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Title: DOUGLAS-FIR BARK: CHARACTERIZATION OF A CONDENSED TANNIN EXTRACT

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The inner and outer barks of Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] were extracted with a solution of acetone-water (70:30 v/v). The resulting solubilized solids were acid hydrolyzed with sulfuric acid. Paper and thin-layer chromatography of the hydrolyzates showed spots for the following monosaccharides: D-glucose, D-mannose, D-galactose, D-xylose, and L-arabinose.

High-performance liquid chromatography using a column packed with Chromex DA-X4-11 anion exchange resin and a borate buffer as eluant resolved the monosaccharides. The detection system utilized a cupric sulfate, dipotassium 2,2'-bicinchoninate dye system which on heating with the monosaccharides produced a lavender color which absorbed at 546 nm. The high-performance liquid chromatographic system was used to quantitatively measure the amount of carbohydrates in the solubilized material. The solubilized fraction from outer bark contained 12.04 % glucan and 4.51 % arabinan. The other sugars resulting from hydrolysis, D-

mannose, D-galactose, and D-xylose were in too small amounts to be measured quantitatively by this method. The solubilized fraction from inner bark contained 15.94 % glucan. The other sugars resulting from hydrolysis, L-arabinose, D-mannose, D-xylose, and D-galactose were in too small amounts to be quantitatively measured.

The solids from the inner bark were methylated and then treated with thioglycolic acid to degrade the condensed tannins to their monomeric units. Raney nickel reduction of the thioglycolic acid reaction products produced tetra-O-methylcatechin and tetra-O-methylepicatechin which were analyzed by gas-liquid chromatography. Analyses showed that 0.12 % catechin and 0.25 % epicatechin resulted from degradation of the original solids. The carbohydrates and other materials in the solubilized solids clearly interfered with the methylation, thioglycolysis, and reduction of the condensed tannins.

The solubilized solids from outer bark were reacted with phloroglucinol in the presence of hydrochloric acid. The reaction mixture was separated on a Sephadex column with 95 % aqueous ethanol as eluant. The collected fractions were monitored by thin-layer chromatography and high-performance liquid chromatography. The presence of free catechin, epicatechin and flavanoid phloroglucinol reaction adducts were established. ^{1}H and ^{13}C-nuclear magnetic resonace spectra showed that the phloroglucinol was attached to the

C-4 position of the catechin and epicatechin moieties. The phloroglucinol reaction illustrated that the solubilized solids contained a condensed tannin comprised of catechin and epicatechin units joined from C-4 of the upper units to probably C-8 or C-6 of the lower units.

Douglas-Fir Bark: Characterization of a Condensed Tannin Extract

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I. INTRODUCTION

Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] is the principal commercial softwood of the Pacific Northwest, accounting for nearly three quarters of the conifer inventory. At advanced ages, Douglas-fir trees may attain diameters of 1.8 meters and heights of 75 meters. These trees typically are grown in a rotation of 50-80 years, and the timber is used for a substantial part of the pulp and paper, lumber, plywood, furniture, and particleboard produced in the United States.

Large amounts of bark are harvested in conjunction with the manufacture of these wood products. These resources receive little utilization other than recovery of fuel values. Douglas-fir bark, however, is a rich source of condensed tannins and the energy crises of the early 1970's stimulated renewed interest in the possibilities for use of these polymers as raw material for exterior wood adhesives.

Also, some studies have produced data to support the use of Douglas-fir bark to replace as much as 20 percent of the wood now used in high quality underlayment-grade

particleboard (88). This use of bark is undoubtedly enchanced by chemical reactions which occur between the condensed tannins in the bark and the formaldehyde resins used in particleboard manufacture.

Although many researchers have attempted to synthesize wood adhesives from condensed tannins as formaldehyde reactive compounds, none has yet found commercal reality. More complete knowledge of the structure and properties of these polymers is needed if further advances in their use are to be made. Although much work has been done to characterize the monomeric flavanoid compounds which comprize the condensed tannins in Douglas-fir bark, little has been done to determine their quantities as monomeric substances.

The isolation of condensed tannins from Douglas-fir bark has involved extraction of the bark with aqueous solvents or aqueous-organic solvents. The extractions invariably solubilize other components in addition to the condensed tannins so that mixtures are obtained. Carbohydrates account for an appreciable amount of the solubilized material and an identification and quantitative evaluation of these carbohydrates is important to an understanding of the extracted solids to improve possibilities for utilization.

The specific objectives of this work were: 1) to identify the carbohydrates and quantitatively determine

the amounts present in on acetone-water extract of Douglas-fir bark; 2) to determine the relative amounts of monomeric polyflavanoids which comprise the condensed tannins in the acetone-water extract; 3) to elucidate the structures of the condensed tannins by reaction with phloroglucinol.

II. HISTORICAL REVIEW

Quantitative analyses of the carbohydrates in the condensed tannin extracts in the present work required an initial acid hydrolysis of the polysaccharides to monosaccharides followed by quantitative measurement of the resulting monosaccharides. Hydrolyses of glucan polysaccharides linked by β (1->4) glycosidic bonds is difficult because of hydrogen bonding between the polymer molecules and because of the stability of the linkages. Monier-Williams and Wickham (53) in 1923 developed a method for the hydrolyses of cellulose which involved dissolution in 72.0 % sulfuric acid followed by dilution of the acid and refluxing. There are a number of more recent modifications of this original procedure. In 1960, Jeffery, Partlow, and Polglase (32) found that 77.0 % sulfuric acid provided better dissolution of cellulosic wood pulps, especially those that contained mannans, than did 72.0 % sulfuric acid. Giertz (22) showed that evacuation of the flask containing the concentrated sulfuric acid and the pulp also improved initial dissolution. Saeman, Moore, and Millet (67) and Jeffery, Partlow, and polglase (32) diluted the concentrated acid hydrolyzate to 6.0 % sulfuric acid followed by reflux. It was later found that dilution to 3.0 % sulfuric acid and a longer reflux

time provided a slower reaction that could be controlled better (47) for maximum monosaccharide yield. Hydrolysis of the solids in the condensed tannin extract in the present work utilized an initial dissolution in 77.0 % sulfuric acid followed by dilution to 3.0 % sulfuric acid followed by reflux for 4.5 hours.

Quantitative analyses of the various monosaccharides, L-rhamnose, L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose that result from acid hydrolyses of plant polysaccharides has evolved through several techniques. Paper chromatography successfully separqtes the monosaccharides (67,68). It is a simple and easy way to qualitatively identify the monosaccharides but it does not work well for quantitative analyses. Thin-layer chromatographic (TLC) methods have been used for both detection of the monosaccharides and their quantitative measurements. However, the quantitative measurements are not too reproducible.

The separation of carbohydrates by gas-liquid chromatography (GLC) has proven difficult because of their heat sentitivity and lack of volatility. These problems can be solved by converting the parent sugars or their methyl glycosides which are more volatile substances (2,10,70). Gunner, Jones, and Perry (23) developed a method of converting the sugars to their alditol acetates. The method was extended by Sawardeker, Sloneker, and Jeanes

(71), by Sloneker (78) and by Albersheim, Nevins, English and Karr (1). The method includes a reduction of the carbonyl group of the monosaccharides to the alcohol(sugar alditol) with sodium borohydride. This prevents the formation of the anomers and ring isomers and provides only one alditol form for each monosaccharide. The alditols are acetylated to give heat stable, volatile derivatives. The method was adapted to wood carbohydrates by Crowell and Burnett (16), by Borchardt and Piper (9), and more recently by Borchardt and Easty (8), McDonald and Garby (51), and Vidal and Pastor (84). The method is excellent, but the formation of the alditol acetates is timeconsuming, complex, and also requires care to obtain quantitative results. It is too complicated to be used as a routine analytical method.

Laine and Sweeley (44,45) and Mawhinney, Feather, Barberro, and Martinez (49,50) prepared the methyloximes of some of the monosaccharides to solve the difficulty of detection of the colorless carbohydrates. These per(trimethylsilyl) or acetyl derivatives were found to be suitable for GLC but also time-consuming and difficult to make quantitatively.

High-performance liquid chromatography (HPLC) has provided the means for the separation and quantitative analyses of carbohydrates without their derivatization. There are two parts to emphasize in the quantitative

analyses of carbohydrates by HPLC. First, there is the required separation of the carbohydrate moieties, and second there is the detection and quantitative measurement of the separated moieties.

Separation of the carbohydrates by HPLC involves both column and elution solvent. In 1974, Bovine and McGinnis (6) reported that water-soluble polysaccharides from wood could be separated by Bio-Glas (granular, porous glass) and Bio-Gel P (polyacrylamide) packed columns with water as the eluting medium. Palmer (62) showed that selected monosaccharides could be separated on a Waters Associates "μ Bondapak/Carbohydrate" column using various proportions of water:acetonitrile as eluant. Although this system separated the pentoses quite well, the hexoses were poorly resolved and the system was less than desirable for wood hydrolyzates.

Several of the equipment manufacturers (3,66) have published data concerning the separation of carbohydrates on prepacked columns. None of these columns and solvent systems, however, completely resolve the monosaccharides resulting from the hydrolyses of wood and wood products. A recent brochure by Bio-Rad Laboratories (7) described the use of their Aminex HPX -87P column to separate the monosaccharides from wood hydrolyzates. The chromatogram shows reasonable resolution for arabinose and mannose and good resolution for glucose, xylose and galactose. Wentz

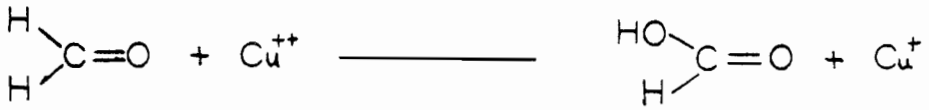
and Marcy (87) reported the separation of wood sugars in pulp and paper industry samples with Aminex HPX-87P series columns. Ladisch, Huebner, and Tsao (42) and Ladisch and Tsao (43) have used cation-exchange resins to separate some carbohydrates. Their systems used water as solvent.

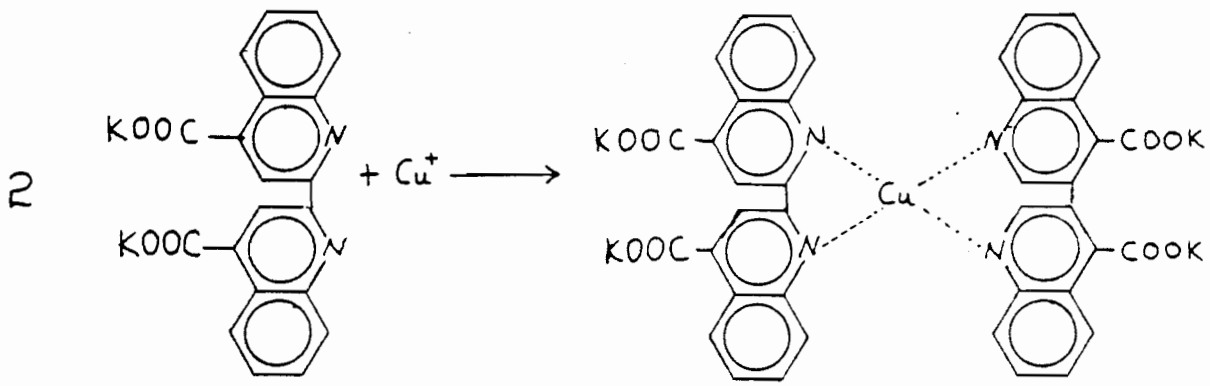
Anion-exchange columns are the most widely used for the analyses of monosaccharides. There are several reports on the use of improved anion-exchange resins and borate complexing to reduced the separation time for the analyses of acid hydrolyzates of wood (55,57,76). Sinner, Simatupang, and Dietrichs (77) published a paper describing the complete separation and quantitative determination of sugars from acidic and enzymatic hydrolyzates of wood polysaccharides via borate complex ion exchange chromatography. Separation of mannose, arabinose, galactose, xylose and glucose can be achieved in 70 to 90 minutes. Ni (60) modified this method to analyze the carbohydrates from commercial wood pulps.

Monosaccarides, when they elute from the column, are colorless and it is difficult to detect them. Detection methods for carbohydrates commonly used in HPLC may be divided into three categories; 1)refractive index detectors (29,33,65,73,82), colorimetric methods using orcinol-sulfuric acid (40,75,83), tetrazolium blue (56), and bicinchoninate-copper (55,58,76), fluorimetric methods using cerate ions (37,38), 2-cyanoacetamide (31), and ethylene-

diamine (57). The detection limits reported therein range widely from 1 ug by refractive index detectors to 2 ng by fluorimetry using 2-cyanoacetamide. In addition to these, a few trials of coulometric (80), and recently amperometric detection (13,84) as electrochemical means have been reported. In the present work the bicinchoninate-copper detection reagent was used. This is a sensitive reagent which has been shown by Ni (60) to work well with borate buffer systems and anion exchange columns.

The dipotassium 2,2'-bicinchoninate-copper reagent had been used by Mopper and Gindler (58) with ethanol as eluant at first. Sinner and Puls (76) reported that this reagent was adapted to an automated borate complex anion-exchange chromatographic system. The color formation is as follows,





The complex has a strong absorption at 546 nm (60). This region of the visible spectrum allows for measurement by simple colorimeters and gives very reproducible and accurate results.

Degradation of the condensed tannins is the first important step in determining their quantitative amounts and understanding their stereochemistry. No information was available relating to the stereochemistry of the polyflavanoid polymer until Betts, Brown, Brown, and Pike (5) degraded a tannin from heather (Calluna vulharis) with thioglycolic acid. Subsequent permethylation gave the methyl s-benzylthioglycolate derivative, which led them to conclude that the condensed tannin was comprised of flavanoid units attached by C-4 benzylic ether linkage since thioglycolic acid was known to cleave such bonds. However, Sears and Casebier (73) later showed that this reagent also cleaved the benzylic C-C bonds in synthetic procyanidins.

Because of the proven value for determination of the structure and stereochemisty of condensed tannins, Betts, Brown, and Shaw (4) investigated in detail the reaction of thioglycolic acid with several flavanoids. They found that when the 2,3-trans-3,4-cis-flavan-3,4-diol (I) was reacted with thioglycolic acid, followed by methylation with diazomethane, it gave a mixture (2.3:1) of the 3,4-cis (II) and the 3,4-trans (III) isomers of methyl 2,3-trans-(3-hydroxy-3',4',5,7-tetramethoxy-flavan-4-ylthio)-

acetate, and reaction of the 2,3-cis-diol (IV) with thioglycolic acid gave only the (+)-2,3-cis-3,4-trans-(3-hydroxy-3',4',5,7-tetramethoxyflavan-4-ylthio)acetate (V) after permethylation with diazomethane (Figure 1). From this result, they concluded that the nucleophilic addition of thioglycolic acid to 4-hydroxyflavans followed an Sn1 mechanism. This conclusion was supported in further work by Brown and Shaw (12) in which they noted that progressive introduction of methoxy groups into the 5 and 7 positions of flavan-4-ols enabled the reaction to be conducted at progressively lower acid concentrations as expected for an Sn1 mechanism. Since then sulfur nucleophiles other than thioglycolic acid, including toluene-α-thiol and benzenethiol (12) have been employed in an effort to improve yields of the thioethers. Fletcher, Porter, Haslam and Gupta (18) used the principle of this reaction to synthesize procyanidin B dimers (X, XI, XII, XIII) using (-)-epicatechin (IV) or (+)-catechin (VII) as the nucleophile that captures carbonium ions (VIII,IX) produced in the reaction of specific condensed tannins or procyanidins with acid. Gupta and Haslam (24) reported that (+)-catechin is chain initiator (terminal lower unit) and the flavanol carbocation is chain extender (upper unit) (Figure 2).

Sears and Casebier (74) studied the degradation products obtained from polyflavanoid polymers of Tsuga heterophylla bark. Reaction of a methylated condensed tannin with

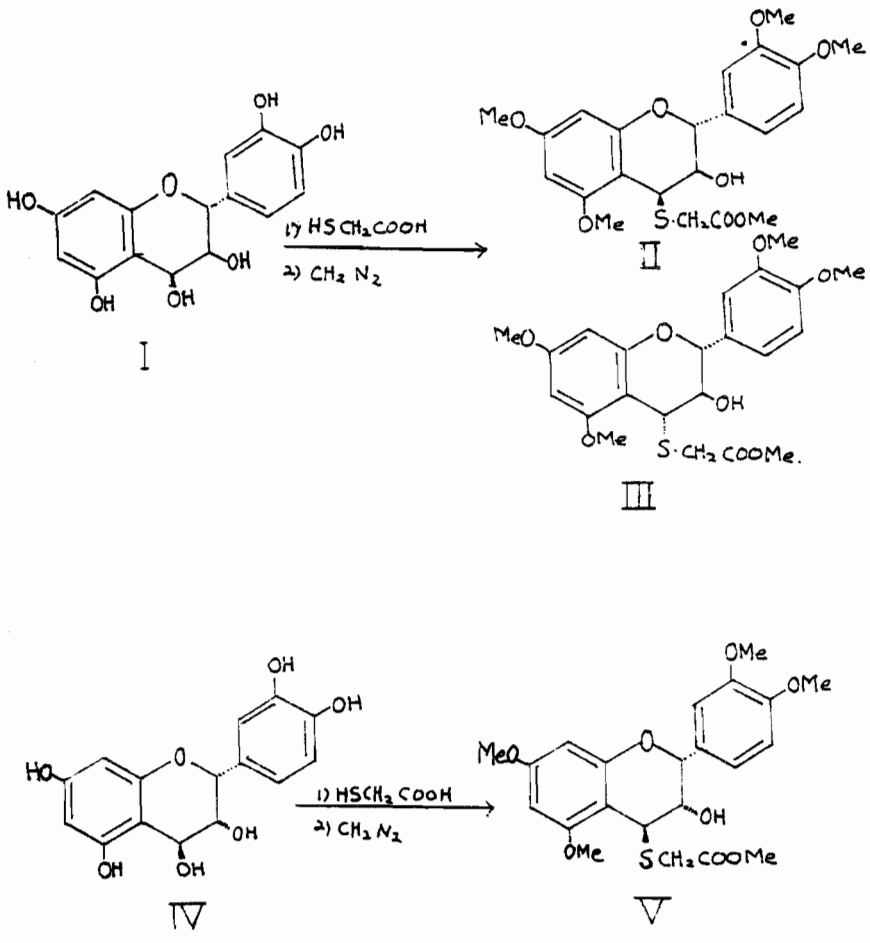


Figure1. Reaction scheme of 2,3-trans-diol and 2,3-cis-diol with thioglycolic acid.

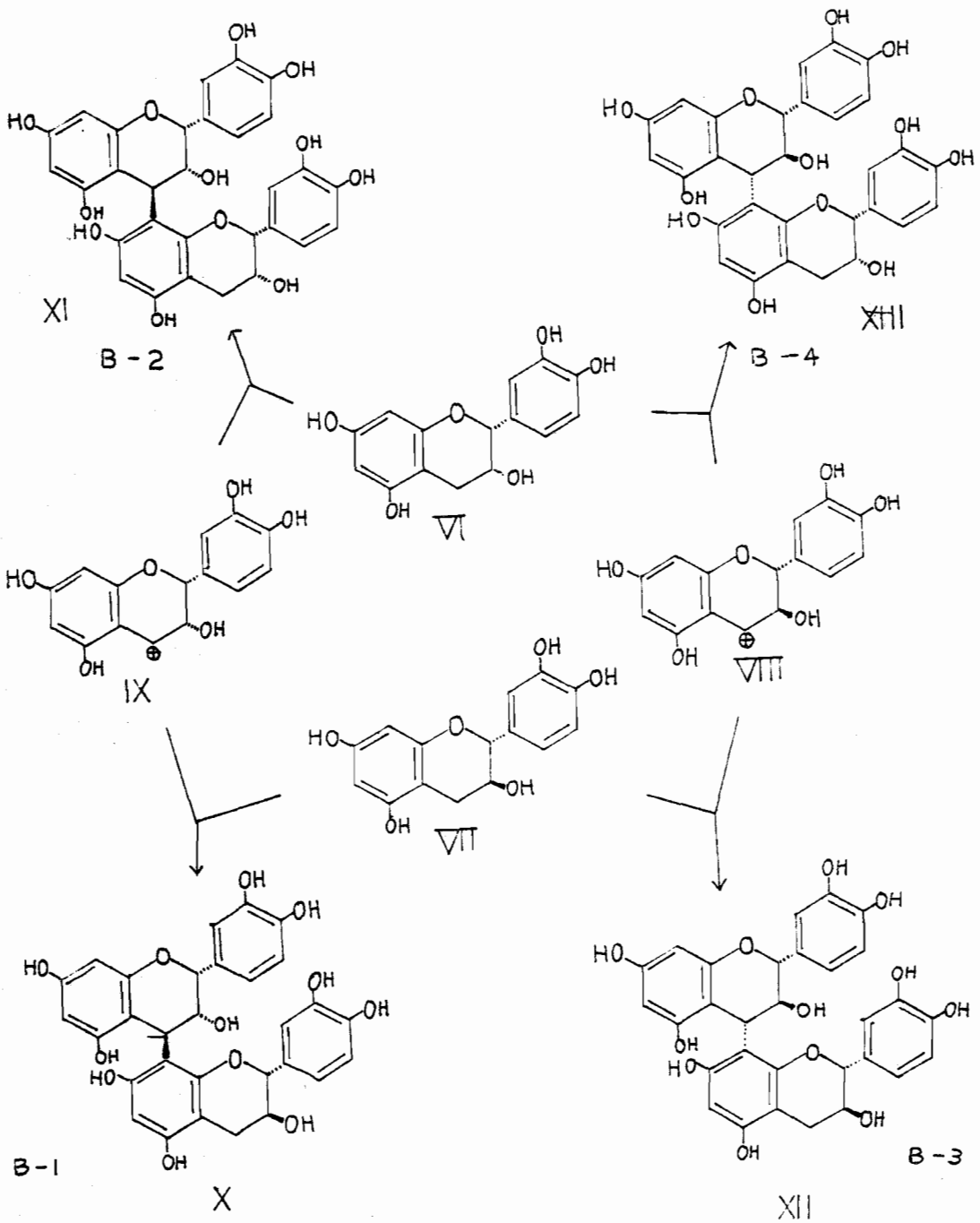


Figure2. Reaction scheme for the synthesis of the procyanidin B dimers.

thioglycolic acid gave equivalent yields of 2,3-*cis* and 2,3-*trans* methyl (3-hydroxy-5,7,3',4'-tetramethoxyflavan-4-ylthio)acetates, suggesting that the condensed tannins were composed of nearly equal proportions of catechin and epicatechin units linked ether C-4 to C-6 or C-8.

Hemingway and McGraw (27,28) examined thioglycolysis products from methylated condensed tannins extracted from tree barks of *Pinus taeda* and *Pinus echinata*. They found epicatechin and catechin thioglycolates in relative yields of about 5 to 1. They also examined the ethylation of these products rather than methylation to determine the polymer structure. No products with aromatic ethoxyl groups were detected.

