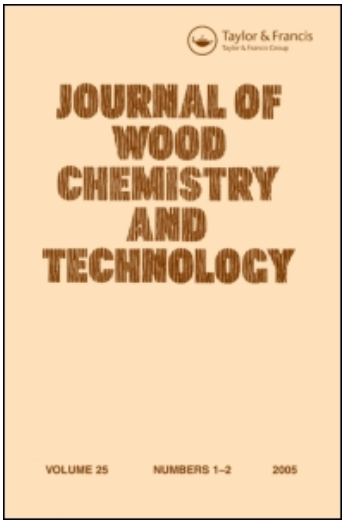

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ANALYSIS OF POLYPHENOL-DERIVED AROMATICS IN EUCALYPT WOODS

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ABSTRACT

Methods for the determination of polyphenols in wood by chemically degrading the wood and measuring the reaction products by gas chromatography (GC) were explored. A four-step procedure, based on permanganate oxidation of ethylated wood, allowed the estimation of polyflavonoids and related polyphenols as the methyl ethoxybenzoates IVa-c. *Eucalyptus marginata* heartwood gave methyl 3,4-diethoxybenzoate (IVb) in 2.7% yield, which was the highest value obtained from the eucalypt woods tested. Estimation of hydrolysable tannins as the methyl esters of tri-O-ethyl gallic acid and 4,4',5,5',6,6'-hexaethoxydiphenic acid, formed by methylation of the alkaline hydrolysis products of ethylated wood with diazomethane, was attempted. Applied to eucalypt woods, the method gave the methyl gallate in yields of up to 0.6%, but the diphenic acid was obtained, in trace amounts, only from wood which had been ethylated for an extended period. The two methods are more suited to the determination of specific polyphenolic structures rather than the total polyphenol content of woods. A second approach to the analysis of diphenic acid moieties in ellagitannins based on alkaline decarboxylation to 2,2',3,3',4,4'-hexahydroxybiphenyl (V) and estimation of the hexahydroxybiphenyl (HHBP) by GC of its silylated derivative, was unsuccessful because the HHBP reacted with wood components.

INTRODUCTION

Polyphenolic extractives in eucalypt woods occur typically as the hydrolysable tannins, ellagitannins and gallotannins (sugar

esters of hexahydroxydiphenic acid (Ia) and gallic acid), condensed tannins (polyflavonoid polymers, e.g. with units of structure IIa), ellagic acid (IIIa), gallic acid and stilbenes.[1] Methods are available for determining some of these substances in wood, and in all cases these rely on examining the extracts. However, in certain *Eucalyptus* woods, the polyphenols cannot be extracted with neutral solvents, and boiling aqueous sodium hydroxide is often required for their complete removal.[2] Under these alkaline conditions, appreciable amounts of lignin and hemicelluloses may be solubilized, particularly from the heartwoods of old trees, and the polyphenols undergo chemical transformations. Thus the amounts and nature of the extracts cannot be used for determining the polyphenol content of these woods.

The method currently used for estimating the polyphenol content of *Eucalyptus* woods was developed by Cohen,[3] who required a correction for the contribution of polyphenols to the Klason lignin analysis. Bland and Menshun[4] extended the procedure to include the acid-soluble components of polyphenols and also those of lignin. Cohen's method is based on the premise that polyphenols do not bear methoxyl substituents, and thus the methoxyl content of Klason lignin from alkali-extracted wood represents the "true" lignin methoxyl content. The corrected Klason lignin and polyphenol contents are then obtained by the expressions below.

$$\% \text{ corrected lignin} = \frac{\% \text{ OMe in Klason lignin from unextr. wood}}{\% \text{ OMe in Klason lignin from extr. wood}} \times \% \text{ Klason lignin from unextr. wood}$$

$$\% \text{ polyphenol} = \% \text{ Klason lignin from unextr. wood} - \% \text{ corrected lignin}$$

