace amounts, it too roughly follows the pattern measured with Folin-Denis reagent (Fig. 4). Determining the chemical compositions of the lost and residual tannin is not merely a geochemical bookkeeping exercise: Hydrolyzable and condensed tannin are very different from each other both structurally and reactively (Fig. 1) (Hemingway, 1988a,b; McGraw, 1988; Laks, 1988; Okuda et al., 1995; Haslam, 1989). Compositional differences among condensed tannins have been shown to influence feeding behavior of herbivores (Clausen et al., 1990) and affect ease of depolymerization (Hemingway and McGraw, 1983), factors that are relevant to tannin degradation and preservation.

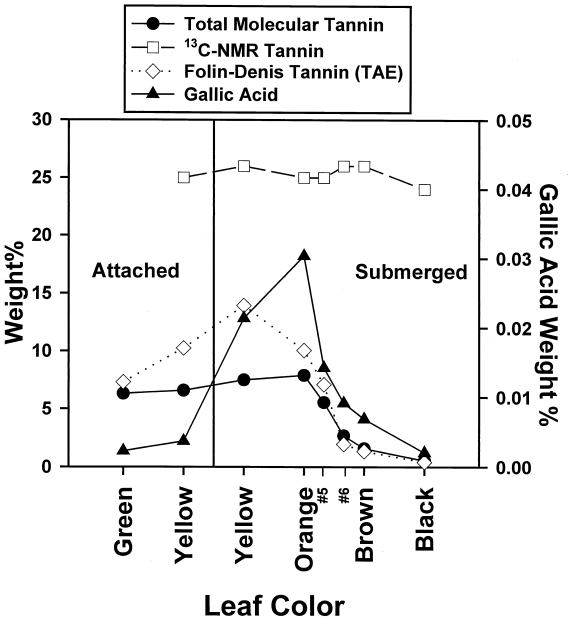

Fig. 4. Bulk estimates of tannin utilizing ^{13}C-NMR, Folin-Denis analyses, total molecular tannin, and gallic acid.

3.6. Quantitative Comparison of Total Molecular Tannin to Other Compound Classes

The mangrove leaf samples are among the most comprehensively-characterized organic materials in the biogeochemical literature with molecular-level analyses of eight different compound classes (tannin, triterpenoids, lignin, polysaccharides, cyclitols, amino acids, cutin, and uronic acid) in addition to solid-state ^{13}C-NMR and several bulk analyses (this study, Benner et al., 1990a,b; Cowie, unpubl. data; Goñi, unpubl. data; Bergamaschi, unpubl. data). In total, 36 to 55% of the leaf tissue can now be accounted for at the molecular level (Fig. 5). There are several striking trends in Figure 5, including the effect of leaf senescence on amino and uronic acids, the predominance of neutral carbohydrates, and increases in amino acids and triterpenoids during diagenesis. However, perhaps the most novel is that the quantitative importance of tannin at the molecular level is directly demonstrated for the first time. In the yellow leaves entering the estuary, measured molecular tannin is second in abundance only to neutral carbohydrates. Hence, tannin alteration is a critical process to consider in the degradation of these leaves and in attending trends in their bulk chemical properties.

3.7. Diagenesis Comparisons to Other Compound Classes

Total molecular tannin shows quite different lability from that evident for other measured biochemicals. Total molecular cyclitols show an immediate and rapid decline between the mangrove leaves on trees and those in the water, eventually dropping to ~1% of the amount found in attached leaves (Fig. 6) (Benner et al., 1990b). Molecular lignin, neutral carbohydrates, uronic acids, cutin, and leaf mass show a steady, parallel decline to 40 to 60% of the amounts found in attached leaves. In contrast, amino acids and triterpenoids appear to accumulate,