Karchesy and Hemingway (35) isolated three different condensed tannins which were an ethyl acetate-soluble polymer, a water soluble tannin and an acetone:water-soluble tannin (XIV) from the inner bark of *Pinus taeda*. Thiolysis of each of these tannins with benzenethiol gave only the epicatechin thioether (XV) and a dimeric epicatechin-epicatechin thioether (XVI) but no catechin products were indicated. Thus the upper units of the polymers (XVII,XVIII) would be expected to be derived from flavan carbocations of a (-)-epicatechin stereochemisty. They also reported that the flavan-3-ol obtained from the lower terminal unit of these tannins was exclusively catechin (XIX); of the momomeric flavan-3-ols, only catechin and no

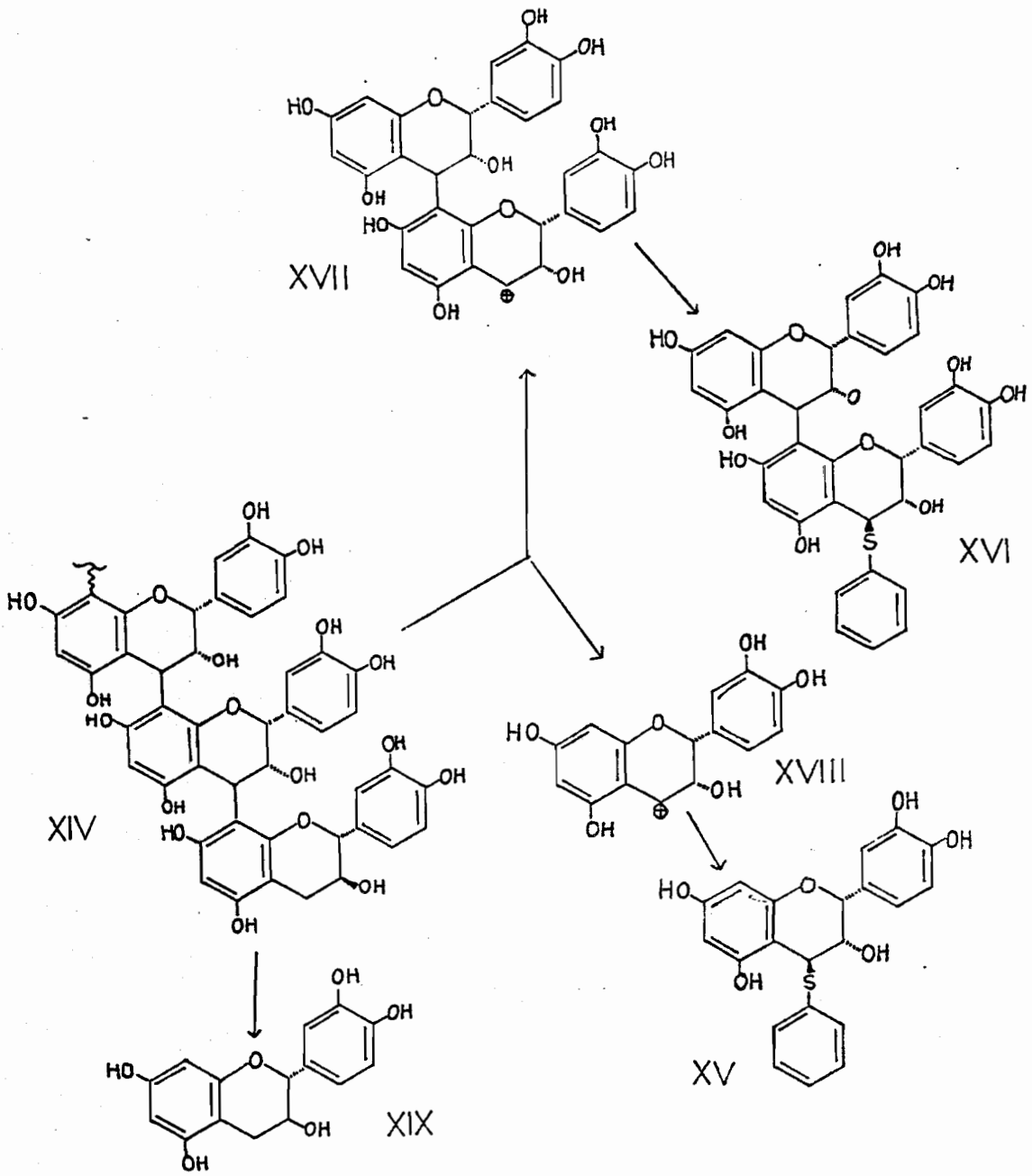


Figure 3. Reaction scheme of the condensed tannin from the inner bark of Pinus taeda with benzenethiol.

epicatechin was detected. This reaction scheme is shown in Figure 3.

Karchesy, Loveland, Laver, Barofsky, and Barofsky (36) studied the condensed tannins isolated from the bark of Pseudotsuga menziesii and Alnus rubra. Treatment of the methylated tannins with propan-2-ol and hydrochloric acid produced 5,7,3',4'-tetramethylcyanidin chloride indicating that a benzyl ether bond was not the dominant linkage. The epicatechin thioglycolate was the only product obtained from Alnus rubra bark. Thiolysis of the methylated tannin from Pseudotsuga menziesii afforded the epicatechin and catechin thioglycolates in a ratio of about 3:1.

Fletcher, Porter, Haslam,and Gupta (18) and Gupta and Haslam (24) used phloroglucinol to degrade some plant proanthocyanidin. Hillis and Yazaki (30) isolated a condensed tannin from Pinus radiata bark by chromatography on an LH-20 Sephadex column using acetone-dimethylformamide solvents. This tannin isolate gave cyanidin chloride on treatment with n-butanol-hydrochloric acid and the infrared spectrum of the tannin was nearly identical with the spectra obtained from procyanidins B-1 and B-3. Fletcher, Porter, Haslam, and Gupta (18) reported quantitative yields of thiolysis products from the ethyl acetate-soluble tannin that was retained on an LH-20 Sephadex column to isolate soluble tannins.

Thompson, Jacques, Haslam, and Tanner (81) and Fletcher, Porter, Haslam and Gupta (18) examined the structures and conformations of various procyanidins through extensive use of ^{1}H-nuclear magnetic resonance(NMR) and ^{13}C-NMR. These researchers reported model structures for the condensed tannins. Foo and Porter (19) reported NMR data for some prodelphinidin polymers. They defined the two basic structural units of condensed prodelphinidin polymers by generation of the C-4 carbocations, corresponding in stereochemistry to (+)-gallocatechin and (-)-epigallocatechin, and trapping them as their phloroglucinol adducts.

Czochanska, Foo, Newman, and Porter (17) isolated homogeneous polymeric proanthocyanidins from 22 plant sources. ^{13}C-NMR spectroscopy was used to calculate the ratio of procyanidin to prodelphinidin monomer units, the average heterocyclic ring stereochemistry of the monomers, and the ratio of monomers to chain terminating units. Porter, Newman, Foo, Wong and Hemingway (64) assigned ^{13}C-NMR chemical shifts to 40 natural and synthetic proanthocyanidins, related flavan-3-ols, and their peracetate derivatives. Thus both the ^{1}H-NMR and ^{13}C-NMR chemical shifts for many flavanoid compounds have been reported.

GLC of flavanoids has received little attention because of the difficulties caused by the low volatility of the hydroxylated flavanoids. Therefore, only a few

papers (21,39,59,61) have reported on the separation of flavanoid compounds. Nordstrom and Kroneld (61) conducted a comparative study of the retention times of trimethylsilyl ether derivatives of flavones and hydroxyflavones. OV-17, a methyl phenyl silicone polymer, was used as the liquid phase. Separation of the trimethylsilyl ethers was better than the corresponding methyl ether derivatives. The methyl ethers also had longer retention times. The trimethylsilyl ethers were easily prepared but were to some degree sensitive to moisture. The methyl ethers, however, were stable and easy to handle.

Colella (15) attempted the quantitative analyses of the catechin type compounds by GLC. He used OV-17 as the liquid phase on GLC columns and made the methyl ether derivatives of samples which had been degradated with thioglycolic acid.

HPLC of polyflavanoids, however, has recently received more attention than GLC. Wulf, and Nagel (89) developed an HPLC technique for the separation and quantitation of three classes of naturally occuring phenolic compounds. They elaborated on the effects of structural variations within the phenolic compounds on elution order and retention. Samejima, and Yoshimoto(67) separated completely the stereoisomers of flavanol monomers and procyanidin dimers with HPLC. HPLC of the thiolysis products from C. japonica polymers showed five distinct peaks corresponding

to (+)-catechin, (-)-epicatechin and three isomers of benzyl thiofavanol. Alnus. firma polymers showed five peaks which were identical to those observed in C. japonica, plus three additional peaks which were identified as pro-delphinidin-thiol derivatives. Several additional investigators (40,47,62) have also presently used HPLC to characterize the extracts from natural sources such as cocoa beans and trees.

III. EXPERIMENTAL

A. Collection of Bark Samples.

Douglas-fir bark was collected by D.A. Charleson (14) from a freshly cut tree taken from McDonald Forest, Benton County, Oregon. The tree was approximately 124 annual rings old and 86.4 cm in diameter at breast height. The outer bark was separated from the inner bark. The outer and inner barks were placed in plastic bags and air-dried in the laboratory.

B. Extraction of Bark Samples.

D.A. Charleson (14) extracted the bark samples and a brief description of his work is presented for clarity. A typical procedure for both inner and outer bark is described. The air-dried bark (1.0 kg) was macerated, then soaked at room temperature for 2 days in 5 liter Erlenmeyer flasks with 3 liters of acetone-water (70:30 v/v) solution. The extraction of the bark was repeated three times, each time with fresh solvent. The acetone-water solution was decanted from the flasks and the acetone was removed under reduced pressure on a rotary evaporator. The remaining aqueous suspension was extracted with diethyl ether (3 times, 500

ml), then filtered through glass wool to remove the sticky, red solids which remained.

The filtrate was extracted with ethyl acetate (3 times, 500 ml) and the aqueous layer was reduced in volume under reduced pressure with a bath temperature of 60°. The freeze-drying of this solution yielded 90 g (9.05% of the original inner bark) and 34 g (3.4% of the original outer bark) of a spongy, tan-colored, tannin material.

C. Acid Hydrolyses of the Bark Extracts.

1. Dilute Acid Hydrolyses

Amounts (500 mg) of the freeze-dried solids which had been extracted from the inner bark and outer bark by Charleson(14) were refluxed with 150 ml of 3.0% sulfuric acid in round-bottomed flasks for 4.5 hr. Each solution was neutralized with saturated aqueous barium hydroxide and the neutralized solution was first centrifuged and then filtered through Whatman No.1 filter paper. The barium sulfate residue was carefully washed with 25 ml of distilled water (3 times). The washings and centrifugates were combined, concentrated on a rotary evaporator at 50° and diluted to 100 ml in a volumetric flask.

2. Concentrated Acid Hydrolyses

Amounts (500 mg) of the freeze-dried solids which had been extracted from the inner bark and outer bark were each dissolved in 5.6 ml of 77.0% sulfuric acid in a beaker for 1.0 hr at room temperature, Each dark-brown, viscous solution was stirred every 10 minutes with a glass rod to enhance dissolution. The viscous paste solutions were each transferred to round-bottomed flasks and diluted to 3.0% sulfuric acid with 145 ml of distilled, deionized water. These were completely mixed and the refluxed for 4.5 hr. The tan-colored solutions were cooled and filtered. Each residue was discarded. The filtrates were diluted with distilled deionized water to 250 ml in volumetric flasks. Aliquots of 10 ml of each solution were neutralized with saturated aqueous barium hydroxide. These prepared samples were analyzed by paper chromatography, TLC, and HPLC on an Aminex HPX-87P cation exchange column.

D. Paper and Thin-Layer Chromatographic Analyses.

Paper chromatographic analyses were carried out on Whatman No.1 paper. TLC analyses were performed on S&S F 1440 cellulose plates. The solvent system employed was ethyl acetate:pyridine:water (8:2:1 v/v) (67,68).

Paper chromatographic analyses employed the descending method and TLC analyses employed the ascending method.

Each chromatogram was run 4 times, air-dried, and sprayed with aniline hydrogen phthalate detection reagent (1.66 g of phthalic acid dissolved in 100 ml of water-saturated n-butanol containing 1.0 ml of aniline) (67,68). Each chromatogram was air-dried and heated at 105° for 10 minutes.

E. High-Performance Liquid Chromatographic Analyses of Carbohydrates in the Bark Estracts.

1. Columns and Equipment.

Two different kinds of chromatographic pumps were used to pump the eluant solvent and the dye reagent. A Waters Associates Co., Model M-45 solvent delivery system pump was used for the eluant solvent. The other pump was a Model A-30-S from Eldex Laboratories Inc., Menlo Park, CA. which pumped the copper dye reagent.

The detection system was an ISCO Model 1840 variable wavelength ultraviolet-visible absorbance monitor. A Hewlett Packard Model 3380A integrator was used to give a recording spectrum of the absorbances. A tee union was used to combine the eluant from the column with the copper dye reagent. The mixed solvent stream then passed through 15 m of 316 stainless steel tubing (0.1" internal diameter) which was heated by an HPLC column heater from Bio-Rad Laboratories. A circulating water bath was used to heat

the column at 57.5°. The block diagram of the total system is illustrated in Figure 8.

The column of anion-exchange resin was prepared according to the manufacturers instructions. The packing material was Chromex DA-X4-11 (Dionex Corporation, Sunnyvale, CA). The column, 250 mm long with a 3.2 mm internal diameter, was equipped with two, dead-column end fittings and two replaceable bed supports constructed of stainless steel frits with a normal pore size of 2 μm. A prepacked column type Aminex HPX-87P (300 mm X 7.8 mm), purchased from Bio-Rad Laboratories also was used. It was packed with 8% crosslinked cation-exchange resin.

2. Solvents

The anion-exchange resin column (Chromex DA-X4-11) was used with a borate buffer solution as an eluant solvent. The borate buffer was prepared as follows. Potassium hydroxide (100.10 g) was completely dissolved in distilled deionized water (6.0 liters). Boric acid (224.96 g) was added to the solution and dissolved. Additional distilled deionized water (1000.0 ml) was added. The final solution had a pH of 9.0 and was 0.52 M in boric acid (H_3BO_3) or 0.13 M in potassium borate ($K_2B_4O_7$) in accordance with the balanced chemical equation:

$$2\ KOH + 4\ H_3BO_3 \text{-----} > K_2B_4O_7 + 7\ H_2O$$

The solution was vacuum filtered through a 0.45 μm pore size filter, degassed in an ultrasonic bath and pumped through the column by the Waters Associates M-45 solvent delivery system (pump A in Figure 8), at a flow rate of 0.2 ml/min. The stock borate buffer solution was agitated by a magnetic stirrer while in use.

The cation-exchange resin column, Aminex HPX-87P from Bio-Rad Laboratories required water as an eluant. Distilled deionized water was filtered through a 0.45 μm filter and degassed before being pumped through the column at a flow rate of 0.6 ml/min. This column was maintained at a temperature of 85° by pumping water of this temperature through a water jacket.

The monosaccharides were detected after elution from the column with a bicinchoninate copper dye reagent. The dye reagent was initially formulated in two parts, "solution A" and "solution B." Solution A contained anhydrous sodium carbonate (215.0 g) and dipotassium 2,2'-bicinchoninate (6.2 g) [$(C_9H_5NCOOK)_2$; F.W. 420.9, from Hach Chemical Co., Ames, Iowa] in 3.45 liters. These two chemicals were sequentially dissolved in distilled deionized water with stirring. Solution B was prepared by adding L-aspartic acid (3.7 g), anhydrous sodium carbonate (5.0 g), and cupric sulfate 5-hydrate (1.0 g) in 150 ml of distilled deionized water and completely dissolving them. The mixing ratio of solutions A to B was 23 to 1. This copper dye

reagent was also filtered through a 0.45 μm filter and degassed. It was stored in a brown bottle wrapped with aluminium foil to protect it from the light because the color of the dye becomes deeper on exposure to light. The dye reagent was pumped into the eluant from the column with a small pump (pump B in Figure 8).

3. Optimum Operating Conditions.

The anion-exchange resin column was heated at 57.5° by a circulating water bath. The flow rates of solvents were 0.2 ml/min of buffer solution and 0.5 ml/min of copper dye reagent. The long tubing after the mixing tee was heated to 100°. A wavelength of 546 nm was used to detect the sugars after their reaction with the copper dye reagent. The sensitivity of the detector was set at 0.5. The integrator settings were: attenuation, 256; slope sensitivity, 0.3 mV/min; chart speed, 0.5 cm/min.

The only differences between the anion-exchange column and the cation-exchange column were the temperatures of the columns and the flow rates of the solvents. The cation-exchange column was heated at 85°. The eluant solvent, water, was pumped at 0.6 ml/min and the flow rate of the copper dye reagent was 1.5 ml/min.

4. Determination of Instrument Calibration Curves.

The method described by Brown (11) was used to determine the difference in detector response between the internal standard (L-rhamnose) and each of the sugars, D-glucose and L-arabinose. Five solutions of various weight ratios of the sugars to the internal standard (L-rhamnose) were prepared. Stock solutions were prepared by dissolving 3.15 mg of the internal standard (L-rhamnose) in 5 ml of water, 4.0 mg of D-glucose (Sigma Chemical Co.) in 5.0 ml of water, 4.0 mg of L-arabinose (Pfanstiehl Chemical Co.) in 5.0 ml of water. The five weight-ratio mixtures were prepared by drawing the appropriate volumes from the stock solutions with a micropipet. The weight ratio mixtures and the volumes of stock solutions used in preparing the mixtures for the Chromex DA-X4-11 anion exchange column are shown in Tables 1 and 2.

On the other hand, for the Aminex HPX-87P cation exchange column, the standard mixture of the sugars was made by directly measuring the weight of each sugar. Tables 3 and 4 show the weight ratio mixtures.

Aliquots of 10 µl each of the standard solutions were injected onto the columns. The areas under the peaks of the resulting spectra were measured with a planimeter. Each instrument calibration curve was obtained by plotting the chromatographic peak area ratios (individual sugar area/internal standard area) as the ordinate versus the

Table 1. Weight ratio mixtures between the internal standard (L-rhamnose) and D-glucose to determine the instrument calibration curve for the Chromex DA-X4-11 anion exchange column.

compound	weight ratio				
	0.38	0.76	1.10	1.52	1.91
internal standard, ml	1.0	1.0	1.0	1.0	1.0*
D-glucose, ml	0.3	0.6	1.0	1.2	1.5

*. From the stock solution.

Table 2. Weight ratio mixtures between the internal standard (L-rhamnose) and L-arabinose to determine the instrument calibration curve for the Chromex DA-X4-11 anion exchange column.

compound	weight ratio				
	0.38	0.76	1.10	1.52	1.91
internal standard, ml	1.0	1.0	1.0	1.0	1.0*
L-arabinose, ml	0.3	0.6	1.0	1.2	1.5

*. From the stock solution.

Table 3. Weight ratio mixtures between the internal standard (L-rhamnose) and D-glucose to determine the instrument calibration curve for the Aminex HPX-87P cation exchange column.

compound	weight ratio						
	0.5	0.7	1.0	1.2	1.5	1.7	2.0
internal standard, mg	5.0	5.0	5.0	5.0	5.0	5.0	5.0
D-glucose, mg	2.5	3.5	5.0	6.0	7.5	8.5	10.0

Table 4. Weight ratio mixtures between the internal standard (L-rhamnose) and L-arabinose to determine the instrument calibration curve for the Aminex HPX-87P cation exchange column.

compound	weight ratio			
	0.3	0.6	0.9	1.2
internal standard, mg	5.0	5.0	5.0	5.0
L-arabinose, mg	1.5	3.0	4.5	6.0

weight ratios (individual sugar weights/internal standard weight) as the abscissa. Tables 5 and 6 for the Chromex DA-X4-11 anion exchange column and Tables 7 and 8 for the Aminex HPX-87P cation exchange column show the area ratios of the sugars which corresponded to the weight ratios.

5. Analyses of the Monosaccharides in the Acid Hydrolyzates of the Bark Extracts.

Aliquots (5.0 ml) of the acid hydrolyzates from the concentrated acid hydrolyses of the bark extracts (section III-C-2 of this thesis) were used for the HPLC analyses. The internal standard (L-rhamnose 1.04 mg, 1.20 mg, inner bark and outer bark respectively) was added to each 5 ml aliquot which was then filtered through a 0.45 μm pore sized filter, degassed and 10 μl was injected into the HPLC using the borate buffer eluant. Each analyses was performed in triplicate using the same HPLC conditions as were used to obtain the instrument calibration factors.

Figure 4 diagrams the HPLC analyses, and the paper chromatographic and TLC chromatographic analyses of the acid hydrolyzates.

F. Analyses of Catechin and Epicatechin Moieties in the Bark Extracts.

Table 5. Area ratios of D-glucose/internal standard(L-rhamnose) peaks which corresponded to their weight ratios determined on the Chromex DA-X4-11 anion exchange column.

	weight ratio*				
	0.38	0.76	1.10	1.52	1.91
	0.29	0.57	0.81	1.12	1.36
area ratio	0.28	0.54	0.78	1.06	1.40
	0.26	0.55	0.83	1.13	1.32

*. From Table 1.

Table 6. Area ratios of L-arabinose/internal standard(L-rhamnose) peaks which corresponded to their weight ratios determined on the Chromex DA-X4-11 anion exchange column.

	weight ratio*				
	0.38	0.76	1.10	1.12	1.91
	0.19	0.39	0.62	0.75	1.00
area ratio	0.22	0.40	0.56	0.78	0.94
	0.18	0.42	0.59	0.76	0.94

*. From Table 2.

Table 7. Area ratios of D-glucose/internal standard(L-rhamnose) peaks which corresponded to their weight ratios determined on the Aminex HPX-87P cation exchange column.

	weight ratio*						
	0.5	0.7	1.0	1.2	1.5	1.7	2.0
	0.72	0.91	1.43	1.72	2.02	2.28	2.73
area ratio	0.82	1.18	1.46	2.14	1.98	2.20	2.70
	0.79	0.96	1.52	1.82	2.09	2.40	2.64

*. From Table 3.

Table 8. Area ratios of L-arabinose/internal standard(L-rhamnose) peaks which corresponded to their weight ratios determined on the Aminex HPX-87P cation exchange column.

	weight ratio*			
	0.3	0.6	0.9	1.2
	0.096	0.166	0.200	0.290
area ratio	0.110	0.182	0.240	0.290
	0.092	0.187	0.230	0.280

*. From Table 4.