An assumption in the method is that the lignin in both the extracted and alkali-extracted woods has the same methoxyl content. However, this assumption must be treated with caution, as

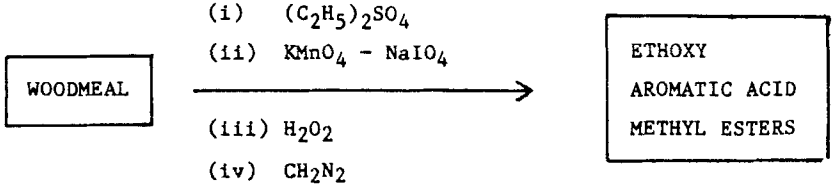
lignin initially extracted from *E. regnans* wood with alkali has been shown to be richer in guaiacyl relative to syringyl groups than the original lignin.[5] It is also possible that polyphenols condense with lignin during the alkaline extraction. Methods for the detection and direct estimation of polyphenols in wood were therefore sought. We present new approaches to the analysis of wood polyphenols which involve degrading ethylated wood either by oxidation with permanganate or by treatment with alkali.

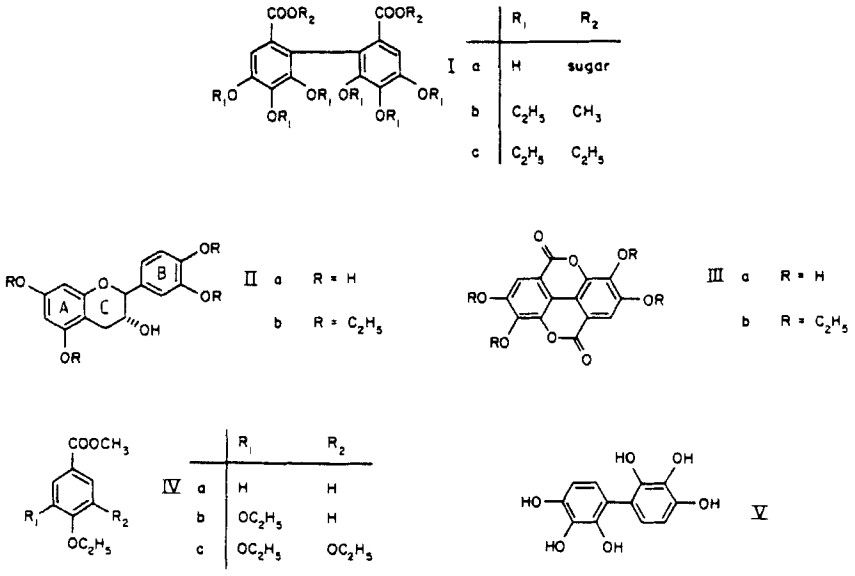
RESULTS AND DISCUSSION

Permanganate Oxidation of Wood

When wood containing polyphenols is oxidized, the polyphenol-derived products can usually be distinguished from those derived from lignin by the absence of methoxyl substituents on the aromatic rings of the former. Exceptions are the *p*-hydroxyphenyl moiety, which may be present in lignin as well as in polyphenolic extractives, and minor amounts of methylated hydroxystilbenes and methylated ellagic acids.

Initial experiments showed that alkaline nitrobenzene and alkaline cupric oxide oxidation at 170°C were not appropriate methods for the analysis of polyphenols in wood, as protocatechualdehyde and protocatechuic acid, typical of the expected oxidation products, were not stable under the reaction conditions. Application of these oxidants to ethylated polyphenols was also not a useful approach, because the tetraethylcatechin IIb, a model for ethylated proanthocyanidins in wood, did not yield products identifiable by GC (gas chromatography).





SCHEME 1. Permanganate oxidation procedure

The adopted method (Scheme 1) is a modification of the permanganate oxidation procedure which has been used for lignin structural studies[6,7] and recently applied to the structure of lignin in kraft pulps.[8] The reaction products of interest were the methyl ethoxybenzoates IVa-c which, with the exception of the 4-ethoxybenzoate IVa, would be derived exclusively from the polyphenols.

