Procyanidins (Condensed Tannins) in Green Cell Suspension Cultures of Douglas Fir Compared with Those in Strawberry and Avocado Leaves by Means of C$_{18}$-Reversed-phase Chromatography[1]

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ABSTRACT

The procyanidins (the most common type of proanthocyanidin or condensed tannin) from cell suspension cultures derived from cotyledons of Douglas Fir have been compared with those isolated from leaves of strawberry and avocado. Seventy per cent methanol (v/v) extracts from 100 milligrams fresh weight samples were analyzed by a combination of C$_{18}$-reversed-phase columns with high-performance liquid chromatography, and normal phase paper chromatography. (−)-Epicatechin and its oligomers were generally retarded longer on C$_{18}$ columns than the corresponding units made of (+)-catechin when eluted with solvents made up of 5% acetic acid alone or mixed with methanol up to 15% (v/v). Douglas fir preparations contained the most complex set of procyanidins and consisted of oligomers of catechin and epicatechin, whereas strawberry and avocado contained mainly (+)-catechin and (−)-epicatechin derivatives, respectively.

Proanthocyanidins (polymeric phenolic compounds also called condensed tannins) are found in numerous gymnosperms and angiosperms in amounts up to 40% of the fresh weight of the tissues extracted. It is assumed that they are generalized chemical defense mechanisms against animal predators and attack by microorganisms (10). The most common type produce cyanidin upon acid hydrolysis and are called procyanidins. They are believed to be formed by the condensation of a precursor diol or a carbocation with already formed catechin or epicatechin monomers or oligomers (5, 9). One example of a dimer is shown in Figure 1.

Recent investigations of the procyanidins in green cell suspension cultures derived from cotyledons of Douglas fir were hampered by apparent low levels of lower mol wt oligomers of procyanidins. Isolation of MeOH[2]-soluble procyanidins by chromatography on paper or Sephadex LH-20 columns (11) or by precipitation with ethyl acetate (7) led to forms that did not migrate in a BAW mixture and streaked on 5% HOAc in two-dimensional chromatography. These problems have now been partially resolved with the development of a rapid small-scale

method of isolation and quantification by means of C$_{18}$-reversed-phase column chromatography combined with normal-phase paper chromatography. Extracts from strawberry and avocado leaves were selected for comparison.

MATERIALS AND METHODS

The three major tissues or cells studied were chamber-grown at about 25 C. The cell suspension cultures of Douglas fir (*Pseudotsuga menziesii*, Franco) were derived from cotyledons (9). The strawberry leaves and green fruits were from an unidentified cultivar of *Fragaria chiloensis* var. *ananassa*, Bailey. Avocado plants (*Persea gratissima*, Gaertn.) were grown from seeds of commercial origin.

Isolation Procedure and Separation on C$_{18}$ Minicolumns. About 100 mg fresh weight of tissues or washed cells were ground in 3- × 1-ml aliquots of MeOH:H$_2$O (70:30, v/v). After evaporation of the methanol under vacuum at 30 to 35 C, the aqueous fraction was extracted with petroleum ether to remove lipids. After re-evaporation to remove traces of petroleum ether, the aqueous extract was diluted to 3 ml with H$_2$O and applied to a 1- × 0.8-cm column (C$_{18}$-Sep-Pak of Waters Associates) previously washed with MeOH:H$_2$O (70:30) and H$_2$O. The yellow-brown color of some of the higher mol wt procyanidins were visible as an adsorbed band in the top 2 to 3 mm of the column. After washing with 5 ml H$_2$O, this minicolumn was eluted sequentially with 3 ml 5% HOAc, 2 ml MeOH:5% HOAc (20:80), and 1 ml MeOH:H$_2$O (70:30). All but traces of the procyanidins were eluted. Columns were routinely cleaned for reuse with butanol followed by 100% MeOH. The three eluant fractions were subsequently analyzed via paper chromatography (two-dimensional) or HPLC using a longer C$_{18}$ column.

Analysis via Two-dimensional Paper Chromatography. Aliquots of the above three fractions were chromatographed on Whatman No. 1 (25 × 30 cm) first with 5% HOAc (v/v) and then with BAW (30:5:10, v/v). Because of concentration effects discussed later, the aliquots applied to the paper were generally too dilute for detection under UV (260 nm). Two sprays were used to detect compounds. One, the 1% vanillin (w/v) in 70% H$_2$SO$_4$ (v/v) spray, produces a pink color with the monomers catechin and epicatechin as well as their oligomeric forms (4). The other spray was a mixture of equal amounts of 1% (w/v) each FeCl$_3$·6H$_2$O and K$_3$Fe(CN)$_6$, which produces a blue color with most phenolics (8).

