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Title THE FLAVONOID COMPOUNDS OF THREE FLORAL PHENOTYPES OF DOUGLAS FIR

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This is the first study of the pigment compounds of flowers of a coniferous species. Red, yellow and green female flowers and red and green male flowers were examined. Flavonoid compounds were removed from the flowers of Douglas fir by extraction with methanol and one percent methanolic hydrochloric acid. Paper chromatographic and ultraviolet spectral techniques were employed for isolation and identification.

The following flavonoid compounds were identified in all the flower phenotypes: quercetin, kampferol, quercetin-3-monoglucoside, kampferol-3-monoglucoside, kampferol-3-diglucoside, dihydroquercitin-3'-monoglucoside, d-catechin, l-epicatechin, eriodictyol-7-monoglucoside, and naringenin-7-monoglucoside. Cyanidin-3-monoglucoside was found only in the red flowers. No differences were found between the male and female flowers of the same

phenotype.

Comparison of the flavonoids present in the flowers of Douglas fir with those reported in other tissues of the tree supports the view that flavonoids are formed in situ. For example, neither kampferol nor naringenin, either as aglycones or glucosides, have been reported in any other tissues of Douglas fir studied, yet were present in the flowers.

Various spray reagents were tested on standard flavonoid compounds. The reaction of sodium borohydride as a reagent for differentiation of flavanones and flavanonols was of particular interest. Flavanones reacted with this spray reagent to give violet or pink colors; flavanonols failed to react.

Leucoanthocyanins were not found in any of the flowers. The presence of a red pigment, cyanidin-3-monoglucoside, in the red flowers supports the recently advanced view that anthocyanins are not formed in vivo from leucoanthocyanins.

Flavanones identified were found to have a different glycoside pattern than the flavonols and the single anthocyanidin. Thus, it appears that flavanones arise through a different biosynthetic route than that of the flavonols and anthocyanidins.

THE FLAVONOID COMPOUNDS OF THREE FLORAL PHENOTYPES OF DOUGLAS FIR

by

TERRY L. HIGHLEY

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INTRODUCTION

The importance of a vast number of simple and complex phenolic compounds in the life cycle of plants is becoming increasingly evident. Of this type of compound, flavonoids represent a particularly interesting group and have received much attention, though their exact physiological function in plants has still not been entirely clarified.

Variation in flavonoid content has been clearly shown to be responsible for the variation of flower color in certain species (17, p. 171; 20, p. 298; 38, p. 220). Color differences may also be the result of cell sap pH variation and change in the availability of metal ions which chelate with certain anthocyanins (12, p. 565). Often too, flavones, flavonols and their glycosides, although yellow or yellow-green themselves, may be present in flowers in which no color (white) is observed, such as the garden snapdragon, Antirrhinum majus (54, p. 109).

Evidence has been presented which suggests that flavonoid compounds may be involved in the physiology of the reproductive process (36, p. 474). The failure of two varieties of Forsythia to

cross-pollinate has been shown to be due to the presence of rutin, the 3-rhamnoglucoside of 3, 3', 4', 5, 7-pentahydroxyflavone, in the pollen of one and quercitrin, the 3-rhamnoside of 3, 3', 4', 5, 7-pentahydroxyflavone, in the other. The stigma of the flower capable of being fertilized by pollen in which rutin is present, contains an enzyme capable of hydrolyzing this glycoside; this enzyme is absent in the flower stigma which is fertilized by the pollen containing quercitrin. This common precursor is transformed into various flavonoid compounds by enzymatic oxidation, reduction, hydroxylation, glycosidation, methylation and acylation (16, p. 269).

Catechins and leucoanthocyanins, important members of the flavonoid class of phenolic compounds, have been shown to be the precursors of certain tannins (12, p. 210). These compounds condense upon heating and in the presence of mineral acids or enzymes to water soluble tannins in the early stages and to insoluble tannins in later stages (12, p. 211).

Many theories (6, p. 279; 39, p. 453; 48, p. 27) regarding the role of flavonoid compounds in the metabolic oxidation-reduction processes of the plant have been advanced but none have been sufficiently verified at the present time to allow their evaluation.

Also some evidence has recently been advanced suggesting that flavonoid compounds behave as growth inhibitors in dormant

peach flower buds (22, p. 798) controlling the emergence of these buds from the rest. Flavonoid compounds as well as other phenolics, such as chlorogenic acid, have been linked to the physiology of plant diseases and disease resistance (11, p. 105). The exact role of these compounds is still little understood.

The exact pathway of flavonoid biosynthesis in plants has still not been clearly established since these compounds occur naturally with various patterns of ring hydroxylation, etherification or glycosidation (sugar molecules attached to hydroxyl groups through a glycosidic bond); various patterns often occurring in the same plant. It has been proposed that flavonoids arise from a common C_{15} precursor of the type shown in Figure 1, which is synthesized by the condensation of hexoses and trioses (44, p. 172).

The C_6 (Ring B)-C_3 (Figure 1) of quercetin, 3, 3', 4', 5, 7-pentahydroxyflavone, and tricin, 5, 7, 4'-trihydroxy-3', 5'-dimethoxyflavone, have been demonstrated to arise from shikimic acid (52, p. 219). This acid has also been assumed to be the precursor of the C_6 (Ring B)-C_3 portion of other flavonoid compounds (9, p. 426). Experiments using radioactive labelled precursors have shown that the A ring (Figure 1) arises from acetate units. C^{14}-labelled sucrose or sodium acetate (9, p. 426) C^{14}-sucrose produced quercetin which was almost uniformly labelled, while

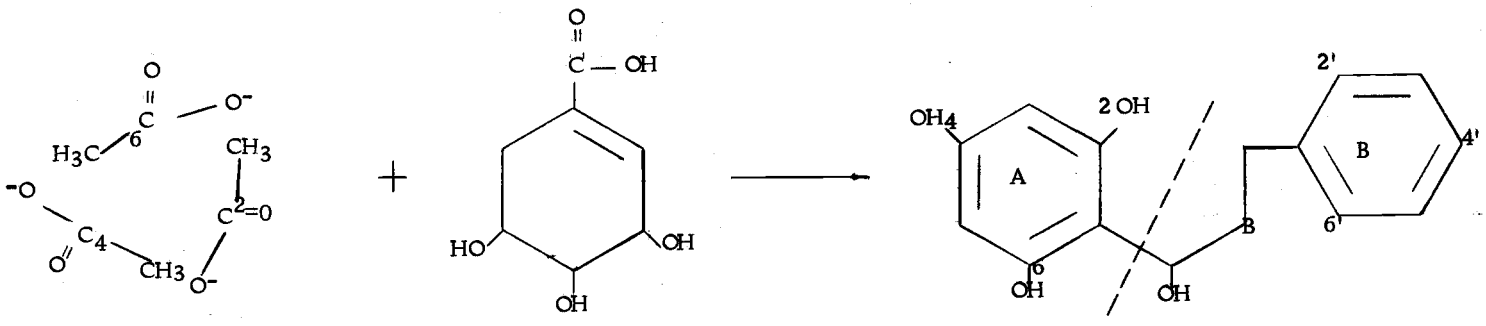


Figure 1. Synthesis of flavonoid precursor.

radioactivity was almost confined to the A-ring of the flavonol when methylated or carboxyl-labelled acetate was fed. The distribution of C^{14} in cyanidin, prepared by the hydrolysis of rubrobrassicin synthesized in red cabbage seedlings, which had been supplied methyl- or carboxyl-labelled acetate, showed that acetate units are probably added to the C_6(Ring B)-C_3 segment by the successive addition of three acetate units as illustrated in Figure 1 (9, p. 427). Radioactivity in carbons 1, 3, and 5 were measured separately from that of carbons 2, 4, and 6 (Figure 1). Carbons 2, 4, and 6, the hydroxyl-bearing carbons, were found to contain eighty-nine percent of the activity of the A-ring of cyanidin.

Harborne (12, p. 614) has postulated that flavonoid biosynthesis follows the general type of pathway indicated in Figure 2. Allowance has been made for the possibility that a common precursor occurs between alternative routes. It has been assumed that one gene controls the production of one enzyme which then mediates a single biosynthetic step in the chain (12, p. 604).

The co-occurrence of the same glycosides of both flavonols and anthocyanidins has been established in a number of plants such as Primula sinensis (20, p. 262), Plumbago rosea (17, p. 171) and Plumbago capensis (17, p. 171), by Harborne. He proposes that glycoside-synthesizing enzymes of three types of specificity exist

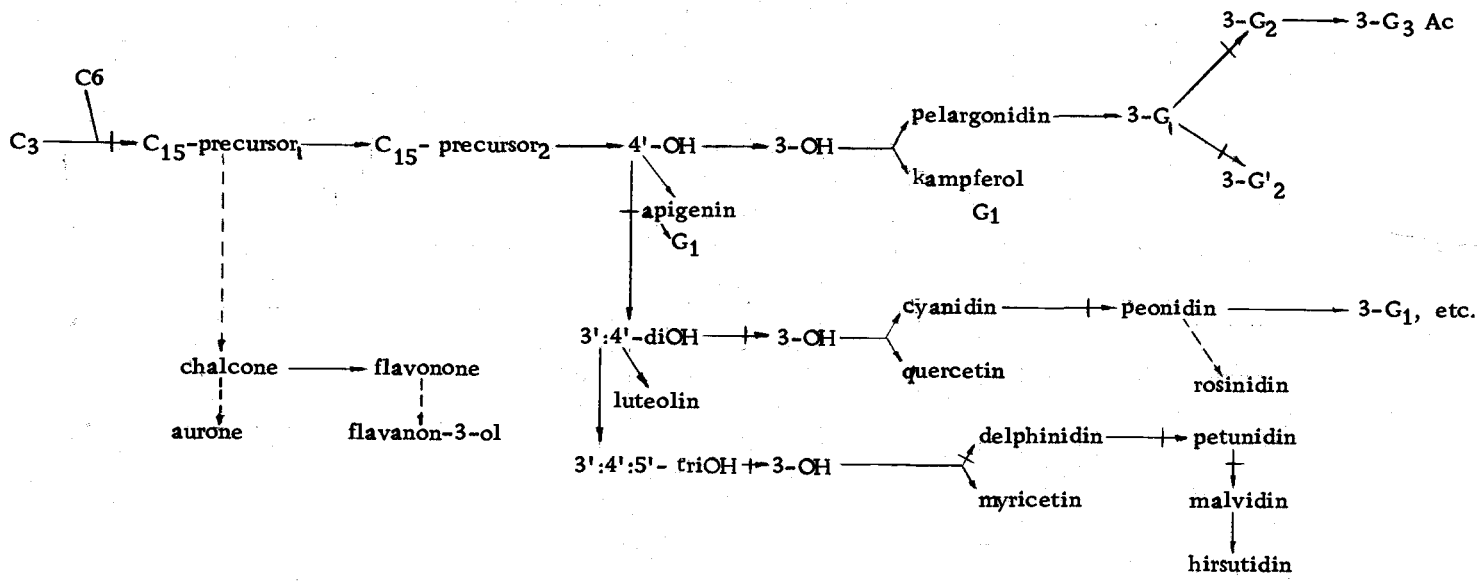


Figure 2. Proposed pathway of flavonoid biosynthesis.

in plants: those that are specific for anthocyanidin substrate, those specific for flavonol substrate and those which can add sugars to either compound.

While investigating the pigments in potato, Harborne (16, p. 269) found that the acylating gene had more than one biochemical effect on pigment production. From this, he concluded that the biochemical processes involved in methylation, acylation, and glycosidation of anthocyanins are sequential steps in biosynthesis. Harborne further stated that these processes probably occur late in synthesis and function in the plant by protecting the pigments from enzymic destruction or for increasing sap solubility of the pigment.

In a later study, concerning the flavonol glycosides of the potato, Harborne (18, p. 104) noted the occurrence of the same di- and tri-saccharides in association with flavonols throughout the tuber. He concluded, therefore, that the mode of linkage of sugars in plant di- and tri-glycosides is a generic character.

Considerable interest has been shown recently in the relation of structure of polyphenols to their presence in various tissues and their movement or "translocation" through the plant, particularly in trees. Several workers (49, p. 178; 53, p. 225) have postulated the cambium as the origin of plant polyphenols; subsequent translocation taking place from there. Hillis and Carle (27, p. 435) tested the

validity of this hypothesis by comparing the composition of the extractives in different tissues of Eucalyptus astringens and Eucalyptus marginata. They felt that if translocation of polyphenols occurs in plants, the polyphenolic extractives of the various tissues examined might be expected to exhibit a biochemical interrelationship. They found that elligitannins were the major monomeric components in the cambium of Eucalyptus astringens and E. marginata. Flavans, which were not related to the elligitannins, were the most important components of the extractives of the bark of the former and the heartwood of the latter. Also, the composition of the extractives of the sapwood and heartwood of E. astringens were shown to differ, not only from each other, but also from the extractives in the cambium and bark. Thus, they concluded that polyphenols are formed in situ probably from carbohydrates.

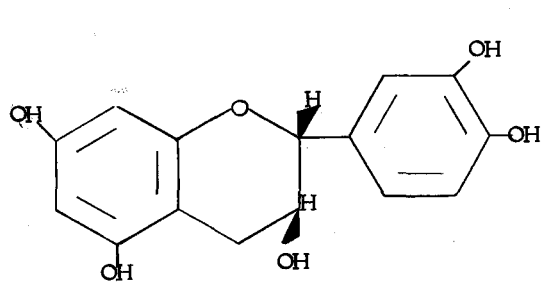
Hillis (26, p. 109) also felt that the work of Zimmerman (55, p. 213), who found the organic solutes in the sieve tubes of phloem of Fraxinus americana to be almost entirely carbohydrates, precluded the possibility that heartwood polyphenols were formed in leaves or phloem.