The initial step in the oxidation procedure outlined in Scheme 1 involves ethylation with diethylsulfate at pH 11 for 24 h. The possible extraction of polyphenolic material from the eucalypt woodmeals during the ethylation was studied by examination of the filtrates from the reaction mixtures by ultraviolet (UV) spectroscopy. The highest absorbance at 260 nm was obtained from the filtrate of the ethylation of unextracted *E. diversicolor* heartwood, and if this was entirely due to gallic acid residues, it

would correspond to a gallic acid content of 0.1%. Thus the loss of polyphenols during ethylation of the woodmeals was insignificant.

The stability of the ester linkages in hydrolysable tannins under ethylation conditions was tested by monitoring the reaction of the tannin model compound, *n*-propyl gallate with 0.5M sodium carbonate solution (pH 11.0) by proton magnetic resonance (PMR) spectroscopy. After 24 h, the PMR spectrum contained a triplet centered at 3.56 δ due to the protons on C-1 of *n*-propanol, in addition to the triplet at 4.22 δ assigned to the protons at C-1 of the propyl group of the ester. Integration of these signals showed that 30% hydrolysis had occurred. Thus although there was no significant loss of polyphenols in the samples examined, some ester hydrolysis in the hydrolysable tannins would be expected to occur during the ethylation.

Oxidation of the tetraethylcatechin IIb by the above procedure gave the methyl benzoate IVb in 86% yield. When the hydrogen peroxide step was omitted, the yield of IVb was reduced to 55%. Compound IVb originates from the B-ring of the ethylated catechin, and no product deriving from the A-ring was identified. This is probably because the C-ring oxygen atom released as a phenoxide ion during the alkaline permanganate treatment would allow further oxidation to take place, leading to opening of the A-ring.

No product was identified from the permanganate oxidation of the tetraethylellagic acid IIIb. The phenoxide groups formed by ring opening of the lactone rings of IIIb under the alkaline oxidation conditions would be the site of further oxidative attack.

When various ethylated eucalypt woodmeals were subjected to the permanganate oxidation procedure, a series of substituted methyl benzoates were obtained. The gas chromatogram of the oxidation products of *E. marginata* heartwood is shown in Figure 1.

The amounts of the ethoxybenzoates IVa–c obtained after permanganate oxidation of ethylated eucalypt woodmeals are given in Table 1. Of the woodmeals from the four eucalypt species, *E.*

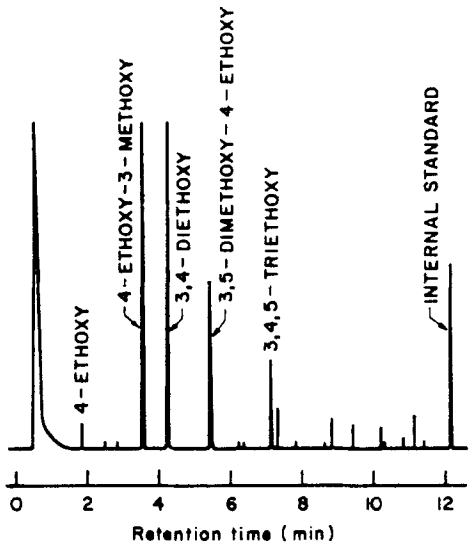


FIGURE 1. Gas chromatogram of substituted methyl benzoate products obtained from permanganate oxidation of ethylated *E. marginata* heartwood

regnans heartwood gave no trace of the diethoxybenzoate IVb, whereas the other species gave 1-3% on oxidation. *E. regnans* is representative of a pale-brown heartwood species and the other three species form part of the red-coloured group of species.[1] The latter group are known to contain proanthocyanidins,[1,9,10] and compound IVb probably derives from the B-ring of catechin units (IIa) of procyanidins. The ethoxybenzoate IVa could arise from lignin, polyflavonoids, e.g. propelargonidins, or hydroxystilbenes. Compound IVc, the gallic acid derivative, may derive from either the polyflavonoids, e.g. prodelphinidins, or the hydrolysable tannins.