HPLC Analysis. An isocratic HPLC (model 204, Waters Associates) was used at RT in conjunction with a 30- × 0.4-cm C$_{18}$-μBondapak reversed-phase column. Generally, 25- to 100-μl aliquots of the three minicolumn fractions were injected and the eluants were detected at 280 nm with automatic recording at 1

[2] Abbreviations: MeOH, methanol; HOAc, acetic acid; BAW, 1-butanol-HOAc-H$_2$O; HPLC, high-performance liquid chromatography; V_E, total elution volume, including V_0; V_0, void volume; cat$_2$, cat$_3$, epi$_2$, and epi$_3$, dimers and trimers of catechin and epicatechin, respectively; cat-epi and epi-cat, mixed dimers; epi$_2$-i, isomer of epi$_2$.

cm/min. Profiles were graphed as $A_{280\,nm}$ against total elution volume (V_E), including a void volume (V_0) of approximately 3 ml, as determined with uracil in MeOH:H$_2$O (70:30). "Fingerprint" elution profiles were made with a low concentration of procyanidins and a solvent flow rate of 2 ml/min. Collection profiles were made with 10 times the concentration and a flow rate of 0.5 ml/min. Sample injection volumes were kept below 100 μl by concentrating at 30 to 50 C under vacuum. Eluant fractions of selected peaks were collected manually and concentrated at 30 to 50 C under vacuum for recyling through the HPLC, for procyanidin analysis, or for paper chromatography.

Areas (cm^2) under the peaks detected at 280 nm were estimated by counting squares on the graph or by planimeter readings. The areas were converted to A units by the following calculation: A_{280} = area (cm^2) × A_{280}/cm × flow rate (ml/cm) ÷ aliquot injected (ml). A_{280} was expressed as the total A/peak isolated from an extract derived from 100 mg fresh weight of tissue in 1.0 ml (A × ml/100 mg fresh weight).

Recovery of standards from the C$_{18}$ column was approximately 100%. Resolution characteristics of the column were routinely checked with mixtures of catechin and epicatechin, or uracil and three peaks in an impure acenaphthene sample.

Procyanidin Analyses. Aliquots in a final volume of 0.1 ml were heated with 1 ml butanol-HCl (95:5, v/v) at about 95 C for 30 min (9). The A was determined at 550 nm, the peak absorption for cyanidin. No delphinidin was detected. Biphasic curves were generally obtained. The first was linear up to an A of about 0.2; the second up to 0.8.

Standard Flavan-3-ols and Lower Oligomers of Procyanidins. Only (+)-catechin and (−)-epicatechin were available commercially (Tridom-Fluka, New York). Samples of the major dimers (cat$_2$, epi$_2$, cat-epi, and epi-cat) and one trimer (epi$_3$), all with the C$_4$-C$_8$ linkage (11), were kindly supplied by Dr. E. Haslam, University of Sheffield, England. The brown solids contained impurities, but all consisted of one major component. Comparable standards were isolated by paper chromatography or HPLC from sources listed by Haslam as his original plant source (Tables I and 6 in ref. 11). Cocoa bean (*Theobroma cacao*) was a source of epi$_2$ and epi$_3$, *Rubus fruticosus* leaves were a source of cat-epi, avocado (*Persea gratissima*) leaves were a source of epi$_2$ and epi$_2$-i, strawberry (*Fragaria* sp) green fruits were a source of cat$_2$, and *Salix caprea* catkins were a source of cat$_3$.

RESULTS

Preliminary Separation into Three Fractions on Minicolumns of C$_{18}$-Reversed-phase Columns. The flavan-3-ols and their 70% MeOH-soluble procyanidins were separated into three fractions on minicolumns of C$_{18}$-bonded silica packings (C$_{18}$-Sep-paks). All procyanidins were tightly held to these C$_{18}$ columns equilibrated
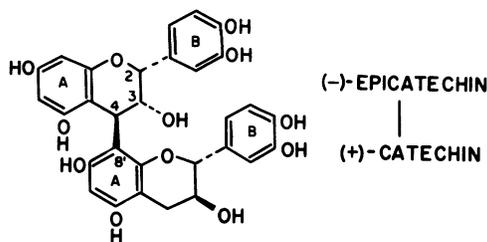
FIG. 1. An example of one of the four major dimers consisting of epicatechin and catechin, linked through carbons at positions 4 and 8. Upon acid hydrolysis, the upper unit is oxidized to the red pigment, cyanidin, whereas the lower unit gives rise to catechin. The basic monomers, (+)-catechin and (−)-epicatechin, are stereoisomers at position 3 but have the same configuration at position 2. The interflavan bond at position 4 is always *trans* to the hydroxyl group at position 3.

with H$_2$O when added as an aqueous extract from which all the MeOH had been removed. A useful sequence for Douglas fir extracts derived from 100 mg fresh weight of cells was to elute first with 5% HOAc, followed by MeOH:5% HOAc (20:80) and finally MeOH:H$_2$O (70:30).

