AN ABSTRACT OF THE THESIS OF

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Date thesis is presented May 3, 1963

Title: The Isolation and the Properties of the Bark Phenolic Acids from Mountain Hemlock, Tsuga Mertensiana (Bong.) Carr.

Abstract approved Redacted for privacy

The portion of "bark lignin" known as "phenolic acid" was removed from the extractive-free outer bark of mountain hemlock (Tsuga Mertensiana (Bong.) Carr.) by extraction for one hour with one percent aqueous sodium hydroxide at 90° C. This crude material consisted of two equal fractions that were markedly different in their colors, solubilities, methoxyl contents and infrared spectra. They were separated on the basis that one was soluble in anhydrous dioxane while the other was not.

These two materials were studied by electrophoresis under the following conditions: room temperature, a field strength of five volts per centimeter, an ionic strength of 0.05, pH's that varied from 6.1 to 10.0, times up to 8.5 hours, and an apparatus of the hanging-strip type. Under these conditions, the two fractions were electrophoretically similar. There was no

significant migration below pH 9.0, and mobility increased as the pH was increased from 9.0 to 10.0. When substantial movement took place, it was confined to a single band that widened as it migrated. The mobility of the two phenolic acids at pH 10.0 was 9.48×10^{-5} square centimeter per volt second.

The anhydrous dioxane-soluble portion of the crude phenolic acid was extracted with benzene and ethyl ether, and divided into six fractions by a solution fractionation scheme which consisted of the extraction of the phenolic acid by the following ketones and their corresponding water-saturated solutions listed in order of use: methyl isobutyl ketone, water-saturated methyl isobutyl ketone, methyl isopropyl ketone, water-saturated methyl isopropyl ketone, methyl ethyl ketone and water-saturated methyl ethyl ketone. The anhydrous methyl ethyl ketone extraction did not dissolve any phenolic acid. The sixth fraction was the residue left from the solution fractionation scheme. Adsorption chromatography demonstrated that these fractions were still a mixture of components.

The six fractions were compared by their chemical and physical properties. The infrared spectra were all very

similar, but did show some meaningful difference. The position of the 1700 $cm.^{-1}$ carboxyl-carbonyl peak indicated that the carboxyl groups were aliphatic. The ultraviolet spectra in dioxane all had a peak or shoulder near 280 millimicrons. The carbon and hydrogen content steadily decreased from the most soluble fraction (63.9 percent carbon and 6.5 percent hydrogen) to the residue (54.4 percent carbon and 5.0 percent hydrogen). Methoxyl content randomly varied from two to four percent. Potentiometric nonaqueous titration using tetrabutylammonium hydroxide as the titrant and dimethylformamide as the solvent was used to determine the phenolic hydroxyl and carboxyl contents. It was found necessary to titrate the phenolic acids in admixture with parahydroxy benzoic acid in order to develop separate breaks in the titration curve for both the phenolic hydroxyl and carboxyl groups. The phenolic content randomly varied from three to four percent and the carboxyl content, again in a random manner, from nine to 12 percent. Vapor pressure osmometry was used to determine the molecular weights in a dimethylformamide solvent system. The values randomly varied from a low of 1000 to a high of 5400.

THE ISOLATION AND THE PROPERTIES OF THE BARK PHENOLIC ACIDS FROM MOUNTAIN HEMLOCK, *TSUGA MERTENSIANA* (BONG.) CARR.

by

JIM DEGNAN WILSON

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1963

APPROVED:

Redacted for privacy

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Date thesis is presented May 3, 1963

Typed by Margaret Hartline

ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation to Dr. E. F. Kurth for his guidance during the course of this investigation.

The research described in this thesis was supported in part by a grant from the National Science Foundation.

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THE ISOLATION AND THE PROPERTIES OF THE BARK PHENOLIC ACIDS FROM MOUNTAIN HEMLOCK, TSUGA MERTENSIANA (BONG.) CARR.

INTRODUCTION

The economic situation in the last few years has been such that the forest products industry has become increasingly aware of the need for the fuller utilization of their raw material (18, pp. 27A-30A). Consequently, a large amount of interest has been directed toward a more complete utilization of conifer barks, the major portion of which is now being burned either as a fuel or as a waste residue (58, p. 100).

This interest has already resulted in the introduction of a number of bark-derived products into the commercial field. Bark is potentially capable of supplying flavonoids in a volume larger than any other known source (22, p. 580), and at least one bark flavonoid, quercetin, is now commercially available. The economic importance of bark and wood flavonoids has been recently reviewed (22, pp. 588-591). A wax extracted from Douglas-fir bark is also com mercially available. The physical components of conifer barks can be used as plastic extenders, in building boards and as soil conditioners. The closely related polymeric polyphenols--phlobatannins, phlobaphenes and phenolic

acids--are already of economic importance in the leather tanning, oil well drilling and adhesive fields (58, p. 100).

The polymeric polyphenols comprise the largest non-carbohydrate fraction of conifer bark, and is the fraction of interest in this investigation.

Great progress has been made in the last ten years in understanding the chemistry and structure of the first of this group. This understanding has come about largely through the discovery that the red color formed when phlobatannin extracts are boiled with dilute mineral acid is due to the formation of anthocyanidins from leuco-anthocyanins, and that these leucoanthocyaninins were monomeric flavan-3-ols, monomeric flavan-3,4-diols and polymeric polyphenols (phlobatannins). Identification of the monomeric compounds present in tannin extracts, the determination of which of these gave the same color reactions and degradation products as purified tannins, and the identification of the flavonoids produced by tannin degradation resulted in the conclusion that most tannins are primarily polymerization products of flavan-3-ols and flavan-3,4-diols. The manner by which these flavonoid units are linked is still in question. This whole field has been reviewed by Hergert (22, pp. 568-577).

The second type polymeric polyphenols present in conifer barks are the phlobaphenes. They are water-insoluble but soluble in alcohol and similar organic solvents, and are closely related to the co-occurring tannins. There are two general types One is very similar to the corresponding tannin, and is believed to be water insoluble only because of a higher molecular weight. The second type is thought to be a condensation product of phlobatannin by the elimination of water (22, pp. 907-910). Becker and Kurth decided that this type of phlobaphene in red fir was a polymer of cyanidin (3, p. 382). The name phlobaphenes is also given to the insoluble products formed by heating phlobatannins with mineral acids. It has been shown that these products are very similar to the natural occurring phlobaphenes (22, p. 578).

The particular polymeric polyphenols of interest in this investigation are those which cannot be extracted with neutral solvents but can be removed by the action of alkaline solutions. The term "phenolic acid" has been applied to this material because it contains both phenolic hydroxyl and carboxyl groups. Throughout this paper, the phrase "phenolic acid" will be used as a generic term, and will not refer to a specific phenolic acid material

unless so specified. It is the major component of the conifer bark and exists as a large potential supply of raw material. It is the opinion of the author and others (22, p. 578) that its economic utilization depends largely upon the elucidation of its structure.

On isolation, phenolic acid is a red-brown, amorphous powder that is soluble in dilute alkalis but insoluble in mineral acids and non-polar solvents. Immediately after isolation and before air drying, it is soluble in acetone, alcohol and dioxane, but is only partially soluble in these same solvents after being dried. In addition to phenolic hydroxyl and carboxyl groups, it contains alcoholic hydroxyl and methoxyl groups. The methoxyl content is lower than that of wood lignin, and at least one phenolic acid has been isolated that was free of methoxyl groups (11, p. 220). Phenolic acid does not give typical wood lignin color reactions.

Structural evidence to date is rather scarce for phenolic acid and, as in the case of wood lignin, is derived largely from degradation reactions. Kurth and Smith (45, p. 131) obtained small amounts of protocatechualdehyde and vanillin by an alkaline nitrobenzene oxidation of Douglas-fir bark phenolic acid. Fahey and

Kurth identified vanillin, phloroglucinol, catechol, para-hydroxybenzaldehyde, para-hydroxybenzoic acid, 5-formyl vanillic acid, 5-carboxy vanillic acid, isovanillic acid, veratric acid and vanilloyl formic acid from alkaline fusion and oxidation reactions on white fir cork phenolic acid (17, pp. 506-512). It is to be noted that this group of compounds contain the guaiacyl nucleus, which is common to wood lignin, and the phloroglucinol nucleus, which is not.

Hergert has shown that amabilis fir and longleaf pine barks contain fractions that are identical with wood lignin. The acidolysis of these barks with dioxane-hydrochloric acid reagent yield compounds that appear to be quite specific for wood lignin. A few of the more specific compounds are: paracoumaraldehyde, vanilloyl methyl ketone and alpha hydroxy propiovanillone. These same products were obtained from Brauns' lignin under the same conditions (33).

There is much evidence showing the similarity between the phenolic acids and the tannins. Kurth and Smith showed that Douglas-fir phenolic acid gave many common tannin reactions, such as precipitation with gelation, bromine and lead acetate, absorption by hide powder, and the formation of an insoluble red-brown precipitate when boiled with

dilute mineral acids (45, p. 127). Infrared studies have shown a marked similarity between the phenolic acids and the corresponding phlobaphenes and phlobatannins (33; 31, p. 337; 40, p. 52 and 63, pp. 62-65). The infrared spectrum of longleaf pine tannin is identical with its phenolic acid except for the presence of a carboxyl group absorption in the spectrum of the latter. However, treating the tannin with dilute caustic under the same conditions as those used in the phenolic acid isolation resulted in a spectrum identical with that of the phenolic acid. This treatment also changed the solubility properties of the tannin to those of the phenolic acid (33). The same spectral phenomenon has been noticed in relation to phlobaphenes (31, p. 336 and 40, p. 52).

Degradation studies (alkaline fusion and oxidation) with longleaf pine bark revealed that the tannin and phenolic acid both gave the same products. These were the same as obtained by other workers with other phenolic acids; that is, phloroglucinol, catechol, protocatechuic acid and compounds containing the guaiacyl nucleus. On chromatographic purification, the tannin was rendered virtually free of methoxyl groups and no longer gave guaiacyl-type degradation products. It was concluded,

therefore, that the unpurified tannin had been contaminated with lignin. Due to its different solubility properties, the phenolic acid could not be purified in the same manner, but it was believed that its methoxyl content was also due to lignin impurities (33).

The accumulated evidence points out an apparent structural similarity between the phenolic acids and the phlobotannins and the phlobaphenes. One possibility is that the phenolic acids are derived from the phlobaphenes in the same manner as the phlobaphenes are derived from the phlobatannins. The observation that a carboxyl group can be introduced into the phlobaphenes and the phlobatannins by a caustic treatment would explain the presence of this group in the phenolic acids. Further evidence that the carboxyl group is an artifact is that Hergert failed to find in the infrared spectrum of extractive-free whole bark any carboxyl group absorption above that due to uronic acids (33). Holmes (36, p. 64) suggests that the pyran ring of a flavonoid structure is cleaved to form carboxyl groups, while Hergert (33) believes they arise from the rupture of a phenolic ring.

The view that the carboxylic group is <u>not</u> formed by the action of alkali during extraction but is an inherent

part of the phenolic acid molecule is held by Lenz and Kurth (49, p. 31). They based their conclusion on the ability of hydrotropic pulping agents to remove phenolic acids from Douglas-fir bark that had approximately the same ratio of phenolic hydroxyls to carboxyl groups as did material removed by the action of one percent sodium hydroxide. "Bark lignins" containing carboxyl groups have also been isolated by dioxane-hydrochloric acid and 72 percent sulfuric acid (41, pp. 17-19).

The resistance of phenolic acid to inert solvent extraction could be due to a high molecular weight, a three-dimensional polymer network or a chemical link with the cellular matter in the bark.

In addition to phenolic acid, extractive-free bark contains a "bark lignin" that can be removed by dioxane-hydrochloric acid. This "bark lignin" has a methoxyl content similar to wood lignin. After the removal of phenolic acid and the dioxane-hydrochloric lignin, the bark still contains a fraction that is freed from cell-wall carbohydrates only by the action of strong mineral acids (41, p. 18 and 10, p. 121).

As previously stated, the author feels that a primary goal in the study of bark phenolic acid is the elucidation

of its structure; however, there are a number of preliminary problems that must be solved before the final problem can be attacked with any degree of certainty. One of these is purification and fractionation.

Smith (63, pp. 29-61) concluded from a paper chromatographic study that Douglas-fir inner bark and cork phenolic acids were largely homogeneous materials with small amounts of impurities. A similar investigation was carried out on white fir cork phenolic acid and, while the results indicated that the preparation was homogeneous, the evidence was not felt to be completely satisfactory (16, pp. 6-10). It appears logical that instead of being homogeneous that the phenolic acids are mixtures of closely related polymers differing only in extent of polymerization. The purpose of this investigation is to explore this hypothesis.

The bark chosen as the source of the phenolic acid to be studied was that of mountain hemlock, Tsuga mertensiana (Bong.) Carr. This bark affords the advantage of being relatively homogeneous, since it has only a very small amount of cork. Also, its phenolic acid has not previously been studied; therefore, its investigation will add to the knowledge of the variance and similarity of phenolic acid among the conifers.

Two previous papers have appeared that dealt with mountain hemlock bark. One dealt with the isolation of holocellulose from this bark (67, pp. 260-263), and the second with its chemical analysis (43, pp. 733-734). The summary analysis from the latter paper appears in Table 1.

TABLE 1

CHEMICAL ANALYSIS OF MOUNTAIN HEMLOCK BARK

Material	Bottom Bark (percent)	Top Bark (percent)	Inner Bark (percent)
Ethyl ether soluble[a]	7.53	7.31	3.90
Ethyl alcohol soluble[a]	26.25	21.65	23.92
Hot water solubles[a]	4.39	10.49	1.59
Sum of extractives[c]	38.17	39.45	29.41
Ash residue[b]	1.83	1.36	2.35
Klason lignin[b]	51.91	49.05	30.58
Pentosans[b]	6.95	10.61	13.11
Acetyl group[b]	0.73	0.94	1.16
Methoxyl group[b]	...	3.44	3.43
1% NaOH soluble[b]	...	43.60	...

a. Quantities based on moisture-free, unextracted bark.
b. Quantities based on moisture-free, extractive-free bark.
c. Extractions were made successively in the order shown.

EXPERIMENTAL

COLLECTION AND PREPARATION OF BARK SAMPLE

The mountain hemlock bark chosen for investigation was collected in August of 1957 from the Big Lake-Sand Mountain region of the Oregon Cascades. This area has an elevation of about 5400 feet. Samples were taken from living trees at a height of two to four feet above the ground. The trees were about 24 inches in diameter, and had a bark thickness of three-fourths of an inch at the four-foot level. The tree size and bark thickness indicated that the trees were at least 100 years old.

Before drying, the inner bark was stripped from the outer bark by means of a pocket knife. After air drying, the outer bark was chopped into small pieces and ground in a Wiley mill using a one-millimeter screen. The ground bark was then sieved through a standard number 40 screen. Those particles not passing the screen were reground and resieved. This procedure was repeated until all but a small amount of the original sample passed the screen. This technique was used in preference to obtaining the desired particle size in one grinding in order to avoid the formation of an extensive amount of

bark dust which, if formed in excess, hampers extraction. The inner bark was ground and stored for other possible investigations.

Extractive-free bark was prepared by exhaustively extracting approximately 300 grams of air-dried, outer bark with 2:1 (V/V) benzene-ethanol, 95 percent ethanol, water, again with 95 percent ethanol and again with water, in the order given. Repeating the alcohol and water extractions was necessary because of the magnitude of the sample and the high extractive content of mountain hemlock bark (43, p. 733). The organic solvent extractions were carried out in a large Soxhlet-type extractor, while water extraction was performed by lowering a cotton bag containing the bark into boiling distilled water. The water was changed periodically. The samples were air dried and stored in air-tight glass jars.

PAPER ELECTROPHORESIS

Being an acidic material, phenolic acid carries an electrical charge that would cause it to migrate under the influence of an electric field and is, therefore, readily suited to an electrophoresis investigation. Paper electrophoresis was chosen over the older moving-boundary method because of the former's simplicity and preparative aspects. This newer technique depends on the electromigration of charged particles on a wet surface of filter paper, rather than electromigration within the body of an aqueous solution. The extent of movement is determined in much the same manner as paper chromatography; that is, the material is located on the paper electrophoretogram by means of color development, etc., after development.

It was hoped that the investigation would not only give new insight into the nature of phenolic acid, but would also lead to a means of fractionation.

Phenolic Acid Isolation

The phenolic acid for the first phase of the investigation was isolated from the bark as follows: extractive-free bark was extracted for one hour in an oil bath maintained at 90° C. with one percent aqueous sodium hydroxide

solution in a bark-to-liquor ratio of 1.14 (W/V). The liquor was then filtered off, the marc washed with distilled water, and the combined washings and liquor centrifuged to remove the last traces of bark particles. The sodium ions were removed from the resulting solution by shaking with an excess amount of a strong cation exchange resin (Dowex 50 X-4) in the hydrogen form. The resulting dark red-brown solution was filtered from the ion exchange resin and centrifuged for two hours to remove any material which may have precipitated as a result of the neutralization of the sodium hydroxide. Only a small amount of material was centrifuged out. The resulting solution had a pH of 3.3 and a solid content of 0.68 percent with 1.5 percent of the solids being ash. The solution was stored at 5° C.