Studying the flavonoids in Douglas fir tissues, Hergert and Goldsmith (23, p. 700) found dihydroquercetin-3'-glucoside and quercetin-3'-glucoside, while the parent flavanonol and flavonol

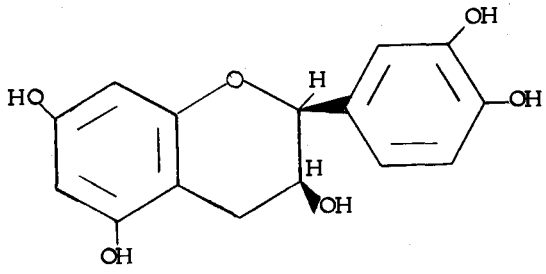
were absent in the needles, inner bark, cambium, and sapwood. This finding prompted their suggestion that dihydroquercetin and quercetin may be synthesized and glycosidated in the leaves, then transported down the inner bark, to the outer bark and through the rays to the heartwood. They propose that the aglycone is released at or near the sapwood-heartwood and inner bark boundaries. They felt, however, that in order to definitely establish the actual site of synthesis that further work using radioactive tracer techniques will be necessary.

Holmes (28, p. 61), also investigating Douglas fir tissue, in particular the newly formed bark, presented evidence which he suggests indicates that dihydroquercetin is formed by the oxidation of the catechins. d-Catechin and l-epicatechin, whose structures are shown in Figure 3, were found in the newly formed and mature inner barks, while d-dihydroquercetin was found in the outer bark. Upon air drying, an increase of d-dihydroquercetin in the mature inner bark in relation to the amount of catechins was noted. However, recent studies employing isotopically labelled catechins (21, p. 243) have definitely demonstrated that this compound is not converted to other flavonoid compounds, but rather to polymeric substances.

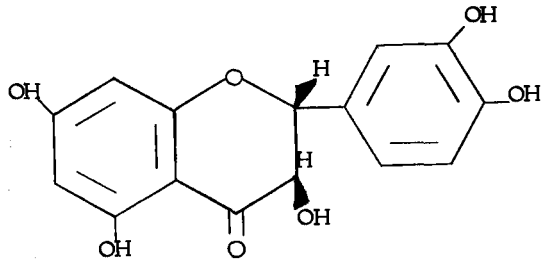
Interest in the inheritance of flower color is certainly not new. However, the relation between flavonoid compounds and flower color



d-Catechin

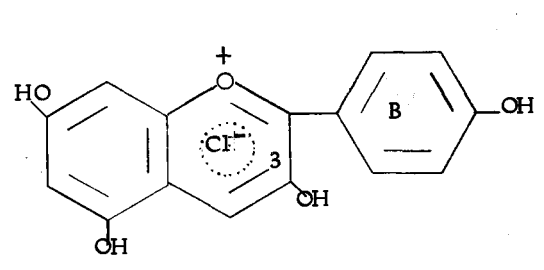


l-Epicatechin

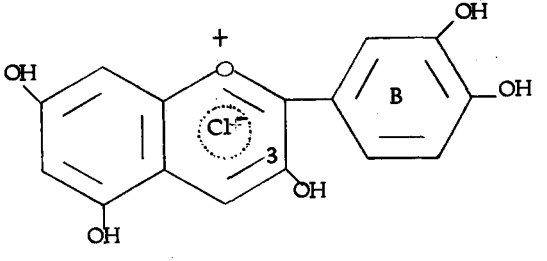


d-Dihydroquercetin

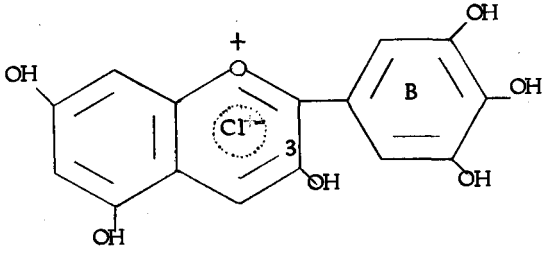
Figure 3. Flavonoids of newly formed bark of Douglas fir



Pelargonidin



Cyanidin



Delphindin

Figure 4. Anthocyanidins present in plants.

was first discovered by Wheldale (54, p. 126) in her study of Mendelian factors controlling flower color in Antirrhinum majus. She isolated apigenin, 5, 7, 4'-trihydroxyflavone, and luteolin, 5, 7, 3', 4'-tetrahydroxyflavone. Crossing white with yellow or ivory varieties produced plants bearing red or magenta flowers. This led her to hypothesize that an anthocyanin was formed from a flavone by the action of some factors contained in the white flower. She further suggested that the anthocyanin is either an oxidation or condensation product of a flavone or both. Scott-Moncrieff (50, p. 117) following up Wheldale's work, isolated cyanidin-3-rhamnoglucoside from Antirrhinum majus and noticed the presence of a pelargonidin-3-pentoseglycoside. No direct relationship existed between the two anthocyanins and the two flavones that had been identified by Wheldale. A thorough reinvestigation of the pigments of A. majus by Geissman and his co-workers (33, p. 72; 51, p. 5685) showed that a close biogenetic relationship did exist between certain of the flavonoids present. In addition to the apigenin and luteolin glycosides originally found, quercetin, kampferol and naringenin glycosides were also isolated and identified.

The chemical structure of anthocyanins was not elucidated until Willstatter (13, p. 168) showed that anthocyanins are 3-hydroxy flavonoid compounds. Later investigations (44, p. 172; 43, p. 732;

6, p. 242) have shown that natural anthocyanidins were of three basic types, based upon the number of phenolic hydroxyl groups in the B ring, as illustrated in Figure 4. A few examples of the occurrence of these pigments in plants are as follows: pelargonidin-3-monoglucoside in fruit of F. virginiana (45, p. 634), pelargonidin-3-rhamnoglucoside in A. majus (12, 598), pelargonidin-3, 5-diglucoside in potato (16, p. 263), cyanidin-3-rhamnoglucoside in A. majus flowers (12, p. 598), cyanidin-3-monoglucoside in Prunus cerasus flowers (37, p. 979) and delphinidin-3-monoglucoside in Vaccinium myrtillus fruit (42, p. 1039). While the basic flavonoid nucleus is present, the sugar moiety may vary as indicated above.

The primary objective of this research was the qualitative determination of the flavonoid components present in the inflorescences of three Douglas fir phenotypes. Knowledge of the structure of all flavonoid components could permit the development of a scheme of biosynthesis of all these compounds in the flowers of Douglas fir. Together with statistical information obtained from cross breeding studies currently under investigation at the Forest Research Laboratory, Oregon State University, it will eventually be possible to develop a sound understanding of the genetic control of flower color in Douglas fir. The complexity of the mixtures of flavonoid compounds present in the flowers of Douglas fir will also be illuminating with regard to the degree of sophistication of this color control mechanism in comparison with that of other species studied.

EXPERIMENTAL

Collection of Flower Samples

In initiating the study, only the red and green flowers of Douglas fir trees, located in Corvallis, Oregon, were collected. One year later, both red and green male and female flowers from different Douglas fir trees in the same vicinity were gathered, in addition to yellow female flowers. The flowers had opened and were ready for pollination. Immediately upon removal from the tree, the flowers were placed in one of the two extracting solutions, methanol or one percent methanolic hydrochloric acid (prepared by adding one ml. of concentrated hydrochloric acid for every 99 ml. of methanol). The solutions containing the flowers were refrigerated overnight.

Extraction of Flowers

Methanol and methanol-acid extractions of all flowers were performed identically. The original extracting solutions, approximately one liter, were removed from the flowers by vacuum filtration. Each flower batch was then treated with additional solvent in a Waring blender until no further color was noted in liquid; generally three extractions with 200 ml. portions of respective solvent

sufficed. The solutions were then evaporated in vacuo to a small volume, about 50-60 ml, at 40 degrees centigrade and were kept in a freezer while not in use.

Hydrolysis

The methanol extracts of the flowers were hydrolyzed by a procedure similar to that used by Nordstrom and Swain (38, p. 220). Twenty ml. of flower solution were mixed with fifty ml. of 2N sulfuric acid and heated on a steam bath for six hours. After cooling, the aqueous solution was extracted four times with fifty ml. portions of ethyl acetate to remove the flavonoid compounds. The combined ethyl acetate extracts were washed twice with fifty ml. of water and then taken to dryness in vacuo at forty degrees centigrade. The residue was dissolved in twenty-five ml. of methanol and stored in a freezer.

The aqueous layer of the extracted hydrolysate was neutralized (to litmus) with solid sodium bicarbonate and the neutral solution was concentrated to about five ml. in vacuo at forty degrees centigrade. This solution was chromatographed to determine the sugars present.

Paper Chromatography

Flavonoid pigments. Both one- and two-dimensional

chromatography, by the descending method, were employed in the analysis of the glycosides and their hydrolysis products. Whatman No. I filter paper and the following solvent systems were used (volume-volume mixtures unless otherwise indicated):

(1) butanol, acetic acid, water (4:1:5) (BAW) upper layer

(2) sixty percent acetic acid (aqueous) (60% HAc)

(3) fifteen percent acetic acid (aqueous) (15% HAc)

(4) water (H_2O)

(5) hydrochloric acid, water (three ml. concentrated hydrochloric acid added to ninety-seven ml. of water) (3% HCl)

(6) two percent acetic acid (aqueous) (2 % HAc)

(7) water, acetic acid, concentrated hydrochloric acid (82:15:3) (H_2O-HAc-HCl)

(8) chloroform, acetic acid, water (8:12:5) ($CHCl_3$-HAc-H_2O) lower layer.

Solvent pairs for two-dimensional chromatography were as follows:

(1) H_2O and BAW (4:1:5)

(2) H_2O and 15% HAc

(3) H_2O and $CHCl_3$-HAc-H_2O (8:12:5)

(4) BAW (4:1:5) and 15% HAc

After spotting, the papers to be developed with solvents having two phases (BAW, chloroform solvents) were equilibrated in an

atmosphere of the solvent for twelve hours before adding the developing solvent. Chromatograms were then air dried, examined under ultraviolet light, both before and after exposure to ammonia fumes, and treated with one of the following spray reagents:

(1) one percent aluminum chloride in methanol

(2) bis-diazotised benzidine (47, p. 65)

(3) vanillin- toluene - p-sulphonic acid (47, p. 65)

(4) one percent aqueous lead acetate

(5) sodium borohydride (29, p. 1733)

(6) three percent toluene-para- sulphonic acid in absolute ethanol (46, p. 973)

(7) cinnamaldehyde (23, p. 703).

Sugars. The sugar solution obtained by hydrolysis of the methanol extract was chromatographed by the same techniques employing the following solvents:

(1) BAW (4:1:5) upper phase

(2) ethyl acetate, water, pyridine (2:2:1) water poor phase

(3) ethyl acetate, water, pyridine (3:3:1) water poor phase

(4) amyl acetate, pyridine, ethanol, water (8:2:2:1)

Solvent mixtures containing ethyl acetate were prepared fresh for each run to insure solvent composition, inasmuch as ethyl acetate is hydrolyzed in the solvent mixture upon standing (32, p. 516).

The developed chromatograms, after air drying, were sprayed with one of the following sugar detection reagents:

(1) ammoniacal silver nitrate (7, p. 132)

(2) aniline hydrogen phthalate (7, p. 133)

(3) aniline hydrogen oxalate (7, p. 133)

(4) diphenylamine-urea (5, p. 58)

Spectroscopic Examination of Flavonoid Compounds

Solutions for measure of the ultraviolet spectra of the flavonoid compounds were obtained by elution of spots with ninety-five percent ethanol from two-dimensional chromatograms, or from bands where possible to obtain pure compounds by one-dimensional chromatography. The solutions obtained by elution of chromatogram spots were measured against a reference solution obtained by elution of a blank area of the chromatogram with ninety-five percent ethanol. Spectral measurements were made with a Beckman DB recording spectrophotometer.

Identification of flavonoid compounds from ultraviolet spectra was accomplished by the methods of Jurd (12, p. 107; 34, p. 376) and Jurd and Horowitz (30, p. 2446; 35, p. 1618). These spectrophotomeric procedures have been used extensively in determination of flavonoid structures (17, p. 176; 18, p. 101; 23, p. 701). These

procedures are particularly valuable in structure determination of the extremely small amounts of material obtained by chromatographic separations of plant extracts.

Isolation of Anthocyanin Pigment

Isolation of the anthocyanin was achieved by the technique suggested by Hayashi (12, p. 255). One hundred ml. of a saturated aqueous solution of lead acetate was added to 250 ml. of the red flower extract and the lead-anthocyanin complex removed by suction. The lead salt was then converted into the chloride by dissolving in cold ten percent methanolic hydrochloric acid. The chloride was precipitated from the filtrate with three times its volume of anhydrous ether. Repetition of the same procedure resulted in an amorphous product, which was dissolved in 20 ml. of 20 percent hydrochloric acid and kept in a refrigerator for three days. The red solid precipitate was recrystallized by dissolution in hot water and addition of an equal volume of seven percent hydrochloric acid. The solution was placed in the freezer and allowed to remain until crystallization occurred. The yield of the pure anthocyanin from the red flower extract was extremely small. Chromatography of the material indicated that it was a single compound.