Table I shows a typical distribution pattern into the three fractions for the total MeOH-soluble procyanidins of three species of green tissues or cells, based on the A at 550 nm due to the cyanidin formed after hydrolysis in a butanol-HCl mixture.

Extracts from these three green tissues fell into three classes, depending on whether they consisted mainly of catechin derivatives (strawberry), epicatechin derivatives (avocado), or a mixture of both (Douglas fir). Strawberry contained the greatest percentage of procyanidins in the first two fractions, indicating the prevalence of more H$_2$O-soluble forms or ones having the least attraction to the C$_{18}$ column. Avocado and Douglas fir contained the least in the 5% HOAc fraction. Instead, equivalent amounts were found in the fractions eluted with MeOH. Douglas fir and strawberry were similar in containing large amounts of the monomer, (+)-catechin, whereas avocado contained only small amounts of the other isomer, (−)-epicatechin. The major dimer differed in each case; *i.e.* epi-cat, epi$_2$-i, and cat$_2$ for the Douglas fir, avocado, and strawberry, respectively.

In the case of Douglas fir, no differences in the distribution of procyanidins into the three minicolumn fractions were detected for cell suspension cultures analyzed 1, 2, or 3 weeks after subculture. Likewise, the distribution patterns in young and fully expanded leaves of strawberry did not differ, although the older leaves contained a higher amount of procyanidins/fresh weight basis. Furthermore, avocado leaves grown in bright or dim light showed a similar distribution pattern among the three fractions, but about twice as much total procyanidin was found in the bright-light grown leaves.

A useful estimate of the purity of these fractions in terms of procyanidins is to compare the ratio of A at 550 nm after acid hydrolysis with the total A at 280 nm before hydrolysis (Table I). Whereas the 550-nm value is based on a specific assay for oligomeric procyanidins, the value at 280 nm represents the A due to the monomers as well as the other unrelated compounds. Strawberry-leaf extracts showed the lowest ratios, although the total amount of procyanidin was similar to that from Douglas fir. The purest preparations of procyanidins obtained thus far have ratios ranging from about 1.5 to 3.0 (see Table IV for further discussion). Some range was expected since trimers and tetramers have been reported to produce larger amounts of cyanidin on a per weight basis than either the dimers or higher molecular weight forms (1).

Analysis of Minicolumn Fractions by Two-dimensional Paper Chromatography. Monomers and oligomeric procyanidins were visualized on duplicate chromatograms with the FeCl$_3$-K$_3$Fe(CN)$_6$ spray, a general spray for most phenolics, and with the vanillin-HCl spray, a relatively specific one for flavan-3-ols and their

Table I. *Distribution of Total Procyanidin as A_{550} in Three Fractions Eluted from C$_{18}$ Minicolumn (from 100 mg Fresh Weight Cells or Tissue in 1.0 ml)*

Values represent single, but reproducible analyses.

Tissue	Total Pro-cyanidin	Elution Solvent		
		5% HOAc	20:80[a]	70:30[b]
	A_{550}	%/fraction		
Douglas fir cell cultures	25.4	11 (1.3)[c]	45 (0.9)	44 (0.8)
Avocado leaves	20.9	6 (0.9)	49 (1.5)	45 (1.3)
Strawberry leaves	28.8	34 (0.8)	39 (0.6)	27 (0.1)

[a] Ratio, MeOH:5% HOAc.
[b] Ratio, MeOH:H$_2$O.
[c] Values in parentheses are ratios of A_{550}/A_{280}.

oligomers (4). Diagrams of the pink, vanillin-positive spots in the MeOH:5% HOAc (20:80) fraction are shown in Figure 2.

