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Tannin geochemistry of natural systems: Method development and application

by

Peter J. Hernes

A dissertation submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

1999

Program Authorized to Offer Degree: School of Oceanography

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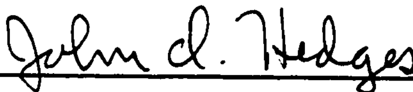

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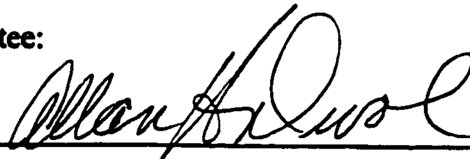

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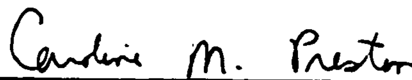

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Abstract

Tannin geochemistry of natural systems:
Method development and application

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Polyphenolic tannin comprises a significant proportion of the leaves, needles, and barks of vascular plant tissues—all major contributors to terrigenous organic matter cycling in aquatic systems. However, virtually nothing is known at the molecular level about tannin diagenesis despite the increased sensitivity and unambiguity of molecular characterizations vs. bulk techniques. Due to the analytical challenges in measuring tannin molecularly, there has been little crossover to ecology from the large body of research in natural products. This thesis presents the development of a new method for molecular-level condensed tannin analysis, a survey of source tissues, and a geochemical application focussed on mangrove leaf diagenesis in a tropical estuary.

The developed method consists of acid hydrolysis (HCl) of samples in acetone, water, and phloroglucinol as a nucleophile for carbocation capture. Analytes are partitioned into ethyl acetate, dried, trimethylsilyl derivatized, and quantified by gas chromatography. The method reproducibly yields intact terminal units and phloroglucinol-adducted extender units, in addition to flavones, flavanones, triterpenoids, and carboxylic acids.

Molecular tannin signatures were obtained from 117 source tissues, including leaves, needles, woods, and barks from tropical and temperate forests, coasts, and grasslands. Conifer needles were distinguished by high prodelphinidin content while dicotyledon leaves alone yielded both triterpenoids and flavones. Barks were distinguished by flavanones and tetracosanoic acid.

Molecular tannin in a degradation sequence of mangrove leaves (*Rhizophora mangle*) revealed several important trends not evident by bulk techniques. Degree of polymerization (average chain length) highlighted early leaching processes and a subsequent shift toward abiotic or microbially-mediated chemical reactions. An increased degree of hydroxylation corresponded to increased degradation rate, a trend that may be indicative of changes in redox or pH conditions leading to quinone formation. The apparent inverse correlation between basic amino acids and molecular tannin might signal Schiff base reactions partially responsible for nitrogen immobilization. Measured molecular tannin was second in abundance only to carbohydrates in the senescent yellow leaves entering the estuarine system. Leaching losses of 30% tannin highlight the potential importance of tannin in dissolved organic matter.

Based on these these studies, molecular level tannin shows great promise as a tool for geochemical studies.

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ACKNOWLEDGEMENTS

In retrospect, my graduate school fate was sealed by a general letter that I sent out to a number of departments at the University of Washington while I was tearing up the hiking trails in isolation at Holden Village. The letter simply stated that I was interested in graduate studies in the (fill in the blank) Department, but wanted to get some experience as a technician first in order to be sure that it was the right field. John Hedges' technician just happened to be leaving, and somebody in Oceanography administration just happened to give John a copy of my letter. John just happened to hire me sight unseen.

On second thought, maybe what REALLY sealed my fate was my first day in Seattle after descending from the mountains. Through common friends, I ended up staying at a certain house where a certain person named Carol Klippstein was living. You see, after nearly three years as a tech, I can't really say that I knew for sure that oceanography was the right field for me, but I was pretty sure that Carol was the right person for me and she was still going to school. I decided that graduate school might be a good way to stick around for awhile, but I still hedged my bet (no pun intended) by taking on a masters project (as part of the U.S. JGOFS EqPac study with John) instead of going for the Ph.D. Oh, yes—Carol and I were married three days before graduate school started.

So now that the masters transformed into a Ph.D., there are many people to acknowledge: John Hedges for taking the initial flier on me and supporting me through three separate projects. Carol for obvious reasons. My parents, Joel and Sonja, for giving me an environment where I always expected to go to college and beyond. My kids, Alexander and Katrina, who are very good at bringing me into their world and away from the sometimes turbulent world of dissertations . . . My older siblings, Jeff and Janet, who set the table for me by going to Luther College and who have both supported me during various crises in my life. My younger sister, Marsha, who always just assumes that I can accomplish whatever I set out to do. The rest of my extended family for showing an interest in me and my family and my studies.

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DEDICATION

I dedicate this dissertation to my wife, Carol, my kids, Alexander and Katrina, and my parents, Joel and Sonja.

CHAPTER 1: INTRODUCTION

Although a considerable body of knowledge now exists about tannin in general, the geochemistry of tannin is in its infancy. The geochemical perspective of tannin is quite different from previous work, taking as its prime motivation the role of tannin in the carbon cycle both quantitatively and in terms of biomarker potential. Net terrestrial and marine primary production is approximately 100 x 10^{17} gC y^{-1}(Hedges, 1992). Of this global primary production, only 0.2 percent passes through the ecosystem and is ultimately preserved in marine sediments. Carbon preservation is of interest because of its link to the atmosphere. For every mole of organic carbon buried, one mole of carbon dioxide is removed, and one mole of oxygen released. Ultimately, all of the oxygen in the atmosphere is due to organic carbon preservation and pyrite formation, and considerable effort has been made to understand all the factors and feedback mechanisms in these relationships (Berner, 1989).

The role of tannin in the carbon cycle and carbon preservation is largely unknown. Riverine input of terrestrial organic carbon to the marine environment is approximately twice that of preservation in the sediments, suggesting that terrestrial organic carbon could potentially comprise a large component of preserved organic carbon. Stable carbon isotopes have shown that this is not the case, however, which presents a paradox: Riverine organic carbon represents the most refractory terrestrial organic carbon and yet more than 80 percent is remineralized upon entering the ocean (Aller et al., 1996; Emerson and Hedges, 1988). Because tannins are quantitatively important (the fourth most abundant component of vascular plant tissue), their study may be an important piece in the solution of this paradox.

In addition to quantitative importance, molecular level tannins represent a unique class of biomarkers both in structure and reactivity that may lead to new understanding of carbon diagenesis. Hydrolyzable tannin, with its carbohydrate component and gallic acid derivatives, is very different from the polyflavanoid nature of condensed tannin (Fig. 1.1). As the name implies, the former is more soluble than the latter, and therefore

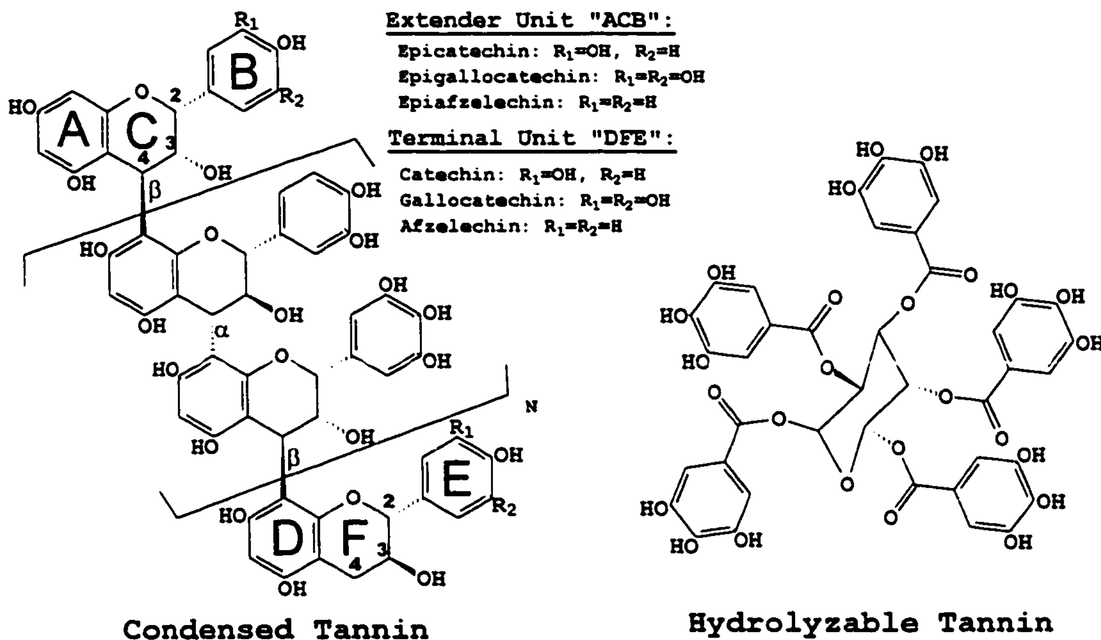

Figure 1.1 Structures of typical condensed and hydrolyzable tannins.

the latter would be more likely to persist. Monomers of condensed tannin are also much more varied and stereochemically active than the basic building blocks of hydrolyzable tannin, and this has lead some to attribute much different functionality of the two types within plants (Zucker, 1983). The combination of lower solubility and greater monomeric diversity highlights the biomarker potential of condensed tannins.

Few geochemical studies are aimed specifically at tannins. Historically, interest in tannins has stemmed from industrial uses. In recent decades, tannin research has expanded to natural products (where much progress has been made in terms of tannin structure and taxonomic surveys) and in a much more limited fashion to forestry. Research in forestry has tended to focus on the delivery of tannins to the soil (via leaves, needles, bark, and wood), the degradation of tannin in the soil, and the impact of tannin on nutrient recycling and organic matter degradation within the soil (Horner et al., 1988). The methods employed are primarily bulk measurements of tannins, including ^{13}C NMR, various spectrophotometric methods, and tannin binding assays. Findings include the loss of 84 percent of extractable tannins from pine litter after 1 year and 92 percent loss after 2 years (Tiarks et al., 1992), little difference between tannin content of

trees vs. logs after 1 year on the ground (Kelsey and Harmon, 1989), and the control of nitrogen release from pine litter by tannin and less effective organic matter degradation when tannins are present (DeMontigny et al., 1993; Northup et al., 1995). The latter is of particular concern to forestry because of its impact on young trees in recent clearcuts and the overall decline in site quality (Preston, in press). Despite the impacts of tannin on soils, little or no research has been done at the molecular level, and even the bulk measurements have been questioned as to their accuracy or meaningfulness (Mole and Waterman, 1987; C. Preston, pers. comm.).

Tannin geochemistry research in riverine and marine systems is virtually nonexistent, limited primarily to the monitoring of tannin leaching or degradation from leaves via bulk measurements (Benner et al., 1990a; Suberkropp et al., 1976). We are unaware of any molecular-level studies, in spite of the fact that tannins constitute up to 20 percent of the leaf tissue that is a major form of terrigenous organic matter cycling in these systems (Benner et al., 1990a). For comparison with other organic biomarkers, lignin accounts for approximately 5 percent of leaf tissue (Benner et al., 1990a; Hedges and Weliky, 1989), carbohydrates 40-50 percent (Benner et al., 1990a: Cowie and Hedges, 1984), cutin 5-10 percent (Goñi and Hedges, 1990), amino acids 5-10 percent (Cowie and Hedges, 1992), lipids 5-10 percent (Suberkropp et al., 1976), and pigments ~1 percent (Tissot and Welte, 1978). Among other sources of organic matter to natural systems, tannin accounted for up to 17 percent of outer bark (western hemlock), 4 percent of inner bark (Douglas-fir), 1 percent of sapwood (Douglas-fir), and 0.6 percent of heartwood (western red cedar) (Kelsey and Harmon, 1989). Tannins are a major component of leaf tissue and bark, and therefore can have a significant impact on the bulk properties of organic mixtures, such as aromaticity, organic carbon:nitrogen ratios, phenolic OH, color, and reactivity. Because of this importance, Benner et al. (1990) 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."

Unlike carbohydrates, lipids, amino acids, and pigments—which are ubiquitous in organic matter and have both marine and terrestrial sources—tannins (along with lignin and cutin) are uniquely terrestrial. Thus, in addition to bulk importance, tannins have the potential to provide source information that is complementary to lignin and cutin. For instance, monocots are not distinguishable based on lignin composition. Although monocots do have a unique cutin signature, this signature can be overwhelmed by nonwoody gymnosperm or dicotyledon tissues (Goñi and Hedges, 1990). Monocots, however, uniquely produce *ent*-epicatechin (2S and (+) optical rotation), a monomer found in condensed tannins (Ellis et al., 1983). *Ent*-epicatechin should be clearly resolvable from (-)-epicatechin with a chiral gas chromatography column. In addition, propelargonidin-containing polymers are much more common in monocots than dicots (Ellis et al., 1983). Hydrolyzable tannins, on the other hand, are notably absent from monocots. Though flavones can be found in angiosperms, they do not appear to be present in gymnosperms. In addition, condensed tannin dimers appear to contain more species-dependent taxonomic information among angiosperms that may be useful in certain environments in which the potential sources are more constrained (Haslam, 1989).

A challenge in measuring tannins at the molecular level in natural samples is that these polyphenols are very reactive and may undergo significant changes once organic matter senesces and becomes part of the litter. For instance, vicinal diols (such as those on the B-ring in many condensed tannins) can be easily oxidized to quinones in basic conditions (Laks, 1989). Quinones, in turn, can participate in many condensation reactions, primarily due to the electron withdrawing nature of the carbonyl functional group. The non-carbonyl carbons become electron-poor and can form bonds very readily with electron-rich carbons (such as those on the A-ring of condensed tannin or other phenols) or any other strong nucleophile. Alternatively, the carbonyl groups on quinones and flavones can react directly with amines via a Schiff's base reaction to form nitrogenous condensation products.

The hydroxy groups in tannin are also responsible for its complex-forming nature. A tannin polymer (either condensed or hydrolyzable) has a plethora of hydroxy

groups to hydrogen-bond with proteins and amino acids and to complex with metals. Interestingly, a condensed tannin must be sufficiently large before significant protein complexation takes place—monomers, dimers, and other small oligomers apparently are not able to form enough cross-bridges to strongly complex with proteins (Jones, 1976).

The ability to complex with proteins and amino acids leads to another geochemically significant trait of tannin mentioned previously—inhibition of organic matter degradation. At the right concentration, tannins are toxic. At 15 mg L^{-1}, tannins have been known to cause fish kills (Field and Lettinga, 1992). Concentrations ranging from 325 to 3,000 mg L^{-1} have been reported to be inhibitory to methanogenic bacteria (Field and Lettinga, 1992). Concentrations of 1-2 percent tannin have been shown to reduce the overall decomposition of organic materials applied to soil (Field and Lettinga, 1992). In addition to complexing bacterial exoenzymes and directly slowing degradation, tannin may also bind up nitrogen sources used by heterotrophs for growth.

Despite the challenges of measuring tannins, there may be a wealth of information to be obtained from them specifically because of their highly reactive nature. Because of their redox and photochemical sensitivity, it may be possible to use tannins as an indicator of the environmental history of associated organic matter. For instance, tannins present in anoxic sediments may be able to tell us whether the sediments have been under constant or intermittent anoxia. In the Rio Negro, there is evidence for significant photochemical degradation in the upper few centimeters of the water column (Amon and Benner, 1996), as well as evidence for a significant fraction of dissolved tannin in the Rio Negro that could account for this degradation.

Finally, tannins have long been suspected as precursors to humic materials via "autoxidation" when neutral to alkaline pH conditions prevail (Field and Lettinga, 1992). Again, this is due to the ease of formation of quinones and subsequent condensation reactions. Because so little is known about molecular-level tannins in natural samples, the role of tannins in humification is still largely theoretical. Perhaps molecular-level tannin analyses of natural samples coupled with isolation of humic substances and bulk characterization by NMR will provide a first look at the empirical relationship of tannins to humification.

METHODS

Several available techniques for measuring tannins can be used in geochemical studies, including (but not limited to) ^{13}C-NMR estimates (Benner et al., 1990a), protein-binding assays (Hagerman, 1987), Folin-Denis spectrophotometric analyses (Folin and Denis, 1915), and anthocyanidin assays (Porter et al., 1986). The strengths and weaknesses of each relative to geochemistry will be briefly discussed. More in-depth discussion of analytical methods can be found in Hagerman and Butler (1989).

^{13}C-NMR is a bulk method for estimating total tannins, either directly by integrating peaks attributable to tannin (Newman and Porter, 1992), or indirectly by subtracting independently measured lignin content from total phenolics (Benner et al., 1990a). When carbon content of natural samples permits, solid-state ^{13}C-NMR can provide this information nondestructively. A primary disadvantage of ^{13}C-NMR is that while it is good for identifying functional groups, structural information on whole samples can be sparse. Preston et al. (1997) showed that exposing a sample to harsh acid treatments can significantly alter the chemistry of the sample without changing the ^{13}C-NMR. ^{13}C-NMR is also subject to interference from paramagnetic metals such as iron and manganese present in soils and sediments (Skjemstad et al., 1994; Preston, 1992; Baldock et al., 1992).

The Folin-Denis colorimetric technique is a bulk method for estimating total tannin that utilizes the oxidizing property of tannin to produce color (Folin and Denis, 1915). An advantage to Folin-Denis analyses is the relative ease with which it can be carried out. Unlike ^{13}C-NMR, this technique does not necessarily measure all of the tannin, but only that readily available as an oxidizing agent toward the Folin-Denis reagent. In this sense, then, the Folin-Denis technique is useful as an estimate of the potential reactivity of the tannin in natural environments, but may be limited in detecting reactions that have already occurred. A primary disadvantage of this method is that it is most often calibrated using tannic acid, when in fact, tannic acid (or other hydrolyzable tannin) is not necessarily a major component of the sample. Another disadvantage is that other compounds capable of oxidizing the Folin-Denis reagent such as ascorbic acid or peptides will lead to overestimates of the tannin content. On the other hand, it is

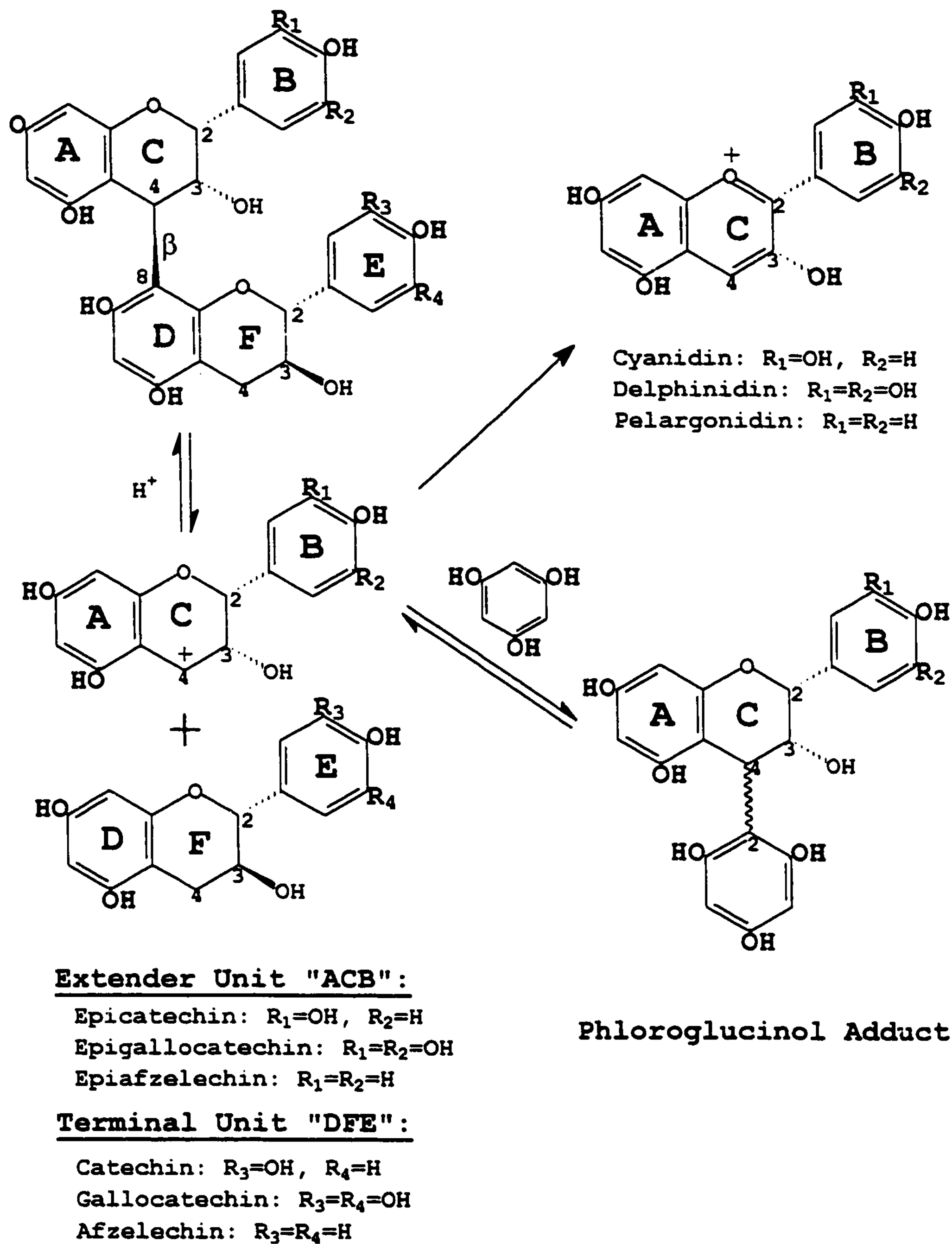

Figure 1.2 Acid hydrolysis of a condensed tannin dimer with and without phloroglucinol.

usually carried out on an extract, as opposed to the whole sample, which can lead to underestimates.

Protein-binding assays (e.g., precipitation of tannin with protein, or radial diffusion of tannin through protein gels) are a third method for measuring total tannin

(Hagerman, 1987). The advantages and disadvantages are similar to Folin-Denis. Protein-binding assays are easy and give an indication of the potential reactivity of the tannin in natural environments, in this case, their ability to complex with proteins. As with Folin-Denis, protein-binding assays may not provide much information about prior tannin reactions. The use of appropriate calibration standards is an important consideration, since different tannin compounds will have different complexing ability. Again, there is potential interference from other compounds present in the samples leading to overestimates, while the use of extracts may lead to underestimates of tannin in a particulate source.

The anthocyanidin assay of Porter et al. (1986) is another colorimetric technique specific for the extender units of condensed tannin oligomers and polymers. This technique relies on acid hydrolysis to release the extender units as carbocations, which then convert to cyanidins, pelargonidins, and afzelechins (Fig. 1.2). The latter can then be detected spectrophotometrically. Because condensed tannins are more likely to persist in natural samples than more labile and soluble hydrolyzable tannins, this technique is applicable to soils and sediments when molecular information is desired (Preston, in press). In principle, cyanidins, pelargonidins, and delphinidins are distinguishable from each other due to respective differences in absorption maxima, but in practice they are not well resolved spectrophotometrically. The primary advantage of this technique is that it provides specific molecular information. Disadvantages are, again, that appropriate standards for calibration must be obtained (preferably a well-characterized oligomer), stereochemical information is lost upon conversion of the carbocations, and the terminal unit of the oligomers or polymers is not detected. The latter is crucial toward calculating degree of polymerization—a potentially important geochemical indicator of environmental history of a sample.

While all these techniques provide valuable information, acid hydrolysis utilizing nucleophilic capture of the extender unit carbocations (Fig. 1.2) provides the least ambiguous measure of tannins and potentially the most diagnostic information. However, because no acid hydrolysis methods had been developed specifically for environmental samples, the initial focus of my research (Chapter 2) was to develop such

a method. Unfortunately, no standard hydrolysis conditions are outlined in the literature. Hydrolysis usually has been carried out on extractions, with many different acids, solvents, and nucleophiles at varying concentrations, temperatures, and times, and utilizing many different chromatographic techniques. Advantages to these techniques are that stereochemistry of the extender units is preserved, and all monomers can be unambiguously resolved. Since both extender and terminal units can be measured, one can calculate an average degree of polymerization by dividing total molecular tannin by the total terminal units. Degree of polymerization may be an important diagenetic parameter because of its relation to leachability, complexation with proteins or metals, or lability. The primary disadvantage is incomplete hydrolysis leading to underestimates of tannin.

With this in mind, my goal for molecular-level analysis of tannins in natural samples included high sensitivity (low background), applicability to various forms and matrices, no analytical artifacts, ease and safety of analysis, quantification, and reproducibility.[30] For ease and safety considerations, I opted to use ~0.25 M phloroglucinol as the nucleophile instead of the more common sulfur nucleophiles. Because of its relative safety and the potential for evaporation if so desired, hydrochloric acid (0.5 M) was chosen for hydrolysis. Instead of dioxane:water solvents, I opted to used acetone:water (70:30, v/v), which was also deemed most appropriate for analyses to be done on whole samples since acetone:water is the typical solvent system used for extracting tannin from whole samples. The hydrolysis is carried out at 30 °C, the reaction mixture extracted with ethyl acetate and run through sodium sulfate drying columns, and then the ethyl acetate evaporated to dryness before redissolving the sample in pyridine. Because gas chromatographs (GCs) are ubiquitous and commonly coupled to mass spectrometers, I felt it was desirable to carry out the chromatography on a GC if feasible, with trimethylsilyl (TMS) derivatization. I used splitless injection on an HP 5890 GC equipped with electronic pressure control and a capillary column with DB35ms or DB5ms liquid phase. Given the appropriate temperature program as outlined in Chapter 2, all compounds are generally eluted by 40 minutes.

After developing a working method, the next step of my project was to conduct a survey of source tissues to evaluate the potential of tannin as a source signature. This work is the subject of Chapter 3, in which nearly 120 samples were analyzed from woods, barks, cones, leaves, needles, and whole plants. Included were temperate species from the Pacific northwest (collected by me and previous investigators) and tropical species from the Amazon rain forest (collected as part of the Carbon in the Amazon River Experiment, or CAMREX, project). In addition to establishing the quantitative importance of tannin in various sources, a goal was to develop source parameters that would complement while extending the two other uniquely terrestrial markers, lignin and cutin.

Chapter 4 details a geochemical application of this method, i.e. the study of tannin diagenesis in mangrove leaves submerged in a tropical estuary. From Benner et al. (1990), it was known that these mangrove leaves contained ~20% tannin, but at the time, only the bulk techniques were available, in this case, ^{13}C-NMR and Folin-Denis. This study marks the first use of molecular-level tannins in geochemistry. Among the questions to be addressed were how leaching affects tannin composition, how degree of polymerization changed with diagenesis, how the source signature of mangrove leaves changed with diagenesis, whether there would be a correlation between degree of hydroxylation on the tannin B-ring and reactivity, and how tannin geochemistry compares to several other major biochemicals previously measured on these samples, including amino acids, lignin, carbohydrates, cutin, and uronic acids.

This work represents nearly five years of "tinkering" in the laboratory. There are many tests and experiments that are not reported here and likely never will be. Mass spectroscopy alone could account for an entire chapter as I endeavoured to identify tannin peaks without standards. A favorite of mine involved a hydrolysis series with phloroglucinol (1,3,5-trihydroxybenzene), methoxyresorcinol (1-methoxy, 3,5-dihydroxybenzene), dimethoxyphenol (1,3-methoxy, 5-hydroxybenzene), and trimethoxybenzene (1,3,5-trimethoxybenzene) as nucleophiles. This particular experiment cemented my identification of the phloroglucinol adducts as well as the fragmentation pathway for the prominent 533 m/z peak in all the adducts. With more

attention to kinetics and yields, it could be a great physical organic chemistry paper. Still, the work that is presented here accomplishes nearly all that I set out to do.

CHAPTER 2: DETERMINATION OF CONDENSED TANNIN MONOMERS IN ENVIRONMENTAL SAMPLES BY CAPILLARY GAS CHROMATOGRAPHY OF ACID HYDROLYSIS EXTRACTS

INTRODUCTION

Tannin is the fourth most abundant biochemical in terrestrial biomass, following cellulose, hemicellulose, and lignin. In rapidly cycling soft tissues like leaves and needles, however, tannin is often more abundant than lignin (Hedges and Weliky, 1989; Benner et al., 1990a), and therefore an important component of the carbon cycle. Tannin consists of two types, condensed and hydrolyzable tannin (Fig. 1.1). The former exists as oligomers and polymers of three-ring flavanols (note the terminology of extender units and terminal units in Fig. 1.1), while the latter is made up of gallic acid often ester-linked to a carbohydrate backbone. Tannin in the environment is of interest because it is quantitatively important and participates in a number of important transformations, including photochemical and redox reactions, nitrogen immobilization and cation complexation. Tannin is also a potential precursors to humic substances via quinone formation and subsequent condensation reactions with proteins and amino acids. Although a number of molecular methods for tannin can be found in the natural products literature, application to environmental samples has been limited almost exclusively to bulk techniques. Because of the potential wealth of information at the molecular level, the aim of this study was to develop a molecular technique suitable for environmental samples. Further objectives were that the technique should utilize commonly available instrumentation, require minimal chemical manipulations or hazardous reagents, and should allow rapid throughput.