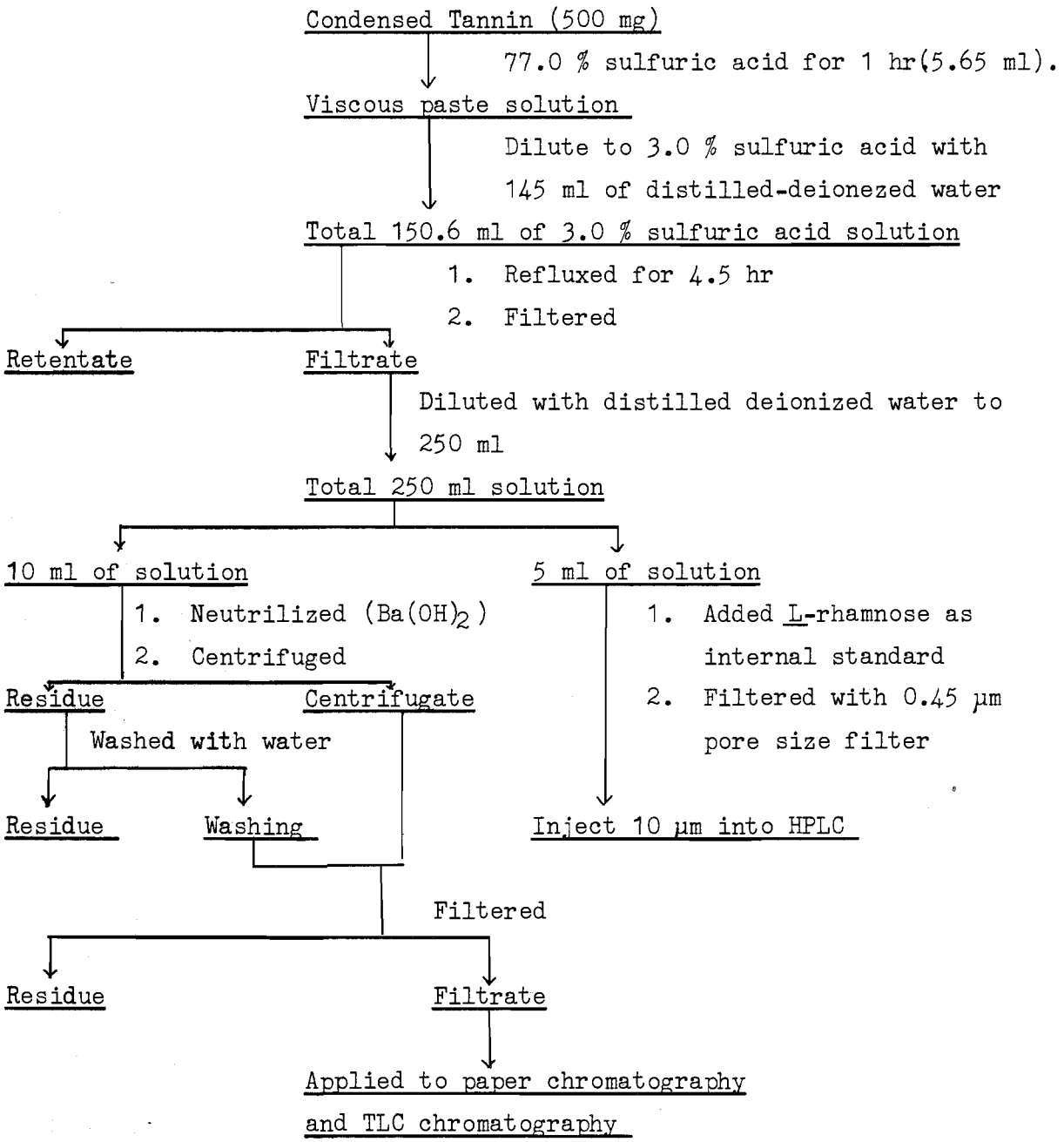


Figure 4. Chromatographic Analysis of Acid Hydrolyzte

1. Methylation and Degradation of the Bark Extracts.

The freeze-dried solids extracted from inner bark (section III-B of this thesis) (2.80 g) in acetone-methanol (5:1 v/v, 160 ml) was refluxed with dimethyl sulfate (8.0 ml) and potassium carbonate (14.0 g) for 18.5 hr under vigorous boiling conditions. The tan-colored reaction mixture was centrifuged and filtered. The residue was washed well with acetone (10 ml, three times) and discarded. The combined filtrate and washings were reduced in volume on a rotary evaporator at 45°. The dark tan-colored oily solution was dissolved in 200 ml of chloroform, washed with water (two 40 ml aliquots) in a separatory funnel. The chloroform layer was dried with anhydrous sodium sulfate, then filtered and reduced in volume on a rotary evaporator. The paste-like solution was evaporated completely under vacuum to give a dark tan-colored granular solid (454 mg). An infrared spectrum (Beckman IR20 Spectrometer) of this solid showed the following major absorbance peaks: Vmax (KBr), cm^{-1}: 3440, 2810, 1680, 1590, 1490, 1440, 1240, 1180, 1100, 1010.

The methylated tannin (439 mg) was degraded by Sears and Casebier's method (73), which involved refluxing for 4 hr under argon with water (7.0 ml) and thioglycolic acid (10 ml) under vigorously boiling conditions. The reaction mixture was cooled and diluted to 50 ml in a volumetric

flask with 95% ethanol.

2. Raney Nickel Reduction Reaction

Raney nickel # 2800 (Davison Specialty Chemical Co.), a finely divided activated catalyst,is similar to the W-2 preparation developed by Mozingo (54). The primary difference is that Raney nickel # 2800 is stored under water, while Raney nickel W-2 is stored under ethanol. Because the hydrogenation of methylated tannin required an organic medium it was necessary to exchange the solvent of Raney nickel # 2800 from water to ethanol. The procedure for the solvent exchange was followed according to Mozingo (54). The catalyst was suspended in water, allowed to settle, and the supernatant decanted. This process was repeated until the supernatant tested neutral to litmus. The same washing process was next repeated three times with 200 ml aliquots of 95% ethanol.

The tannin thioglycolate prepared earlier was divided into five 10 ml portions. Excess Raney nickel in ethanol was added to a 10 ml portion of the thioglycolate and reacted at room temperature for 2 hr. The reaction mixture was filtered under vacuum with Whatman # 50 filter paper.

The filtrate was tested by TLC. TLC analyses revealed two spots under ultraviolet light: R_f = 0.30 due

to tetra-O-methylcatechin and R_f = 0.34 due to tetra-O-methylepicatechin. The solvent system was benzene : acetone (9:1 v/v).

The filtrate was concentrated to dryness and the resulting solid was dissolved in 10 ml of dichloromethane, and 2.47 mg of a 5-hydroxy-3',4',7-trimethoxyflavanone was added as internal standard. The suspended solution was centrifuged and filtered in order to eliminate the insoluble material and dried in air. The light yellow solid was dissolved in an accurately measured 1.0 ml of dichloromethane.

3. Preparation of Tetra-O-Methylcatechin.

A solution containing 10 g of commercial (+)-catechin (Sigma Chemical Co.) of 80% or greater purity in 700 ml of distilled deionized water was prepared at 60° under a nitrogen atmosphere. The solution was decolorized at this temperature with 10 g of 20/40 mesh activated charcoal for 20 minutes. The solution was filtered through Whatman # 1 filter paper into flasks and stored in a refrigerator overnight. The first white (+)-catechin crystals were isolated from the solution by centrifugation. These crystals were redissolved, decolorized, filtered, cooled and centrifuged in the same way to yield the second purified (+)-catechin crystals. The supernatant was

poured off and the wet second (+)-catechin crystals were freeze-dried providing a 55% yield. The freeze-dried (+)-catechin crystals were dried for one day over phosphorus pentoxide at 105° in vacuo. The melting point of the (+)-catechin was 153-155° [Karchesy and Hemingway: 150-151° (36)].

The freeze-dried (+)-catechin (1.0 g) crystals were dissolved in 40 ml of ethanol in order to synthesize the tetra-O-methylcatechin (2R:3S)-5,7,3',4'-tetra-O-methyl-flavan-3-ol. This solution was methylated three times with excess ethereal diazomethane at a temperature below 5 for over 36 hr. The ethereal diazomethane was prepared from Diazald (99% N-methyl-N-nitroso-P-toluenesulfonamide) according to the instructions of the mmanufacturer (Aldrich Chemical Co,Inc.). Needle-like crystals of tetra-O-methylcatechin were obtained after filtration. The crystals were air-dried for 3 hr, then dried over phosphorus pentoxide in vacuo for one day. The melting point of the crystals was 143-145° [Gupta and Haslam: 144-145° (24)]. The GLC analyses of this product yielded a single peak.

4. Gas-Liquid Chromatographic Analyses of Catechinic Materials.

a. Determination of Optimum Conditions.

GLC analyses were performed on a Hewlett-Packard 5751B Research Chromatograph equipped with dual flame ionization detectors. The conditions were: column 3' x 1/8" O.D. stainless steel, packed with 3% OV-17 on Gas Chrom Q 100/200 mesh; injection port, 250°; detector, 245° ; column temperature 230°, isothermal; carrier gas, helium, flow rate 30 ml/min; oxygen flow rate 40 ml/min; attenuation setting, 32; range setting, 10 . Peaks were recorded on an HP 7127A recorder and peak areas were measured by a planimeter.

b. Determination of Instrument Calibration Curves.

The method described by McNair and Bonelli (52) was used to determine the difference in detector response between the internal standard (5-hydroxy-3',4',7-trimethoxyflavanone), and each of the tetra-O-methylcatechin and tetra-O-methylepicatechin compounds. Five solutions of various weight ratios of the compounds to the internal standard were prepared. The stock solutions were prepared by dissolving 10 mg of each of the three compounds, internal standard, tetra-O-methylcatechin and tetra-O-methylepicatechin in 3.0 ml of dichloromethane. The five weight ratio mixtures were prepared by drawing the appropriate volumes from the stock solutions with a micropipet. Table

9 shows the weight ratio mixtures and the volumes of stock solutions used in preparing the mixtures. Amounts of 3 µl of each samples were injected into the gas chromatograph (three times each). Each instrument calibration curve was obtained by plotting the area ratios of each peak (individual tetra-O-methyl derivative area/internal standard area) versus the weight ratios (individual tetra-O-methyl derivative weight/internal standard weight). Tables 10 and 11 show the area ratios of these compounds which correspond to the weight ratios.

c. Analyses of Methylated Degradation Products.

Amounts of 3.0 µl of sample were injected into the gas chromatograph under conditions which were used to determine the calibration curves. Figure 5 shows the analyses scheme from methylation to injection of the sample into the gas chromatograph.

G. Phloroglucinol Degradation of Outer Bark Extracts.

The crude water-soluble tannin (5.0 g) was dissolved in 75 ml of dioxane-water (1:1, v/v). Phloroglucinol dihydrate (8.0 g) was added, followed by concentrated hydrochloric acid to adjust the acidity of the solution to 0.5 M. The solution was kept at 20° ± 2° for 60 hr with

Table 9. Weight ratio mixtures between the internal standard (5-hydroxy-3',4',7-trimethoxyflavanone) and the tetra-O-methyl derivatives of catechin and epicatechin to determine the instrument calibration curves.

compound	weight ratio				
	0.2	0.4	0.6	0.8	1.0
internal standard	0.5	0.5	0.5	0.5	0.5*
tetra-0-methyl-catechin	0.1	0.2	0.3	0.4	0.5
tetra-0-methyl-epicatechin	0.1	0.2	0.3	0.4	0.5

* ml from the stock solution.

Table 10. Area ratios of tetra-O-methylepicatechin corresponding with the weight ratios between the internal standard (5-hydroxy-3',4',7-trimethoxyflavanone) and tetra-O-methylepicatechin.

	weight ratio*				
	0.2	0.4	0.6	0.8	1.0
	0.40	0.85	0.96	1.66	1.92
area ratio	0.40	0.68	1.14	1.56	2.17
	0.40	0.70	1.24	1.80	2.03

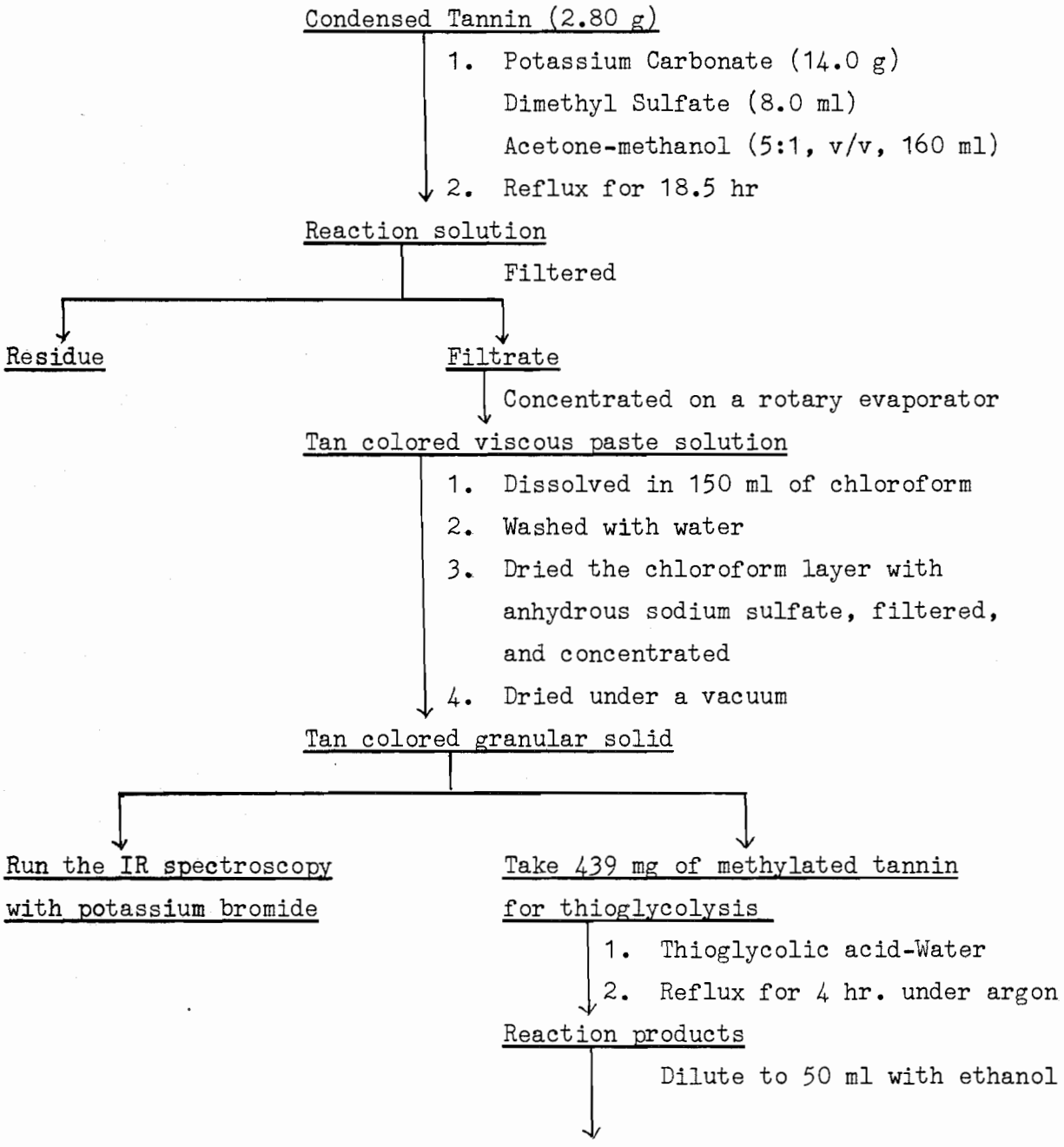
*. From Table 9.

Table 11. Area ratios of tetra-O-methylcatechin corresponding with the weight ratios between the internal standard (5-hydroxy-3',4',7-trimethoxyflavanone) and tetra-O-methylcatechin.

	weight ratio*				
	0.2	0.4	0.6	0.8	1.0
	0.33	0.70	0.85	1.56	1.76
area ratio	0.32	0.64	0.96	1.28	2.07
	0.35	0.60	1.00	1.63	1.78

*. From Table 9.

Figure 5. GLC Analysis of Catechin and Epicatechin



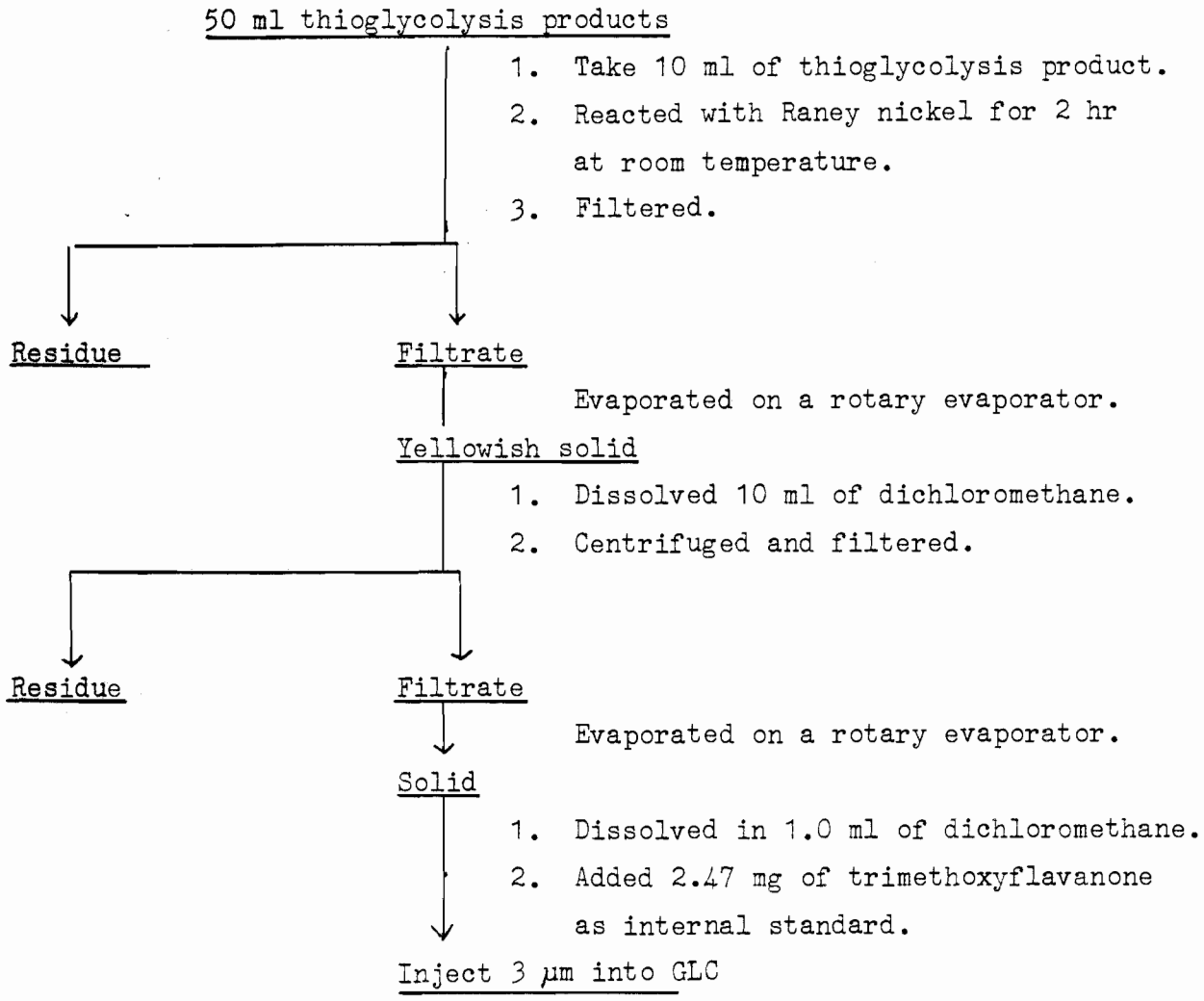


Figure 5 continued

continuous stirring (23). The reaction product was poured into 200 ml of water. The solution was extracted with 125 ml of ethyl acetate (six times). The ethylacetate-soluble fraction was dried with anhydrous sodium sulfate and evaporated on a ratary evaporator at 40°. A yellow-orange colored solid was obtained, which was dissolved in 10 ml of distilled 95% ethanol.

H. Thin-Layer Chromatographic Analyses of the Phloroglucinol Degradation Products.

Analyses were performed on S & S F 1440 cellulose TLC plates cut to 10 cm squares. The plates were developed in the first dimension with t-butanol:acetic acid: water (3:1:1 v/v), air dried and then developed in the second dimension with 6% acetic acid. After air drying the plates were sprayed with vanillin-hydrochloric acid-ethanol (600 mg:1.5 ml:60 ml) as a detection reagent and heated at 95° for five minutes.(25)

I. Adsorption Column Chromatographic Analyses.

The phloroglucinol degradation products were applied to a column of Sephadex LH-20 (3' x 2" diameter) in 95% ethanol. The column was eluted with distilled 95% ethanol. The first fractionation of sample was based on the visual

color bands which were separated into 5 fractions. The elution was detected by a U.V. detector (Model III B, Gilson Co.) at 280 nm. This detector was used to indicate roughly the elution of the compounds. After the five fractions were completely eluted, the column was washed with methanol.

Before the fractions were rechromatographed on the same column, each fraction was applied to TLC plates under the same conditions as mentioned in section III-H. Fraction III showed catechin, epicatechin and residual phloroglucinol. Fraction V showed the hloroglucinol-flavanoid derivatives with some residual phloroglucinol. These two fractions were rechromatographed separately on the same column with the same solvent and fractions (25 ml) were collected.

The fraction III-19-32 gave (+)-catechin and (-)-epicatechin. These fractions were analyzed with three different TLC systims and by HPLC on a C-18 reverse phase column. all analyses gave the same result as authentic (+)-catechin and (-)-epicatechin.

The R_f values on TLC analyses are shown in table.

	R_f of (+)-catechin	R_f of(-)-epicatechin
6 % acetic acid	0.45	0.36
chloroform:ethylacetate: formic acid(5:4:1 v/v)	0.41	0.37
two dimensional chromatogram		
1. 6 % acetic acid	0.40	0.36
2. t-butanol:AcOH: water(3:1:1 v/v)	0.42	0.41

HPLC analyses of (+)-catechin and (-)-epicatechin were performed on a C-18 analytical column supplied by Waters Associatates Co. with methanol:water (1:1 v/v) as solvent. The flow rate was 1.0 ml/min. Fraction III-19-32 gave the same retention times as authentic (+)-catechin and (-)-epicatechin.

Fraction V was also rechromatographed on the Sephadex column under the same conditions as before. One hundred fractions were collected (25 ml) and every third fraction was analyzed by TLC. Fraction V-1-67 should residual phloroglucinol and some unknown compounds. Fraction V-70-100 should a mixture of phloroglucinol-flavanoid derivatives. These fractions were separated by HPLC on a C-18 reverse phase column with methanol:water (45:55 v/v) after concentration on a rotary evaporator. Four fractions were collected and then each fraction was freeze-dried. The freeze-dried samples were run on ^{1}H-NMR and ^{13}C-NMR with deuterated acetone as solvent. The internal standard was tetramethylsilane.