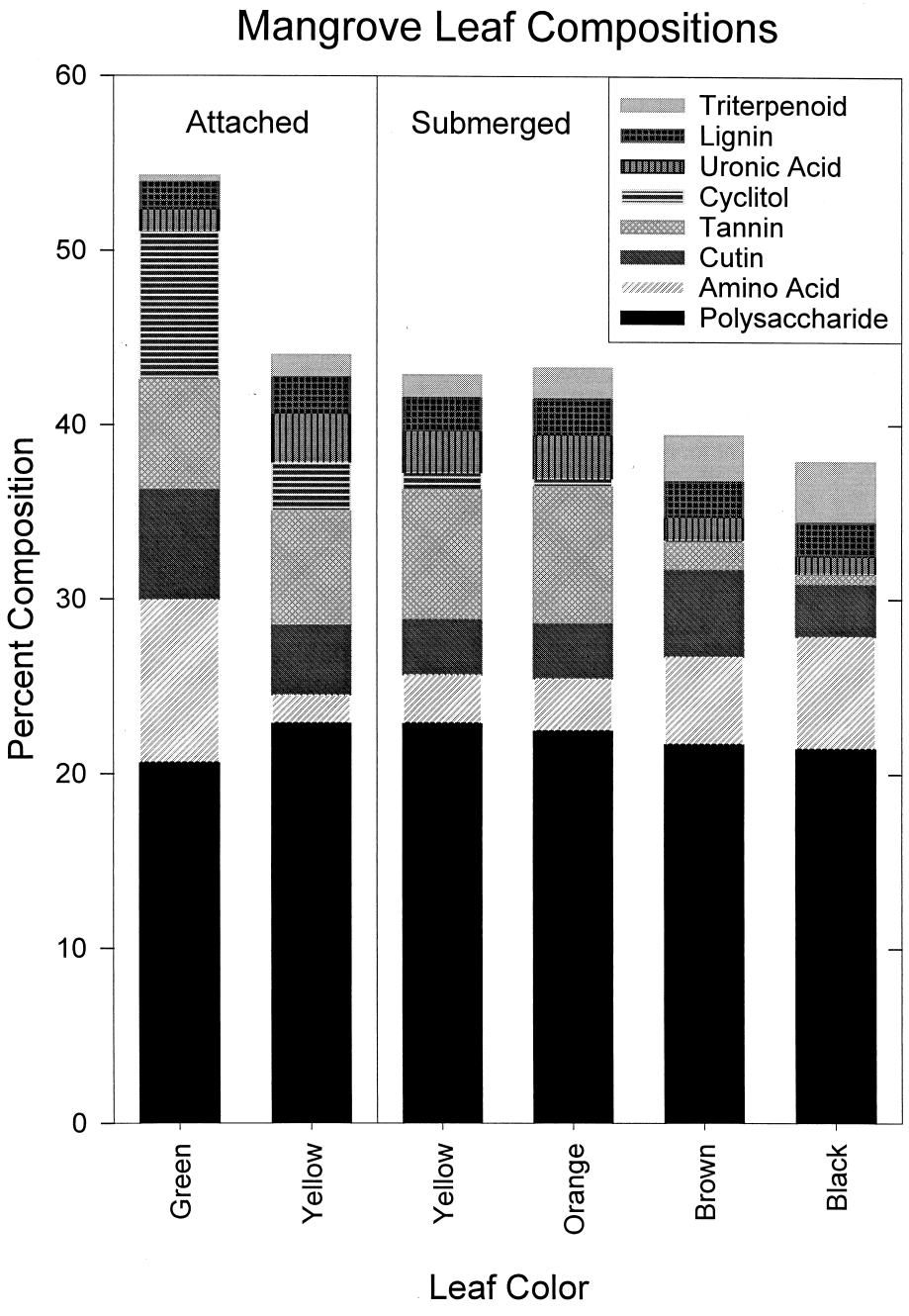

Fig. 5. Percent of organic matter (i.e., AFDW-based) occurring in mangrove leaves as total triterpenols, lignin, uronic acids, cyclitols, tannin, cutin, amino acids, and polysaccharides (all measured chromatographically at the molecular level). Compound types are stacked in the same order as they appear in the legend. Not shown is the chromatographically uncharacterized fraction of total organic matter remaining at each degradation stage.