Existing methodologies for tannin analyses contain many drawbacks either inherent in the method (Mole and Waterman, 1987; Hagerman and Butler, 1989), or when applied to environmental samples (Mole and Waterman, 1987; Hagerman and Butler, 1989; Appel, 1993). Briefly, these include lack of appropriate standards, analyses of extracts instead of whole samples, unpleasant or hazardous reagents, lack of

reproducibility or quantification, and complicated chromatography or less common instrumentation. Among the molecular techniques, while nearly all utilize acid hydrolysis with capture of the carbocation by a nucleophile to form an adduct (Fig 2.1),

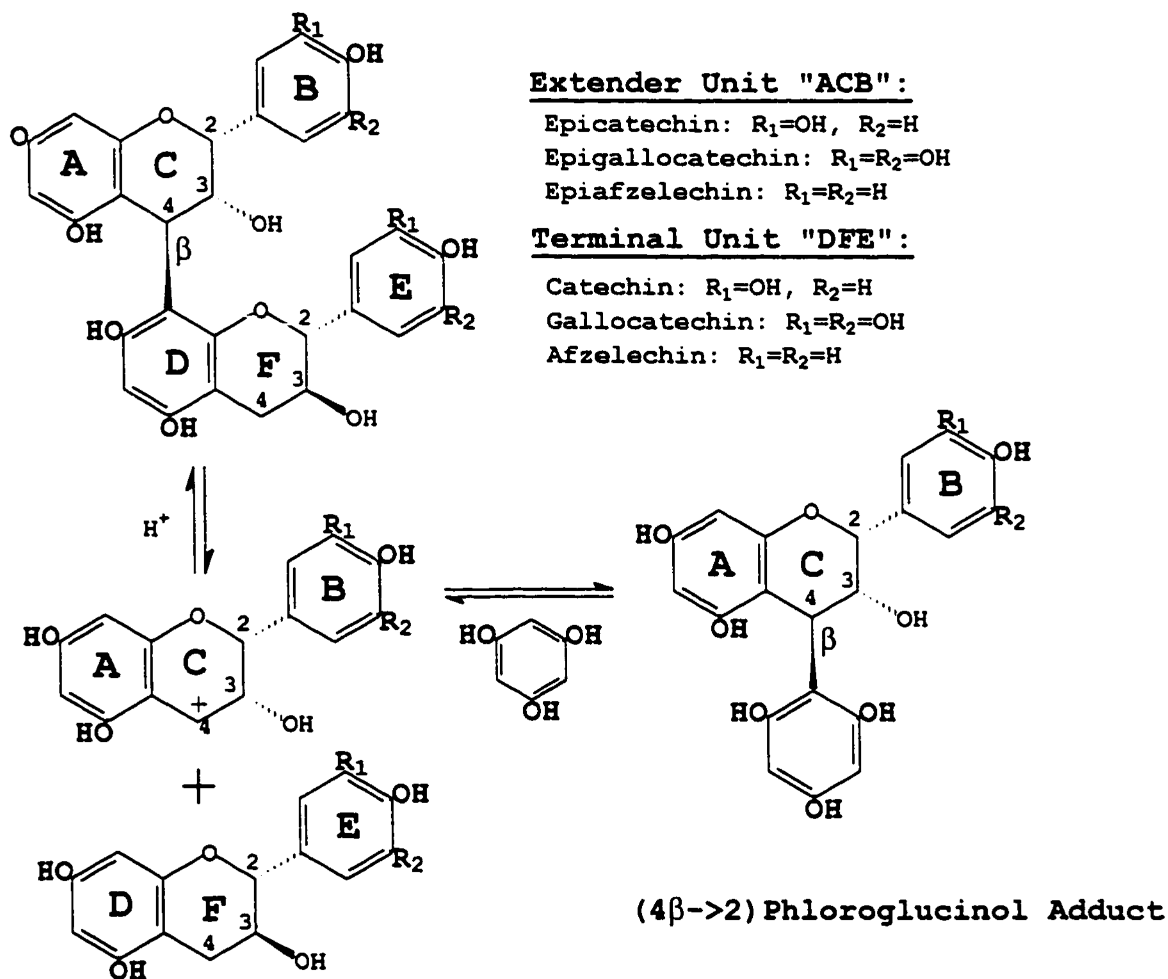

Fig. 2.1 Acid hydrolysis of a condensed tannin dimer in the presence of phloroglucinol.

there is a lack of standardization among hydrolysis conditions. The various techniques utilize hydrolysis times from 15 minutes to 50 hours, temperatures from room temperature to >100 °C, acid strengths from 0.02 to 3.5 M, and nucleophile concentrations from 0.3 to 2.4 M. Several different acids have been tried, including acetic, sulfurous, phosphoric, hydrochloric, and several carboxylic acids. The

nucleophile predominantly consists of benzyl mercaptan or phloroglucinol. Solvents include ethanol, methanol and water, and dioxane and water.

Various chromatographic techniques have been utilized for separation of hydrolysis mixtures, including paper chromatography, column chromatography, liquid chromatography, and gas chromatography (GC). The hydrolysates are often methylated prior to chromatography which involves either explosive or toxic reagents. Less common for GC is the formation of trimethylsilyl (TMS) derivatives, which avoids the safety hazards of methylation but increases molecular weight significantly. Nevertheless, with today's high temperature gas chromatographic columns and electronic pressure controllers, increased molecular weight is less of an issue.

The analytical procedure described here was developed utilizing many of the hydrolysis conditions referred to above, but using acetone:water (70:30 v/v) as the solvent. Acetone:water is the most commonly used extraction solvent for condensed tannin prior to analysis in nearly all techniques, and was a natural choice since we desired a method appropriate for whole samples. Although benzyl mercaptan often gives higher yields of adducts than phloroglucinol (Matthews et al., 1997), it is also unpleasant to work with and so we opted for the latter. For the acid, we chose hydrochloric because its volatility allows easy removal after hydrolysis if so desired. Finally, we chose TMS derivatization and capillary gas chromatography coupled with an FID for detection. TMS derivatization is easy, the derivatives are stable, and GC's offer relatively high resolution and ready mass spectral analysis.

A primary reason for analyzing whole samples instead of tannin extracts is the potential for measuring non-tannin compounds within our analytical window. Two compound classes that can be measured by this technique include carboxylic acids and triterpenoids. The former include short-chain carboxylic acids as well as some cutin acids. The latter are routinely quantified, and include unique terrestrial biomarkers. Together with tannin, they offer a powerful tool for geochemical studies.

EXPERIMENTAL SECTION

Sample Preparation. Soils, sediments, and leaf samples are freeze-dried and ground to pass through a 0.351-mm sieve. Green leaves from *Camellia* sp. and dripline

soil were collected in Seattle, WA. Sediments were collected from Dabob Bay, WA. Whole brown leaves were collected from the litter of pin oaks (*Quercus palustris)* on the University of Washington campus in Seattle, WA.

Condensed Tannin Hydrolysis. Approximately 50 mg of sample and 100 mg of phloroglucinol is weighed into a 50-mL Pyrex centrifuge tube (12-24 samples at time depending on heating bath capacity) with a Teflon-lined phenolic screw cap. After addition of a Teflon-coated magnetic stir bar, 0.9 mL of 0.334 M HCl and 2.5 mL of acetone (both Ar-sparged for ten minutes) is added to each tube. Each tube is purged with Ar (thereby evaporating ~0.4 mL of acetone for a final total volume of ~3 mL) and capped before being placed in a constant temperature bath (30° C) equipped with a magnetic stirrer. Samples are hydrolyzed for 24 hours.

Sample Extraction and Cleanup. Upon removal from the constant temperature bath, internal standard is added along with 10 mL of deionized water. Approximately 4 mL of ethyl acetate is added to each tube. Tubes are shaken for 30+ seconds, centrifuged briefly, and then the ethyl acetate fraction is drawn off the top with a Pasteur pipette and dripped through anhydrous $NaSO_4$ columns to remove water. The columns drain into 1-dram vials under a stream of N_2. After three extractions, a two-thirds split is removed from each sample and placed in another 1-dram vial. All samples are taken to near dryness and then placed in a vaccuum dessicator overnight to remove any residual water, HCl, or solvent.

Derivatization and Gas Chromatography. Samples (one third splits) are redissolved in 200 µL pyridine and an aliquot (20-30 µL) is placed in an autosample vial containing a 200 µL insert. An equal volume of Regisil (bis(trimethylsilyl)trifluoroacetamide) + 1% trimethylchlorosilane (Regis Chemical Co.) is added to each vial, the vial is capped, the contents mixed with a syringe, and the vials placed in a 60° C heating block for 15 minutes.

A Hewlett-Packard 5890 gas chromatograph fitted with a flame ionization detector (FID) or interfaced to a Hewlett-Packard 5970 Mass Selective Detector is used for all analyses. The injection port and detector is maintained at 310° C. The injection port is set up for "splitless" injection using a 2 mm gooseneck liner. Analyses are made

by using 30 m by 0.25 mm i.d. fused-silica capillary columns, coated with either DB35ms or DB5ms liquid phase (J&W Scientific Inc.).

After initial injection with the oven temperature held at 70° C, the purge valve is kept in the closed position to allow column loading. After two minutes, the purge valve is opened to flush any remaining organic vapors out of the injection port. For the DB5ms, the oven temperature is ramped up to 250° C at 25° C per minute. The temperature ramp is then decreased to 3° C per minute until 295° C. Finally, the temperature ramp is decreased to 1.5° C per minute until 325° C at which point the temperature is held until the end of the run, usually 20-30 minutes. For the DB35ms, following column loading, the oven temperature is ramped up to 200° C at 25° C per minute. The temperature ramp is then decreased to 4° C per minute until 330° C, at which point the temperature is held until the end of the run, usually 20-50 minutes. If the gas chromatograph is equipped with an electronic pressure controller (EPC), the initial column head pressure used for the DB5ms is 20 psi. After 9.2 minutes (i.e. when the oven reaches 250° C), the pressure is increased 0.5 psi per minute to 35 psi and held until the end of the run. For the DB35ms, initial pressure is held at 13 psi for 2 minutes, then ramped at 1 psi per minute to 30 psi and maintained until the end of the run. A DB5ms chromatogram of TMS-treated hydrolysate from pin oak leaf litter can be found in Fig. 2.2a, while the same sample injected on a DB35ms is shown in Fig. 2.2b.

Peak Identification and Quantification. Most peak identities were determined by comparing mass spectra to those of available authentic standards (epicatechin, catechin, epigallocatechin, α-amyrin, oleanolic acid, ursolic acid), and to those in a library of trimethylsilyl-derivatized natural compounds generated by W. Greenaway and colleagues (stigmast-5-en-3β-ol, β-amyrin). These compound structures are shown in Fig. 2.3. Phloroglucinol adducts were identified by mass spectra fragmentation patterns relative to unadducted parent monomers, relative peak areas, elution orders, and hydrolysis of a catechin (4α→8) catechin dimer (also designated as B-3) standard in the presence of phloroglucinol. Each extender unit when hydrolyzed to a carbocation can form two adducts at C-4 (see extender unit ACB in Fig. 2.1), which IUPAC rules designate as α and β (β as shown in Fig. 2.1 is "up" from the plane of the molecule as

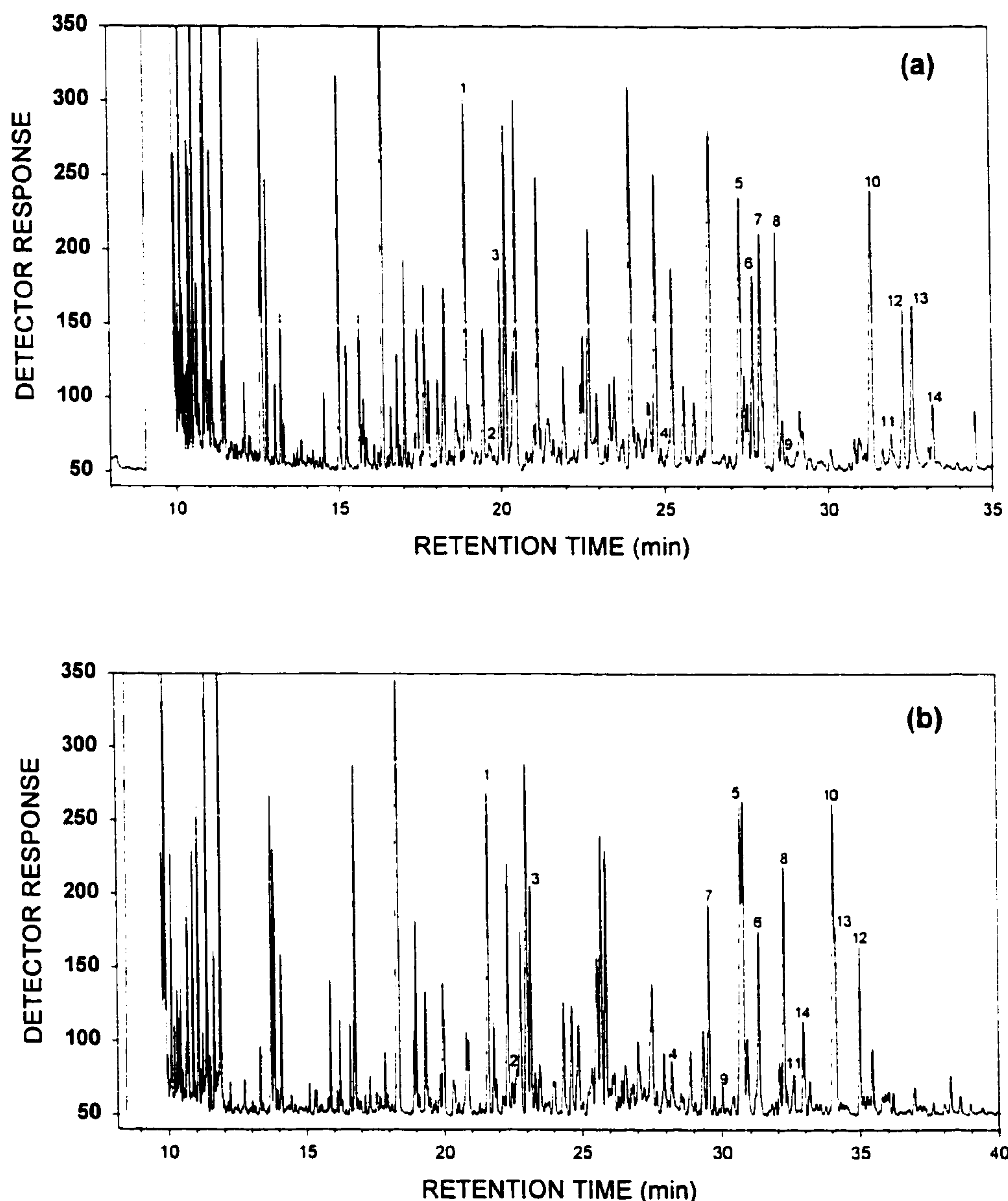

Figure 2.2. Gas chromatographic traces on (a) DB5ms and (b) DB35ms liquid phases of the trimethylsilyl derivatives of tannin and triterpenoid compounds obtained by acid hydrolysis of pin oak leaf litter using the standard hydrolysis conditions. Gas chromatographic equipment and conditions are described in the text. Identifications: 1 = Hematoxylin (internal standard); 2 = Epicatechin; 3 = Catechin; 4 = Epigallocatechin; 5 = Stigmast-5-en-3β-ol; 6 = β-Amyrin; 7 = Epicatechin (4β→2) phloroglucinol; 8 = α-Amyrin; 9 = Catechin (4β→2) phloroglucinol; 10 = Oleanolic Acid; 11 = Catechin (4α→2) phloroglucinol; 12 = Ursolic Acid; 13 = Epigallocatechin (4β→2) phloroglucinol; 14 = Gallocatechin (4α→2) phloroglucinol.

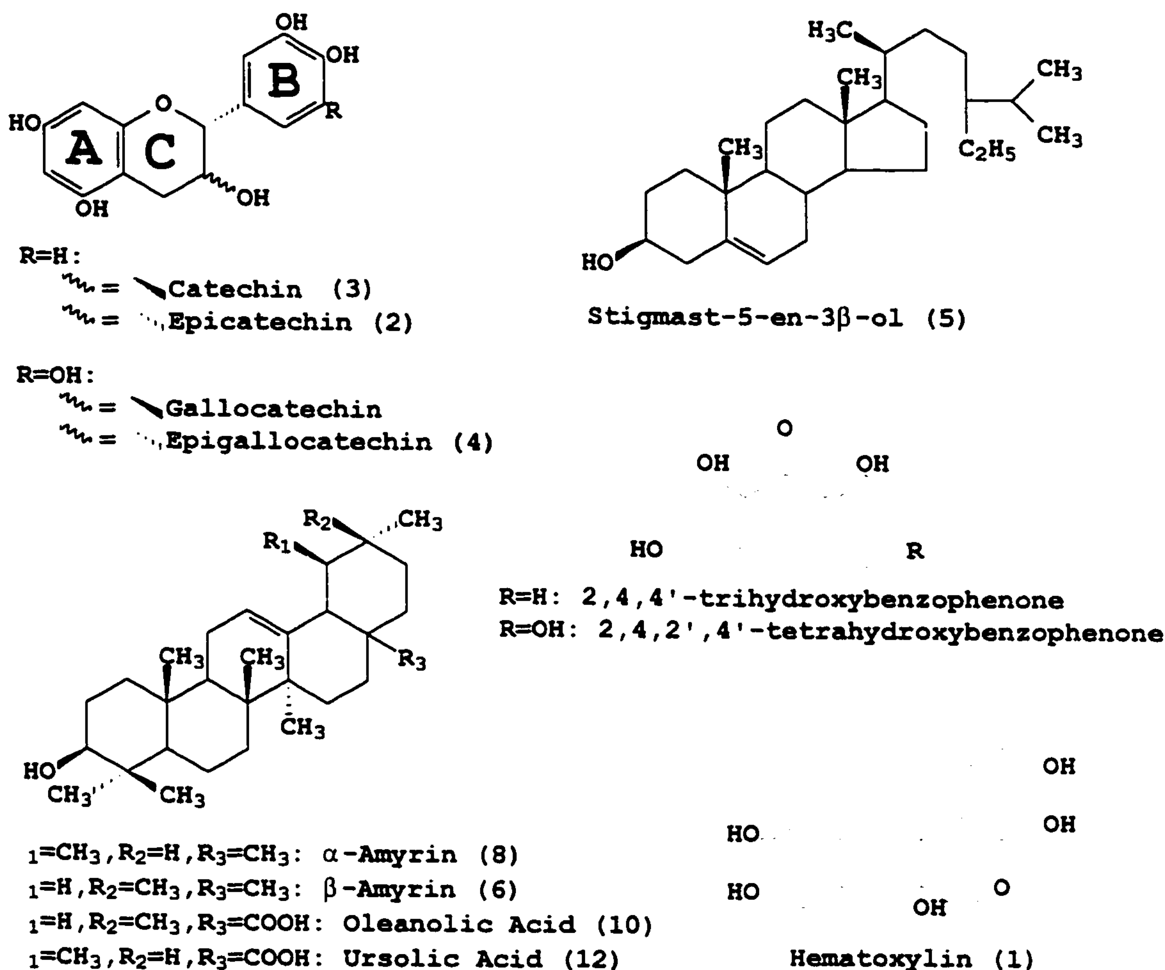

Figure 2.3. Structure (underivatized forms) of compound identified and quantified, numbered according to elution order on DB5ms where appropriate.

typically drawn). Thus, the isomeric pair catechin/epicatechin, or procyanidin (PC), gives rise to four adducts, as does gallocatechin/epigallocatechin, or prodelphinidin (PD). Mass spectral fragmentation patterns for the four adducts in each pair are nearly identical. Steric hindrance from the C-3 hydroxyl group is the primary factor that determines the orientation of the adduct, although stereochemistry of the B-ring at C-2 (which is almost always α) will also be a factor. When both the C-3 hydroxyl group and the C-2 B-ring are *cis* to each other (as in all the epi- forms), then the β position should be highly favored by the adduct: If the C-3 hydroxyl group and C-2 B-ring are *trans* to each other, then a mixture of α and β adducts is possible. In this study, three PC adducts and two PD adducts were identified from mass spectra fragmentation patterns. Two PC

adducts, catechin (4β→2) phloroglucinol and catechin (4α→2) phloroglucinol, were identified from hydrolysis products of the catechin (4α→8) catechin dimer. The third PC, then, is epicatechin (4β→2) phloroglucinol. The elution order as determined for the PC adducts is applicable to the PD adducts as well, which were identified as gallocatechin (4α→2) phloroglucinol and epigallocatechin (4β→2) phloroglucinol. Primary mass spectral fragments for all routinely quantified compounds are given in Table 2.1. Identified compounds for which commercial standards are unavailable were quantified using the area response of the internal standard.

Table 2.1. Diagnostic Mass Spectral Fragments of Trimethylsilyl Derivatives[a].

Compound	Fragments
Tannin	
Catechin/Epicatechin (PC)	368 (B-ring), 355 (A-ring), M^+ 650
Afzelechin/Epiafzelechin (PP)	280 (B-ring), 355 (A-ring), M^+ 562
Gallocatechin/Epigallocatechin (PD)	456 (B-ring), 355 (A-ring), M^+ 738
Gallocatechin/Epigallocatechin (PD) acetone derivative	409/424 (B-ring), 355 (A-ring), M^+ 706
Catechin 4β Adduct	368 (B-ring), 533 (A-ring+adduct), 648
Epicatechin 4α and PC Adduct	368 (B-ring), 533 (A-ring+adduct)
PP Adduct	280 (B-ring), 533 (A-ring+adduct)
PD Adduct	456 (B-ring), 533 (A-ring+adduct)
PD Adduct acetone derivative	409/424 (B-ring), 533 (A-ring+adduct)
Triterpenoid	
α-Amyrin	218, 189, 203, M^+ 498
β-Amyrin	218, 203, 189, M^+ 498
Stigmast-5-en-3β-ol	357, 396, 381, M^+ 486
Oleanolic Acid	203, 320, 482, 189, 585, M^+ 600
Ursolic Acid	203, 320, 482, 189, 585, M^+ 600

[a]Abbreviations: PC, procyanidin; PP, propelergonidin; PD, prodelphinidin. See Figs. 2.1 and 2.3 for structures.

RESULTS AND DISCUSSION

A primary issue in method development is the choice of representative sample material. Preliminary experiments were conducted using whole green leaves from

Camellia sp. as well as an extracted and purified condensed tannin from the same leaves. The following routine hydrolysis conditions were established: 1.0 M HCl in acetone:water (70:30 v/v), 0.26 M phloroglucinol, 30 °C for 24 hours. Other reported hydrolysis conditions when using phloroglucinol include 0.2 M HCl in dioxane:water (1:1 v/v), 0.13 M phloroglucinol, 80 °C for 20 min. (Matthews et al., 1997); 0.3 M HCl in ethanol, 0.04 M phloroglucinol, ambient temperature overnight (Koupai-Abyazani et al., 1992); and 0.3 M HCl in ethanol, ~0.7 M phloroglucinol, ambient temperature for 30 min. (Foo and Karchesy, 1989). The basis for how these conditions were chosen was not reported or limited in nature. In our preliminary experiments, yield efficiencies from extracts were noticeably lower than the whole samples (~5% vs. an estimated 20-50%), which raised concerns about chemical alteration during extraction and the overall suitability of extracts as proxies for environmental samples. Subsequently, the emphasis was shifted toward whole samples, since the ability to analyze the latter was a primary objective of this study. Hydrolysis series were conducted by holding three of the conditions constant and varying the fourth. All analyses were done in duplicate and the results presented are averages with error bars indicating the range. The acid strength series was carried out on both green camellia leaf material (CLM) and pin oak leaf litter (POLL). Only POLL was used for the remaining experiments for a variety of reasons, including the presence of both PC and PD tannin, the presence of triterpenoids which also fall in our analytical window, and the fact that litter more closely resembles the degraded environmental samples that we ultimately wish to analyze.

Acid Strength. Acid concentrations were varied from 0 M to 2 M HCl for both CLM and POLL. Based on the 0 M HCl results, CLM contained nearly 2 wt% catechin + epicatechin as monomers (Fig. 2.4) while POLL contained no tannin in the monomeric form (Fig. 2.5). Catechin and epicatechin were present as both terminal units and extender units in CLM, while POLL contained catechin, epicatechin, gallocatechin, and epigallocatechin terminal and extender units. Maximum tannin yields from CLM were achieved at 0.2-0.5 M HCl, while for POLL, maximum yields were achieved at 1.0 M HCl. Considering the degradation resistance needed for tannin to persist into the litter stage, the higher acid strength necessary for POLL is not surprising. The fact that tannin

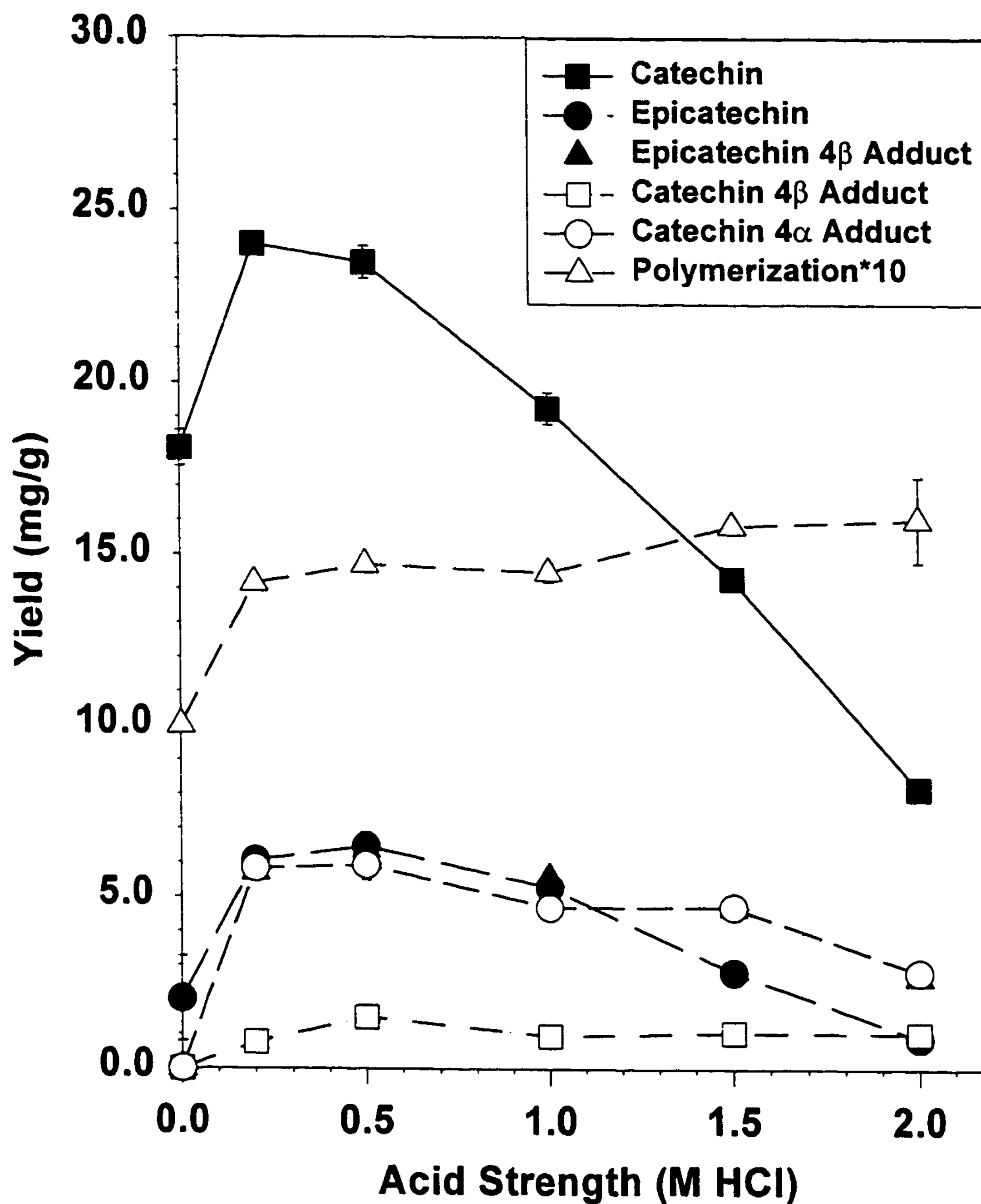

Figure 2.4 Hydrolysis of CLM with varying acid strengths. Note that in order to plot on the same scale degree of polymerization was multiplied by 10.