Conversion of Dihydroquercetin-3'-Monoglucoside to Quercetin-3'-Monoglucoside

The eluted (in ninety-five percent ethanol) dihydroquercetin-3'-monoglucoside solution was evaporated to dryness *in vacuo* at 40 degrees centigrade. The residue was dissolved in eight ml. of aqueous twenty percent sodium bisulfite solution and heated for three hours on a steam bath (23, p. 703). After cooling, the aqueous solution was extracted five times with five ml. portions of ethyl acetate. The ethyl acetate extract was washed once with ten ml. of water and taken to dryness *in vacuo* at 40 degrees centigrade. The residue was dissolved in five ml. of ninety-five percent ethanol and analyzed by spectral and chromatographic procedures.

RESULTS

Standard Compounds

Paper chromatography has been an exceptionally effective technique in the separation and identification of flavonoid compounds, particularly in plant tissue studies where their concentration is, quite often, very small. The development of ultraviolet spectral methods for structural determination, particularly of flavonoid compounds, has greatly augmented chromatographic micro analytical procedures. Combination of the two analytical tools has achieved wide use in the investigation of the chemical content of plant tissues.

To adapt these procedures for use in this laboratory, it was necessary to develop standard procedures of chromatography and "calibrate" standard compounds to this procedure. Also, since color reactions with various spray reagents are often diagnostic of the group of compound present, it is necessary to record these colors for standard compounds as analyzed by the chromatographic techniques and solvents employed.

In Tables I and II are presented the R_f values in solvents employed and the color reactions of authentic flavonoid compounds which were used in this investigation. The R_f values of flavonoid

Table I. Flavonoids; R_f values.

Compound	Solvent Systems 1	2	3	4	5
Flavones					
Luteolin	--	.38	.55	.84	.57
Flavonols					
Kampferol	--	.10	.53	.85	.63
Queretin	--	.06	.41	.75	.40
Flavonol Glycosides					
Astralagin (K-3-Mono)	.17	.52	.79	.61	.59
Isoquercitrin (Q-3-Mono)	.12	.42	.72	.55	.44
Peonoside	.50	.76	.79	.29	.53
Quercetin-3'-Monoglucoside	.02	.11	.49	.37	.42
Quercetrin	.25	.57	.79	.83	.54
Rutin	.25	.42	.78	.36	.47
Dihydrochaleones					
Phloretin	.10	.30	.79	.93	.68
Phloridzin	--	.64	.82	.72	.61
Flavanes					
d-Catechin	.36	.59	.76	.65	.34
d-Epicatechin	.25	.42	.62	.52	.28
l-Epicatechin	.33	.47	.66	.55	.28
Flavanones					
Eriodictyol	.12	.35	.74	.88	.68
Hesperetin	.14	.42	.83	.93	.85
Homoeriodictyol	.18	.48	.82	.93	.85
Naringenin	.16	.41	.85	.93	.81
Flavanonols					
Dihydrokampferol	.32	.59	.83	.92	.63
Dihydromorin	.36	.61	.79	.85	.60
Dihydroquercetin	.33	.58	.75	.83	.54
Pinobanksin	.38	.63	.86	.93	.86
Flavanone Glycosides					
Naringin	.63	.85	.82	.53	.70
Sakuranin	--	.44	.85	.61	.75
Flavanonol Glycosides					
Astilbin	.50	.71	.80	.74	.51
Dihydroquercetin-3'-Monoglucoside	.50	.70	.80	.54	.50

1. Water 2. 15 percent acetic acid 3. 60 percent acetic acid 4. Butananol-acetic acid-water (4:1:5) 5. Chloroform-acetic acid-water (8:12:5)

Table II. Flavonoids; colors produced by sprays.

Compound	Chromogenic spraying reagent																	
	1		2		3		4		5		6		7		8		9	
	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.
Flavones																		
Luteolin	pY	B	--	--	--	Y	--	YG	Y	YB	Y	Y	YB	OB	Y	bY	--	--
Flavonols																		
Kampferol	pY	bY	--	--	--	Y	--	YG	Y	YG	Y	bY	Y	YG	Y	bY	--	--
Quercetin	pY	bY	--	--	--	Y	--	YG	Y	bY	Y	bY	Y	OY	Y	bY	B	--
Flavonol Glycosides																		
Astralagin	pY	B	--	--	--	--	--	YG	pY	bY	Y	bY	Y	Y	Y	YG	--	--
Isoquercitrin	pY	B	--	--	--	--	--	Y	pY	bY	Y	bY	YB	OY	Y	bY	pB	--
Peonoside	pY	B	--	--	--	--	--	Y	pY	bY	Y	bY	YB	OY	Y	bY	pB	--
Quercetin-3'-Monoglucoside	pY	bY	--	--	--	pY	--	pY	Y	bY	Y	bY	Y	OY	Y	bY	B	--
Quercetrin	pY	B	--	--	--	pY	--	Y	pY	YB	Y	bY	dY	YB	Y	bY	pB	--
Rutin	pY	B	--	--	--	Y	--	YG	Y	YB	Y	bY	Y	OB	Y	bY	pB	--
Dihydrochalcones																		
Phloretin	--	pB	--	--	P	--	VB	--	--	Y	--	Y	--	--	--	--	dB	--
Phloridzin	--	pO	--	--	P	--	VB	--	--	BlG	--	--	--	--	--	--	dB	--
Flavanes																		
d-Catechin	--	--	OB	--	bP	--	VB	--	--	--	--	--	--	--	--	--	dB	--
d-Epicatechin	--	--	OB	--	bP	--	VB	--	--	--	--	--	--	--	--	--	dB	--
l-Epicatechin	--	--	OB	--	bP	--	VB	--	--	--	--	--	--	--	--	--	dB	--

Table II (Continued) Flavonoids; colors produced by sprays.

Compound	Chromogenic spraying reagent																	
	1		2		3		4		5		6		7		8		9	
	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.	vis.	uv.
Flavanones																		
Eriodictyol	--	pB	--	--	pP	--	VB	--	--	pY	--	pY	--	--	V	--	B	--
Hesperitin	--	pB	--	--	pP	--	VB	--	--	YG	--	pY	--	--	V	--	B	--
Homoeriodictyol	--	pB	--	--	P	--	VB	--	--	YG	--	Y	--	--	V	--	OB	--
Naringenin	--	pB	--	--	P	--	pB	--	--	YG	--	YG	--	--	P	--	B	--
Flavanonols																		
Dihydrokampferol	--	pB	--	--	P	--	pVB	--	--	Y	--	YB	--	YB	--	--	dB	--
Dihydromorin	--	pB	--	--	P	--	VB	--	--	Y	--	Y	--	Y	--	--	dB	--
Dihydroquercetin	--	pB	--	--	pP	--	VB	--	--	Y	--	--	--	--	--	--	B	--
Pinobanksin	--	--	--	--	--	--	--	--	--	pY	--	YB	--	pY	--	--	B	--
Flavanone Glucosides																		
Naringin	--	pB	--	--	P	--	VB	--	--	YG	--	YG	--	--	P	--	OB	--
Sakuranin	--	--	--	--	P	--	VB	--	--	--	--	Y	--	--	V	--	--	--
Flavanonol Glycosides																		
Astilbin	--	pB	--	--	--	--	--	--	--	Y	--	--	--	--	--	--	OB	--
Dihydroquercetin-3'-Monoglucoside	--	--	--	--	--	--	--	--	--	pY	--	YB	--	pY	--	--	B	--

B - Brown
Bl - Blue
G - Green
O - Orange
P - Pink
V - Violet

Y - Yellow
d - Dark
p - Pale

1. Untreated
2. Cinnamaldehyde
3. Vanillin
4. Toluene-para-sulphonic acid
5. Aluminum chloride (1% methanolic)
6. Ammonia
7. Lead Acetate (1% neutral)
8. Sodium borohydride
9. Bis-diazotised benzidine

compounds obtained when chloroform-acetic acid-water (8:12:5) was used were found to be especially sensitive to temperature, freshness of solvent and extent of equilibration of the filter paper with respect to the volatile portion of the solvent. Although fresh solutions of chloroform-acetic acid-water (8:12:5) were used and the chromatogram paper equilibrated, temperature was not controlled. Results with the other solvents used were more reproducible than chloroform-acetic acid-water (8:12:5). Standard compounds were always run along with the extracts to cancel out any variations in runs, such as temperature and paper saturation.

Recently the use of sodium borohydride as a detecting reagent for flavanones has been reported (30, p. 2446). While a series of flavanones was included in this report, only a single flavanonol, dihydroquercetin, was studied. It was found that this compound did not react to give a color with this reagent. In the study of standard compounds and their color reactions, particular emphasis was given this reagent, and a number of flavanones and flavanonols were tested. Flavanones were found to react with sodium borohydride to give pink or violet colors and all flavanonols tested failed to react (Table II).

Comparison of Flower Extracts

When the extracts of the red, yellow and green flowers of

Douglas fir were compared by two-dimensional paper chromatography, only one difference was noticed. Present in the methanolic-acid extract of both the red male and female flowers was an anthocyanin, subsequently identified as cyanidin-3-monoglucoside, which was not present in the methanolic-acid extract of the other flower phenotypes. No difference was detected between the male and female flowers of the same phenotype. The methanol and methanolic-acid extracts of each flower phenotype were identical except that the methanolic-acid extract contained the sugar-free compounds, the aglycones, kampferol and quercetin.

Identification Studies of Hydrolysis Products

During the hydrolysis procedure, a distinct difference between the red, green and yellow extracts was noticeable. The red extract turned tomato red when 2N sulfuric acid was added whereas the green and yellow extracts turned yellow-green. During the heating period, all three extracts turned dark red-brown. The immediate change in color observed in the red flower methanol solution upon addition of heat is probably due to hydrolysis of cyanidin-3-monoglucoside to cyanidin. The red-brown color developing in the yellow and green solutions as the heating progressed could be due to formation of the highly colored phlobaphenes due to condensation of the

catechins; reactions these compounds are known to undergo upon acid treatment (12, p. 211). The disappearance of these compounds during the acid treatment can be interpreted as further confirmation. Both catechins are present in all extracts prior to hydrolysis, but are absent in the extracts after acid hydrolysis. Cyanidin did not appear when the red ethyl acetate extract was chromatographed since it is unstable in the solvents used in chromatography and fades on the chromatogram.

Five flavonoid compounds were detected on the paper chromatogram when the ethyl acetate extract was chromatographed. In Table III are listed R_f values obtained with the solvents used and color reactions with spray reagents. A typical two-dimensional chromatogram is illustrated in Figure 5.

Identification of quercetin and kampferol. The ethyl acetate hydrolysate, when chromatographed, contained two spots (spots nos. 1 and 2 in Figure 5) which fluoresced yellow under ultraviolet light and had low R_f values in aqueous solvents, indicating they were flavonols. R_f values for the two spots were identical with quercetin and kampferol when co-chromatographed.

Spectral analysis (Figures 6 and 7) of the two compounds provided further proof of their structure. Spot no. 1 (quercetin) had absorption maxima of 256 millimicrons and 373 millimicrons in 95

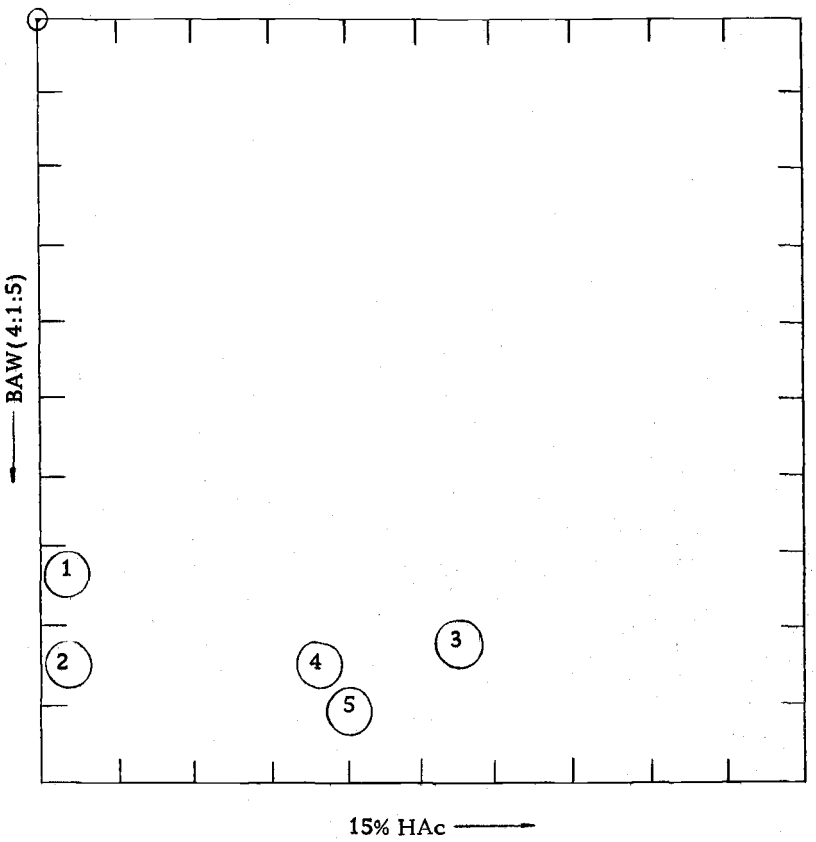
Table III. Color reactions and R_f values of flavonoid compounds obtained by hydrolysis of flower extracts.