In general, the 5% HOAc fraction contained mainly (+)-catechin and its simpler oligomers cat_2 and cat_3. The MeOH:5% HOAc (20:80) fraction still contained some of these components, but had, in addition, (−)-epicatechin, epi_2, and epi_3 as well as the mixed dimers, cat-epi and epi-cat. All of the above migrated to some extent in the BAW solvent. R_F values were slightly higher with Haslam's solvent mixture containing less H_2O and 2-butanol instead of 1-butanol (11). The "overlap" between the catechin components in the 5% HOAc and the subsequent MeOH:5% HOAc (20:80) fraction was minimized by a more exhaustive extraction (15 ml rather than 3 ml) with 5% HOAc. Other major procyanidin constituents found in both the MeOH:5% HOAc (20:80) and MeOH:H_2O (70:30) fractions did not migrate significantly in the BAW solvent and formed a "smear" with no well-defined spots up to at least an R_F of 0.5 in the 5% HOAc solvent. Reversal of the order of the solvents made no difference in this streaking. These vanillin positive areas were assumed to be oligomers above
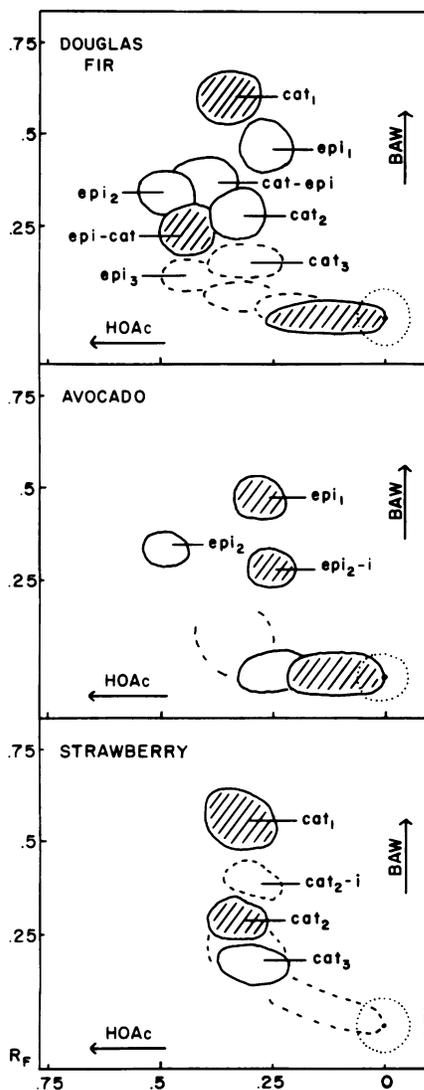
FIG. 2. Tracings of two-dimensional paper chromatograms of the MeOH:5% HOAc (20:80) fractions eluted from the C_{18} minicolumn. Only vanillin-HCl-positive pink spots are shown, to indicate the presence of the monomers, catechin and epicatechin, and their oligomeric derivatives. Approximate amounts are designated as major (diagonal lines), minor (——), and trace (– – –) amounts.

the trimer level, possibly combined with forms modified by oxidation or conversion to branched forms (3). As discussed later with the HPLC profiles, concentration effects were observed; there was a tendency for the lower mol wt oligomeric forms to become more H_2O-soluble or to form reversible complexes with the higher oligomers when high concentrations were applied to chromatograms.

Strawberry leaves contained the simplest pattern of vanillin positive spots, but the array of other phenolic compounds detected with the $FeCl_3$-$K_3Fe(CN)_6$ was complex. Two major yellow flavonoids were present in the MeOH:H_2O (70:30) fraction. The pink, vanillin positive spots were identified as (+)-catechin, cat_2, and cat_3, plus small amounts of the mixed dimers (Table II). Haslam reported major amounts of catechin and cat_2, with minor amounts of the cat_2 isomer and epi-cat (11).

Extracts from avocado leaves contained little evidence of any vanillin or $FeCl_3$-$K_3Fe(CN)_6$-positive spots in the 5% HOAc fraction at the levels routinely examined, although analysis indicated the presence of procyanidins. The MeOH:5% HOAc (20:80) fraction, however, showed an array of vanillin positive areas, identified as epicatechin, epi_2, and possibly epi_3, plus relatively large amounts of a form tentatively identified as the structural isomer of epi_2 (B-5 of Haslam), in which the interflavan linkage is between C_4-C_6 instead of C_4-C_8 (5). Haslam (11) reported major amounts of epicatechin, epi_2, and epi-cat, but only minor amounts of epi_2-i in leaves.

Extracts of Douglas fir cell suspension cultures contained the most complex array of vanillin-positive spots, with relatively few other phenolics detected with the $FeCl_3$-$K_3Fe(CN)_6$ spray. Both catechin and epicatechin derivatives were present.