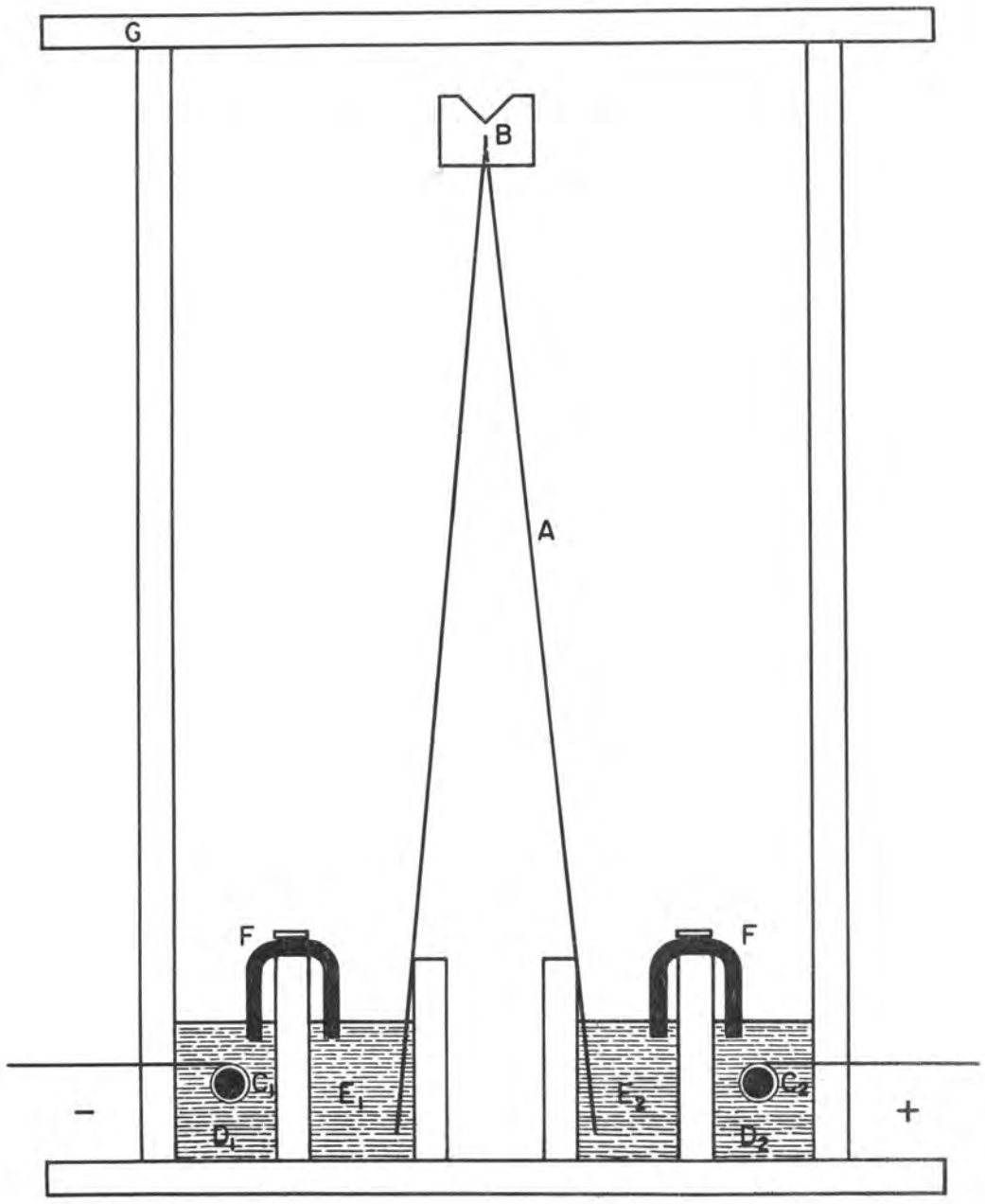
Although the above preparation appeared to be a true solution, it is more likely that the method of preparation resulted in the formation of a stable colloidal suspension, as phenolic acid is generally considered to be water insoluble. In trying to wash freshly precipitated Douglas-fir bark phenolic acid, Smith found that it appeared to dissolve, unless a relatively high concentration of inorganic ions was maintained in the wash water. He assumed

that true solution was not taking place but rather, the formation of a colloidal suspension in the absence of electrolytes (63, p. 18). The tendency to peptize in water in the absence of electrolytes has been reported by others (11, p. 38; 46, pp. 4-5 and 54, p. 55).

Electrophoresis Procedure

The electrophoresis apparatus employed was patterned after Durram according to a design by Flynn and de Mayo, and manufactured by Shandon Scientific Company (47, p. 37). This apparatus is of the hanging strip type, where the center of the paper strip is elevated above both of its ends, thus forming an inverted V. The lower portion of the electrophoresis chamber is divided into three main compartments. Each outside compartment is, in turn, divided into an electrode compartment, and a compartment into which one end of the paper dips. These two compartments are connected by a wick system, thereby preventing electrode products from reaching the ends of the paper strip. The entire arrangement is incased in a vapor-tight Lucite cell. See Figure 1.

It may be difficult to visualize such an apparatus giving satisfactory electrophoresis analyses, but it has



A filter paper
B suspension wire
C_1 and C_2 carbon electrodes
D_1 and D_2 electrode buffer compartments
E_1 and E_2 buffer compartments for the paper strips
F wicks connecting compartments D and E
D glass lid

FIGURE I. ELECTROPHORESIS APPARATUS

been shown to give a linear mobility-time relationship (6, p. 523) and results identical with those obtained by more complex methods (47, p. 37).

The apparatus was prepared for operation by first filling the outside compartments with a buffer of desired pH and ionic strength. Three by 15 centimeter strips of Whatman No. 1 filter paper were then creased in the center and suspended as shown in Figure 1. Capillary action between the paper and inside wall of the buffer compartment was sufficient to keep the paper taut. A uniform liquid level was established between all compartments to prevent capillary siphoning through the paper. The paper was wetted by allowing two to four milliliters of buffer to flow freely from a pipette onto each side of the paper strip. The system was then closed and equilibrated for one hour with the current on.

After equilibration had been attained, the phenolic acid solution was applied to the apex of the paper by dragging a hypodermic syringe filled with solution, but without a plunger, across the width of the paper and allowing capillary forces to pull the liquid from the needle onto the paper. The system was again closed and run for the desired length of time. The phenolic acid

appeared brown against the white background of the paper, and consequently its movement could be followed visibly.

After the strips had been removed from the tank and air dried, they were dipped through Barton's (2, p. 249) phenol indicator, followed by a dipping in six normal hydrochloric acid and washed with water. This treatment gave a permanent chromatogram with the areas where phenolic materials were present colored Turnbull's blue.

The conditions of electrophoresis were room temperature, a field strength of five volts per centimeter, an ionic strength of 0.05, and pH's that varied from 6.1 to 10.0. The field strength was the maximum obtainable from the Shandon apparatus, and gave better results than the lower field strengths that were tried. An ionic strength of 0.05 was chosen because of the necessity of a low ionic strength for high migration velocities. Mobility studies also require a low ionic strength. The temperature in the Lucite cabinet rose only slightly above room temperature during the course of the run. The various buffer systems are listed in Table 2.

The capacity of the buffers tried above pH 10.0 were too small at the low ionic strength used for them to maintain a constant pH under the conditions employed, and

therefore 10.0 was the highest successful pH employed.

TABLE 2

BUFFER SYSTEMS FOR ELECTROPHORESIS

pH	Buffer System
7.1-8.0	Sodium hydroxide Dibasic potassium phosphate
8.5-9.0	Sodium hydroxide Boric acid
10.0	Sodium hydroxide Boric acid Potassium chloride

The results of this phase of the investigation appear in Table 3. The distance of migration was measured from the band front to the point of application. Here, as throughout this study, all movement was toward the positive electrode.

TABLE 3

PAPER ELECTROPHORESIS OF PHENOLIC ACID

pH	Time (hr.)	Distance of Migration (cm.)	Band Width (cm.)
6.1	3.0	1.0	1.0
6.1	6.0	1.0	1.0
7.1	4.0	3.0	3.0
7.1	6.0	3.0	3.0
8.0	4.0	7.0	7.0
8.0	6.0	Diffused	Diffused
8.5	4.0	3.0 8.0 (very light)	3.0 2.5
8.5	6.0	3.5 Diffused	3.5 Diffused
9.0	4.0	2.5 (very light) 6.5	2.5 1.5
9.0	6.0	9.0	2.2
10.0	4.0	7.0	2.0
10.0	6.0	10.0	3.5
10.0	8.5	14.5	5.0

The tabulated data reveal that at pH 6.1, 7.1 and 8.0 all the material was confined to a single area that extended from the origin to the furthermost point of

migration. The width of this area increased with increasing pH, but did not seem to be affected by extending the time to six hours. The fact that the bands extended from the origin to the point of furthest movement means that at these lower pH's the phenolic acid was strongly adsorbed by the paper. At pH 8.5, a small amount of material seemed to break away and move as a distinct band; however, it was not until pH 9.0 that any substantial movement was observed. At this pH, the majority of the phenolic acid migrated down the paper as one strong band that became wider as it moved. Some material still remained near the origin, but faded out with time. The same type of migration continued at pH 10.0 with an increased velocity and the absence of any band at the origin.

When the voltage gradient was reduced to 2.5 volts per centimeter at pH 10.0 the main band moved as before, only slower, and a very weak band appeared a short distance in front of it.

In summary, phenolic acid shows no significant movement below pH 9.0, its mobility increases as the pH is increased from pH 9.0 to 10.0, and when substantial movement does take place it is confined to a single, strong band that widens as it migrates.

Mobility of Phenolic Acid

A plot of time versus distance at pH 10.0 (Figure 2) results in a straight line, and demonstrates that the conditions required for mobility studies had been met. The mobility at this pH was calculated to be 9.48×10^{-5} square centimeters per volt-second, and appeared to be reproducible. This mobility remained unaltered over a wide range of concentrations, although at the higher concentrations it was evident that the center of the band was darker than its front and bark portions.

Rather than delve into the controversy of the validity of mobilities determined by paper electrophoresis versus those determined by the moving-boundary method (6, p. 501 and 52, p. 434), the author will simply quote Hugh J. McDonald, a leader in the electrophoresis field .... "whether or not the mobilities obtained by ionography [1] agree exactly, in all cases, with those obtained by the moving-boundary technique is not really important. What is important is that data from ionographic measurements be

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1. Suggested name for electrophoresis on solid supports.

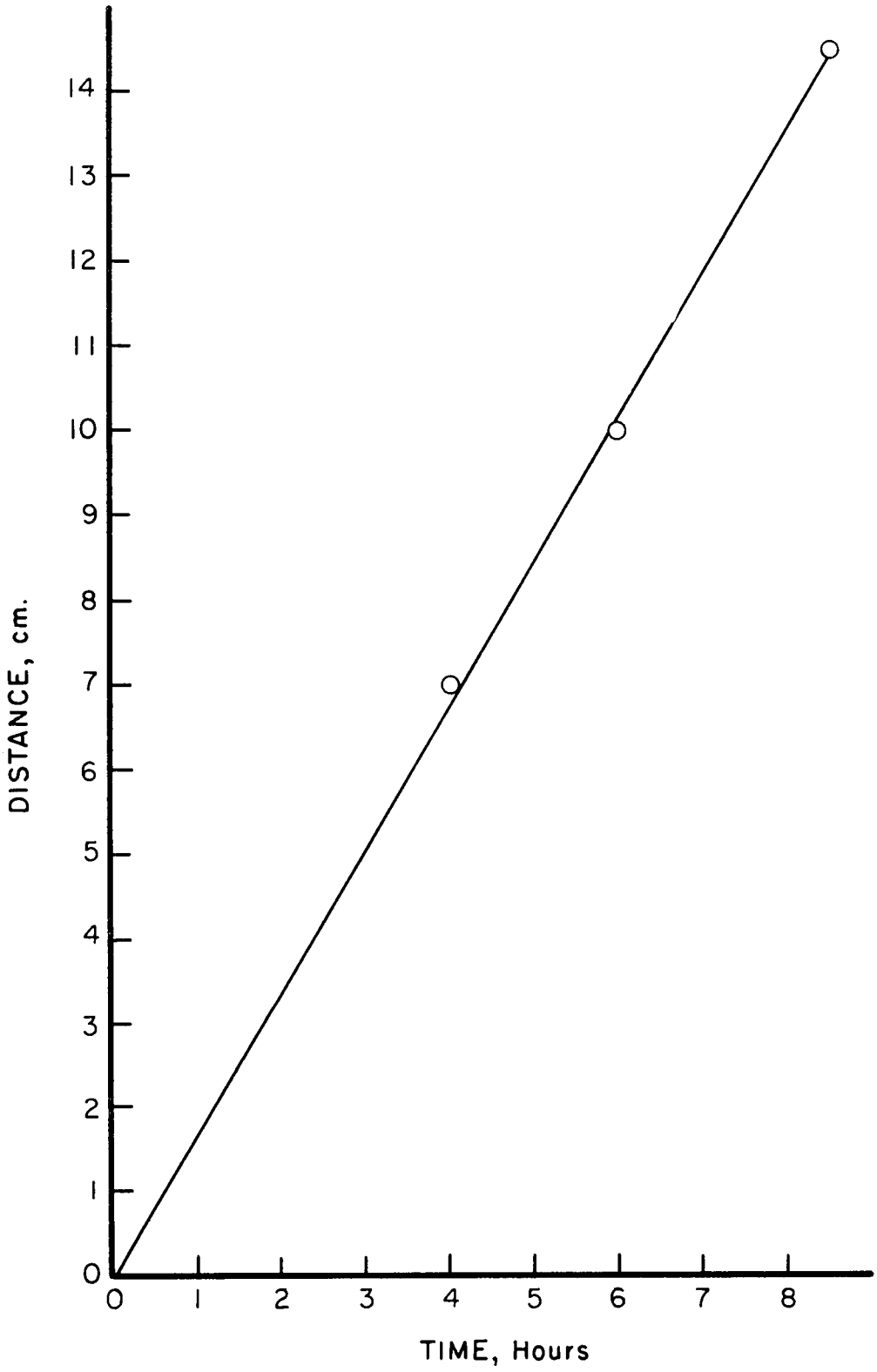

FIGURE 2. MOBILITY STUDY

reproducible" (6, p. 434).

Reference to a study of vegetable tannins (quebracho, wattle mangrove and gambier) by Putnam and Bowles (61, pp. 343-349) shows that the paper electrophoresis behavior of purified tannins is very similar to that of phenolic acid in regard to pH and mobility.

Electrophoresis of Fractionated Phenolic Acid

Phenolic acid was next fractionated by a simple solvent scheme and the separate fractions compared by paper electrophoresis. The scheme is represented in Figure 3.

The extractive-free bark was extracted with a one percent aqueous sodium hydroxide solution, and the extract treated with Dowex 50 X-4 ion exchange resin as previously described. On centrifuging the resulting solution, a small amount of brown, amorphous material was obtained. After being washed with water, this precipitate was air dried and labeled Fraction 1.

The clarified solution (Fraction 2) was acidified with dilute hydrochloric acid to pH 2.0 and the resulting brown precipitate removed by centrifuging, and then washed once with distilled water. The moist precipitate was then agitated with dioxane and again centrifuged. The material that was insoluble in the moist solvent was washed with

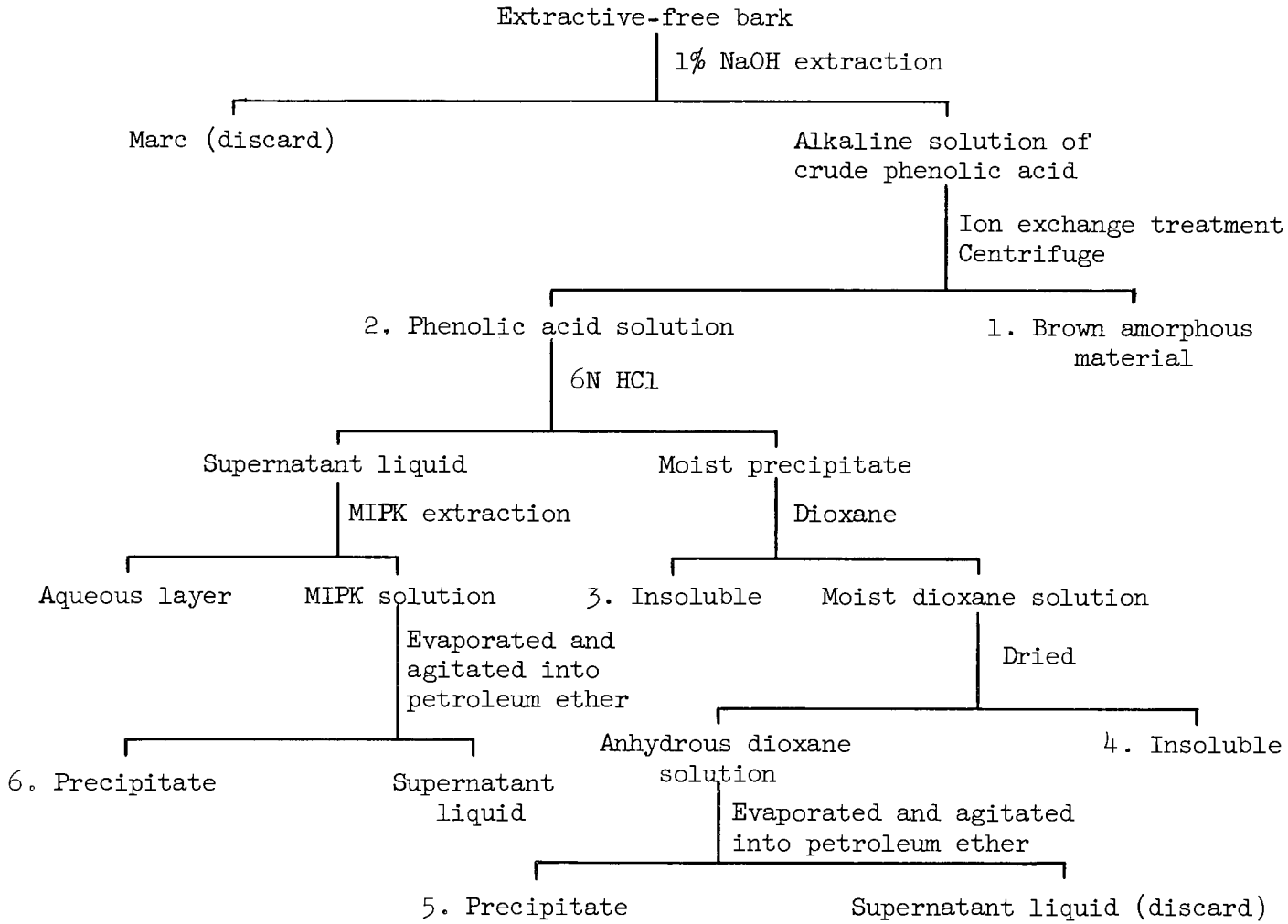

FIGURE 3. FRACTIONATION OF PHENOLIC ACID

dioxane followed by petroleum ether, air dried, and designated Fraction 3. When this fraction was dissolved in sodium hydroxide and again precipitated with hydrochloric acid, it was still insoluble in moist dioxane, regardless of its water content. The dioxane was termed as "moist" because of the introduction of water into the solvent through the precipitate that contained 90 percent water.

The moist dioxane solution was next evaporated under vacuum at between 25 and 30° C. in a rotary evaporator to a small volume. The evaporation had the effect of drying the dioxane solution and caused the precipitation of a fraction that, while soluble in moist dioxane, was insoluble in anhydrous dioxane. If a little water was added to the dioxane, the insoluble material again dissolved. This fraction was centrifuged off, washed with dioxane followed by petroleum ether, air dried, and designated Fraction 4. The dioxane solution was further evaporated to a thin syrup and precipitated into petroleum ether in the manner of Brauns (9, pp. 742-744). The resulting amorphous precipitate was collected, redissolved in dioxane, centrifuged, and again precipitated into petroleum ether. It was washed with petroleum ether, air dried, and labeled Fraction 5. After air drying, it was

still soluble in anhydrous dioxane.

Fraction 6 was obtained by extracting the supernatant liquid from the acid precipitation exhaustively with methyl isopropyl ketone (MIPK). The ketone solution was evaporated down and precipitated into petroleum ether in the same manner as Fraction 5. However, a gummy solid resulted instead of a flocculent precipitate. The gummy solid was dissolved in a small amount of moist acetone and stored as a solution. All fractions were stored at 5° C.

While the weights of the various fractions were not determined, it was observed that Fractions 4 and 5 were of about equal magnitude and together comprised the vast majority of the original phenolic acid. Fractions 3, 4 and 5 were present in only minor amounts.

It is of interest to note the colors of Fractions 3, 4 and 5. Fraction 3 was reddish-brown, 4, light orange-brown, and 5, light orange-tan. That is, the more soluble the fraction, the lighter it was in color.

These various components were compared electrophoretically under the following conditions: pH 10.0, field strength of five volts per centimeter, 0.05 ionic strength and a time of six hours. Fractions 1, 3, 4 and 5 were

applied as one percent solution in dilute base, and Fractions 2 and 6 as their original solutions. Results are reported in Table 4.

Inasmuch as separation is often enhanced at higher ionic strengths, the above was repeated for Fractions 4 and 5, at an ionic strength of 0.1. Longer times were also necessitated because of the slower mobility at higher ionic strengths. See Table 5.