with nearly three times the amount present in black than in senescent yellow leaves. With its initial increase in yellow and orange leaves in the water and rapid drop off in the brown and black leaves, molecular tannin straddles all other biochemical trends. Geochemically, tannin appears to be unique relative to the other biomarkers in its intermediate lability, and therefore may be particularly valuable in determining early diagenetic mechanisms.

3.8. Leaching

In mangrove swamps, leaching has been shown to be an important process in the initial stages of leaf degradation (Benner et al., 1988). The Benner et al. (1990b) leaching experiment resulted in 21% overall mass loss from yellow senescent leaves after 20 h. Mass balance for total molecular tannin indicates nearly complete recovery of the senescent leaf tannin between

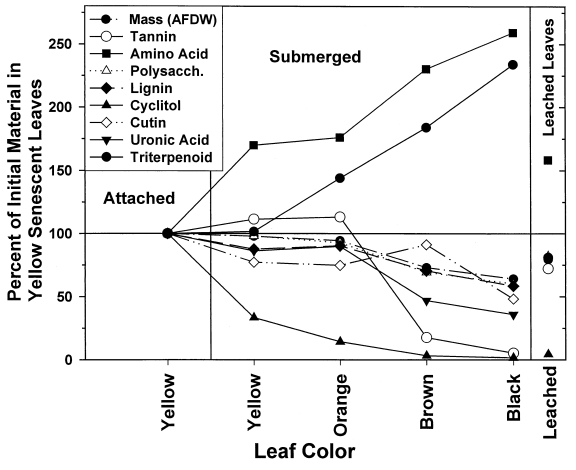

Fig. 6. Percent of initial measured masses of triterpenols, lignin, uronic acids, cyclitols, tannin, cutin, amino acids, and polysaccharides present in yellow senescent leaves along the degradation sequence.

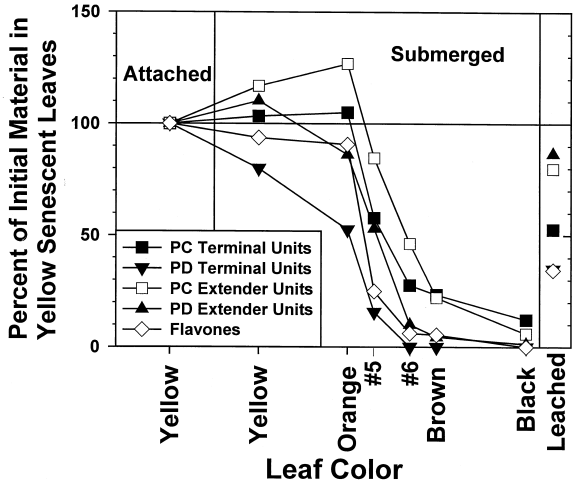

Fig. 7. Percent of initial procyanidin (PC) and prodelphinidin (PD) tannin masses for both terminal and extender units, along with flavones.

the leachate and leached leaf fractions (Table 1). Overall, tannin loss from the leached leaf material was ~30%, with higher losses among the PC extender units and lower losses among the PD extender units. Just the opposite leaching pattern was observed for terminal tannin units. Coupled with the lower degree of polymerization in the leachate (4.1 vs. 9.2 in the residue; see Table 2), this trend indicates that the smaller oligomers are made up of a higher proportion of PC extender units and a higher proportion of PD terminal units than the larger oligomers. The significance of this observation is that PD tannin, with its three vicinol triols on the B-ring, is more reactive than PC tannin. Thus, in the absence of any protective matrices, the tannin remaining in the leached leaves is potentially more diagenetically labile than the leached tannin. Gallic acid showed > 70% loss, which may indicate that the hydrolyzable tannin in these leaves is more soluble than condensed tannin, if indeed gallic acid is representative of hydrolyzable tannins. The flavones also exhibited higher losses (~40%), which suggest weak associations with the original plant matrix. The triterpenols were not appreciably leached, yet did not exhibit a commensurate increase in the leached leaves as would be expected for this conservative behavior.

