

ISOLATION AND IDENTIFICATION OF POLYPHENOLS  
OF HEMICELLULOSE EXTRACTS

by

HSIN-CHUAN TSAI

B. S., Taiwan Provincial College of Marine  
and Oceanic Technology (Taiwan), 1971

---

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

in

FOOD SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY  
Manhattan, Kansas

1975

Approved by:

  
Major Professor

LD  
2668  
T4  
1975  
T77  
C.2  
Document

ii

## TABLE OF CONTENTS

	Page
INTRODUCTION. . . . .	1
LITERATURE REVIEW . . . . .	2
Chemical Composition of Wood. . . . .	2
Wood By-Products. . . . .	3
Phenols and Tannins . . . . .	6
Determination of Total Polyphenol in Plant. . . . .	9
Isolation of Polyphenols from Plant Material. . . . .	11
Identification of Polyphenols . . . . .	12
MATERIALS AND METHODS . . . . .	15
Total Polyphenol Content in Hemicellulose Extract . . . . .	15
Extraction of Polyphenols from Hemicellulose Extract. . . . .	17
Testing for the Presence of Polyphenols . . . . .	22
Column Chromatographic Separation of Ethyl Acetate Extracts. . . . .	26
Paper Chromatographic Studies of Ethyl Acetate Extracts of Hemicellulose Extract and Fractions Separated by Dialysis . . . . .	26
Thin-Layer Chromatographic Studies of Ethyl Acetate Extracts of Hemicellulose Extract and Fractions by Dialysis and Column Separation. . . . .	27
High Pressure Liquid Chromatographic Studies of Ethyl Acetate Extracts of Hemicellulose Extract and Fractions by Dialysis and Column Separation . . . . .	28
RESULTS AND DISCUSSION. . . . .	29
Total Polyphenol Content in Hemicellulose Extract . . . . .	29
Isolation of Polyphenols from Hemicellulose Extract . . . . .	42

	Page
Evidence for the Presence of Polyphenols. . . . .	46
Column Separation of Ethyl Acetate Extracts . . . . .	46
Paper Chromatographic Studies of Ethyl Acetate Extracts of Hemicellulose Extract and Fractions Separated by Dialysis . . . . .	47
Thin-Layer Chromatographic Studies of Ethyl Acetate Extracts of Hemicellulose Extract and Fractions by Dialysis and Column Separation. . . . .	47
High Pressure Liquid Chromatographic Studies of Ethyl Acetate Extracts of Hemicellulose Extract and Fractions by Dialysis and Column Chromatography . . . . .	50
SUMMARY . . . . .	72
LITERATURE CITED. . . . .	74
ACKNOWLEDGMENTS . . . . .	81

## INTRODUCTION

Wood by-products have been nutritionally neglected, but interest has developed in the use of these materials in animal feed. This utilization by ruminants has the potential of sparing conventional foods for man. Additionally, they may be more economical than conventional nutrient sources. Wood by-products could serve as a source of energy in livestock rations. Initially, the by-products of fiber production were drained into rivers and lagoons for disposal. Many are now raised in cattle feed because of their carbohydrate content.

Hemicellulose extract (HCE), a by-product of the production of Masonite board is a water soluble wood extract resulting from the steam pressure processing of wood chips. The wood fibers after initial processing are washed with water and water soluble materials are removed. The water soluble components from the processing are referred to as hemicellulose extract and contain about 5-7% solids. After concentration, the dark viscous liquid contains approximately 65% solids. The viscous liquid is a complex mixture containing wood sugars, hemicellulose inert solids, polyphenol complexes, and some fine fibrous materials. One such commercial product, HCE, has found widespread use in animal feeds, even though its chemical composition is not completely understood.

Phenolic compounds occur commonly in wood, though often in rather small amounts (1). However, there is considerable evidence of biological activity by phenolic compounds. Evidence indicates phenolic compounds have the potential for affecting animal performance. The objective of this study was to determine: 1) Total polyphenol in HCE, and 2) Isolation and identification of some of the polyphenols of HCE.



## LITERATURE REVIEW

### Chemical Composition of Wood

The chemistry of wood has been one of the most difficult tasks to elucidate. Compounds present are very complex and undergo changes according to age, climate and environmental conditions. Browning (2) classified the components that are present in woods as follows:

a. Carbohydrates. The carbohydrates are chiefly polysaccharides. They include cellulose, hemicellulose, starch, pectic substances, and water-soluble polysaccharides such as arabino-galactans. Cellulose and hemicellulose are present in relatively high proportion in wood cell walls.

b. Phenolic substances. Aromatic materials characterized by the presence of phenolic hydroxyl groups (partly methylated) comprise a diverse group of substances. Lignin is the major source of phenolic substances and is a complex, relatively inert polymer having a phenylpropane unit as the basic repeating structure. Other phenolic compounds present in wood include tannins, phlobaphenes, coloring material and lignans.

c. Terpenes. The terpenes and terpenoid compounds include volatile constituents and resin acids. This group amounts to about 5% in softwoods, but is present in very small amounts or practically absent in hardwoods.