TABLE 4

PAPER ELECTROPHORESIS
OF FRACTIONED PHENOLIC ACID

Fraction Number	Distance of Migration (cm.)	Band Width (cm.)
1	0.3	0.3
	8.6 (very light)	1.8
2	10.0	2.7
3	0.6 (very light)	0.6
	7.9	2.7
4	9.8	2.9
5	9.8	2.5
6	11.0	3.7

TABLE 5

PAPER ELECTROPHORESIS OF MAJOR COMPONENTS

Fraction Number	Time (hr.)	Distance of Migration (cm.)	Band Width (cm.)
4	8	6.2	2.5
5	8	5.5	3.2
4	12	11.0	4.6
5	12	9.4	4.0

It can be seen from the data in Table 4 that Fractions 1, 3 and 6 were all electrophoretically different and were, in turn, different from 4 and 5, which were electrophoretically similar. Fraction 2, which was composed of all the fractions but Fraction 1, showed only one strong band corresponding to 4 and 5. This means that the other fractions were present in too low a concentration to be detected, or were masked by the 4 and 5 band.

Some difference between 4 and 5 is evident at the higher ionic strength, but it was not enough to achieve clear-cut separation. At this higher buffer concentration, the band produced by Fraction 4 was darker and sharper than that produced by Fraction 5, showing that 4 was probably the more homogeneous material.

In conclusion, it may be stated that mountain hemlock phenolic acid is composed of two main fractions that, while exhibiting different solubility characteristics, are electrophoretically similar, and a number of lesser fractions that show both solubility and electrophoretic differences.

The original hypothesis that phenolic acid is a mixture of closely related polymers was supported by this investigation in that as the material migrated, its band grew progressively wider. This is the expected behavior of a spectrum of polymeric material.

Paper electrophoresis does not seem to hold much promise as a means of fractionating phenolic acid. It can be assumed that by employing a continuous electrophoresis apparatus and higher field strengths, phenolic acid could be separated to the point that arbitrary fractions could be taken, but even this seems to offer no advantage over other methods.

SOLVENT FRACTIONATION OF PHENOLIC ACID

It was decided to separate the crude phenolic acid into its two major fractions (Fractions 4 and 5 in Figure 3), and further fractionate the more soluble of these two by a solvent extraction scheme.

Preparation of Major Fractions

The phenolic acid was extracted from the bark in much the same manner as was described in the paper electrophoresis study. Extractive-free bark was extracted with one percent sodium hydroxide in the ratio of one part of air-dried bark to 14 parts of alkaline solution for one hour at 90° C. The extraction apparatus consisted of a one-liter wide-mouth Erlenmeyer flask, containing 50 grams of bark and 700 milliliters of sodium hydroxide solution, immersed in a two-liter stainless steel beaker containing mineral oil. The oil was heated in such a manner that extraction was carried out within ±1° C. of the desired temperature. A mechanical stirrer provided consistent stirring of the suspension. At the end of the extraction period, the liquor was removed by centrifuging and the marc washed with distilled water (6 x 200 ml.). The last wash was still highly colored. The liquor and washes were combined and acidified with six normal hydrochloric acid to a pH of approximately two. The brown flocculent precipitate formed upon acidification was centrifuged off, washed once with distilled water, and then with 0.02 normal hydrochloric acid (3 x 200 ml.). The washes, after the first, had to contain a substantial amount of

electrolyte or the phenolic acid would dissolve (or peptize) in the water. Smith and Kurth (45, p. 127) used five percent sodium chloride for this purpose, but very dilute hydrochloric acid was believed to be better, since it resulted in a more or less ash-free sample. The 0.02 normal concentration of acid used was found to be the lowest permissible. All washes were pale-yellow. The supernatant liquid from the acid precipitation was quite red in color, but no additional precipitate was formed by the addition of excess acid or sodium chloride.

A total of 150 grams of bark was extracted in 50 gram batches, and the resulting moist phenolic acid precipitates combined.

Centrifuging was the preferred means of separating a liquid from a solid in the above scheme, because experience has shown that attempts to filter phenolic acid from a solvent for which it had any affinity (water, dioxane, etc.) always resulted in a plugged filter.

The crude phenolic acid precipitate was next divided into its two major components. The wet precipitate was dissolved in 1.5 liters of dioxane, and the resulting solution evaporated to a small volume under vacuum at approximately 25° C. in a rotary evaporator. Anhydrous

dioxane was then added and the solution again evaporated. This procedure was repeated until all the water had been removed. The precipitate resulting from the drying process was centrifuged off, washed with anhydrous dioxane followed by petroleum ether, and air dried. It was then ground to a fine powder in a mortar, and washed with distilled water until the washings gave a negative chloride test with silver nitrate and a negative carbohydrate test with Molish reagent; it was then again air dried. This material was insoluble in dioxane and acetone, and only slightly soluble in either moist dioxane or moist acetone. Its color was dark red-brown.

The anhydrous dioxane solubles were recovered by pouring the concentrated dioxane solution into petroleum ether by the method of Brauns (9, pp. 742-744). The method consists of directing a fine stream of solution into 200 milliliters of mechanically agitated petroleum ether contained in a 250 milliliter centrifuge bottle. The resulting suspension may then be centrifuged (three or four minutes at 1000 rpm), the petroleum ether poured off, fresh ether added, and another portion of dioxane solution precipitated into the same bottle, thus building up the precipitate in a single centrifuge bottle. Or, each batch

of precipitate may be filtered onto a Buchner funnel with slight suction, being careful to keep the residue always covered with petroleum ether, and in this manner build up the precipitate on the funnel. The former method was suited for preparing small amounts of material, but the latter was more convenient for larger preparations. After all the dioxane solubles had been precipitated, the collected material was extensively washed with petroleum ether and air dried. At this point, the material was coarse and dark-brown in color. The phenolic acid material was redissolved in anhydrous dioxane to form a five percent solution and reprecipitated as before, but this time a fine, light-brown powder was obtained. If a ten percent solution was reprecipitated, a coarse, dark precipitate was again formed.

A number of factors influenced the formation of the desired precipitate. If too large a volume of dioxane solution was added to the petroleum ether, or if its phenolic acid concentration was too high, a gum formed instead of the desired product. Two hundred milliliters of petroleum ether would tolerate only seven to eight milliliters of dioxane solution. A gum also formed if either the dioxane solution or petroleum ether was

insufficiently dry. It was necessary to dry the petroleum ether by some standard method before a satisfactory precipitation was achieved.

Petroleum ether was a more satisfactory, but a more difficult, precipitating medium than the commonly used ethyl ether. When ethyl ether was used, some phenolic acid was lost by solution and peptization in the ether. The supernatant liquid from an ethyl ether precipitation was cloudy and yellow, and was not cleared by extensive centrifuging. The petroleum ether supernatant liquid, on the other hand, was completely clear and colorless. The loss resulting from the use of ethyl ether has also been noted by Kiefer (40, p. 33). The same problem has been encountered with benzene (46, pp. 4-5).

Seventeen grams of dioxane solubles and 17 grams of dioxane insolubles were recovered from the original 150 grams of extractive-free bark. The dioxane-insoluble portion was designated Fraction 4' because, while the majority of it was Fraction 4 of Figure 3, it also contained Fraction 3. Any water-insoluble carbohydrates that were extracted from the bark by the sodium hydroxide, and subsequently precipitated with the hydrochloric acid, would also be in this fraction. The dioxane solubles were

designated Fraction 5, as in Figure 3. Inasmuch as carbohydrates are insoluble in dioxane, this fraction would be carbohydrate-free (63, p. 21). Here, as throughout the investigation, the fractions were stored in a refrigerator.

Solvent Scheme

Preliminary work had shown that Fraction 5 could be partially fractionated with a solvent extraction scheme which included a series of ketones and their corresponding water-saturated solutions. The series consisted of the following solvents listed in their order of use: benzene, ethyl ether, methyl isobutyl ketone, water-saturated methyl isobutyl ketone, methyl isopropyl ketone, water-saturated methyl isopropyl ketone, methyl ethyl ketone and water-saturated methyl ethyl ketone. Benzene was employed to remove any lipid material that was liberated by the action of sodium hydroxide on the small amount of cork present in the extractive-free bark (31, pp. 335-339 and 34, pp. 59-66) and carried along with the phenolic acid. Ethyl ether was used to extract any polyphenols that might have been present.

The extractions were carried out in the following manner. Sixteen grams of Fraction 5 were placed in a 250-milliliter centrifuge bottle along with 125 milliliters of

solvent, and the suspension mechanically stirred for a period of time that varied from 0.5 hour to two hours. The suspension was then centrifuged, the supernatant liquid drawn off and replaced with fresh solvent. When the extractions became lightly colored or successive extractions remained the same shade over a number of extractions, extraction with that solvent was stopped and extraction with the next solvent started. The number of extractions for each solvent varied from 12 for benzene and ether to 30 for the ketones.

When a water-saturated solvent was followed by an anhydrous solvent, the water remaining in the marc was removed by washing with the anhydrous organic portion of the water-organic system. For example, before the marc was extracted with methyl isopropyl ketone, it was washed with anhydrous methyl isobutyl ketone to remove the residual water. This wash was treated as part of the water-methyl isobutyl ketone extraction.

In every case except with anhydrous methyl ethyl ketone, the first extraction with a new solvent was very much darker than the last extraction of the previous solvent. Anhydrous methyl ethyl ketone did not dissolve any of the phenolic acid, as was evidenced by the fact

that it did not become colored. The color of the first extractions varied from a very dark-red to a red-orange color. The last extractions were always some shade of light yellow.

The fractions were recovered by replacing the extraction solvents with dioxane and then precipitating into petroleum ether as follows. The separate solutions from a given extraction series were combined and evaporated in a rotary evaporator at approximately 25°C. In the case of the water-organic solvents, the evaporation resulted in the formation of two layers as the more easily evaporated organic compound was removed first and this, in turn, caused extensive precipitation of the solute. When a convenient volume was reached, anhydrous dioxane was added and evaporation continued. This cycle was repeated until the extraction solvent had been replaced with dioxane. The dioxane solution was centrifuged to remove any insoluble matter, concentrated to a small volume, and precipitated into petroleum ether as previously described. A satisfactory precipitation was, however, difficult to achieve. A gummy precipitate usually formed on the first attempt and had to be redissolved and reprecipitated. As many as three reprecipitations were needed to obtain the

desired results. The fraction designations, yields and color of each fraction are presented in Table 6.

The residue remaining after the last solvent extraction was washed with anhydrous methyl ethyl ketone and petroleum ether and air dried.

The yields in Table 6 total 12 grams, which leaves four grams unaccounted for. Part of this is represented by the benzene and ethyl ether extractables. The benzene solubles were not investigated, but the color of the extractions indicated that only a small amount of material was removed. It was noted, however, that a cream-colored waxy material that was acetone-insoluble was extracted by benzene. Ethyl ether dissolved a fair amount of material, but this fraction was unfortunately lost. The remainder of the unaccounted for material was lost in handling and accidents.

TABLE 6

SOLVENT EXTRACTION FRACTIONS

Extraction* Solvent	Fraction Designation	Color	Yield, (percent)
Benzene	-	....	....
Ethyl Ether	-	....	....
MIBK	A	Tan	13.8
H_2O-MIBK	B	Tan-Brown	10.0
MIPK	C	Tan-Brown	5.0
H_2O-MIPK	D	Tan-Brown	12.5
MEK	No material	extracted	
H_2O-MEK	E	Red-Brown	5.6
Residue	F	Brown	28.1

* MIBK - methyl isobutyl ketone.
MIPK - methyl isopropyl ketone.
MEK - methyl ethyl ketone.

The colors of Fractions A to F show the same trend that was noticed in the electrophoresis study; that is, the more soluble the fraction the lighter it was in color. Fractions B, C and D were all recorded as tan-brown, but C was darker than B and D darker than C.

The scheme of extraction just described involved a good deal of handling and an extended period of time. The use of a Soxhlet extractor would have been more convenient.

but would have exposed the extracted material to long periods of time at elevated temperatures in the boiling flask and would have resulted in the condensation, resinification or insolubilization of the extracted phenolic acids. The unstable nature of the phenolic acid is shown by the fact that over one-fourth of the original dioxane-soluble phenolic acid was recovered as a dioxane-insoluble residue.

The fact that the anhydrous dioxane-soluble portion of mountain hemlock phenolic acid can be fractionated by a rather simple solvent extraction scheme is strong evidence that the material is not completely homogeneous, but a mixture of components. The remainder of this study will be concerned with the comparison and investigation of the fractions separated by this scheme.

ADSORPTION COLUMN CHROMATOGRAPHY

Column chromatography using cellulose powder has been suggested as a possible means of fractionating the phenolic acid component of barks (63, p. 103), and was here employed in an attempt to further fractionate Fractions A through E. The most promising method found, an adsorption chromatography procedure, is described below.

Column Packing

Whatman chromatography grade cellulose was first tried as the adsorbent, but preliminary experiments showed that the eluting solvents removed an impurity that absorbed in the ultraviolet region. Inasmuch as the fractions separated by the column were to be compared through their ultraviolet spectrum, it was necessary to remove this impurity before the cellulose could be used. This was accomplished by extracting the cellulose in a Soxhlet extractor with acetone-water mixtures ranging from 80 to 100 percent water (55, p. 754). The extraction was continued until no ultraviolet absorbing material was removed, whereupon the cellulose was air dried and screened through a standard 100-mesh sieve.

Attempts to pack the cellulose in the chromatographic column by procedures found in the literature were unsatisfactory (1, p. 47). The dry packing method, where layers of dry adsorbent are packed one upon another, resulted in the formation of air pockets as soon as solvent was added. In an alternate procedure, an adsorbent-solvent suspension is added to a head of solvent, and the adsorbent allowed to settle as the solvent slowly drains out the bottom of the column. This

procedure gave a very loosely packed column with an excessive amount of channeling. The method finally devised was a combination of the above two. A small amount of cellulose-petroleum ether slurry was added to a column filled with petroleum ether that was slowly draining out of the bottom of the column. When a sufficient amount of cellulose (one to two centimeters) had settled onto the bottom of the column, it was gently packed by tamping lightly with a glass stirring rod. Another portion of slurry was added, and the suspension in the column stirred to integrate the newly added cellulose with the upper portion of the already packed adsorbent. After settling, the cellulose was packed as before and the procedure was repeated until the entire column was built up. The liquid level was never allowed to drain below the top of the packed column. The resulting column had the appearance of being very uniform and tightly packed. Petroleum ether was used because its low density gave a fast settling rate. The column was finally prepared for use by washing thoroughly with anhydrous acetone.

The column was 1.3 centimeters in diameter, 16 centimeters long and held 12 grams of cellulose.

The test samples were prepared by dissolving ten milligrams of a phenolic acid fraction in a small amount

of acetone and evaporating it onto 250 milligrams of cellulose powder. The liquid in the column was then allowed to run until its surface was just broken by the column packing. The cellulose powder-phenolic acid mixture was then added to the top of the packing and tamped down with a glass rod. A piece of filter paper was placed at the top of the test sample to avoid its disturbance by the developing solvents.

Development

The column was developed with nine 50-milliliter portions of acetone-water solutions. Each portion contained 2.5 percent more water than the previous one, progressing from 0.0 to 20.0 percent water. Five milliliter fractions were collected by means of an automatic fraction collector, and each collection tube stoppered soon after collection to avoid evaporation. The rate of flow decreased as the amount of water increased in the developing solvent.

Peaks were located by plotting the absorbency at 400 millimicrons of each tube against its tube number. The wave length of 400 millimicrons was arbitrarily chosen as

a convenient wave length in a range of strong absorption, as the visible spectrum of phenolic acid is a nondescript curve showing only increasing adsorption with decreasing wave length. The customary wave length for this type of material and this type of plot is 280 millimicrons, the phenol peak, but it could not be used in this case because of the absorption of acetone in the ultraviolet region. The plot for Fraction A is shown in Figure 4. The small arrows indicate the tube number that was being collected when the eluent containing the percentage of water, shown just above the arrow, was added to the top of the column.

Column chromatography of the other soluble phenolic acids (Fractions B-E) gave isotherms very similar to that of Fraction A, with the only variations being in the height of the different peaks. These variations, however, were no greater than those between duplicate runs.

The shape of the isotherms and the equal spacing of the peaks suggests that each solvent completely desorbed some fraction of the adsorbate, and that there were no characteristic migration rates resulting from the adsorption-desorption equilibrium often associated with this type of cnromatography.

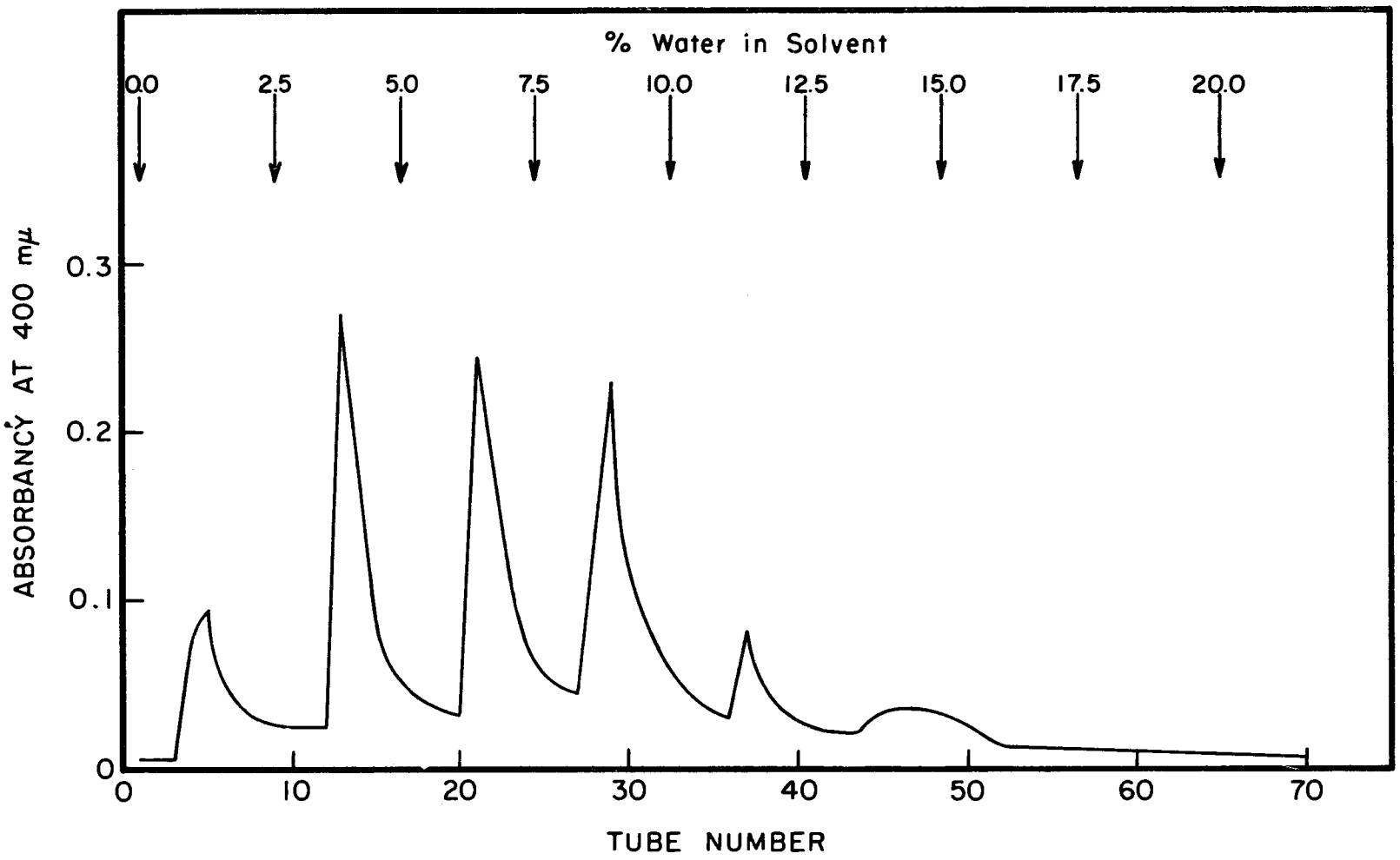

FIGURE 4. COLUMN CHROMATOGRAPHY OF FRACTION A

Silicic acid was also tried as an adsorbent, but proved to have no adsorbing power for the phenolic acid.

