termined in situ nondestructively.

The ability of the technique to rapidly accumulate sequential scans with time adds an even more important dimension. When the changes in proton signal across the imaging plane are followed with time, this measures the changes in density, hence a direct, noninvasive drainage pattern for every point in the foam. We are no longer limited to simply measuring liquid as it exudes from the bottom of the foam. MRI analysis can measure precisely the drainage rates throughout the foam.

This advance in technology has several immediate benefits. One of the important technical limitations in the measurements of foam breakdown until now is the inability to characterize the processes of drainage and bubble collapse independently. The ability of the imaging technique to distinguish distinct breakdown behaviors can be seen from analyses of two quite different foams: beer and egg white (Figure 5). Egg white is typically more stable than beer foam, and measurement rates were altered accordingly. In addition to the drainage rates, the drainage profiles are quite different. This is most clearly seen in an expanded view of the top of the foams (Figure 6). Liquid drains from the egg foam but maintains a finite bubble structure even to very low densities. Alternatively, the beer foam collapses completely as soon as the drainage reaches a critical liquid content. We interpret this as a reflection of the relatively poor ability of beer to form stable bubble films compared to the protein-stabilized films of egg white.

The MRI system has proven in initial studies to be extremely valuable in the description of the dynamic behavior of aqueous foams. Densities, drainage rates, and structural collapse can be readily calculated from digitized signal intensity profiles generated noninvasively from actual foods. The development of new velocity measurement pulse sequences such as spin-echo two-dimensional Fourier transform flow-compensated experiments should provide the techniques necessary to measure velocities within the foam (Majors et al., 1988). This combined with new developments, which may allow for collection of an entire image within several milliseconds (Mansfield, 1987), would allow for a complete understanding of both the structure and drainage of a foam. The breadth of physical and chemical information accessible to this technique suggests its use in the analysis of a variety of colloidal systems we are currently pursuing.

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Tannins in Wood: Comparison of Different Estimation Methods[1]

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Different estimation methods of tannins—proanthocyanidins or hexahydroxydiphenoyl esters—have been compared and applied to polyphenols of various wood extracts. In the case of proanthocyanidins, reaction with vanillin in the presence of sulfuric acid is more sensitive than estimation by anthocyanidin formation. It is more specific than the assay by formaldehyde precipitation. Reaction with nitrous acid affords a selective estimation of hexahydroxydiphenoyl esters. These methods are applied to tannin estimation in wood extracts of 5 gymnosperms and 12 angiosperms and the values obtained compared to total phenols amounts.

Tannins are found in leaves, fruits, bark, and wood of most trees (Hillis, 1962; Scalbert et al., 1988). In woods such as quebracho (Hillis, 1962), they may account for over 20% of the dry matter. They are currently extracted from quebracho wood in South America and from chestnut wood in Europe and used in the leather industry.

Many of the tannin determination methods are based on their ability to form complexes with proteins (Deshpande et al., 1986; Hagerman, 1987). However, these methods do not take into account the structural heterogeneity of tannins. Indeed, tannins can be classified into two groups (Haslam, 1981): the proanthocyanidins (or condensed tannins) and the polyesters of gallic acid and (or) hexahydroxydiphenic acid (hydrolyzable tannins, re-

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spectively, gallo- and ellagitannins).

Tannins of the first group were identified in both gymnosperms and angiosperms. The occurrence of tannins of the second group (hydrolyzable tannins) is limited to some orders of dicotyledons. In these orders, the cooccurrence of both kinds of tannins in a same plant or in a same plant tissue is often observed (Hillis, 1987; Scalbert et al., 1988). Furthermore, they offer, according to their structure, different utilities (Roux et al., 1980) and give to the plant materials some of their characteristic properties, such as their durability (Hillis, 1987; Scalbert and Haslam, 1987).

In this work, different methods, known to afford the selective estimation of the two groups of tannins, are applied to wood extracts.

For the proanthocyanidins: formation of anthocyanidins by acid catalysis in butanol without (Bate-Smith, 1977) or with addition of ferrous ions (Govindarajan and Mathew, 1965) or ferric ions (Porter et al., 1986), condensation of vanillin with the phloroglucinol ring catalyzed by hydrochloric acid (Broadhurst and Jones, 1978) or sulfuric acid (Swain and Hillis, 1959), and precipitation by formaldehyde (Singleton, 1974).