On the diagenetic scale (as indicated by leaf color), the overall mass loss in the leached leaves (21%) places them between the orange (6%) and brown leaf (27%) stages, although more closely aligned with the latter. Molecular tannin yields from the leached leaves also fall between the orange and brown leaf stages (Table 1; Fig. 7), although more closely aligned with the former. Thus, leaching is likely not the only process involved in tannin loss from submerged mangrove leaves. Because this transition from orange to brown leaves is such an important stage in tannin diagenesis, Benner et al. (1990b) original Orange #5 and Brown #6 leaf samples (referred to above) were analyzed to provide greater coverage and shed additional light on the alteration processes involved. In terms of mass loss, mass-to-area ratios of the Orange #5 and Brown #6 samples indicate an 18% mass loss, which is comparable to the 21% loss in the leaching experiment. As would be expected, tannin measurements from these two samples fall between the orange and brown values (Table 1; Fig. 7). Once again, however, the results are split, as the PC tannin in leached leaves aligns with the Orange #5 samples, whereas PD tannin is more similar to the composite orange sample. One interpretation of this pairing would be that PC tannin loss is entirely accounted for by leaching, while additional processes are necessary to explain PD tannin loss. This result is not surprising, since PD tannin (due to its three vicinol hydroxyl groups) should be more reactive diagenetically than PC tannin. Because the orange and brown composite samples include a component of Orange #5 and Brown #6, respectively, a mathematical correction to Figure 7 would increase all the orange leaf values and decrease the brown leaf values.

Finally, we can evaluate tannin leaching in the context of other compound classes. Notably, every molecular total (with the exception of the triterpenols) indicates mass loss from leaching that falls between those of submerged orange and brown leaves (Fig. 6). The amino acid measurements are perhaps the most surprising. They point to increases in protein in the early stages of diagenesis that are not necessarily due to accumulating microbial biomass or metabolites, but simply unmasking (or "softening") of protein that is already present in the leaves. Although it is clear from the tannin data that individual compounds do not all follow the same leaching/diagenetic pattern, the molecular totals make a convincing argument for leaching as the dominant factor in the first 1 to 2 weeks of diagenesis.

3.9. Chemical Alteration and Diagenetic Parameters

While leaching clearly accounts for early losses through the orange leaf stage, chemical alteration and degradation become prominent in the brown and black stages. A number of predictions can be made about relative reactivities of molecular tannin based on structure, including polymer length, polymer makeup, and hydroxylation patterns.

3.9.1. Degree of polymerization

The first of these parameters is the average length of the tannin oligomers (or polymers) present in the sample, which is

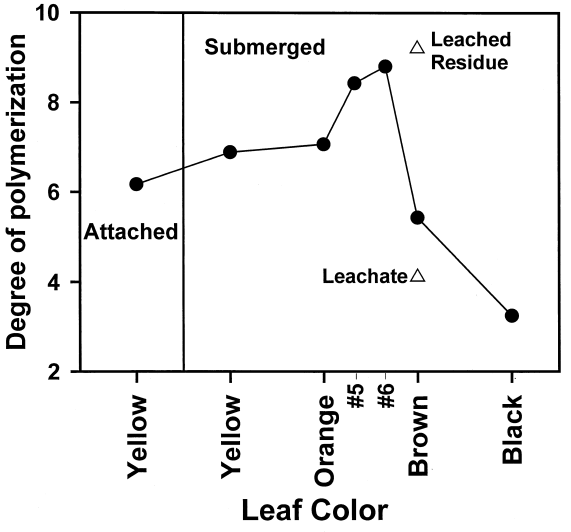

Fig. 8. Degree of polymerization in mangrove leaves along the degradation sequence. Included for reference are values for leached leaves and the leachate from laboratory experiment.