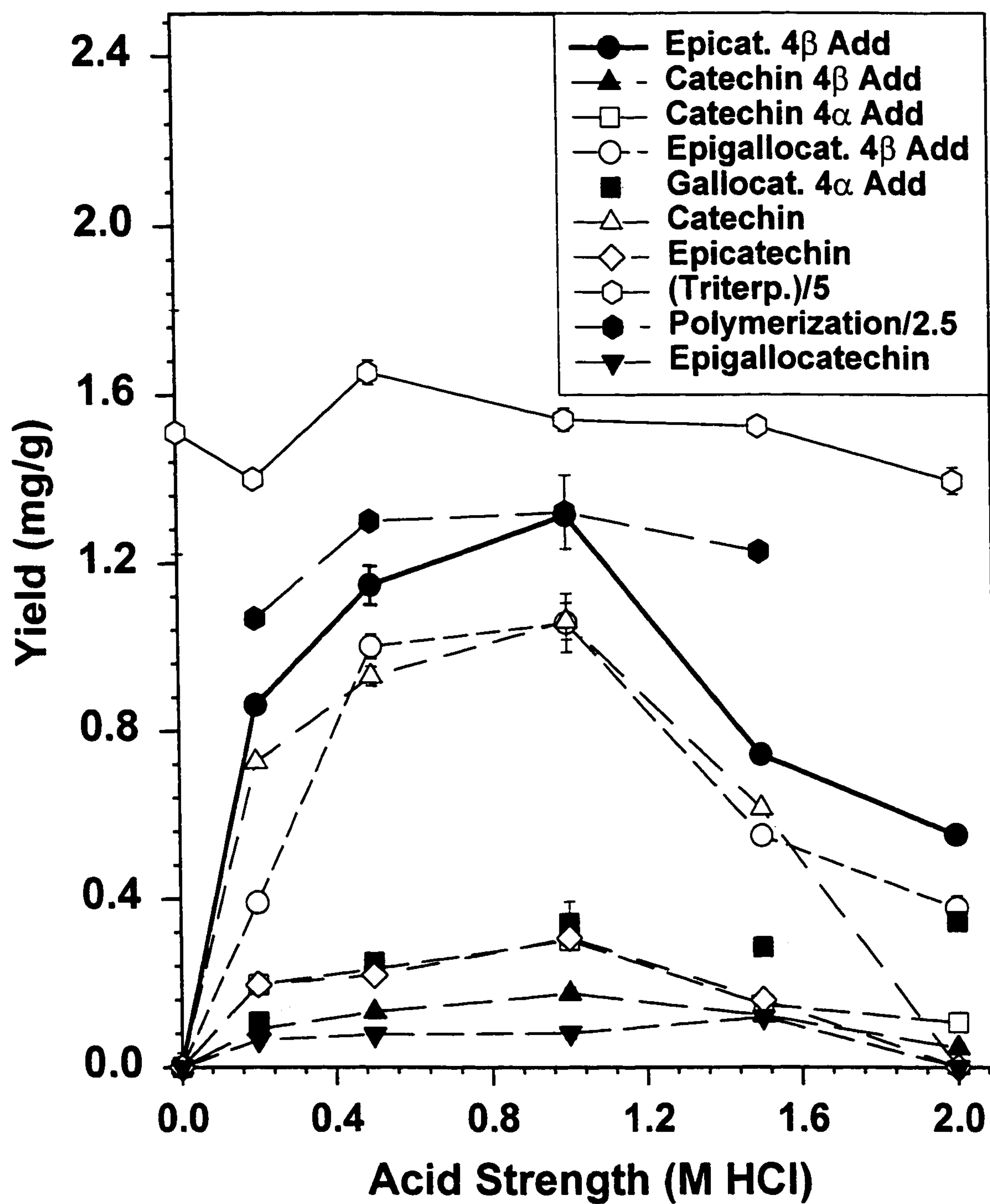

Figure 2.5. Hydrolysis of POLL with varying acid strengths. Note that in order to plot on the same scale, in this and all following plots, degree of polymerization was divided by 2.5 and triterpenoids were divided by 5.

yields decrease at higher acid strengths in both sample types (especially monomeric catechin in CLM) indicates a competing degradation reaction. A further problem with higher acid concentrations is the increased yield of phloroglucinol reaction products in the blank that can potentially co-elute with peaks of interest. The primary interference occurs between monomeric catechin and the peak immediately preceding it in Fig. 2.2b. Below 1.0 M HCl, catechin can be resolved cleanly on the DB35ms column. At 1.5 M and 2 M HCl, the phloroglucinol reaction product peak was larger than catechin and made resolution difficult. For this reason, the DB5ms proved to be a better overall choice for POLL. Given the complexity of the chromatograms resulting from this technique in general, dual column analyses may be appropriate.

Only two other studies were found that tested acid concentrations for phloroglucinol hydrolysis. Matsuo et al. (1984) found that kaki-tannin yields increased with increasing acetic acid concentration, but stopped at 2.5 M before a plateau was reached. Matthews et al. (1997) tested 0.05 M and 0.1 M HCl at ambient temperatures and 80 °C and found that the higher acid concentration resulted in a 20-25% yield increase. Given the increases seen in this study at up to 1.0 M HCl, it would be informative to see the experiments of Matthews et al. repeated at higher HCl concentrations.

In addition to individual tannin compounds, the degree of polymerization (extender plus terminal units divided by terminal units—an indication of chain length) is shown for CLM in Fig. 2.4. Because shorter polymers are generally more labile than longer polymers, degree of polymerization is a potential diagnostic parameter for diagenesis in environmental samples. In CLM, the measured degree of polymerization increased with increasing acid strength. At 0.2 and 0.5 M HCl, this is simply indicative of the higher release of extender units by acid hydrolysis. At higher acid strengths, it appears that catechin and epicatechin are more susceptible to degradation during acid hydrolysis than the adducted extender units. This may be because they are, in fact, more readily degraded. Kinetics also likely come into play, however, as nearly 80% of the catechin is present in CLM as a monomer and immediately available for degradation, whereas the extender units are released over time.

Degree of polymerization in POLL shows a similar trend to CLM, initially increasing to 1.0 M HCl, before exhibiting a slight decrease at 1.5 M (Fig. 2.5). At 2.0 M HCl, no terminal units were detected, therefore a degree of polymerization could not be calculated. As with CLM, though, the adducted extender units persist. Because both terminal and extender units are released at the same time, this appears to indicate that adduction imparts increased stability.

Finally, the sum of the triterpenoids β-amyrin, α-amyrin, oleanolic acid, and ursolic acid show little trend (Fig. 2.5. Note division by 5 to plot on the same scale). They do not appear to be covalently bound to the plant matrix, given that the yield at 0 M HCl is similar to the yields when acid is present. Extra care must be taken when quantifying the triterpenoids given the chromatographic conditions used in this method. In particular, response factors relative to the internal standard can vary considerably when column overloading occurs with the triterpenoids, and the amount of standards injected on the GC must be matched more carefully to the amount present in the sample injected.

Because it gave the highest yields in POLL, 1.0 M HCl was used for the remainder of the experiments.

Hydrolysis Time. POLL was hydrolyzed from 1-48 hours. Nearly half the total yield was achieved within the first hour, and a plateau was reached by 24 hours (Fig. 2.6). This pattern shows the reactivity of condensed tannin to relatively mild acid hydrolysis, and indicates that the advanced reaction system is stable. Since adduction of the carbocation in theory is reversible, while decomposition of the carbocation to a cyanidin or delphinidin is not, yields might have been expected to decrease with time. At ambient temperatures, Matthews et al. (1997) found that >200 hours were needed to achieve a plateau with their hydrolysis conditions. Again, our acid strength was a factor of ten greater, which likely explains the difference.

The degree of polymerization also reaches a plateau at 24 hours (Fig. 2.6). The initial value is 2.4 increasing to 3.5. If all interflavan bonds were equally accessible or hydrolyzable, then this ratio should not change. The observed increase suggests that in this sample, terminal units on average are more easily hydrolyzed than the extender unit.

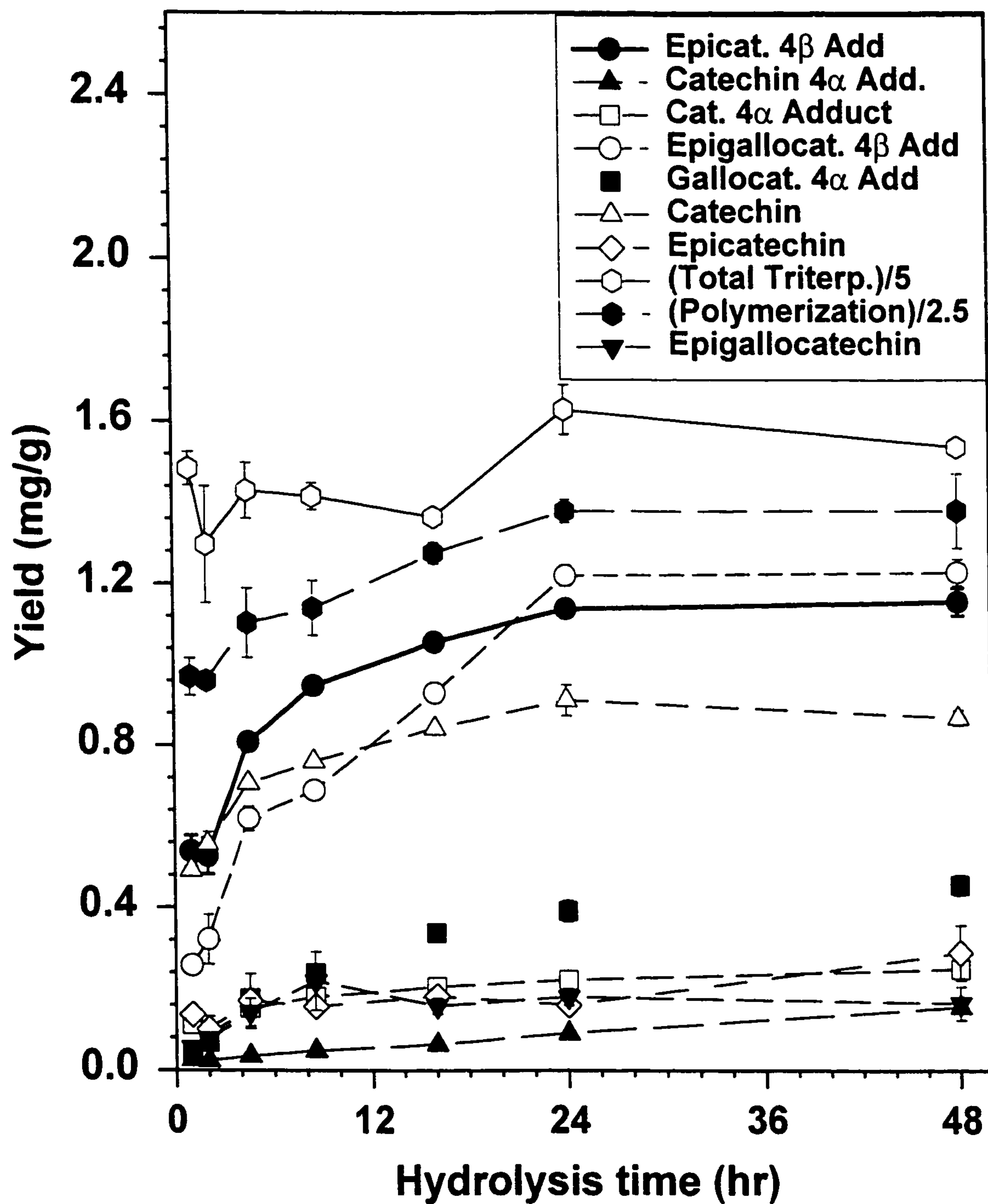

Figure 2.6. Hydrolysis of POLL with varying hydrolysis times.

It is possible that the ends of the polymer are equally hydrolyzable (one terminal and one extender unit), and that hydrolysis of internal extender units is inhibited until the ends are released. Since the actual conformation of condensed tannin polymers is not known, it isn't clear whether the difference in hydrolyzability is due to hindrance by the tannin itself, or by compounds associated with tannin.

Based on the plateaus in yields and degree of polymerization, 24 hours is an appropriate choice for hydrolysis times and was used in the remainder of the experiments.

Hydrolysis Temperature. Hydrolysis temperatures were varied by ~10 °C from room temperature (22 °C) to 50 °C. Highest yields were achieved at 30 °C (Fig. 2.7). As with increased acid strength, harsher temperature conditions appear to decrease yields. Again, terminal units are less stable than adducted extender units, as evidenced by the large decrease in catechin and the large increase in the degree of polymerization (3.4 to 5.9) under harsher conditions. Higher temperatures also lead to larger numbers and quantities of phloroglucinol reaction products in the blank, which resulted in coelution problems.

As mentioned previously, Matthews et al. (1997) conducted experiments at ambient temperatures and at 80 °C. While hydrolysis times were very different (>200 hours vs. 20 min.), total yields were similar.

Although steric hindrance is the primary factor in determining relative proportions of α and β adducts, temperature can also likely play a role. A case in point appears to be the formation of PD adducts. At 22 °C and 30 °C, only two PD adducts (epigallocatechin (4$\beta\rightarrow$2) phloroglucinol and gallocatechin (4$\alpha\rightarrow$2) phloroglucinol) give quantifiable peaks. A third PD adduct is detectable by mass spectroscopy in trace amounts. However, at 40 °C, a distinct peak is evident (likely gallocatechin (4$\beta\rightarrow$2) phloroglucinol), and at 50 °C it is of the same magnitude as gallocatechin (4$\alpha\rightarrow$2) phloroglucinol.

In many studies, ambient temperature is used for hydrolysis with phloroglucinol. However, in order to reduce variability, a higher controllable temperature is desirable if it doesn't sacrifice yields. And in fact, 30 °C gave higher yields, and so was chosen.

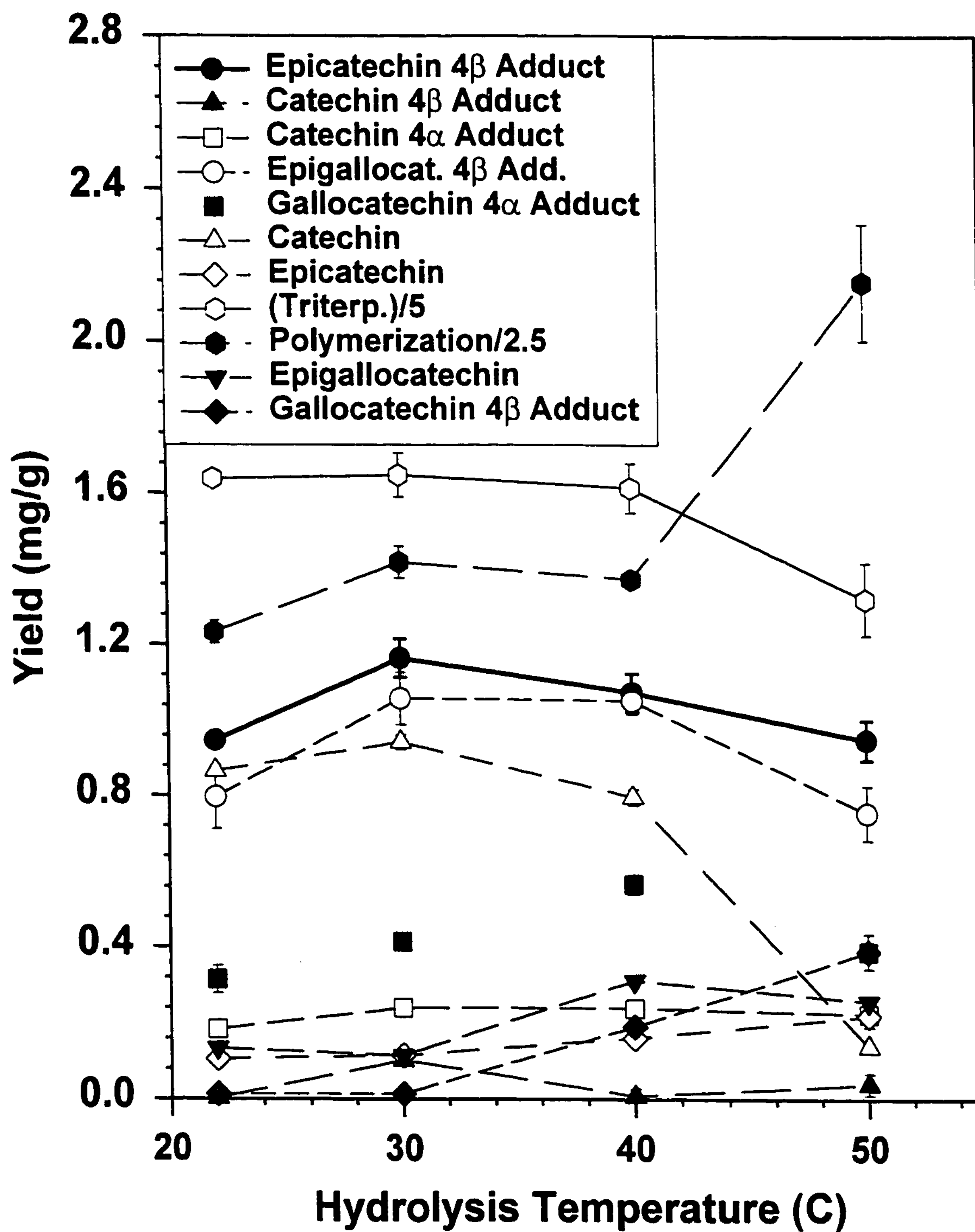

Figure 2.7. Hydrolysis of POLL with varying temperatures.

Nucleophile Concentration. Phloroglucinol concentrations were varied over six concentrations from 0.066 M to 0.53 M (as compared to the 0.04 M to ~0.7 M range in the studies cited above). For 50 mg POLL, this is stoichiometrically in excess of the condensed tannin extender units by a factor of 250-2000. Tannin yields increased up to 0.26 M phloroglucinol before tailing slightly (Fig. 2.8). The tailing may be in part related to the noticeable viscosity increase observed in the samples with higher phloroglucinol concentrations throughout sample workup. For example, redissolving the one-third split from a 0.26 M phloroglucinol hydrolysis sample in less than 200 μL pyridine often lead to derivatization problems. Thus, the 0.39 M and 0.53 M samples required a larger volume of pyridine and introduced variability into the procedure. Degree of polymerization increased with increasing phloroglucinol. This is as expected, given that an increased nucleophile concentration should lead to more efficient capture of the extender carbocations. Because adduct yields decrease at the highest phloroglucinol concentrations, obviously capture efficiency is not the only factor.

Choosing the best nucleophile concentration is perhaps the least obvious of the four hydrolysis conditions varied. 0.13 M, 0.195 M, and 0.26 M all appear to be reasonable choices. 0.26 M was adopted because it gave the highest yield of epicatechin (4β→2) phlorolglucinol. Epicatechin extender units from which it is derived are the most abundant condensed tannin.

Linearity. Hydrolysis-yield linearity tests were carried out on POLL, the results of which are presented as correlation coefficients (r^2) in Table 2. Sample sizes ranged over two orders of magnitude: 1, 5, 10, 25, 50, and 100 mg. When the entire range is included, all correlation coefficients exceeded 0.98, with the exception of epicatechin at 0.90. When plotted, however, compounds from the 100 mg samples all fall below the linear regression line. If linear regressions are calculated for only the 1-50mg samples, correlation coefficients are similar to those obtained for 1-100mg, but sample weight percents increase by as much as 15%. Based on the weight percent of the lowest two compounds, epicatechin and catechin (4β→2) phloroglucinol, and the 1 mg sample size, GC detection limits for this technique are at least as low as 130 ng of tannin.

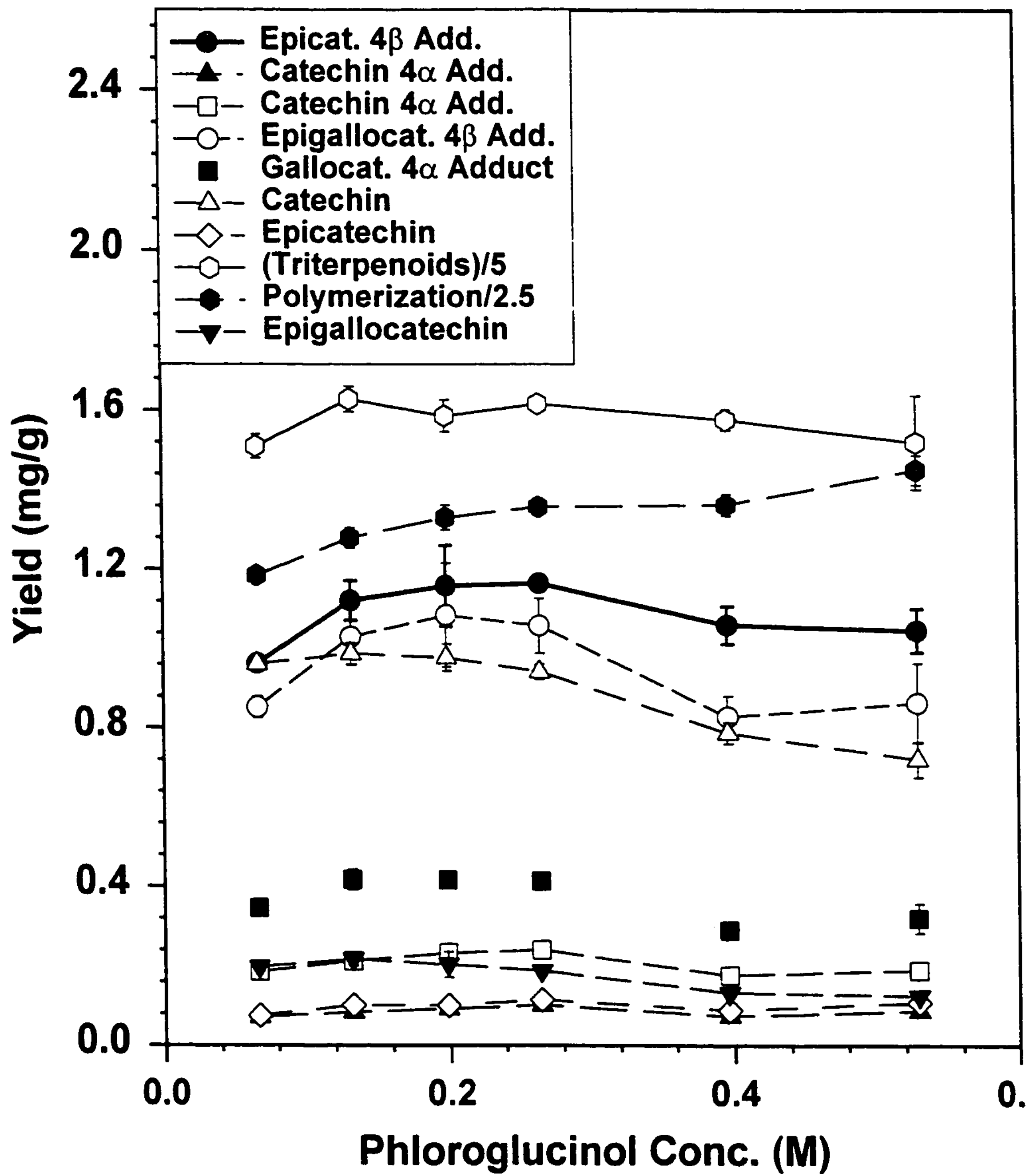

Figure 2.8. Hydrolysis of POLL with varying nucleophile (phloroglucinol) concentrations.

Table 2.2. Hydrolysis-Yield Linearity Tests[a]

Tannin	1 to 50 mg samples corr (r^2)	Wt%	1 to 100 mg samples corr (r^2)	Wt%
Catechin	0.997	0.087	0.997	0.080
Epicatechin	0.663	0.014	0.895	0.014
Epigallocatechin	0.980	0.020	0.989	0.021
Epicatechin 4β Add.	0.998	0.171	0.988	0.143
Catechin 4β Add.	0.994	0.013	0.994	0.012
Catechin 4α Add.	0.994	0.032	0.993	0.030
Epigallocat 4β Add.	0.989	0.177	0.997	0.170
Gallocat. 4α Add.	0.963	0.053	0.990	0.056
Triterpenoid				
α-Amyrin	0.999	0.182	0.991	0.156
β-Amyrin	0.998	0.130	0.996	0.118
Stigmast-5-en-β-ol	0.995	0.210	0.991	0.181
Oleanolic Acid	0.992	0.461	0.991	0.400
Ursolic Acid	0.988	0.147	0.994	0.135

[a]Hydrolysis-yield linearity for pin oak leaf litter (duplicates of 1, 5, 10, 25, 50, and 100 mg samples, 45.1% OC). Correlation coefficients (r^2) calculated by linear regression.

Internal Standards and Standard Recoveries. Acquisition of appropriate standards is challenging. Catechin, epicatechin, and epigallocatechin have been the only commercially available condensed tannin standards until very recently. Numerous flavone and triterpenoid standards are commercially available, as are a few flavanones. Acquiring compounds analogous to the condensed tannins for use as internal standards is also difficult. Historically, quercetin has been used as an internal standard when GC or liquid chromatography has been used with purified condensed tannins. However, because quercetin is present in many of the tissue types that we have analyzed, it is inappropriate for whole samples. To date, three polyphenolic compounds have been used as internal standards: 2,4,4'-trihydroxybenzophenone, 2,4,2'4'-tetrahydroxybenzophenone, and hematoxylin (Fig. 2.3). The primary qualities desirable in an internal standard are similar recoveries relative to the compounds of interest, clean

chromatographic elutions, and similar elution times to the compounds of interest. In this method, the former is largely dependent on solubility in water as compounds are partitioned from the aqueous phase into ethyl acetate. In general, compounds with a higher number of hydroxyl groups will have lower recoveries into ethyl acetate and vice versa. In triplicate partition experiments involving the internal standards, if the recovery of tetrahydroxybenzophenone is assigned 1.00, recovery of trihydroxybenzophenone was 1.14, and hematoxylin with five hydroxyl groups was 0.93, showing the predicted trend. On this scale, most hydroxylated flavones, flavanones, and flavanols also range from 0.9-1.1. Triterpenoids and unhydroxylated flavone and flavanone ranged from 1.2-1.4. If the amount of water added after hydrolysis was reduced, all recoveries would converge toward 1.0. The tradeoff in this case would be more complex chromatography. In terms of partitioning, all three internal standards appear to be reasonable choices. A second factor in recovery, however, is reactivity with the sample, and as will be shown below, hematoxylin is problematic when mineral matrices are involved.

Chromatographically, all three internal standards elute before any of the compounds of interest. Ideally, a late-eluting internal standard could also be found. Because of a partial coelution problem with trihydroxybenzophenone on the DB35ms column, hematoxylin was used exclusively in the hydrolysis series of this study. With the incorporation of the DB5ms, however, coelution does not appear to be a problem with the benzophenones, and therefore either is an appropriate choice.

As stated earlier, acetone:water (70:30 v/v) was chosen as the hydrolysis solvent because of its common use in extracting tannin. An unexpected result of this is the quantitative conversion of PD tannins in a reaction involving acetone and one of the hydroxyl groups on the B-ring. Mass spectra indicates the addition of 56 mass units with a readily lost methyl group. Thus, instead of a 456 fragment analogous to the 368 fragment of PC tannin and the 280 fragment for propelargonidin (PP) tannin, what is actually seen is a 409/424 doublet (Table 2.1). Based on experiments with epigallocatechin standard, the rate of reaction is on the order of minutes, the conversion quantitative, and the product stable at least through a 24-hour hydrolysis.