IV. RESULTS AND DISCUSSION

A. Collection of Bark Samples.

D.A. Charleson (14) collected the bark samples from a freshly fallen tree to reduce chances of contamination from organisms and forest debris. The samples were collected in the Spring of the year when it was easy to separate the outer bark from the inner bark. In this way clean samples of outer bark and inner bark were collected for comparison of their extractive contents.

B. Extraction of Bark Samples.

There are numerous solvent systems which can be used to extract condensed tannins from bark. As explained by Suomi-Lindberg (79) the extraction solvent has a strong effect on the yield and the chemical nature of the polyphenols removed. Some typical extraction systems include water to which has been added either sodium hydroxide, sodium sulfite, sulfur dioxide or methanol. D.A. Charleson (14) used acetone-water (70:30 v/v) primarily because Karchesy, Loveland, Laver, Barofsky and Barofsky (36) used this system in their structural studies of the condensed tannins from Douglas-fir bark. The results of their work gave direction and provided reference for the

present work.

C. Acid Hydrolyses of the Bark Extracts.

1. Dilute Acid Hydrolyses

A fundamental aspect of polysaccharides is the component monosaccharides which are linked together to form the polymer chain. Often polysaccharides contain linkages which are resistant to acid cleavage. The bark extracts contained some carbohydrates which were soluble in an aqueous solvent. If the polysaccharides under investigation were water soluble and if hydrolysis could be accomplished under mild acid conditions, treatment with strong acid (77% sulfuric acid) would not be desirable because of degradative side reactions. Since the carbohyrates of bark extracts were water soluble, a mild acid hydroysis (3 % sulfuric acid) was compared to the 77% sulfuric acid hydrolysis.

2. Concentrated Acid Hydrolyses.

A concern that some of the more resistant glycosidic linkages in the polysaccharides may not have been hydrolyzed by the dilute 3.0% sulfuric acid prompted an hydrolysis with concentrated 77.0% sulfuric acid. The

method of Jeffery, Partlow and Polglase (32) as modified by Laver, Root, Shafizadeh, and Lowe (47) was used because it was developed to hydrolyze resistant glycosidic bonds. After hydrolysis, a tan-colored precipitate formed in the bottom of the containers during storage in the refrigerator. The solids were removed by filtration with Whatman # 1 filter paper. They were not investigated further.

D. Paper and Thin-Layer Chromatographic Analyses

The mixtures of monosaccharides which resulted from both mild and concentrated acid hydrolyses were well resolved by paper chromatography and TLC. Figures 6 and 7 show the results. The hydrolyzates from the 3.0% sulfuric acid treatment contained five monosaccarides, glucose, arabinose, mannose, galactose, and xylose. Both inner and outer bark showed the same pattern.

The hydrolyzates from the strong acid treatment, however, contained what appeared to be different amounts of arabinose by both paper and thin-layer chromatography. The visual appearance of the arabinose spots showed that the solids extracted from the outer bark contained more arabinose than the solids extracted from the inner bark.

E. High-Performance Liquid Chromatographic Analyses of Carbohydrates in the Bark Extracts

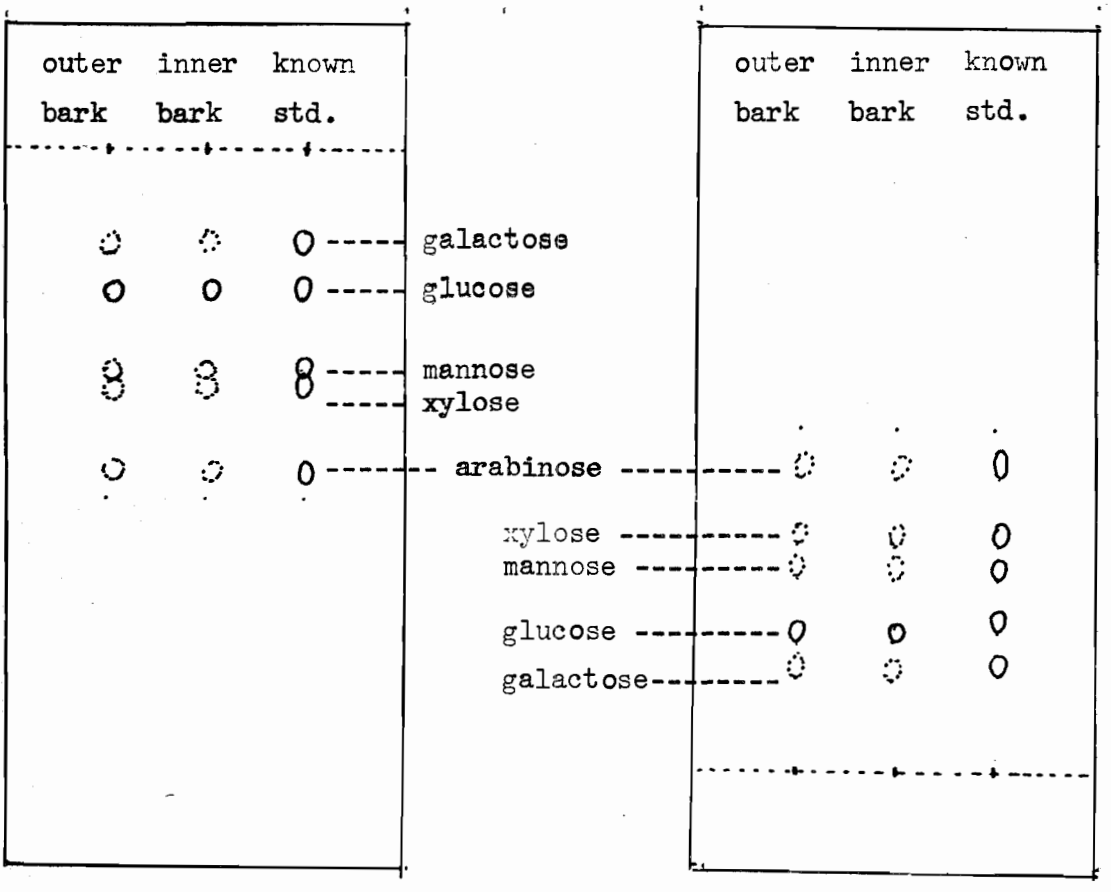


Figure 6

Paper Chromatogram of the Acid Hydrolyzates in Bark Extracts.

Figure 7

Thin-Layer Chromatogram of the Acid Hydrolyzates in Bark Extracts.

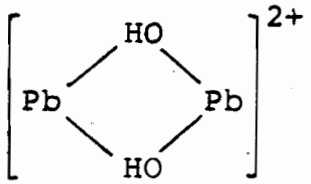
Paper chromatography and TLC are excellent methods for the identification of monosaccharides but they are not accurate enough for quantitative analyses. Therefore, HPLC was used to quantitatively measure the amount of each monosaccharide in the acid hydrolyzates.

1. Columns and Equipment

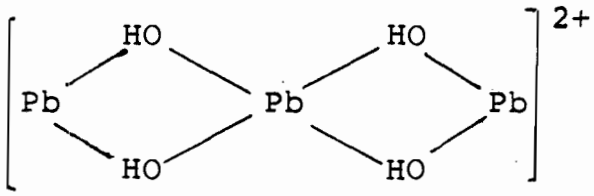
The HPLC column was packed with Chromex DA-X4-11 anion exchange resin from Dionex Corporation, Sunnyvale, CA.. This is a spherical resin copolymer of divinylbenzene crosslinked with quaternary amine functional groups. It is supplied fully hydrated in the chloride form and typically contains 50% water, but this depends on the degree of crosslinking. It was transformed from the chloride form by washing with the borate buffer solution that was used as the eluant for separation. The column was packed under vacuum with hand vibration to remove air bubbles from the slurry as instructed by the manufacturer.

The cation exchange column, Aminex HPX-87P, was supplied by Bio-Rad Laboratories and is prepacked with an 8% crosslinked cation exchange resin with lead ionic forms. Lead forms two series of basic salts frequently formulated as Pb(OH)X and PbX:2Pb$(OH)_2$ (86), a bivalent cation (XX and XXI respectively) was present in a solution

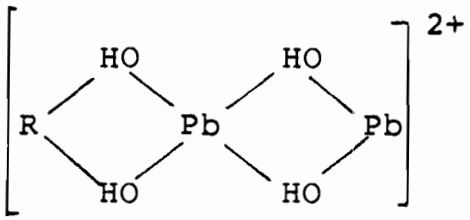
of salts of both series. It is thus possible that, in the cationic complexes formed from a polyhydroxy compound and basic lead acetate, a diol grouping of the former coordinates with the lead atom (XX, XXI, XXII).



XX



XXI



XXII

2. Solvents.

The separation of monosaccharides by complexing with borate ions is well established (20,86) and the ability of the various monosaccharides to complex with the borate ions in the eluant buffer was introduced by Sinner, Simatupang, and Dietrichs (77) in the anion exchange column chromatographic method. The borate ion is able to complex with those compounds in which the oxygen atoms of at least two hydroxyl groups are separated by, or can approach each other to a distance of 2.4 A (59).

The ease of complex formation and the stability of the complexes are undoubtedly due to the stereochemistry of the sugar molecules with respect to their hydroxyl groups. The monosaccharides are thus separated in relation to their stereochemistry.

Figure 8 shows a schematic diagram of the apparatus used for carbohydrate analyses. A detailed description was given in the experimental section except for the detection system. Both cation and anion exchange column systems were used with an ultraviolet-visible detector.

Because the reducing sugars are colorless, the detection method is a critical point in the quantitative analyses. In a comparison of the two different column efficiencies, it is important to choose the correct detection reagents. The 2,2'-bicinchoninate reagent was suitable for this purpose according to Sinner and Puls's report (76) because the differences of the column eluant does not affect the detection.

3. Optimum Operating Conditions

The 2,2'-bicinchoninate-copper complex reagent is both light and heat sensitive. The reaction with reducing sugars requires a constant temperature, 100° in the present work. The eluant from the column was mixed with the copper dye in a three-way valve. The reaction coil was a

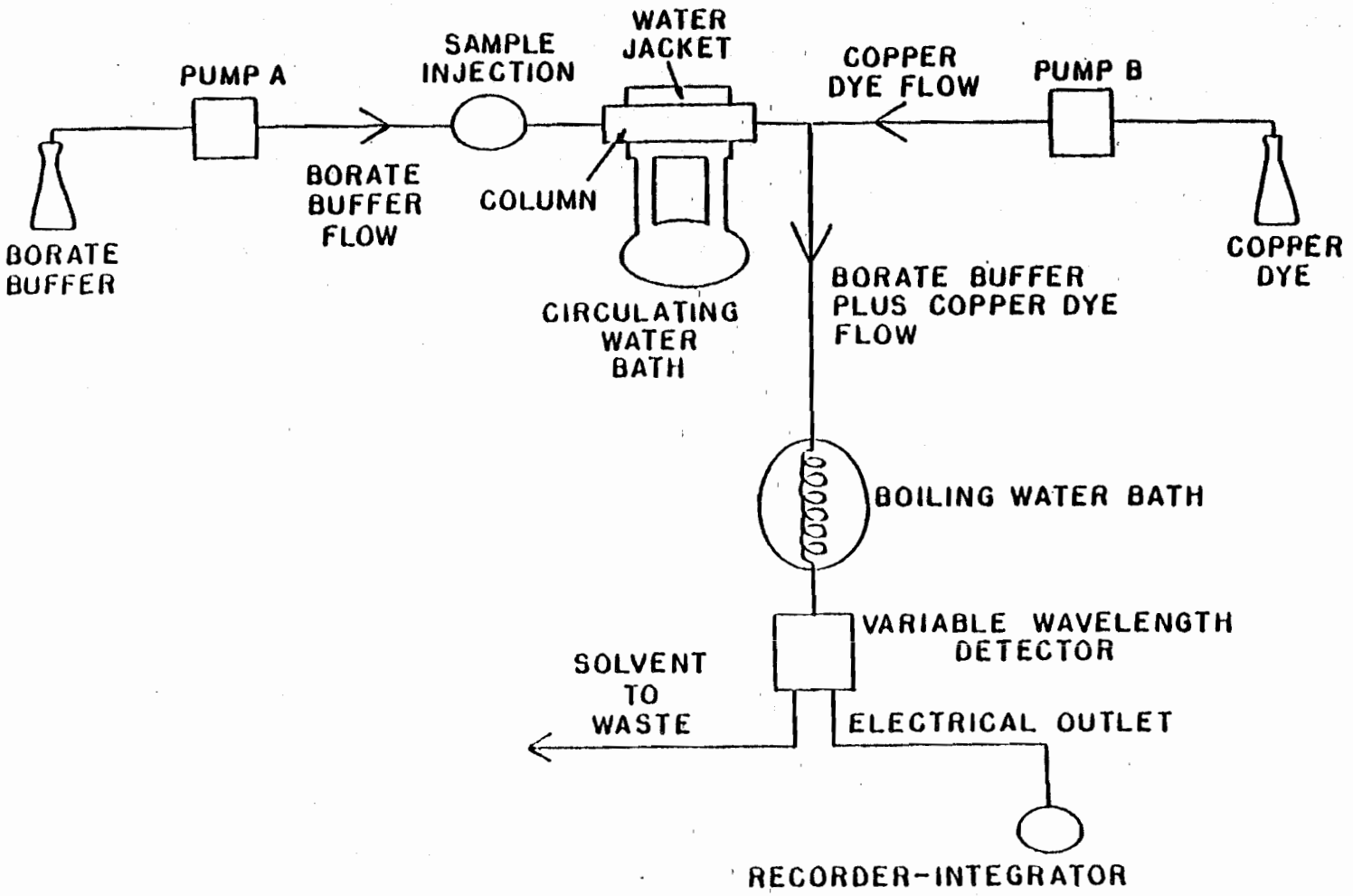


Figure 8. Schematic diagram of the apparatus used for carbohydrate analyses.

15 m length of steel capillary tubing of 0.1" internal diameter. The tubing carried the mixed column eluant and dye through the heating equipment.

Since the reaction between the sugars and the dye strongly depended on heating time and temperature, it was critical that all conditions of the analyses be maintained constant. If the length of tubing in the heating equipment were changed and the temperature of the heating equipment were changed, the amount of color developed would be changed and the absorption results would be erroneous. To maintain a good baseline on the recorder it also was critical to maintain a constant temperature without fluctuation. The internal standard method was used in order to give the same situation for quantitative analyses in all experiments.

4. Determination of Instrument Calibration Curves.

Detectors respond differently to different compounds. These response factors must be known to obtain quantitative results. In addition, the recorder is also a possible source for error when the chromatogram is used for quantitative results. A good way to reduced these sources of error is to add an accurately weighed amount of an internal standard to the mixture to be analyzed and compare the peak areas of the compounds to be measured

against the peak area of the internal standard. As mentioned earlier, it is a good method to eliminate equipment variation. In the present work L-rhamnose was used as the internal standard.

Figure 9 shows the separation by HPLC on the anion exchange column (Chromex DA-X4-11) with borate buffer of L-rhamnose, D-mannose, L-arabinose and D-glucose. The monosaccharides are well resolved by this system.

Figure 10 shows the separation of D-glucose, D-xylose, L-arabinose, D-mannose, and L-rhamnose on the Aminex HPX-87P column. The monosaccharides were not as well separated on this column as on the Chromex DA-X4-11 anion exchange column (Figure 9) but this system had the advantage that the eluant was water instead of a borate buffer, and the retention times were less.

The monosaccharides differ from each other in stereochemical configuration. As mentioned in section IV-E-2 borate ions react with monosaccharides to form complexes and the ease of complex formation and the stability of the complexes are undoubtedly due to the stereochemistry. It has been established (20) that with reducing sugars the greater the number of hydroxyl groups in the axial position, the less stable the formed complex will be. It has been found that D-glucose, which possesses all of the hydroxyl groups in the equatorial position, moves fastest in electrophoretic analyses with borate buffer.(20) These

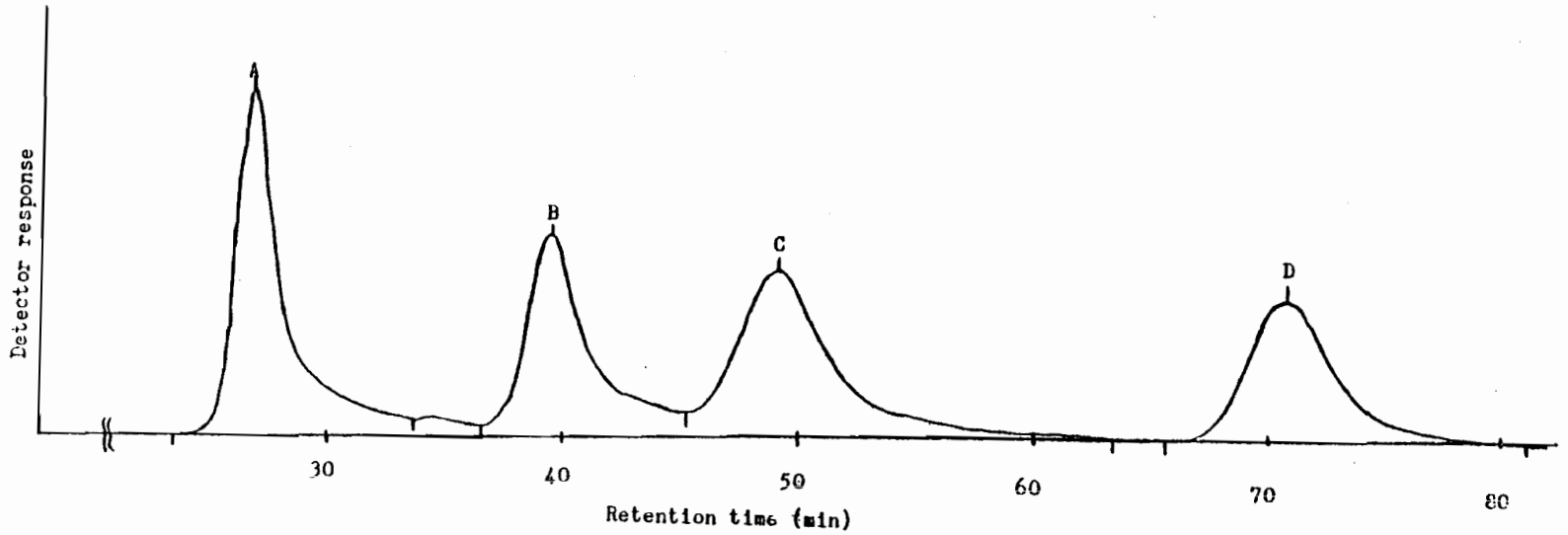


Figure 9. HPLC spectrum of the authentic sugar mixture. Peak A is from L-rhamnose, B is from D-mannose, C is from L-arabinose, and D is from D-glucose. Conditions: column, Chromex DA-X4-11; eluant, 0.13 M potassium borate buffer; flow rate, 0.2 ml/min detector, copper dye and absorbance monitor set at 546 nm.

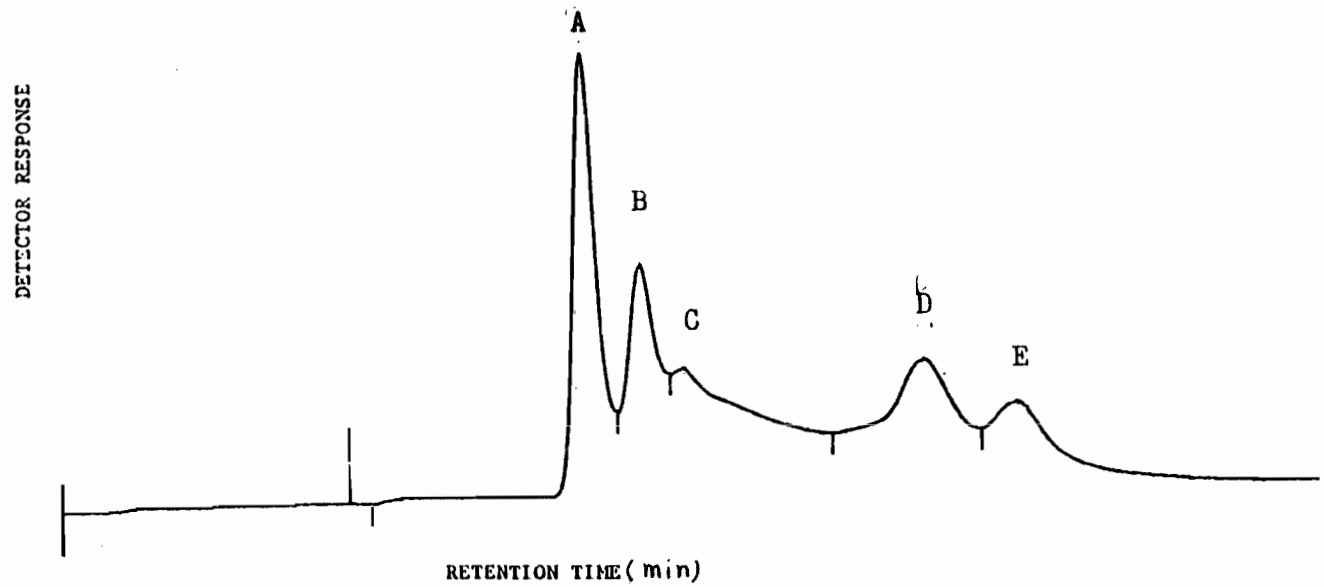


Figure 10. HPLC spectrum of the authentic sugar mixtures on the cation exchange column. Peak A is glucose, B is xylose, C is arabinose, D is mannose, and E is rhamnose. Conditions: column, Aminex HPX-87P cation exchange column; eluant, water; flow rate, 0.6 ml/min; detector, copper dye and absorbance monitor set at 546 nm.

findings indicate that the borate buffer complexes with trans-hydroxyl groups easier than with cis-hydroxyl groups and once formed a more stable complex results. Figure 9 shows the separation of a mixture of authentic monosaccharides on the anion exchange column Chomex DA-X4-11. On the other hand, the chromatogram from the cation exchange column Aminex HPX-87P (Figure 10) shows elution in essentially the reverse order. The acid hydrolyzates from both the inner and outer bark extracts showed a predominance of glucose with some arabinose and trace amounts of xylose, mannose and galactose. Instrument calibration curves were obtained for only glucose and arabinose because the other sugars were present in amounts too small for accurate measurements.

The response of HPLC to L-arabinose and D-glucose was determined by analyzing varying weight ratios of each authentic sugar with the internal standard, L-rhamnose (Tables 5, 6, 7, 8). The instrument calibration curves for L-arabinose and D-glucose were obtained by plotting the ratio of the peak area of each sugar to the area of the internal standard against the ratio of the weights. The results of these plots are shown in Figure 11 and 12 for the anion exchange column system (Chromex DP-X4-11) and Figure 13 and 14 for the cation exchange column system (Aminex HPX-87P).