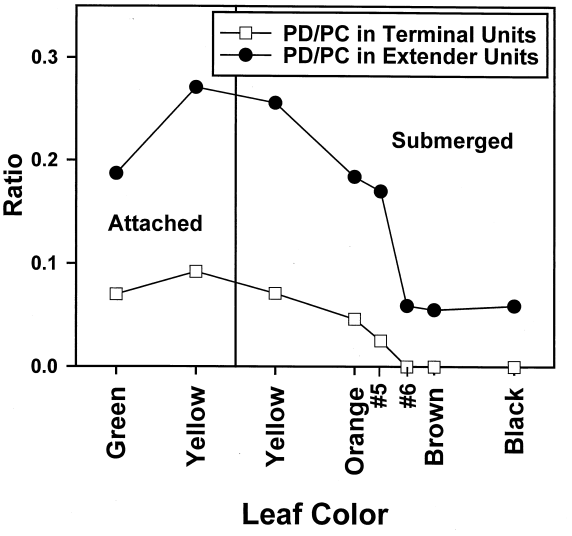

Fig. 9. Ratio of PD to PC in extender and terminal units of tannin in mangrove leaves along the degradation sequence.

indicated by the degree of polymerization as defined above. Typically a higher degree of polymerization should correspond to lower solubility and greater resistance to degradation. The former is certainly borne out by results from the leaching experiment in which the degree of polymerization in the leachate was only 4.1, whereas in the leached leaves it was 9.2 (Table 2). In fact, in the early stages of diagenesis, when leaching predominates, the degree of polymerization increases from 6.2 in the senescent yellow leaves to 8.8 in the submerged Brown #6 leaves (Fig. 8).

The relationship of degree of polymerization to degradation, however, is less straightforward. Microbial degradation studies on condensed tannin generally show the predicted trend, i.e., that larger polymers are more resistant to degradation (Grant, 1976; Field and Lettinga, 1991). However, as degradation progresses and the more labile components are removed, eventually the larger polymers remaining will also be degraded, and this may be a factor in the decrease in degree of polymerization in the brown and black stages of mangrove leaf diagenesis. One point to keep in mind is that phenolic carbon (as detected by ^{13}C-NMR) remains quantitatively constant, an indication that while tannin is clearly reacting, it is not being completely remineralized by microbial utilization. Thus, although microbial degradation likely plays some role in tannin conversion in these mangrove leaves, it may not be the direct role indicated in the studies of Grant (1976) and Field and Lettinga (1991). An abiotic explanation might also be plausible. A decrease in degree of polymerization suggests that terminal units are more degradation-resistant than extender units. This is certainly true abiotically under acidic conditions, as depolymerization will leave the terminal unit intact while forming carbocations from extender units that can then undergo any number of reactions (including capture by a nucleophile). Under basic conditions in the presence of oxygen, both the extender unit and terminal unit are subject to degradation and rearrangement (e.g., Ferreira et al., 1992). Thus, although marine waters are generally alkaline, the decrease in degree of polymerization measured in the brown and black leaves could result from an acidic microenvironment characteristic of decaying organic matter (Sagemann et al., 1999). However, as will be shown below, the overall degradation pattern of tannin is more consistent with an alkaline environment.

A second factor in the observed pattern is the relationship between degree of polymerization and composition. The leaching experiment indicated enrichment in PD tannin and an increase in degree of polymerization in the leaf residue relative to the whole leaf, thus suggesting that larger polymers in the mangrove leaves are enriched in PD tannin. Since PD tannin should be more reactive than PC tannin, the larger PD-enriched polymers may also be more reactive, which could explain why the degree of polymerization subsequently decreases. However, hydroxylation cannot be the primary factor involved, as the Brown #6 sample has a PC:PD ratio almost identical to the brown and black leaves, but a degree of polymerization of 8.9 vs. 5.4 and 3.3 (Table 2).