Evaluation of Chromatographic Fractions

The materials represented by the various peaks were compared by means of their ultraviolet spectra. The collected samples corresponding to the apex of each peak were evaporated under vacuum in a rotary evaporator at approximately 25° C. Purified (19, pp. 284-285) dioxane was added, and the solution again evaporated. The cycle was repeated a number of times to remove all the acetone.

Since the concentration of the resulting solutions was not known, and inasmuch as concentration difference could result in the same material giving a slightly different spectrum, the following procedure was used. Before its spectrum was determined, each solution was diluted so that the percent transmittance at 320 millimicrons was 30 percent. It was reasoned that solutions of identical substance would have the same concentration at the same percent transmittance at a given wave length. Therefore, if the spectra were different, the substances in solution would be different. The converse would not always hold true, however.

The spectra were determined with a recording Beckman

DB spectrophotometer. It is a characteristic of this instrument that the wave length is plotted decreasing from right to left. The spectra from the chromatography of Fraction A are shown in Figure 5.

Examination of the figure shows that the materials represented by the various chromatographic peaks all gave their own characteristic spectra. The curves differed in the development of the peak at 280 millimicrons and the absorbency of the entire curve.

The results from Fraction A are exemplary of those from the other phenolic acid fractions. No correlations were found between materials isolated from the same peaks but from different phenolic acids.

While ultraviolet spectroscopy has shown that cellulost, adsorption column chromatography effected some degree of true fractionation, it is still somewhat disconcerting that all the phenolic acid fractions gave such similar chromatographic isotherms. It must be remembered, however, that phenolic acids are polymers, and consequently those properties responsible for the chromatographic separations are present in all of its fractions.

Large Scale Column Chromatography

Fraction A was chromatographed on a large scale in an

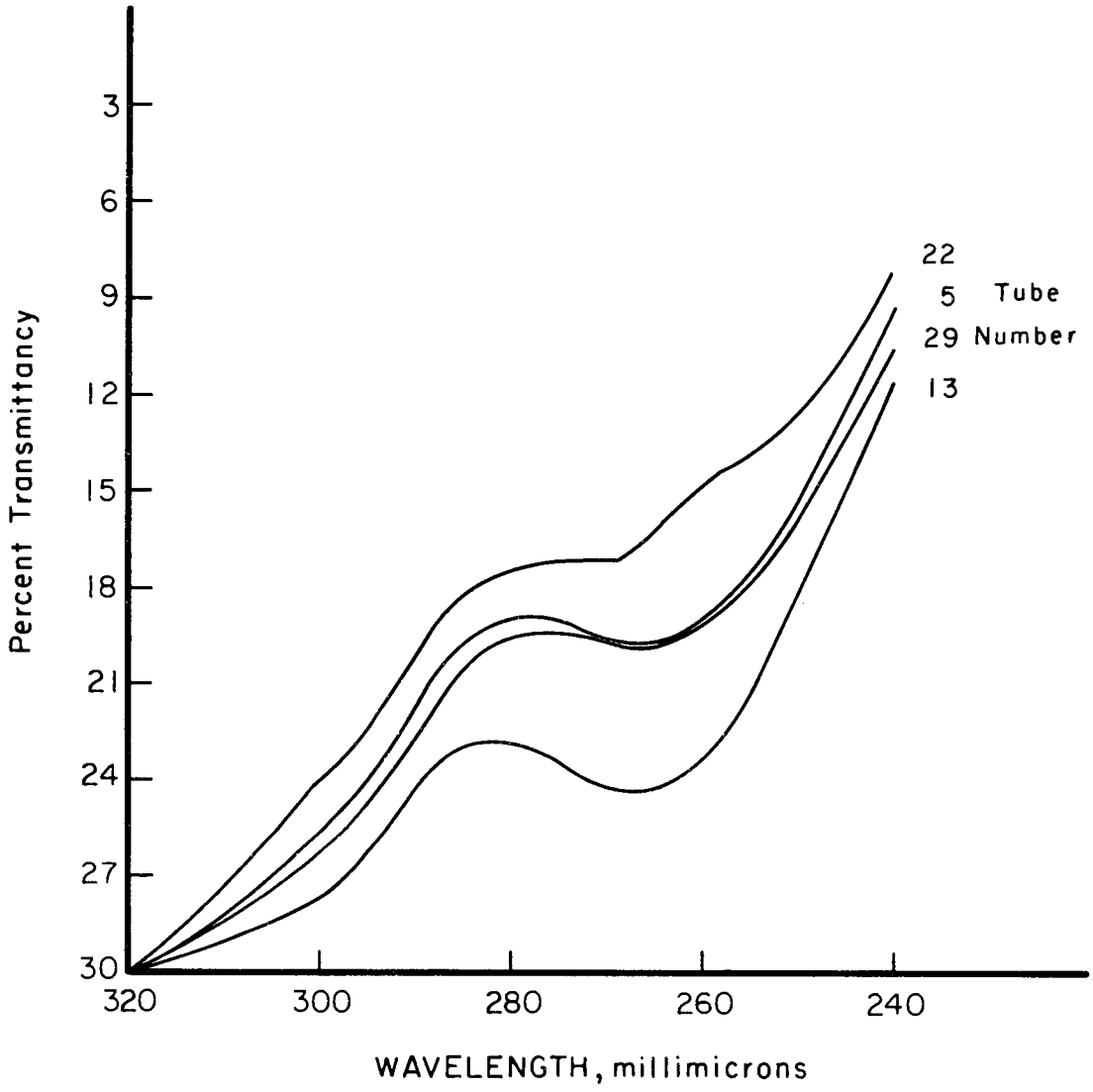

FIGURE 5. ULTRAVIOLET CURVES OF CHROMATOGRAPHIC FRACTIONS FROM A

effort to isolate the different fractions shown to be present by the above experiment. The ratio of adsorbent to sample in the small-scale chromatographic fractionation was 1200:1; thus, enlarging the procedure on the same scale so that even a gram of material could be chromatographed was considered impractical. Consequently, the ratio was cut to a tenth of the above ratio, which was still above the ratio of many acceptable procedures. Otherwise the conditions were the same, except on a larger scale.

Instead of breaking into three major peaks as before, the majority of material came through with the first eluent. The other peaks did develop, but to a minor extent compared to the first peak. The collected fractions corresponding to the initial peak were combined, concentrated by evaporation, the acetone replaced with dioxane and dioxane solution precipitated into petroleum ether, all in the manner already described. The fraction was designated A' and amounted to 58 percent of the original material chromatographed. The second peak, eluted with acetone containing 2.5 percent water, was also recovered but it amounted to only five percent of the original material.

It is evident that the large-scale chromatography did not give the same results as the small scale. The

reason was undoubtedly the reduction of the adsorbent to adsorbate ratio.

Fraction A' was chromatographed on a small scale, in the same manner already described, to see if the passage through the large column had resulted in any fractionation. A comparison of the fraction number versus absorbency curve for Fraction A' (Figure 6) with that for Fraction A (Figure 4) shows that no definite fractionation had been obtained, but there was a concentration of material in the first peaks.

Since large-scale chromatography had not given the desired fractionation, no further work was done with it. However, Fraction A' appeared to be a more purified product than Fraction A and was, therefore, used in place of Fraction A in the remainder of this study.

SPECTROSCOPIC STUDY

The infrared and ultraviolet spectra of the phenolic acid fractions were determined as a means of comparison, and as a means for gaining molecular structural information.

Infrared Spectra

The infrared spectra of the various fractions were

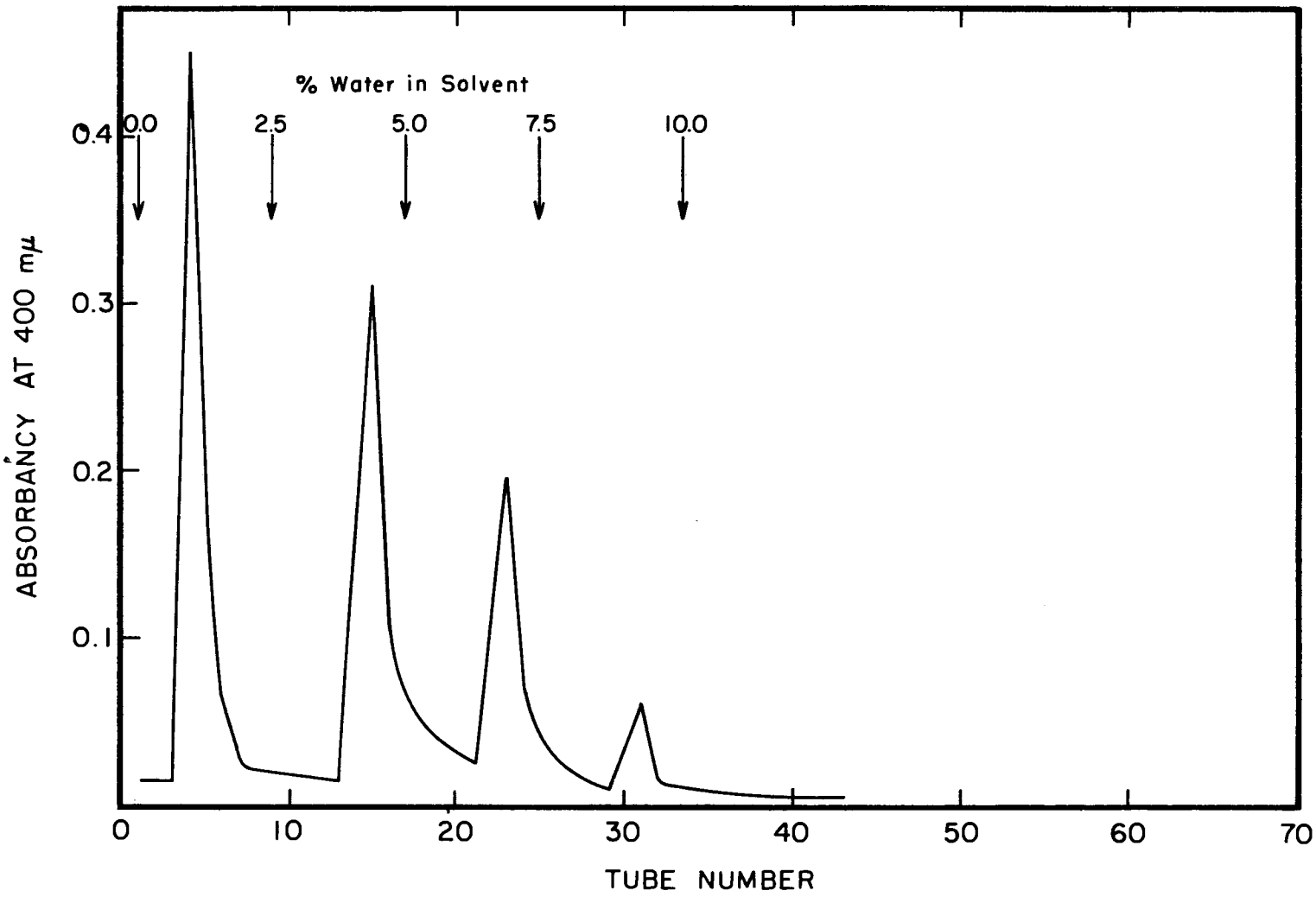

FIGURE 6. COLUMN CHROMATOGRAPHY OF FRACTION A'

obtained using the potassium bromide pellet method with a Perkin-Elmer Model 21, double-beam, infrared spectrophotometer with a sodium chloride prism. The sample contained 1.5 milligrams of phenolic acid and 300 milligrams of salt. The resulting pellet was a dark reddish-brown color. The spectra of crude mountain hemlock bark tannin and phlobaphene were also determined for comparison. The spectra are recorded in Figures 7-11.

The simplicity of the polymeric polyphenols spectrum was not unexpected, as this is a known characteristic of the spectra of high polymers. The major peaks, while strong, were rather broad and diffused, and some were only humps in areas of strong absorption.

Structural interpretation. Infrared spectroscopy has been extensively employed for the recognition of structural units of unknown compounds, and was so applied to the spectra of the phenolic acids. Structural correlations and peak assignments were made under the assumption that spectral behavior was normal, and with heavy reliance placed upon the knowledge of what structural units were most likely to be present. The peaks and assignments are listed in Table 7. The recorded frequency have been corrected as determined from a calibration curve using polystyrene as the standard.