In the anion exchange column (Chromex DA-X4-11)

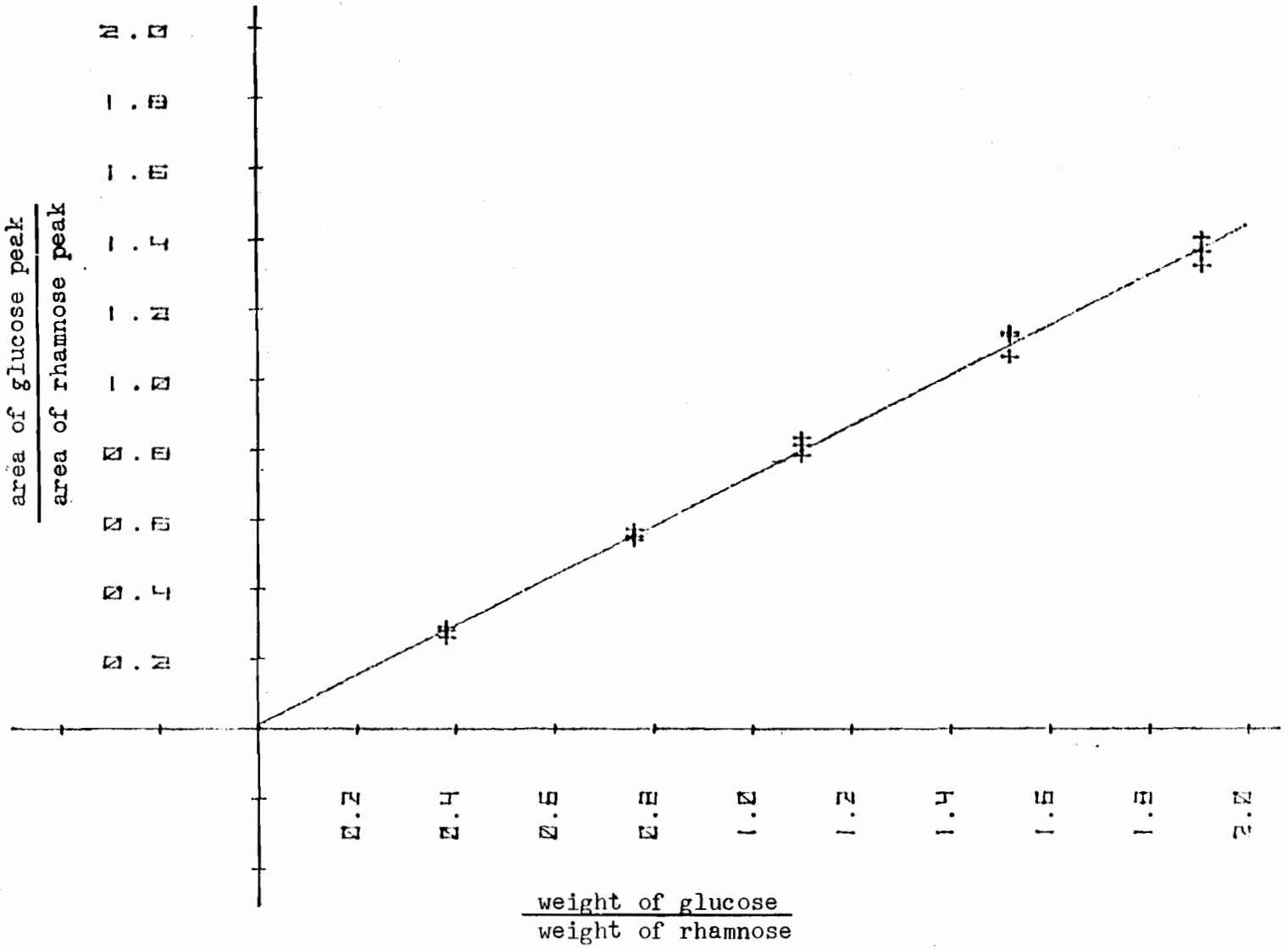


Figure 11. Recovery of authentic D-glucose to determine an " Instrument K factor " on the anion exchange column Chromex DA-X4-11.

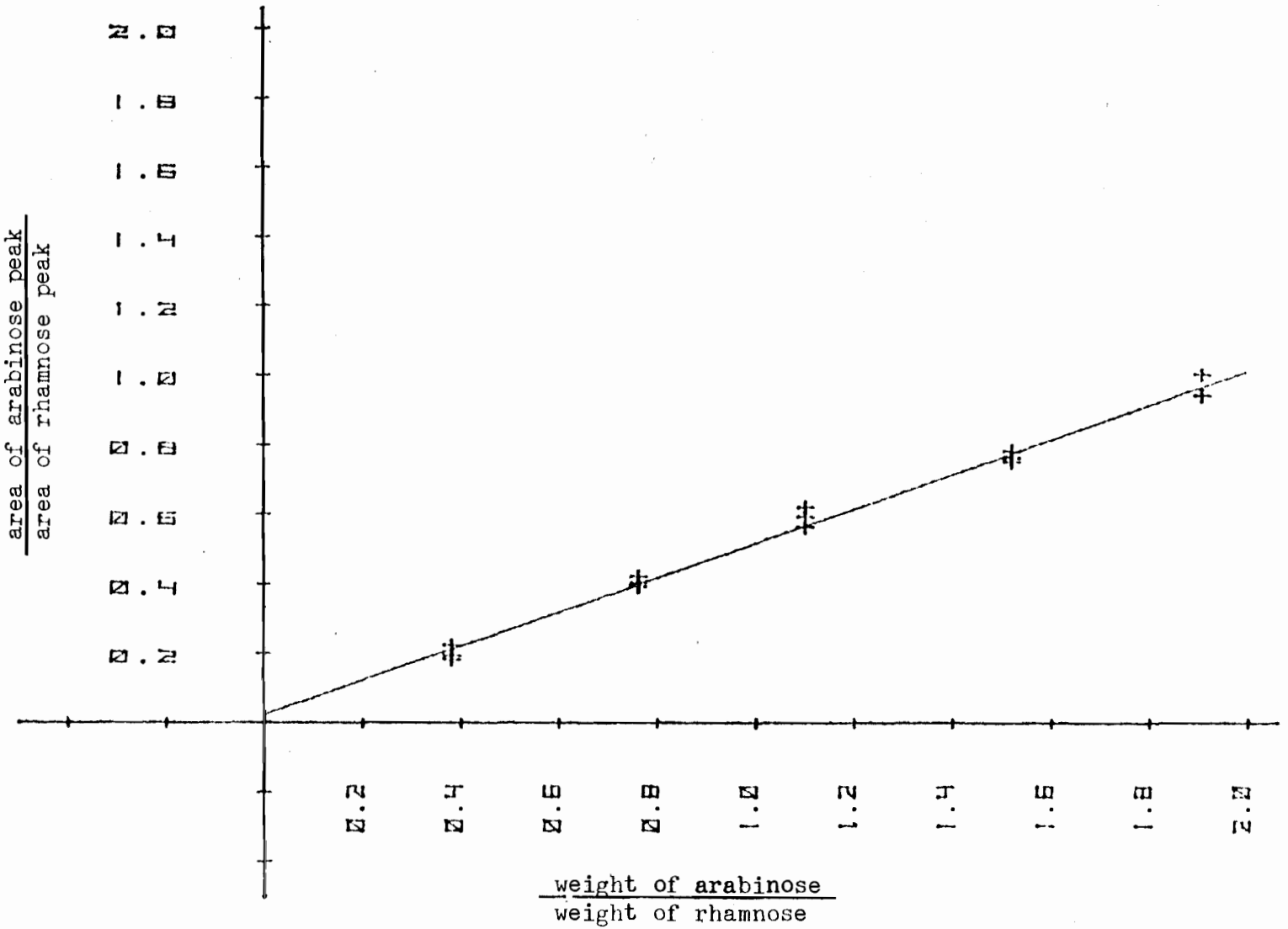


Figure 12. Recovery of authentic L - arabinose to determine an " Intrument K factor " on the anion exchabge column Chromex DA-X4-11.

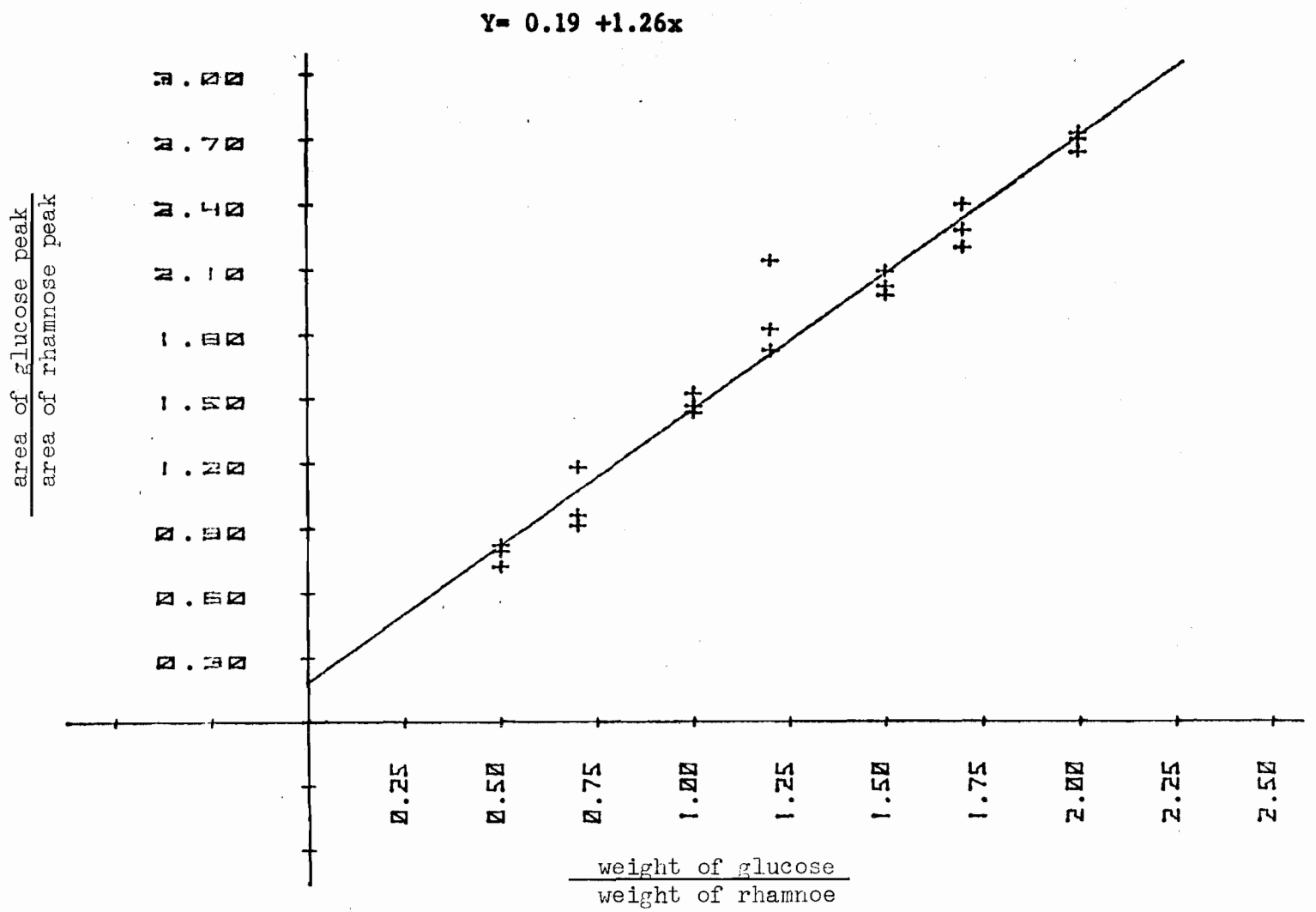


Figure 13. Recovery of authentic D-glucose to determine an " Instrument K factor " on the cation exchange column Aminex HPX-87P.

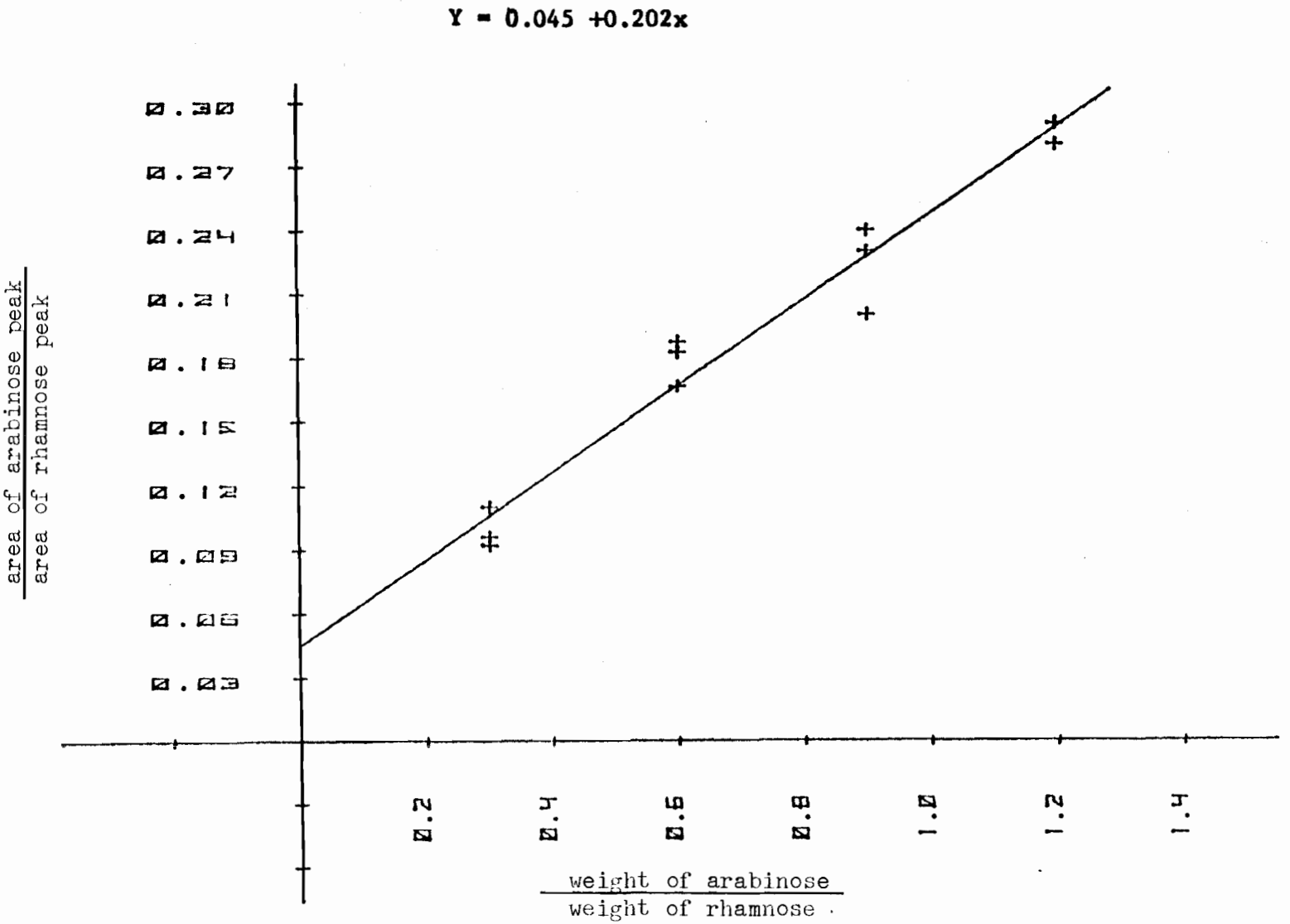


Figure 14. Recovery of authentic L-arabinose to determine " Instrument K factor" on the cation exchange column Aminex HPX-87P.

system, the overall response of both sugars was linear over the entire range of weight ratios tested (Figures 11 and 12). The resulting line equations were as follows.

For glucose (Figure 11) $Y = 0.01 + 0.71X$ ---------- (1)

$R = 0.99$

For arabinose (Figure 12) $Y = 0.02 + 0.49X$ ---------- (2)

$R = 0.98$

Where Y = area ratio; dependent variable.

X = weight ratio; independent variable.

Though the curves do not pass exactly through the origin, the correlation coefficient of each linear regression line was greater than 0.98 and these are good linear curves between weight ratios of 0.4 and 2.0 of both sugars, which is the range where the weight of both sugars were found in the bark extracts. These instrument calibration curves (Equations 1 and 2) were used in subsequent calibrations.

In the cation exchange column (Aminex HPX-87P) system, the overall response of the sugars was linear between weight ratios of 0.5 and 2.50 for glucose (Figure 13) and between 0.3 and 1.20 for arabinose (Figure 14), which is the range where the weights of both sugars were found in the bark extracts.

The resulting equations were as follows;

For glucose, (Figure 13) $Y = 0.19 + 1.26\ X$ -------- (3)

$R = 0.96$

For arabinose, $Y = 0.045 + 0.202\ X$ -------- (4)
(Figure 14)

$R = 0.94$

where, Y = area ratio; dependent variable.

X = weight ratio; independent variable.

Figure 10, however, shows poor resolution and reproductibility except for glucose. Thus the Aminex HPX-87P system proved unsuitable for quantitation of the monomeric carbohydrates in douglas-fir bark tannin extracts. The instrument calibration curves (Equations 3 and 4) were not used in subsequent calibrations.

5. Analyses of the Monosaccharides in the Acid Hydrolyzates of the Bark Extracts.

Five monosaccharides were identified in the acid hydrolyzates of the bark extracts of Douglas-fir by paper chromatography and TLC (Figures 6 and 7). These monosaccharides migrated the same distance as authentic D-glucose, D-mannose, D-galactose, D-xylose, and L-arabinose on co-chromatography. Since earlier work by Laver, Chen, Zerrudo and Lai (46) had definitely established that the polysaccharides of the inner bark of Douglas-fir were comprised of monosaccharides having the configurations of D-glucose, D-mannose, D-galactose, D-xylose, and L-arabinose it is considered that the monosaccharides in the present acid hydrolyzates also possess these configurations.

Figures 15 and 16 show the HPLC spectra of the

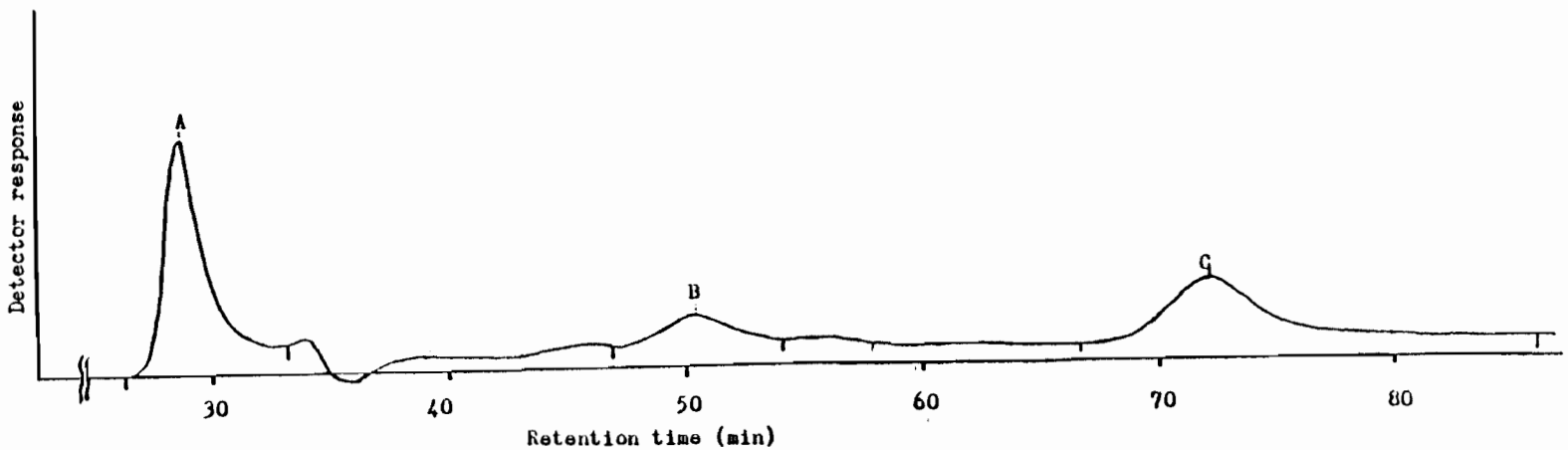


Figure 15. HPLC spectrum of the acid hydrolyzate of outer bark extract. Peak A is the internal standard, L-rhamnose, B is L-arabinose, C is D-glucose. Conditions: column, Chromex DA-X4-11; eluant, 0.13 M potassium borate buffer; flow rate, 0.2 ml/min; detector, copper dye,and absorbance monitor set at 546 nm.

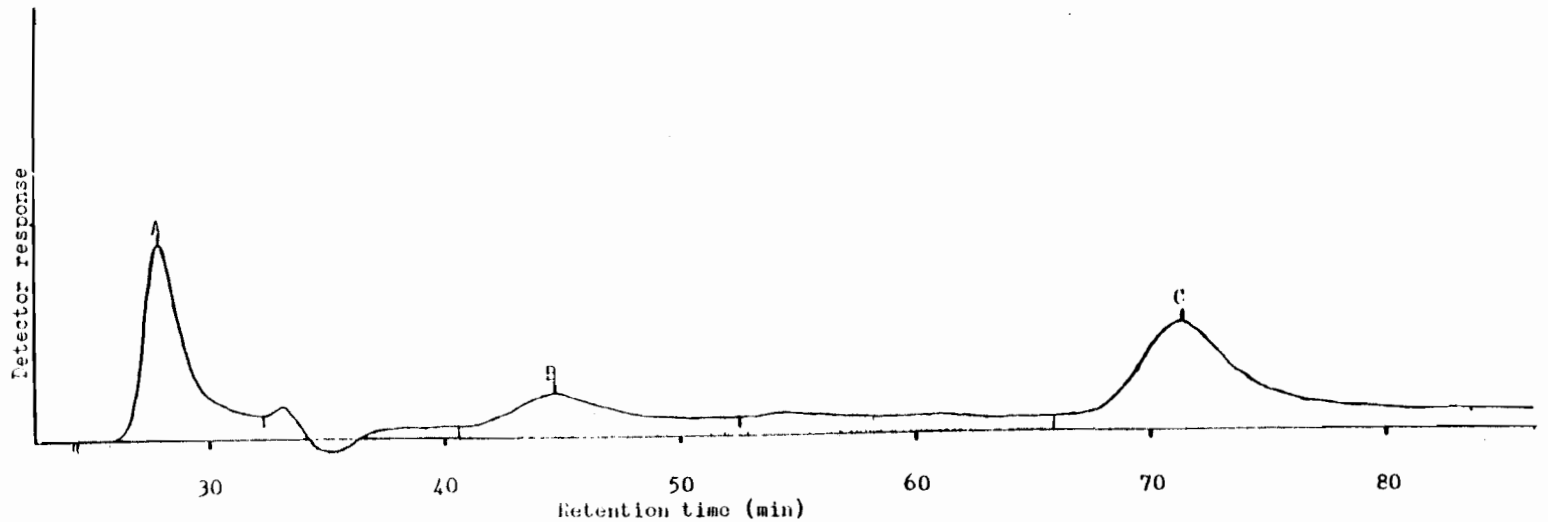


Figure 16. HPLC spectrum of the acid hydrolyzate of inner bark extract. Peak A is the internal standard, L-rhamnose, B is unidentified, and Cis D-glucose. Conditions: column, Chromex DA-X4-11; eluant, 0.13 M potassium borate buffer; flow rate, 0.2 ml/min; detector, copper dye, and absorbance monitor set at 546 nm.

hydrolyzates of outer bark and inner bark respectively on the Chromex DA-X4-11 anion exchange column. As the figures show, a significant amount of D-glucose was present and a small amount of L-arabinose was detected. The other sugars, D-xylose, D-galactose, and D-mannose which were detected in trace amounts by paper chromatography and TLC were not present in large enough quantities relative to D-glucose and L-arabinose to be measured quantitatively. Table 12 shows the areas of D-glucose and L-arabinose in the acid hydrolyzates of the outer bark extracts.