Matrix Effects. Matrix effects were evaluated by the addition of 100-1000 mg sediment from Dabob Bay, Washington to ~50 mg of POLL, or approximately 0.3 mg of tannin (Fig. 2.9). A single addition of 1000 mg soil to 50 mg of POLL was also done for comparison. The effective tannin concentration relative to the sediment, then, was 0.3-0.03 wt% tannin, and 0.03 wt% relative to the soil. Because hematoxylin proved to be very reactive toward the sediment and soil, quantitation was carried out by normalizing to the sum of the two amyrins, oleanolic and ursolic acid since the latter were shown to be relatively unchanged in various conditions. Not surprisingly, the PD tannins showed the highest reactivity, decreasing by more than a factor of two with the addition of 100 mg of sediment. All PC tannins remained unchanged. At 250 mg, all tannin showed considerable decreases. In contrast to harsher hydrolysis conditions in the earlier experiments, the degree of polymerization decreased by a factor of two with the addition of sediment. While many mechanisms are possible, larger molecules have generally been shown to be more reactive than smaller molecules toward mineral surfaces in sorption experiments (Thimsen and Keil, 1998), and the trend in degree of polymerization is consistent. Tannin reactivity toward the soil appears to be less than that of the sediment. The effect of the addition of 1000 mg soil corresponds approximately to 400 mg addition of sediment. The overall implication of this experiment is that unreacted tannin may be detectable by GC in soils and sediments at least as low as 0.03 wt%. Analysis of a soil sample taken from within the dripline of the CLM camellia tree showed the presence of tannin at 0.002 wt% as estimated by GC/MS, which shows greater sensitivity than GC.

In order to evaluate mechanisms for the matrix effects shown above, POLL was hydrolyzed in the presence of combusted sediment (to remove organic carbon), desalted combusted sediments, and sodium chloride in amounts equivalent to that present in 1000 mg of sediment. Sodium chloride did not affect yields, while both the combusted sediment additions resulted in no tannin. This suggests that tannin is very mineral reactive, and that organic coatings might aid preservation. In addition to these matrix effects, protein/tannin interactions were also tested by the addition of 50 mg bovine serum albumin to POLL prior to hydrolysis. Yields were not affected.

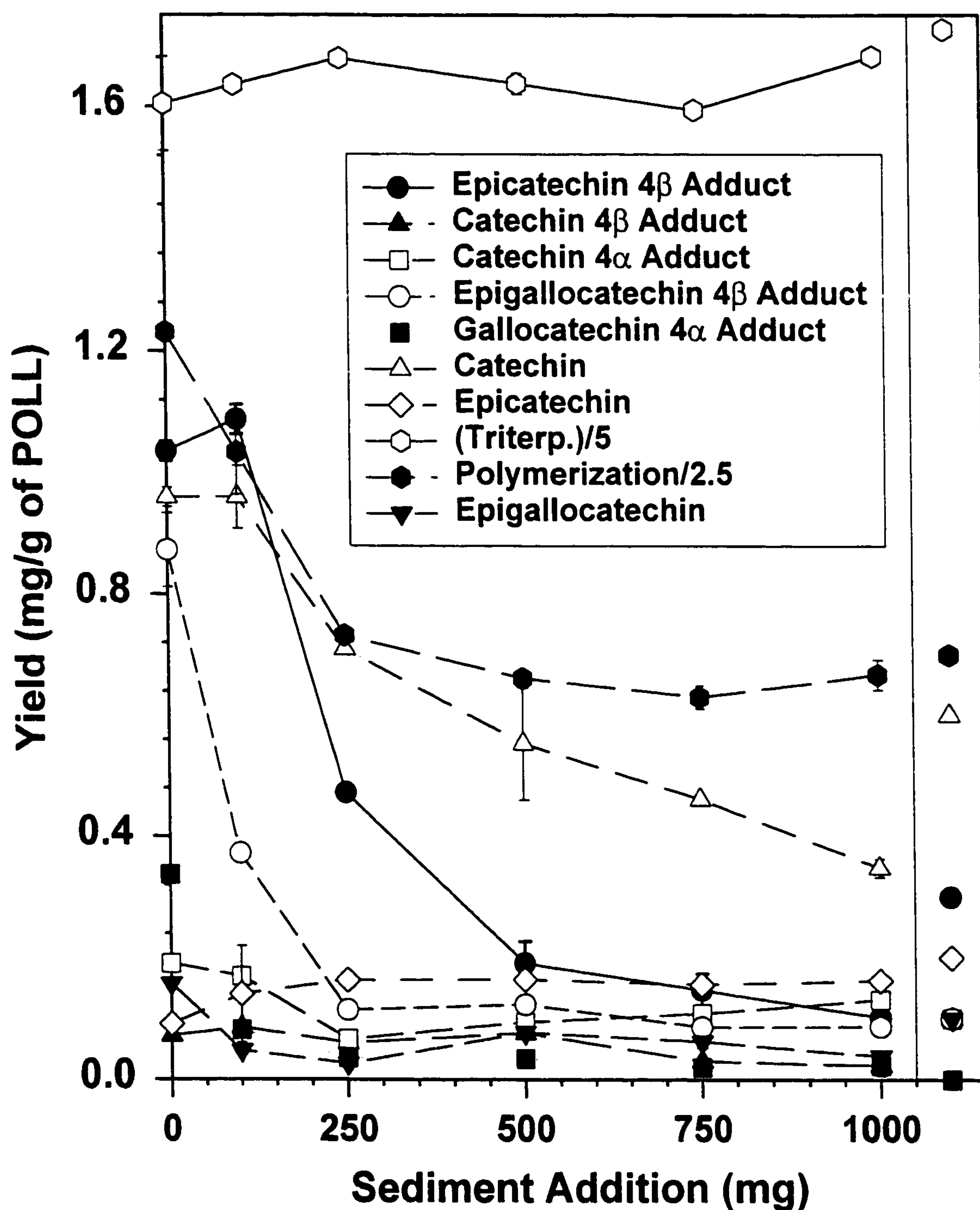

Figure 2.9. Hydrolysis of POLL with addition of sediment. Yields are given relative to the amount of POLL hydrolyzed in order to allow direct comparisons between samples, i.e. the decreases shown are real and not a dilution effect.

Our experience with mineral matrix effects was similar to that of Schofield et al. (1998). They were unable to detect tannin in soils underlying willow leaf litter, despite evidence that tannin was being leached from the leaves. Even when purified *Sorghum* tannin was added to the soil at up to 0.2 wt%, they were unable to extract any measurable tannin by several different techniques, including sorptive techniques and direct hydrolysis in the presence of phloroglucinol.

Clearly, mineral matrix effects and the ability to quantify tannin in soils and sediments are areas that need further study. There are a number of factors to consider, the primary one being whether tannin is sorbed by the minerals (and associated organics) but remains intact, or whether it is chemically altered. If it is the former, then it may be possible in the future to find a method for desorption. If it is the latter, the challenge will be to determine how it is altered and how to quantify it. In either case, if a means of extraction ultimately is not possible, then solid phase ^{13}C-NMR or pursuing variations of stronger degradative techniques like pyrolysis or CuO oxidation may be necessary for direct quantification. Mechanistically, sorption studies involving purified tannin, soils, and sediments are in order.

Comparisons to Other Methods. Analysis of a variety of sources allows for direct comparisons to be made between this and other tannin methods. Complete discussions can be found in (Hernes et al., in prep) and (Hernes and Hedges, in prep). In a mangrove leaf study, total tannin estimates and measurements were made with ^{13}C-NMR and the Folin-Denis reagent (Benner et al., 1990a) as well as with the method reported here (Hernes et al., in prep). In green and senescent yellow mangrove leaves, for instance, total molecular tannin via this method was measured at 6% in both leaf types, 7% and 10% with the Folin-Denis reagent, and ~20% by ^{13}C-NMR. Quantitative comparisons can also be made for bark tannins of the same species analyzed by this method on whole samples (Hernes and Hedges, in prep) and thioacidolysis (hydrolysis in the presence of toluene-α-thiol) on extracts (Matthews et al., 1997b). Barks from *Pinus contorta* and *Pseudotsuga menziesii* were measured at the molecular level in both studies, the latter method yielding 3.2% and 3.3% total tannin, respectively, while this method gave 3.3% and 3.1%. The analysis by this method, however, also included

flavanones, which constituted about half the tannin measured in these two samples. While part of the difference is likely directly attributable to the higher efficiency reported for thioacidolysis (Matthews et al., 1997a), preservation is also likely a factor. Tannin in samples has been shown to become more hydrolysis resistant with age (Matthews et al., 1997a). The barks analyzed by thioacidolysis were freshly collected (Matthews et al., 1997b) whereas those by this technique were >10 years old.

Compositional comparisons between the barks can also be made. In the *P. contorta* and *P. menziesii* barks, the degree of polymerization was measured at 4.7 and 3.7, respectively, by thioacidolysis (Matthews et al., 1997b) vs. 4.4 and 2.5 for this technique (12). A commonly measured parameter is the ratio of 2,3-*cis* (i.e. epicatechin and epigallocatechin) monomers to 2,3-*trans* (i.e. catechin and gallocatechin) monomers. This ratio was determined to be 60:40 and 81:19, respectively, by thioacidolysis (Matthews et al., 1997b) vs. 60:40 and 64:36 by this technique (Hernes and Hedges, in prep). Discrepancies in *P. menziesii* can be attributed to the considerable variability found in inner and outer barks (Matthews et al., 1997b). Finally, <20% of the condensed tannin in *P. contorta* bark as measured by thioacidolysis was PD tannin, as compared to a previous literature value of 69% (Porter, 1989). The measurement of 34% PD tannin by this technique is intermediate (Hernes and Hedges, in prep).

Compositional characteristics of tannin are commonly measured by ^{13}C-NMR on extracted and purified tannin. Results from seed cones analyzed by this method (Hernes and Hedges, in prep) can be compared to those obtained by ^{13}C-NMR (Eberhardt and Young, 1994). ^{13}C-NMR results from five *Pinus sp.* seed cones indicated that 2,3-*cis* conformations made up 65-81% of condensed tannin. Six seed cones were analyzed by this method, with four species showing 62-90% 2,3-*cis*, and 37% and 38% in *Sequoia sempervirens* and *Tsuga heterophylla*, respectively. The only common species between the two sample sets was *Pinus ponderosa*. In this species, ^{13}C-NMR indicated 74% 2,3-*cis* vs. 62% by this method. Degree of polymerization in the seed cones ranged from 5.3-8.5 by ^{13}C-NMR vs. 1.8-7.4 by this method (5.4 vs. 2.4 for *Pinus ponderosa*). This latter difference is expected given that monomers and smaller oligomers are eliminated in extraction and purification procedures. On the whole, then, this method gives results

that are consistent with other established techniques for tannin, but without extraction procedures and with much greater throughput.

Further Considerations. From the beginning of this project, commercial availability of condensed tannin standards has been a limiting factor in the overall method development. The catechin-catechin dimer used to identify catechin-phloroglucinol adducts, for instance, has only recently become available. Propelargonidin standards do not exist. Authentic standards of phloroglucinol adducts are still needed in order to improve quantification. In addition, authentic standards of condensed tannin oligomers or polymers are needed in order to evaluate hydrolysis efficiency. The catechin-catechin and catechin-epicatechin dimers can certainly be useful in addressing this question. However, the effect of chain length on hydrolysis efficiency remains to be seen, and epicatechin and catechin extender units are known to have different kinetic rates of hydrolysis (Hemingway and McGraw, 1983).

One measure of the effectiveness of a technique is how well it performs under a given set of conditions. The purpose of this study has been to conduct such an evaluation. This technique allows rapid, reproducible analyses of tannin and triterpenoids on whole samples. An equally important measure, however, is the potential of the method for improvement and adaptation. For instance, identification and quantification of the carboxylic acids will certainly increase the overall usefulness of the method. Tetracosanoic acid as quantified by this method has already been shown to be a potential indicator for barks (Hernes and Hedges, in prep). Possible modifications to the method to increase sensitivity include on-column injection, drying down the hydrolysate followed by direct extraction, or the use of photo ionization detectors tuned specifically to detect phenolics.

Tannin in soils and sediments, and the overall issue of mineral matrix effects remains to be evaluated. However, this method has already shown utility in the study of early diagenesis of mangrove leaves (Hernes et al., in prep) and in characterizing nearly 120 source tissues at the molecular level for tannin (Hernes and Hedges, in prep). As such, it may prove useful in many geochemical studies involving terrestrial plant material as well as in natural products.

CHAPTER 3: TANNIN SIGNATURES OF BARKS, NEEDLES, LEAVES, CONES, AND WOOD AT THE MOLECULAR-LEVEL

INTRODUCTION

The environmental reactivity of tannin, combined with its abundance and ubiquity in vascular plants (e.g. Haslam, 1989), makes it a prime candidate for biogeochemical studies. In terrestrial biomass, tannin trails only carbohydrates (in the form of cellulose and hemicellulose) and lignin in overall abundance, as the latter are the primary components in woody tissues. However, in soft tissues such as leaves, needles, and bark, tannin is often more abundant than lignin (Hedges and Weliky, 1989; Kelsey and Harmon, 1989; Benner et al., 1990a). Because these tissues cycle more rapidly than woods, tannin can be quantitatively, as well as qualitatively, important in early diagenesis (eg. Benner et al., 1990a; Hernes et al., in prep).

Tannin is found only in vascular plant tissues and occurs in both condensed and hydrolyzable forms (Fig. 1.1). Condensed tannins are composed of polymers and oligomers of three-ring flavanols. The components are typically labelled "extender" or "terminal" units depending on their location (Fig. 1.1). Hydrolyzable tannin is made up of gallic acid units, often ester-linked to a carbohydrate backbone (Fig. 1.1), but can also exist just as polymers of gallic acid. With their diverse structures and phenolic character, tannins participate in a number of important reactions, including photochemical and redox transformations, nitrogen immobilization and cation complexation. Tannins are also potential precursors of humic substances via quinone formation and Schiff base reactions.

While the natural products literature contains numerous molecular-level characterizations of tannin from differenct source materials (e.g. Thompson et al., 1972; Harborne, 1988; Mole, 1993), the results of these analyses are difficult to use as source indicators in biogeochemical studies due to lack of a standardized analytical procedure, representation among the biogeochemically important tissue types, and definitive quantification. Nevertheless, several molecular trends are evident in the tannin

literature that might be useful and complementary to applications of lignin and cutin (also uniquely terrestrial) as biomarkers. For instance, lignin is not used to distinguish monocotyledons and dicotyledons, whereas *ent*-epicatechin is unique to monocotyledens, and propelargonidin-containing polymers are much more common in monocotyledons than dicotyledons (Ellis et al., 1983). In addition, hydrolyzable tannin is only found in dicotyledons. Although the geochemistry of bark is poorly studied, some barks have been shown to contain large quantities of flavanones (Hergert, 1989). Barks are also important industrially and anthropogenically, as debarking waste generated by the timber industry often contains toxic levels of tannin (Field and Lettinga, 1992). Finally, in certain environments where potential sources are more constrained, condensed tannin compositions often provide more species-dependent taxonomic information (Haslam, 1989).

This study utilizes a new molecular tannin method (Hernes and Hedges, in prep) that combines reproducible quantification along with rapid throughput in order to evaluate the source potential of various plant tissues in biogeochemical studies. We analyzed 117 different source materials from tropical and temperate monocotyledons, dicotyledons, and conifers, including 18 barks, 16 woods, 7 cones and seedpods, and over 70 leaves, needles, and whole plants. In addition to molecular tannin compounds, several triterpenoids and carboxylic acids also fall within the analytical window and provide additional source information.

EXPERIMENTAL SECTION

Sample Collection and Preparation.

Samples were obtained from a variety of sources. All temperate samples (including ferns, conifers, monocotyledons, and dicotyledons) were collected in Washington state. Green leaf samples for *Camellia* sp., *Malus* sp., *Rosa* sp., *Rubus* sp., *Quercus palustris*, and *Tsuga heterophylla* cones and bark were gathered in Seattle, WA. Monocotyledons were obtained around Dabob Bay, WA as described in Cowie and Hedges (1984). Some conifer species were also collected near Dabob Bay, WA as described by Hedges and Weliky (1989). The remainder of the temperate samples were found in the University of Washington main campus or arboretum as described in Goñi

and Hedges (1990). All the Amazon samples (whole monocotyledons, green dicotyledon tree leaves, dicotyledon wood and bark) were collected in the Amazon River basin and identified as described in Hedges et al. (1986). All samples were oven-dried at 50-60 °C or freeze-dried and ground to pass a 42-mesh (350-μm) sieve.

Analytical Procedure.

A complete description of the tannin analytical procedure can be found in Hernes and Hedges (in prep). Briefly, ~50 mg of plant material (12-24 samples at a time are routinely analyzed) was hydrolyzed in a 1.0 M HCl, 0.26 M phloroglucinol solution of acetone:water (70:30 v/v), total volume 3 mL. The hydrolysis was carried out at 30 °C for 24 hours, after which 100-150 μg of internal standard, hematoxylin, was added. The hydrolysate was then diluted with 10 mL water, and extracted 3x with ethyl acetate. After passing through anhydrous Na_2SO_4 drying columns to remove water, the ethyl acetate solution was split (two thirds for archive, one third for analysis), dried under a stream of N_2, and the samples placed in a vacuum dessicator overnight. The next morning, the one-third split was redissolved in 200 μL pyridine. A subsample (20-30 μL) was placed in an autosampler vial (containing a 200 μL insert) along with an equal volume of Regisil, (bis(trimethylsilyl)trifluoroacetamide) + 1% trimethylchlorosilane (Regis Chemical Co.), and heated to 60 °C for 15 minutes.

All samples were analyzed using a Shimadzu AOC-14 autoinjector coupled to a Hewlett-Packard 5890 gas chromatograph. Quantification was carried out using a flame ionization detector (FID) while peak identification was done with a Hewlett-Packard 5970 Mass Selective Detector. Analyses were made by using splitless injection on a 30 m by 0.25 mm i.d. fused-silica capillary column coated with DB35ms liquid phase (J&W Scientific Inc.). The oven temperature was held at 70 °C for two minutes 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 maintained for 30 minutes. Electronic pressure control was also used during FID detection, 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 Fig. 3.1 for senescent *Rhizophora mangle* leaves. Compound structures of identified peaks are shown in Fig. 3.2.

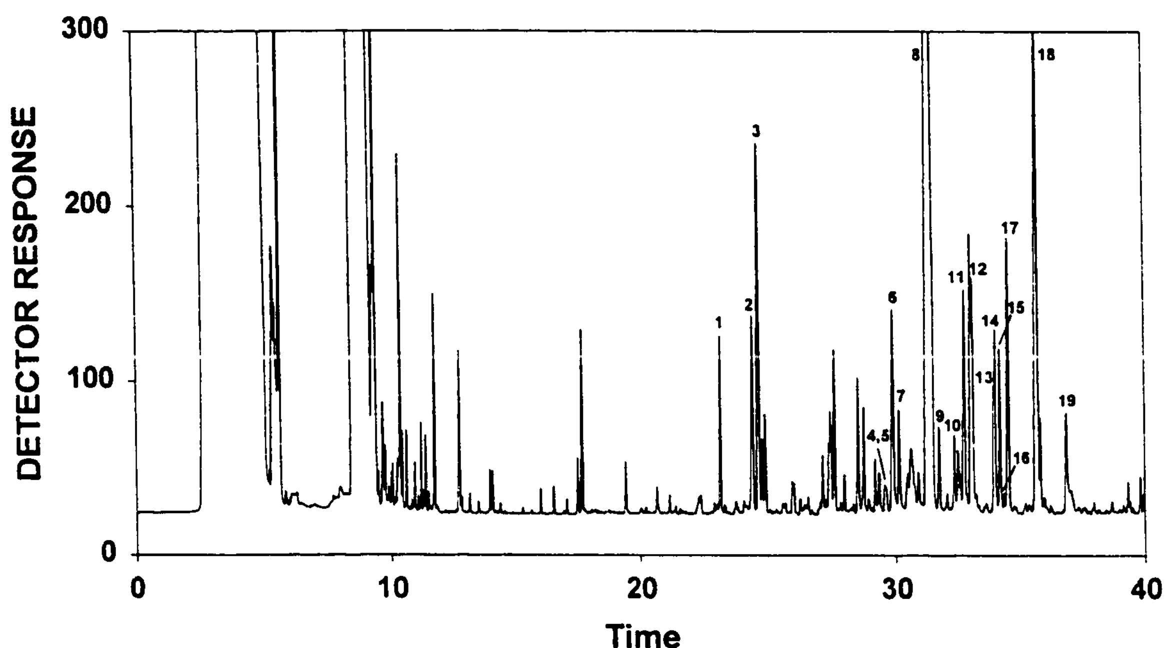

Fig. 3.1 GC trace of *Rhizophora mangle* yellow leaves. 1=Hematoxylin, 2=Epicatechin, 3=Catechin, 4=Epigallocatechin, 5=Gallocatechin, 6=Quercetin, 7=Myricetin, 8=Epicatechin(4α→2)phloroglucinol, 9=Catechin(4β→2)phloroglucinol, 10= Stigmast-5-en-3β-ol, 11=Taraxerol, 12=β-Amyrin, 13=Epicatechin(4β→2)-phloroglucinol, 14=α-Amyrin, 15=Catechin(4α→2)phloroglucinol, 16=Epigallocatechin(4α→2)-phloroglucinol, 17=Epicatechin-derivative(4→2)-phloroglucinol, 18=Gallocatechin-(4β→2)phloroglucinol, 19= Epicatechin-derivative(4→2)phloroglucinol.

RESULTS AND DISCUSSION

A challenge in any study involving tannin is that this macromolecular material "resists" broad taxonomic classification. There are well over 4000 unique flavanoid structures that have been isolated and identified in the literature (Harborne et al., 1975). Given the many different biochemical and ecological functions ascribed to tannins and polyphenols (Appel, 1993 and references therein), this molecular diversity is not surprising. The number of flavanoids incorporated into condensed tannin is considerably smaller, but the overall diversity (combined with multiple functions) means that individual species can have quite different tannin suites. Thus, there is great potential for distinguishing individual source contributions in a variety of natural settings. Nevertheless, if any condensed tannin is present in a sample, there is a great

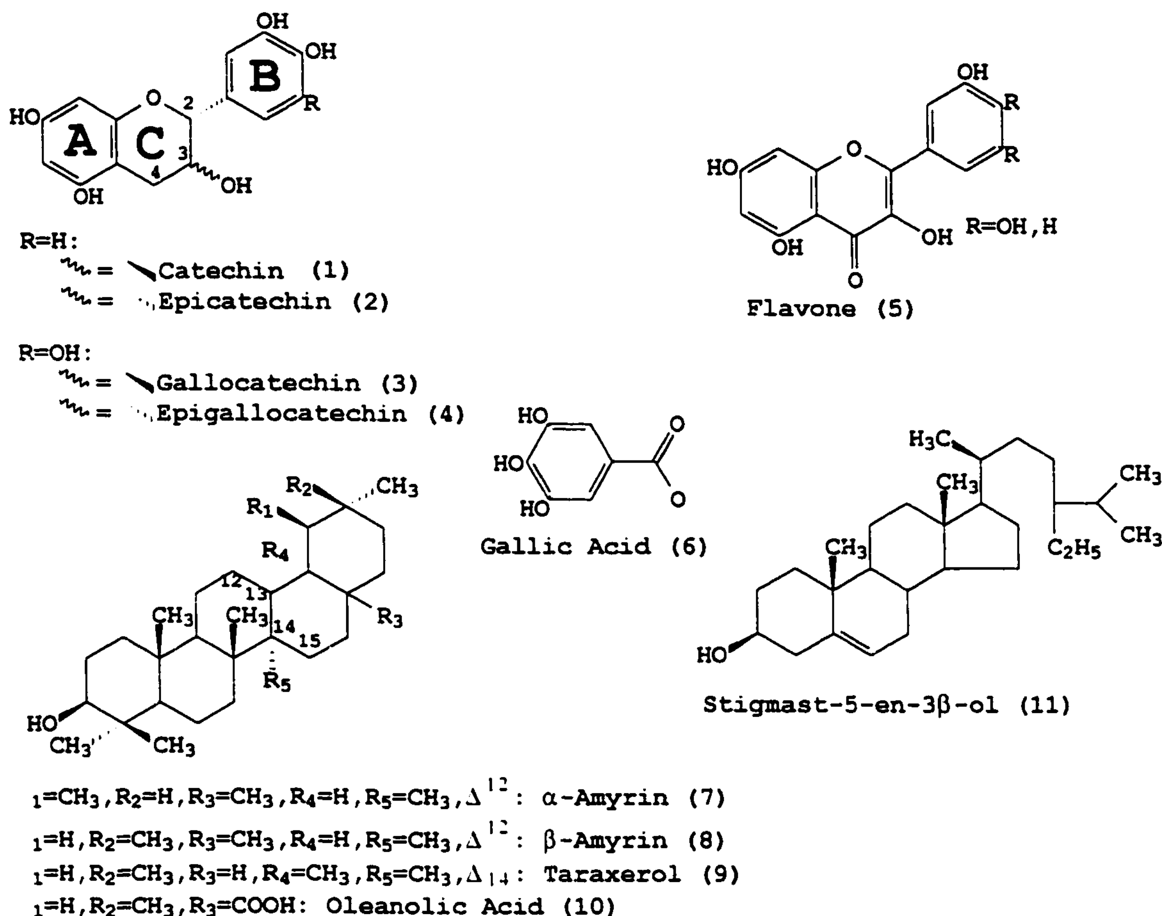

Fig. 3.2 Structure of measured phenols and triterpenols.

likelihood that a component of it will be procyanidins (PC) in the form of catechin and epicatechin. For instance, of the 117 samples analyzed in this study, 89 contained tannin, and 88 of those contained at least one PC tannin. In comparison, 50 contained prodelphinidin (PD) tannin in the form of gallocatechin and epigallocatechin, and 24 contained propelargonidin (PP) tannin in the form of afzelechin and epiafzelechin. In addition, 22 samples contained flavones (i.e. flavanols with a 2,3 double bond and a carbonyl at C-4), and six had flavanones (i.e. flavanols with a carbonyl at C-4. See Fig. 3.2 for structural differences). The complete molecular data set can be found in Appendix I. Commonly measured parameters for all samples are found in Table 3.1.

As mentioned previously, a number of triterpenoids also fall within the analytical window of the method. The predominant triterpenoid, stigmast-5-en-3β-ol (synonyms

Table 3.1. Tannin and triterpenoid parameters.