For hexahydroxydiphenoyl esters: oxidation by nitrous acid (Bate-Smith, 1972).

The selectivity of these methods for each group of tannins is evaluated, and the influence of some parameters (solvents, temperature, reaction duration) is detailed.

The methods most suitable to woods are applied to the extracts of 5 gymnosperms and of 12 angiosperms. The amounts of tannins measured are compared to the amounts of total phenols.

EXPERIMENTAL PROCEDURES

Materials. Gallic acid hydrate (Fluka), (+)-catechin tetrahydrate (Serlabo), ellagic acid (Sarsynthèse or Fluka), cyanidin chloride, and delphinidin chloride (Sarsynthese) were used as standards without further purification.

Wood samples were air-dried after trees were felled.

Wood Extraction. Wood chips (0.5 g) are reduced to powder in a vibratory ball mill (Dangoumeau) for 5–15 min according to the toughness of the wood and then extracted with methanol/water (4:1, 3 × 10 mL). The methanol is removed under reduced pressure; the aqueous solution is acidified by 6 N hydrochloric acid (pH 2 ± 0.5) and extracted by freshly distilled diethyl ether (3 × 5 mL). Low molecular weight phenols like (+)-catechin (which would interfere with proanthocyanidins in their determination by reaction with vanillin) and ellagic acid (nearly insoluble in aqueous solutions) are thus separated from tannins. After the mixtures are dried with sodium sulfate, diethyl ether is removed and the ether-soluble material is dissolved in 1 mL of methanol. The volume of the residual aqueous solution is made to 10 mL.

Estimation of Total Phenols. Total phenols are estimated colorimetrically by the Folin–Ciocalteu method (Singleton and Rossi, 1965). The extracts are diluted 5–10 times by methanol (ether extracts) or water (aqueous extracts) to obtain a final absorbance below 0.5. A 2.5-mL portion of Folin–Ciocalteu reagent (Merck, diluted 10 times by water) and 2 mL of a sodium carbonate solution (75 g/L) are added to 0.5 mL of the diluted extract. Sodium carbonate is added 30 s to 8 min after the Folin–Ciocalteu reagent. The assay tubes are kept 5 min in a water bath at 50 °C and then transferred to cold water. If necessary, the mixture is centrifuged before absorbance is read at 760 nm. Results are expressed in gallic acid equivalents per amount of wood extracted. Calibration is achieved with gallic acid aqueous solutions (8–80 μg/mL).

Determination of Proanthocyanidins. Anthocyanidin formation is run as described by Govindarajan and Mathew (1965). A 5-mL portion of an acidic solution of ferrous sulfate (77 mg of $FeSO_4 \cdot 7H_2O$ dissolved in 500 mL of 2:3 HCl (d = 1.18)/n-BuOH) is added to 0.5 mL of aqueous extract. The tubes are loosely covered and placed in a water bath at 95 ± 0.5 °C during 15 min. Absorbance is read at the maximum around 530 nm. Results are expressed as cyanidin equivalents (ϵ_{mol} = 34 700; Fuleki and Francis, 1968) per amount of wood extracted. Each result is the mean of three measures.

Vanillin assays are carried out as recommended by Swain and Hillis (1959). A 2-mL portion of a freshly prepared solution of vanillin (1 g/100 mL) in 70% sulfuric acid is added to 1 mL of aqueous extract. The reaction proceeds in a water bath at 20 ± 0.5 °C and then in the temperature-controlled cells of the spectrophotometer. After exactly 15 min of reaction, the absorbance is read at the maximum around 500 nm. Results are expressed as (+)-catechin equivalents per amount of wood extracted. They are the average of three measurements.

Proanthocyanidins are also assayed by precipitation with formaldehyde (Singleton, 1974). A 2-mL portion of an aqueous solution of wood aqueous extract and phloroglucinol (0–5 mol equiv for one gallic acid equivalent of wood extract as measured by the Folin–Ciocalteu method) is mixed with 1 mL of a 2:5 hydrochloric acid (d = 1.18)/H_2O solution and 1 mL of an aqueous solution of formaldehyde (13 mL of 37% HCHO diluted to 100 mL by H_2O). After one night, the unprecipitated phenols are estimated in the supernatent by the Folin–Ciocalteu method. Phloroglucinol is always totally precipitated.