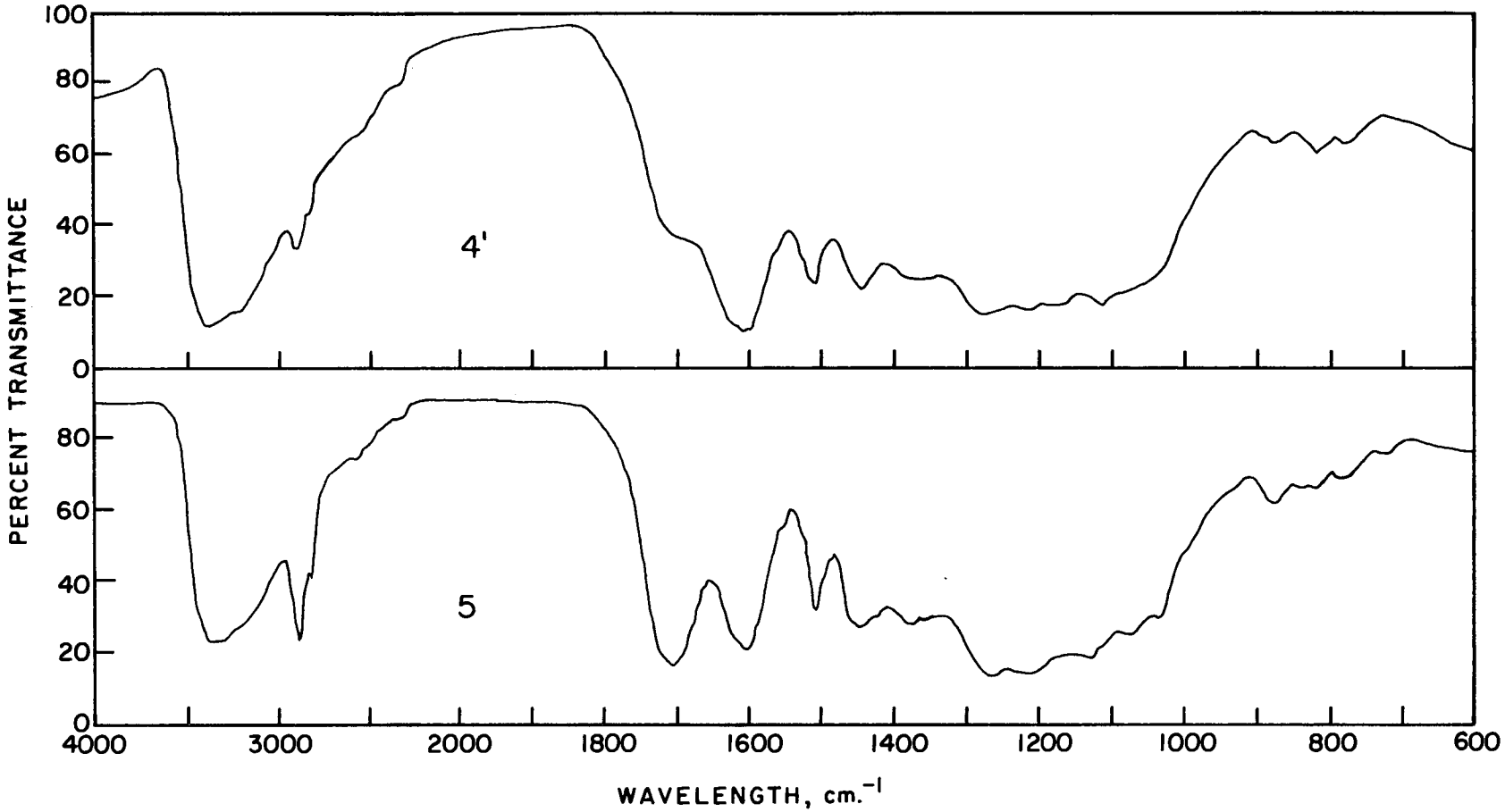

FIGURE 7. INFRARED SPECTRA OF FRACTIONS 4' AND 5

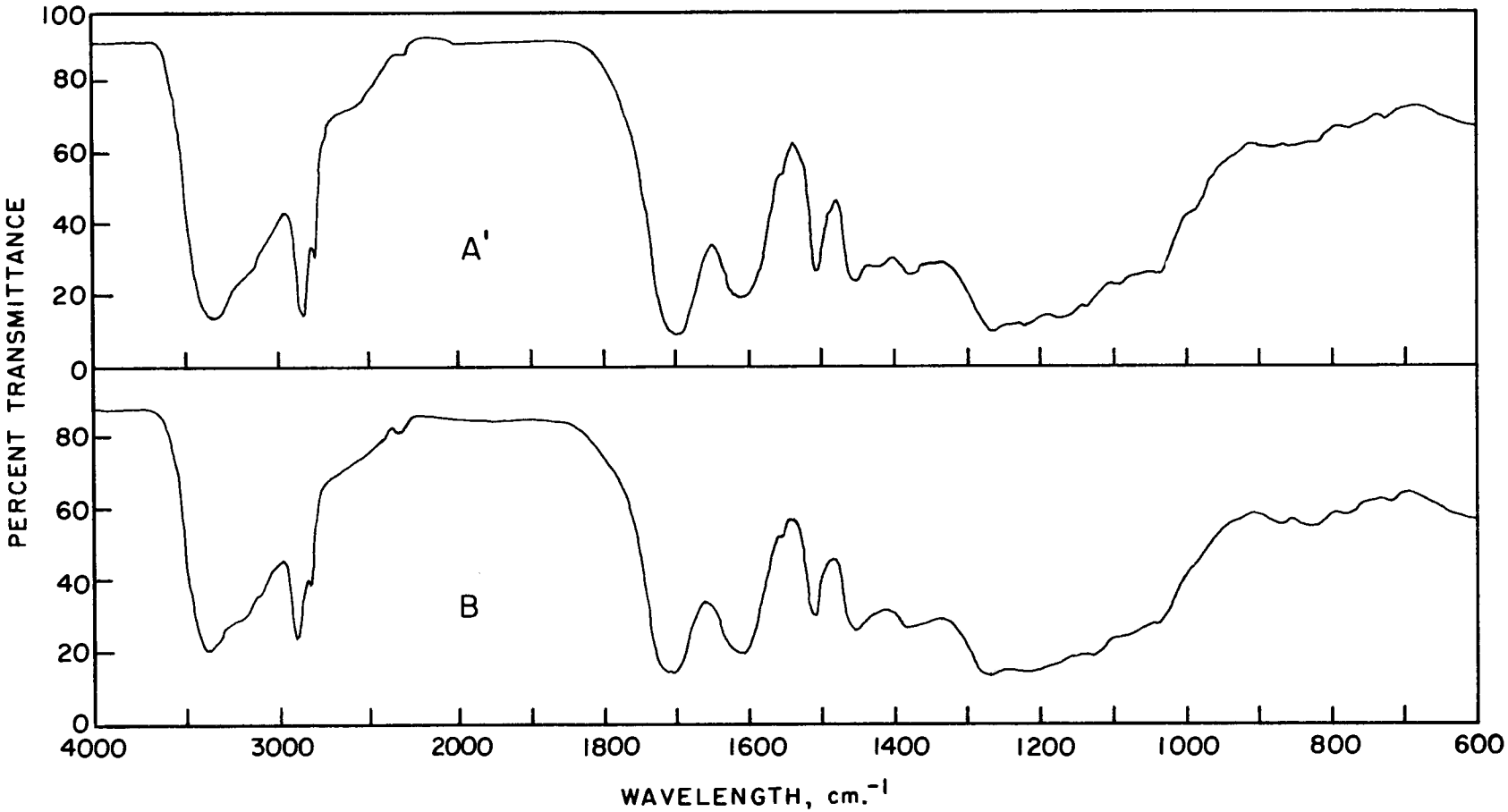

FIGURE 8. INFRARED SPECTRA OF FRACTIONS A' AND B

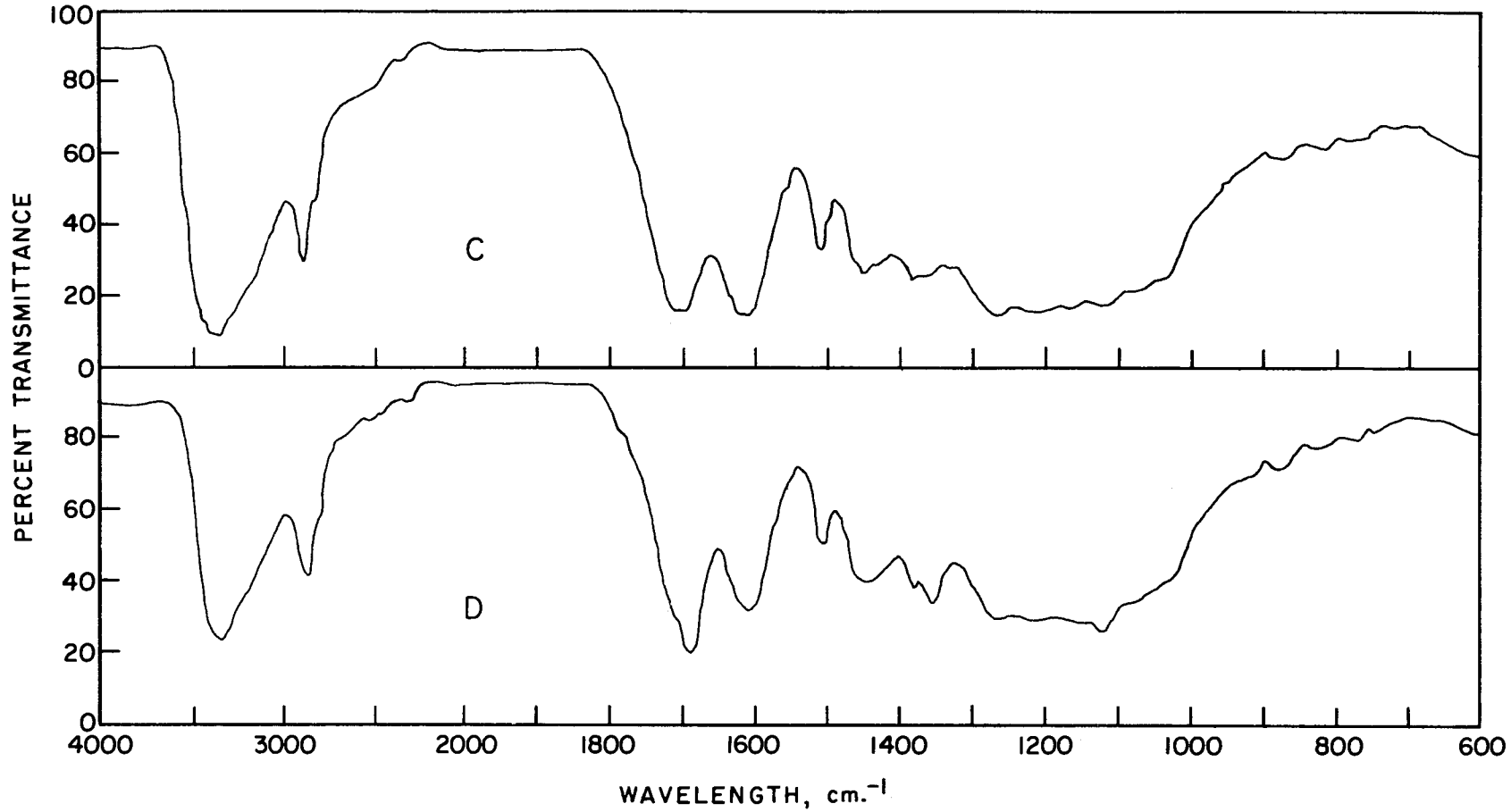

FIGURE 9. INFRARED SPECTRA OF FRACTIONS C AND D

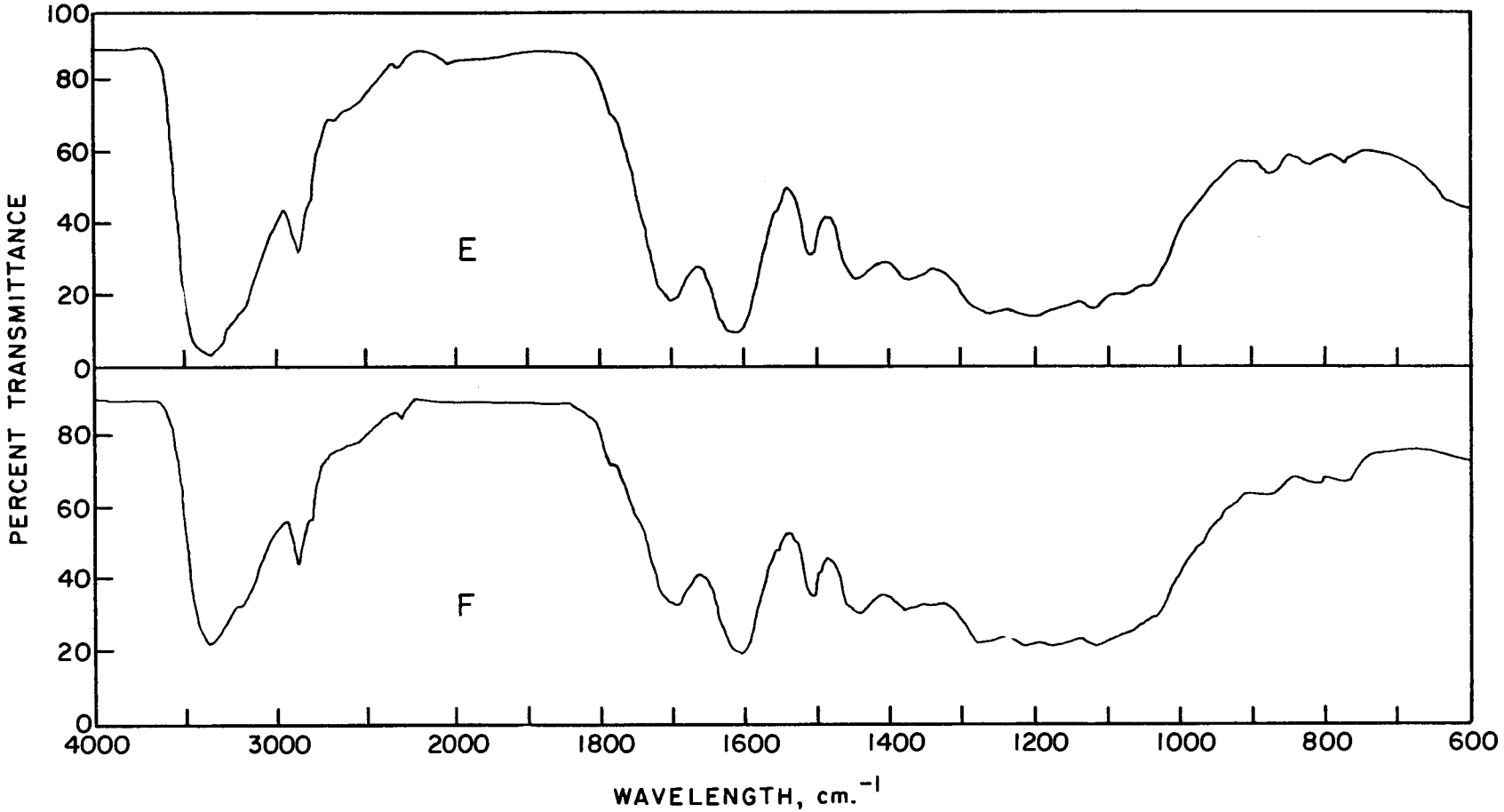

FIGURE 10. INFRARED SPECTRA OF FRACTIONS E AND F

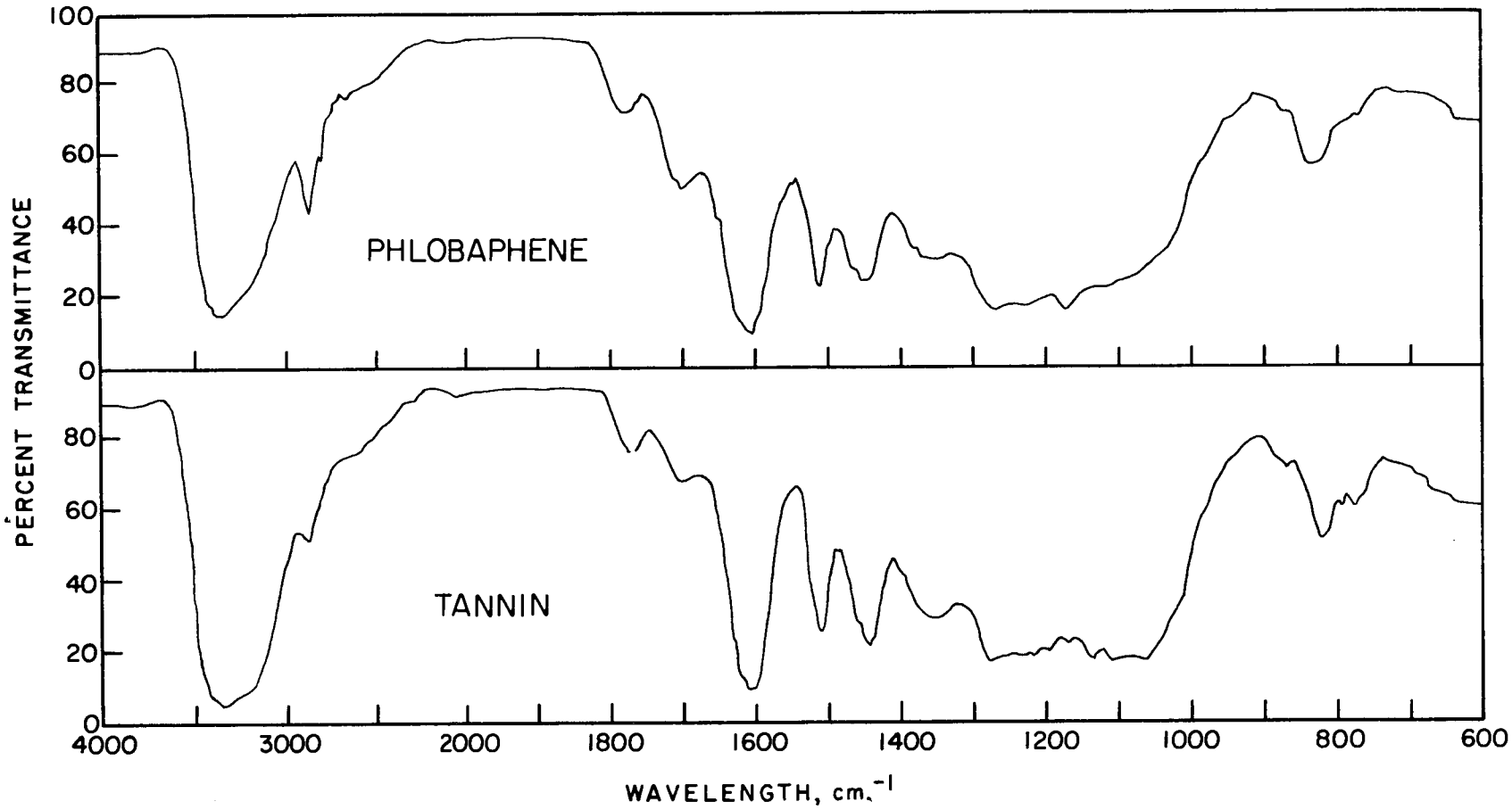

FIGURE 11. INFRARED SPECTRA OF PHLOBAPHENE AND TANNIN

TABLE 7

INFRARED FREQUENCY ASSIGNMENTS
($cm.^{-1}$)

Assignment	Fraction A'	B	C	D	E	F	Tannin	Phlobaphene	4'
OH stretching (H-bonded hydroxyl)	3410	3415	3410	3400	3430	3430	3430	3410	3435
C-H stretching (aliphatic)	2910	2920	2915	2903	2935	2935	2925	2905	2935
C-H stretching (aliphatic)	2840	2850	2845	2841	2850	2862		2835	2865
Unassigned	2351	2351	2352	2338	2360	2340			2357
Unassigned						1795	1784	1784	
C=O stretching (unconjugated)	1710	1715	1710	1695	1710	1710	1707	1707	1700
C=O skeletal stretching (aromatic)	1617	1614	1617	1611	1623	1615	1612	1609	1617
C=O skeletal stretching (aromatic)?	1552	1559	1555	1555	1559	1561			1555
C=C skeletal stretching (aromatic)	1511, 1492	1512	1511, 1502	1506	1512	1513	1516	1511	1511
C-H deformation	1455	1454	1449, 1464	1447	1449	1446	1448, 1460	1445	1443
Unassigned	1429		1431						
C-H deformation (symmetric)	1375	1382	1378	1378, 1353	1375	1375	1354	1345	1360
C-O stretching (aryl ethers?)	1260	1265	1265	1260	1270	1270	1277	1266	1275
C-O stretching (phenol?)	1215		1210		1207	1217			1212
Unassigned	1175		1165				1172	1170	
Unassigned (ether?)	1130	1130	1120	1120	1120	1119	1137		1100
Unassigned	1035	1040	1040						
Unassigned	985								
C-O out-of-plane deformation									
(One H, aromatic)		873	880	880	875		865		875
(One or two H, aromatic)		833	820	825	825	817	820	830	817
(Three aromatics)?	775	785	775	775	775	782	775, 793		780
(One or three H, aromatics)?	725	720		747?					

The strong, broad band near 3400 $cm.^{-1}$ arises from the hydrogen-bonded, O-H-stretching vibration of alcohols and phenols. The broad shape of the band is usually attributed to the association of the molecules into various polymeric forms by the hydrogen-bonded alcohol groups (5, p. 99).

The C-H stretching vibrations of methylene groups are responsible for the two absorption peaks at 2900 and 2850 $cm.^{-1}$. The intensity of these bands indicates that the phenolic acid structure must include a high ratio of aliphatic constituents. A weak shoulder near 2980 $cm.^{-1}$ in the spectrum of Fraction A' indicates that a masked peak for the methyl group might be present.

Carbonyl absorption of the carboxylic acid is responsible for the strong band at 1710 $cm.^{-1}$. Since aryl conjugation of this group usually lowers the position of this band to below 1700 $cm.^{-1}$ it seems likely that the carboxyl group of the phenolic acids are not attached to an aromatic ring (5, pp. 168-169).

The three bands at 1615, 1510 and 1450 $cm.^{-1}$ are characteristics of the semi-unsaturated carbon-carbon bond of the aromatic ring. The last peak of the series would be expected to be overlaid by strong C-H deformations of the CH_2 group and, as in the case of lignin (32, p. 410),

has been given the aliphatic assignment.

The aromatic structure is also characterized by a fourth C=C stretching vibration that normally appears in the 1600-1560 cm.$^{-1}$ region as a weak shoulder on the main 1600 cm.$^{-1}$ peak. If the ring is conjugated, this normally weak band is considerably enhanced and becomes a prominent peak of medium intensity (5, pp. 71-72). Although well below its normal position (usually near 1580 cm.$^{-1}$), this adsorption could be the origin of the weak shoulder near 1555 cm.$^{-1}$ in the phenolic acids spectra. The weakness of this shoulder, or if the band is erroneously assigned, lack of absorption in this area, is added weight to the conclusion that the carboxylic acid group is aliphatic in nature.

The relatively greater height of the 1615 cm.$^{-1}$ band in regard to the 1510 cm.$^{-1}$ band is to be noted, as it has been stated that this is a characteristic of materials containing the phloroglucinol and catechol nuclei (32, p. 407).

The peak near 1375 cm.$^{-1}$ was assigned to the C-H symmetrical deformation of the C-CH group, even though no definite band was found for the methyl group in the 2960 cm.$^{-1}$ region. The asymmetric deformation of this group is

probably also a contributor to the 1450 cm.$^{-1}$ peak. It is noted that the C-H symmetrical deformation absorption for the methoxyl group of native lignin and guaiacyl model compounds appears at 1365 cm.$^{-1}$ (32, p. 410), rather than 1375 cm.$^{-1}$. The absence of a large amount of methoxyl was confirmed by chemical analysis.

The area between 1300 and 908 cm.$^{-1}$ is associated with the C-O stretching vibration, but also encompasses part of the O-H bending region and the C-C skeletal frequencies. As would be expected from a complex, highly oxygenated material, the phenolic acids show strong absorption in this region. The spectra are very diffused here, and consequently peak assignments are somewhat arbitrary. Because of the large number of bonds that can absorb in this area, interpretation must be made with caution.

There are at least three groups that can be contemplated as giving rise to a C-O stretching peak near 1260 cm.$^{-1}$: carboxyl, phenol, and the unsaturated carbon-to-oxygen ether group. While carboxylic acids are known to give a strong band in this region (5, p. 162), a reduction in the carbonyl peak (1710 cm.$^{-1}$) in the tannin and

phlobaphene spectra is not accompanied by a reduction in the 1260 cm.$^{-1}$ peak. Thus, it was assumed that this group was not the major contributor to the peak. It could, however, account for the peak at 1237 cm.$^{-1}$ in the A' spectrum. Inasmuch as the phenol peak is usually nearer 1210 cm.$^{-1}$, the 1260 cm.$^{-1}$ peak was assigned to the aromatic ether group. This group is not unlikely, as the aromatic ether linkage is the basis of one proposed structural theory of tannins (22, pp. 575-577).