Analysis of Minicolumn Fractions with HPLC using a C_{18}-μBondapak Column. HPLC chromatography of standards indicated that epicatechin and its oligomeric derivatives were generally more retarded on the C_{18}-μBondapak column than are the catechin derivatives. Furthermore, the distance between the members of the epicatechin series was greater (Table III). These characteristics are summarized in Figure 3 in which the log k' is plotted against increasing amounts of MeOH present in the 5% HOAc solvent. In similar plots by Wulf and Nagel (12) for a variety of C_6-C_3 and C_6-C_1 phenolic acids, the line for catechin was much steeper than

Table II. *Comparison of Amounts of Monomers and Lower Mol Wt Oligomeric Procyanidins in Douglas Fir Cell Suspension Cultures with Those Found in Avocado and Strawberry Leaves*

Estimates represent the sum of A_{280} values/peak in both the 5% HOAc and MeOH:5% HOAc (20:80) minicolumn fractions. In addition, extracts of all three species contained unidentified higher oligomeric forms in this 20:80 fraction.

Compound		Douglas Fir	Avocado	Strawberry
(+)-Catechin		++++[a]	0[b]	++++
Cat₂	(B-3)[c]	++	+	++++++
Cat₃	(C-2)	tr[d]	0	++
Epi-cat	(B-1)	+++	tr	0
Cat-epi	(B-4)	+	+	+
(−)-Epicatechin		+	+	tr?
Epi₂	(B-2)	+	+	0
Epi₃	(C-1)	tr	+	0
Epi₂ − i	(B-5)	0	++[e]	0

[a] Each + equals an A_{280} value of approximately 1.0/100 mg fresh weight sample of tissue in 1.0 ml.

[b] Zero means not detectable via paper chromatography or HPLC.

[c] Identification system used by Haslam (11).

[d] tr, trace.

[e] Based on an assumption that 50% of the A_{280} of the peak is due to an impurity.

Table III. *Relative V_E from C_{18}-μBondapak Columns of Standards*
V_0 was 3.0 ml.

Compound	HPLC Solvents				
	5% HOAc	5:95[a]	10:90[a]	20:80[a]	70:30[b]
(+)-Catechin[c]	21.5	14.5	10.9	6.1	3
Cat$_2$[d, e]	13.9	9.1	6.8	4.4	3
Cat$_3$[e]	16.0	10.6	7.4		
(−)-Epicatechin[c]	49.5	33.0	21.8	9.4	3
Epi$_2$[d, e]	34.2	21.7	13.4	6.0	3
Epi$_3$[d, e]	110.0	47.5	25.0	7.9	3
Epi-cat[d, e]	19.6	11.8	8.0	4.6	
Cat-epi[d, e]	23.8	14.3	9.6	5.2	
Caffeic acid[c]	29.4	21.6	17.1	10.1	
Chlorogenic acid[c]	38.4	23.6	16.5	8.1	

[a] Ratios, MeOH:5% HOAc.
[b] Ratio, MeOH:H_2O.
[c] Commercial samples.
[d] Standards from E. Haslam (personal communication).
[e] Standards isolated from tissues cited by Haslam (11).
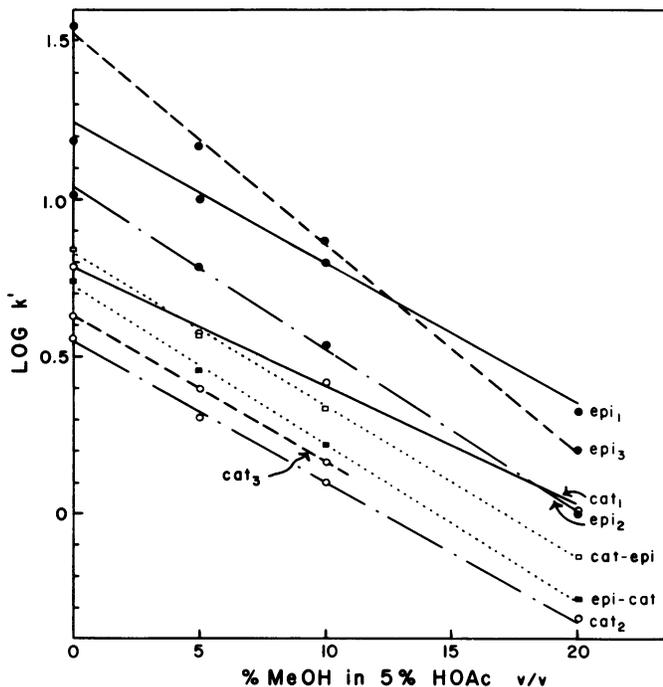
FIG. 3. Effect of per cent MeOH content (v/v) on the capacity factor or partition ration, k', of flavan-3-ols and procyanidins [$k' = V_E - V_0$ ÷ V_0, ($V_0 - 3.0$)]. Solvents and data as in Table II. (O), cat series; (●), epi series; (——), monomers; (—·—·—), dimers; (— — —), trimers; (□·····□), cat-epi; (■·····■), epi-cat.

for the other phenolic compounds studied. The parameter of k' is a measure of the capacity of the column and is comparable to an R_F value in normal phase paper chromatography. The plot of log k' versus per cent MeOH tends to be linear with nonionizing compounds (6). In the catechin series, the lines tend to be parallel to each other, whereas, in the epicatechin series, the slopes of the dimer and trimer lines are increasingly steeper than that of the monomer. Although straight lines have been drawn through the points, some nonlinearity may possibly occur. The plots for the mixed dimers tend to be parallel to the cat$_2$ line but show evidence of a biphasic portion as the aqueous component approaches 100%.