Determination of Hexahydroxydiphenoyl Esters (Ellagitannins). They are estimated by oxidation with nitrous acid according to Bate-Smith (1972). In a tube sealed with a Teflon-lined screw cap, 0.2 mL of aqueous extract (1 mL if the extract is weakly concentrated) is added to 1.8 mL of 1:1 methanol/water (1 mL of 9:1 methanol/water if the sample is weakly concentrated) and 0.16 mL of aqueous 6% acetic acid. Nitrogen is bubbled for 5–10 min and 0.16 mL of an aqueous solution of 6% sodium nitrite is added. Nitrogen is bubbled during a few more seconds, and the tube is sealed and kept 100 min in a water bath at 25 °C. The absorbance is read at the maximum around 590 nm. Results are expressed as 4,6-hexahydroxydiphenoyl-glucose (ϵ_{mol} = 2169; Bate-Smith, 1972) per amount of extracted wood.

Chromatographic Methods. Cyanidin and delphinidin are separated on a C-18 Novapak column (5 μm; Waters) with a linear gradient 0–100% B in 20 min; solvent A, H_2O/MeOH/H_3PO_4, 940:50:1; solvent B, MeOH/H_3PO_4, 990:1. Flow rate is 1.7 mL/min. Two wavelengths are selected for detection, 280 and 530 nm. Identification of the compounds is made by cochromatography with commercial standards. Retention times are the following: delphinidin, 13.9 min; cyanidin, 14.8 min; ellagic acid, 14.2 min.

Ellagic acid is also detected by high-performance thin-layer chromatography on cellulose (Merck) using 4:1:5 n-BuOH/H_2O/AcOH (upper layer) as eluant. It is detected under UV (±NH_3). R_f value is 0.5.

RESULTS AND DISCUSSION

Polyphenol Extraction. The most widely used solvents for polyphenol extraction are methanol/water or acetone/water mixtures. Extraction of *Quercus robur* L. heartwood with 4:1 methanol/water at room temperature or with 89:11 acetone/water under reflux provided similar amounts of polyphenols. Absorbances at 275 nm calculated for 1 g of wood extracted by 1 L of solvent (E(1%) were, respectively, 30 and 28. Furthermore, when the residue of aqueous methanol extraction was extracted by 89:11 acetone/water under reflux, it provided very little polyphenols (E(1%) = 1.5). It can thus be concluded that ellagitannins of *Q. robur* L. heartwood (Table I) are similarly extracted by cold aqueous methanol or by hot aqueous acetone. Similar results were obtained with proanthocyanidins of *Salix* leaves (Julkunen-Tiito, 1985) or proanthocyanidins of Ponderosa pine wood (Anderson, 1946).

Diethyl ether extraction allowed the quantitative extraction of (+)-catechin and ellagic acid from the acidified aqueous extract, as checked by HPLC. Geissman and Dittmar (1965) used the same fractionation procedure to remove the bulk of catechins. On the other hand, the dimers of proanthocyanidins they identified remained in the aqueous phase.

Table I. Polyphenol Contents of Various Woods and Bark (mg/g)