Spot No. (Figure 5)	Chromogenic spraying reagent										R_f value			
	1		2		3		4		5		H_2O	15% HAc	BAW (4:1:5)	$CHCl_3$ (8:12:5)
	vis.	u. v.	vis.	u. v.	vis.	u. v.	vis.	u. v.	vis.	u. v.				
1	[b]pY	bY	pB	--	--	Y	--	OY	Y	dY	.00	.06	.75	.40
2	pY	bY	--	--	--	Y	--	YG	Y	dY	.00	.09	.85	.62
3	--	--	B	--	--	--	--	pY	--	--	.31	.57	.82	.54
4	--	--	pB	--	V	--	--	pY	--	--	.12	.35	.88	.68
5	--	--	pB	--	P	--	--	pYG	--	--	.16	.41	.93	.81

1. No reagent
2. Bis-diazotised benzidine
3. Sodium borohydride
4. Aluminum chloride (1% methanolic)
5. Ammonia

B - Brown
G - Green
O - Orange
P - Pink
V - Violet
Y - Yellow

b - Bright
p - Pale
d - Dark



1. Quercetin
2. Kampferol
3. Dihydroquercetin
4. Eriodictyol
5. Naringenin

Figure 5. Schematic chromatogram of hydrolytic products of flower extracts.

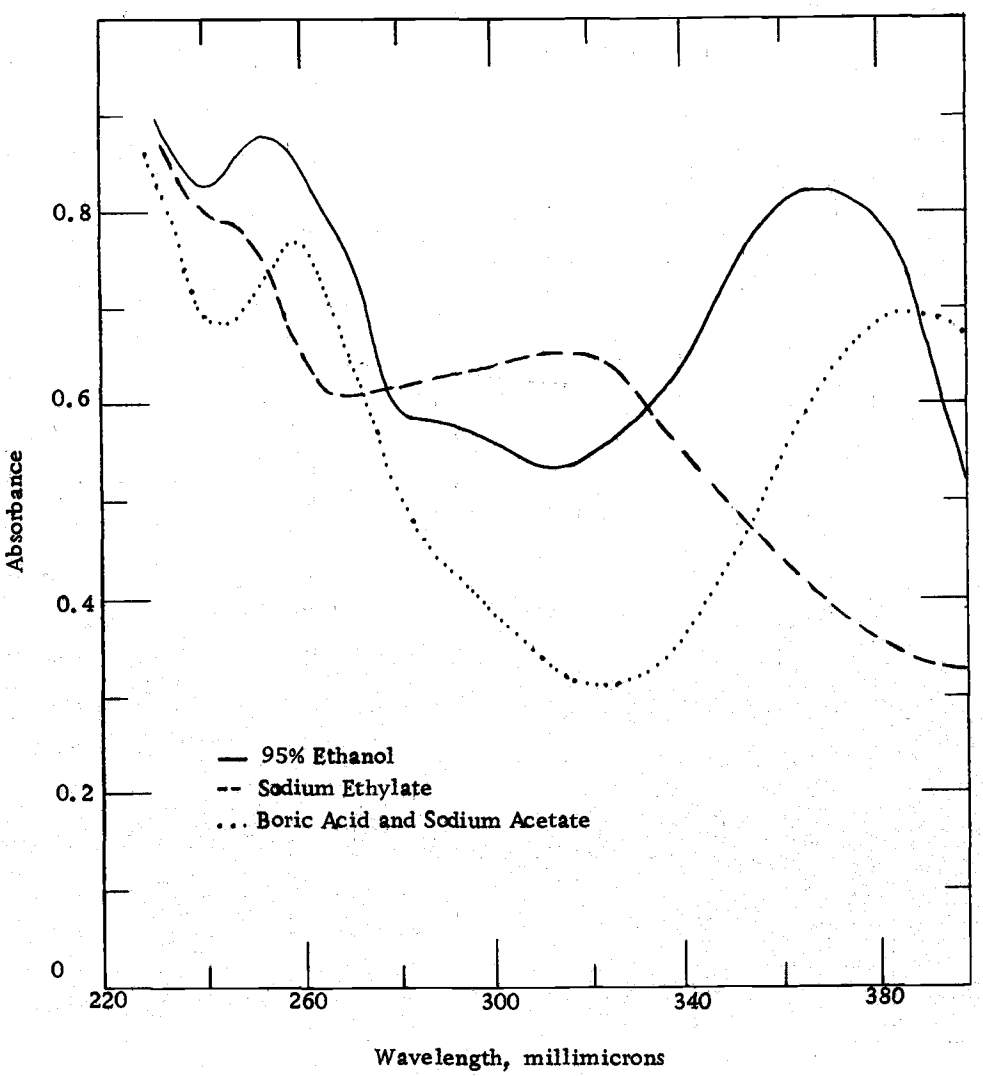


Figure 6. Ultraviolet absorption spectra of quercetin.

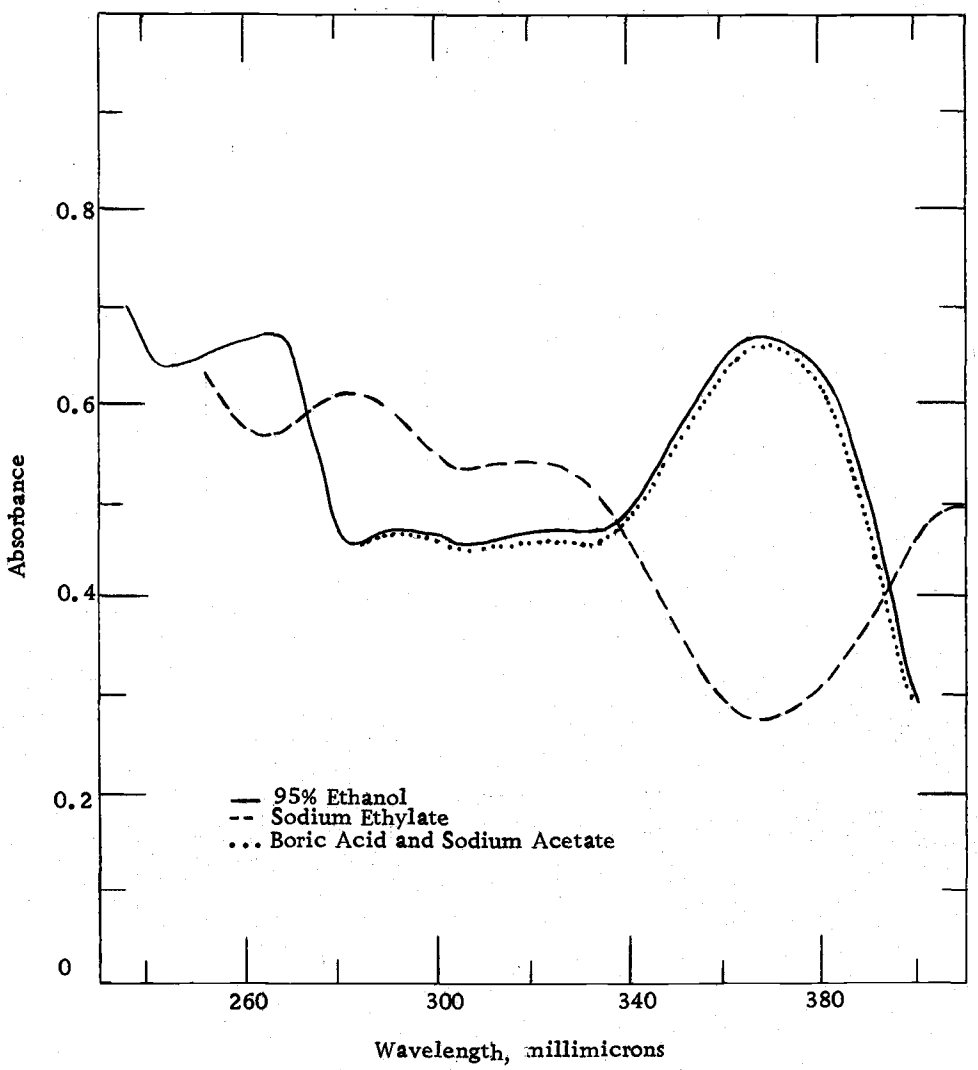


Figure 7. Ultraviolet absorption spectra of kampferol.

percent ethanol. When sodium ethylate was added, the ethanol solution turned yellow and the absorption maximum shifted from 373 millimicrons to 320 millimicrons. No shift of the lower wavelength maximum occurred. The shift observed indicates the presence of 3 and 4'-dihydroxyl groups in the flavone nucleus. A shift of the 373 millimicron maximum to 390 millimicrons, with no shift of the lower wavelength maximum, was observed when boric acid and sodium acetate were added, indicating the presence of o-dihydroxyl groups in the B ring of this flavonol.

When the same analysis was repeated with the 95 percent ethanol elute of spot no. 2 (kampferol), the following was observed: maximum absorption in ethanol at 256 millimicrons and 370 millimicrons; a shift of the 370 millimicron absorption maximum to 320 millimicrons when sodium ethylate was added, indicating unsubstituted 3 and 4'-dihydroxyl groups; no shift in the presence of boric acid and sodium acetate, indicating that o-dihydroxyl groups were not present in the B ring of this flavone.

Identification of dihydroquercetin, eriodictyol and naringenin. The remaining three flavonoid compounds (spots nos. 3, 4, and 5 in Figure 5) were not visible under ultraviolet light. However, their color reactions with sodium borohydride were quite diagnostic in their structure determination. Spot no. 3 (dihydroquercetin),

though reacting with bis-diazotised benzidine, gave no color with sodium borohydride, whereas spot no. 4 (eriodictyol) gave a violet color and spot no. 5 (naringenin) reacted to give a pink color. Authentic dihydroquercetin gave no color with sodium borohydride whereas authentic eriodictyol and naringenin gave violet and pink colors respectively. These three spots lined up consistently with dihydroquercetin, eriodictyol and naringenin, when the hydrolysate was co-chromatographed with the three standards.

Absorption spectra of the three compounds are illustrated in Figure 8. Dihyroquercetin displayed a maximum absorption at 290 millimicrons, eriodictyol at 289 millimicrons and naringenin at 288 millimicrons.

Sugars

Only one sugar, glucose, was detected in the aqueous layer hydrolysate. This was identified by color reaction comparison and co-chromatography with sugar standards. The following color reactions were noted:

(1) ammoniacal silver nitrate - brown

(2) aniline hydrogen phthalate - brown

(3) aniline hydrogen oxalate - brown

(4) diphenylamine-urea - pink.

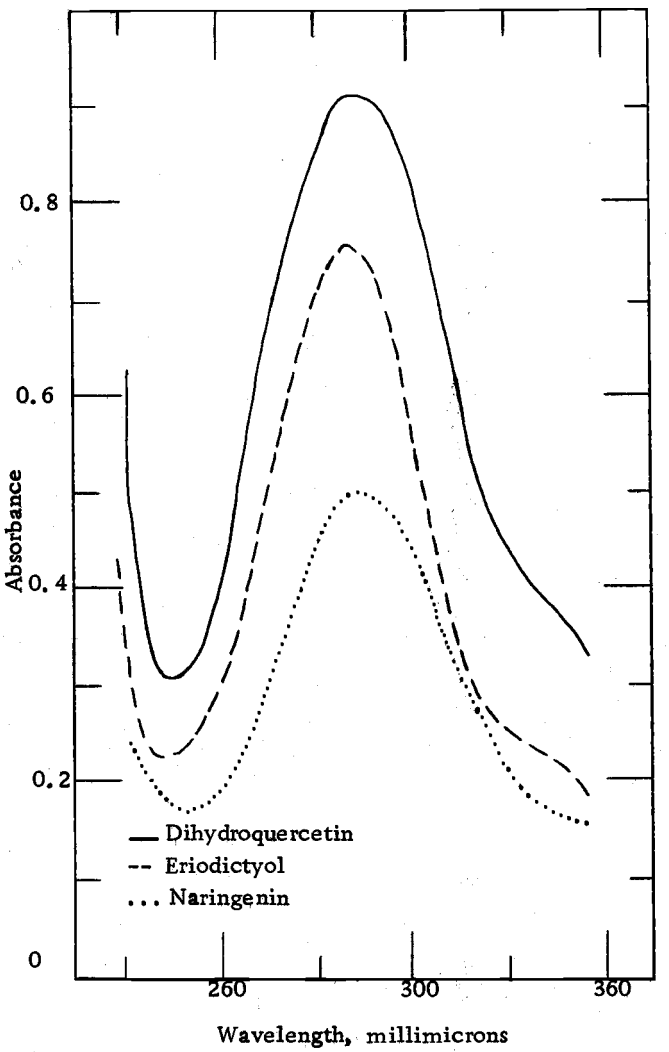


Figure 8. Ultraviolet absorption spectra of dihydroquercetin, eriodictyol and naringenin.