Latin Name	LBL	TISS.	ΣTannin	Xn	Terminal *cis/trans*	Extender *cis/trans*	PC:PD:PP	ΣTerp.
Acer macrophyllum	d	BK	0.09	2.57	1.00	0.78	100:0:0	
Alnus rubra	d	BK	0.49	2.49	0.49	0.88	93:7:0	1.25
Artemisa tridentata	d	BK					::	
Quercus garrayana	d	BK	0.47	1.39	0.13	0.61	88:12:0	0.10
Thuja plicata	gc	BK	1.48	2.44	0.28	0.22	80:3:17	
Abies holophylla	gp	BK	0.20	1.17	0.46	1.00	100:0:0	
Pinus contorta	gp	BK	1.14	4.38	0.08	0.60	66:34:0	
Pinus ponderosa	gp	BK	1.55	2.37	0.11	0.74	97:3:0	
Pseudotsuga menziensii	gp	BK	0.83	2.50	0.56	0.64	100:0:0	
Tsuga heterophylla	gp	BK	10.57	1.42	0.56	0.68	69:2:28	
Aniba guianensis	td	BK	0.48	2.09	0.42	0.79	100:0:0	
Clarisia racemosa	td	BK	0.06	2.60	0.90	0.68	27:0:73	0.88
Crescentia amazonica	td	BK					::	
Genipa americana	td	BK	0.00	1.00	1.00		100:0:0	0.01
Nectandra amazonium	td	BK	1.33	2.52	0.56	0.83	98:2:0	
Neoxythece elegans	td	BK	0.65	10.91	0.29	0.36	14:86:0	3.21
Xylosma intermedium	td	BK	0.05	1.71	0.00	0.64	89:11:0	0.03
Zanthoxylum compactum	td	BK					::	2.00
Larix occidentalis	gp	C	0.87	5.35	0.25	0.85	97:3:0	
Picea sitchensis	gp	C	0.30	1.82	0.08	0.64	75:25:0	
Pinus ponderosa	gp	C	0.66	2.39	0.06	0.62	100:0:0	
Pseudotsuga menziensii	gp	C	2.00	5.00	0.54	0.90	100:0:0	
Tsuga heterophylla	gp	C	3.13	7.37	0.23	0.39	38:61:0	
Sequoia sempervirens	gT	C	1.81	5.15	0.32	0.37	32:68:0	
Alnus rubra	d	S	1.87	5.25	0.80	0.93	100:0:0	4.53
Acer macrophyllum	d	GL	0.63	2.22	0.25	0.75	77:23:0	0.23
Alnus rubra	d	GL	0.96	2.97	0.53	0.87	95:5:0	0.36
Artemisa tridentata	d	GL					::	
Avicennia germinans	d	GL					::	
Camellia spp.	d	GL	3.72	1.46	0.80	0.52	100:0:0	0.03
Ilex aquifolium	d	GL	0.03	1.24	1.00	1.00	100:0:0	3.40
Malus spp.	d	GL	0.89	6.99	0.82	1.00	59:0:41	1.10
Populus trichocarpa	d	GL	2.13	5.32	0.04	0.65	77:23:0	
Quercus garrayana	d	GL	2.22	1.74	0.41	0.66	84:16:0	0.20
Quercus palustris	d	GL	0.59	1.67	0.00	0.84	100:0:0	0.74
Quercus robur	d	GL	0.44	1.81	0.00	0.75	92:8:0	0.09
Quercus rubra	d	GL	0.64	1.74	1.00	0.93	100:0:0	0.40
Rhizophora mangle	d	GL	5.23	7.33	0.28	0.79	87:13:0	0.06
Rosa spp.	d	GL	1.70	1.99	0.10	0.84	98:2:0	0.11
Rubus spp.	d	GL	0.09	1.27	1.00	1.00	100:0:0	
Vitis spp.	d	GL	4.67	4.28	0.55	0.52	49:51:0	
Aniba guianensis	td	GL	0.59	2.65	0.48	0.94	100:0:0	
Aspidosperma rigida	td	GL					::	
Bothriospora lorymbosa	td	GL	0.17			1.00	100:0:0	0.08
Buchenavia oxycarpa	td	GL	0.02			0.61	61:39:0	0.10
Campsiandra comosa	td	GL	1.33	1.58	0.29	0.76	70:4:6	
Caraipa grandifolia	td	GL	0.70	3.05	0.82	0.87	91:9:0	0.18
Clarisia racemosa	td	GL	1.61	3.70	0.50	0.90	37:0:63	0.20
Genipa americana	td	GL	0.09	1.43	0.66	1.00	100:0:0	1.55
Glycydendron amazonicum	td	GL	1.19	10.66	0.00	0.50	43:56:1	
Guarea rubiflora	td	GL	1.30	5.32	0.73	0.56	55:45:0	0.06
Guarea trichilioides	td	GL	1.53	3.32	0.47	0.80	99:1:0	
Lecointea amazonica	td	GL	0.03			0.88	88:12:0	0.14
Leonia racemosa	td	GL					::	
Malouetia furfuracea	td	GL	2.09	3.10	0.56	0.74	41:5:54	2.74
Nectandra amazonium	td	GL	0.63	2.79	0.53	0.98	100:0:0	
Neoxythece elegans	td	GL	1.13	3.60	0.56	0.17	18:82:0	0.20
Pterocarpus amazonicus	td	GL	0.52	6.29	0.76	0.37	30:70:0	0.06
Symmeria paniculata	td	GL	1.57	2.55	0.91	0.97	99:0:1	
Tabebuia barbata	td	GL					::	1.86
Vatairea guianensis	td	GL	0.37	1.53	0.07	1.00	36:0:64	0.16
Vitex cymosa	td	GL					::	0.90

Table 3.1. Tannin and triterpenoid parameters (continued).

Latin Name	LBL	TISS.	ΣTannin	Xn	Terminal *cis/trans*	Extender *cis/trans*	PC:PD:PP	ΣTerp.
Zanthoxylum compactum	td	GL	0.99	2.88	0.69	0.99	100:0:0	
Araucaria araucana	ga	GL	2.49	5.08	0.28	0.48	45:51:5	
Thuja plicata	gc	GN	5.05	3.22	0.22	0.56	43:55:3	
Abies marocana	gp	BN	6.02	2.72	0.14	0.42	42:57:1	
Abies nebrodensis	gp	BN	6.20	2.82	0.07	0.36	34:66:0	
Picea engelmannii	gp	GN	2.35	3.53	0.16	0.74	82:18:0	
Picea sitchensis	gp	BN	1.36	3.00	0.20	0.95	98:1:0	
Pinus contorta	gp	GN	9.98	4.53	0.07	0.35	26:73:1	
Pinus ponderosa	gp	GN	8.63	5.69	0.08	0.26	27:73:1	
Pseudotsuga menziensii	gp	GN	5.58	7.82	0.42	0.45	45:54:1	
Tsuga heterophylla	gp	GN	4.63	12.82	0.32	0.30	27:73:0	
Sequoia sempervirens	gT	GN	5.49	7.04	0.19	0.33	27:73:0	
Sequoiadendron giganteum	gT	GN	2.77	6.45	0.20	0.44	43:57:0	
Taxodium distichum	gT	GN	4.23	6.95	0.14	0.45	43:57:0	
Taxodium distichum	gT	GN	2.77	7.42	0.14	0.45	46:54:0	
Alnus rubra	d	W	0.61	2.31	0.06	0.77	92:8:0	
Thuja plicata	gc	W					::	
Picea sitchensis	gp	W	0.01			1.00	100:0:0	
Picea spp.	gp	W	0.01			1.00	100:0:0	
Pseudotsuga menziensii	gp	W					::	
Bothriospora lorymbosa	td	W					::	0.14
Buchenavia oxycarpa	td	W					::	
Campsiandra comosa	td	W	0.41	1.75	0.09	0.67	74:0:0	
Clarisia racemosa	td	W					::	
Leonia racemosa	td	W					::	
Neoxythece elegans	td	W	0.01	3.97	1.00	0.32	49:51:0	
Symmeria paniculata	td	W	0.60	1.80	0.55	0.94	100:0:0	
Tabebuia barbata	td	W					::	
Vitex cymosa	td	W					::	
Xylopia callophylla	td	W					::	
Xylosma intermedium	td	W	0.03	4.43	1.00	1.00	100:0:0	
Rhacomitrium aciculare	B	WP					::	
Rhytidiadelphus loreus	B	WP					::	
Lycopodium sitchense	C	WP	0.02			1.00	100:0:0	
Polystichum acrostichoides	F	WP	1.09	6.84	0.78	0.93	86:8:6	
Pteridium aquilinium	F	WP	0.93	7.75	1.00	0.73	63:30:7	
Equisetum telmateia	H	WP	1.24	37.10	0.85	1.00	58:0:42	
Alaria fistulosa	K	B					::	
Alaria fistulosa	K	ST					::	
Laminaria longpipes	K	WP					::	
Thalasiophylum	K	WP					::	
Agrostis alba	m	WP	0.02			0.21	21:79:0	
Avena spp.	m	WP					::	
Carex spp.	m	WP					::	
Salicornia spp.	m	WP					::	0.12
Spartina alterniflora	m	WP					::	0.10
Zostera spp.	m	WP					::	
Echinochloa polystachya	tm	WP	0.00			1.00	100:0:0	
Eichornia crassipes	tm	WP	0.42	6.74	1.00	1.00	67:0:33	
Gynerium sagittatum	tm	WP	0.01	2.20	0.00	0.00	100:0:0	
Paspalum repens	tm	WP	0.03	1.91	1.00	1.00	100:0:0	
Pistia stratoides	tm	WP	0.01			1.00	100:0:0	
Salvinia auriculata	tm	WP	1.46	11.28	1.00	0.99	91:0:9	

Abbreviations: LBL=plant type, TISS.=tissue type, ΣTannin=total molecular tannin, Xn=degree of polymerization, *cis/trans*=2,3-cis conformation to 2,3-trans conformation ratio, PC=procyanidin, PD=prodelphinidin, PP=propelargonidin, ΣTerp.=total triterpenoids.

Plant types: d=temperate dicotyledons, td=tropical dicotyledons, ga=gymnosperms family Araucariaceae, gc=gym. f. Cuppresaceae, gp=gym. f. Pinaceae, gT=gym. f. Taxodiaceae, B=mosses, C=club mosses, F=ferns, H=horsetails, K=kelps, m=temperate monocotyledons, tm=tropical cotyledons

Tissue types: BK=bark, C=cone, S=seedpod, GL=green leaves, GN=green needles, BN=brown needles, WP=whole plant, W=wood, B=blade, ST=stipe

β-sitosterol and 24-ethylcholesterol), was present in 94 of the samples. Stigmast-5-en-3β-ol is one of a class of steroids that are widespread in angiosperms, gymnosperms, and ferns (Gershenzon and Croteau, 1991) and is also present in marine sources (Volkman, 1986). The primary diagnostic triterpenoids are the amyrins, which were present in 27 of the samples, and oleanolic and ursolic acids, at least one of which was present in 17 samples. The amyrins as well as oleanolic and ursolic acids are uniquely terrestrial, widespread in angiosperms, and are thought to be bonded to carbohydrates (Gershenzon and Croteau, 1991). Although not definitively known, their function is believed to be similar to many of the polyphenols, i.e. for defense against herbivores.

There are a number of valid ways in which the analyzed samples can be grouped for comparison and source distinction, e.g. tropical vs. temperate, woody vs. non-woody, angiosperm vs. gymnosperm. For the purposes of discussion, we chose to group them according to tissue types.

Bark

The molecular contribution of bark to carbon cycling is not well known. Our data set certainly represents one of the largest characterizations to date of this tissue type. Using a molecular acid thiolysis method, Matthews et al. (1997b) analyzed eleven barks for PC and PD tannin, of which two are present in our sample set—*Pinus contorta* and *Pseudotsuga menziesii*. Whereas Matthews et al. measured 3.2% and 3.3% of total PC and PD tannin, we obtained 3.3% and 3.1%, respectively (Table 3.1). Part of this close agreement may be fortuitous, however, since our analysis also includes flavanones, which constitute about half the tannin measured in these two samples. Degree of polymerization (total tannin divided by total terminal units—a measure of average tannin chain length) was higher in their samples: 4.7 and 3.7, respectively, vs. 4.4 and 2.5. Compositionally, Matthews et al. found a 60:40 and 81:19 percent ratio of 2,3-*cis* (i.e. the epi- forms epicatechin, epigallocatechin, and epiafzelechin) to 2,3-*trans* (catechin, gallocatechin, and afzelechin) conformations, respectively, while our ratios were 60:40 and 64:36. These authors found <20% of the condensed tannin in *Pinus contorta* bark was of PD type, as compared to a previous literature value of 69% (Porter, 1989). Our measurement of 34% PD tannin falls in between these values. No

PD tannin was measured in *Pseudotsuga menziessi* bark in either study. Finally, our analysis of *Tsuga heterophylla* bark yielded 10.6% tannin vs. a value of 12.6% reported by Hergert (1989).

A number of trends stand out in our measured molecular compositions (Appendix I, Table 3.1). Perhaps the most noteworthy is the unique presence of measurable flavanones in the three conifers, *Pinus contorta* (0.58 wt% dihydroquercetin or taxifolin, 1.25 wt% dihydromyricetin or ampelopsin), *Pinus ponderosa* (0.60 wt% taxifolin), and *Pseudotsuga menziensii* (2.28 wt% taxifolin). Trace levels of flavanones were detected in needles and wood of *Pseudotsuga menziensii*. Bark flavanones are believed to occur in condensed tannin (Hergert, 1989), but because of the carbonyl function on C-4, can only be present as terminal units.

Quantitatively, *Tsuga heterophylla* bark yielded nearly 10.6 wt% tannin phenols, whereas total tannin yields from the other 17 samples ranged from undetectable (three samples) to 3.7 wt%. Tannin yields from conifer barks tended to be higher than from barks of either the tropical or temperate dicotyledons. Although PC tannin was the dominant flavanol found in the barks, once again individual species exhibit substantial variation. *Tsuga heterophylla* bark showed the highest yield (>3%) of PP tannin of any samples in the entire data set, whereas bark of the tropical dicotyledon, *Neoxythece elegans*, yielded nearly 90% PD tannin. *Neoxythece elegans* also showed the highest degree of polymerization with a chain length of 11. Degree of polymerization by itself is not likely to provide much source information, but can reflect degradation downstream of the source. On average, however, the measured barks of tropical dicotyledons showed a higher degree of polymerization (1.7 to 11) than the conifer barks (1.2-2.4). Finally, *Pinus contorta* and *Quercus garrayana* yielded small amounts of flavones. Hergert (1989) also reports their presence in the barks of *Tsuga heterophylla* and *Picea sitchensis*.

Other than stigmast-5-en-3β-ol, triterpenoids were not found in any of the conifers. On the other hand, many were measured in the angiosperms. Again, *Neoxythece elegans* stands out with 1.1 wt% each of α- and β-amyrin, and 0.84 wt% of oleanolic acid. In addition, *Alnus rubra* and *Zanthoxylum compactom* yielded roughly 1

and 2 wt%, respectively, of a compound with a mass spectrum similar to the amyrins, but with an additional 131 m/z fragment. The only other tissue in the entire sample set yielding a similar product was *Alnus rubra* seed pods, indicating a potentially unique marker for *Alnus rubra* tissues. In general, barks were elevated in carboxylic acids. Most were not quantified, except for tetracosanoic acid, which again was detected only in bark tissues, namely the angiosperm, *Alnus rubra*, in trace amounts (0.03 wt%), and the conifers *Abies holophylla*, *Pinus contorta*, and *Pinus ponderosa* in greater amounts (about 0.25 wt%).

Cones and Seedpods

Tannin yields from six seed cones and one angiosperm seed pod (*Alnus rubra*) ranged from 0.3 to 3.1 wt%. Compositionally, the only distinguishing feature was the presence of triterpenoids in *Alnus rubra* tissues and their absence in all of the conifers—a pattern similar to what was found in the barks. In addition to the "131-amyrin" noted above, the *Alnus rubra* seed pods yielded more than 4% of an unidentified triterpenoid with a prominent 587 m/z fragment. The only other plant tissue in the entire sample set giving a similar compound was *Alnus rubra* green leaves. Given the 587 peak in *Alnus rubra* seedpods and green leaves combined with the "131-amyrin" in seedpods and barks, red alder sources should be readily discernable in natural environments.

Our results on the seed cones can be compared to the study of Eberhardt and Young (1994), who analyzed purified condensed tannins from five *Pinus spp.* seed cones with ^{13}C-NMR. They found that 2,3-*cis* conformations made up 65-81% of condensed tannin. Our results are in good agreement, with 2,3-*cis* constituting 62-90% in four species, and 37% and 38% in *Sequoia sempervirens* and *Tsuga heterophylla*, respectively. The only common species between the two sets of seed cones was *Pinus ponderosa*, in which they measured 74% 2,3-*cis* vs. 62% in this study. Degree of polymerization ranged from 5.3-8.5 in Eberhardt and Young's study, and 1.8-7.4 in this study (5.4 vs. 2.4 for *Pinus ponderosa*). This difference may be explained by the fact that the purification procedure employed by Eberhardt and Young eliminates monomers and smaller oligomers. None of the seed cones from *Pinus spp.* measured in either study contained any PD tannin. However, we found >60% PD tannin in cones of

Sequoia sempervirens and *Tsuga heterophylla*. The latter is interesting, because of the contrast with *Tsuga heterophylla* bark which contained only 3% PD tannin. As in the bark, *Pseudotsuga menziensii* seed cones, as well as *Alnus rubra* seed pods, contained no PD tannin.

Conifer Needles

Of the fourteen needles analyzed, twelve were virtually identical compositionally, the only exceptions being the two *Picea spp.* Among the twelve, percent of PD tannin ranged from 54-74%. Among temperate species, conifer needles were the only tissues to exhibit >23% PD, with the exception of *Vitis spp.* green leaves (51%), and the bracken fern *Pteridium aquilinium* (33%). Thus, PD tannin might be a useful marker of conifer needles. In thirteen of the species, gallocatechin ranged from 70-100% of the PD tannin. Gallocatechin was predominant in nearly all tissues that contained PD tannin. In all needle samples, percent catechin of PC tannin ranged from 8-21%. This composition is consistent with results for conifer seed cones and barks. Finally, eight of the needle samples yielded measurable PP tannin. Among temperate non-conifer species analyzed, PP was found in only one angiosperm (*Malus spp.*), both ferns, and the single horsetail (Table 1). Thus, PP may also be useful as an indicator of conifer needles.

Degree of polymerization in conifer needle tannin ranged from 2.7-12.8, the latter in *Tsuga heterophylla*. Values are generally comparable to that for conifer seed cones, but at least a factor of two greater than that in conifer barks. Total tannin in the conifer needles ranged from 1.4 wt% to 10.1 wt%, with half greater than 5% and the two *Picea spp.* giving the lowest values. The 10.1% value was measured in *Pinus contorta*, 8.6% in *Pinus ponderosa*, while *Pseudotsuga menziensii* was 7.1%. In addition to distinct signatures, the tannin of conifer needles should be abundant enough to carry their signal into litters and soils.

Dicotyledon Leaves

Leaves were analyzed from 16 temperate and 24 tropical dicotyledons. In all, 14 of the temperate and 20 of the tropical species contained tannin. All tropical leaves yielded less than 2.1 wt% total tannin, while the temperate leaves gave as high as 6.6

wt% (*Vitis spp.)*. Other notably high yields were 5.6% from *Rhizophora mangle*, and 4.3% from *Camellia spp.* In contrast to conifer needles, 25 of the dicotyledon leaves yielded triterpenoids with total yields as high as 3.5 wt% in *Ilex aquifolium.*

Compositionally, there were some differences between leaves from the tropical and temperate dicotyledons. Whereas eleven out of 16 temperate species contained quercetin (as high as 1.6 wt% in *Malus spp.*), it was measured in only five of 24 tropical species. Leaves from five tropical species gave PP tannin, compared to only one temperate. Tropical species were more likely to yield high proportions of PD tannin relative to total tannin, with five species ranging from 39-82% PD. Only one temperate leaf yielded greater than 23% PD tannin. In both temperate and tropical leaves, epicatechin was the primary extender unit, similar to what is reported in the literature.

One of the more intriguing leaf samples is from the tropical species, *Campsiandra comosa*, which was the only species in the entire sample set that contained proguibourtinidin or profisetinidin tannin (i.e. with only one hydroxyl group on the A-ring—see Fig. 3.2). Such tannins are comparitively rare, and reportedly more resistant to acid hydrolysis when present as extender units. Since these compounds were only found in the extender units, the 0.27 wt% yield from *Campsiandra comosa* might be indicative of considerably more precursor in the leaf.

Leaves from six species of dicotyledons contained compounds corresponding to hydroxy-oleanolic and hydroxy-ursolic acid. The position of the hydroxy group on the triterpenoid structures could not be determined by mass spectra alone. In five of the six leaves, the parent compounds, oleanolic and ursolic acid, were also present.

Finally, two compounds corresponding to amyrins with the addition of a methyl group were detected in leaves of two tropical species, *Clarisia racemosa* and *Malouetia furfuracea*. One of the compounds was also detected in *Clarisia racemosa* bark. Notably, these two species also contained >1 wt% PP tannin, which was more than half the tannin present in these samples. *Vatairea guianensis* was the only other tropical species to yield more than 6% PP of the total tannin.

Monocotyledons

Whole plants from six species each of temperate and tropical monocotyledons were analyzed. All tropical monocots yielded some tannin, ranging from <0.01 wt% up to 1.46%. Only one temperate grass, *Agrostis alba*, yielded measurable tannin (0.02 wt%), and was also the only monocotyledon to yield PD tannin. *Zostera spp.* have been shown to have phenolic content as measured by Folin-Denis reagent (Harrison and Durance, 1989), but similar to McMillan (1984), we found no condensed tannin. Molecular data for the tropical water hyacinth, *Eichornia crassipes*, can be found in Ellis et al. (1983). Whereas they report PC:PD:PP ratios of 56:19:25, we measured 67:0:33. Ellis et al. found 2,3-*cis*/*trans* ratios in *Eichornia crassipes* of 40:60, while no *trans* was measured in this study.

Monocotyledons were distinct from the dicotyledons in that no triterpenoids were detected in the former, other than the ubiquitous stigmast-5en-3β-ol.

Woods

Only half of the 16 wood samples analyzed in this study contained measurable tannin. *Alnus rubra*, a temperate dicotyledon, and *Symmeria paniculata*, a tropical dicotyledon, each gave 0.6 wt% tannin. *Campsiandra comosa* yielded 0.4% tannin, and all others were 0.03% or less. No compositional feature stands out for woods. Individually, *Campsiandra comosa* wood yielded proguibourtinidin and profisetinidin tannin, just as the leaves did. As in most tissues, the *cis* form of the tannin was more abundant than the *trans*.

Ferns, Horsetails, Kelps, Mosses, and Clubmosses

No tannin was measured in the three kelps and two mosses, and only 0.02 wt% was measured in the single club moss, *Lycopodium sitchense*. On the other hand, the two ferns, *Polystichum acrostichoides* and *Pteridium aquilinium*, yielded about 1% tannin, and the horsetail, *Equisetum telmateia*, yielded 1.2% tannin. All three contained PP tannin and both ferns contained PD tannin. All three tissues contained >90% of the *cis* form of tannin, and relatively high degrees of polymerization at 6.8, 7.8, and 37.1, respectively. Although compositional trends cannot be established on such a small sampling, it is clear that ferns and horsetails can be possible sources of tannin. In particular, the PD and PP tannin content might be mistaken as conifer in origin.

Parameters for Source Distinctions

All told, there are at least 18-20 different categories of plant materials represented by the 117 samples analyzed in this study, based on temperate vs. tropical habitats, taxonomic distinctions, and tissue type. As such, there is no universal parameter for distinguishing all of them in an unknown environmental sample such as soils or sediments on the basis of tannin composition alone. A more useful concept might be something akin to a dichotomous key, as is commonly used in biological sciences to "type" organisms. In this sample set, the first key might be the geographic location of the sample, i.e. temperate vs. tropical since most species are not likely be present in both settings. Further keys could be based on chemical composition parameters, including those presented here for tannin, triterpenoids, and other compounds, and those presented for the terrestrial counterparts cutin (Goñi and Hedges, 1990) and lignin monomers and dimers (Hedges and Mann, 1979; Goñi and Hedges, 1992).

Two types of parameters are generally used for source distinctions. The first denotes the presence of a specific compound (or compound class) unique to a subset of the source possibilities. Examples from this data set include the 1) flavanones, which were only measured in conifers and primarily in barks, 2) tetracosanoic acid in barks, 3) triterpenoids in angiosperms, and 4) PP tannin, which was only yielded by non-woody tissues of conifers, tropical species, ferns, and horsetails. Given a comprehensive survey, these types of distinction are robust in that one can generally infer the presence of the source from detection of the compound class. However, the converse is not always true, i.e. the absence of these compounds does not necessarily mean the absence of the sources.

The second type of parameter involves a compound (or compound class) that is present in all or most source types, but in varying compositional ratios. An example from this data set would be the fraction of 2,3-*cis* stereochemistry in the extender units of tannin. The presence of 2,3-*cis* extender units alone carries no source information, but in relation to 2,3-*trans* extender units, it carries a great deal. For instance, the fraction of 2,3-*cis* extender units is less than ~0.5 in all conifer needles analyzed except

Picea spp. On the other hand, the fraction is greater than 0.5 from all tissue types in temperate or tropical angiosperms except the tropical dicotyledons, *Neoxythece elegans* and *Pterocarpus amizonicus*, and the temperate monocotyledon, *Agrostis alba*. The latter, however, contained only 0.02 wt% total tannin and would be essentially invisible in a tissue mixture.

While the first type of unique biomarker can provide unambiguous evidence of a single source, abundance estimates of multiple sources are problematic unless there are unique markers for each endmember. The second type of parameter, on the other hand, lends itself readily to endmember mixing calculations because each endmember contributes to the bulk signal. In a gymnosperm/angiosperm mixture, for example, a measurement of 0.25 for the fraction of 2,3-*cis* extender units indicates both the predominance of gymnosperms and the absence of angiosperms. A value of 0.8 would indicate both the absence of gymnosperms and the predominance of angiosperms.

While there are many examples of source-specific compounds of the first type, few span broad enough cross-sections of a category to be generally representative. For instance, flavanones, PP tannin, PG and PF tannin, tetracosanoic acid, the 587 peak and 131 "amyrin" of *Alnus rubra*, hydroxyoleanolic and hydroxyursolic acid, and methyl amyrins all might be powerful source markers in certain systems, but cannot be generalized to trace an entire tissue type. On the other hand, PD tannin, flavones, and total triterpenoids are found in enough species and tissue categories to warrant possible generalizations.

Parameters for Conifers

The primary reason that conifer needles yielded a lower fraction of 2,3-*cis* extender units is the presence of large amounts of PD tannin, which can be more than 90% trans. PD tannin yields obviously also directly impact PC:PD:PP percentages. Thus, conifers can be distinguished by any one of three parameters related to PD tannin: 1) weight percent PD tannin, which falls into the first category discussed above and only gives information about the presence of PD-bearing sources, 2) the fraction of 2,3-*cis* extender units, and 3) PC:PD:PP percentages, the latter two which fall into the second category and give information about all tannin-bearing sources. Although PD

tannin is not exclusive to conifers, their tissues generally give the highest yields. A plot of weight percent PC vs. PD tannin illustrates this pattern (Fig. 3.3), in which conifer needles (except *Picea spp.*) are clearly distinguishable from all other sources.

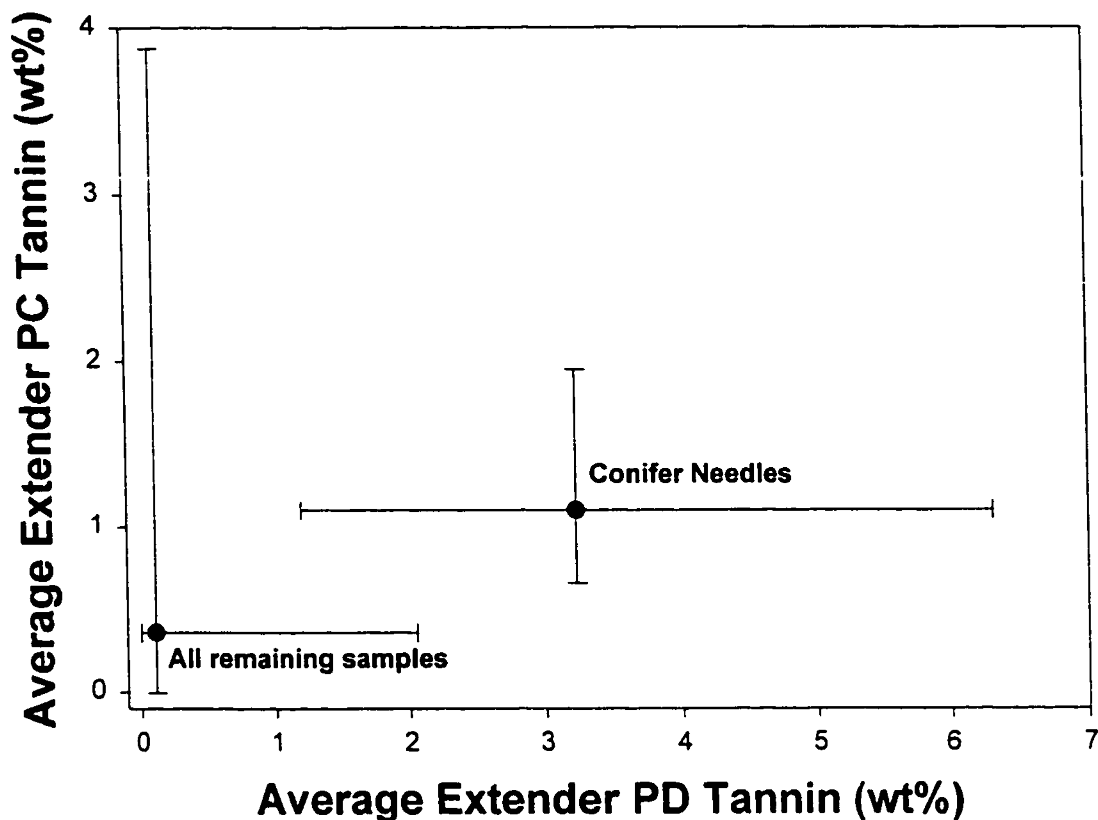

Fig. 3.3 Weight percent PC tannin vs. PD tannin for extender units.