	aqueous extract				ether extract	
		proanthocyanidins				
	total phenols	BuOH/HCl/Fe(II)	vanillin/H_2SO_4	ellagitannins	total phenols	ellagic acid
		Woods				
		Gymnosperms				
Pinus sylvestris L.	0.8	a	0.08	nd[b]	5.9	-
Cedrus atlantica Manetti	2.4	a	0.33	nd	5.8	-
Pseudotsuga menziesii (Mirb.)	2.0	a	0.13	nd	11.6	-
Picea sitchensis (Bong.) Carr.	1.6	a	0.08	nd	1.7	-
Tsuga heterophylla (Raf.) Sarg.	2.4	a	0.09	nd	1.9	-
		Angiosperms				
Ulmus campestris Mill.	1.0	a	0.08	nd	0.2	-
Populus x euramericana cv 1214	1.0	a	0.06	nd	0.5	-
Juglans regia L.	15.5	a	0.23	9.7	1.0	+
Quercus petraea (Matt.) Liebl.	39.3	0.28	0.28	47.2	1.5	+
Quercus robur L.	62.6	0.29	0.29	75.4	2.1	+
Quercus rubra L.	24.5	0.19	0.27	5.3	10.1	+
Castanea sativa Mill.	53.4	0.16	0.18	60.4	2.7	+
Fagus sylvatica L.	1.6	0.12	0.17	nd	0.3	-
Carpinus betulus L.	4.5	a	0.22	nd	1.4	-
Prunus avium L.	14.6	0.86	4.8	nd	14.1	-
Eucalyptus globulus Labill.	24.0	a	0.24	21.3	2.6	+
Fraxinus excelsior L.	13.7	a	0.28	nd	1.1	-
		Bark				
Quercus robur L.	25.2	2.3	7.3	9.5	3.5	+

[a] Values less than 0.12 mg/g. [b] nd = not detected.

Determination of Total Phenols. The Folin–Ciocalteu method has first been tested on model compounds. Methanol (0.5 mL) in the reaction mixture sometimes induces the formation of a precipitate, as was observed with gallic acid, ellagic acid, or (+)-catechin. It is thus advisable to introduce the sample in a water solution when this is possible. When compounds to be estimated are poorly soluble in water but soluble in methanol, as with ellagic acid, the color reaction is run in centrifugation tubes and the precipitate eliminated before reading the absorbance. The reproducibility of the measurement is not affected.

The molar absorbances obtained for gallic acid and (+)-catechin are, respectively, 25.2×10^3 and 37.3×10^3. They are in good agreement with those published by Singleton (1974). On the other hand, for ellagic acid, the molar absorbance measured (39.5×10^3) is nearly twice the one published by this same author, whatever the commercial origin of the ellagic acid. The value presently found is however in good agreement with the stoichiometry described by Singleton (1974): absorbance of about 20×10^3/phenolic residue in the molecule.

The standard curve established for gallic acid (Figure 1) shows that the Beer–Lambert law is followed for absorbances inferior to 0.5.

Determination of Proanthocyanidins by Anthocyanidin Formation. The original method, as described by Bate-Smith (1977), lacks sensitivity. Different authors have tried to improve it by addition of ferrous (Govindarajan and Mathew, 1965) or ferric (Porter et al., 1986) ions. They reported an increase of sensitivity by a factor of 2. We compared the three methods on wood extracts without observing such important differences of absorbance. However, the shape of the spectra differed significantly (Figure 2): The maximum around 550 nm is more pronounced without metal ions (Figure 2a) and even more with ferrous ions (Figure 2b).

The influence of some parameters relative to the method using ferrous ions was thus detailed. Reproducibility is improved if the temperature is rigorously controlled.

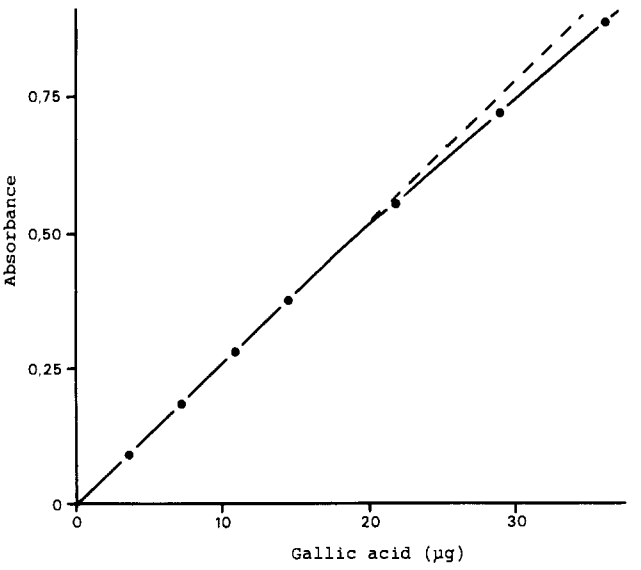

Figure 1. Standard curve for determination of total phenols by the Folin–Ciocalteu method.