d. Aliphatic acids. The higher fatty acids occur in all woods, mostly as esters. Acetic acid esterified with a portion of the polysaccharides is present to the extent of 1 to 5%.

e. Alcohols. These include aliphatic alcohols and sterols.

f. Proteins. Proteins represent a significant portion of the developing tissue, but in raw wood the amount as judged by nitrogen content

is only about 1%.

g. Inorganic constituents. The inorganic constituents amount to less than 0.5% in most woods of the temperate zone, although a few woods and particularly those of the tropics may have ash contents of 1 to 5% or more.

h. Many other organic substances occur in wood of only a few genera or species. These include cyclitols, aldehydes, hydrocarbons and alkaloids.

Isolation and separation of the chemical components according to Browning's classification is not feasible. Wenzl (3) indicated that analytical separation of individual wood components is very difficult, for several reasons. For one, specific agents that permit the selective isolation of only one component are rare. In spite of the elucidation of the constitution of the single cell-wall compositions, the type and combinations of the cell-wall components are not sufficiently known. Thus one reagent will react with several components rather than an individual one. In spite of this, it has been possible to carry out analysis for various groups and to sum the group data to reflect the general composition of wood.

The summative analysis of wood is based on the isolation and identification of certain groups of wood compositions and therefore doesn't deal with the determination of a chemically uniform substance. These groups are chiefly cellulose, hemicellulose, lignin and extractives. The proportions of cellulose, hemicellulose, and lignin present in oven-dry wood is 20% hemicellulose in softwoods and 15-35% in hardwoods; lignin, 25-35% in softwoods and 17-25% in hardwoods. The proportion of pectic substances is small.

#### Wood by-products

Wood wastes are being given more attention as feed for ruminants; however, the feeding of wood products to livestock is not new. Pulped wood

materials were fed to livestock in Germany and the Scandinavian countries during both world wars (4). Recently, in the U. S., the search for inexpensive roughages which can be handled in mechanized feeding systems and the need to alleviate pollution problems while more fully utilizing natural resources have enhanced the interest in feeding wood waste materials.

Sawdust has the desired handling characteristics for mechanized feeding systems and also appears to enhance ruminal function when small amounts are added to concentrate diets. An associated effect on dietary digestibility has been noted (5). That is, the dry matter digestibility of the concentrate was increased by the presence of sawdust. Steers fed ad libitum concentrated diets containing 20, 35 and 50% sawdust showed no differences in average weight gain over a 20-day interval (6). Millett et al. (7) reported an in vitro digestibility study, where southern pine kraft unbleached pulp residue was substituted for timothy hay in diets fed to steers. There were small changes in ruminal fluid pH and relative percentage of volatile fatty acids. The percentage of dietary wood pulp increased from 0 to 65%; but there was no change in pattern either in number or morphology of rumen microbes. Steers fed a diet of 50% wood gained 70% as much as those fed a 72% timothy hay diet.

Of the waste paper products, news print has received the most interest as a feedstuff (8). Newspapers are readily available and are approximately 30% digestible. Average daily gain of steers was not significantly affected by incorporating 8% newsprint in the diet, but gains declined markedly with feeding of 16 and 24% newsprint. Newsprint did not alter the protozoal population in ruminal fluid indicating no adverse effect from the wood. Office paper and paper board also has been included in experimental

diets (9, 10). Both materials proved more beneficial than newsprint in terms of promoting feed intake and growth.

Interest has recently developed in using wood by-products in ruminant feeds. Hemicellulose extract is a by-product of hardboard production and is commercially available to the feed industry. It is the concentrated soluble product obtained from steam treatment of wood at elevated temperature and pressure without acids, alkali, or salts. The liquid HCE has a total carbohydrate content of ca. 55% (10% simple sugars and 37% of a mixture of pentosans and hexosans). Hemicellulose extract is also available in dried form and is prepared by spray drying. No carrier is used. The dry product contains ca. 18% simple sugars and ca. 57% of a mixture of pentosans and hexosans. The liquid and dry products have the following manufacturer's guaranteed analysis:

	Liquid (%)	Dry (%)
Protein	0.5	0.5
Fat	0.5	0.5
Fiber	0.5	1.0
NFE	55.0	84.0
Ash	6.0	4.0
Moisture	35.0	3.0

University research with ruminant animals showed HCE was equivalent to molasses based on feed intake and feed efficiency. Perry (11) reported that HCE was apparently equal to cane molasses at 10% of the total ration for fattening beef cattle and at 7% of the total ration for fattening lambs. Boren et al. (12) self fed a roughage concentrate ration containing 10% of either HCE or cane molasses to beef steers for 138 days. They concluded

that type of molasses had no influence on rate of gain, feed consumption, or carcass quality. Bartley et al. (13) concluded that either liquid or dry hemicellulose extract, when comprising 10% of the grain ration, was equal to cane molasses for lactating dairy cows. They found no statistically significant differences in milk production, milk composition, grain intake, palatability, hay intake or body weight. Williams et al. (14) compared HCE, cane molasses and mixtures of these on urea utilization in lambs. They observed that microbial synthesis of protein was most rapid when cane molasses was the liquid energy source. Good protein synthesis occurred when cane molasses and HCE were in 50:50 ratio. Hartnell & Satter (15) reported HCE tended to increase the amount of soy protein escaping ruminal degradation as compared to cane molasses, using two different in vitro systems. Treating soybean meal with HCE (10% HCE-90% SBM) increased the amount of protein escaping by 10 to 20%.