R_f values were as follows:

(1) butanol, acetic acid, water (4:1:5) - 0.18

(2) ethyl acetate, water, pyridine (2:2:1) - 0.19

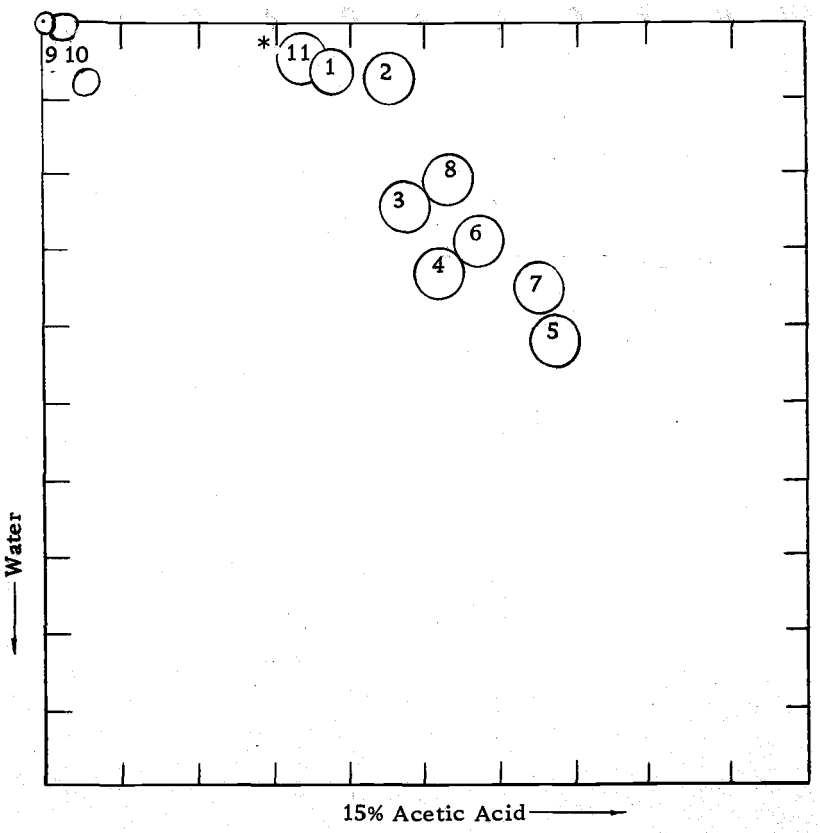
(3) ethyl acetate, water, acetic acid (3:3:1) - 0.10

(4) amyl acetate, pyridine, ethanol, water (8:2:2:1) - 0.03.

Identification of Flavonoid Compounds in the Methanol and Methanolic Acid Flower Extracts

Two-dimensional chromatography was used exclusively in the separation and identification of the flavonoid compounds of the methanol and methanolic-acid extracts of the flowers. Schematic representations of these chromatograms are shown in Figures 9 and 10. The color reactions and R_f values for these compounds are listed in Table IV.

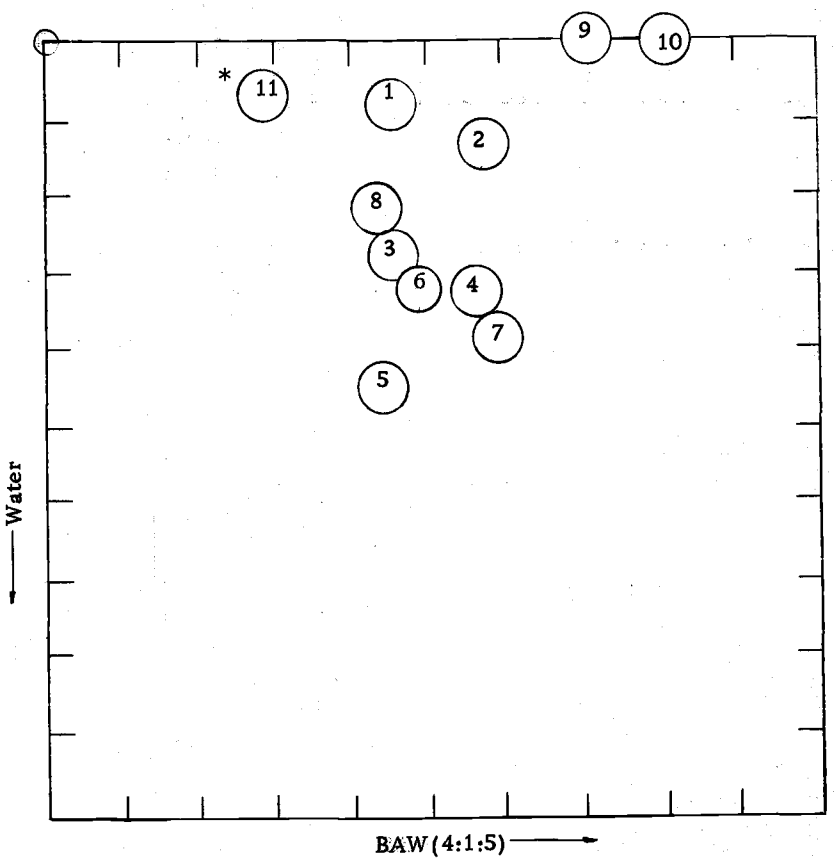
Identification of quercetin and kampferol. On the two-dimensional chromatogram of the flower extracts, two spots fluoresced yellow under ultraviolet light. Using the same procedure employed in the identification of quercetin and kampferol in the hydrolysate, these compounds were also identified in the methanolic-acid extracts of all flowers.



1) Quercetin-3-Monoglucoside
2) Kampferol-3-Monoglucoside
3) l-Epicatechin
4) d-Catechin
5) Dihydroquercetin-3'-glucoside
6) Eriodictyol-7-glucoside
7) Naringenin-7-glucoside
8) Kampferol-3-diglucoside
9) Quercetin
10) Kampferol
11) Cyanidin-3-Monoglucoside

*Found only in acid extract of red flower

Figure 9. Schematic chromatogram of red, green, and yellow flower extracts of male and female flowers.



1) Quercetin-3-Monoglucoside
2) Kampferol-3-Monoglucoside
3) l-Epicatechin
4) d-Catechin
5) Dihydroquercetin-3'-glucoside
6) Eriodictyol-7-glucoside
7) Naringenin-7-glucoside
8) Kampferol-3-diglucoside
9) Quercetin
10) Kampferol
11) Cyanidin-3-Monoglucoside

* Found only in acid extract of red flower

Figure 10. Schematic chromatogram of red, green and yellow flower extracts of male and female flowers.

Table IV. Color reactions and R_f values of flavonoid pigments present in the flowers of Douglas fir.

Spot no. (Figures 9 and 10)	Chromogenic spraying reagent												R_f value			
	1		2		3		4		5		6		H_2O	15% HAc	BAW (4:1:5)	$CHCl_2$ (8:12:5)
	vis.	u. v.	vis.	u. v.	vis.	u. v.	vis.	u. v.	vis.	u. v.	vis.	u. v.				
1	Y	bY	pB	--	--	bY	--	--	--	--	--	YG	.13	.41	.54	.44
2	--	bY	pB	--	--	bY	--	--	--	--	--	YG	.17	.52	.68	.59
3	--	--	B	--	--	--	OB	--	P	--	VB	--	.30	.47	.52	.28
4	--	--	B	--	--	--	OB	--	P	--	VB	--	.36	.59	.63	.34
5	--	pYG	B	--	--	--	--	--	--	--	--	--	.50	.71	.54	.49
6	--	pYG	pB	--	V	--	--	--	--	--	--	--	.36	.61	.53	.53
7	--	pYG	pB	--	P	--	--	--	--	--	--	--	.44	.69	.65	.69
8	--	pY	--	--	--	bY	--	--	--	--	--	--	.28	.56	.45	.61
9	Y	--	pB	--	--	Y	--	--	--	Y	-	YG	.00	.06	.75	.40
10	Y	--	pB	--	--	Y	--	--	--	Y	--	YG	.00	.09	.85	.62
11	--	--	--	--	--	--	--	--	--	--	--	--	.10	.36	.26	--

1. Aluminum chloride (1% methanolic)
2. Bis-diazotized benzidine
3. Sodium borohydride
4. Cinnamaldehyde
5. Vanillin
6. Toluene-para-sulphonic acid

B - Brown
G - Green
O - Orange
P - Pink
V - Violet
Y - Yellow

b - Bright
p - Pale

Identification of quercetin-3-monoglucoside, kampferol-3-monoglucoside and kampferol-3-diglucoside. Three spots (nos. 1, 2 and 8 in Figures 9 and 10) appeared brown on the paper chromatogram under ultraviolet light and were mobile in aqueous solvents indicating flavonols glucosidated in the three-position. The three-glycosides of flavonols are mobile in aqueous solvents (15, p. 583) and are brown on chromatogram paper when illuminated with ultraviolet light (Table II). Spot no. 8 travelled further on paper than the other two indicating it probably had more than one glucose unit attached to the flavonol nucleus. Greater glycosidation increases the distance travelled on paper in aqueous solvents.

Kampferol and quercetin were the only flavonols identified in the hydrolysate, thus the three spots had to be glucosides of these compounds. When the flower extracts were co-chromatographed with authentic flavonol glucosides, spot no. 1 had the same R_f values as quercetin-3-monoglucoside and spot no. 2 travelled the same as kampferol-3-monoglucoside. It was not possible to obtain authentic kampferol-3-diglucoside, but reported R_f values of this compound were identical to those of spot no. 8 and spectral analysis provided confirmation.

Spectral data of the three compounds are shown in Figures 11, 12 and 13. The ultraviolet spectrum of spot no. 1 (quercetin-3-

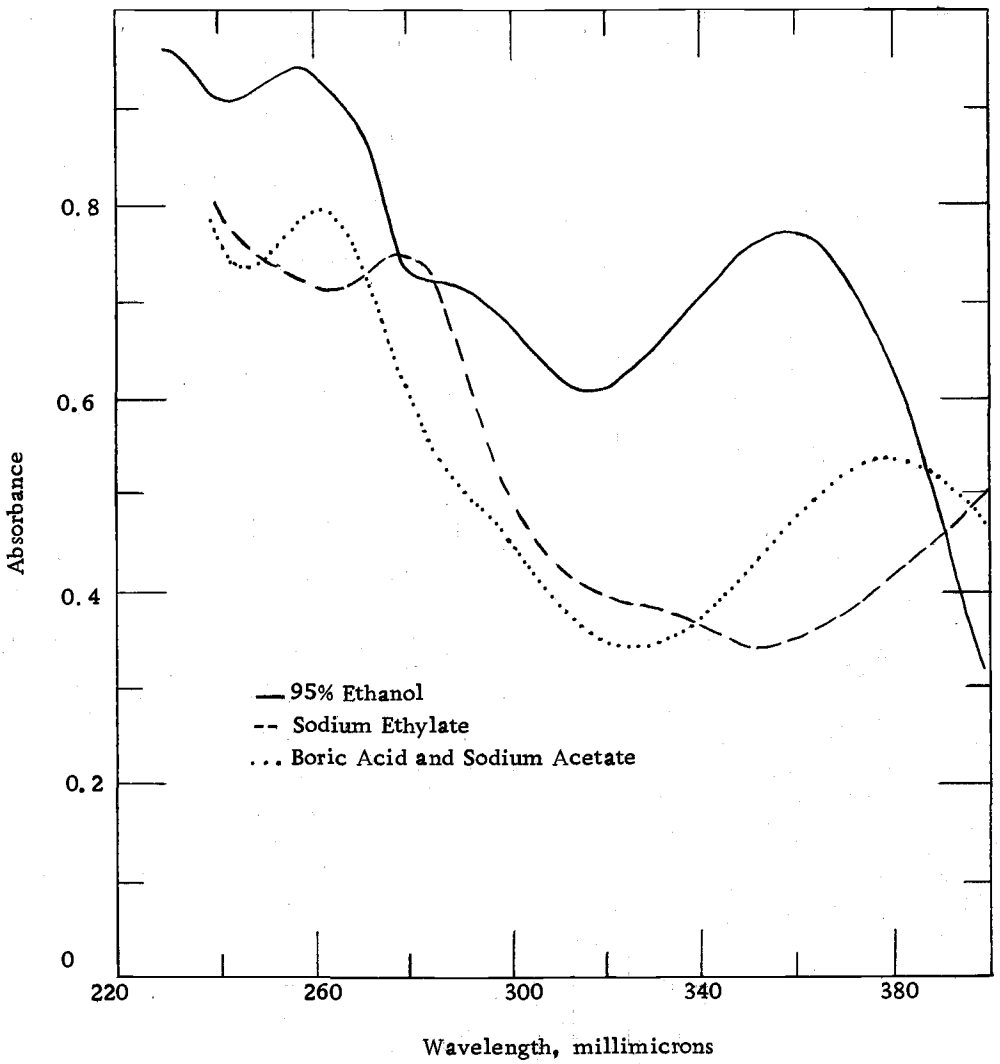


Figure 11. Ultraviolet absorption spectra of quercetin-3-monoglucoside.