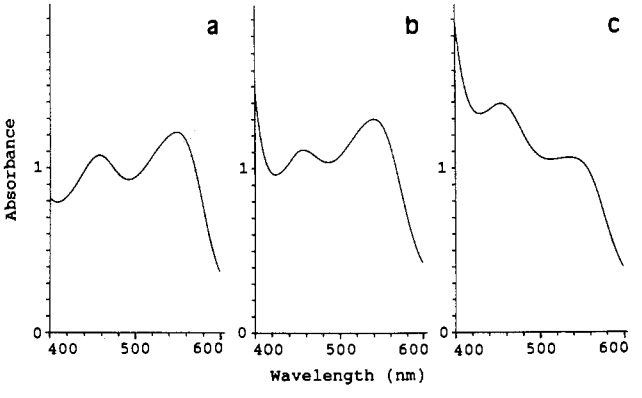

Figure 2. Absorption spectra of aqueous extracts obtained from the bark of *Q. robur* L. and treated by acidified butanol (a) with no addition of salts, (b) with ferrous salt, and (c) with ferric salt.

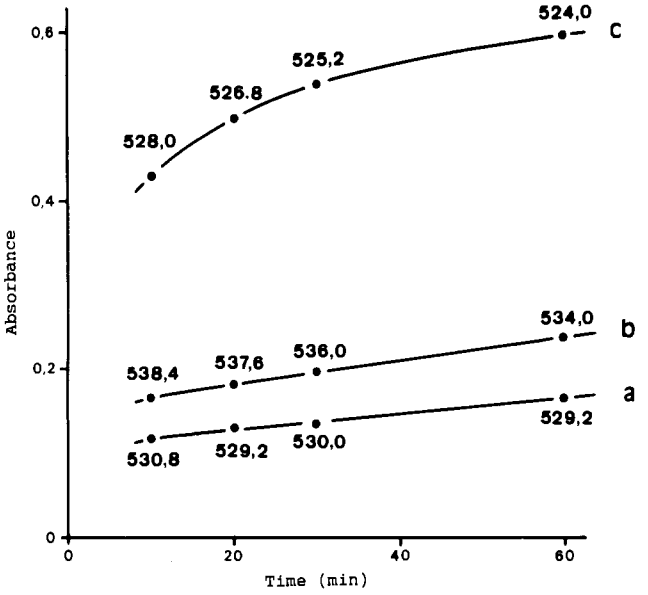

Figure 3. Dependence of absorbance on the reaction duration of aqueous wood extracts with acidified butanol containing ferrous salt: (a) *Q. rubra* L.; (b) *Q. petraea* (Matt.) Liebl.; (c) *P. avium* L. Figures correspond to the wavelength at the absorption maximum.

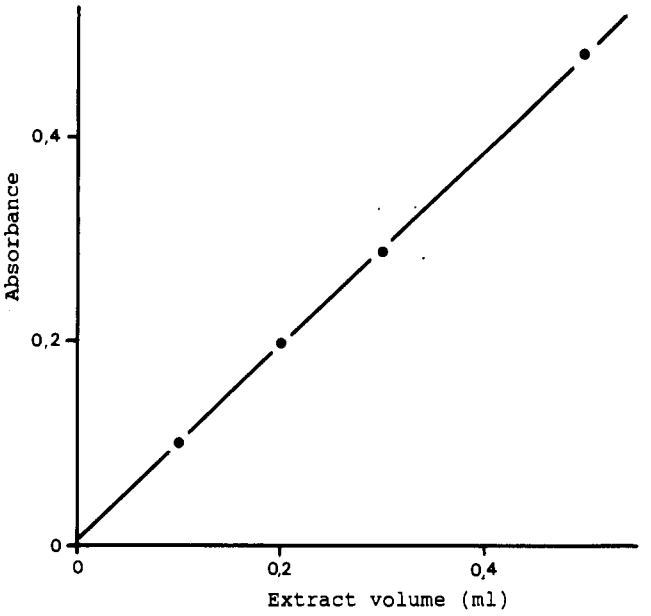

Figure 4. Dependence of absorbance on the concentration of *P. avium* L. aqueous extract treated by acidified butanol containing ferrous salt.