As discussed, the fraction of 2,3-*cis* extender units and PC:PD:PP percentages are closely related. When plotted against the fraction of 2,3-*cis* terminal units, either parameter is capable of differentiating conifer needles (less *Picea spp.*) from other tannin sources (Fig. 3.4a and b, respectively). PC:PD:PP, however, gives tighter clustering. Also of note is that ferns and horsetails (other sources of PD and PP tannin) are well removed from conifers on these plots, because their fractions of 2,3-*cis* terminal units are roughly double that of conifers.

Parameter for Leaves, Needles and Barks

The abundances of flavones and triterpenoids may be similarly useful for distinctions between leaves, needles, and barks (Fig. 3.5). While there is considerable

Fig. 3.4 (a) The fraction of 2,3-*cis* structures in extender vs. terminal units, and (b) the fraction of 2,3-*cis* terminal units vs. PD percent of tannin. Abbreviations are as in Table 3.1.

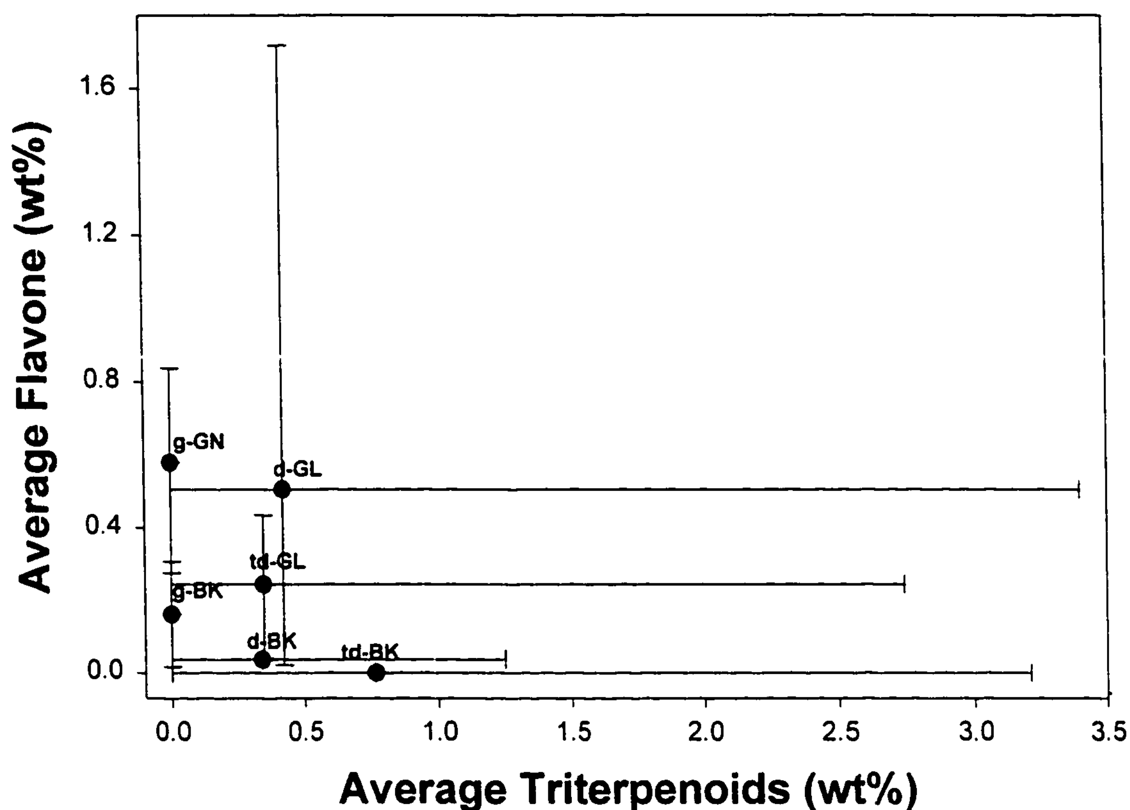

Fig. 3.5 Weight percent flavones vs. weight percent triterpenoids. Abbreviations are as in Table 3.1.

overlap between tropical and temperate dicotyledons, it is unlikely that both would be simultaneously present in natural samples. Also, only species that yielded flavones and triterpenoids were included in these plots. Temperate dicotyledons contained flavones in a much higher percentage of species than any other category.

Comparisons to Lignin and Cutin

Hedges and Mann (1979) and Goñi and Hedges (1992) established source parameters for lignin monomers and dimers, respectively, while Goñi and Hedges (1990) established parameters for cutin. Because lignin and cutin are also unique to terrestrial plants, the yields and compositional parameters they provide are directly comparable to those of tannin (and the identified triterpenoids). Quantitatively, lignin is by far the most abundant of the three biopolymers in woods (cutin is not present in wood), and Hedges and Mann found that angiosperm and gymnosperm woods were

readily distinguishable based on syringyl phenol (S) to vanillyl phenol (V) ratios (the former is not obtained from gymnosperms).

Among conifer needles and angiosperm leaves, cutin and tannin are more abundant than lignin. With cutin, gymnosperm/angiosperm distinctions are made primarily by the presence of 14-hydroxytetradecanoic acid, which is only yielded by gymnosperm tissues, although not all. In this study, gymnosperm (conifer) needles are distinguishable based on their low fraction of 2,3-*cis* extender units as well as their high PD tannin content, while angiosperm leaves are distinguishable based on flavone and triterpenoid content. Similar to woods, leaf/needle distinctions with lignin are made using S/V vs. cinnamyl (C) to vanillyl (V) phenol ratios.

Among other tissue types, cutin remains the only one of the three biopolymers that can distinguish monocots from dicots. Cutin also appears to be uniquely useful for distinguishing club mosses and ferns. On the other hand, cutin is not present in cones or barks, and neither have been characterized for lignin. Thus, the tannin, triterpenoid, and tetracosanoic acid content measured in this study are the strongest established markers for these often abundant tissue types. Finally, cutin and lignin do not have nearly the overall diversity of compounds that can be found in the flavonoids or triterpenoids, and as such, do not contain unique source markers as, for instance, were found in *Alnus rubra, Campsiandra comosa*, or the barks. Thus, the strength of the described "tannin" method might lie in its potential to expand.

Overview

A summary of the diagnostic compounds and parameters discussed here is given in Table 3.2. Clearly, conifers as a whole are the most readily identifiable (as well as quantitatively important) tannin sources. Thus, the analytical method utilized in this study shows great promise for studies involving litter and carbon cycling in conifer forests and downstream environments. In addition, flavanones and tetracosanoic acid might provide useful biomarkers in studies related to debarking in the timber industry.

The detection and measurement of triterpenoids in conjunction with tannin provides a powerful complementary tool at two levels of source distinctions. While high triterpenoid yields are indicative of dicotyledons as a whole, triterpenoid

Table 3.2. Source identification compounds and parameters.

Source tissue	Species or type	Compound/parameter
Needles	Conifers	PD tannin, 2,3-*cis*/*trans* ratios, PC:PD:PP percent
Leaves	Dicotyledon	Flavones and triterpenoids
	Alnus rubra	587 triterpenoid
	Campsiandra comosa	PG and PF tannin
	Clarisia racemosa and *Malouetia furfuracea*	Methyl amyrins
Bark	Across types	Tetracosanoic Acid
	Conifer	Flavanone
	Alnus rubra and *Zanthoxylum compactom*	Amyrin with 131 mass spectra fragment
Seeds	*Alnus rubra*	Triterpenoid w/ prominent 587 ms fragment
Wood	*Campsiandra comosa*	PG and PF tannin

Abbreviations: PC=procyanidin, PD=prodelphinidin, PP=propelargonidin, PG=proguibourtinidin, PF=profisitinidin.

compounds unique to individual species (e.g. the "587 compound" from *Alnus rubra*) may show great utility in tracing materials from these specific sources.

Finally, in order to fully evaluate these parameters, the effects of diagenesis must be studied. With all other factors equal, the extent of hydroxylation of the B-ring should parallel reactivity, with PD tannin more reactive than PC tannin, which in turn should be more reactive than PP tannin. This inference appears to be the case for *Rhizophora mangle* (Hernes et al., in prep), but will be critical to evaluate in the case of conifers as the primary source of PD tannin. However, if the alteration products of the different tannin sources can eventually be identified and quantified, they may provide a powerful tool in the study of organic matter degradation. In a similar vein, degree of polymerization, while not particularly useful as a source indicator, may have utility in

diagenetic studies because smaller compounds are generally more labile than larger counterparts. If so, plots of the fraction of 2,3-*cis* structures in terminal vs. extender units would be skewed toward the composition of extender units. The lability of the triterpenoids detected by the method used in this study is also relatively unknown. Killops and Frewin (1994) provide evidence for differential protection of these triterpenols within cutin and wax matrices of mangrove leaves, as well as persistence into the sediments. Volkman et al. (1987) detected many of the same triterpenols in a variety of soils and sediments, also indicating protection or resistance to degradation. Finally, compared to tannin, one study indicates that triterpenoids may be more resistant to degradation than tannins (Hernes et al., in prep). In any case, these and other potential effects of diagenesis must be assessed before parameters and source signals introduced in this study can be used confidently as geochemical source indicators.

CHAPTER 4: TANNIN DIAGENESIS IN MANGROVE LEAVES FROM A TROPICAL ESTUARY:A NOVEL MOLECULAR APPROACH

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 in riverine and marine systems—and 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. While 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 in much the same stage as that of its phenolic "cousin", lignin, nearly three decades ago. The similarities are considerable: each is phenolic, polymeric, unique to vascular plants, and rich in natural products history. However, the contrasts are also considerable: whereas lignin polymers are highly branched, tannin macromolecules are highly linear; whereas lignin predominates in woody tissue, tannin is often more abundant in leaves and needles; whereas lignin is relatively refractory, tannin is very reactive. 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. 1.1). A third type known as phlorotannin (due to the basic building block, phloroglucinol) is found in brown algae. Hydrolyzable tannin is primarily made up of gallic acid, which can either combine with itself to form gallotannins, or with sugars as shown in Fig. 1.1. As the name implies, hydrolyzable tannin is more soluble than condensed tannin and therefore less likely to persist in vascular plant detritus. The building blocks for condensed tannin are three-ring flavanols. At least a dozen variations of these stereochemically active compounds are known to occur in condensed tannin. The structural contrast to hydrolyzable tannin has lead some to attribute much different functionality within plants of the two polyphenols (Zucker, 1983). The greater

variety, in addition to lower solubility, of condensed tannin offers much more potential as a biomarker than hydrolyzable tannin.

Unlike carbohydrates, lipids, amino acids, and pigments—which are ubiquitous in organic matter and have both marine and terrestrial sources—condensed tannins (along with lignin and cutin) are uniquely terrestrial. Thus, tannins have 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, 1991). *Ent*-epicatechin, on the other hand, is unique to monocotyledons, and propelargonidin-containing polymers are much more common in monocotyledons than dicotyledons (Ellis et al., 1983). On the other hand, hydrolyzable tannin is only found in dicotyledons. Tannin 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 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 reactivity unlike the other major biochemicals, e.g. 1-5 years 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 Fig. 1.1, 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 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. 1.1) to terminal units. Smaller polymers and oligomers are more water soluble than larger, 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 (*Rhizophora 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 to estuarine food webs (Odum and Heald, 1975). In addition, utilizing bulk ^{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 utilized, Benner et al. (1990a) concluded "Clearly, molecular-level methods for the characterization and quantification of tannins . . . in nonwoody vascular plants 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, in prep). 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.

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. All leaves were oven-dried at temperatures below 60 °C and ground 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 24 hours. Both dissolved and particulate fractions were freeze-dried and ground. Elemental, ^{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). Neutral aldoses plus cyclitols were released by acid hydrolysis and also quantified by GC as TMS derivatives (Cowie and Hedges, 1984). Previously unpublished yields of cutin acids and uronic acids were measured by the GC methods of Goñi and Hedges (1990) and Walters and Hedges (1988), respectively. Amino acid compositions were measured by HPLC versus charge-matched recovery standards as outlined by Cowie and Hedges (1992).

Molecular-level tannin analyses were done in duplicate by acid hydrolysis in the presence of excess phloroglucinol to capture the released carbocations. Details can be found in Hernes and Hedges (in prep). Briefly, ~50mg of bulk organic matter was hydrolyzed in 3 mL acetone:water (70:30 vol%) with an acid strength of 1.0M HCl and ~0.25M 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. 2.1). The carbocation is then captured by phloroglucinol either alpha or beta to the C-ring at C-4, producing a monomer-phloroglucinol adduct (Fig. 2.1). The hydrolysis was carried out at 30 °C for 24 hours in culture tubes purged with Ar. All solvents were sparged with Ar. After 24 hours, 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 (see Hernes and Hedges, in prep).

The following day, samples were redissolved in ~200 µL pyridine and small aliquots derivatized with equal amounts of Regisil™ (bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane) by heating for 10 minutes at 60 °C. A Shimadzu AOC-14 autoinjector introduced samples (2 µL) onto an HP 5890 gas chromatograph equipped with a 0.25 mm x 30 m capillary column coated with DB35ms liquid-phase (J&W Scientific Inc.). Splitless injection was carried out using a column-loading time of two minutes. 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 minutes. 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 for mangrove leaves is shown in Fig. 3.1.

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. While standards exist for many monomeric compounds, only very recently have phloroglucinol adducts become commercially available. For this study, catechin adducts were identified by the hydrolysis of a catechin-catechin dimer (Leuven Bioproducts) in the presence of phloroglucinol. Remaining adduct identities are based upon 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.

Quantification was done using the hematoxylin internal standard and relative responses to available standards. Where standards were unavailable, the hematoxylin response was used as a default. The reproducibility of all the major compounds was ±5-10% mean deviation, while trace compounds were within ±10-30%.

RESULTS AND DISCUSSION

Bulk Decomposition Indeces

In the early diagenesis of leaves, color is often a useful marker of 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. estimated that these losses corresponded to approximately one week submerged in water for orange leaves, 4 weeks for brown leaves, and 6-7 weeks for black leaves. Relative mass-to-area ratios proved useful as a means of normalizing molecular data between color types when discussing diagenetic trends. Much of our discussion also adopts this format, in which [Compound X] 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 originally). Given the potential relationship between tannin and nitrogen, this becomes an important trend to keep in mind when interpreting the tannin molecular data.

Tannin Yields and Composition

Four terminal units and four extender units of condensed tannin were identified overall in the acid hydrolysis mixture from the mangrove leaf samples analyzed (Table 4.1). 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

Table 4.1. Tannin and triterpenol compositions and yields from decaying mangrove leaves[1].

Leaf Color	Yield (mg g^{-1} tissue AFDW)														
	Condensed Tannin														
	Terminal Units				Extender Units				Tannin-related		Triterpenols				
	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	
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	
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	
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	
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	
Brown #6	2.09	0.86			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			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			0.3	3.31	0.21	0.00	0.02		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	
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			
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	

[1] 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.

subgroups and Brown #6 which represents the least degraded of the three brown leaf subgroups. Rationale for inclusion of the latter two samples will be discussed below. The structural precursors of all analyzed compounds (prior to trimethylsilyl derivatization) are shown in Fig. 4.1. Because alpha and beta adducts from the extender

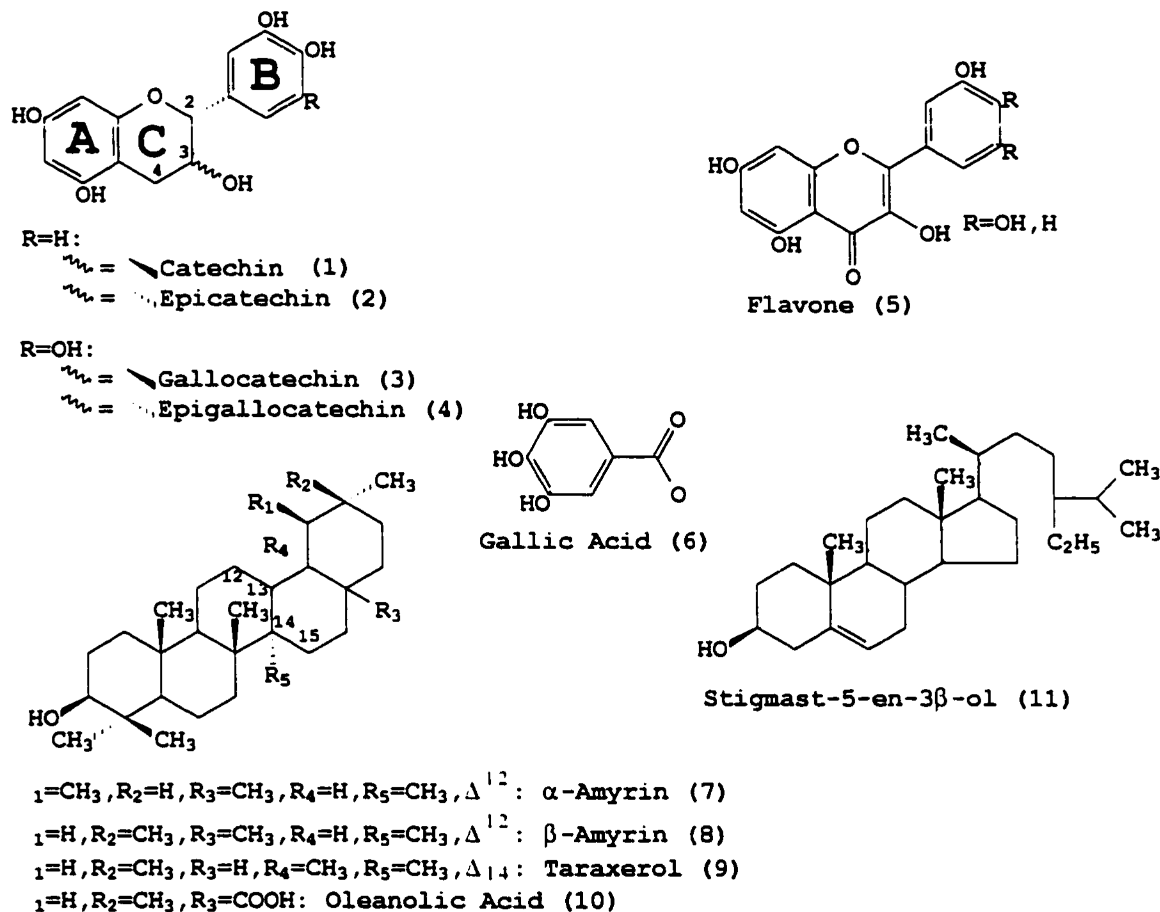

Fig. 4.1 Structures (underivatized forms) of measured phenols and triterpenols.

units are a result of the carbocation chemistry and not 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 (6), which is potentially derived from hydrolyzable tannin, and flavones (5), which are structurally related to condensed tannin (Fig. 4.1). Gallic acid was a minor constituent, ranging from 0.02 to 0.30 mg g^{-1} AFDW, while total flavones ranged from undetected to 6.58 mg g^{-1} AFDW.

Compositionally, condensed tannin consisted of predominantly catechin (1) 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 7.9-19.9% and 3.4-10.8%, respectively, of the total condensed tannin, while catechin and epicatechin extender units represented 4.8-6.9% and 60.6-78.6%. The remainder of the condensed tannin consisted of gallocatechin (3) and epigallocatechin (4). Together, these prodelphinidin (or PD) tannins ranged from undetected to 0.4% and undetected to 1.0%, respectively, in the terminal units and 3.8-17.5% and undetected to 0.9%, respectively, in the extender units. Generally, tannin compositions remained stable through to the orange stage (approximately one week in the water), before undergoing noticeable change in the brown and black stages.

Triterpenols

In addition to tannins, five triterpenols were recovered from the acid hydrolysis mixture: α-amyrin (7) and β-amyrin (8) (0.24-6.90 mg g^{-1} AFDW and 0.47-13.7 mg g^{-1} AFDW, respectively), taraxerol (9) (1.82-6.80 mg g^{-1} AFDW), oleanolic acid (10) (undetected to 4.05 mg g^{-1} AFDW), and stigmast-5-en-3β-ol (11) (0.66-2.73 mg g^{-1} AFDW) (Table 4.1). All triterpenols were found in higher concentrations in the brown and black leaves than the yellow leaves, indicating preferential preservation or chemical release. Total triterpenol yields ranged from 3.2-34.2 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 *Rhizophora 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 present in these mangrove leaves are very unreactive, and in fact, show absolute increase recoveries relative to yellow senescent leaves. Killops and Frewin (1994) reported that β-amyrin in *Rhizophora mangle* leaves was largely confined to the epicuticular wax, while taraxerol appeared to be a cutin component. They further suggest that the resistence of cuticular membranes to degradation allows such triterpenols to persist into the sediments, where Killops and Frewin were able to detect them. Thus, the absolute increase in triterpenol recoveries in this study may be a result of weakening of the cuticular membrane structure. 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. 4.1 for relative structures. 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 4.1). If oxidation were an important mechanism, then the oxidative counterpart of α-amyrin (ursolic acid) would be expected in the brown and black leaves as well. 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 triterpenol in vascular plant tissues and shows a weak increasing trend.

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 4.2), as compared to gymnosperm needles which typically have PD contents >50% (Hernes and Hedges, in prep). Another 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. 4.1). 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 4.2). This pattern also is typical of dicotyledon leaves, as compared to gymnosperm needles in which 2,3-*cis* forms compose <50% of all extender units (Hernes and Hedges, in prep). Another diagnostic source indicator is the degree of polymerization. This parameter corresponds to the total yield of condensed tannin divided by the yield of

Table 4.2. Tannin compositional parameters from decaying mangrove leaves[1].

		Percent PC:PD tannin			Percent 2,3-*cis*:2,3-*trans*		
Leaf Color	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

[1] 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.

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 than most other dicotyledon leaves by a factor of three (Hernes and Hedges, in prep). Flavones are more common in dicotyledon leaves than gymnosperm needles (Hernes and Hedges, in prep), 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 (Hernes and Hedges, in prep; 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 analyzed.

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 4.1 and 4.2). Thus, while the composition of the black leaves is still recognizable as dicotyledon in origin, it would be difficult to attribute it specifically to mangroves if the source was unknown.

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 (*Rhizophora mangle*) leaves utilizing ^{13}C-NMR and Folin-Denis analyses. While ^{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 is much more soluble in water than condensed tannin (Zucker, 1983), Benner et al. hypothesized that the extractable tannin measured using the Folin-Denis reagent was primarily hydrolyzable, and that the residual "tannin" (i.e. the difference between the ^{13}C-NMR and Folin-Denis results) in the leaf complex was condensed. While this may be partly true, it is worth noting that on a mass-balance basis, the residual "tannin" actually increased by 15-25% in the latter stages of decomposition, such that at least that much of the residual "tannin" 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 contributors include lignin and lignan 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.

Our molecular-level tannin data (Table 4.1) do not definitively confirm or refute the hypothesis of Benner et al. (1990a) for selective loss of hydrolyzable tannin. On the 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 of the ^{13}C-NMR and colorimetric analyses (~8% to ~20%). The solvent system (i.e. 70:30 v/v acetone:water) used in our hydrolysis, however, is identical to that used by Benner et al. for extraction prior to Folin-Denis analysis. In addition, the pattern we measured along the color series is similar to the pattern Benner et al. determined using Folin-Denis reagent (Fig 4.2), 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, follows the pattern measured with Folin-Denis reagent (Fig 4.2). 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. 1.1; Zucker, 1983). Compositional differences among condensed tannins have been shown to influence feeding behavior (Clausen et al., 1990), and affect ease of hydrolysis (Hemingway and McGraw, 1983).

Quantitative Comparison of Total Molecular Tannin to Other Compound Classes

These 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 ^{13}C-NMR and several bulk analyses (this study, Benner et al., 1990b; Cowie, unpublished data; Goñi, unpublished data; Bergamaschi, unpublished data; Benner et al., 1990a). In total, 36-55% of the leaf tissue can now be accounted for at the molecular level (Fig. 4.3).

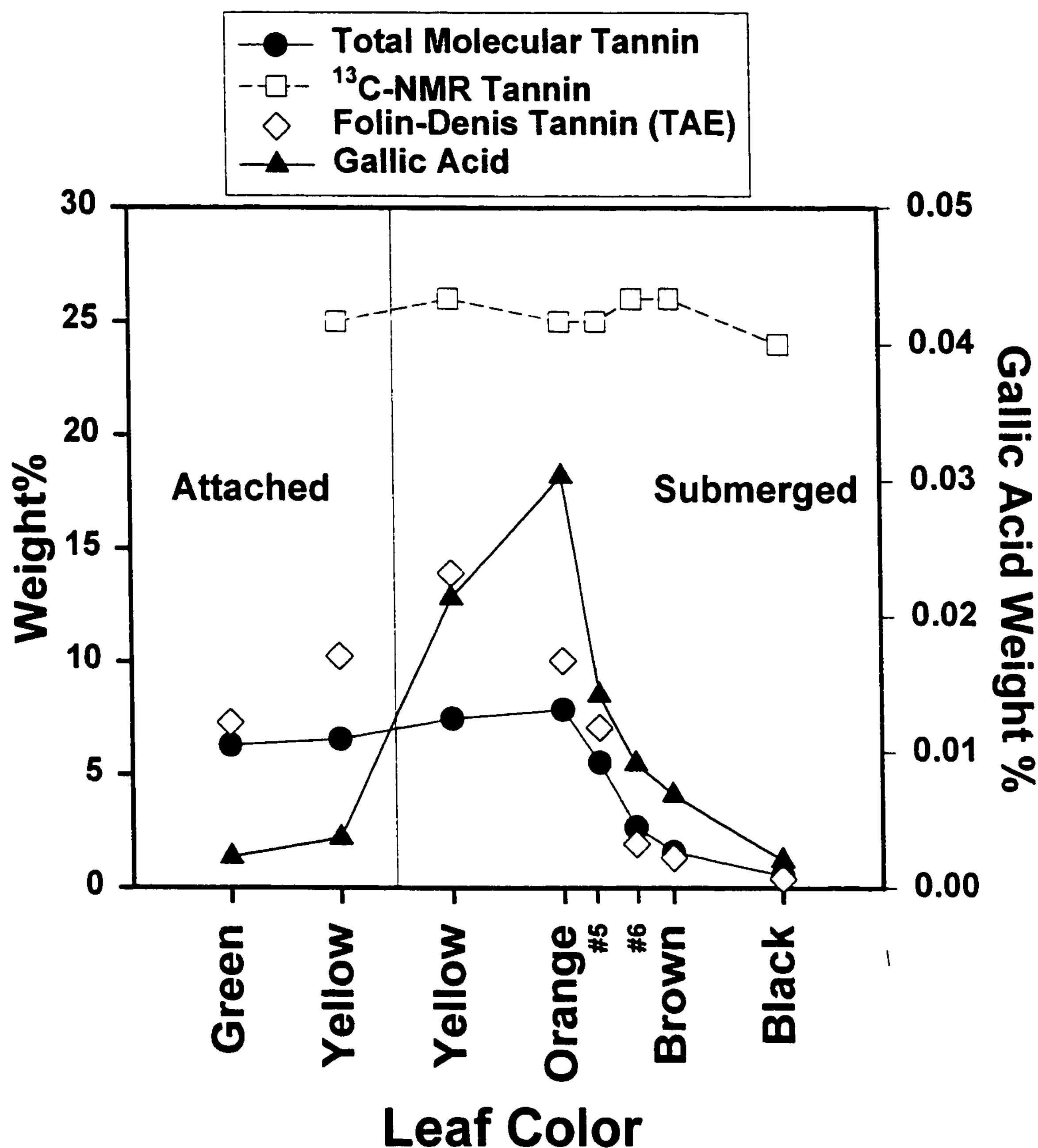

Fig. 4.2 Bulk estimates of tannin utilizing ^{13}C-NMR, Folin-Denis analyses, total molecular tannin, and gallic acid.