The average area ratio of D-glucose to L-rhamnose of three injections of the acid hydrolyzate of outer bark was 0.73. Substituting this area ratio of 0.73 as Y into the equation $Y = 0.01 + 0.71X$ derived from Figure 11 the value of X, or the weight ratio of D-glucose to L-rhamnose, is 1.01. Based on the oven-dry weight of the starting bark extract this calculated to a glucan content of 12.04% in the extract from outer bark.

The average L-arabinose to L-rhamnose area ratio of three injections of the acid hydrolyzate from outer bark was 0.21. Substituting this area ratio of 0.21 as Y into the equation $Y = 0.02 + 0.49X$ derived from Figure 12 the value of X, or the weight ratio of L-arabinose to L-rhamnose, is 0.39. Based on the oven-dry weight of the starting bark extract this calculates to an arabinan content of 4.51%. Thus the total amount of measureable

Table 12. Areas of D-glucose and L-arabinose in the 77.0 % sulfuric acid hydrolyzate of outer bark extract.

Area under L-rhamnose peak	Area under D-glucose peak	Area ratio D-glucose/ L-rhamnose peaks	Area under L-arabinose peak	area under L-arabinose/ L-rhamnose peaks
0.223	0.162	0.727	0.050	0.224
0.225	0.160	0.711	0.046	0.204
0.216	0.159	0.736	0.042	0.195

Table 13. Areas of D-glucose in the 77.0 % sulfuric acid hydrolyzate of inner bark extract.

Area under L-rhamnose peak	Area under D-glucose peak	Area under D-glucose/L-rhamnose peak
0.217	0.235	1.083
0.193	0.216	1.119
0.195	0.223	1.144

polysaccharide in the outer bark extract was 16.55%. There were also trace amounts of D-galactose, D-mannose and D-xylose containing polysaccharides.

The HPLC chromatogram (Figure 16) of the hydrolyzate of the inner bark extract contained a large amount of D-glucose and a trace aamount of the other monosaccharides. Table 13 shows the areas of D-glucose in the acid hydrolyzate of the inner bark extract. The average area ratio of D-glucose to L-rhamnose of three injections of the acid hydrolyzate of the inner bark was 1.10. Substituting this area ration of 1.10 as Y into the equation $Y = 0.01 + 0.71X$ derived from Figure 11 the value of X, or the weight ratio of D-glucose to L-rhamnose, is 1.55. Based on the oven-dry weight of the starting bark extract, this calculated to a glucan content of 15.94% in the extract from inner bark. There were also trace amounts of D-galactose, D-mannose, L-arabinose and D-xylose.

F. Analyses of Catechin and Epicatechin Moieties in the Bark Extracts.

The quantitative determination of the polyphenolic materials in the bark extracts is critical to their utilization in formaldehyde resins. The polyphenolic materials which comprise the condensed tannins are the catechin and epicatechin monomers. The amounts of catechin

and epicatechin polymers (condensed tannins) in the bark extracts determine their reactivety to formaldehyde in resin systems. If there are large percentages of catechin and epicatechin polymers in the extracts, then the reactivity with formaldehyde should be high and the resulting resins should be strong. On the other hand, if the quanti ty of catechin and epicatechin polymers are low then the reactivity to formaldehyde will be low and the resin systems will be weak. Another system of extraction should be considered if the percentage of catechin and epicatechin polymers are low.

1. Methylation and Degradation of the Bark Extracts.

The free phenolic forms of the condensed tannins are reactive and sensitive to both acid and base conditions. Therefore, the phenolic groups must be protected before the polymer is degraded to its catechin and epicatechin monomers. In the present work the phenolic groups were protected by methylation which formed stable methyl ethers. The methylation reaction was monitored by infrared spectroscopy and methylation was shown to have occured because the infrared spectra (Figure 17) matched that reported by Karchesy (34). It also closely resembled the infrared spectrum of authentic tetra-O-methylepicatechin (Figure 18). Methylation of the catechin-epicatechin

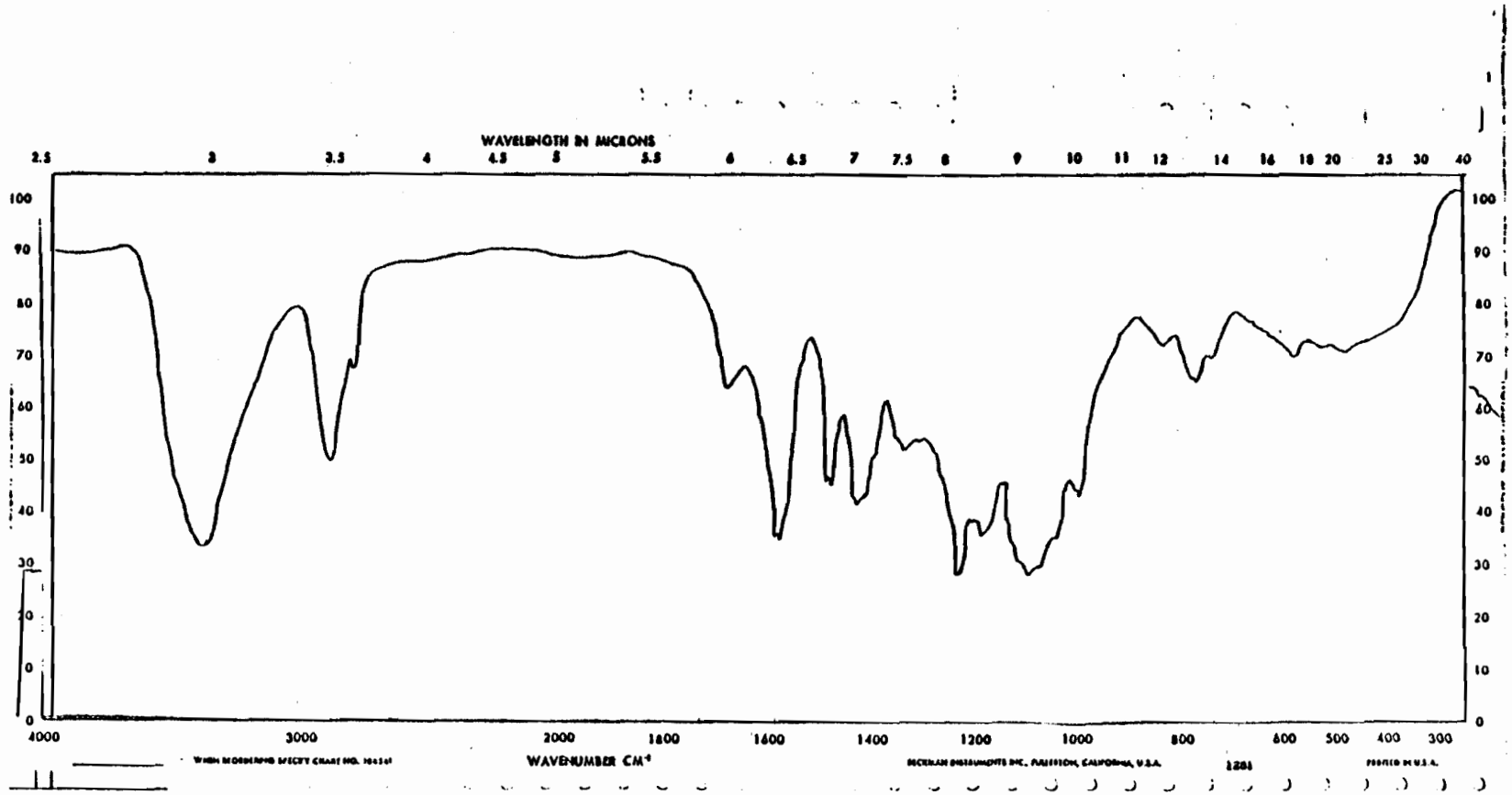


Figure 17. Infrared spectrum of methylated bark extract.

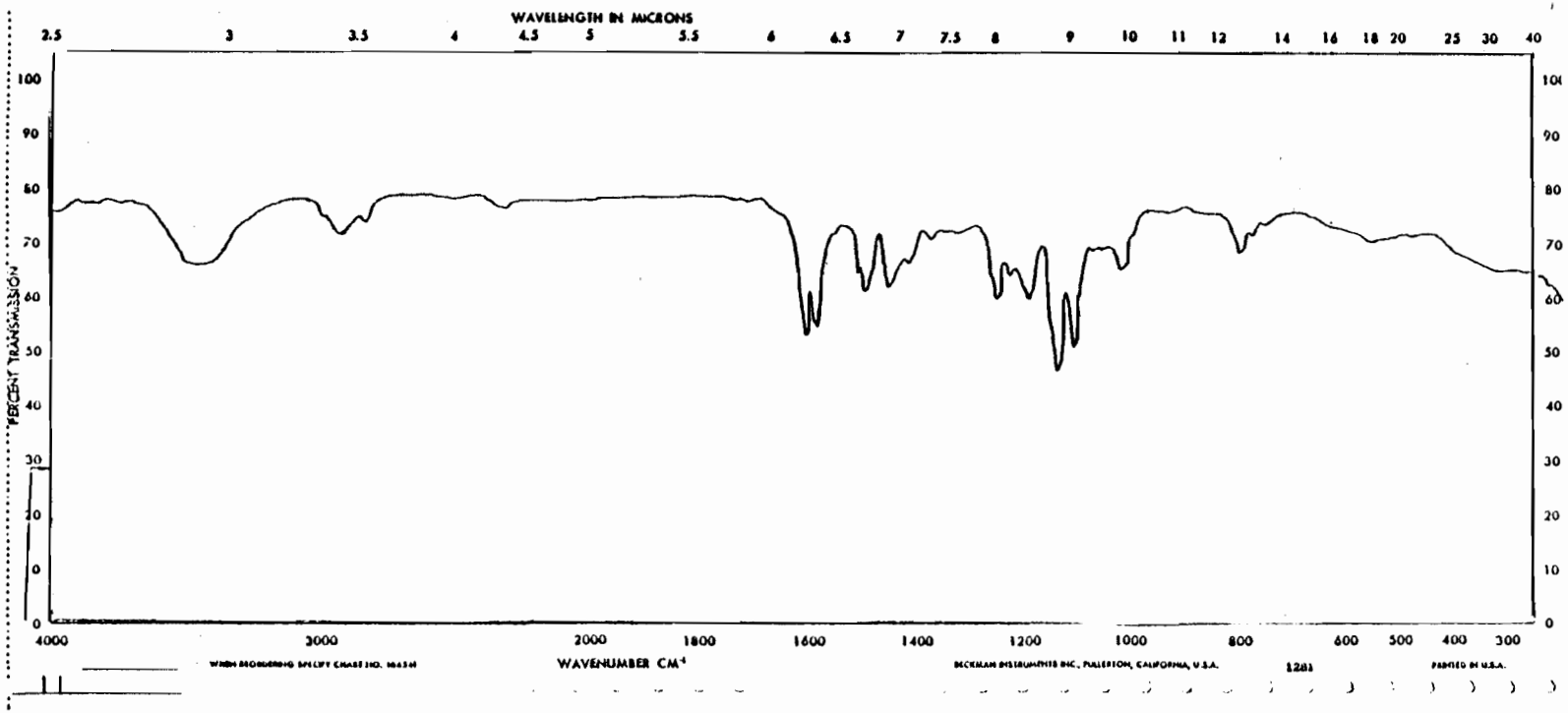


Figure 18. Infrared spectrum of authentic tetra-O-methylepicatechin.

condensed tannin polymers also had the added advantage of resulting in tetra-O-methylcatechin and tetra-O-methylepicatechin which are volatile under conditions of gas-liquid chromatography.

The methylation reaction was performed with dimethylsulfate and potassium carbonate according to the procedure of Sears and Casebier (73). Potassium carbonate is not a strong enough base to bring about methylation of the aliphatic hydroxyl at the 3 position and only the phenolic hydroxyls are methylated. Therefore, upon degradation of the methylated condensed tannin tetra-O-methylcatechin and tetra-O-methylepicatechin are released.

Degradation of the methylated tannin was accomplished by thioglycolic acid. Nucleophilic substitution reactions of thioglycolic acid on the polyflavanols have been well documented (3,12). The nucleophilic attack of thioglycolic acid on the C-4 positions of the condensed tannins follows an Sn 1 mechanism with substitution of the thioglycolic acid in the equatorial position being favored (4,12). In this way the condensed tannin polymers are degraded to their monomeric derivatives.

2. Raney Nickel Reduction Reactions

The thioglycolic acid derivatives of catechin and

epicatechin resulting from the thioglycolic acid degradation of the condensed tannins were readily reduced by activated Raney nickel catalysts without prior isolation. Excess Raney nickel was used and the solution was allowed to react for two hours at room temperature. The solutin was filtered directly into a round-bottomed flask and the solvent evaporated on a rotary evaporator. The resulting residue was dissolved in dichloromethane and 2.47 mg of and internal standard, 5-hydroxy-3',4'7-trimethoxyflavanone, was added. The suspension was centrifuged, filtered, and dried in air. The resulting light-brown solid was redissolved in a carefully measured 1.0 ml amount of dichloromethane and analyzed by gas-liquid chromatography. The scheme of the overall reaction is shown in Figure 19.

3. Preparation of Tetra-O-Methylcatechin

Authentic tetra-O-methylcatechin was needed as a standard for quantitative analyses. It was prepared by methylation of catechin with diazomethane. Diazomethane is a strong enough reagent to yield methyl ether groups on the phenolic hydroxyl groups which are quite acidic but is not a strong enough methylating reagent to methylate the aliphatic hydroxyl group on C-3. A sample of authentic tetra-O-methylepicatechin had beed previously prepared by Dr. Joseph J. Karchesy (34) and the internal standard, 5-

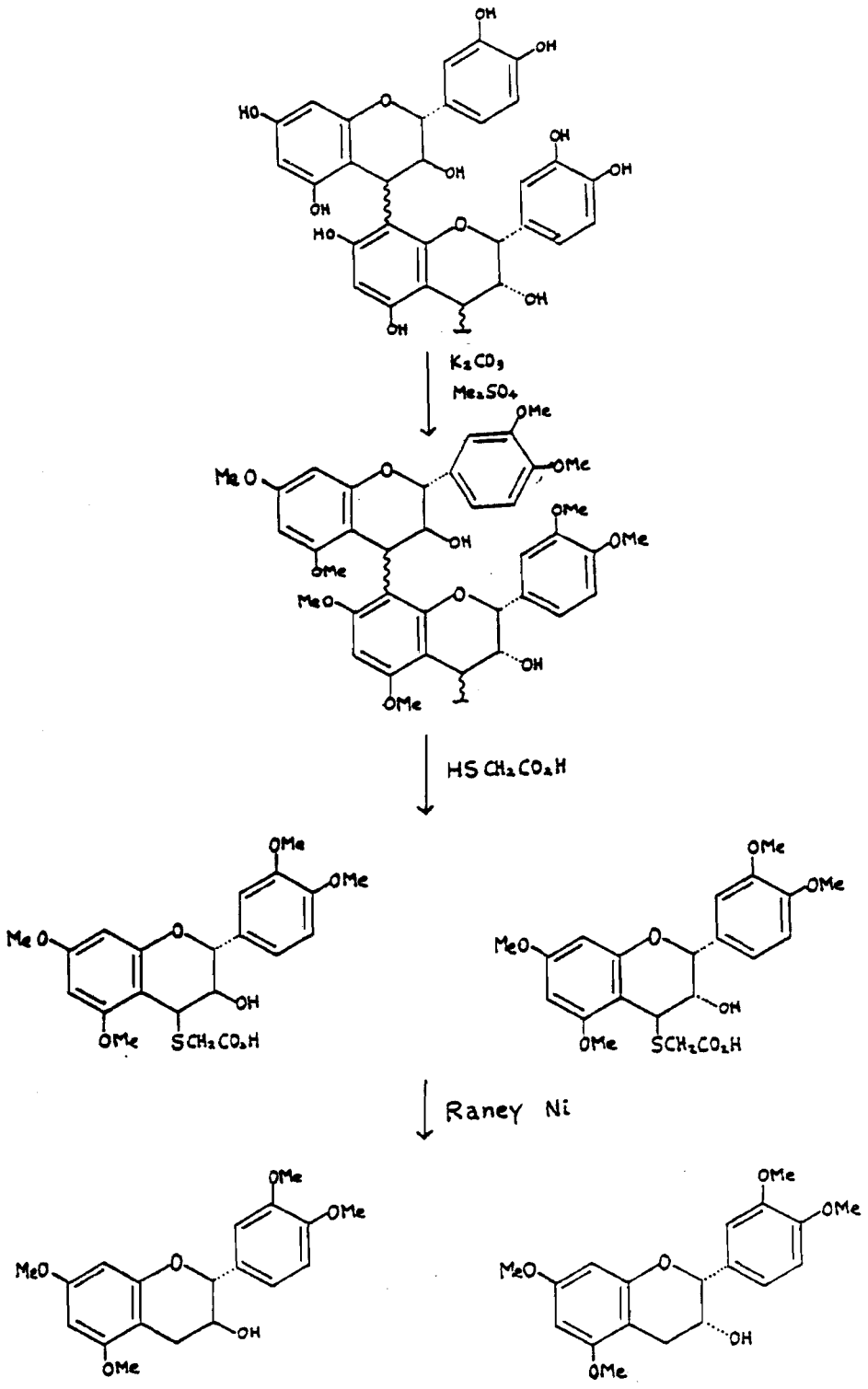


Figure 19. Reaction pathway for obtaining tetra-O-methylcatechin and tetra-O-methylepicatechin from condensed tannins.

hydroxy-3',4',7-trimethoxyflavanone, had been previously prepared by Robert J. Colella (15).

4. Gas-Liquid Chromatographic Analyses of Catechin Materials.

a. Determination of Optimum Conditions

There are many parameters which can be varied in GLC. The resolution of the peaks can be affected by changes in the carrier gas flow, length of the column, and changes in the column temperature. Peak heights and peak areas can be changed by changes in injection port and detector temperatures. Several conditions were systemically changed until the optimum conditions, which are reported in the experimental section of this thesis, were determined.

Identification of the compounds by GLC was realized by comparing the time required for the unknown material to pass through the column with the time required for a sample of the authentic material to pass through the column. These times are called "retention times".

Authentic, crystalline, tetra-O-methylcatechin and tetra-O-methylepicatechin were injected to determine the retention times and instrument response for qualitative

and quantitative analyses. Each of the authentic samples was passed through the gas chromatograph and the retention time was determined. The authentic samples were them mixed and passed through again to ascertain resolution. The resulting spectrum is shown in Figure 20. Retention times for the internal standard (5-hydroxy-3',4',7-trimethoxyflavanone), tetra-O-methylcatechin and tetra-O-methylepicatechin, measured at the center of the peak were 21.3 minutes, 26.8 minutes, and 31.7 minutes, respectively.

b. Determination of Instrument Calibration Curves.

Gas-chromatographic instruments respond differently to different compounds. These response factors must be known to obtain quantitative results. A good way to reduce these sources of error is to add an accurately weighed amount of an internal standard to the mixture to be analyzed and compare the peak areas of the compounds to be measured against the peak area of the internal standard. In this work, 5-hydroxy-3',4',7-trimethoxyflavanone was used as the internal standard.

The response of the gas chromatograph to tetra-O-methylcatechin and tetra-O-methylepicatechin was determined by analyzing varying weight ratios of each authentic tetra-O-methyl derivative with the internal standard. An instrument calibration curve for tetra-O-

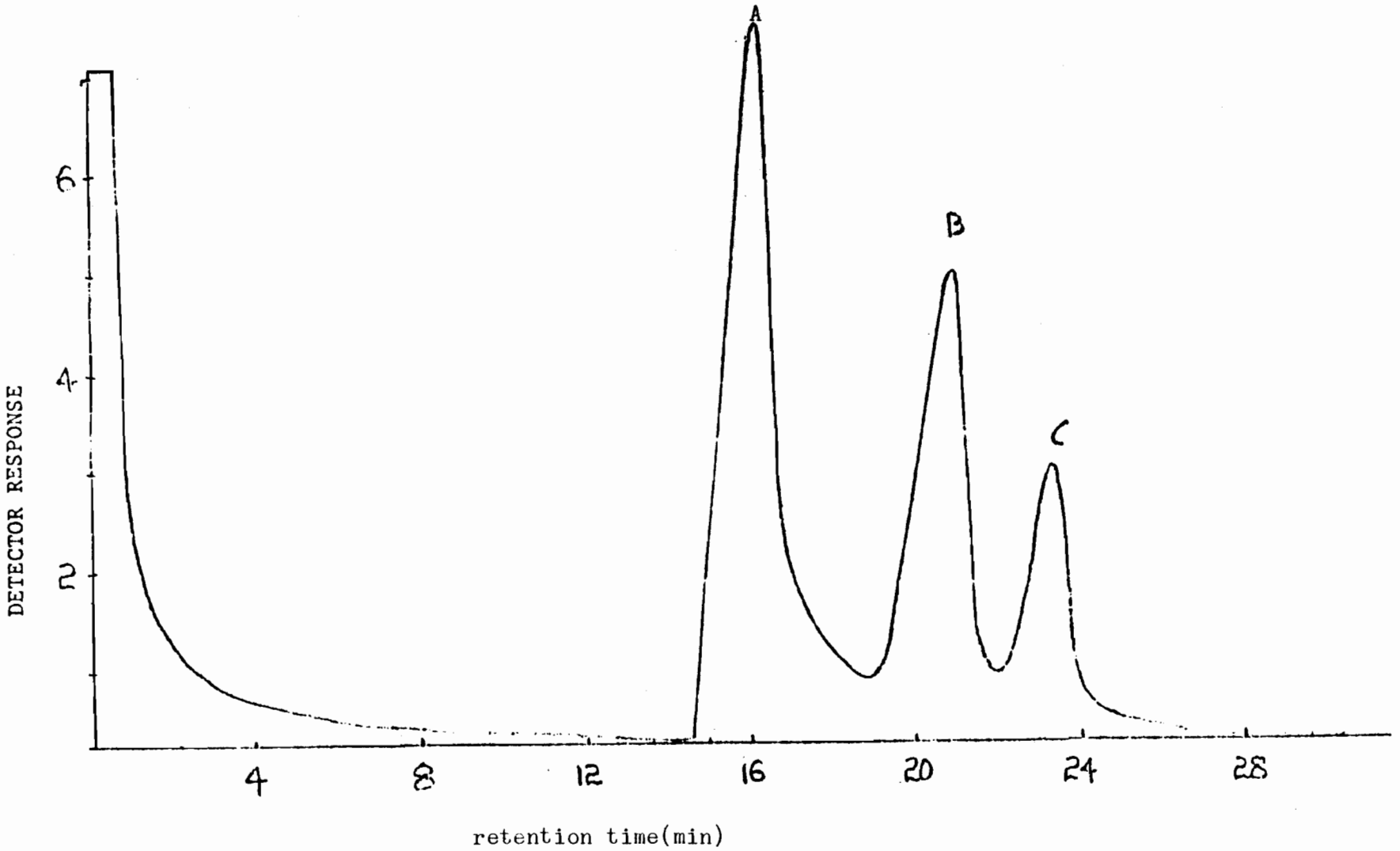


Figure 20. GLC spectrum of a mixture of authentic tetra-O-methylactechin, tetra-O-methylepicatechin, and 5-hydroxy-3',4',7-trimethoxyflavanone. Peak A is the trimethoxyflavanone, B is tetra-O-methylcatechin, and C is tetra-O-methylepicatechin. Conditions: column, 3% OV-17 on Gas Chrom Q 100/200 mesh, 3ft x 1.8in O.D. stainless steel; injection port, 250°; detector, 245°; column temperature, 230°, isothermal; helium, flow rate 30 ml/min; range setting 10^2, attenuation 32.

methylcatechin and tetra-O-methylepicatechin was obtained by plotting the ratio of the peak area of each tetra-O-methyl derivative to the area of the internal standard against the ratio of the weights (Tables 10 and 11).