As just mentioned, the 1210 cm.$^{-1}$ peak was assigned to the C-O stretching vibration of the phenol group. Peaks are not recorded for tannin and phlobaphenes at this wave length but are still believed to be present, if obscured.

The peak near 1170 cm.$^{-1}$ was not assigned. A peak in this area (1155 cm.$^{-1}$) in the spectra of lignins has been tentatively assigned to 1:2:4-substitution of the aromatic ring (32, p. 410), but 1:3, 1:2:3- and 1:3:5-substitution also have weak bonds between 1175 and 1125 cm.$^{-1}$.

Alcohols as well as alkyl ethers are known to absorb between 1150 to 1060 cm.$^{-1}$. Jones (39, p. 169) felt that absorption in this region was at least suggestive of ether groups in lignin. Hergert (32, pp. 410 and 412) associates the 1150-1125 cm.$^{-1}$ bands in lignin to aromatic aliphatic ether linkages, and bands near 1040 cm.$^{-1}$ to primary hydroxyl groups.

In wood lignin, bands in the range 870-890 cm.$^{-1}$ have been thought to be suggestive of tri-substituted phenyl groups (42, p. 691). However, it is more specifically due to the rocking vibration of single isolated, aromatic ring, hydrogen atoms (5, p. 79) that can arise from 1:3-, 1:2:4-, 1:3:5-, 1:2:3:5-, 1:2:4:5- or penta-substituted aromatics. In the case of 1:3:5-tri-substituted materials, additional strong bands are also found in the range 865-810 cm.$^{-1}$ and between 675 and 730 cm.$^{-1}$. There is some evidence that these correlations also hold for tetra- and penta-substituted materials (5, p. 79). The spectra of the phenolic acids show a band in all three of these areas, but other possibilities exist for the latter two bands. The peak near 830 cm.$^{-1}$ could be due to two adjacent aromatic ring hydrogens, and the one near 720 cm.$^{-1}$ to a secondary band of three adjacent hydrogens (5, p. 78).

The main absorption for three adjacent hydrogens is found between 810-750 cm.$^{-1}$ and could be responsible for the phenolic acid peak at 775 cm.$^{-1}$.

The presence of lactone groups is a possible explanation for the unassigned peak near 1790 cm.$^{-1}$.

The shoulder near 1430 cm.$^{-1}$ that appears in some spectra was not assigned, but could be abscribed to methylene deformations adjacent to a carbonyl (5, p. 23)

or absorption arising from the carboxylic acid group (5, p. 170).

Comparison of infrared spectra. As would be expected from a fractionated high polymer, the spectra of the phenolic acid fractions are all very similar. The tannin and phlobaphene spectra closely resemble those of the phenolic acids, with the main difference being a reduction of intensity of the 1710 $cm.^{-1}$ peak, the presence of a peak at 1790 $cm.^{-1}$ and stronger absorption between 750 and 850 $cm.^{-1}$. The spectrum of Fraction 5 was a composite of the spectra of all the fractions separated from it.

Although all the phenolic acids gave similar spectra, a number of interesting differences can still be noted when the various spectra are compared.

While the trend is admittedly slight, the spectra become generally more diffused in progressing from Fraction A' to F. The trend is most evident with the aliphatic C-H stretching peaks at 2900 and 2850 $cm.^{-1}$. These two peaks are strongest and sharpest in the spectrum of A', and become progressively weaker and more diffused until in the spectra of F the 2850 $cm.^{-1}$ absorption is only a weak shoulder on a moderate 2900 $cm.^{-1}$ peak. To a much lesser

extent, the same trend is observed in the area between 1525 and 1375 cm.$^{-1}$. A progressively more diffused spectrum would be expected as a polymer became increasingly more polymerized.

The 1710 cm.$^{-1}$ band becomes progressively weaker in going from A' to F. In Fraction 4', this band is only a wide shoulder on the strong 1615 cm.$^{-1}$ peak.

A region of particular interest is near 1790 cm.$^{-1}$. No disturbance is noted in the spectra of A', B or C, but in D and E an inflection is detected and in F a definite shoulder has developed. As already mentioned, this is the region of lactone carbonyl absorption, but if such a group is present it is difficult to explain why it appears only in Fraction D, E and F.

Mountain hemlock tannin and phlobaphene show absorption of moderate intensity at both 1790 and 1710 cm.$^{-1}$. The infrared spectra of white fir and Douglas-fir bark phlobaphenes have peaks near 1710 cm.$^{-1}$, but show no absorption at 1790 cm.$^{-1}$ (35, p. 114; 40, p. 57 and 63, p. 65). Neither the spectrum of spruce, Douglas-fir, western hemlock nor white fir bark tannin has peaks at either 1710 or 1790 cm.$^{-1}$ (31', p. 613 and 35, p. 114). At this time it is not known whether the 1790 cm.$^{-1}$ mountain hemlock phlobaphene peak and the 1790 and

1710 cm.$^{-1}$ mountain hemlock tannin peaks are inherent parts of the spectra or are due to contamination.

Whereas the 1265 cm.$^{-1}$ band is relatively constant, the region between 1050 and 1200 cm.$^{-1}$ grows increasingly stronger from Fractions A' to F until the whole area between 1000-1265 cm.$^{-1}$ becomes a broad plateau in the more insoluble fractions. Since this is the area where ether groups absorb, it might be theorized that the increased intensity was the result of polymerization through ether linkages.

Another area of difference is in the low frequence region, where a slight tendency can be noted for the three dominant peaks (880, 825 and 775 cm.$^{-1}$) to become better defined as solubility decreases. These peaks are most prominent in the spectrum of Fraction 4'. Tannin and phlobaphene show stronger absorption in this area than do the phenolic acids.

There are a number of minor peaks that are resolved in the spectra of the more soluble phenolic acid fractions but are lost in the spectra of the more insoluble fractions. The minor peaks at 1550 and 2350 cm.$^{-1}$, on the other hand, remain relatively consistent throughout.

Some of the finer details discussed in this section

may not be as readily apparent in the spectra in Figures 7-11 as they were in the original infrared charts.

Ultraviolet Spectra

The ultraviolet spectra of Fractions 5, A', B, C, D and E were determined in dioxane with a Beckman DB recording spectrophotometer at a concentration of 20 milligrams solute per 100 milliliters of solvent. The dioxane was purified by the method of Fieser (19, pp. 284-285). Resulting spectra, recalculated to the absorbency of a one percent solution in a one centimeter cell, are reproduced in Figures 12 and 13.

Examination of the figures shows that the spectrum of Fraction A' is the most characteristic, having a maximum at 281 millimicrons and a high minimum at 270 millimicrons. In the spectrum of Fraction B, the trough has been filled to the point that there is no peak, only a plateau between 270 and 275 millimicrons. The spectra of Fractions C, D and E do not show even a plateau, but only an inflection in the 275 to 285 millimicron region. Here, as in the infrared spectra, the more characteristic curves are those of the more soluble fractions.

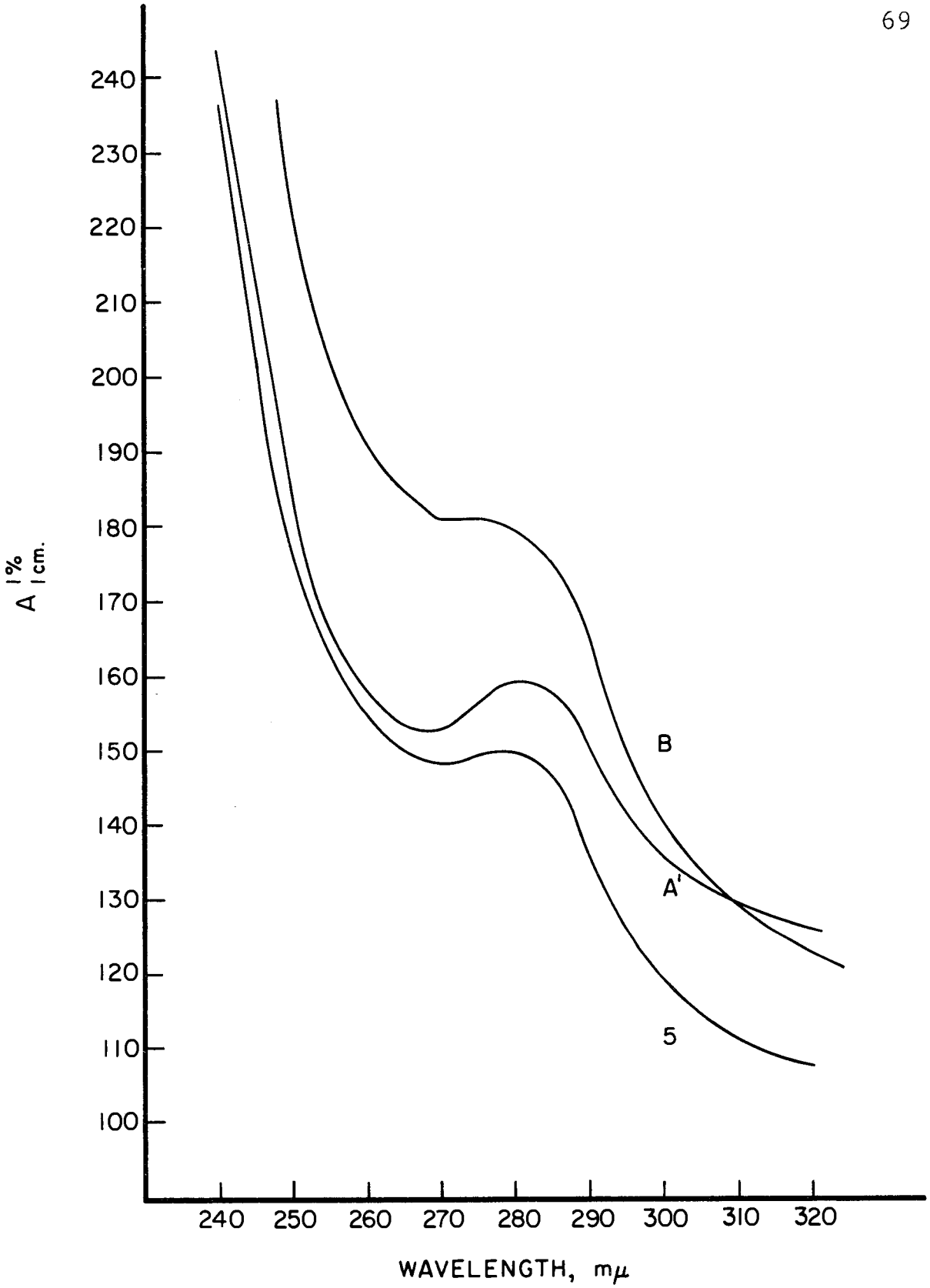

FIGURE 12. ULTRAVIOLET SPECTRA OF FRACTIONS A', B AND 5

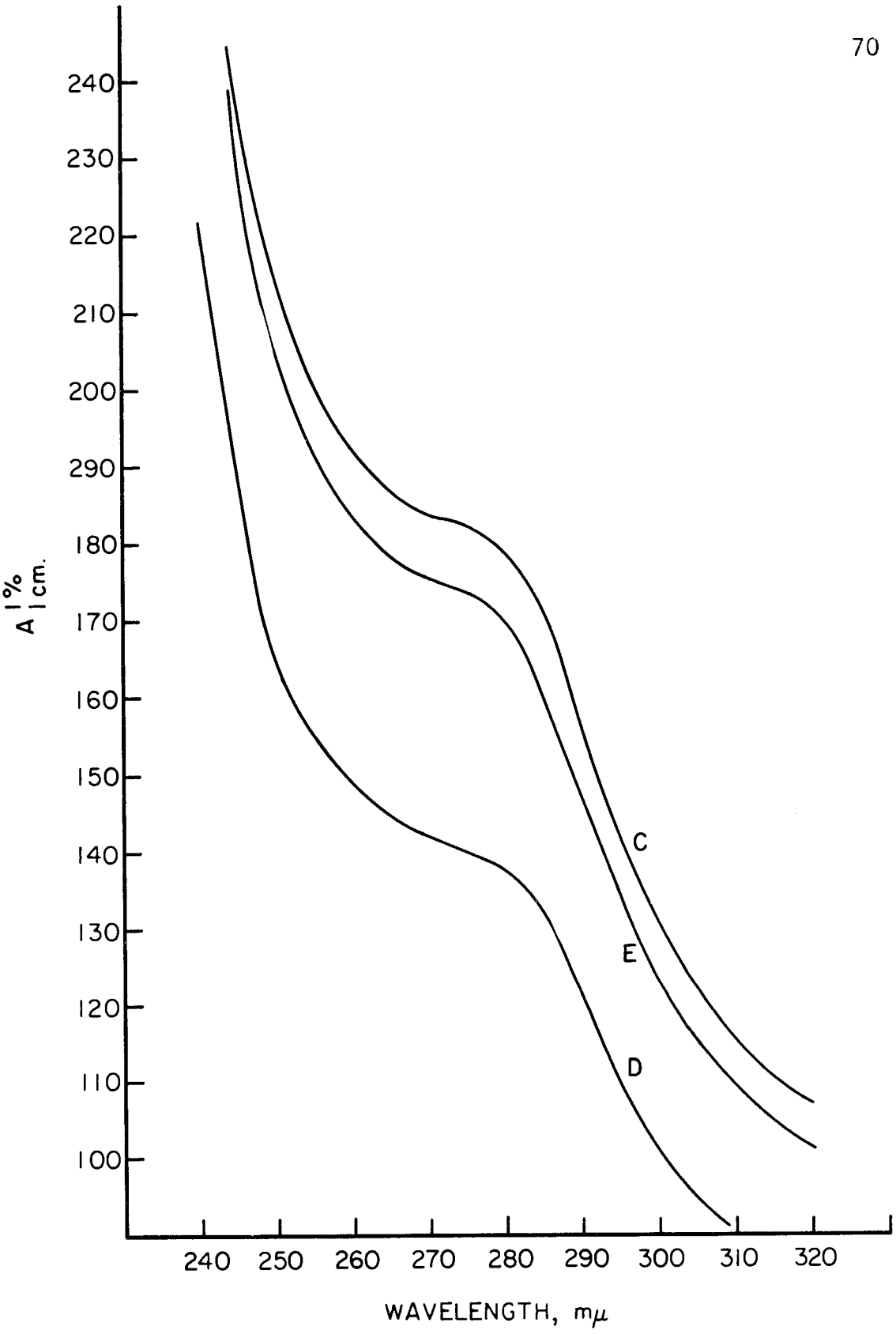

FIGURE 13. ULTRAVIOLET SPECTRA OF FRACTIONS C, D AND E

Unlike infrared spectroscopy, ultraviolet spectra do not lend themselves readily to the identification of specific groups; however, some general comments can still be made. In wood lignins, the peak at 280 millimicrons is generally attributed to an oxygen-substituted aromatic nucleus (10, p. 203), and is probably also responsible for the 280 millimicron peak of the phenolic acids. The strong absorption below 260 millimicrons is caused by the characteristic E_1-band of benzenoid compounds (8, pp. 155-158). It is of interest to note that, unlike lignin spectra (9, p. 225), the phenolic acids spectra fall off rapidly above 285 millimicrons. Lignin and related compounds show a shoulder in this area that has been ascribed to either a carbonyl or ethylenic double bond in conjugation with a benzene ring (59, p. 251 and 57, p. 1867).

COLOR REACTIONS

The phenolic acid of mountain hemlock bark was further characterized by subjecting it to a few common color reactions. All tests were performed on Fraction 5.

The phenolic acid failed to produce any color with phloroglucinol-hydrochloric acid, which is in agreement with results from other phenolic acids (10,p. 121; 41,p. 17 and 45,p. 127). The purple-red color produced by this reagent

and wood lignin is attributed to the presence of a coniferyl aldehyde group in the lignin structure (10, pp. 36-46). Other compounds with a conjugated carbonyl group also give colors with phloroglucinol-hydrochloric acid. The results of this test with phenolic acids would then suggest the absence of this group. This is not surprising, as the infrared spectra did not give any evidence of a conjugated carbonyl group.

Another common lignin reaction, the Maule test (10, pp. 51-56 and 66, p. 421) also failed to give any characteristic color with the phenolic acid. The sample was treated with chlorine-water for a few minutes at room temperature, washed, and moistened with six normal ammonium hydroxide. The color formed (a reddish-brown) could not be distinguished from a control where the sample was simply moistened with ammonium hydroxide. The same results were obtained when the chlorine-water was replaced by a one percent potassium permanganate solution-12 percent hydrochloric acid treatment.

A green color was formed with ferric chloride, but only with some difficulty. No color was noted when two drops of five percent ferric chloride solution was added to a few milligrams of phenolic acid suspended in one milliliter of

water in the usual manner (62, p. 112). The color was obtained, however, when a few drops of the reagent was added directly to the phenolic acid on a spot plate and allowed to stand a few minutes. The difficulty of obtaining a positive phenol test with phenolic carboxylic acids has been noted by Soloway and Wilen (64, p. 983), who concluded that the failure of the test in the presence of carboxyl groups was due to the competition of two groups for the reagent.

While a green color produced with ferric chloride suggests the presence of the catechol nucleus, and while this group may well be present, this test should not be taken as absolute proof of its existence in the phenolic acid molecule.

Quinone monochlorimide couples with phenols that are free in the para position to form indophenols. If the para position is occupied, the reaction takes place only if the substituent group is split off. When this reaction is carried out on wood lignin, a blue color is produced that is believed to be the result of the splitting off of para, alpha hydroxyl groups and the subsequent formation of guaiacyl indophenol (23, p. 1319 and 50, p. 104). When this test was applied to the phenolic acid, only a nondescript green-yellow color was formed. Some reaction was

believed to have taken place, however, as the color was quite intense, and much different than the orange-brown color of a blank run in conjunction with the test.

The test was also performed on a few model compounds in order to gain some background in the color produced by groups thought to be present in phenolic acid. A quarter of a milligram of the sample was added to one milliliter of quinone monochlorimide alcohol solution (one gram per liter), followed by one milliliter of 0.4 normal sodium hydroxide solution. The colors were usually produced at once, and of such intensity that dilution was necessary to determine their true color. The compounds and the color they formed appear in Table 8.

TABLE 8

QUINONE MONOCHLORIMIDE TEST

Compound	Color
Phenol	Blue
Catechol	Purple
Phloroglucinol	Red-brown
Catechin	Purple
p-Hydroxybenzoic acid	Green-blue
vanillyl alcohol	Blue
Guaiacol	Blue
Phenolic acid	Green-yellow

Quinone monochlorimide is seen to produce a number of different colors with various phenols. It seems likely that a number of colored indophenols are formed with phenolic acid and the combination results in the non-descript hue. If this is true, the identification of the indophenols formed would certainly give additional insight into the structure of phenolic acid. Some work has already been done along this line with wood lignin (24, pp. 257-262).

CHEMICAL ANALYSIS

Carbon and hydrogen, methoxyl, phenolic hydroxyl and carboxyl analyses were performed in order to further investigate the relationship among the various fractions, and as a means of comparing mountain hemlock phenolic acid with phenolic acids obtained from other species.

Elementary Analysis

The phenolic acid fractions were analyzed for carbon and hydrogen by the commercial laboratory of Wieler and Strauss, Oxford, England. The averages of duplicate analyses appear in Table 9. The percentage of carbon and hydrogen is seen to steadily decrease from Fraction A' through F with a subsequent increase in oxygen. The trend is somewhat disarrayed by Fractions C and D. The tendency

for the more soluble fractions to have the higher carbon and hydrogen content persist between Fractions 4' (anhydrous dioxane insoluble) and 5 (anhydrous dioxane soluble).