The diphenic acid derivative Ib, an expected product of ethylated ellagitannins, was not detected in the reaction mixtures from the ethylated woodmeals. This could be due to conformational constraints restricting access of the diethylsulfate to the 2,2' hydroxyl groups of the Ia moieties in wood, particularly in the ellagitannins in which both carboxyl groups of the diphenic acid are

TABLE 1

Permanganate Oxidation of Woodmeals

Wood sample	% yield (w/w) of methyl benzoate derivative		
	IVa	IVb	IVc
E. regnans			
heartwood	0.1	-	trace
E. tetrodonta			
heartwood	0.1	1.1	0.3
E. diversicolor			
heartwood	0.3	2.2	0.6
boiling water extracted	0.5	2.0	trace
acetone-methanol extracted	0.3	2.0	trace
cold alkali extracted	-	0.2	-
boiling alkali extracted	trace	0.2	-
E. diversicolor			
sapwood	0.2	1.2	0.1
boiling water extracted	0.2	0.8	trace
acetone-methanol extracted	0.1	0.8	-
cold alkali extracted	-	0.2	-
boiling alkali extracted	trace	0.1	-
E. marginata			
heartwood	0.2	2.7	0.7
cold alkali extracted	0.1	0.9	0.2
boiling alkali extracted	trace	0.2	-
E. marginata			
sapwood	0.1	1.2	1.5
boiling water extracted	0.1	0.8	0.4

esterified with the same sugar unit. Also, hydrolysis/lactonization of the diphenic acid esters to ellagic acid could be a competing reaction to ethylation.

The wood components which give rise to ethoxybenzoates IVa and IVb are only extracted to a limited extent with boiling water or acetone-methanol, and require an alkaline extraction for their

TABLE 2

Alkaline Hydrolysis Products of Ethylated Woodmeals

Wood sample	% yield (w/w) of methyl benzoate derivative IVb	IVc
E. regnans sapwood	trace	trace
E. regnans heartwood	trace	trace
E. diversicolor sapwood	0.2	0.1
E. diversicolor heartwood	0.1	0.3
E. marginata sapwood	0.1	0.6
E. marginata heartwood	0.1	0.1
E. tetrodonta heartwood	trace	0.4

removal (Table 1). This extraction behaviour parallels that of the polyflavonoids. The substances which yield the gallic acid derivative IVc are removed with boiling water or organic solvents, and are thus more likely to be gallotannins, ellagitannins or gallic acid itself rather than prodelphinidins.

The amounts of methyl benzoates listed in Table 1 may be used as a measure of the polyphenol content of the woods. Assuming the ethylation of phenolic hydroxyl groups in the woodmeal samples to be complete and an 85% conversion of ethylated catechin moieties to compound IVb, the content of proanthocyanidins based on catechin is obtained by multiplying the yield of IVb by a factor of 1.5. Similar considerations for the amounts of the triethoxybenzoate IVc would be more difficult, as its genesis is less certain.

Alkaline Degradation of Polyphenols

1. Alkaline Hydrolysis of Ethylated Wood

Estimation of the aromatic groups in the hydrolysable tannins in wood was attempted by a method in which ethylated woodmeals were

treated with boiling 0.1M sodium hydroxide, and the liberated acids after methylation were measured as their methyl esters by GC. The diphenic acid derivative Ib and the methyl triethylgallate IVc would be the expected products from the ethylated ellagitannins and gallotannins.