The usefulness of HPLC and the longer C_{18}-μBondapak column

with its greater resolving power can best be shown by examining profiles of the 280-nm absorbing materials from the 20:80 minicolumn fraction (Fig. 4). Each profile shows the position of the elution peaks, and the total $A_{280 \text{ nm}}$/peak can be calculated from the area under the curve. Analysis of these fractions was always carried out in conjunction with paper chromatography. A useful initial solvent was MeOH:5% HOAc (10:90), although the catechin oligomers were not well-separated.

Strawberry leaves showed the simplest A_{280} elution pattern, and the major procyanidins were clustered in the peaks V_E, 7–8ml consisting of cat$_2$ and cat$_3$. The avocado leaf profile was more complex with some significant peaks containing nonprocyanidin material. Such a contaminant accounts for the very high peak at about V_E, 17 ml containing approximately equal amounts of the procyanidin epi$_2$-i and an unidentified blue fluorescing compound ("x"). Except for the absence of this epi$_2$-i peak at 17 ml, the procyanidin profile from Douglas fir preparations was a composite of those from strawberry and avocado. Except for peaks prior to a V_E, 7 ml, relatively few non-procyanidin compounds were detected.

Cat$_2$, cat$_3$, and epi-cat (V_E, 6–9 ml) are not well-resolved in the MeOH:5% HOAc (10:90) solvent. However, this complex area can be separated into at least three components by paper chromatography or by recycling it through the HPLC using a 5% HOAc solvent.

Recycling of eluted peaks with the HPLC using solvents containing a lower per cent of MeOH, was a much more effective separation method than by paper or TLC, although they were still useful as a purity check. Examples of this are shown in Table IV for some of the dimers isolated with the ratio A_{550}/A_{280} as the criterion of purity. Comparisons were made with available standards which were also expressed as $E^{1\%}_{550}$ values. Values obtained from an ethyl acetate-precipitable procyanidin and a residue insoluble in 100% MeOH, but soluble in 70% MeOH isolated as reported previously (9) are also shown. In general, dimers gave a ratio A_{550}/A_{280} of about 1.5, whereas trimers and tetramers gave ratios closer to 3.0.

70:30 Fraction from C_{18} Minicolumn. After two-dimensional paper chromatography, a non-butanol-soluble component ($R_F = 0$ in BAW) was seen as a continuous streak in 5% HOAc from $R_F = 0$ to about 0.5. During chromatography on the μBondapak column, a complex profile was obtained in a step-wise elution, first with 24 ml MeOH:5% HOAc (20:80) followed by 12 ml MeOH:H_2O (70:30). Approximately 50% of the total A_{280} was eluted in the 20:80 solvent as a series of poorly resolved peaks between V_E, 3–24 ml, including a long tail; the remaining 50% was eluted in MeOH:H_2O (70:30) or (100:0) as a single peak in the void volume.

About 18% A_{280} of the elution profile in the 20:80 solvent was due to a major unidentified non-vanillin-positive peak at about V_E, 20 ml, which migrated close to epicatechin in paper chromatography. The streaking effect of the remainder of this 20:80 solvent profile from V_E, 4–24 ml, was due not to a complex breaking down during chromatography but to the presence of a series of poorly resolved forms. Upon paper chromatography, practically all of the vanillin-positive material migrated as a streak in 5% HOAc from about an R_F of 0.1 to 0.5 and did not move in the BAW solvent. Only traces of epicatechin and its oligomers, which are somewhat soluble in BAW, were found.

The portion eluted subsequently as one major peak in 100% MeOH also did not migrate in the BAW solvent and moved to an R_F of about 0.1 in the 5% HOAc solvent. Thus far, a solvent mixture has not been found that permits an effective resolution of individual components of the 70:30 minicolumn fraction.