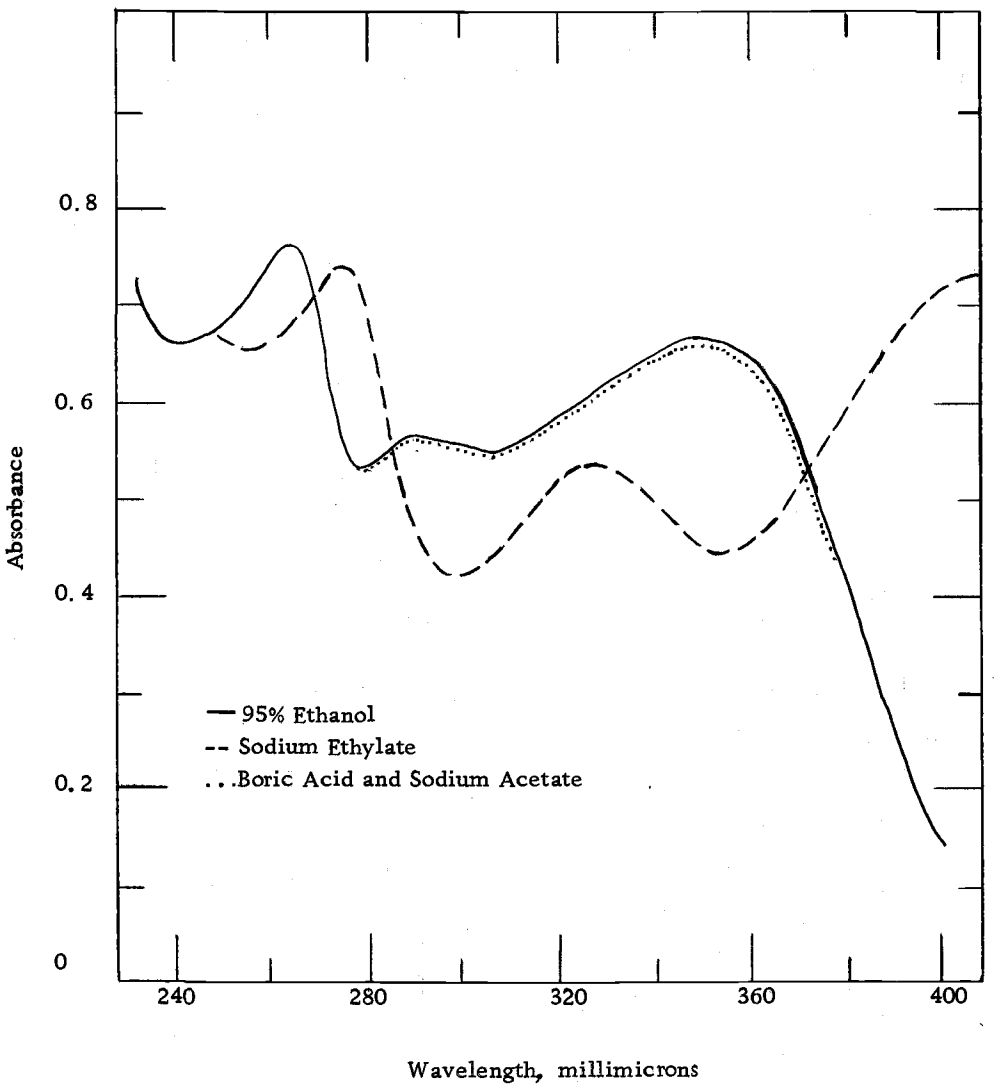


Figure 12. Ultraviolet absorption spectra of kampferol-3-monoglucoside.

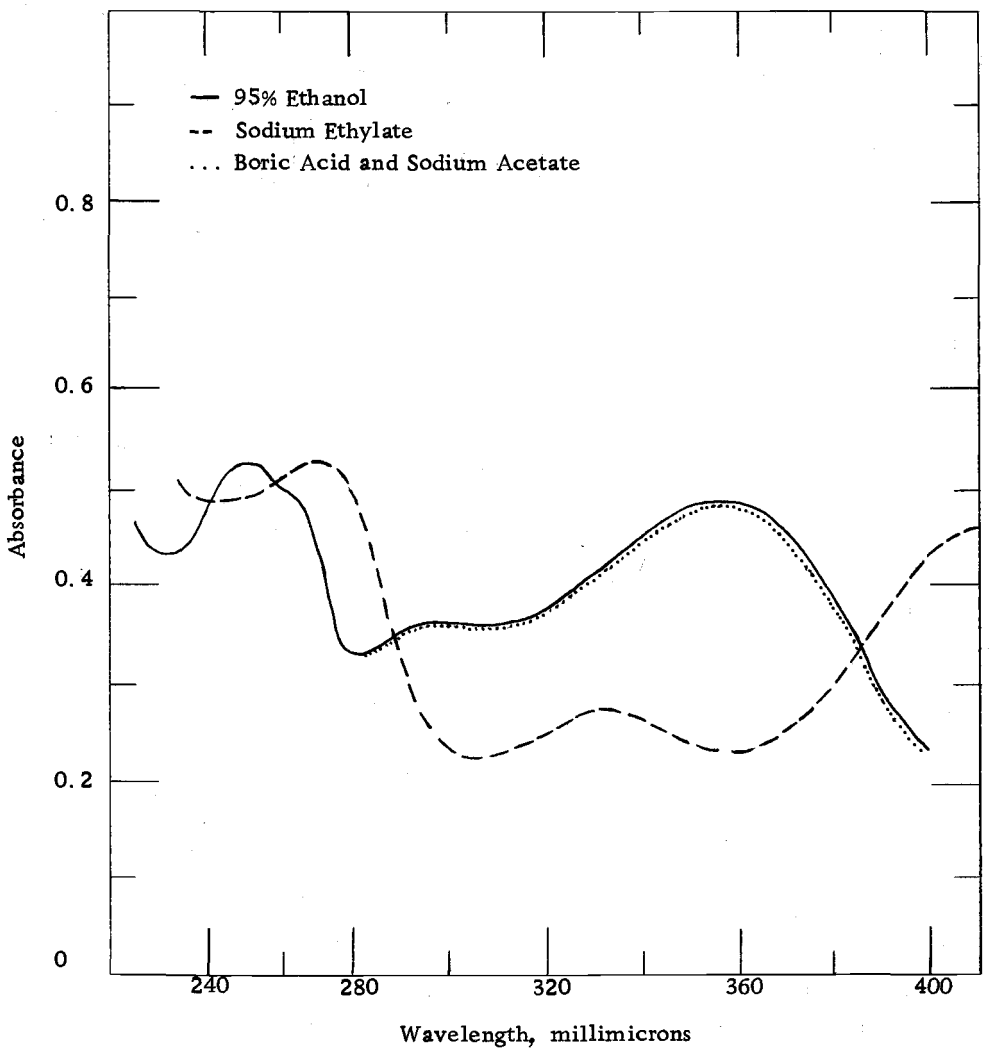


Figure 13. Ultraviolet absorption spectra of kampferol-3-diglucoside.

monoglucoside), measured in 95 percent ethanol, had maximum absorption at 256 and 360 millimicrons. Sodium ethylate caused a shift of the spectral maximum at 360 millimicrons to 415 millimicrons, indicating substitution at either the 3- or 4'-hydroxyl groups of the molecule. Saturated boric acid and excess sodium acetate shifted the 360 millimicron maximum to 380 millimicrons, signifying the presence of o-dihydroxyl grouping in the B ring. This indicated that the 4' position was unsubstituted and hence sugar attachment was at the 3-hydroxyl position.

Repeating the above procedure with spot no. 2 (kampferol-3-monoglucoside) produced the following results: maxima at 266 millimicrons and 352 millimicrons in 95 percent ethanol; a shift of the 266 millimicron maximum to 415 millimicrons upon addition of sodium ethylate, showing the absence of unsubstituted 3, 4'-dihydroxyl groups; no shift was observed with boric acid and sodium acetate, indicating no o-dihydroxyl group in the B ring.

The following spectral data were observed for spot no. 8 (kampferol-3-diglucoside) using the same technique as above: maxima at 260 millimicrons and 352 millimicrons in 95 percent ethanol; a shift of the 352 maximum to 415 millimicrons upon addition of sodium ethylate, showing the absence of unsubstituted 3, 4'-dihydroxyl groups; no shift was noted with boric acid and sodium acetate,

indicating the absence of a o-dihydroxyl group in the B ring.

Identification of cyanidin-3-monoglucoside. From chromatographic and spectral data, the isolated red pigment appeared to be cyanidin-3-monoglucoside. Co-chromatography and infra-red spectral comparison with an authentic sample proved its identity. The R_f values found were:

(1) 15% acetic acid - 0.36

(2) butanol, acetic acid, water (4:1:5) - 0.31 (reported 0.38 (14, p. 473))

(3) 2% acetic acid - 0.11

(4) hydrochloric acid, water (3:97) - 0.080 (reported 0.070 (14, p. 473))

(5) water, acetic acid, hydrochloric acid (82:15:3) - 0.27 (reported 0.26 (14, p. 473)).

The spectrum of a 0.01 percent hydrochloric acid solution is shown in Figure 14. Maximum absorption occurred at 282 millimicrons and 536 millimicrons. The ratio of the optical density at 440 millimicrons to that at absorption maximum is a useful means of distinguishing 3-glycosides and 3, 5-diglycosides (12, p. 134), the ratio being substantially higher for anthocyanidin-3-monoglycosides. The ratio calculated for cyanidin-3-monoglucoside was 22.9 percent. It is reported as 22 percent (12, p. 133). The reported

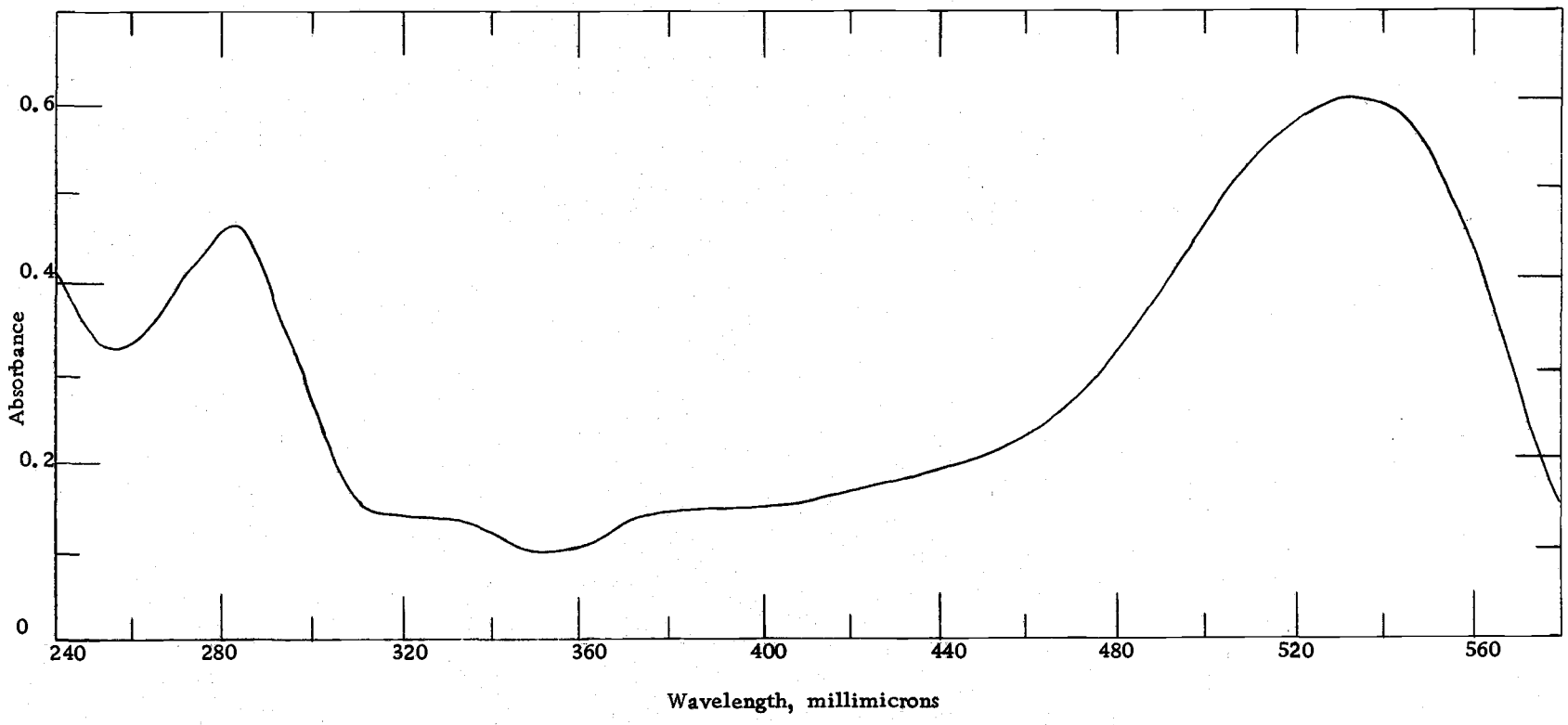


Figure 14. Ultraviolet absorption spectrum of cyanidin-3-monoglucoside.

value for cyanidin-3, 5-diglucoside is 13 percent.

Identification of d-catechin and l-epicatechin. Comparison of color reactions with authentic d-catechin and l-epicatechin was particularly important in the identification of spots nos. 3 and 4 (Figures 9 and 10). Spots nos. 3 and 4 were the only two spots to react with cinnamaldehyde, giving an orange-brown color. Only authentic d-catechin and l-epicatechin, among the standard compounds tested, gave this color reaction with this reagent. The two spots also reacted rapidly, giving intense colors with bis-diazotised benzidine, as did the authentic compounds. Co-chromatography with the standard compounds gave identical R_f values in all solvents.

Spectra of the two spots are shown in Figure 15. Maximum absorption for both compounds occurs at 280 millimicrons.

Identification of dihydroquercetin-3'-monoglucoside. Since dihydroquercetin was detected as one of the hydrolysis products and was not found in any of the flower extracts prior to hydrolysis, it must then be present in the flower extracts as a glucoside. Spot no. 5 (Figures 9 and 10) produced similar color reactions as dihydroquercetin and had an identical ultraviolet spectrum but markedly different R_f values. Authentic dihydroquercetin-3'-monoglucoside had the same R_f values in all solvents.