3.9.2. Degree of hydroxylation

Tannin reactivity stems from the number of hydroxyl groups present. In the case of the B-ring, more vicinol hydroxyl groups lead to greater potential for quinone formation. Therefore, one B-ring hydroxyl group, as in propelargonidin (PP) tannin, should be less reactive than two vicinol hydroxyl groups, as in PC tannin, and both should be less reactive than trihydroxylated B-rings, as in PD tannin. However, only two of the three types are represented in this data set: PC and PD. After an initial increase between green and senescent yellow leaves, PD:PC ratios for both terminal and extender units decrease through the brown and black stages (Fig. 9). This consistent trend supports the notion that more extensively hydroxlated tannins are more susceptible to oxidative alteration, even though normalization to a PP "control" is not possible.

*3.9.3. 2,3-*cis*:2,3-*trans *ratios*

It is not clear from theory or the literature whether the stereochemistry at C-2 and C-3 would be expected to have any impact on reactivity of tannin monomers. However, in tannin oligomers (and polymers), it has been shown that the depolymerization cleavage rate of 2,3-*cis* forms of extender units is nearly a factor of two greater than the cleavage rate of 2,3-*trans* forms (Hemingway and McGraw, 1983). This contrast could lead to a diagenetic decrease in *cis*:*trans* ratios. On the other hand, PD extender units measured in this study are enriched in 2,3-*trans* monomers, whereas PC extender units are enriched in 2,3-*cis* monomers; therefore, it might be expected that as PD tannin decreases, the *cis*:*trans* ratio would increase. From Table 2, it is clear that the *cis*:*trans* ratio of extender units increases, which suggests in this case that hydroxylation extent is a more important factor than stereochemistry. Terminal units in general are much more variable in PC and PD tannin content, and as such, exhibit no clear diagenetic trend.

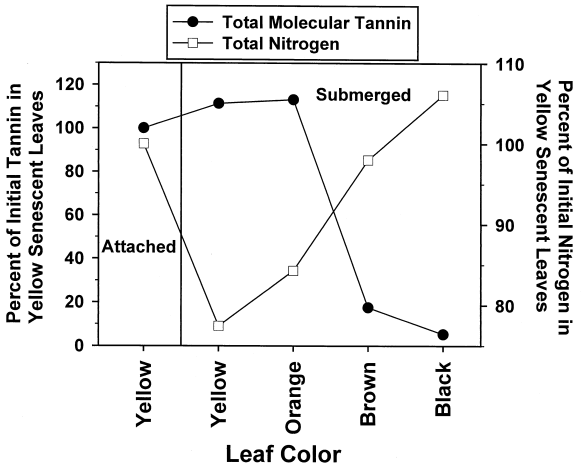

Fig. 10. Percent of initial tannin and total nitrogen in yellow senescent mangrove leaves and submerged counterparts.

3.10. Potential Role of Tannin in Nitrogen Immobilization

When vascular plant material decomposes in aqueous environments, researchers have consistently noted an initial leaching phase in which nitrogen content decreases, as Benner et al. (1990a) observed in this sample set. However, as exhibited by this sample set, leaching is typically followed by an absolute increase in the nitrogen content of the degrading material (often termed nitrogen immobilization) beyond what is possible with conservative behavior (e.g., Rice, 1982; Melillo et al., 1984). Neither the exogenous sources of this nitrogen nor the processes by which it becomes refractory are known. The dilemma surrounding its exogenous source has been delineated clearly in a number of investigations of plant tissue decomposition in different environments, including coastal salt marshes (White and Howes, 1994; Benner et al., 1991; Rice, 1982; Rice and Tenore 1981; Rice and Hanson, 1984), coastal mangroves (Rice, 1982; Rice and Tenore 1981; Rice and Hanson, 1984; Zieman et al., 1984; Benner et al., 1990a), streams (Qualls, 1984), and rivers (Melillo et al., 1984; Bowden, 1986). Whereas most investigators advocate a microbial source for the immobilized nitrogen, direct molecular or isotopic evidence for this origin has not been presented. All indications are that microbial biomass can account directly for only a few percent of the nitrogen that accumulates on decaying tissues (Lee et al., 1980).