There are several striking trends in Fig. 4.3, including the effect of leaf senescence on amino and uronic acids, the predominance of neutral carbohydrates, and the 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

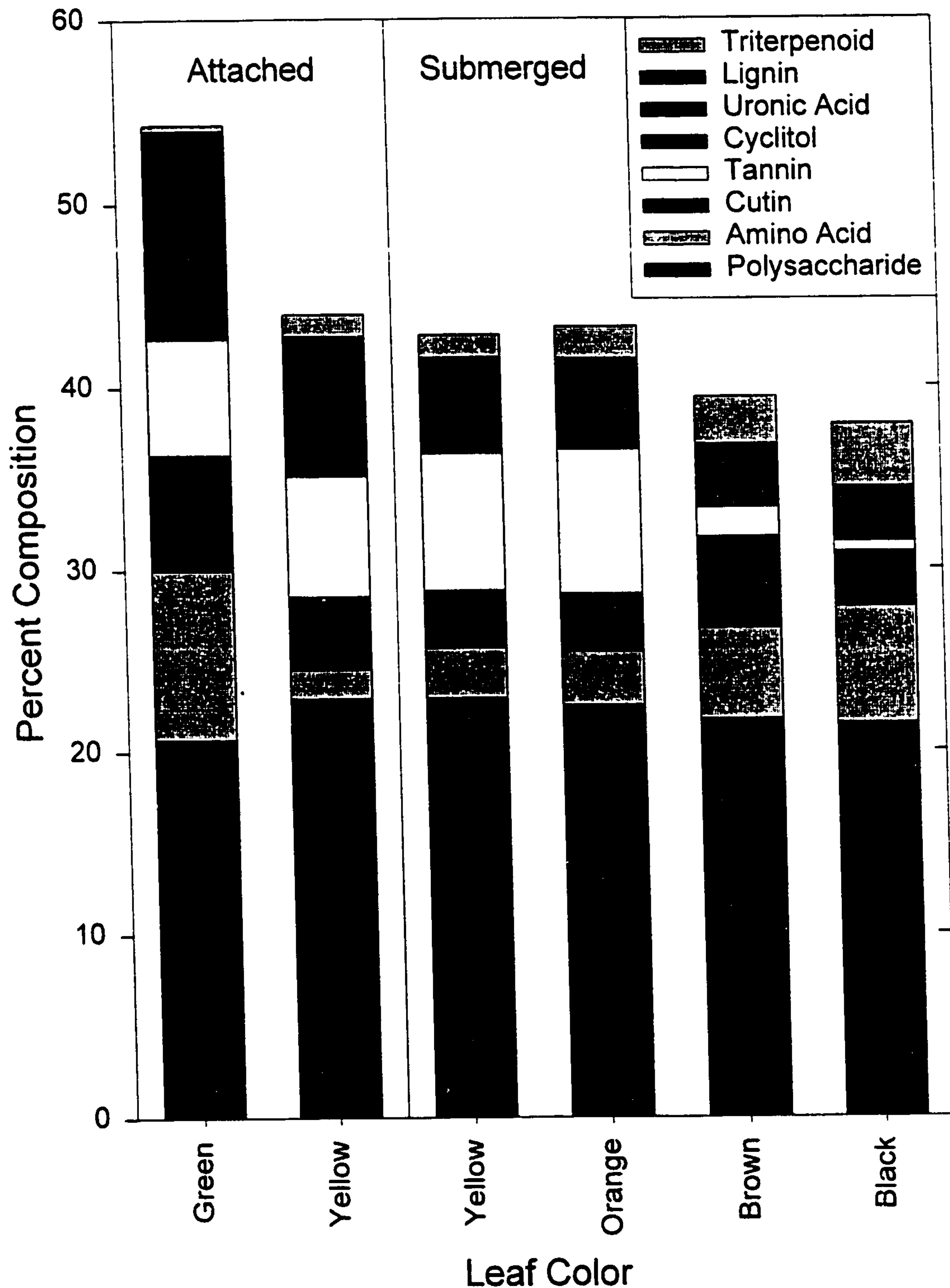

Fig. 4.3 Percent of organic matter (i.e. AFDW-based) occurring in mangrove leaves as total triterpenols, lignin, uronic acids, cyclitols, tannin, cutin, amino acids, and polysaccharides (all measured at the molecular level). Bars are stacked in the same order as they appear in the legend.

for the first time. In the yellow leaves entering the estuary, measured molecular tannin is second only to neutral carbohydrates in abundance. Hence, tannin alteration is a critical process to consider in the degradation of these leaves and in attending trends in their bulk chemical properties.

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 24 hours . Mass balance for total molecular tannin indicates nearly complete recovery of the senescent leaf tannin between the leachate and leached leaf fractions (Table 4.1). Overall, tannin loss from the leached leaf material was approximately 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—Table 4.2), this trend indicates that the smaller oligomers are made up of a higher proportion of PC extender units and 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 the tannin remaining in the leached leaves is likely more diagenetically reactive. Not surprisingly, gallic acid showed >70% loss, as would be expected if indeed it represents hydrolyzable tannin. The flavones also exhibited higher losses (~40%), which suggests 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 in 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 4.1, Fig. 4.4), 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

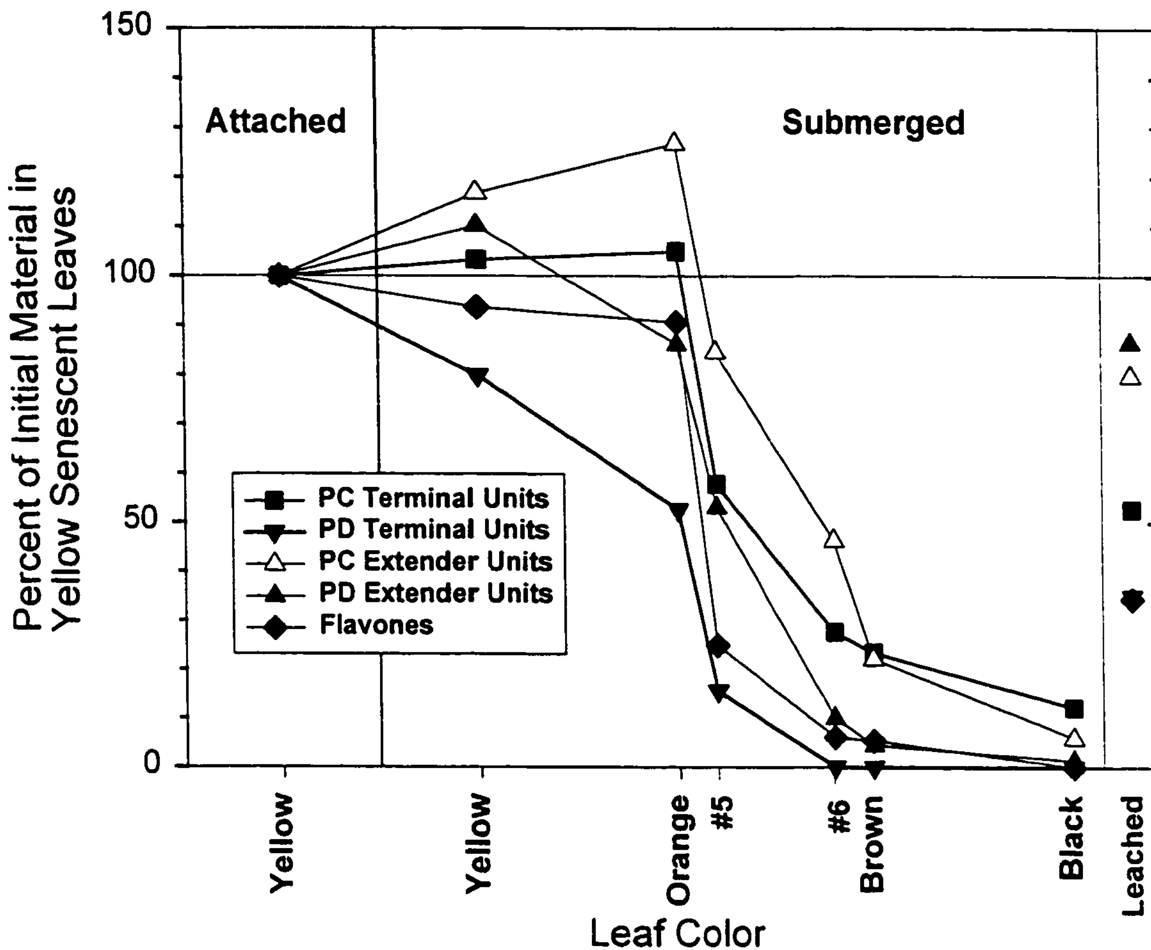

Fig. 4.4 Percent of initial procyanidin (PC) and prodelphinidin (PD) tannin for both terminal and extender units, along with flavones.

transition from orange to brown leaves is such an important stage in tannin diagenesis, Benner et al's. (1990b) original Orange #5 and Brown #6 leaf samples (referred to above) were analyzed in order to provide greater coverage in this critical stage and shed further light on the alteration processes involved. In terms of mass loss, mass-to-area ratios the Orange #5 and Brown #6 samples both indicate an 18% mass loss, which is comparable with 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 4.1 and Fig. 4.4). Once again, however, the results are split, as the PC tannin in leached leaves aligns with the Orange #5 samples, while 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

account for 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 Fig. 4.4 would increase all the orange leaf values and decrease the brown leaf values.

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.

Degree of Polymerization. The first of these parameters is the average length of the tannin oligomers (or polymers) present in the sample, which is indicated by the degree of polymerization as defined above. Typically, a higher degree of polymerization should correspond to a 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 while in the leached leaves it was 9.2 (Table 4.2). And 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. 4.5).

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). The fact that degree of polymerization decreases in the brown and black stages of mangrove leaf diagenesis suggests, however, that larger polymers are not necessarily always more degradation-resistant. 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, while microbial degradation likely plays some role in tannin conversion, it may not be a direct role for mangrove leaves as the studies of Grant (1976) and Field and Lettinga (1991) might indicate. An abiotic explanation might be

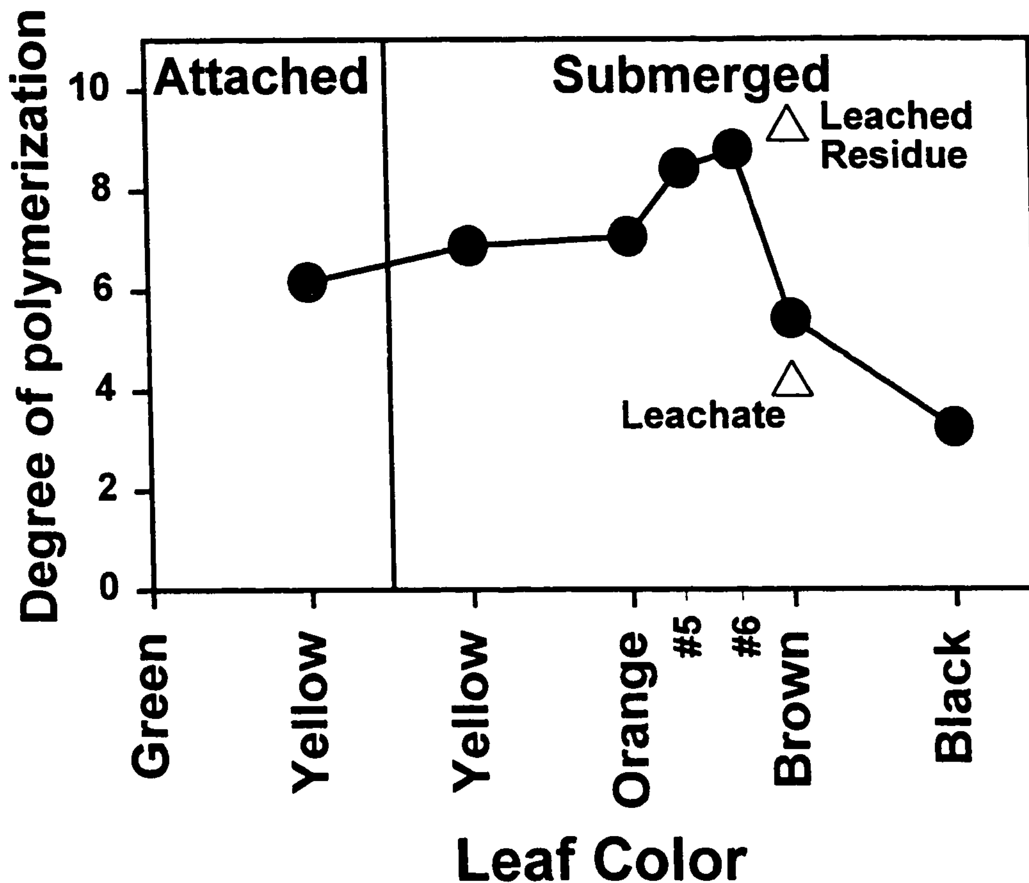

Fig. 4.5 Degree of polymerization in mangrove leaves along the diagenetic path. Included for reference are values for leached leaves and the leachate from laboratory leaching experiment.

more in order. A decrease in degree of polymerization suggests that terminal units are more degradation-resistant than extender units. This is certainly true abiotically in acidic conditions, as hydrolysis 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, the decrease in degree of polymerization measured in the brown and black leaves may simply be an indication of an acidic microenvironment, as shown by Sagemann et al. (1999) to occur in decaying organic matter.

A second factor is the relationship between degree of polymerization and composition. The leaching experiment indicated an 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 are enriched in PD tannin. Since PD tannin is more reactive than PC tannin, these larger polymers would be more reactive, explaining in part why the degree of polymerization subsequently decreases. However, hydroxylation cannot be the primary factor involved, as the Brown #6 sample has a PD content almost identical to the brown and black leaves, but a degree of polymerization of 8.9 vs. 5.4 and 3.3.

A third factor may be tannin-nitrogen interactions. Most theories about the ecological function of tannin involve interactions with nitrogen. These processes include inhibition of herbivore and microbial enzymes as a general defense, sequestering nitrogen in nitrogen-poor environments, and many other observed and suspected reactions (see Zucker, 1983 and Appel, 1993 and references therein). Thus, the inverse correlation between total molecular tannin and total nitrogen as shown in Fig. 4.6 is intriguing. As nitrogen content in the leaves increases in the brown and black stages, measured tannin decreases and the degree of polymerization decreases. Jones et al. (1976) found that for complexation of tannin with proteins, the oligomeric tannin length had to be at least four. Larger tannin oligomers become complexed whereas smaller ones do not. Once complexed, covalent bonds could form between the tannin and nitrogen, perhaps in analogy to Schiff base reactions. Alternatively, hydrolysis of interflavan bonds (perhaps due to more acidic microenvironments within the leaves as degradation progresses) would lead to carbocations that could react directly with nitrogenous compounds. Yet another plausible mechanism for overall tannin alteration might be indicated by the leaf color itself: the change from orange to brown apparently signals the penetration of molecular oxygen and oxidation of leaf pigments. Tannin should also be exposed to O_2, in which case quinone formation would readily take place. The quinones, in turn, would be highly reactive toward amino functions in protein or free amino acids. As suggested previously, oxidation could also account for the appearance of oleanolic acid in the brown and black leaf stages. It is not clear, however, how oxidation alone would lead to a decrease in degree of polymerization.

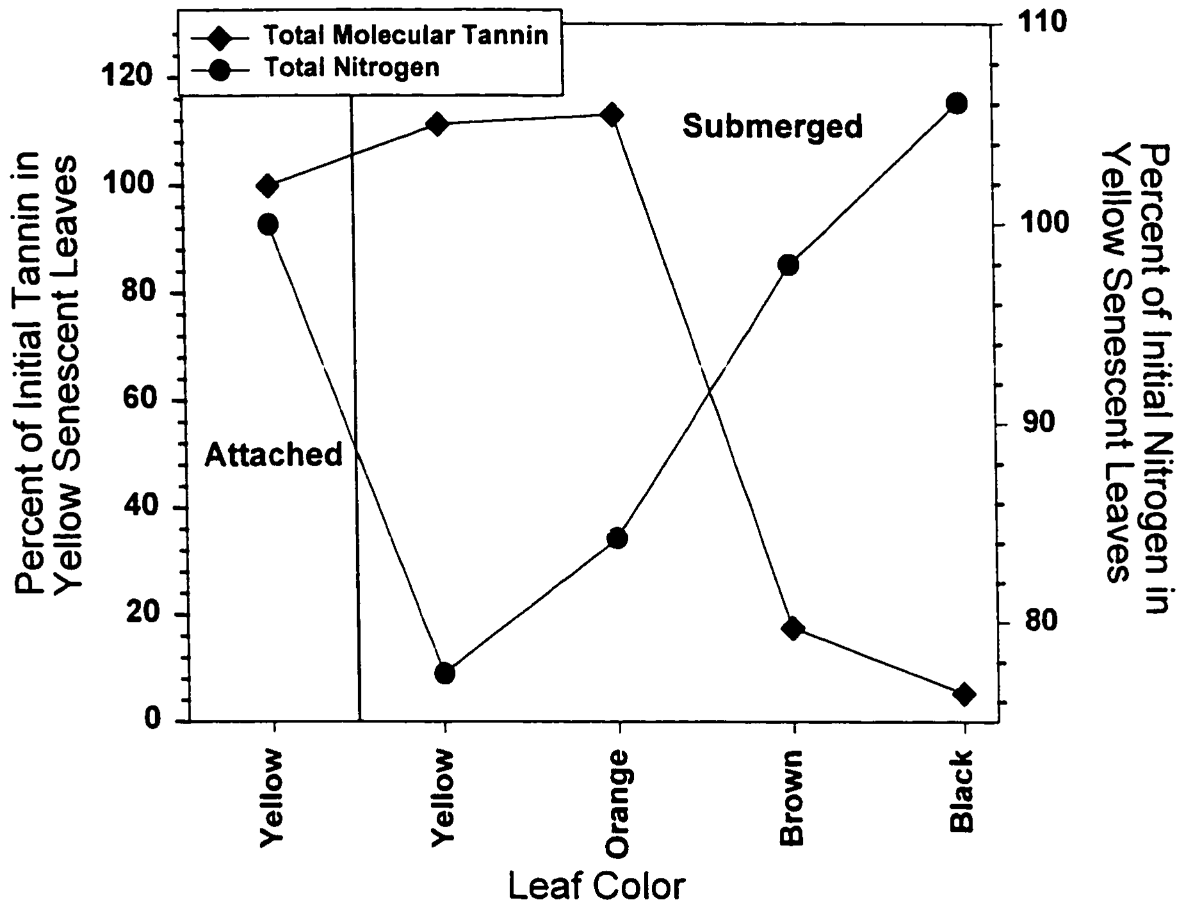

Fig. 4.6 Percent of initial tannin and total nitrogen in yellow senescent mangrove leaves and submerged counterparts.

If tannin is reacting with proteins, the primary candidates would be the polyamino basic amino acids which have the highest affinity toward the hydroxyl groups of tannin. Although correlation does not necessitate causation, the breakdown of basic, neutral, and acid amino acids in relation to tannin (Fig. 4.7) is eye-catching. The rapid decrease in tannin between the orange and brown leaves is matched by a rapid increase in amino acids. However, there is a distinctive shift in the makeup of the measurable amino acids, as basic amino acids are depleted relative to acidic and neutral amino acids. Mechanistically, this is exactly what would be predicted for protein-tannin interactions, and counter to the typical enrichment in basic amino acids with increasing degradation (e.g. Keil et al., in press, and references therein). Regarding degree of polymerization, if complexation were to occur prior to condensation, larger oligomers would be more

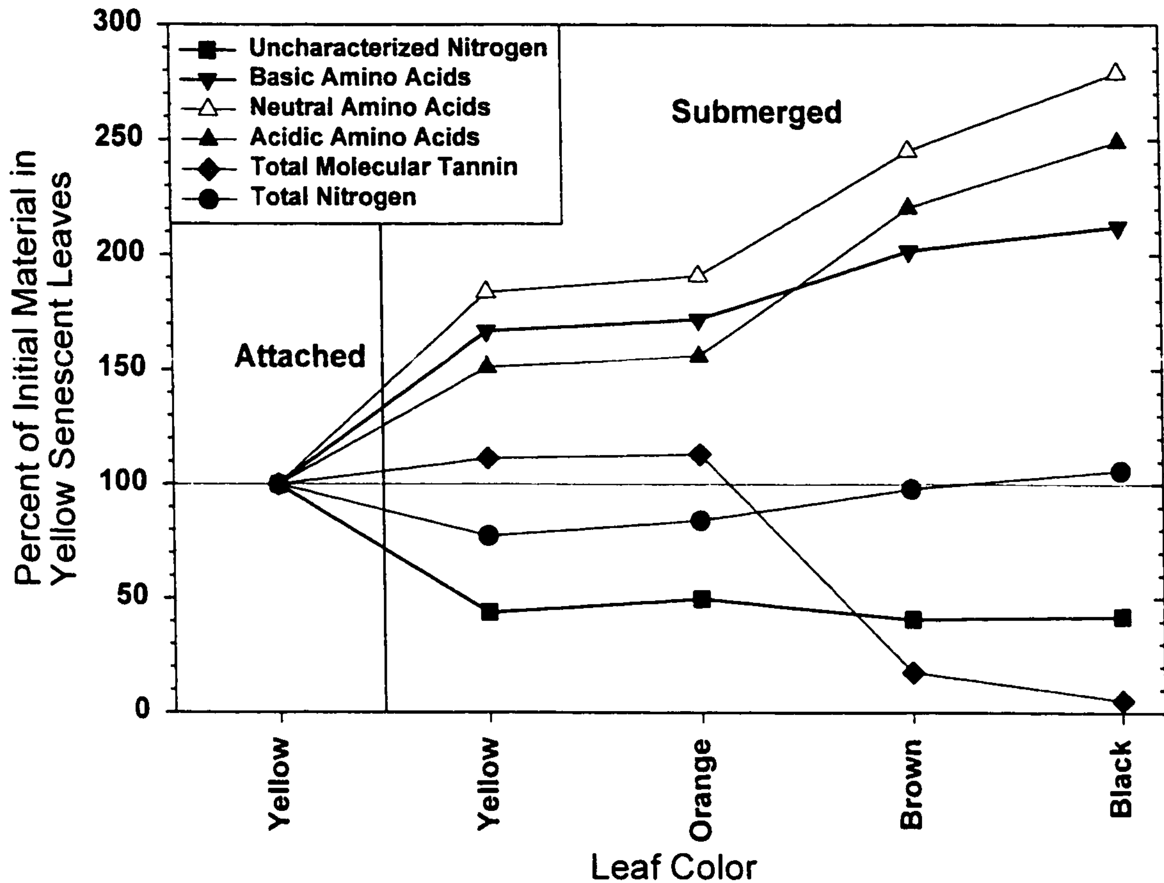

Fig. 4.7 Percent of initial tannin, amino acids, total nitrogen, and uncharacterized nitrogen in yellow senescent mangrove leaves and submerged counterparts.

reactive toward nitrogen because of their greater size and number of hydroxyl groups (Jones et al., 1976). If hydrolysis of tannin and subsequent carbocation formation is the primary step, larger oligomers would simply have a higher proportion of extender units and hence more interflavan bonds that can be broken.

Degree of Hydroxylation. Tannin reactivity stems from the number of hydroxyl groups present. More hydroxyl groups leads to stronger complexes. 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 three hydroxyl groups, 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. 4.8). This consistent trend supports

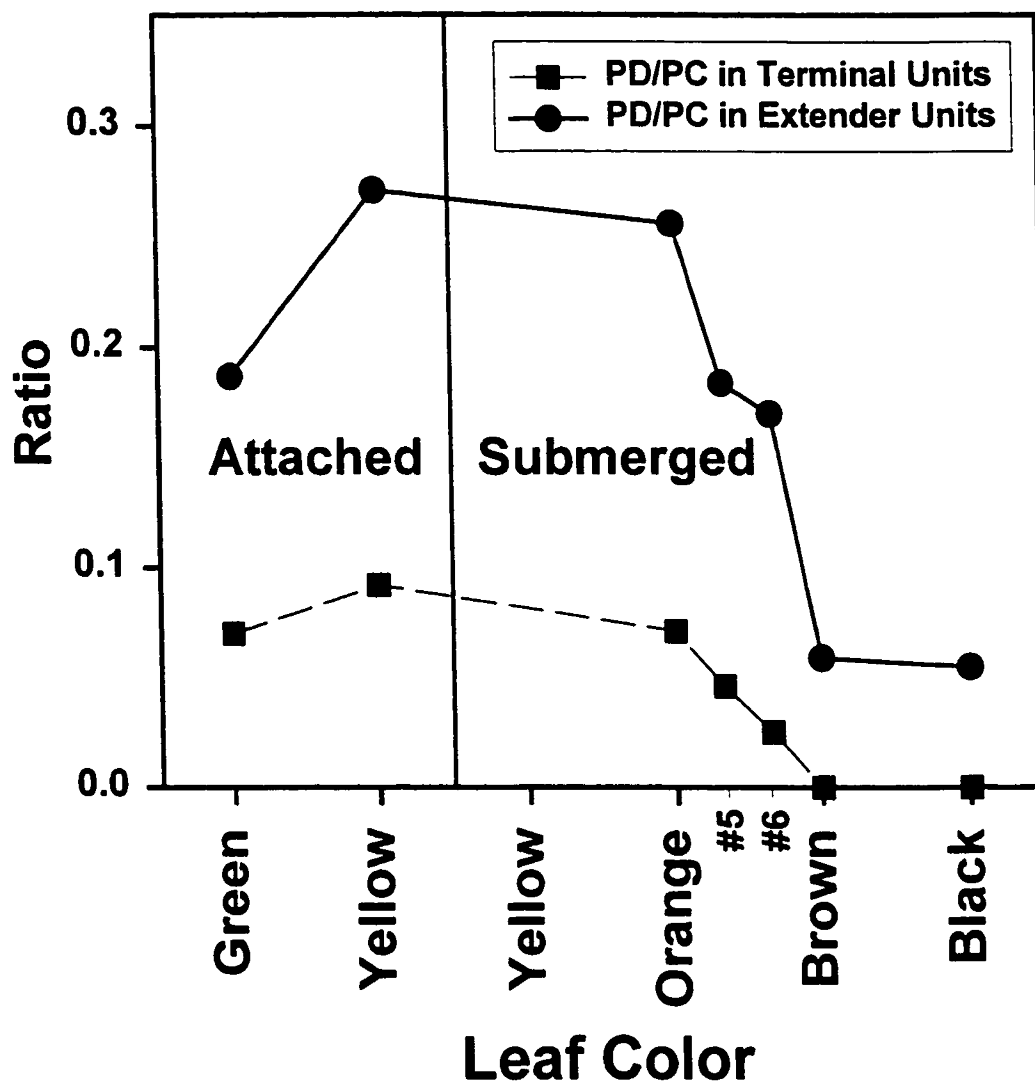

Fig. 4.8 Ratio of PD to PC in extender and terminal units of tannin in mangrove leaves along the degradation sequence.

the notion that more extensively hydroxlated tannins are more susceptible to oxidative alteration, even though normalization to a PP "control" is not possible.

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 hydrolysis 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 could lead to a decrease in *cis/trans* ratios. On the other hand, PD extender units measured in this study are enriched in 2,3-*trans* monomers while PC extender units are enriched in 2,3-*cis* monomers, and therefore it might be expected that as PD tannin decreases, the *cis/trans* ratio would increase. From Table 4.2, the *cis/trans* ratio of extender units increases, which suggests that the latter is a more important factor. Terminal units in general are much more variable in PC and PD tannin content, and as such, exhibit no clear diagenetic trend.

Diagenesis Comparisons to Other Compound Classes

Total molecular tannin shows quite different reactivity 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 about 1% of the amount found in attached leaves (Fig. 4.9; Benner et al., 1990b). Molecular lignin, neutral carbohydrates, uronic acids, cutin, and leaf mass show a steady parallel decline to 40-60% of the amounts found in attached leaves. On the other hand, amino acids and triterpenoids appear to accumulate, with nearly three times the amount of each present in black leaves as opposed to 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 reactivity, and therefore may be extremely valuable in determining early diagenetic mechanisms.