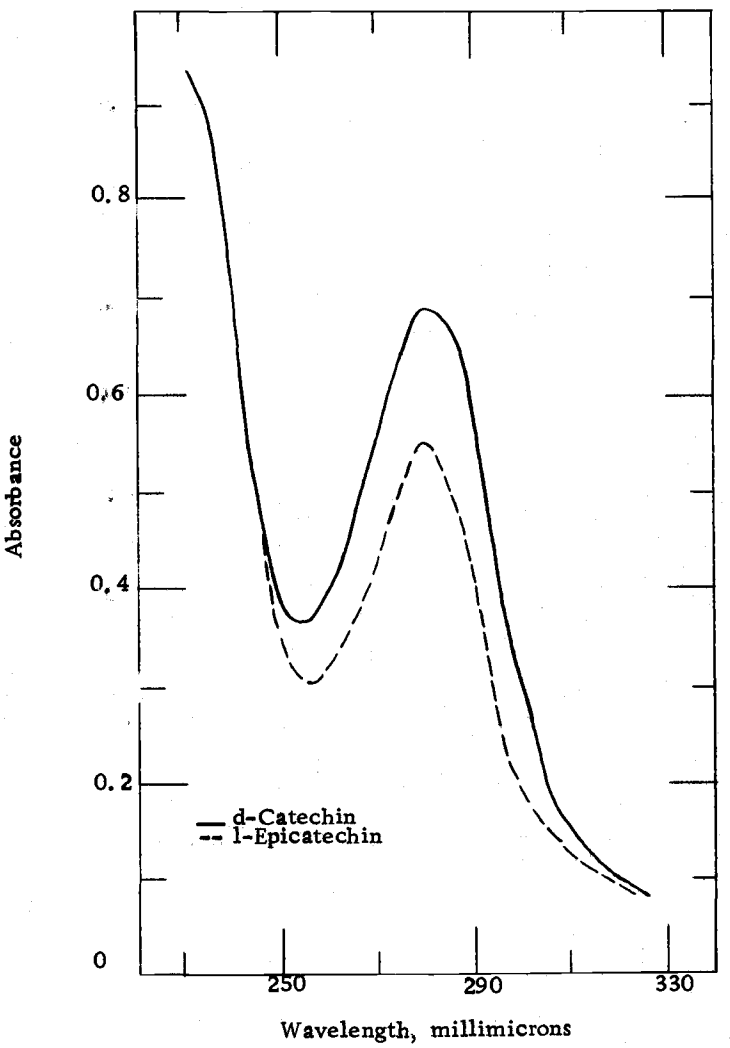


Figure 15. Ultraviolet absorption spectra of d-catechin and l-epicatechin.

In order to varify the position of the sugar, ultraviolet spectral shifts with various chemical agents was measured (Figure 16). The absorption maximum in the 95 percent ethanol solution occurred at 290 millimicrons. Addition of sodium acetate shifted the spectral maximum to 330 millimicrons, indicating the seven-hydroxyl group was unsubstituted (30, p. 2447). The spectrum of dihydroquercetin in 95 percent ethanol, to which sodium acetate had been added, is shown in Figure 16. Spectral maximum were observed at 248 millimicrons and 330 millimicrons in the presence of 0.006N potassium hydroxide; identical to those observed with dihydroquercetin-3'-monoglucoside upon addition of 0.006N potassium hydroxide (23, p. 701). Aluminum chloride added to the 95 percent ethanol solution shifted the spectral maximum to 310 millimicrons, indicating an unsubstituted five-hydroxyl group.

Aqueous sodium bisulfite solutions are known to convert flavanonols to the corresponding flavonols (23, p. 702); the reaction does not occur with flavanones or flavanonols in which the three-hydroxyl position is substituted (2). When spot no. 5 was treated with 20 percent aqueous sodium bisulfite, the solution turned yellow indicating conversion to the flavonol-glucoside. This meant that the sugar moiety was not attached to the three-hydroxyl group. Co-chromatography of the product with authentic quercetin-3'-monoglucoside

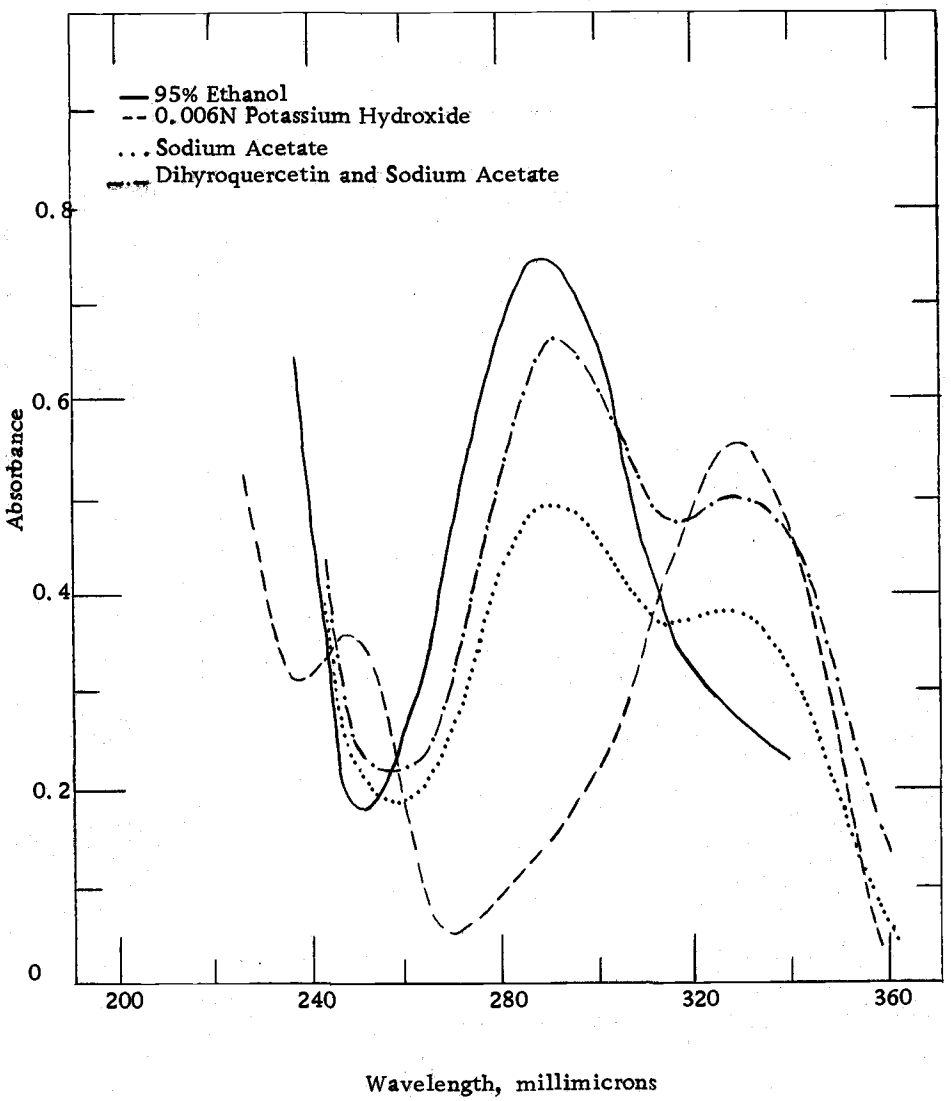


Figure 16. Ultraviolet absorption spectra of dihydroquercetin-3'-glucoside.

showed they were identical.

Identification of eriodictyol-7-monoglucoside and naringenin-7-monoglucoside. Spots no. 6 and no. 7 (Figures 9 and 10) gave violet and pink colors respectively when sprayed with sodium borohydride. Since the two spots had markedly different R_f values than those spots in the hydrolysis extract giving violet and pink colors with this reagent, they must be glucosides of eriodictyol and naringenin. No glucosides of eriodictyol and naringenin for co-chromatographic comparison were available. Spot no. 7 had identical R_f values as those reported for naringenin-7-monoglucoside (15, p. 591). R_f values for standard eriodictyol glucosides were not available. Since the R_f values of these two glucosides were low in aqueous solvents compared to reported values of naringenin and eriodictyol di-glycosides (15, p. 59), it was concluded that they must be monoglucosides.

To verify the position of the sugars in these two compounds, spectral shifts with chemical agents were measured (Figures 17 and 18). Absorption maximum of the 95 percent ethanol elute of spot no. 7 was at 285 millimicrons. Addition of sodium acetate to the elute produced no shift of this maximum, indicating the 7-hydroxyl group was substituted (30, p. 2447). Only 4'-hydroxy-7-alkoxy- or 4'-hydroxy-7-glucosdoxyflavanones form chalcones (broad maximum

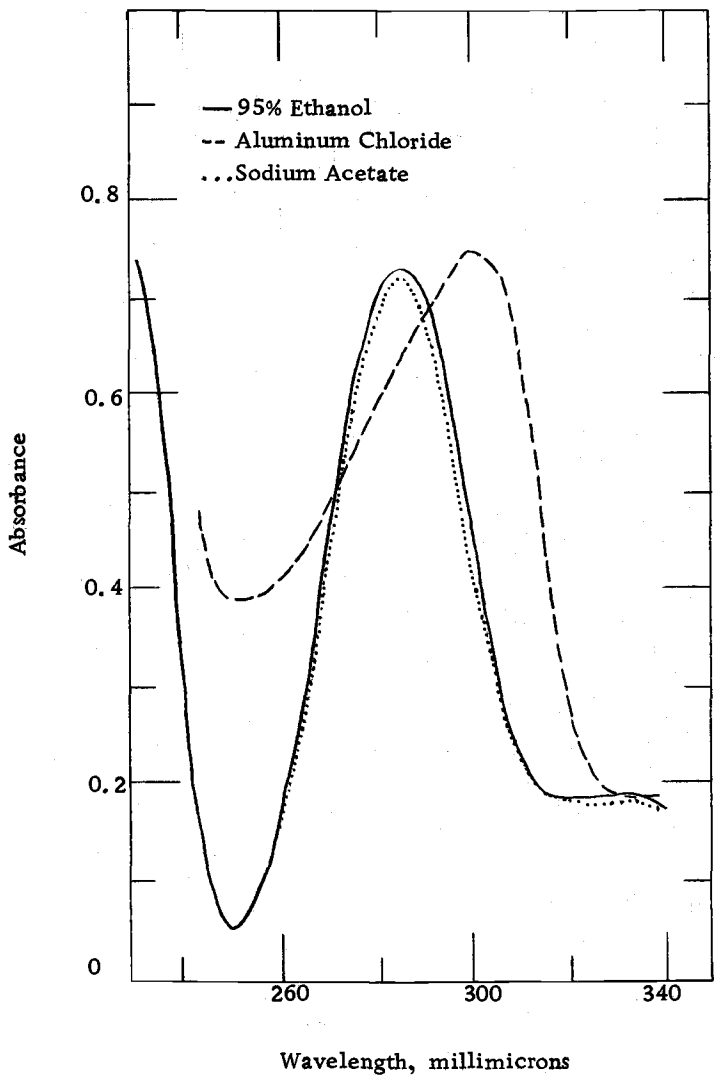


Figure 17. Ultraviolet absorption spectra of eriodictyol-7-glucoside.

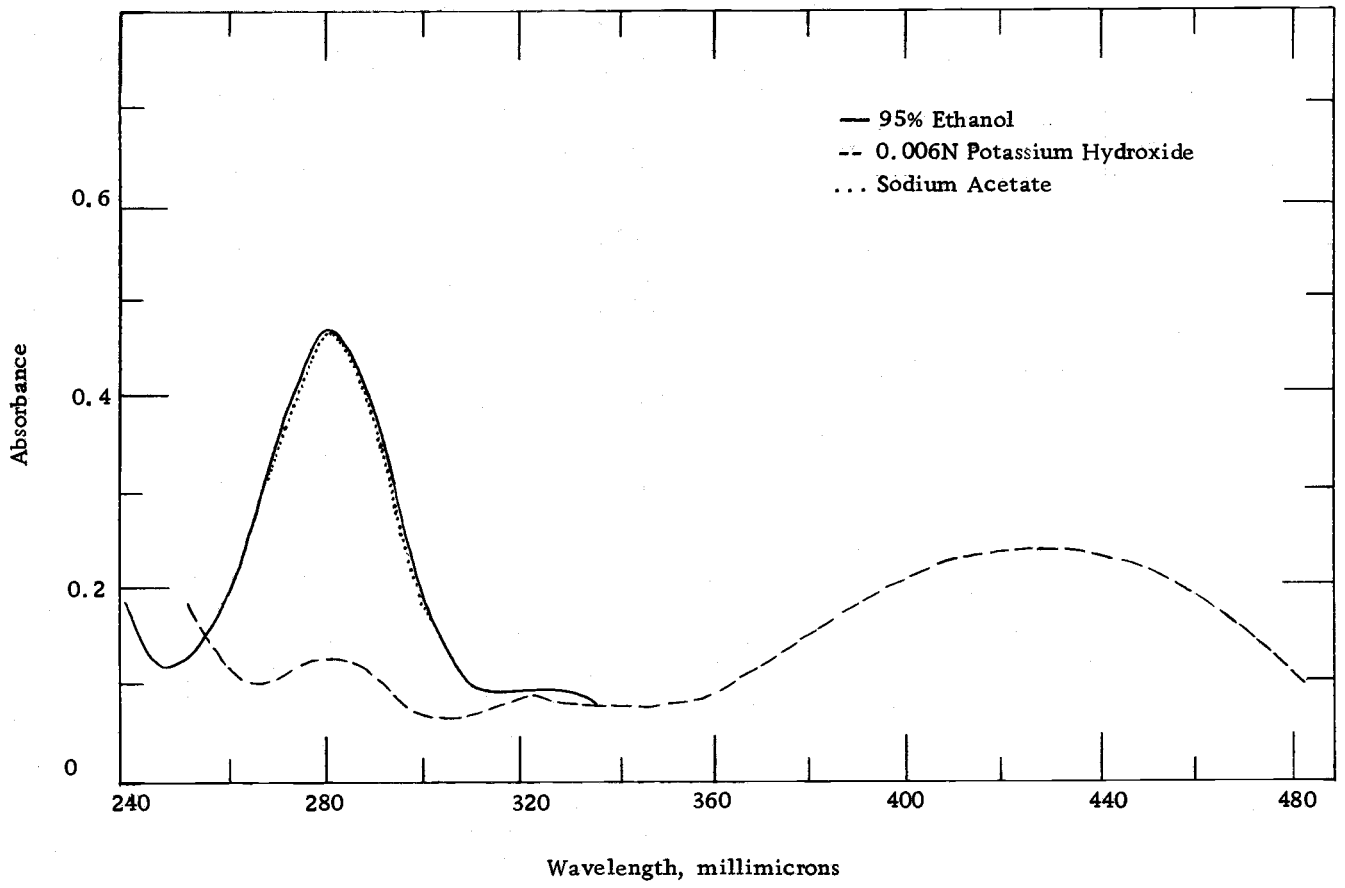


Figure 18. Ultraviolet absorption spectra of naringenin-7-glucoside.

absorption in the region of 400-450 millimicrons) rapidly in very dilute alkali (30, p. 2447). The spectral maximum of the elute of spot no. 7 was shifted to 430 millimicrons in 0. 006N potassium hydroxide, indicating that the unsubstituted hydroxyl group is in the B ring at the 4' position and thus the 7-hydroxyl group is substituted. A shift of the maximum to 306 millimicrons with aluminum chloride signified the 5-hydroxyl group is unsubstituted. Thus, it was concluded that spot no. 7 is naringenin-7-monoglucoside.