TABLE 9

ELEMENTARY COMPOSITION OF PHENOLIC ACIDS

Fraction	Percent by Weight Carbon	Hydrogen	Oxygen (by difference)
A'	63.88	6.54	29.58
B	61.84	6.23	31.93
C	59.93	5.84	34.23
D	59.97	6.35	33.68
E	57.64	5.34	37.02
F	54.44	4.95	40.61
4'	58.30	5.35	36.35
5	59.92	6.12	33.96

In general, the carbon and hydrogen content of mountain hemlock phenolic acids are comparable to those reported for other phenolic acids (see Table 12). However, the values for Fractions A' and B are somewhat high and closely approximate the analysis of native wood lignins (9, p. 236 and 10, p. 63).

Methoxyl Analysis

Methoxyl analysis is extensively used in lignin chemistry as a means of characterizing various wood lignins, and this practice has been carried over into the field of bark phenolic acid. Methoxyl determinations were performed on the phenolic acid fractions by Wieler and Strauss, and the averages of the duplicate analyses are shown in Table 10. No trend in methoxyl content is evident, but it is noted that in the two cases where an anhydrous extraction was followed by extraction with a water-saturated solution of the same solvent (A-B and C-D), the two anhydrous extractions removed fractions that were higher in methoxyl content than fractions from either water-saturated extractions. Quite a large difference in methoxyl is seen to exist between the anhydrous dioxane solubles (Fraction 5) and insolubles (Fraction 4').

TABLE 10

METHOXYL CONTENT OF PHENOLIC ACIDS

Fraction	Methoxyl (percent*)
A'	3.06
B	2.64
C	3.20
D	2.62
E	4.24
F	2.08
4'	1.26
5	3.46

* By weight.

The methoxyl values for the mountain hemlock bark phenolic acids are low, and thus in general agreement with those reported for other phenolic acids (see Table 12).

Phenolic Hydroxyl and Carboxyl Analysis

Because of their probable participation in the condensation of lignin-building stones, the quantitative determination of phenolic hydroxyl groups has played an ever-increasing role in the chemistry of wood lignin (10, pp. 244-258). It is not surprising to find this same interest,

expanded to include carboxylic acid groups, carried over into the study of the bark phenolic acids. Not only should the quantitative determination of these two groups serve as a basis for the comparison of various phenolic acids, but should also contribute to the elucidation of their structure.

The means available for the determination of acidic groups in lignin include methylation, chemosorption, conductometric titration, spectrophotometry and, of late, nonaqueous titration. The latter method is based on the fact that acids too weak to be titrated in aqueous systems are titratable in basic solvents that enhance their acidity (4 and 21). Because of its speed and simplicity, and because it has shown to give results comparable to other methods with both wood lignins (13, p. 1470; 29, p. 281 and 51, p. 791) and bark phenolic acids (48, p. 41 and 40, p. 30), nonaqueous titration was the method chosen for acid group analysis.

The method is able to differentiate between carboxyl and phenolic groups, usually in the form of separate breaks in a potentiometric titration curve. In some cases, differentiation between different phenolic hydroxyls is possible (26, p. 459 and 53, pp. 784-788). Nonaqueous titration has the disadvantage that other acidic groups

such as enols, imides, etc., are also titratable, while incomplete titration can result from steric hindrance, chelation or hydrogen bonding. In the case of phenols, substitution can cause some groups to become so acid as to be confused with carboxyl groups, or so weak that they are not titrated at all (3, pp. 1469-1470).

The titration apparatus employed (Figure 14) was a modification of that suggested by Willard and Baldyreff (21, p. 26 and 65, pp. 471 474). The contact between the two electrodes is achieved by having the burette tip immersed into the solution being titrated. This necessitates having a very small hole in the burette tip in order to reduce the mixing of the titrant and the solution being titrated. The reference electrode is mounted in a manner that permits its continual flushing, thereby avoiding diffusion effects. The electrode system used was platinum-platinum (30, p. 785) in connection with a Beckman Zeromatic pH meter acting as a potentiometer. Isolation from the atmosphere is necessary to prevent the absorption of carbon dioxide by the solvent. Stirring was provided by a magnetic stirrer that was grounded to minimize spurious potential readings.

The titrant was 0.04 normal tetrabutyl-ammonium

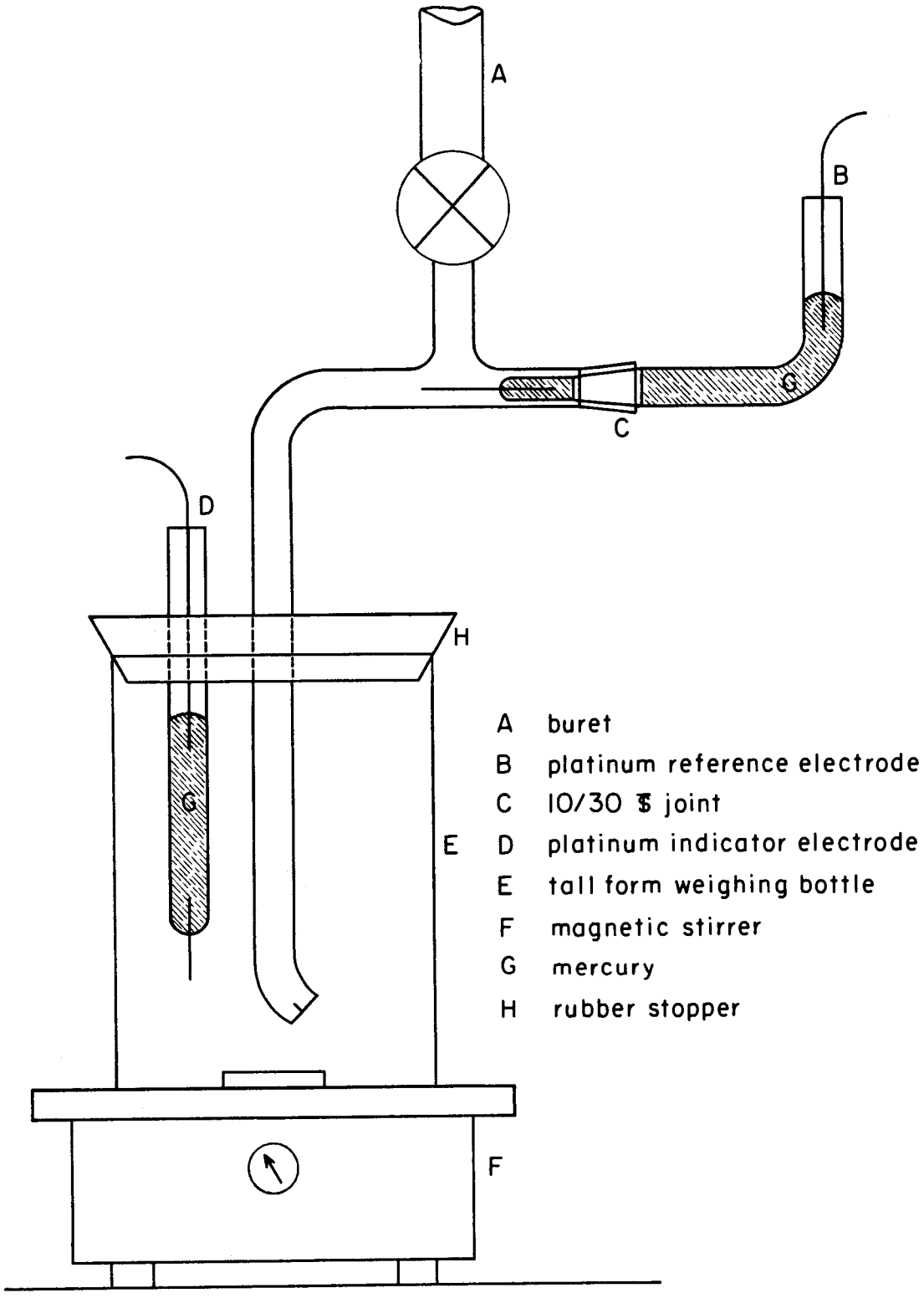

FIGURE 14. NONAQUEOUS TITRATION ASSEMBLY

hydroxide in benzene-methanol (10/1, V/V). It was prepared by treating an anhydrous methanol solution in tetrabutyl ammonium iodide with silver oxide, filtering off the precipitated silver iodide and excess silver oxide, and diluting the filtrate to volume with dry benzene. The solution was then flushed with carbon dioxide-free nitrogen and stored in a glass container under dry, carbon dioxide-free air (4, p. 34). The solvent system was redistilled Eastman Kodak white-label dimethylformamide.

The tetrabutyl-ammonium hydroxide solution was standardized against pure benzoic acid using the apparatus just described. Three drops of 0.3 percent (W/V) of thymol blue in methanol was added to 25.00 milliliters of dimethylformamide contained in the titration vessel, and the acidic impurities neutralized by titrating to the full blue color of the indicator. The accurately weighed benzoic acid (about 12 milligrams) was immediately introduced into the vessel and the solution titrated until the full blue color was again obtained. The precision of three determinations was better than one part per hundred. The titration was also followed potentiometrically.

The phenolic acid samples were titrated as follows. A weighed amount of sample was added to the titrating

vessel along with 25.00 milliliters of dimethylformamide. The vessel was then fitted snugly to the rubber stopper containing the indicator electrode and burette tip. The magnetic stirrer was put in place and the solvent stirred for a short while to dissolve the sample. The titrant was added in 0.1 milliliter amounts at the extremes of the titration, and 0.05 milliliter amounts in the region of the end points. The latter amount of titrant was the smallest volume that could be added and still observe a noticeable change in the potentiometer reading. The potential was recorded after each addition. The solution was stirred continuously by means of the magnetic stirrer. At the beginning of the titration, the dimethylformamide was dark-red in color, but became darker as titrant was added and was black by the end of the titration.

The titration curve of the phenolic acids had only one break, with no differentiation between phenolic hydroxyl groups and carboxylic acid groups. A typical curve is shown in Figure 15.

It was discovered that if the titration was carried out in admixture with parahydroxy benzoic acid (PHB) two sharp breaks were obtained, each being in excess of the calculated amount of titrant needed for the

parahydroxybenzoic acid alone. It was concluded that the parahydroxybenzoic acid served as a "carrier" for hitherto unresolved phenolic and carboxylic groups in the phenolic acid.

The use of an additive to produce more well-defined titration curves has been used by Greenhow and Smith (26, pp. 457-461) in the study of the phenols found in coal tar. They found that the nonaqueous titration of dihydroxy benzenes gave a diffused titration curve that showed slight breaks for each phenolic group. When the titration was carried out in admixture with 2,6-xylenol, however, a single, sharp inflection resulted corresponding to the total phenolic hydroxyl content.

Fractions A', B, C, D and E (F was insoluble in dimethylformamide) were titrated in conjunction with parahydroxybenzoic acid in the manner already described. Approximately six milligrams of reagent parahydroxybenzoic acid and 15 milligrams of a given phenolic acid fraction, both accurately weighed, were the amounts used. The phenolic acid fractions were dried under vacuum for three days at room temperature over phosphorous pentoxide prior to titration. A typical titration curve for this system along with curves for each component titrated separately,

are shown in Figure 15.

The first break of the right hand curve of Figure 15 corresponds to the titration of the strongly acid groups (carboxylic) of both components, and the second break to the titration of the total acids present (carboxylic plus phenolic). The Beckman Zeromatic pH meters potentiometric scale is adjustable, consequently the ordinate of Figure 15 is only relative.

After correcting for the parahydroxybenzoic acid added and the acid impurities present in the solvent as determined from a blank, the percentages of carboxyl and phenolic hydroxyl groups present in the phenolic acids were determined. The average values from three determinations, along with equivalent weights and the ratio of phenolic to carboxyl groups, appear in Table 11.

It was noted that when Fraction A' was titrated alone, the single break in the titration curve corresponded to an equivalent weight of 240. When titrated in admixture with parahydroxybenzoic acid, and ignoring the first break, a value of 230 was obtained. This means that in the former case, the break represents the total acid content, and that the addition of parahydroxybenzoic serves only to distinguish between strong and weak acids, but does not

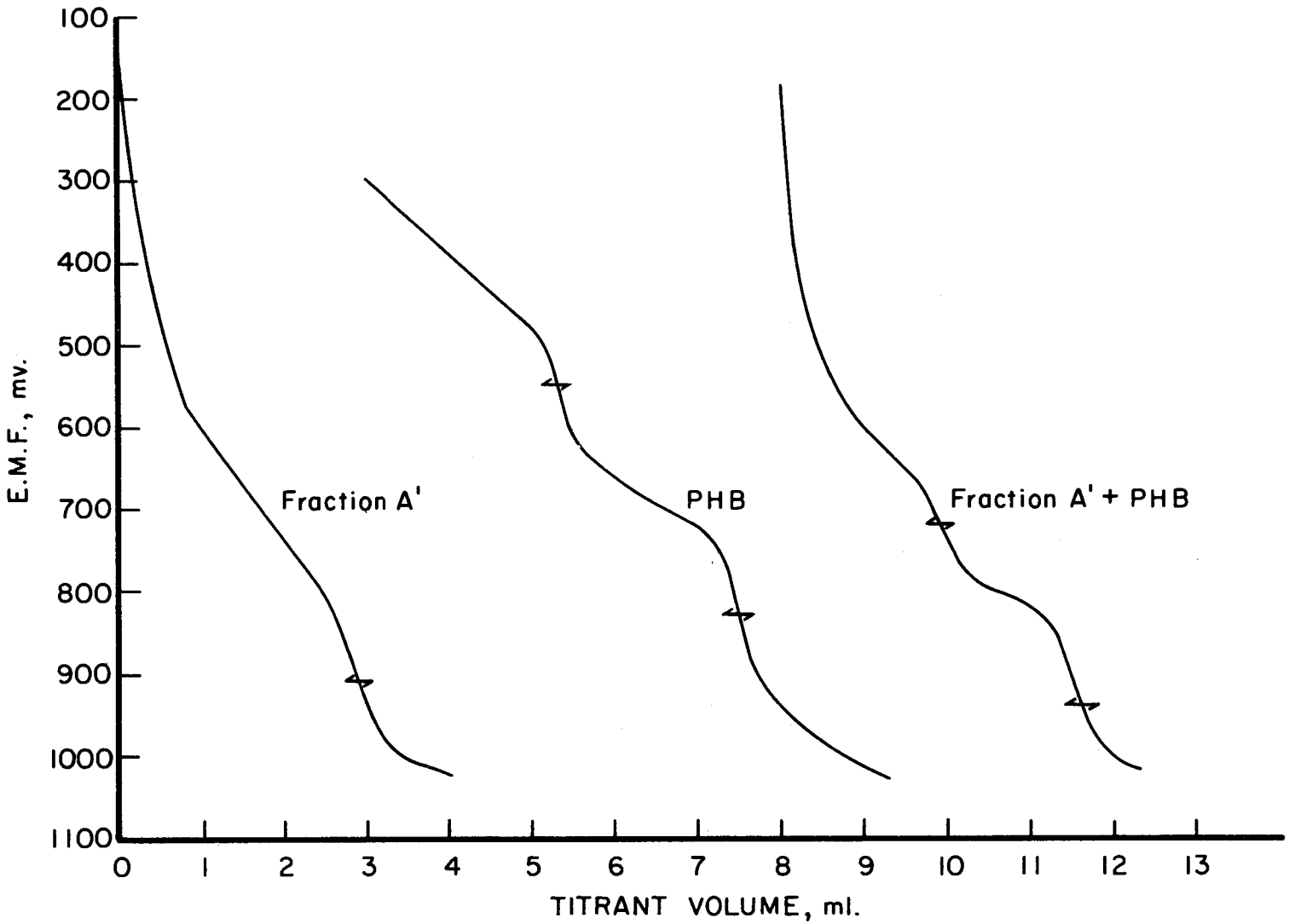

FIGURE 15. NONAQUEOUS TITRATION CURVES

alter the determination otherwise.

The data shown in the Table 11 reveal that no trend exists in either the phenolic hydroxyl or carboxyl content of the various phenolic acids. The ratio of the two groups is shown to be somewhat lower in the first two fractions, however. Compared to phenolic acids from other conifer barks, mountain hemlock phenolic acid has a phenolic hydroxyl content somewhat lower than general, and a carboxyl content in agreement with two of the values present in Table 12. The ratios, however, are lower than any yet reported. The lower values for the ratio and phenolic hydroxyl content could be an inherent property of this particular phenolic acid, or could reflect purification. Noncarboxyl polyphenolic contaminants, such as lignin, phlobaphene, etc., would increase both of these values.

TABLE 11

PHENOLIC HYDROXYL AND CARBOXYL CONTENT OF PHENOLIC ACIDS

	Phenolic Hydroxyl (P)		Carboxyl (C)		
Fraction	(percent*)	Equivalent Weight	(percent*)	Equivalent Weight	Ratio P/C
A'	3.1	550	11.3	400	0.7
B	3.3	520	11.7	380	0.7
C	4.2	400	10.8	420	1.0
D	3.0	570	9.1	500	0.9
E	3.8	450	11.6	390	0.9

* By weight.

Other solvents and titrants were tried but were found to be unsatisfactory. The commonly used ethylenediamine (4, p. 30) could not be used as a solvent as the phenolic acids were only partially soluble in it. Sodium amino-ethoxide in ethanolamine was not satisfactory as a titrant because it caused the precipitation of the phenolic acid being titrated. Acetone has been suggested as a solvent for differentiating titrations (4, p. 30), but failed to give a satisfactory curve with the phenolic acids when titrated with tetrabutyl-ammonium hydroxide.

TABLE 12

COMPOSITION OF BARK PHENOLIC ACIDS

Phenolic Acid Source	Carbon	Hydrogen	Methoxyl	Phenolic Hydroxyl (P)	Carboxyl (C)	Ratio P/C
	percent, by weight					
Douglas-Fir Bast Fibers (41, pp. 17-18)	54.12	5.33	4.34	8.3	5.3	4.1
White Fir Cork (17,p. 507)	60.2	5.7	3.56	4.4-6.2	10.9-13.3	1.1-1.2
Red Pine (10,p. 121)	60.5	4.81	1.71	9.23	2.2	11.1
White Spruce (54, p. 56)	58.38	4.97	1.46	-	7.22	-
Redwood Bast Fibers (11, p. 36)	-	-	2.7	7.8	4.4	4.7
Redwood Fines (11, p. 38)	57.1	5.0	0.0	8.6*	11.0*	2.1

* Calculated from methylation data of authors.

Molecular Weights

To date, there has been no report of the physical determination of the molecular weights of bark phenolic acids. The only information available has been calculated on the basis of functional group analysis. Kiefer and Kurth

(41, p. 17) calculated a value of 850 for the minimum molecular weight of Douglas-fir bast fiber phenolic acid on the assumption that it was monocarboxylic. Their proposed molecule also contained one methoxyl group, four phenolic hydroxyl groups and two aliphatic hydroxyls. Using the same approach, an apparent value of 1600-1840 has been obtained for the same material derived from a different source (9, p. 31). A minimum molecular weight ranging from 785 to 1000 was calculated for redwood bark phenolic acid depending on whether methoxyls, phenolic hydroxyls or carboxyls were taken as the basis (11, p.220).