The eucalypt wood samples subjected to the above procedure gave the ethoxybenzoates IVb and IVc (Table 2). The ellagitannin-derived diphenic acid Ib was only detected from wood which had been ethylated for an extended period (3 days). The detection of the diethoxybenzoate IVb was unexpected, as esters of protocatechuic acid are not known structural features of lignin or polyphenols. The triethylgallate IVc may derive either from tannins or from free gallic acid in the woods. The higher yield of IVc from the sapwood than the heartwood of *E. marginata* was unusual, as heartwoods normally contain more polyphenols than sapwoods.[2]

When an alkali-extracted ethylated wood sample was spiked with the esters IVc and Ic and subjected to the hydrolysis-extraction-methylation procedure, compounds IVc and Ib were recovered in yields of 85 and 94%, respectively. Thus the method may be used for determining the aromatic residues in ethylated hydrolysable tannins providing the ethylation step could be accomplished satisfactorily.

2. Alkaline Treatment of Ellagitannins in Wood

An attempt was made to estimate the hexahydroxydiphenic acid moieties in ellagitannins as the HHBP (hexahydroxybiphenyl) (V), based on the observation of Hemingway and Hillis[11] that ellagic acid treated with 0.5M sodium hydroxide at 160°C gave the decarboxylated product V. The present approach would allow the measurement of the combined content of ellagic acid and the diphenic acid groups in ellagitannins as the HHBP (V). The method would complement that of Hillis *et al.*,[12] in which the free ellagic acid content of wood was determined by GC of its silylated derivative.

Reaction of ellagic acid with 0.5M potassium hydroxide at 105°C for 48 h gave the HHBP (V) (estimated by GC as the trimethylsilyl

ether) in 76% yield. However, when various eucalypt woods were digested with alkali at 105°C or 170°C, neither the HHBP (V) nor pyrogallol, which could arise from decarboxylation of gallic acid, were identified as products. Furthermore, when the decarboxylation reaction of ellagic acid was carried out in the presence of eucalypt woods or alkaline extracts of these woods, the yield of V was reduced to *ca.* 5%. As the eucalypt woods would be expected to contain ellagitannins, the HHBP (V) formed by hydrolysis/decarboxylation probably undergoes alkali-catalysed condensation reactions with lignin extracted from the woods. The approach for analysis of ellagitannins was not pursued further.

Concluding Remarks

None of the proposed methods were applicable to the analysis of hexahydroxydiphenic acid groups of ellagitannins, nor was there a single method which gave values for the total polyphenolic content of wood. Bland and Menshun obtained values of 1.7-18.2% for the content of polyphenols in nine eucalypt woods by the Klason lignin methoxyl content method.[4] Application of the method to the woods examined in the present study gave a similar range of values for the polyphenol contents; 1.4% for *E. regnans* heartwood to 17.5% for the heartwood of *E. marginata*.[13] These values are generally significantly higher than the combined values for polyphenol-derived aromatics obtained in the present study. This is at least partly due to the lack of methods for estimation of hexahydroxydiphenic acid groups in wood. Thus the procedures discussed in the present study are more valuable for estimating specific polyphenol groups, e.g. the B-ring hydroxylation pattern of proanthocyanidins, than for determining the polyphenol content of wood.

EXPERIMENTAL

Air-dried woodchip samples were Wiley milled to pass through a screen with 1 mm apertures. Four different methods were used to extract the woodmeals; (a) 0.1M sodium hydroxide at 20°C for 24 h

(cold alkali), (b) boiling water for 1 h, (c) boiling 0.1M sodium hydroxide for 1 h, and (d) acetone and methanol sequentially for 18 h in a Soxhlet extractor.

Chemical Syntheses

The methyl ethoxybenzoates IVa-c were prepared by ethylation of *p*-hydroxybenzoic, protocatechuic and gallic acids with diethylsulfate, and subsequent esterification with boron trifluoride-methanol.[14]

5,7,3',4'-Tetra-O-ethylcatechin (IIb) was obtained by ethylation of (+)-catechin (Koch-Light Laboratories Ltd) with diethylsulfate at pH 11 and crystallized from aqueous ethanol as *needles*, m.p. 92-3°C Found: C, 68.7; H, 7.2; OC_2H_5, 41.8. $C_{23}H_{30}O_6$ requires C, 68.6; H, 7.5 and OC_2H_5, 44.8%.