Another important comparison is how the leaching of tannin compares to other compound classes. Incredibly, every molecular total (with the exception of the triterpenols) indicates mass loss from leaching falls between those of submerged orange and brown leaves. 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

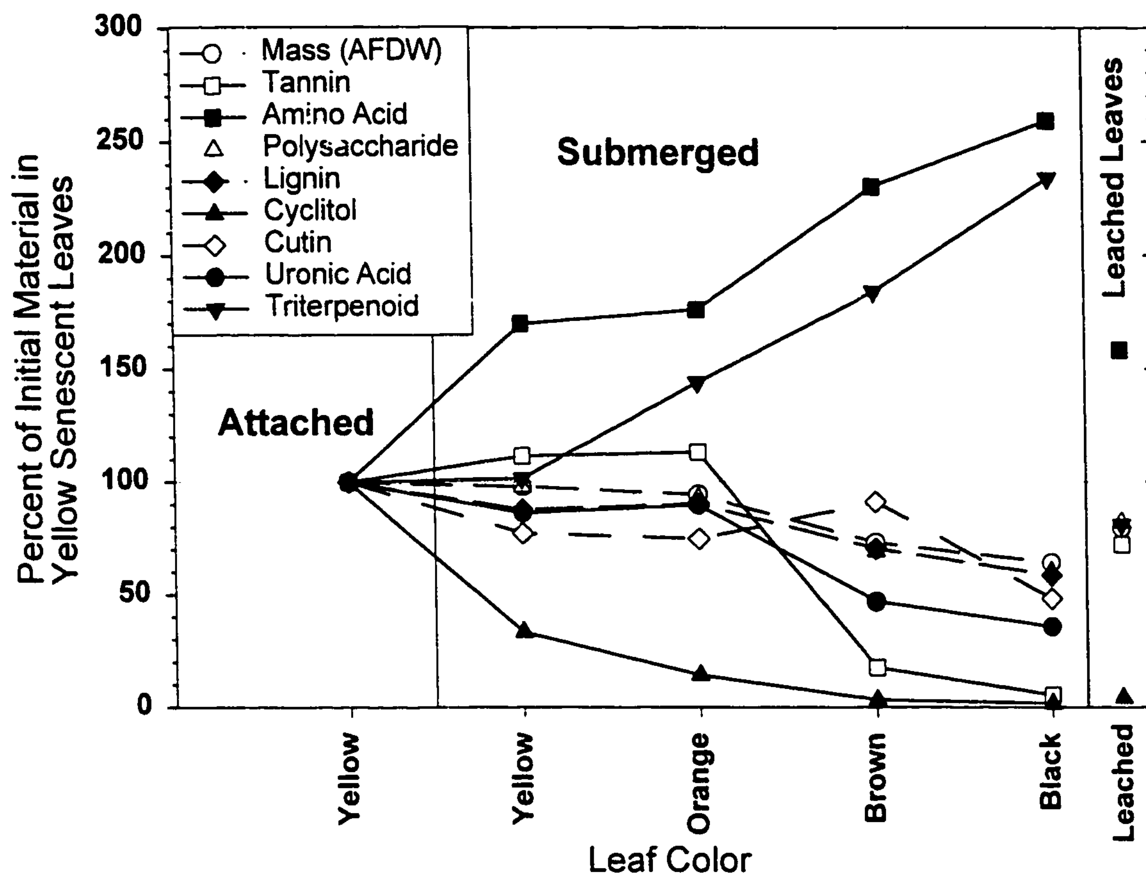

Fig. 4.9 Percent of initial measured triterpenols, lignin, uronic acids, cyclitols, tannin, cutin, amino acids, and polysaccharides present in yellow senescent leaves along the degradation sequence.

pattern, the molecular totals make a convincing argument for leaching as the dominant factor in the first 1-2 weeks of diagenesis.

CONCLUSIONS

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

1) Molecular level analyses confirm the quantitative importance of tannin previously suggested in other studies by bulk techniques. In this study, 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 reactivity not shown by any other biomarker in this study. 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 apparent inverse correlation observed here between amino acids and tannin might be indicative of at least one mechanism by which nitrogen immobilization occurs.

6) The ability to calculate a degree of polymerization for tannin based on molecular analysis makes it unique among commonly measured biochemicals. In this study, degree of polymerization highlights both 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 both 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). Tannin/nitrogen interactions (in particular interactions with basic amino acids) remain a critical area to study both in terms of early tannin diagenesis and

nitrogen immobilization. If quinone formation from tannin is a precursor to tannin-nitrogen reactions, then the degree of hydroxylation and oxidative history become important factors to consider. 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 within the dissolved phase (including phototransformations), and to evaluate the importance of tannin as a component of dissolved organic matter pools in rivers, lakes, and possibly the ocean.

CHAPTER 5: OVERVIEW

The goal of this project was to lay the groundwork for molecular tannin research in geochemistry. The key to meeting this goal was the development of a molecular technique for condensed tannin (Chapter 2) that is sensitive, facile, allows reproducible quantification and detailed characterization, and is capable of rapid throughput. The concomitant measurement of triterpenoids provides even more valuable information.

Once method development was completed, a survey of 117 source tissues (Chapter 3) established the quantitative importance and compositional signature of tannin in many sources. In addition to individual species compositions, the resulting source parameters provide a background for any future molecular tannin work. Conifer needles and barks, in particular, are rich sources of tannin that contain unique structural signatures. As such, tannin is important to consider in any study involving organic matter from conifer forests.

Finally, the method was applied to the study of early diagenesis of mangrove leaves in a tropical estuary (Chapter 4). This molecular tannin study is the first of its kind, and highlights the diagenetic behavior of tannin at the molecular level. The primary effects of the initial leaching were seen in an increase in degree of polymerization as smaller oligomers were leached from the leaves. Further degradation, however, was evident by a decrease in the degree of polymerization as well as a decrease in degree of hydroxylation. In addition, interactions with nitrogen are suggested by the inverse correlation of total tannin to total nitrogen and the correlation between total tannin and nitrogen-rich basic amino acids. In comparison to the other major biochemicals, tannin appears to occupy a unique niche of intermediate reactivity and may provide an invaluable tracer for organic matter processing.

The attractiveness of tannin research lies in the structural diversity of tannin as well as the diversity of reactions with other organic substances, nitrogen, and minerals at changing pH's, oxygen levels, and light conditions. Many of these traits were exhibited in this study. The structural diversity is seen in the unique source signatures from

Chapter 3 and the differing diagenetic behavior of procyanidin (PC) and prodelphinidin (PD) tannin in Chapter 4. Mineral interactions were studied in the method development described in Chapter 2. The effects of pH are shown in the method itself (also Chapter 2), which capitalizes on acid hydrolysis of the condensed tannin. The mangrove study in Chapter 4 shows the interactions of tannin with nitrogen, and possibly the effects of oxygen exposure as evidenced by changing leaf color and tannin recoveries. One notable absence is the effects of photochemistry on tannin reactions. Given the 30% loss of tannin from the mangrove leaves due to leaching, it will be important in the future to assess phototransformations of tannin as a loss term in the dissolved phase. With careful laboratory experiments, it may be possible to identify photochemical reaction products of tannin that could serve as actinometers.

Along with phototransformations, several other areas for future research are suggested by the work in this dissertation. From Chapter 2, it is clear that tannin interacts or reacts strongly with minerals. As suggested by this study (and Schofield et al., 1998), mineral interactions may be a significant sink for tannin in soils and sediments. Therefore, more work is needed on the nature of these interactions, including a means of better quantifying mineral-associated tannin. Also from Chapter 2, hydrolysis efficiency remains an issue, both in absolute terms for quantifying tannin, and in relative terms between whole samples and purified condensed tannin. As with CuO oxidation for molecular measurements of guaiacyl lignin, the amount of tannin measured at the molecular level likely represents a third of what is actually present in the samples.

The primary area for future research as indicated in Chapter 3 is the effect of diagenesis on source signatures. In particular, differential degradation between PC and PD tannins as indicated in Chapter 4 has ramifications for high PD sources such as the conifers. And in fact, given the quantitative importance of tannin in the conifers and the importance of conifer forests overall to carbon cycling, more molecular tannin work needs to be done in these systems.

The mangrove leaf study in Chapter 4 highlights many areas that merit further research. It has been shown that PD tannin with three hydroxyl groups on the B-ring is more labile than PC tannin with only two. Does this trend also extend to

propelargonidin (PP) tannin with only one hydroxyl? *Tsuga heterophylla* bark would be an excellent candidate for degradation studies in this regard, given its high tannin content (>10 wt%), of which nearly 30% is PP tannin. The mangrove study also hints at the importance of tannin/nitrogen interactions, and in particular interactions with basic amino acids. Finally, the 30% loss term due to leaching indicates that the dissolved phase is an important component of tannin diagenesis, suggesting the potential importance of tracing tannin into the dissolved organic matter pools in rivers, lakes, and possibly the ocean.

With these and other studies and applications that will become apparent, the future of tannin geochemistry is exciting. Tannin geochemistry may still be in its infancy, but the molecular method developed in this project, in combination with the source and mangrove studies, provide an important first step toward a promising future.

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APPENDIX I. Table A. Molecular data for all plant tissues (Chapter 3) in weight percent.

		PA Monomers						Phloroglucinol Adducts								Flavanone, Flavone, Carboxy A.						Triterpenoids									
Latin Name	TISS.	C	EC	GC	EGC	A	EA	EC	C	EGC	GC	EA	A	PG	PF	T	AP	FS	Q	MR	TCS	bA	aA	MbA	MaA	131A	587	OA	UA	HOA	HUA
Acer macrophyllum	BK	-	0.04	-	-	-	-	0.04	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alnus rubra	BK	0.10	0.10	-	-	-	-	0.25	-	-	0.04	-	-	-	-	-	-	-	-	-	0.03	0.23	-	-	-	1.00	-	0.02	-	-	-
Artemisa tridentata	BK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Quercus garrayana	BK	0.29	0.05	-	-	-	-	0.04	0.04	0.04	0.02	-	-	-	-	-	-	-	0.04	-	-	0.04	0.06	-	-	-	-	-	-	-	-
Thuja plicata	BK	0.38	0.17	-	-	0.05	-	0.13	0.50	-	0.05	0.06	0.13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Abies holophylla	BK	0.09	0.08	-	-	-	-	0.03	-	-	-	-	-	-	-	-	-	-	-	-	0.26	-	-	-	-	-	-	-	-	-	-
Pinus contorta	BK	0.19	0.02	0.05	-	-	-	0.48	0.06	0.05	0.29	-	-	-	-	0.58	1.25	-	0.02	0.31	0.24	-	-	-	-	-	-	-	-	-	-
Pinus ponderosa	BK	0.58	0.08	-	-	-	-	0.66	0.19	-	0.04	-	-	-	-	0.60	-	-	-	-	0.27	-	-	-	-	-	-	-	-	-	-
Pseudotsuga menziesii	BK	0.15	0.19	-	-	-	-	0.32	0.18	-	-	-	-	-	-	2.28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tsuga heterophylla	BK	2.18	2.24	0.01	0.02	1.07	1.94	2.11	0.77	-	0.23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aniba guianensis	BK	0.13	0.10	-	-	-	-	0.20	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clarisia racemosa	BK	-	0.01	-	-	0.00	0.01	0.00	0.01	-	-	0.02	0.00	-	-	-	-	-	-	-	-	0.03	0.16	-	0.07	-	-	-	-	0.63	-
Crescentia amazonica	BK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Genipa americana	BK	-	0.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-
Nectandra amazonium	BK	0.23	0.29	-	-	-	-	0.67	0.10	-	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neoxythece elegans	BK	-	0.00	0.04	0.01	-	-	0.06	0.03	0.15	0.35	-	-	-	-	-	-	-	-	-	-	1.10	1.10	-	-	-	-	0.84	-	0.16	-
Xylosma intermedium	BK	0.03	-	-	-	-	-	0.01	0.00	-	0.01	-	-	-	-	-	-	-	-	-	-	0.01	0.01	-	-	-	-	0.01	-	-	-
Zanthoxylum compactum	BK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02	-	-	-	1.98	-	-	-	-	-
Larix occidentalis	C	0.12	0.04	-	-	-	-	0.60	0.08	-	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea sitchensis	C	0.14	0.01	0.01	-	-	-	0.05	0.02	0.03	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pinus ponderosa	C	0.26	0.02	-	-	-	-	0.24	0.14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudotsuga menziesii	C	0.18	0.22	-	-	-	-	1.44	0.16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tsuga heterophylla	C	0.26	0.07	0.06	0.02	-	0.01	0.68	0.18	0.38	1.46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sequoia sempervirens	C	0.20	0.09	0.03	0.02	-	-	0.15	0.13	0.39	0.78	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alnus rubra	S	0.07	0.29	-	-	-	-	1.41	0.10	-	-	-	-	-	-	-	-	-	-	-	-	0.17	0.04	-	-	0.20	4.13	-	-	-	-
Acer macrophyllum	GL	0.19	0.07	0.02	-	-	-	0.22	0.01	0.04	0.08	-	-	-	-	-	-	-	0.22	-	-	0.05	0.19	-	-	-	-	-	-	-	-
Alnus rubra	GL	0.15	0.17	-	-	-	-	0.56	0.03	-	0.05	-	-	-	-	-	-	-	0.93	-	-	0.10	-	-	-	-	0.25	-	-	-	-
Artemisa tridentata	GL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Avicennia germinans	GL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Camellia spp.	GL	0.50	2.04	-	-	-	-	0.62	0.56	-	-	-	-	-	-	-	-	-	0.58	-	-	0.01	0.02	-	-	-	-	-	-	-	-
Ilex aquifolium	GL	-	0.02	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	0.08	0.62	-	-	-	-	0.51	2.19	-	-
Malus spp.	GL	0.02	0.10	-	-	-	-	0.39	-	-	-	0.37	-	-	-	-	-	-	1.57	-	-	-	-	-	-	-	-	0.15	0.77	0.05	0.13
Populus trichocarpa	GL	0.33	0.01	0.05	-	-	-	1.13	0.18	-	0.43	-	-	-	-	-	-	-	0.09	-	-	-	-	-	-	-	-	-	-	-	-
Quercus garrayana	GL	0.72	0.52	0.03	-	-	-	0.46	0.17	0.16	0.18	-	-	-	-	-	-	-	0.42	-	-	-	-	-	-	-	-	0.15	0.05	-	-
Quercus palustris	GL	0.36	-	-	-	-	-	0.20	0.04	-	-	-	-	-	-	-	-	-	0.02	0.33	-	0.21	0.28	-	-	-	-	0.14	0.11	-	-
Quercus robur	GL	0.25	-	-	-	-	-	0.15	0.02	-	0.03	-	-	-	-	-	-	-	-	-	-	0.03	0.07	-	-	-	-	-	-	-	-
Quercus rubra	GL	-	0.37	-	-	-	-	0.25	0.02	-	-	-	-	-	-	-	-	-	-	-	-	0.21	0.19	-	-	-	-	-	-	-	-
Rhizophora mangle	GL	0.49	0.18	0.02	0.02	-	-	3.56	0.31	0.01	0.63	-	-	-	-	-	-	-	0.26	0.06	-	0.04	0.02	-	-	-	-	-	-	-	-
Rosa spp.	GL	0.75	0.08	0.03	-	-	-	0.71	0.14	-	-	-	-	-	-	-	-	-	0.23	-	-	-	-	-	-	-	-	0.04	0.03	0.02	0.02
Rubus spp.	GL	-	0.07	-	-	-	-	0.02	-	-	-	-	-	-	-	-	-	0.23	0.73	-	-	-	-	-	-	-	-	-	-	-	-
Vitis spp.	GL	0.33	0.43	0.16	0.17	-	-	1.42	0.11	0.45	1.60	-	-	-	-	-	-	0.17	1.72	-	-	-	-	-	-	-	-	-	-	-	-
Aniba guianensis	GL	0.12	0.11	-	-	-	-	0.35	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspidosperma rigida	GL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

APPENDIX I. Table A. (continued).

Latin Name	TISS.	PA Monomers						Phloroglucinol Adducts								Flavanone, Flavone, Carboxy A.						Triterpenoids									
		C	EC	GC	EGC	A	EA	EC	C	EGC	GC	EA	A	PG	PF	T	AP	FS	Q	MR	TCS	bA	aA	MbA	MaA	131A	587	OA	UA	HOA	HUA
Bothriospora lorymbosa	GL	-	-	-	-	-	-	0.17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.05	0.03	-	-
Buchenavia oxycarpa	GL	-	-	-	-	-	-	0.01	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	0.03	-	-	-	-	0.04	0.01	0.02	-
Campsiandra comosa	GL	0.53	0.23	-	-	0.07	0.02	0.17	-	-	0.05	-	-	0.19	0.07	-	-	-	0.23	0.30	-	-	-	-	-	-	-	-	-	-	-
Caraipa grandifolia	GL	0.04	0.19	-	-	-	-	0.41	-	-	0.06	-	-	-	-	-	-	-	-	-	-	-	0.13	-	-	-	-	-	0.02	0.03	-
Clarisia racemosa	GL	0.13	0.08	-	-	0.08	0.13	0.37	-	-	-	0.68	0.12	-	-	-	-	-	-	-	-	0.02	0.03	0.04	0.11	-	-	-	-	-	-
Genipa americana	GL	0.02	0.04	-	-	-	-	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.14	1.41	-	-
Glycydendron amazonicum	GL	0.08	-	0.03	-	-	-	0.34	0.09	0.18	0.46	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Guarea rubiflora	GL	0.06	0.17	0.00	0.00	-	-	0.46	0.02	0.13	0.44	-	-	-	-	-	-	-	-	-	-	0.02	0.04	-	-	-	-	-	-	-	-
Guarea trichilioides	GL	0.24	0.22	-	-	-	-	0.88	0.20	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lecointea amazonica	GL	-	-	-	-	-	-	0.03	-	-	0.00	-	-	-	-	-	-	-	-	-	-	0.05	0.09	-	-	-	-	-	-	-	-
Leonia racemosa	GL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Malouetia furfuracea	GL	0.20	0.28	0.06	0.03	0.04	0.06	0.30	0.09	-	-	0.76	0.27	-	-	-	-	-	-	-	-	0.12	0.65	0.05	0.31	-	-	0.26	1.35	-	-
Nectandra amazonium	GL	0.11	0.12	-	-	-	-	0.40	0.01	-	-	-	-	-	-	-	-	-	0.21	-	-	-	-	-	-	-	-	-	-	-	-
Neoxythece elegans	GL	-	0.06	0.14	0.12	-	-	0.14	-	-	0.68	-	-	-	-	-	-	-	0.04	0.43	-	0.13	0.06	-	-	-	-	-	-	-	-
Pterocarpus amazonicus	GL	-	0.05	0.02	0.02	-	-	0.11	-	0.05	0.28	-	-	-	-	-	-	-	-	-	-	-	0.06	-	-	-	-	-	-	-	-
Symmeria paniculata	GL	0.05	0.56	-	-	-	-	0.91	0.03	-	-	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tabebuia barbata	GL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.42	1.14	0.11	0.18
Vatairea guianensis	GL	0.09	0.02	-	-	0.13	-	0.02	-	-	-	0.10	-	-	-	-	-	-	-	-	-	0.03	0.13	-	-	-	-	-	-	-	-
Vitex cymosa	GL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.03	0.06	0.27	0.54
Xylopia callophylla	GL	0.07	0.32	-	-	-	-	0.97	0.02	-	-	-	-	-	-	-	-	-	0.18	-	-	-	-	-	-	-	-	-	-	-	-
Xylosma intermedium	GL	0.14	0.02	-	-	-	-	0.10	0.02	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.04	-	-	-	-	-	-	-	-
Zanthoxylum compactum	GL	0.11	0.24	-	-	-	-	0.64	0.01	-	-	-	-	-	-	-	-	-	0.31	-	-	-	-	-	-	-	-	-	-	-	-
Araucaria araucana	GL	0.31	0.07	0.03	0.03	0.02	0.03	0.66	0.07	0.23	0.97	0.07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thuja plicata	GN	1.18	0.32	-	-	0.05	0.02	0.40	0.26	1.50	1.27	0.06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Abies marocana	BN	1.20	0.21	0.68	0.11	0.01	-	0.78	0.33	0.75	1.89	0.06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Abies nebrodensis	BN	1.12	0.12	0.92	0.04	-	-	0.66	0.21	0.79	2.35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea engelmannii	GN	0.51	0.11	0.05	-	-	-	1.24	0.08	-	0.37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea sitchensis	BN	0.36	0.09	-	-	0.00	-	0.86	0.03	-	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pinus contorta	GN	1.04	0.14	0.99	0.02	-	-	1.01	0.40	1.67	4.64	0.07	-	-	-	0.08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pinus ponderosa	GN	0.83	0.09	0.57	0.03	-	-	1.24	0.16	0.59	5.08	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudotsuga menziesii	GN	0.33	0.22	0.08	0.08	-	-	1.81	0.14	0.35	2.52	0.04	-	-	-	0.03	-	0.66	0.84	-	-	-	-	-	-	-	-	-	-	-	-
Tsuga heterophylla	GN	0.24	0.10	-	0.02	-	-	0.86	0.05	0.40	2.96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sequoia sempervirens	GN	0.44	0.06	0.19	0.09	-	-	0.70	0.26	0.86	2.88	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sequoiadendron giganteum	GN	0.28	0.06	0.06	0.03	-	-	0.79	0.07	0.24	1.24	-	-	-	-	-	-	-	0.28	-	-	-	-	-	-	-	-	-	-	-	-
Taxodium distichum	GN	0.44	0.09	0.08	-	-	-	1.18	0.11	0.45	1.89	-	-	-	-	-	-	-	0.54	-	-	-	-	-	-	-	-	-	-	-	-
Taxodium distichum	GN	0.27	0.05	0.05	-	-	-	0.88	0.06	0.21	1.25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alnus rubra	W	0.23	0.02	0.02	-	-	-	0.27	0.05	-	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thuja plicata	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea sitchensis	W	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Picea spp.	W	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

APPENDIX I. Table A. (continued).

Latin Name	TISS.	PA Monomers						Phloroglucinol Adducts*								Flavanone, Flavone, Carboxy A.						Triterpenoids									
		C	EC	GC	EGC	A	EA	EC	C	EGC	GC	EA	A	PG	PF	T	AP	FS	Q	MR	TCS	bA	aA	MbA	MaA	131A	587	OA	UA	HOA	HUA
Pseudotsuga menziesii	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bothriospora lorymbosa	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.14	-	-	-
Buchenavia oxycarpa	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Campsiandra comosa	W	0.21	0.02	-	-	-	-	0.05	0.02	-	-	-	-	0.01	0.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clarisia racemosa	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leonia racemosa	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neoxythece elegans	W	-	0.00	-	-	-	-	0.00	-	-	0.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Symmeria paniculata	W	0.15	0.18	-	-	-	-	0.25	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tabebuia barbata	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vitex cymosa	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylopia callophylla	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylosma intermedium	W	-	0.01	-	-	-	-	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhacomitrium aciculare	WP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhytidiadelphus loreus	WP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lycopodium sitchense	WP	-	-	-	-	-	-	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Polystichum acrostichoides	WP	0.04	0.10	-	0.02	-	-	0.80	-	-	0.06	0.07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pteridium aquilinium	WP	-	0.07	-	0.03	-	0.02	0.51	-	0.03	0.22	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Equisetum telmateia	WP	-	-	-	-	0.00	0.03	0.72	-	-	-	0.49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alaria fistulosa	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alaria fistulosa	ST	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Laminaria longpipes	WP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thalasiophylum	WP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Agrostis alba	WP	-	-	-	-	-	-	0.00	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Avena spp.	WP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carex spp.	WP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicornia spp.	WP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.12	-	-	-
Spartina alterniflora	WP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02	0.08	-	-	-	-	-	-	-	-
Zostera spp.	WP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Echinochloa polystachya	WP	-	-	-	-	-	-	0.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eichornia crassipes	WP	-	0.04	-	-	-	0.03	0.24	-	-	-	0.11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gynerium sagittatum	WP	0.00	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Paspalum repens	WP	-	0.02	-	-	-	-	0.02	-	-	-	-	-	-	-	-	-	-	0.34	-	-	-	-	-	-	-	-	-	-	-	-
Pistia stratoides	WP	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salvinia auriculata	WP	-	0.08	-	-	-	0.05	1.23	0.02	-	-	0.08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*4α and 4β adducts for each compound have been summed.

Abbreviations

Compounds: C=catechin, EC=epicatechin, GC=gallocatechin, EGC=epigallocatechin, A=afzelechin, EA=epiafzelechin, PG=proguibourtinidin, PF=profisitinidin, T=taxifolin (dihydroquercetin), AP=ampelopsin (dihydromyricetin), FS=fisetin, Q=quercetin, MR=myricetin, TCS=tetracosanoic acid, bA=β-amyrin, aA=α-amyrin, MbA=methyl-β-amyrin, MaA=methyl-α-amyrin, 131A=amyrin with 131 mass spectra fragment, 587=triterpenoid with prominent 587 mass spectra fragment, OA=oleanolic acid, UA=ursolic acid, HOA=hydroxyoleanolic acid, HUA=hydroxyursolic acid

Tissue types: BK=bark, C=cone, S=seedpod, GL=green leaves, GN=green needles, BN=brown needles, WP=whole plant, W=wood, B=blade, ST=stipe

Curriculum Vitae

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EDUCATION

- Doctor of Philosophy, Chemical Oceanography, September 1999. University of Washington, Seattle, Washington 98195.
- Master of Science, Chemical Oceanography, June 1994. University of Washington, Seattle, Washington 98195.
- Bachelor of Arts (summa cum laude), Chemistry and Computer Science, May 1987. Luther College, Decorah, Iowa 52101.

RESEARCH EXPERIENCE

- Post-Doctoral researcher, University of South Carolina, Columbia, South Carolina 29208, October 1999 to present.
- Doctoral candidate, University of Washington, Seattle, Washington 98195, April 1996 to September 1999.
- Research Associate, University of Washington, Seattle, Washington 98195, September 1994 to April 1996.
- Office of Naval Research Fellow, University of Washington, Seattle, Washington 98195, September 1991 to August 1994.
- Oceanographer, University of Washington, Seattle, Washington 98195, August 1988 to June 1991.
- Student Fellow, Argonne National Laboratory, Argonne, Illinois 60439, January 1988 to May 1988.
- Technician, Sports Medicine Laboratory, Indiana University-Purdue University Indianapolis, Indianapolis, Indiana 46202, June 1987 to August 1987.
- Student Summer Fellow, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, June 1986 to August 1986.

TEACHING EXPERIENCE

- Teaching Assistant, Introductory Oceanography, University of Washington, Seattle, Washington 98195, March 1993 to June 1993.

PUBLICATIONS

1. Hernes P.J. and J.I. Hedges (in press) Geochemistry of tannin: methods and applications. *In*: Gross, G.G.; Hemingway, R.W.; Yoshida, T. (eds). Plant polyphenols 2: chemistry and biology. Plenum Press, New York.

2. Hernes P.J., J.I. Hedges, M.L. Peterson, S.G. Wakeham, and C. Lee (1996) Neutral carbohydrate geochemistry of particulate material in the central equatorial Pacific. *Deep-Sea Research II*, **43**, 1181-1204.

3. Hernes P.J., M.L. Peterson, J.W. Murray, S.G. Wakeham, C. Lee, and J.I. Hedges (submitted) Particulate carbon and nitrogen fluxes and compositions in the central equatorial Pacific. *Deep-Sea Research II*.

4. Hernes P.J., R. Benner, G.L. Cowie, M.A. Goñi, B.A. Bergamaschi, and J.I. Hedges (in prep) Tannin diagenesis in mangrove leaves from a tropical estuary: A novel molecular approach.

5. Hernes P.J. and J.I. Hedges (in prep) Determination of condensed tannin monomers in plant tissues, soils, and sediments by capillary gas chromatography of acid hydrolysis extracts.

6. Hernes P.J. and J.I. Hedges (in prep) Tannin signatures of barks, needles, leaves, cones, and wood at the molecular level.

7. Wakeham S.G., J.I. Hedges, C. Lee, M.L. Peterson, and P.J. Hernes (1997) Compositions and transport of lipid biomarkers through the water column and surficial sediments of the equatorial Pacific Ocean. *Deep-Sea Research II*, **44**, 2131-2162.

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PRESENTATIONS AT PROFESSIONAL CONFERENCES

1. "Molecular-level characterization of tannins in mangrove leaves from a tropical estuary." Third Tannin Conference, July 1998, Bend, OR.

2. "Particulate fluxes of carbon and nitrogen in the central equatorial Pacific." (poster) Ocean Sciences Meeting, February 1998, San Diego, CA.

3. "Molecular-level characterization of tannins in mangrove leaves from a tropical estuary." (poster) GSA Annual Meeting, October 1997, Salt Lake City, UT

4. "Molecular-level analyses of tannins in natural samples." American Chemical Society Annual Meeting, September 1996, Orlando, FL.

5. "Fluxes, elemental, and neutral carbohydrate compositions of sediment trap and sediment core materials from the equatorial Pacific." (poster) Ocean Sciences Meeting, February 1994, San Diego, CA

RESEARCH SKILLS

- Extensive experience with molecular-level analysis of tannin, neutral carbohydrates, and lignin, and interpretation of results. Familiar with amino acid analysis and interpretation.

- Elemental analysis of particulate samples, as well as dissolved organic carbon analysis.

- Extensive experience with capillary gas chromatography, including split and splitless injection.

- Extensive experience with gas chromatography/mass spectroscopy (Hewlett Packard benchtop quadrupole mass selective detector).

- Miscellaneous laboratory and field techniques, including sediment coring, net tows, deployment and retrieval of sediment traps, design and construction of wood and plexiglass lab and field equipment.