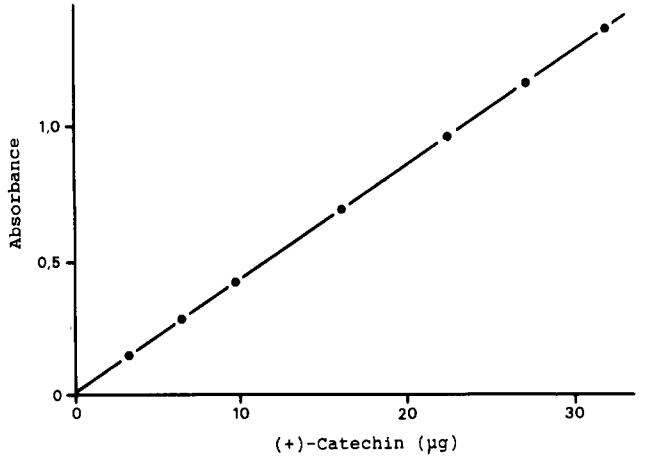

Figure 5. Standard curve for determination of proanthocyanidins by condensation with vanillin in sulfuric acid.

Kinetic study with different wood extracts (Figure 3) shows that the absorbance goes on increasing, even after 60 min. At the same time, the wavelength at the maximum of absorption decreases, sometimes by several nanometers; for several of the different woods tested, the maximum progressively becomes a shoulder. Analysis of the reaction products obtained from woods of *Q. robur* L. and *Quercus rubra* L. by HPLC showed that anthocyanidins (cyanidin and delphinidin were the main pigments formed) reach a maximum after 15 min and that their amounts keep stable during at least the following 2 h of reaction. Some other compounds with conjugated aromatic rings (like ellagic acid in *Q. robur* wood) are more slowly released in the reaction medium and accumulate even after 60 min. These compounds contribute to an increase in the absorbance at 550 nm and make this maximum ill-defined. For this reason, it is preferred to read the absorbance after exactly 15 min of reaction when the maximum is more pronounced.

The standard curve is shown on Figure 4. For absorbances less than 0.07, the absorption band is usually too weak to form an absorption maximum and give reliable results. Only the woods of Fagaceae species (*Quercus* sp., *Castanea sativa* Mill., *Fagus sylvatica* L.) and of *Prunus avium* L. give a well-defined maximum (Table I).

The values calculated with cyanidin as a standard (Table I) are underestimated because the yields of proanthocyanidins degraded into anthocyanidins usually do not exceed 50–60% (Porter et al., 1986). These yields are even lower for proanthocyanidins with shorter chains, because of the increase in proportion of terminal units, which do not form anthocyanidins. For a dimer, for example, the calculated values would thus represent only one-fourth of the actual proanthocyanidin amount.

Determination of Proanthocyanidins by Condensation with Vanillin. The reaction is run in an acid. Previous investigators have used accordingly hydrochloric acid (Broadhurst and Jones, 1978) or sulfuric acid (Swain and Hillis, 1959). The method with sulfuric acid gave absorbances 10 and 30 times higher with, respectively, (+)-catechin and a wood extract of *Q. robur*. This is probably explained by the instability of the colored carbonium ions in hydrochloric acid (Swain and Hillis, 1959). Furthermore, the method with sulfuric acid gives a linear standard curve with (+)-catechin (Figure 5), which was not observed for the method using hydrochloric acid (Price et al., 1978).

The method with sulfuric acid has been optimized. With the (+)-catechin, it was observed that a maximum absorbance is reached after 7 min of reaction (Figure 6). With wood extracts, the absorbance still increases after 100 min. The reaction duration has been fixed to exactly 15 min.

This method can be compared to the one based on the formation of anthocyanidins (Table I). With equal extract concentration in the reaction mixture, the vanillin method gives absorbances 10–20 times higher, and in all cases, the absorbance maximum is well-defined. The vanillin method is thus more suitable for samples containing low amounts of proanthocyanidins. Table I shows however that the values obtained by the two methods are not always comparable. This is probably explained by structural differences of proanthocyanidins in each of the wood samples, related to their degree of polymerization, type of intermonomeric linkage, and nature of monomeric units (Porter et al., 1986; Goldstein and Swain, 1963; Butler et al., 1982).