Major concentration effects have been observed with this 70:30 C_{18} minicolumn fraction when it was eluted from the larger C_{18}-μBondapak column in a 20:80 solvent mixture. When increasing
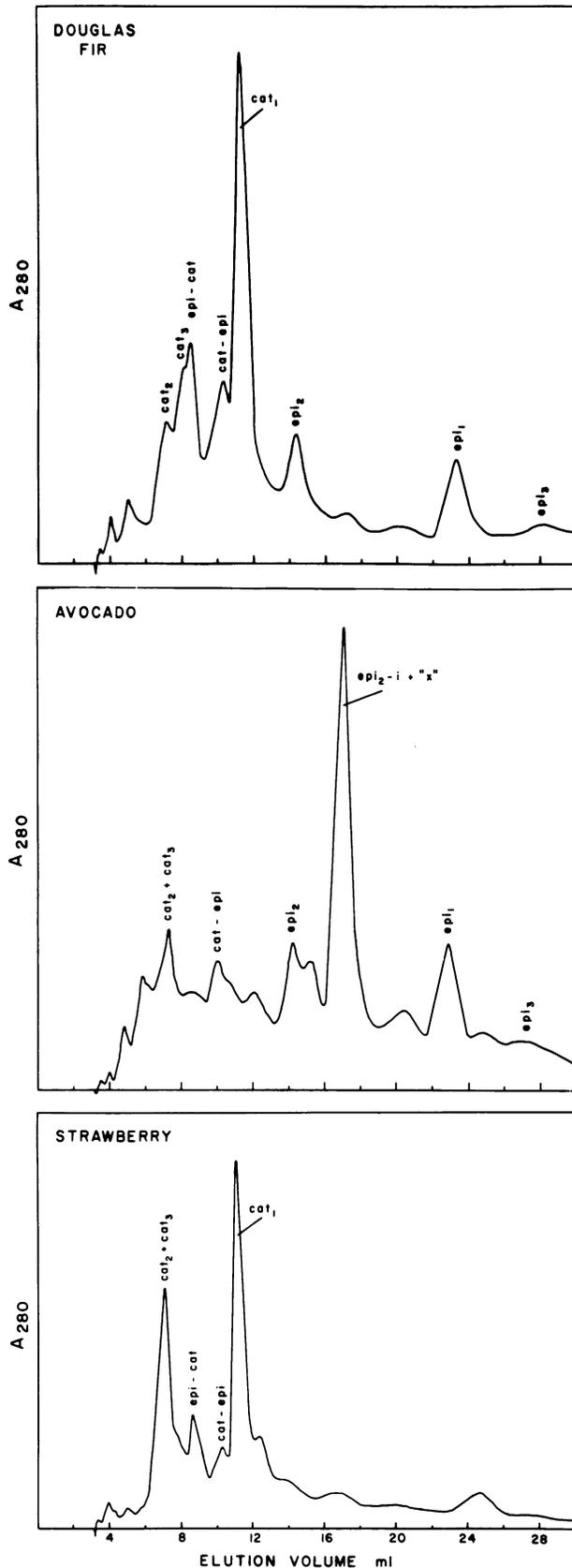
FIG. 4. Elution profiles in MeOH:5% HOAc (10:90) of the MeOH:5% HOAc (20:80) eluants from the C_{18} minicolumn. A_{280} is plotted against V_E (total) elution volume. The pump rate was 2 ml/min and chart speed was 1 cm/min, so that a V_E of 12 ml = t of 6 min.

Table IV. *Ratio of A_{550}/A_{280} and $E_{550}^{1\%}$ Values for Standards and Purified Fractions*

Procyanidin	A_{550}/A_{280}	$E_{550}^{1\%}$
Cat2[a]	1.2–1.7 (1.7)[b]	64 (90–106)[c]
Cat3	2.6	
Epi2	1.6–1.9	70
Epi3	2.7	147
Epi-cat	1.0	54
Cat-epi	1.1	64
Dimer mix	(2.5)[b]	136[c]
Tetramer	(2.9)[b]	140–180[c]
Isolated fractions (HPLC)		
Cat2 + cat3[a]	1.9	
Epi-cat	1.6–2.0	
Cat-epi	1.5	
Cat2	1.3–2.3	
Ethyl acetate precipitate[d]	1.5–3.2	77–146
Residue insoluble in 100%		
MeOH, soluble in 70% MeOH	1.9–2.8	132–176

[a] Compound sources as noted in Table III.

[b] Estimated from data of Bate-Smith (1), based on the assumption of $E_{280}^{1\%}$ of 55 as determined from some of Haslam's samples (personal communication).

[c] Data of Bate-Smith (1).