3,3',4,4'-Tetra-O-ethylellagic acid (IIIc) was the product of ethylation of ellagic acid (Fluka, pract.) with diethylsulfate at pH 11, and was obtained from dimethylsulfoxide as *needles*, m.p. 289-90°C Found: C, 63.8; H, 5.5; $C_{22}H_{22}O_8$ requires C, 63.8; H, 5.3%.

4,4',5,5',6,6'-Hexaethoxydiphenic acid was prepared from compound IIIc by dissolution in warm 5M sodium hydroxide and ethylation with diethylsulfate. Crystallization from aqueous ethanol gave the diacid as *plates*, m.p. 218-9°C Found: C, 61.8; H, 7.1; $C_{26}H_{34}O_{10}$ requires C, 61.5; H, 6.8%.

The dimethyl ester Ib crystallized from aqueous ethanol as *plates*, m.p. 74-5°C Found: C, 62.4; H, 6.8. $C_{28}H_{38}O_{10}$ requires C, 62.9; H, 7.2%.

The diethyl ester Ic was obtained from aqueous methanol as *plates*, m.p. 75-6°C Found: C, 64.1; H, 7.7. $C_{30}H_{42}O_{10}$ requires C, 64.0; H, 7.5%.

2,2',3,3',4,4'-Hexahydroxybiphenyl (V) was prepared by the decarboxylation of ellagic acid. A suspension of ellagic acid (1.0 g) in 4M sodium hydroxide (10 mL) was sealed under nitrogen in a 20

mL steel autoclave and was kept at 170°C for 2 h in a rocking air bath. After cooling, the contents of the autoclave were immediately acidified to pH 2 with 5M hydrochloric acid and the precipitate which formed on cooling (405 mg) was recrystallized from water and gave colourless needles of the HHBP (V), m.p. > 350°C, hexa-O-methyl ether, m.p. 123-4°C (lit.[15] m.p. 124-5°C).

The gas chromatography standards, tetramethylpyromellitate and disyringylmethane, were obtained by methylation of pyromellitic acid with boron trifluoride-methanol,[14] and reaction of 2,6-dimethoxyphenol with formaldehyde in alkali,[16] respectively.

Ethylation of Woodmeals[6]

Woodmeal (1.0 g) was suspended in a mixture of 30 mL methanol-dimethoxyethane-water (21:21:18, v/v/v) adjusted to pH 11.0 with 2M sodium hydroxide, and 2 mL diethylsulfate was added. The mixture was kept under nitrogen at 20°C for 24 h and the pH was maintained at 11.0 by periodic addition of 2M sodium hydroxide with the aid of an automatic titrimeter. After acidification with 1M hydrochloric acid, the mixture was kept at 80°C for 30 min. The mixture was cooled, filtered and the ethylated woodmeal was washed with cold water and dried. The filtrates were made up to 150 mL with water, and the UV absorbance at 260 nm was measured.

Additional woodmeal samples were ethylated in the manner above for 24 h at pH 11.0, and for a further 48 h period at pH 11.5.

Permanganate Oxidations[6]

(a) A suspension of ethylated woodmeal (200 mg) in *t*-butanol (10 mL), 2M sodium hydroxide (10 mL), 0.03M potassium permanganate (20 mL) and 0.12M sodium periodate (50 mL) was shaken and then heated under reflux for 6 h. Additional solid potassium permanganate was added periodically so that the mixture remained purple in colour. After cooling, ethanol (10 mL) was added and the mixture was left to stand for 18 h. The treated woodmeal was

collected by filtration and washed with 1% sodium carbonate solution.