The results of these plots are shown in Figures 21 and 22. In both cases, the overall response of the chromatographic system was linear over the entire range of weight ratios tested except the range from 0 to 0.3 of tetra-0-methylepicatechin. The resulting equations showed good fits, with correlation coefficents of 0.99 and 0.98 for tetra-O-methylcatechin and tetra-O-methylepicatechin, respectively. The equations are as follows;

For tetra-O-methylcatechin: $Y = 2.03X$ ---------(5)
(Figure 21)
For tetra-O-methylepicatechin: $Y = -0.12 + 1.96X$ (6)
(Figure 22)

where; Y = area ratio; dependent variable

X = weight ratio; independent variable

These instrument calibration curves were used in subsequent calculations.

c. Analyses of the Methylated Degradation Products.

Figure 23 shows a GLC spectrum of the solution from the thioglycolic acid degradation of the bark extracts. Peak A is the internal standard, 5-hydroxy-3',4',7-trimethoxyflavanone, peak B is tetra-O-methylcatechin and peak C is tetra-O-methylepicatechin. The other peaks in the

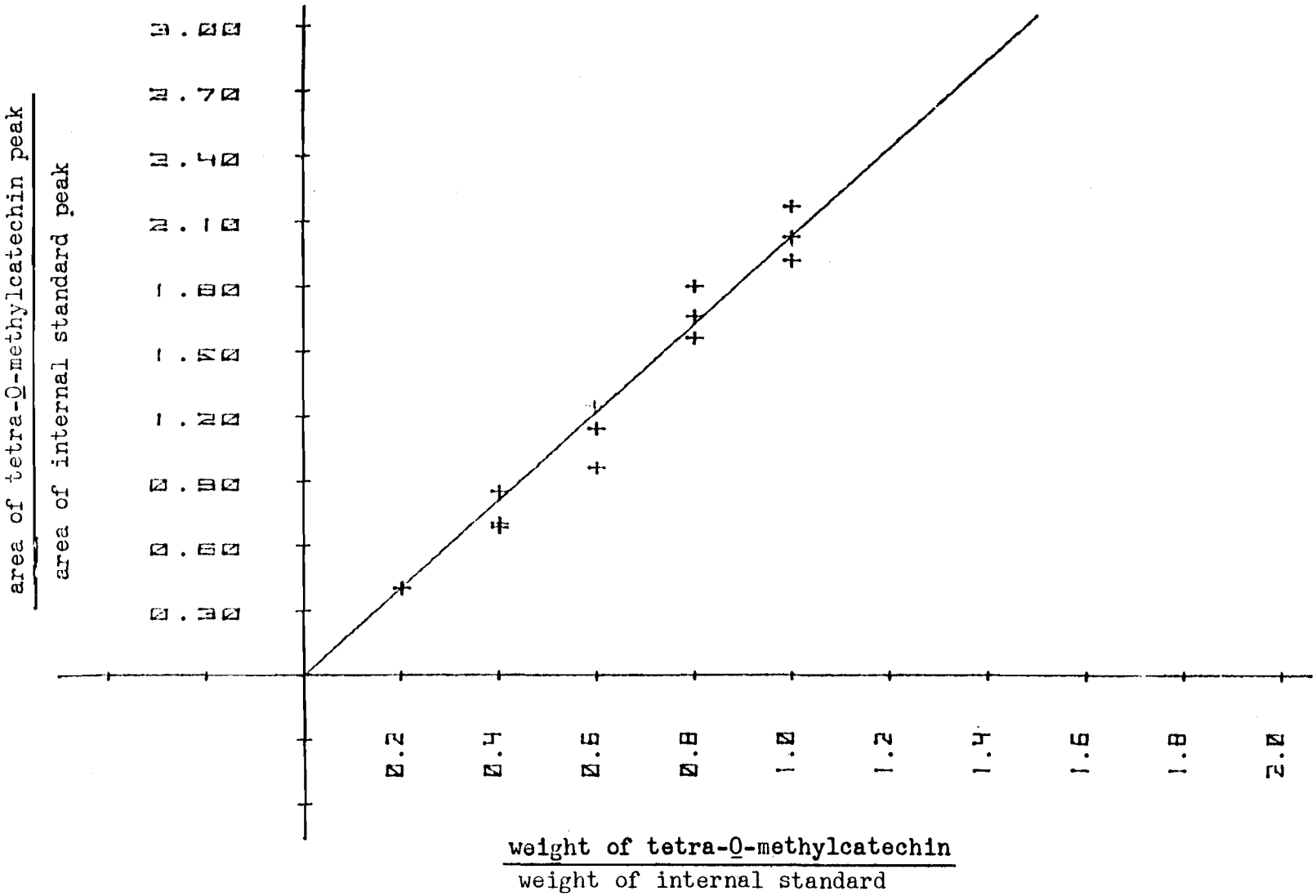


Figure 21. Recovery of authentic tetra-O-methylcatechin to determine an " Instrument K factor "

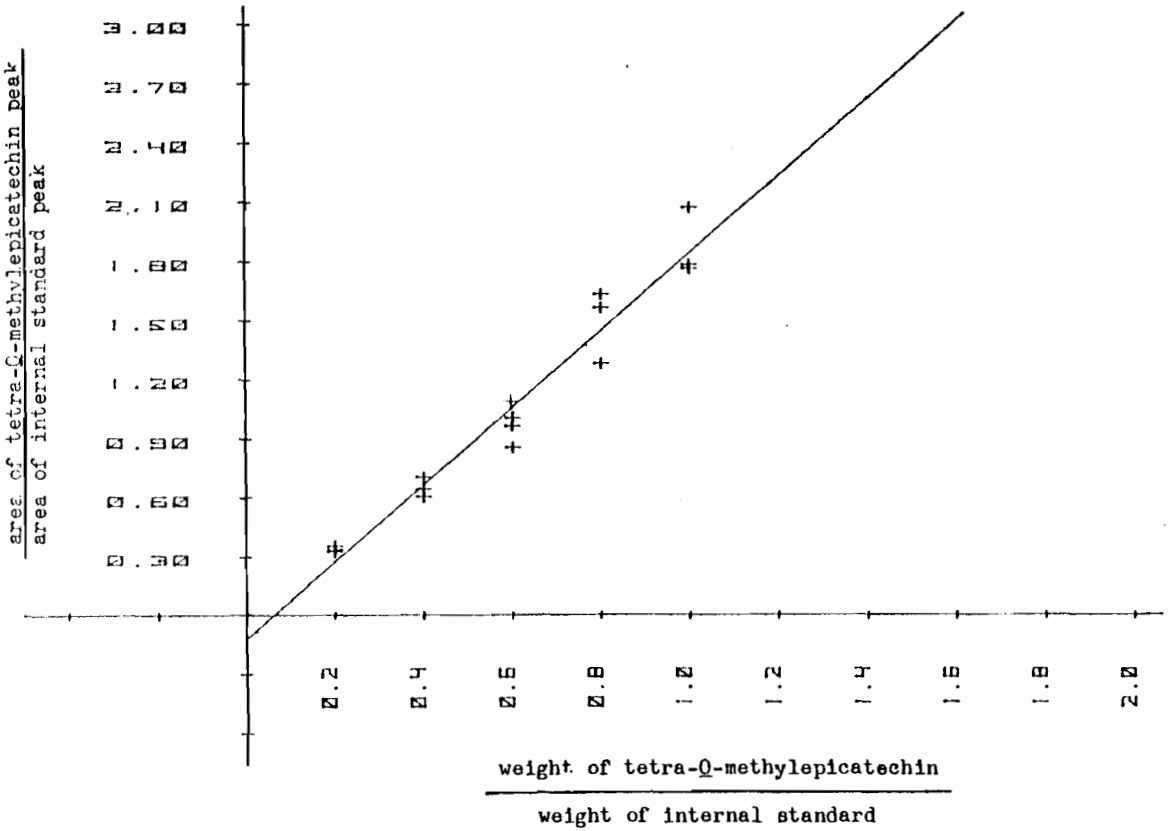


Figure 22 Recovery of authentic tetra-Q-methylepicatechin to determine an " Instrument K factor "

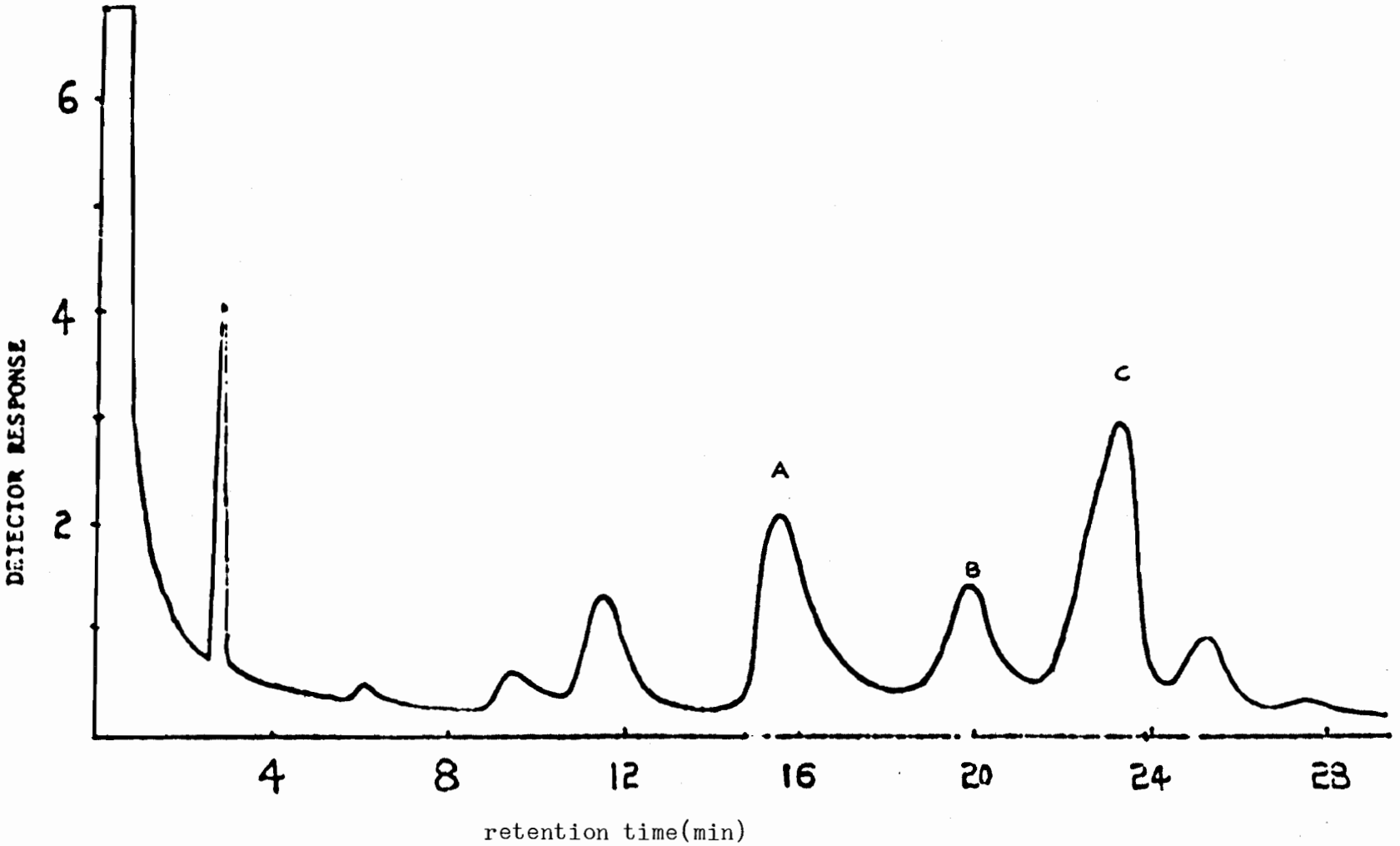


Figure 23. GLC spectrum of methylated degradation product of the inner bark extract. Peak A is the trimethoxyflavanone, B is tetra-O-methylcatechin, and C is tetra-O-methyl epicatechin. Conditions: column, 3% OV-17 on Gas Chrom Q 100/200 mesh, 3ft x 1.8in O.D. stainless steel; injection port, 250°;detector, 245°; column temperature, 230°, isothermal; helium,flow rate 30 ml/min; range setting, 10^2; attenuation,32.

spectrum are unidentified. These possibly result from substances in the bark extracts other than the condensed tannins such as carbohydrates and lignin fragments.

The average tetra-O-methylcatechin peak area ratio to the internal standard of three sample injections was 0.578. Substituting this value as Y into the equation Y = 2.03X derived from Figure 21 then X = 0.285 which is the weight ratio. This calculated to an amount of 3.14 mg of tetra-O-methylcatechin in the solution from the thioglycolysis of the bark extracts. Considering dilution of the sample this calculated to 2.95 mg of catechin in the 2.55 g of inner bark extract (oven-dry weight basis) or 0.12%.

The average tetra-O-methylepicatechin area ratio of three sample injections was 1.072. This resulted in an average weight ratio of 0.608 calculated from the equation Y = - 0.12 + 1.96X derived from Figure 22. This calculated to an amount of 7.51 mg of tetra-O-methylepicatechin in the solution from the thioglycolysis of the bark extracts. This calculated to 6.29 mg of epicatechin in the 2.55 g of inner bark extract (oven-dry weight basis) or 0.25%. The ratio of epicatechin to catechin was 2.1 to 1.

The total amount of 0.37% of catechin and epicatechin from the bark extracts is obviously a very small analytical value. It is not representative of the amount of condensed tannin in the bark extract. Clearly the methylation reaction and the thioglycolic acid reaction gave very poor

yields. Hemingway and McGraw (28) also found that thioglycolic acid gave very poor yields of product even when the molecular weights of the condensed tannins were reasonably high, in the order of 7-8 repeating monommer units (26). The carbohydrates and other extractives must have interfered with these reactions.

G. Phloroglucinol Degradation of Outer Bark Extracts.

The extracted solids were reacted with selected reagents to provide derivatives which could be characterized so that an improved understanding of the condensed tannins could be realized.

The preferred way to degrade condensed tannins and prepare known derivatives is to treat them with phloroglucinol in the presence of hydrochloric acid. This results in cleavage of the carbon-carbon bond at C-4 with the addition of phloroglucinol at the C-4 position. The resulting catechin and epicatechin phloroglucinol derivatives have been quite well characterized.

H. Thin-Layer Chromatographic Analyses of the Phloroglucinol Degradation Products

The bark extracts were reacted with phloroglucinol and hydrochloric acid according to the procedure of Gupta

and Haslam (24) and Fletcher, Porter, Haaslam, and Gupta (18). The progress of the reaction was monitored by TLC. Every 12 hr an aliquot was applied to the bottom corner of an S & S F 1440 cellulose thin-layer plate cut to 10 cm by 10 cm in size. The plates were developed in the first dimension with 2- butanol-acetic acid-water (3:1:1v/v) (system A) and in the second dimension with 6% acetic acid. This irrigation system had a shorter developing time than t-butanol-acetic acid-water (3:1:1 v/v) (system B) (77) by about one hour. The resolution of the compounds were no different by system A than by system B except that the R values were different. However, the pattern of the spots was the same. The spray reagent, vanillin-hydrochloric acid-ethanol (600 mg:1.5 ml:120 ml), produced red or reddish-pink spots with the flavanoid derivatives and an orange spot with unreacted phloroglucinol. Color was a way of identifying the compounds because the R values were not always dependable.

After 24 hr of reaction time, the thin-layer plate showed spots for free epicatechin, some flavanoid derivatives, excess phloroglucinol and some unreacted polymeric material near the origin. After 48 hr much of the polymeric material had reacted but there was still some left. After 60 hr of reaction, no residual polymeric material was detected on the thin-layer plates. It was considered that the phloroglucinol reaction was complete. The reac-

tion time of 60 hr was longer than the reaction time of 48 hr reported by Gupta and Haslam (24).

The thin-layer plate of the reaction products of the bark extract with phloroglucinol after 60 hr is shown in Figure 24. Figure 24 shows spots for phloroglucinol (A), phloroglucinol derivatives (B), catechin (C), and epicatechin (D). The remaining spots were unidentified. The plate matched well the results of Hemingway (25).

I. Adsorption Column Chromatographic Analyses

Sephadex columns were used in an effort to isolate some of the catechin and epicatechin phloroglucinol derivatives. The eluants were monitored by TLC. Fraction III showed the presence of excess phloroglucinol, catechin and epicatechin. Fraction V showed the presence of phloroglucinol derivatives, some unknown material, and a small amount of phloroglcinol.

Fraction III was rechromatographed on the same Sephadex column and the phloroglucinol was separated from the catechin-epicatechin mixture. The catechin and epicatechin were identified by TLC and by HPLC by co-chromatography with authentic samples (Figure 25).

Fraction V of the original Sephadex separation was also rechromatographed on the same Sephadex column and 100 fractions were collected. The phloroglucinol was

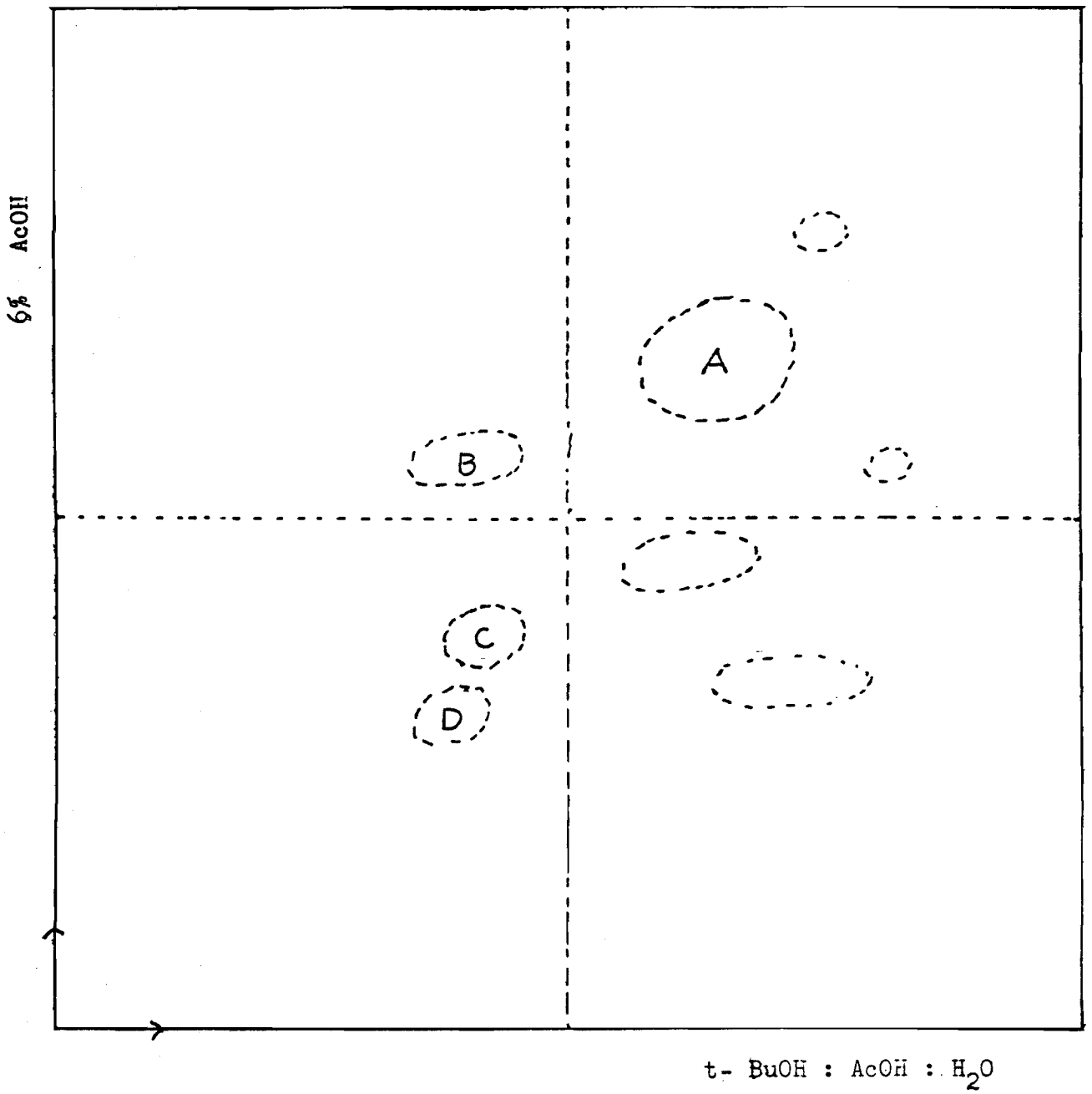


Figure 24. Thin-layer chromatogram of the phloroglucinol degradation products. Spot A is excess phloroglucinol, B is phloroglucinolderivatives, C is catechin, and D is epicatechin. The other spots are unidentified.