[d] From reference 7.

amounts were added to the column, the peaks in the elution profile shifted towards the V_0, an indication of a conversion to a more H_2O-soluble form(s) or ones less retarded on the C_{18} column. For instance, a very small peak at the V_0 of 3 ml increased about 20-fold, whereas the area between V_E, 4–9 ml about doubled. Upon recycling as diluted injections, this V_0 peak at 3 ml was shifted to more retarded ones, indicating that the concentration effect was reversible. Similar shifts to the 3.0-ml peak have been observed when samples of cat2 and epi2 were increased from 5 to 50 μg. These concentration effects were also observed with the ethyl acetate precipitable high mol wt component isolated according to Karchesy et al. (7) and reported previously (9).

Upon aging, two opposite effects occurred. All of the minicolumn fractions turned yellow, but this was more apparent in the 70:30 fraction. There was a shift of some of the components, including the yellow color, originally eluted in the 20:80 profile to forms that were subsequently eluted only in 70 to 100% MeOH. A second aging phenomenon was a significant shift to more H_2O-soluble or less retarded forms as shown by an increase in the peaks V_E, 3–10 ml in the 20:80 solvent. Further aging led to prevoid volume peaks, presumably due to the exclusion from the small pore volume of the column because of the presence of aggregated forms of very high mol wt. Such prevoid volume peaks were reported previously for the ethyl acetate precipitable procyanidin product (9). Since aging starts immediately, it is important to analyze the preparations as rapidly as possible. Storage under N_2 does not alleviate the problem.

DISCUSSION

In a previous report (9), the dimeric and trimeric forms of Douglas fir procyanidins were considered to be present only in very small amounts. They have now been found in amounts equivalent to about 35% of the total MeOH-soluble procyanidins. The difficulties in detecting them were due to a variety of factors; the most important were the complexity of these procyanidins and the apparent formation of reversible complexes of the smaller oligomers with themselves or with larger mol wt forms, especially in concentrated and aged solutions. The methods described here minimize these problems and are more rapid than those used by Haslam and co-workers (11), thus lessening the formation of

modified forms. Use of HPLC permits an easy quantitative estimation of the relative amounts of the major components as they are being separated. Both normal phase paper (or thin-layer) chromatography and column chromatography using reversed-phase C_{18} columns are necessary. The ratio of absorption at 550 nm (after acid hydrolysis) to 280 nm is a useful estimate of the purity of the fractions isolated. Resolution of the mol wt forms above the tetrameric level is still unsatisfactory.

The nature of solute retention on C_{18} columns is a subject of considerable dispute. Adsorption, partitioning, hydrogen bonding, polarity, and the mol wt can be involved (2, 6). The addition of MeOH to a 5% HOAc phase showed a dramatic difference in the log k' when catechin was compared to other cinnamic acid derivatives (12). This comparison has been extended to include the other stereoisomer and the lower mol wt oligomeric forms of both catechin and epicatechin.

The adsorption of the flavan-2-ols and their oligomers in an aqueous extract to C_{18} columns equilibrated with H_2O was surprising since these compounds are soluble in H_2O at the concentrations used. Hydrogen bonding to the uncovered glass surface could be involved, but these sites should have been quickly saturated. Hydrophobic factors may be more important in C_{18}-reversed-phase chromatography (8), whereas hydrogen bonding to cellulose may predominate in normal phase chromatography. This could explain the difficulty in predicting retention characteristics on the C_{18} column from R_F values on paper even when the solvent was 5% HOAc in both cases.

Haslam (5) postulates that these oligomeric forms are constructed as rigid threads due to restricted rotation around the interflavan bond. The o-diphenolic B-rings extend out in a different plane from the thread-like core made up of the A- and central heterocyclic ring of each C_{15} unit. The B-rings of catechin oligomers form a right-handed helix, in contrast to a left-handed one for the epicatechin oligomers, because of the difference in stereochemistry at position C_3 of the heterocyclic ring. Haslam (5) indicated the possibility of random coiling and folding of these oligomeric threads to form globular aggregates with the phenolic groups distributed on the surface of the molecule. Such more polar aggregates might account for some of the concentration effects and the exclusion of some procyanidins from the small molecule pore volume of the μBondapak column.

The procyanidins of the green cells from Douglas fir, avocado, and strawberry form an interesting trio. Oligomeric forms found in strawberry leaves are derived from catechin, whereas those of avocado derive from epicatechin. Douglas fir cell cultures contain both types, but the mixed dimer, epi-cat, predominates. High mol wt oligomers isolated from Douglas fir bark consist of a 3:1 ratio of epicatechin to catechin (7). Factors controlling the biosynthesis of the catechin and epicatechin monomers and oligomers are unknown. The study of these three sources will aid in testing Haslam's hypothesis (5) that two stereoisomeric carbocations condense with already formed pools of catechin or epicatechin to form these oligomers and that NADPH concentrations favor the accumulation of monomers in contrast to the oligomers.

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