(b) The combined filtrate and washings were acidified to pH 4.0, concentrated to *ca.* 30 mL in a rotary evaporator, and after addition of sodium carbonate (900 mg) and 30% hydrogen peroxide (5 ml), kept at 50°C for 10 min. The solution was cooled, and manganese dioxide (100 mg) was added. After the evolution of gas ceased, the mixture was filtered and the residue washed with 1% sodium carbonate. The filtrate was acidified to pH 2 with 5M sulphuric acid and extracted with 50% acetone-chloroform (3 x 50 mL). The extracts were dried, and the solvent evaporated.

(c) A large excess of diazomethane in diethyl ether was added to a solution of the oxidized product in methanol (2 mL), and the mixture was kept at 4°C for 18 h. After evaporation of the solvent, dichloromethane (1 mL) containing pyromellitic acid tetramethyl ester (6.0 mg) as internal standard was added. The GC analysis was carried out with a SE30 bonded phase vitreous silica capillary column (12 m x 0.22 mm ID) (SGE Scientific, Melbourne) with a split ratio of 100:1, and detection was by flame ionization. Injector and detector temp: 250°C. Oven temp: 150°C for 3 min, then programmed at 5°C/min to 230°C and 10 min at 230°C. Retention times and response factors (in brackets): IVa, 1.8 min (1.6); IVb, 4.3 min (1.7); IVc, 7.2 min (1.5); pyromellitic acid tetramethyl ester (internal standard) 12.2 min; Ib, 21.3 min (1.0).

The ethylated catechin (IIb) (25 mg) oxidized by the above procedure gave compound IVb in 86% yield (GC estimation).

The gas chromatogram of the oxidation mixture of the tetraethylellagic acid (IIIb) showed no product peaks.

Treatment of *n*-Propyl Gallate with 0.5M Sodium Carbonate

A solution of *n*-propyl gallate (Aldrich Chemical Co., 5 mg) in 0.5M sodium carbonate in deuterium oxide (1.0 mL) was kept at 20°C for 24 h. Examination of the solution by PMR spectroscopy (Bruker

AM-100 instrument) showed signals at 3.56 δ (triplet, HO-CH_2-CH_2- of *n*-propanol) and 4.22 δ (triplet, -O-CH_2-CH_2- of *n*-propyl gallate). The ratio of the integrals of the respective signals were 29:71.

Alkaline Treatment of Ethylated Woodmeals

Samples of ethylated woodmeal (200 mg) were heated under reflux with 0.1M sodium hydroxide (20 mL) for 1 h. The woodmeal was collected by filtration and washed with warm water. The combined filtrate and washings were acidified to pH 2 and extracted with chloroform (3 x 30 mL). The extracts were dried and the residue after evaporation of the solvent was methylated with diazomethane and examined by GC after addition of pyromellitic acid tetramethyl ester as the internal standard as described above.

The gallic and diphenic acid derivatives IVc and Ic (2.5 mg) treated as above were recovered as IVc (2.1 mg) and Ib (2.2 mg), respectively.

Estimation of Ellagic Acid as the HHBP (V)

Ellagic acid (12-50 mg) and 0.5M potassium hydroxide (5 mL) were sealed under nitrogen in 15 mL glass vials, and the vials were kept at 105°C for periods of 18-96 h. After opening the vials, the contents were immediately acidified to pH 4, transferred to 100 mL flasks and freeze-dried. Methanol (5 ml) containing disyringylmethane (6 mg) as internal standard, was added and the mixture shaken. An aliquot (*ca.* 2 mL) was evaporated under nitrogen and the residue was silylated with N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) (100 L) at 100°C for 24 h. Excess BSTFA was removed under nitrogen, and dichloromethane (1 mL) was added prior to GC analysis on a SE30 bonded phase vitreous silica capillary column as described above. Oven temp: 230°C. Retention times and response factor (in brackets): disyringylmethane (internal standard), 7.2 min; HHBP (V), 7.8 min (1.7).

The decarboxylation reaction of ellagic acid was also carried out in the presence of woodmeals, and alkaline extracts of woodmeals. Alkaline treatment of woodmeals alone was also undertaken.

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