The second component of the nitrogen immobilization dilemma is the mechanism of preservation. Again, most investigators suspect that humification reactions are involved due to the fact that humic substances are enriched in nitrogen relative to vascular plant tissues. The best evidence for this model is the positive correlation linking an increase in humic substances to an increase in nitrogen content during immobilization (Rice, 1982). The general model proposed for humification involves the breakdown and oxidation of phenolic and carbohydrate materials from the plant source material, which then condenses with nitrogenous materials of microbial origin such as exoenzymes. Direct evidence for such nitrogen immobilization has previously been limited to a study of *Spartina alterniflora* in which lignin was inversely correlated with the increase in nitrogen (Benner et al., 1991). However, molecular evidence has been difficult to obtain because methods to look at the immediate precursors to these condensation reactions have been unavailable. On the microbial side, nitrogenous materials that are condensed likely include basic amino acids, but the source in general is uncharacterized, and therefore it is unknown to what extent amino acids might be involved. On the vascular plant side, quinones derived from phenols are believed to be predominant precursors for condensation reactions. However, the most commonly measured phenol, lignin, must be converted into quinones through microbial means. Any intermediates in this conversion are likely to be reactive and thus undetectable by the cupric oxide oxidation method typically used for measuring molecular lignin. On the other hand, condensed tannin may be the ideal substrate for studying humification, due to the susceptibility of the monomers to abiotic quinone formation, as well as the reactivity differences related to hydroxylation pattern. As shown in the previous study of Benner et al. (1991) for lignin and nitrogen in *Spartina*, there is an inverse correlation between tannin and the increase in nitrogen in mangrove leaves, which may be indicative of humification reactions (Fig. 10).

If condensation reactions are occurring in these mangrove leaves, several trends should be evident. ^{13}C-NMR shows constant phenolic concentrations in the leaf samples at $\sim$20 wt.%, while total molecular tannin decreases from 7 to $<$ 1% between the orange and brown/black stages (Fig. 4). This trend suggests that tannin is being transformed and not remineralized, which would be consistent with quinone formation in the slightly alkaline, oxygenated estuarine waters. As indicated earlier in the molecular data, trihydroxylated PD tannin was more reactive than dihydroxylated PC tannin in the mangrove leaves, again, consistent with quinone formation. The overall reactivity of the B-ring hydroxyl groups is evident in the ^{13}C-NMR traces from Benner et al. (1990a), as the peak attributed to B-ring phenolic carbons largely disappears in the brown and black stages, while the A-ring peak is still evident. Finally, in laboratory experiments it has been shown that basic amino acids have a much higher reactivity than neutral and acidic amino acids in condensation reactions with quinones (Hedges,

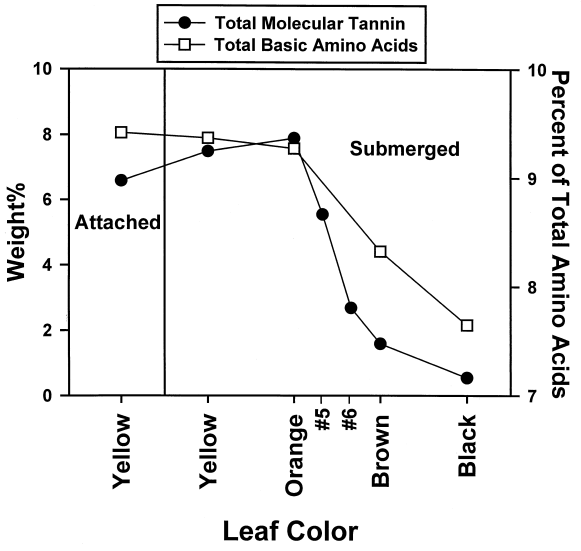

Fig. 11. Weight percent tannin and basic amino acids as a percentage of total amino acids along the degradation sequence.