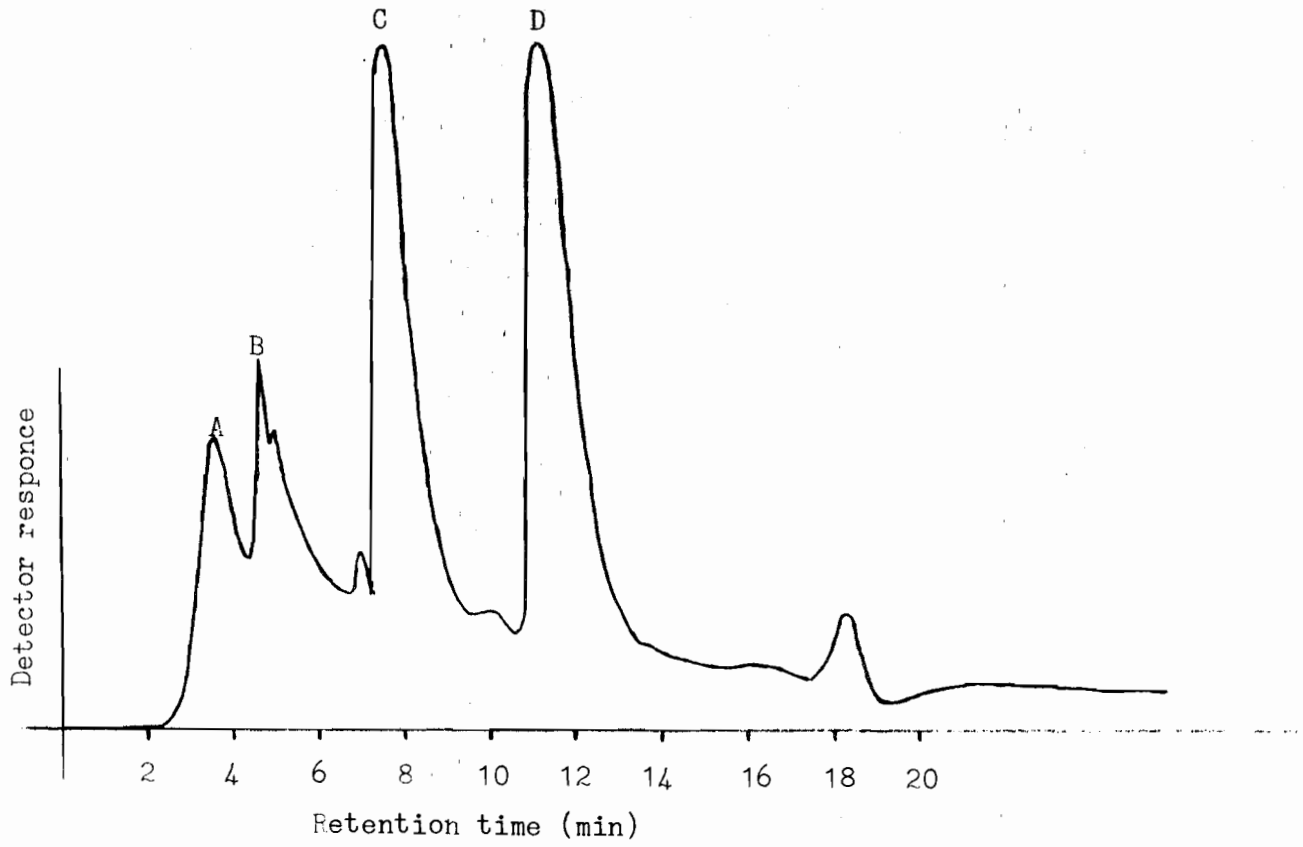


Figure 25. HPLC spectrum of catechin and epicatechin from the phloroglucinol reaction product with authentic (+)-catechin and (-)-epicatechin. Conditions: column, C-18 reverse phase; eluant, methanol:water (1:1 v/v) flow rate, 1.0 ml/min; detector, absorbance monitor set at 280 nm. Peaks A and B are from phloroglucinol, peaks C and D are from catechin and epicatechin, respectively.

separated from the phloroglucinol-flavanoid derivatives. These were collected in fractions 70-100. The fractions were combined and separated by HPLC on a C-18 reverse phase column. Four peaks were resolved (Figure 26). EAch peak was collected and characterized by TLC and ^{1}H-NMR (Figure 27). The peaks showed identical TLC's and similar ^{1}H-NMR spectra because the resolution was still not perfect. However, the TLC's and the ^{1}H-NMR spectroscopy demonstrate the presence of phloroglucinol catechin-epicatechin reaction products.

A ^{13}C-NMR spectrum of a second collection from the HPLC showed the presence of a (-)-epicatechin-4-phloroglucinol adduct and a (+)-catechin-4-phloroglucinol adduct (Figure 28). This ^{13}C-NMR spectrum matched well the data reported by Porter, Newman,Foo, Wong, and Hemingway (63).

The four peaks in the HPLC spectrum (Figure 26) could be due to: (2R,3R,4R)-4-(2,4,6-trihydroxyphenyl) flavan-3,3',4',5,7-pentaol (XXV); (2R,3R,4S)-4-(2,4,6-trihydroxyphenyl)flavan-3,3',4',5,7-pentaol (XXVI) derived from the flavn carbocation (XXIII) with a (-)-epicatechin stereochemistry; (2R,3S,4S)-4-(2.4,6-trihydroxyphenyl)flavan-3,3',4',5,7-pentaol (XXVII), and (2R,3S,4R)-4-(2,4,6-trihydroxyphenyl)flavan-3,3',4',5,7-pentaol (XXVIII) derived from the flavn carbocation (XXIV) with a (+)-catechin stereochemistry.

From these results, it was shown that the bark

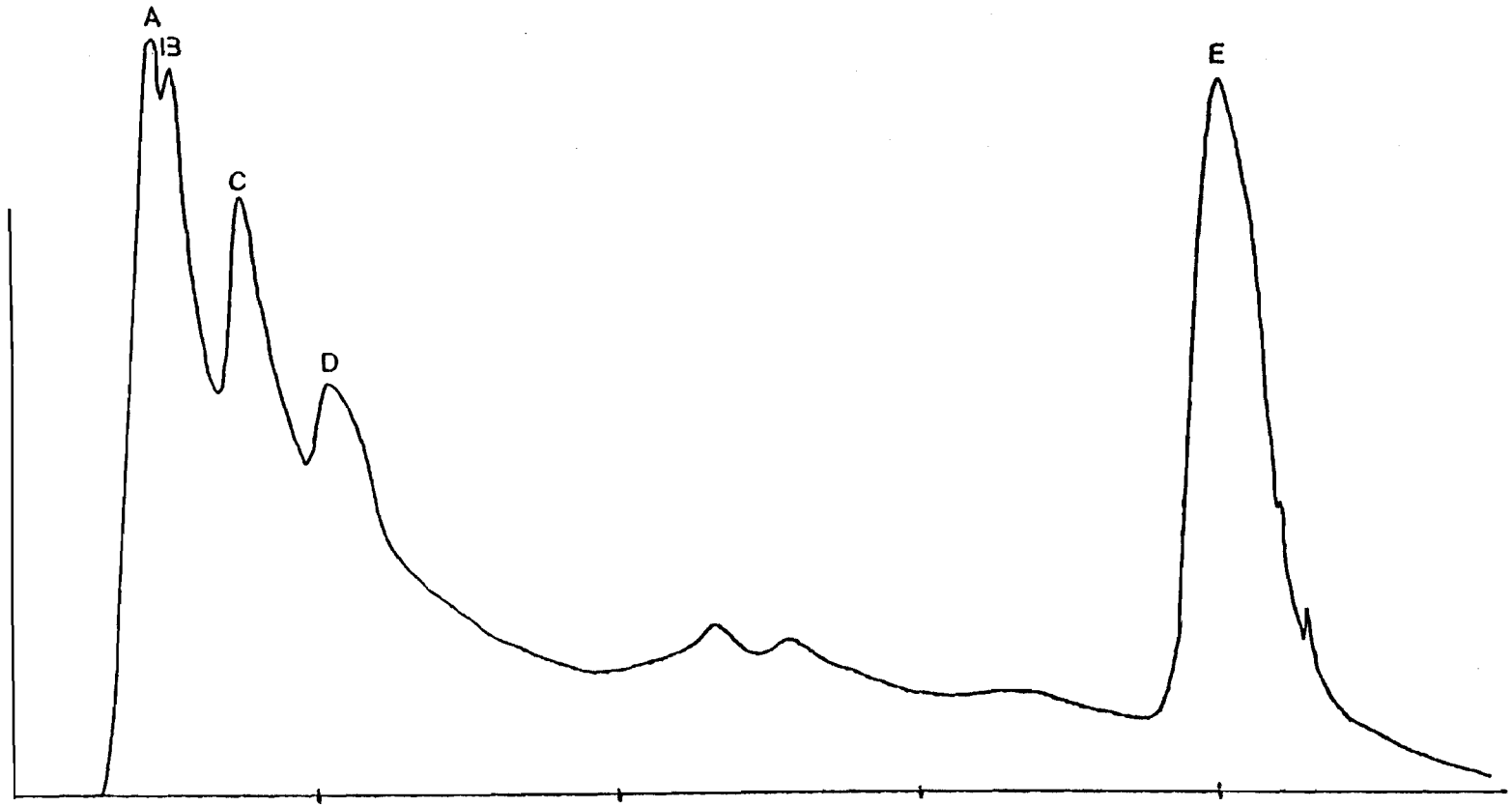


Figure 26. Liquid chromatographic spectrum of phloroglucinol-flavanoid derivatives. Peak A,B,C, and D are phloroglucinol - flavanoid derivatives. Peak E is unidentified. Conditions; Column, C - 18 reverse phase; eluant, methanol;water (45;55 v/v) ; flow rate 1.0 ml/min; detector, absorbance moniter set at 280nm.

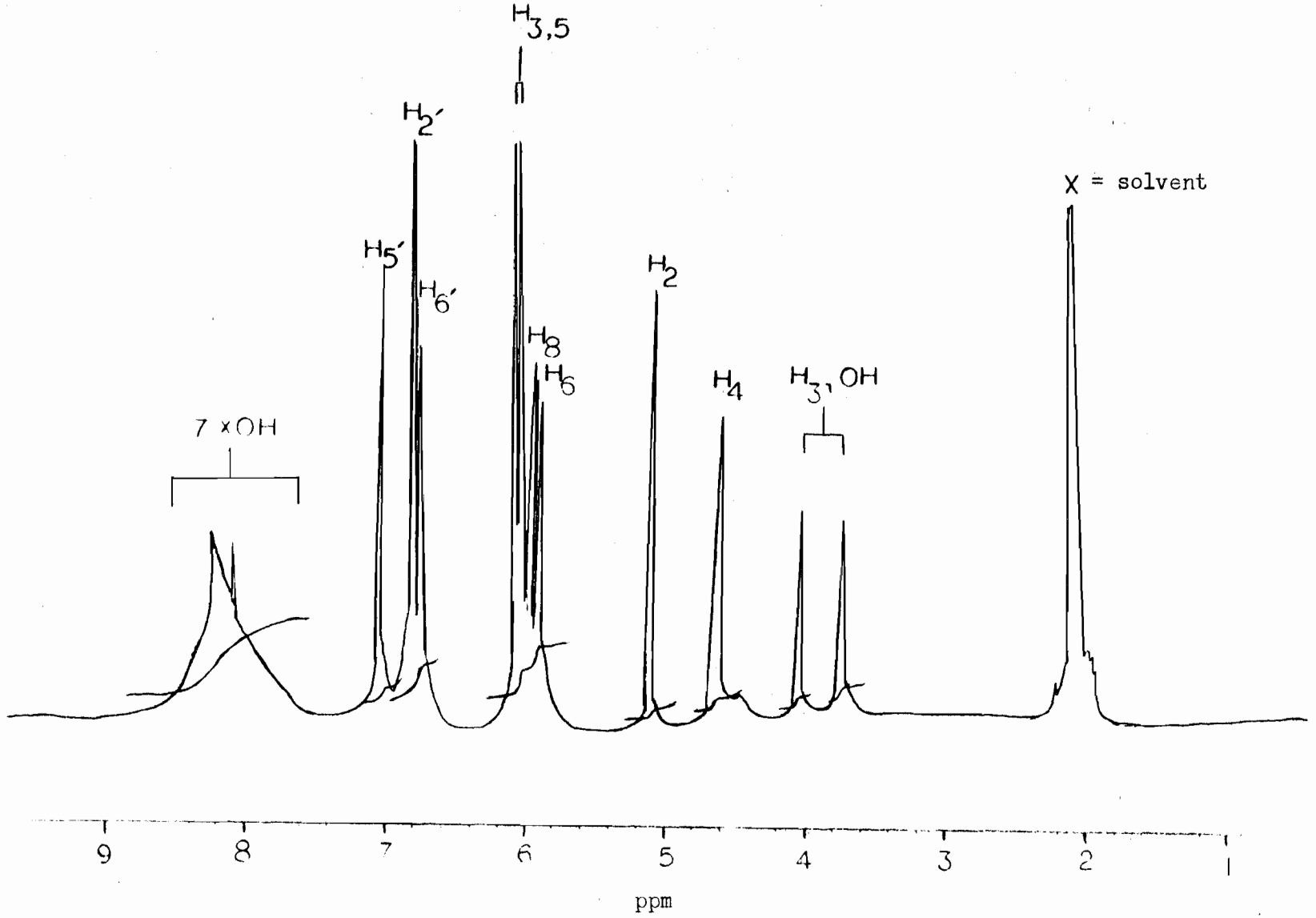


Figure 27. The ^{1}H-NMR spectrum of the phloroglucinol-flavanoid derivatives in acetone-d_6.

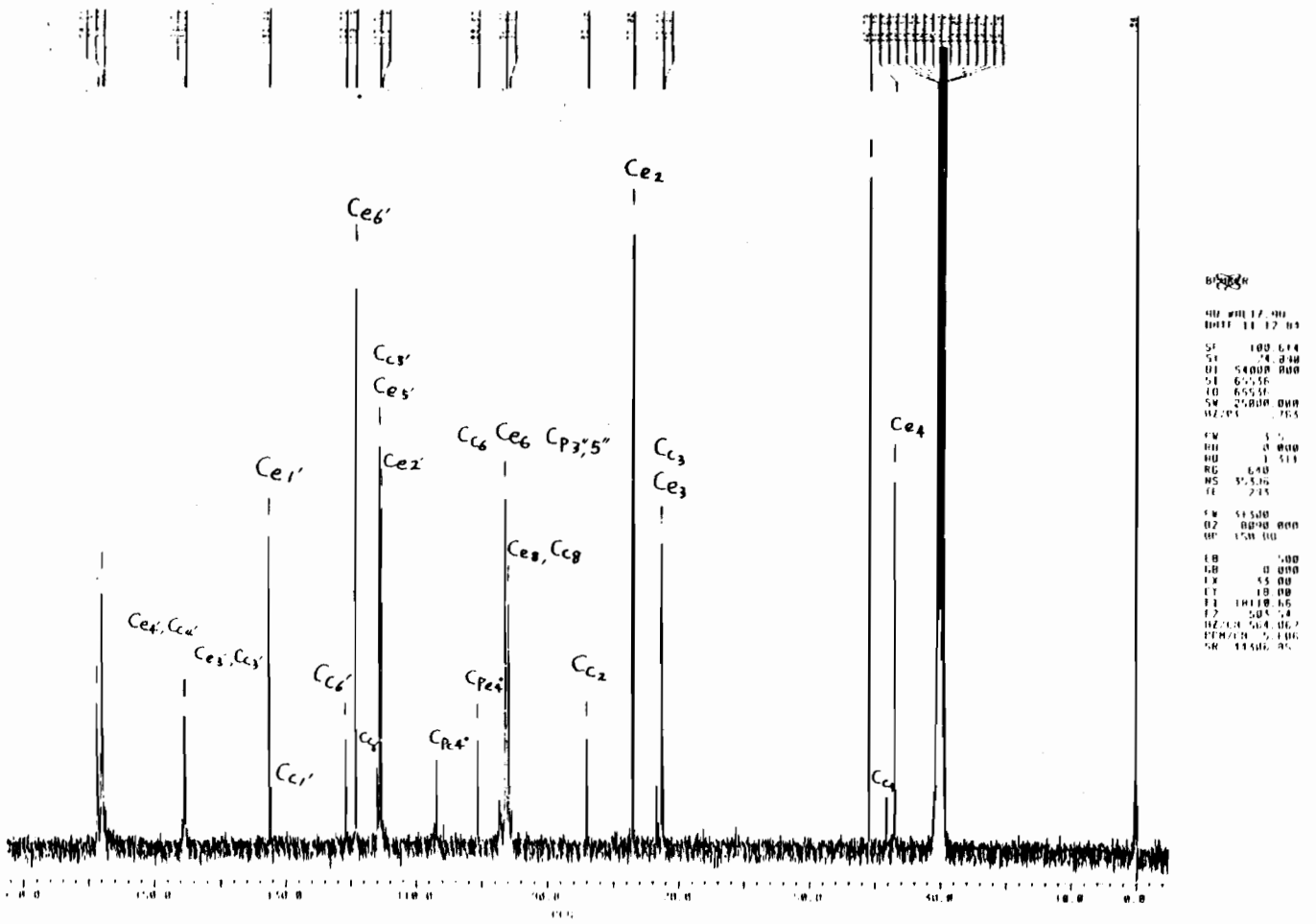


Figure 28. The ^{13}C-NMR spectrum of the phloroglucinol-flavanoid derivatives in acetone-d$_6$.

extract contained condensed tannins comprised of catechin and epicatechin units. Catechin and epicatechin also form the chain terminal units at the bottom of the polymers, because free catechin and epicatechin was formed from the reactions with phloroglucinol. Figure 29 shows the overall reaction scheme.

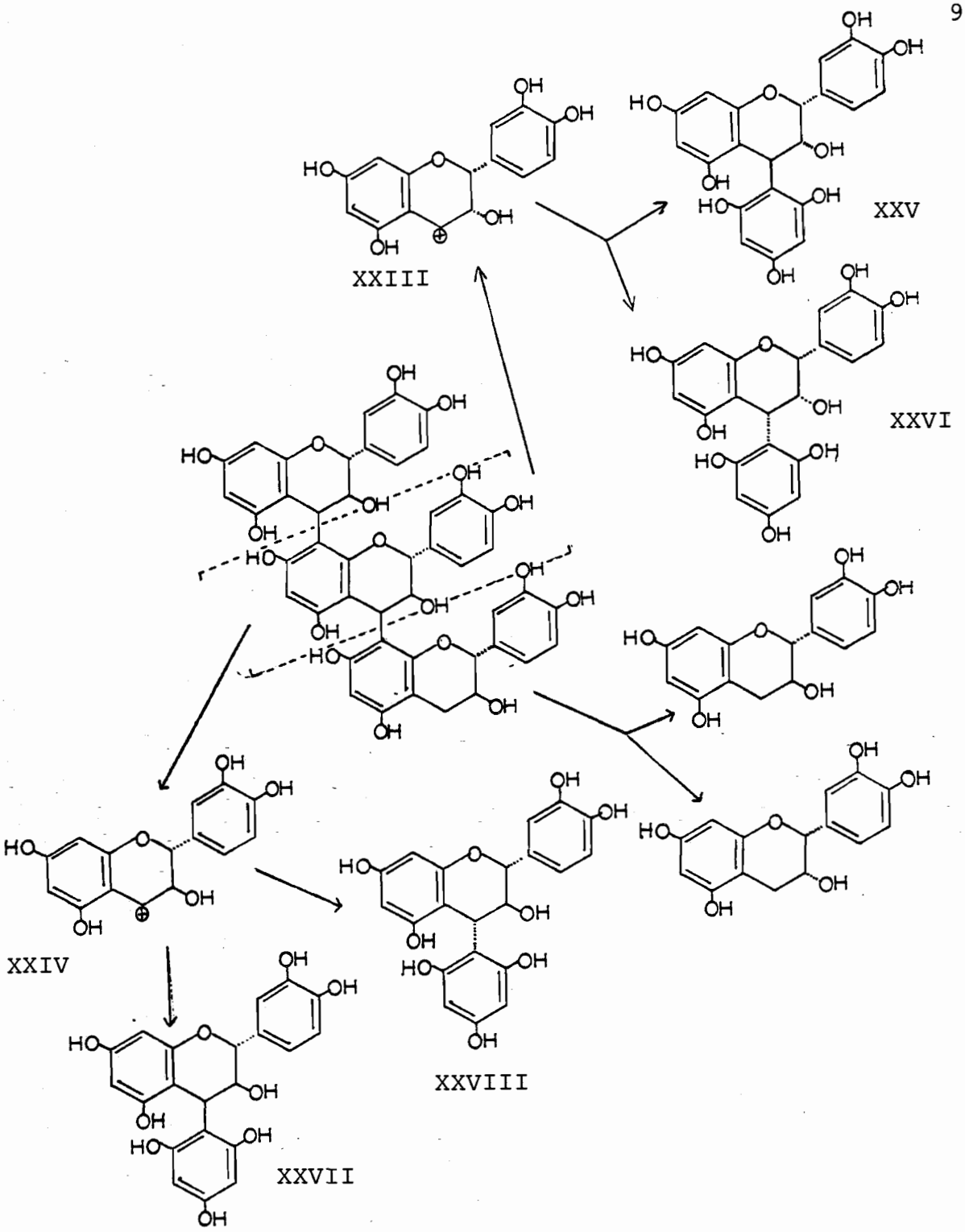


Figure 29. Scheme depicting the products from the reaction of phloroglucinol with a condensed tannin.

V. SUMMARY AND CONCLUSIONS

1. Samples of inner and outer barks from Douglas-fir were extracted with acetone-water (70:30 v/v).
2. The solubilized solids of both bark samples contained polysaccharides comprised of D-glucose, D-mannose, D-galactose, D-xylose, and L-arabinose.
3. The solubilized fraction from outer bark contained 12.04% glucan and 4.51% arabinan. The other sugars were in trace amounts.
4. The solubilized solids from inner bark contained 15.94% glucan. The other sugars were in trace amounts.
5. The solubilized solids from both outer and inner bark thus contained considerable amounts of carbohydrate material.
6. The solubilized solids from inner bark were methylated, degraded with thioglycolic acid, and reduced with Raney nickel to yield tetra-O-methylcatechin and tetra-O-methylepicatechin in quite small amounts. The presence of the carbohydrates and other substances clearly interfered with the reaction sequence.
7. Treatment of the solubilized solids from outer bark with phloroglucinol resulted in free catechin and epicatechin as well as flavanoid-phloroglucinol reaction products. This reaction illustrated that the

solubilized solids contained a condensed tannin comprised of catechin and epicatechin units joined from C-4 of the upper units to probably C-8 or C-6 of the lower units.

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