It can be expected that attempts to physically measure the molecular weights of phenolic acids will be plagued by the same problems encountered with wood lignins, with the additional complications associated with carboxyl groups. Some of these problems are: tenaciously adsorbed solvents, retention of ash, temperature sensitivity, association and ionization.

Determination by Vapor Pressure Osmometry

Thermoelectric osmometry depends upon the vapor pressuring lowering of a solvent by the addition of a solute. When a solvent and a solution of the same solvent

are placed in a thermostated system that is saturated with vapor, condensation of the vapor takes place on the two liquids at a rate according to their vapor pressures. The heat of condensation results in a difference in temperature that remains steady for an appreciable period of time. The temperature difference is proportional to the vapor pressure lowering, that is in turn a colligative property that is proportional to solute concentration (7, pp. 263-268 and 56).

In the model 301A Mechrolab osmometer, single drops of liquid are suspended from each of two matched thermistor beads that form opposite arms of a Wheatstone bridge. The delta resistance arising from the temperature difference between the two beads, when one is covered with pure solvent and the other with a solution, is measured by balancing the bridge. This instrument is capable of measuring a difference of 0.0001° C. The osmometer is calibrated for a given solvent with a solute of known molecular weight, and the molar concentration of a solution of the unknown sample determined.

The molecular weights of Fractions A' through E were determined by the method just described using dimethylformamide as the solvent. It was hoped that the use of a strongly polar solvent would minimize the problem of

association. The fractions were extensively dried under vacuum at room temperature over phosphorous pentoxide prior to the determination.

The method of calculation was as follows. A calibration curve of benzil in dimethylformamide was plotted as log molar concentration versus log delta resistance for the concentration range 0.20 to 0.025 molar. The delta resistance for three concentrations (25, 50 and 100 grams per liter) was determined for each unknown sample, and the apparent molar concentration determined from the calibration curve. The weight concentration for each dilution was then divided by the apparent molar concentration to obtain the reciprocal of the apparent molecular weight. This value was then plotted against weight concentration and the curve extrapolated to zero concentration. According to Huggins (37, pp. 151-158) and Flory (20, pp. 51-61), this type of plot is linear to a fairly high weight concentration. The reciprocal of the value at zero concentration was taken as the number average molecular weight. The values determined by this method, along with the apparent molecular weight at the various concentrations, are recorded in Table 13. The extrapolated curves appear in Figure 16.

The molecular weights as determined in dimethylformamide were found to be very concentration dependent, and to increase with decreasing concentration. While concentration dependence is common with polymers, this particular direction of dependence is somewhat unusual. Of the three conditions most likely to lead to nonideality--association, disassociation and solvation (or molecular compound formation)--, the latter would be expected to behave in this manner and the other two in just the opposite. When a solute combines with the solvent in some way (solvation) so as to decrease the amount of solvent available to act as free solvent, then the effective concentration will be increased and will lead to a low apparent molecular weight. As the solution is diluted, the percent solvent that was unavailable would be decreased and the apparent concentration will then be decreased more than is obvious from simple dilution. Molecular weights determined in such a system would seem to increase with decreasing concentration. A strong dipole-dipole attraction might be expected to exist between phenolic acid and dimethylformamide. While extrapolation to zero concentration will generally correct for nonideal behavior, this is not always the case. The accuracy of the extrapolation was further limited by the scattering of the points of the

reciprocal molecular weight plot.

The extrapolated molecular weights are in the range expected from previous functional group analysis molecular weights, and the molecular weights found for such related materials as tannin (15, pp. 142-148 and 66, p. 619) and soluble wood lignins (28, p. 519). The value of Fraction A' is high when compared to the other four fractions. The extrapolation for this fraction is more uncertain than the others, as only two points were available. The sensitivity of the instrument was evidently not sufficient to detect the concentration of its last dilution. Still, the other two points show that Fraction A' has an apparent molecular weight well above the others.

Ethylene carbonate has proven to be a very good solvent for cryoscopic determination of the molecular weights of soluble wood lignins (14, pp. 518-521), but was found unsatisfactory for phenolic acid. The solvent dissolved phenolic acid initially but after a period of time a precipitate began to form, indicating a reaction with the solvent. During the course of this investigation, dimethyl sulfoxide was found to be a very good solvent and, as it has a freezing point just below room temperature, it might be a good cryoscopic solvent. It was tried as vapor pressure osmometric solvent, but apparently its vapor pressure was too low.

TABLE 13

NUMBER AVERAGE MOLECULAR WEIGHTS OF PHENOLIC ACIDS

Fraction	Concentration (grams/liter)	Molecular Weight
A'	0*	5400
	26.0	..
	51.9	1860
	103.9	1370
B	0*	1560
	25.6	1420
	51.2	1250
	102.5	1060
C	0*	1000
	25.4	920
	50.8	1020
	101.6	880
D	0*	1790
	25.7	1560
	51.3	1170
	102.6	930
E	0*	1390
	24.8	1210
	49.7	1170
	99.75	1060

* By extrapolation.

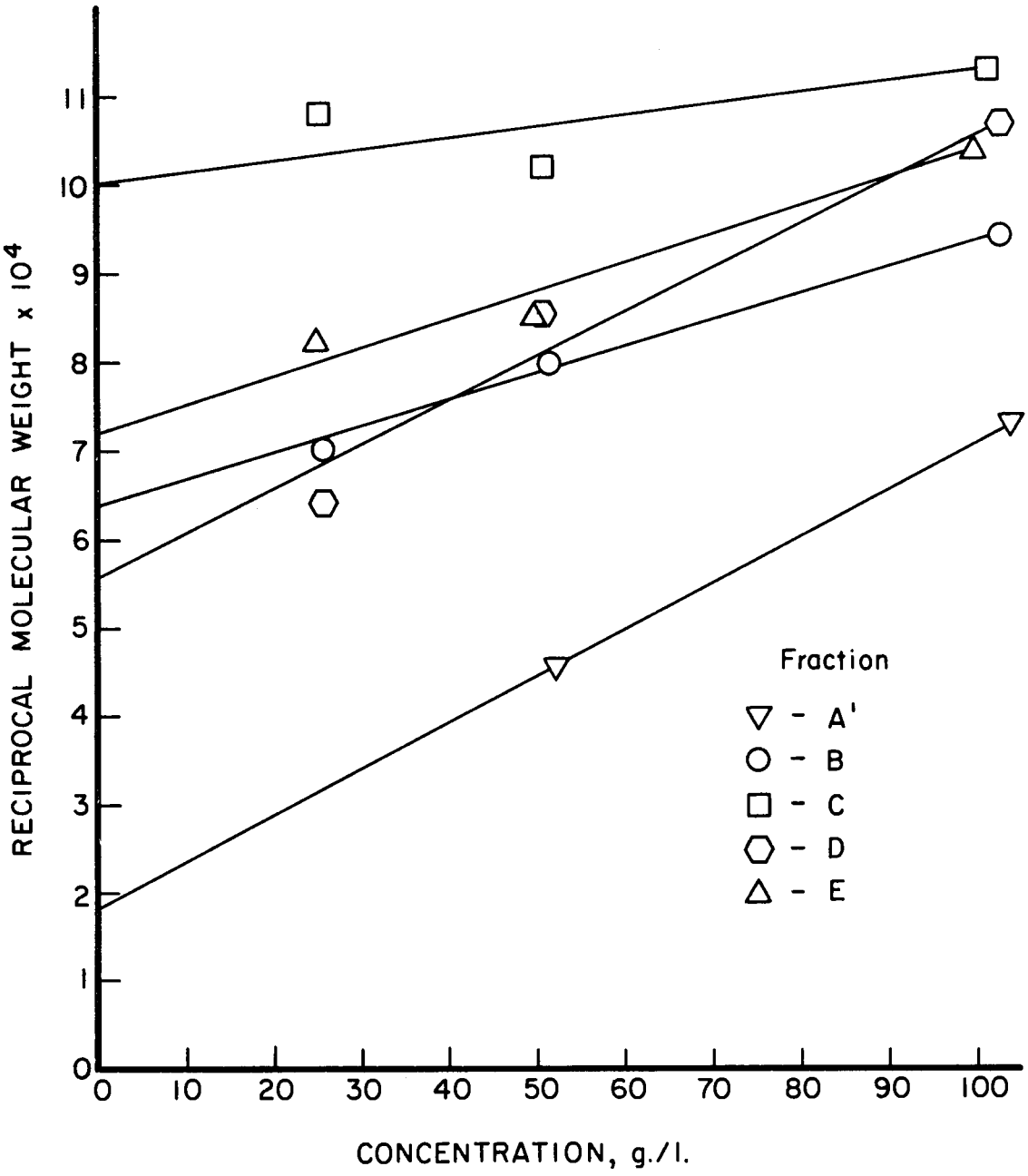

FIGURE 16. MOLECULAR WEIGHT EXTRAPOLATION

DISCUSSION

Under the conditions employed, paper electrophoresis was unable to separate crude mountain hemlock phenolic acid into definite fractions. Some electrophoretic difference could be observed, however, between the main portion of the phenolic acid and a number of minor fractions once separation had been achieved by a very simple solvent scheme. This, coupled with the fact that small amounts of material separated from the main electrophoretic band in the pH range 8.5 to 10.0, suggest the possible use of continuous electrophoresis as a means of purification. In view of the results of this entire investigation of mountain hemlock phenolic acid, it is doubted that even at higher field strengths that more than a continuum of phenolic acid fractions could be obtained by paper electrophoresis. Paper electrophoresis is able to distinguish vegetable tannins from one another (61, p. 344), and could prove useful in this capacity for conifer bark phenolic acids.

Crude mountain hemlock phenolic acid consists of two equal fractions that differ significantly in their colors, solubilities, methoxyl contents and infrared spectra. One is soluble in anhydrous dioxane before and after the fraction is dried, while the other is <u>insoluble</u> in

dioxane, but soluble in moist dioxane before the fraction is dried but not after. It is mentioned a number of times in the literature that phenolic acids are soluble in organic solvents during isolation while still moist, but are only slightly soluble in the same solvents after the material had been dried (34, p. 65; 41, p. 17; 45, p. 127 and 49, p. 30). Inasmuch as this would be the behavior of mountain hemlock phenolic acid if the two fractions were not separated, it appears likely that these other phenolic acids could also be separated into these same types of fractions.

The anhydrous dioxane-soluble portion of the crude phenolic acid was capable of being divided into a number of fractions by a solvent extraction scheme employing a series of ketones and their corresponding water-saturated solutions. Cellulose adsorption column chromatography demonstrated that these fractions were not completely homogeneous but were comprised of a mixture of components, as would be expected from even a fractionated high polymer.

The infrared spectra of the fractions separated by the solvent extraction scheme were all very similar, but did show some definite differences. The dissimilarities were most apparent in the resolution of aliphatic C-H stretching peaks, and the height of the 1710 $cm.^{-1}$ peak

relative to the 1615 cm.$^{-1}$ peak. Carboxyl analysis revealed that the strength of the 1710 cm.$^{-1}$ absorption was not necessarily a reflection of carboxyl content. The position of this peak would indicate that the carboxyl groups in these phenolic acids were aliphatic in nature. The spectra also indicated the presence of ether linkages in the phenolic acids.

The infrared spectra of mountain hemlock bark phenolic acids are in general agreement with those reported for the phenolic acids of other conifers (31, p. 336; 33; 35, p. 142; 40, pp. 48-59; 48, p. 55 and 63, pp. 62-74). This is to be expected, as the material has a diffused spectrum with only a limited number of peaks, and a considerable difference in the structure of a given phenolic acid would have to exist before its spectrum differed greatly for those of other phenolic acids. The dissimilarities that did exist were in the strength or weakness of various peaks, but these same variances existed between the spectra of the different mountain hemlock phenolic acid fractions. A peak of particular interest is the 1700 cm.$^{-1}$ carboxyl-carbonyl peak. Some conifer bark phenolic acids give a peak at this wave length as strong or stronger than the neighboring 1600 cm.$^{-1}$ (31, p. 336; 35, p. 142 and 63, pp. 68-77), while others exhibited only a shoulder on a strong 1600 cm.$^{-1}$ band

(33 and 48, p. 55). For an accurate comparison of the infrared spectra of the phenolic acids from different conifers, samples should be prepared in the same manner and the same technique employed for determining the spectra.

A number of ultraviolet spectra of phenolic acids have been reported, and they are all characterized by a broad peak near 280 millimicrons and a trough filled to varying degrees near 260 millimicrons (31, p. 337; 34, p. 63; 40, p. 58; 48, p. 53 and 63, pp. 75-76). Some spectra also showed inflections near 250 millimicrons (31, p. 337; 34, p. 63; 48, p. 53 and 63, p. 75) and/or near 320 millimicrons (31, p. 337 and 34, p. 63). Reference to Figure 5 shows that the column chromatography of Fraction A separated a material (tube number 22) whose ultraviolet spectrum had inflections in both of these areas from fractions whose spectra did not. This would suggest that absorption in these areas is due to impurities. While the origin of the maximum of 280 millimicrons is fairly well understood, the reason for the filling of the 260 millimicron trough is more obscure. It has been demonstrated that this trough in the spectra of guaiacyl-type lignin model compounds is filled by substitution of the aromatic ring in the five position with oxygen or phenyl groups, thereby giving

symmetrical electron denoting 3:4:5-substitution (59, pp. 250-252). The ultraviolet spectra of tannins and phlobaphenes (31', p. 614; 40, p. 58, and 63, p. 76) are similar to those of phenolic acids except that the trough is much deeper. It should be determined whether treatment of these materials with aqueous caustic causes this trough to fill, and thereby give spectra identical to the corresponding phenolic acids, as is the case with the infrared spectra.

The methoxyl analysis is of particular interest because the role of this group in phenolic acid has not been clearly demonstrated. It seems certain that conifer barks contain a fraction that is the same as, or very similar to, wood lignins, and that part of this fraction is contained in phenolic acid preparations. What is not known is whether this fraction is an inherent part of the phenolic acid structure or is a separate constituent of bark that contaminates the phenolic acid. One would think that if the methoxyl content of phenolic acid was due to lignin impurities, they would have been concentrated in one or the other of the mountain hemlock anhydrous soluble phenolic acid fractions (A'-F) instead of being

fairly evenly distributed throughout. It may be of significance that the methoxyl content of Fraction 4' was so much less than Fraction 5. It is also possible, of course, that methoxyl groups are an inherent part of a phenolic acid structure that is completely devoid of wood lignin type units. Nevertheless, a determined effort should be made to isolate a phenolic acid free from methoxyl groups, or at least one that does not give guaiacyl-type degradation products.

The relative consistency of the content of methoxyl, phenolic hydroxyl and carboxyl groups in Fraction A' through F indicates that the basic monomer of the phenolic acids was the same in all fractions.

Examination of all the data collected on Fractions A'-F does not readily indicate on what basis the fractionation took place. It was initially concluded that the solubilities, color and the trends noted in the infrared and ultraviolet spectra were the result of the increasing molecular weights of Fractions A' to F. The molecular weight data, however, did not bear this out, and indicated that the ketone water-saturated ketone scheme of extraction did not fractionate the phenolic acids in order

of molecular weight, although each fraction did differ in this property. It is especially disconcerting that the molecular weight of Fraction A' was so much higher than the other fractions. While the solution fractionation of high polymers generally takes place on the basis of molecular weights, this is not always the case. Separation actually proceeds according to solubility, and such properties as branching, polarity, functional group content, etc. affect solubility characteristics. The periodic introduction of water into the extraction scheme also may have disarrayed any normal molecular weight trend.

It is suggested that the feasibility of determining the molecular weights of methylated or acetylated phenolic acids be investigated. This has been done with tannins (15, pp. 142-148), and should reduce the difficulties caused by the strongly polar phenolic hydroxyl and carboxyl groups of phenolic acids. A comparison of the physically determined molecular weights of methylated or acetylated phenolic acids and untreated phenolic acids would help determine whether extrapolation to zero concentration corrected for the nonideal behavior of the

system employed in this study.

The carbon and hydrogen content was the only chemical property determination that progressively changed with the fractions. The percentages of these two elements steadily decreased from Fraction A' to F with a subsequent increase in oxygen. The increase in oxygen was not reflected in any similar increase in the oxygen-containing groups determined (methoxyl, phenolic hydroxyl and carboxyl). However, the increase was accompanied by increasing absorption in the area of the infrared spectra (1200-1050 cm.$^{-1}$) associated with ether and aliphatic alcohol groups. It can only be speculated whether or not the carbon and hydrogen content reflect any property upon which fractionation could have taken place.

An area of research that might help clarify the relation between tannin and phenolic acid is alcoholic acid cleavage. A large amount of work has already been done on tannins in this respect, and it is known that they yield flavan-3-ols and flavan-3,4-diols (22, pp. 563-573; 31', pp. 610-616 and 33). It has been determined that extractive-free amabilis fir and longleaf pine barks give such compounds on treatment with acidulated n-propanol,

but no work has been reported on either phenolic acid or caustic-treated tannin. Such a study could go far in determining whether phenolic acid is an artifact resulting from the caustic treatment of a tannin-like material present in extractive-free bark.

In conclusion, mountain hemlock phenolic acid was found to consist of two main fractions that differed significantly as to their solubilities, infrared spectra and methoxyl contents. The more soluble of these was further shown to be a mixture of materials that were similar in regard to their chemical and physical properties, but characteristic enough in these same properties as to establish their differences. The original hypothesis that phenolic acids are mixtures of closely related polymers differing only in extent of polymerization was essentially supported. Mountain hemlock phenolic acid was also found to be similar to the phenolic acids of other conifers.

SUMMARY

A crude phenolic acid was obtained from mountain hemlock bark by extraction with one percent aqueous sodium hydroxide at 90° C. A paper electrophoresis study was undertaken, but did not succeed in separating the crude material into definite fractions.

Crude phenolic acid was found to be made up of two equal fractions. One was soluble in anhydrous dioxane, while the other was insoluble in anhydrous dioxane, but soluble in moist dioxane before the fraction was dried but not after. The two fractions were quite different in regard to color, solubility, infrared spectrum and methoxyl content. The anhydrous dioxane-soluble fraction was divided into six fractions (five soluble fractions and a residue) by a solution fractionation scheme employing a series of ketones and their corresponding water-saturated solutions. Cellulose adsorption chromatography revealed that these fractions were still a mixture of components. The six fractions were compared by their physical and chemical properties. The infrared spectra of all six were very similar, but did show some meaningful differences. The position of the 1700 $cm.^{-1}$ carbonyl carboxyl peak

indicated that the carboxyl groups in the mountain hemlock phenolic acids were aliphatic in nature. All gave ultraviolet spectra showing a shoulder or peak near 280 millimicrons. The carbon and hydrogen content of the six fractions steadily decreased from the most soluble fraction (63.88 percent carbon and 6.54 percent hydrogen) to the most insoluble fraction (54.44 percent carbon and 4.95 percent hydrogen). The methoxyl content randomly varied from two to four percent. Nonaqueous titration was used to determine the phenolic hydroxyl and carboxyl contents of the first five fractions. The phenolic hydroxyl content randomly varied from three to five percent, and the carboxyl content from nine to 12 percent. The molecular weights of the same five fractions varied, again in a random manner, from a low of 1000 to a high of 5400 as determined by vapor pressure osmometry.

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