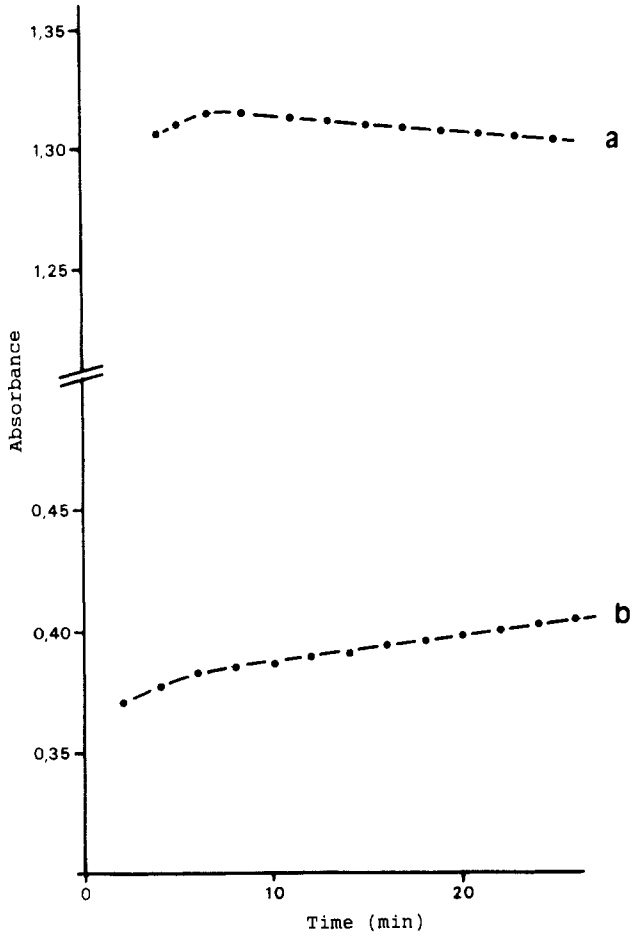

Figure 6. Dependence of absorbance on reaction duration of vanillin in sulfuric acid with (a) (+)-catechin and (b) aqueous extract of *Q. petraea* (Matt.) Liebl. wood.

Determination of Proanthocyanidins by Precipitation with Formaldehyde. Singleton (1974) proposed a method of proanthocyanidin determination by selective precipitation with formaldehyde. He suggested to add, if necessary, up to 5 mol equiv of phloroglucinol to improve the precipitation efficiency. This method has been applied to a wood extract of *Q. robur*, to gallic acid, and to (+)-catechin.

With wood extract, without addition of phloroglucinol, none of the tannins are precipitated. With addition of phloroglucinol, some tannins are precipitated and the precipitated fraction increases with the concentration of phloroglucinol. With 20 equiv of phloroglucinol, 90% of the oak polyphenols are precipitated. This value is far in excess of the amount of proanthocyanidins present in the extract (Table I).

With (+)-catechin, 89% precipitate without phloroglucinol and 100% with 5 equiv of phloroglucinol. Gallic acid does not precipitate without addition of phloroglucinol, whereas 46% precipitate with 5 equiv.

These observations show that the formaldehyde does not selectively precipitate proanthocyanidins and polyphenols containing phloroglucinol rings; it also precipitates polyphenols with pyrocatechol rings, such as gallic acid or the oak wood ellagitannins. This method cannot thus be applied to determination of proanthocyanidins in wood extracts. The reagent used here is similar to the Stiasny reagent (Roberts, 1962). It implies that the widely used Stiasny method cannot provide reliable measurements of condensed tannins.

Determination of Hexahydroxydiphenoyl Esters (Ellagitannins). The only selective method of ellagitannin determination is based on their oxidation by nitrous acid which, in the absence of oxygen, gives a blue color (Bate-Smith, 1972). The maximum of absorbance is reached after about 100 min (Figure 7). The color remains stable during several hours. Standardization achieved with a wood extract of *Q. robur* shows that a linear curve is obtained for absorbance values as high as 2. For absorbances less than 0.2, the reliability of the measure is reduced and it is recommended to concentrate the sample.