Similar treatment of the alcohol elute of spot no. 6 (eriodicityol-7-monoglucoside), produced the following results: absorption maximum at 285 millimicrons; no shift of the maximum with sodium acetate, indicating the 7-hydroxyl was substituted; no shift of the maximum with 0. 006N potassium hydroxide further verifying substitution of the 7-hydroxyl group and that there must be another hydroxyl group in the B ring (23, p. 700); a shift of the maximum to 306 millimicrons with aluminum chloride, indicating an unsubstituted 5-hydroxyl group. From this spectral and chromatography data, it was concluded that spot no. 6 is eriodictyol-7-monoglucoside.

Test for the presence of leucoanthocyanins. To test for the occurrence of leucoanthocyanins, earlier believed to be the direct precursors of red pigments, the chromatograms were sprayed with toluene-para-sulphonic acid (46, p. 973) and cinnamaldehyde (23,

p. 700), which react to give brown and orange-brown colors respectively with these compounds. The two spray reagents developed only two spots, which were subsequently identified as d-catechin and l-epicatechin. However, before any chromatographic studies were made, the absence of leucoanthocyanins was suspected since the yellow and green flower extracts did not turn red upon addition of acid in the hydrolysis. Acid will convert leucoanthocyanins to anthocyanins causing the solution to turn red (24, p. 263).

DISCUSSION

Only the following flavonoid compounds were found in the three flower phenotypes of Douglas fir analyzed: kampferol, quercetin, quercetin-3-monoglucoside, kampferol-3-monoglucoside, kampferol-3-diglucoside, dihydroquercetin-3'-monoglucoside, eriodictyol-7-monoglucoside, naringenin-7-monoglucoside, d- catechin, and l-epicatechin. The red extract alone contained cyanidin-3-monoglucoside.

Previous studies of pigmentation in flowers have been confined almost entirely to members of the angiosperm class. Ten anthocyanins and twelve glycosides of kampferol, myricetin and quercetin have been reported in the flowers of the cultivated potato (18, p. 100). In the flowers of Salvis splendens, seven anthocyanins have been found (4, p. 586). Fifteen anthocyanins, six glucosides of quercetin, kampferol and myricetin and one dihydroflavonol were identified in Primula sinensis (41, p. 115). From these few examples cited, it is apparent that flavonoid mixtures present in the flowers of plants belonging to the angiosperm class are much more complex than in Douglas fir flowers, where only one anthocyanin, three flavonol glucosides, three flavanone glucosides, and d-catechin and l-epicatechin were found.

In phylogenetic classification of plants, the primary trend of evolution has been from organisms relatively simple in structure to those of greater complexity. Since gymnosperms are considered by botanists to be more primative than angiosperms, it is not surprising that the compounds associated directly with the color of the flowers of Douglas fir are more simple than those found in the flowers of angiosperms.

The reason for differences in appearance of the yellow and green flowers of Douglas fir is not apparent from this study. While no qualitative differences in the flavonoid content were found, quantitative differences, which might account for the variation in color, were not determined in the flower phenotypes.

The relative quantities of the flavonoid compounds appeared to be uniform within the flower phenotypes themselves. Isoquercetrin, quercetin-3-monoglucoside, appeared to be the prominent flavonoid. Astralagin, kampferol-3-monoglucoside, was present in much smaller quantity and kamperol-3-diglucoside could be considered a trace compound. There appeared to be more quercetin than kampferol in the flowers. The order of decreasing quantity of the flavanone glucosides was dihydroquercetin-3'-monoglucoside, eriodictyol-7-monoglucoside and naringenin-7-monoglucoside. The concentration of both d-catechin and l-epicatechin in all flower phenotypes was great

in relation to the flavanone glucosides.

A good deal of interest has been shown in regard to the site of biogensis of polyphenols in plant tissues. It has been suggested that flavonoids and other polyphenols are synthesized at one site in the plant, such as the cambium of leaves, and are subsequently translocated through the plant (23, p. 700; 49, p. 176). A contrary view, that these compounds are formed in situ in the various tissues, has also been advanced (27, p. 435). From comparison of the flavonoids identified in the Douglas fir flowers with those reported in other tissues of the tree, it should be possible to make some comment with regard to these theories.

The flavonoid extractives of the various tissues of Douglas fir should exhibit a biochemical interrelationship if translocation of flavonoids occurs in the tree. Since flowers and leaves originate from the same type of tissue, the apical meristem, and leaves provide the carbohydrate from which the common C_{15} precursor is derived, comparison of the flavonoids present in these tissues would be of particular interest.

In a study of the flavonoid compounds present in the leaves, Hergert positively identified dihydroquercetin-3'-monoglucoside and quercetin-3'-monoglucoside. In a cursory examination of other tissues, cambium, inner bark and sapwood, he was able to demonstrate

the presence of these compounds. This led him to suggest that these compounds are synthesized in the leaves and translocated down the inner bark to the outer bark and through the rays to the heartwood. The sugar is removed at or near the sapwood-heartwood and inner-outer bark boundaries giving the aglycones which are found in both heartwood and outer bark.

If such translocation takes place, then both dihydroquercetin-3'-monoglucoside and quercetin-3'-monoglucoside should also be present in the flowers of Douglas fir. Although dihydroquercetin-3'-monoglucoside was found in the flowers of Douglas fir, quercetin-3'-monoglucoside was not. None of the other flavonoid compounds present in the flowers were glucosidated in the 3'-position. These results tend to make the theory of translocation of flavonoid compounds in the Douglas fir tree questionable.

The presence of dihydroquercetin-3'-monoglucoside in both the leaves and flowers might be interpreted as meaning that it is translocated to the leaves from the flowers. However, dihydroquercetin-3'-monoglucoside appears to be a common compound in tissue of Douglas fir and is not related to the other flavonoids present. If dihydroquercetin-3'-monoglucoside is transported to the flowers, a highly selective translocation system would have to be involved. While such a phenomenon is possible, it seems unlikely. It might

be better argued that dihydroquercetin-3'-monoglucoside is a product of flavonoid metabolism in all living tissues of Douglas fir and is formed in situ.

The chromatographic pattern of flavonoid compounds found in the leaves of Douglas fir, as reported by Hergert (23, p. 700), differs greatly from that of the flowers, when the same solvents are employed. Initial studies at this laboratory indicate that the compounds present in the leaves of Douglas fir are different than those in the flowers. On cursory examination, it appears that the leaves do not contain either kampferol (or its glucoside), or naringenin and eriodictyol (or their glucosides).

Structural interrelationship of the flavonoids identified in Douglas fir flowers with those reported in other tissues of the tree tends to further support the in situ formation of flavonoids in various Douglas fir tissues. The flavanones naringenin and eriodictyol, either as aglycones or glycosides, have not been reported previously in any tissue of Douglas fir, although some evidence which suggest the presence of eriodictyol in the bark has been reported (29, p. 1733). The occurrence of eriodictyol in the bark of Douglas fir and other members of the Pseudotsuga genus has definitely been established recently (2). Thus, it would appear that synthesis and glucosidation of both naringenin and eriodictyol takes place in the flowers of Douglas fir.

Current theory of flavonoid biosynthesis postulates that enzymes add hydroxyl groups to the flavonoid nucleus in sequential steps (12, p. 613). Thus, kampferol, 3, 3', 5, 7-tetrahydroxyflavone, having one less hydroxyl group, may be considered the precursor of quercetin, 3, 3', 4', 5, 7-pentahydroxyflavone. Also, naringenin, 5, 7, 4'-trihydroxyflavanone, may be considered the precursor of eriodictyol, 5, 7, 3'4'-tetrahydroxyflavanone. Since kampferol has not been reported in other tissues of Douglas fir and both kampferol and quercetin are present in the flowers in the form of aglycones and glucosides, suggests that formation and glucosidation of these compounds takes place in the flowers.

The presence of d-catechin has been reported in all tissues of Douglas fir which have been examined (23, p. 700; 28, p. 58; 2). The relationship of this compound to the other flavonoid compounds in a biosynthetic scheme is not clear. While it may be synthesized at one site in the tree and translocated, it seems unlikely. The structure of this compound (Figure 3) suggests that it is not involved in the same biosynthetic pathway as the anthocyanins, flavones and flavanones. Harborne (12, p. 614) also does not include it in his hypothetical biosynthesis scheme (Figure 2). Compounds that are transported in the sap of the tree are usually glycosidated to increase their solubility. Glycosides of d-catechin and l-epicatechin have

not been reported in tissues of Douglas fir. Thus, the presence of d-catechin and l-epicatechin in the flowers could probably be best explained by the assumption that they are also products of metabolism in Douglas fir and are formed *in situ*. They may also be involved in the subsequent synthesis of phlobaphenes and tannins in these tissues.

Leucoanthocyanins, earlier postulated as direct precursors of anthocyanins, were not detected in the flowers of Douglas fir, although an anthocyanin was found in the red flowers. This is in agreement with the current view that anthocyanins are not derived from leucoanthocyanins *in vivo* (19, p. 85).

Anthocyanins almost always have the sugar moiety attached in the 3- or 3- and 5-positions; flavonols in the 3- or 3- and 7-positions (12, p. 610). Early investigations of plant pigments failed to indicate a biogenetic relationship between flavonols and anthocyanidins present, due primarily to the fact that methods for isolating and identifying compounds present in trace amounts were not available at this time. However, recent investigations employing paper chromatography, have shown that a closer biogenetic relationship exists between flavonols and anthocyanidins than between anthocyanidins and dihydroflavonols in plants (41, p. 115).

For example, while studying the pigments in the flowers of

P. sinensis, three flavonols were found which were related in their hydroxylation pattern to pelargonidin, cyanidin and delphinidin (41, p. 115). The glycosidic pattern of the anthocyanins and flavonols were very similar, but dihydrokampferol, the only dihydroflavonol present, was glycosidated in the seven-position.

The glycosidation pattern of the two flavanones and the single flavanonol identified in the flowers of Douglas fir was different than that of the anthocyanin. However, the flavonol glucosides found had sugar moieties in the three-position, the same position as that of the anthocyanin. This also tends to support the current contention that flavanone formation involves a synthetic sequence apart from the main pathway of flavonol and anthocyanin production (12, p. 64) as indicated in Figure 2.

The anthocyanin with its sugar in the three-position is most closely related to the quercetin-3-monoglucoside, which may be a precursor for enzymatic biogensis of this pigment. Kampferol, on the other hand, was present as both the 3-mono- and 3-diglucoside. Since no anthocyanin was present related to the kampferol-3-monoglucoside, it is evident that this compound could not undergo further enzymatic development to an anthocyanin. Yet an enzymatic system was present which added a second glucose unit; an enzyme system apparently selective for this compound, since quercetin-3-diglucoside

was not found.

The optical activity of the flavonoid compounds identified was not measured. However, it is very likely that the dihydroquercetin-3'-monoglucoside found is d-dihydroquercetin-3'-monoglucoside, since d-dihydroquercetin is found in Douglas fir bark when removed and purified by methods which prevent total racemization (1, p. 1958). The optical activity of these compounds raises the question of the optical activity and steric configuration of the flavanones, naringenin and eriodictyol, which are present as glucosides in the flowers. These compounds have only a single optical center (carbon 2), whereas the dihydroquercetin has two asymetric carbon atoms (carbons 2 and 3). Thus, it may be possible to determine the steric configuration about carbon 2 in these compounds. In further studies, these two glucosides should be isolated, enzymatically hydrolyzed and the optical activity of the flavonoids measured and the configuration determined (3, p. 73). In the isolation of these compounds, column chromatography using cellulose and the solvents 15 percent acetic acid and chloroform-acetic acid-water (8:12:5) would possibly offer maximum separation (from R_f values found using paper).

This study is the first involved with pigmentation in the flowers of a coniferous species. Quantitative analytical methods will be necessary in further flower studies. Examination of flavonoid

content in other tissues of Douglas fir would provide further information regarding the general metabolic pattern and physiological functions of flavonoid compounds. For example, some understanding of the relation of these compounds to tissue differentiation could be obtained from analysis of developing buds.

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