1978), and thus one would expect to see a correlation between tannin degradation and basic amino acid compositions. Again, the evidence bears this out, as basic amino acids (as a percentage of total amino acids) decrease in the brown and black stages along with total molecular tannin (Fig. 11). Alternate explanations for the relative depletion in basic amino acids may exist. However, in marine systems the typical pattern observed is enrichment in basic amino acids with increasing degradation (e.g., Keil et al., 2000). Although these trends are not definitive evidence for covalent bonding between tannin and nitrogen, the potential is clearly shown for tannin measurements to break new ground in research on nitrogen immobilization and humification.

4. CONCLUSIONS

This study highlights the geochemical importance and potential of molecular tannin analyses for any ecosystems in which vascular plant tissue is a significant source of organic carbon:

1. Molecular level analyses confirm the quantitative importance of tannin suggested previously in other studies by bulk techniques. In these mangrove leaves, measured molecular tannin was second in abundance only to carbohydrates in the senescent yellow leaf material entering the estuarine system.
2. The mangrove leaves in this study exhibit a strong source signature. However, that signal is altered by diagenesis, which highlights the importance of considering the processing history of a sample when identifying and quantifying source contributions.
3. In addition to their source information, triterpenoids as a whole offer a much less reactive counterpart to tannin. However, the appearance of oleanolic acid in brown and black leaves might be an indicator of redox history.
4. Tannin exhibits an intermediate lability not shown by any other biomarker in these samples. If this pattern also applies to other systems in which vascular plant tissues are involved, tannin may be uniquely suited as a tracer of early diagenesis.
5. Nitrogen immobilization in leaf material entering aquatic environments is a commonly observed but poorly understood phenomenon. The molecular tannin and amino acid data, along with ^{13}C-NMR data, provide the first molecular level evidence for humification during nitrogen immobilization.
6. The ability to calculate the degree of polymerization for tannin based on molecular analysis makes it unique among commonly measured biochemicals. In this study, degree of polymerization highlights the early leaching process involved in diagenesis and the subsequent shift toward abiotic or microbially mediated chemical reactions.
7. The degree of hydroxylation (i.e., PD:PC ratios) shows potential as a diagenetic indicator. Reactivity related to hydroxylation patterns may provide clues as to abiotic vs. microbial processes.

There are many areas of tannin diagenesis that merit further research in mangrove leaves and in general. PD tannin (three B-ring hydroxyl groups) was shown to be more labile in the mangrove leaves than PC tannin (two B-ring hydroxyl groups). The question remains as to whether this trend also extends to PP tannin (one B-ring hydroxyl group), which was found to be abundant in several gymnosperm barks and angiosperm green leaves (Hernes and Hedges, 1999). Tannin-nitrogen interactions (in particular, interactions with basic amino acids) remain a critical area to study in terms of early tannin diagenesis and nitrogen immobilization. If quinone formation from tannin is a necessary step toward tannin-nitrogen reactions, then the degree of hydroxylation and oxidative history become important factors in nitrogen uptake. Finally, the 30% loss term due to leaching indicates that the dissolved phase is important for tannin degradation. Further studies are warranted to investigate the various sinks for tannin (including phototransformations) within the dissolved phase and to evaluate the importance of tannin as a component of dissolved organic matter pools in rivers, lakes, and possibly the ocean.

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