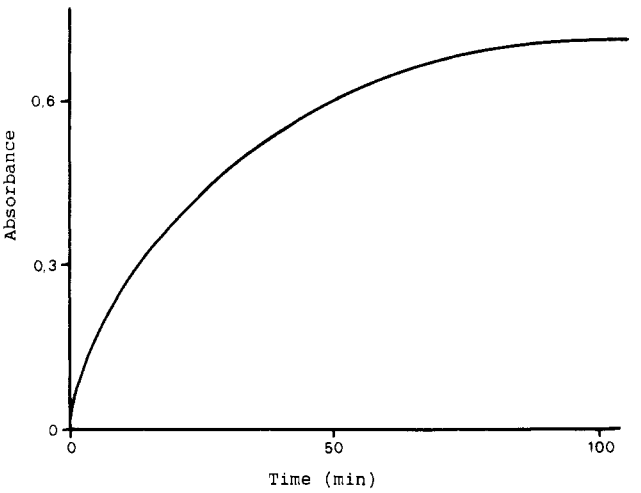

Figure 7. Dependence of absorbance on reaction duration of nitrous acid with aqueous extract of *Q. petraea* (Matt.) Liebl. wood.

All the woods giving the blue color with nitrous acid also showed the presence of ellagic acid in their diethyl ether extract (Table I). On the other hand, in all samples not showing this blue color, no traces of ellagic acid could be detected. The reaction is thus specific of the ellagitannins.

Determination of Polyphenols in the Extracts of Different Woods. Polyphenols have been estimated in the diethyl ether and aqueous extracts of 5 woods of gymnosperms and of 12 woods of angiosperms (Table I). A sample of bark from *Q. robur*, very rich in proanthocyanidins, was also analyzed for comparison.

Gymnosperm woods contain few water-soluble phenols, but they are relatively rich in diethyl ether soluble phenols, particularly those of *Pinus sylvestris* L., *Cedrus atlantica* Manetti, and *Pseudotsuga menziesii* (Mirb.).

They contain no ellagitannins and few proanthocyanidins. Literature data, in agreement with the present ones, have shown that the main phenols identified in gymnosperm woods are lignans, stilbenes, or simple phenols, which are usually soluble in diethyl ether (Fengel and Wegener, 1984).

The angiosperm woods studied are generally richer in water-soluble phenols than those of gymnosperms. For *Q. robur* and *Quercus petraea* (Matt.) Liebl., *C. sativa*, and *Eucalyptus globulus* Labill., these phenols are mainly ellagitannins. Ellagitannins are also found in significant amounts in *Juglans regia* L. and *Q. rubra*. They were not detected in the woods of the other species analyzed.

The amounts of proanthocyanidins present in the woods analyzed are low, except for *P. avium* where they are in concentration nearly as high as in *Q. robur* bark. The ratios of the values obtained by both estimation methods tend to indicate that the proanthocyanidins of *P. avium* wood have a lower molecular weight than those of *Q. robur* bark, as the ratio (BuOH + HCl) to vanillin decreases with molecular weight (Goldstein and Swain, 1963). Contrary to the gymnosperms, and with exception of *Q. rubra* and *P. avium* woods, in angiosperms, phenols soluble in diethyl

ether are present in much lower amounts than the ones soluble in water.

The application of different methods of polyphenol estimation to woods has shown that reaction of vanillin in sulfuric acid and oxidation with nitrous acid are the most suitable methods for proanthocyanidin and ellagitannin determination, respectively. Both provide selective and sensitive assays for these two groups of tannins.

The occurrence of gallotannins, although seldom reported in woods, cannot be excluded (Seikel et al., 1971). A reagent specific of gallotannins, potassium iodate, was proposed by Bate-Smith (1977). Although it can be used reliably to detect gallotannins on paper chromatograms, giving a characteristic pink color, we could never observe this color with crude wood extracts. This could be explained by either low amounts of gallotannins in the samples, or by the presence of other compounds that would interfere with the reaction. Gallotannins have also been assayed by acid hydrolysis and colorimetric determination of the resulting gallic acid (Inoue and Hagerman, 1988). However, this method is unable to differentiate true gallotannins from an ellagitannin containing one or several galloyl residues or from other galloyl esters and was not used in this study.

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