#### Phenols and Tannins

There is no real difference between phenols and tannins since both are phenolic in character (3). Swain and Bate-Smith (16) define tannins as phenolic compounds having a molecular weight between 500 and 3,000, and which, in addition to the classical properties of phenols, precipitate alkaloids and gelatin and other proteins. Any plant polyphenolic substance with a molecular weight greater than about 500 can be considered to be a tannin (17). Two distinctive groups are the hydrolyzable and the condensed tannins.

Tannic acid is typical of the hydrolyzable tannins (18). It is readily hydrolyzed enzymatically or hydrolyzes spontaneously to glucose and gallic acid with about 7 or less gallic acid units per glucose unit (17).

Other tannins of this group may yield as hydrolysis products ellagic acid, replacing gallic acid, or quinic acid, which replaces glucose (18). The condensed tannins are polymeric flavonoids composed predominantly of leuco-anthocyanidin units linked carbon to carbon from the 4-position of one unit to the 6- or 8-position of the next (19).

Phenolic compounds embrace a wide range of compound bearing hydroxy substituent, including their functional derivatives. The phenolics are usually in groups of substances called extractives that are soluble in natural organic solvents, cold water, hot water or are volatile with steam (2, 3, 19). The principal phenolic constituents are not present in a free state in nature, but are found in the form of esters or, more generally, as glycosides (16). There are two main groups of phenolic compounds: simple phenolics and flavonoids. Simple phenolics are phenols, such as catechol and resorcinol; phenolic acids, such as protocatechuic acid, syringic acid and cinnamic acid (e.g., caffeic acid), and their lactone derivatives, the coumarins (20). the flavonoids comprise the widely occurring water-soluble plant pigments, the anthocyanins and flavones, and a number of related substances (e.g., isoflavones, catechins, tannins, and biflavonyls) (20).

Hergert (21) investigated tannins and polyphenols extracted from conifer wood and bark. Jurd (22) extracted ellagic acid, methyl gallate, gallic acid and a tannin from the pellicle of the walnut. The distribution of phenolic substances of *Eucalyptus gigantea* and *Eucalyptus sieberiana* were described by Hillis and Clarke (23). Mayer (24) studied the structure of some tannins which were isolated from chestnut wood and oak wood. Sixteen phenolics were separated and identified from northern red oak by Seikel et al. (25). Gallic acid and catechin were identified as the major phenols in

clear tissue of both red maple and sugar maple by Terry A. Tattar and A. E. Rich (26).

Ringrose and Morgan (27) and Alumot et al. (28) attributed reduced growth of chicks to appetite-depressing effects of tannin in the diet, whereas Mueller (29) and Vohra et al. (30) suggested additional toxic effects. Tannins reportedly bind proteins into indigestible complexes which contribute to the growth inhibitory properties of mature oak leaves for vertebrates (31) and moth larvae (32). Tannin also inhibits fungal growth and virus transmission (33), thus providing many plants with a broad spectrum defense against both animal and microbial enemies (34). McDonald (35) showed that some proteins are readily degraded by rumen micro-organisms to ammonia which may then be absorbed and excreted as urea. The rate of proteolysis of dietary proteins in the rumen is reported to be decreased by protecting the protein from microbial attack. One protecting treatment has been the use of tannin. A. Driedger and E. E. Hatfield (36) reported rates of ammonia nitrogen production of soybean meal treated with 5, 10, 15, 20, 25% of tannin. The amount of ammonia nitrogen produced was used as a measure of the proteolytic action of the rumen micro-organisms on soybean meal protein; all levels of tannin treatment depressed ammonia nitrogen production. A. Driedger and E. E. Hatfield (36) also indicated that tannin significantly depressed pancreatin digestion of the protein. Similarly, Driedger, Hatfield and Garrigus (37) and Driedger and Hatfield (38) found improved nitrogen retention when lambs were fed soybean meal treated with tannic acid. Therefore, feed efficiencies and nitrogen balances appear to be enhanced due to tannin treatment.

The biological activity of other polyphenols has also been studied.

Jolyn and Glick (39) recorded a higher growth depressing effect for gallic acid than tannic acid in rats at 4 and 6% levels in the diet. Dollahite et al. (40) observed pyrogallol to be more toxic than either tannic acid or gallic acid for rabbits. Rayudu et al. (41) reported that mortality was 100% with pyrocatechol and 95% with pyrogallol at 2% levels in the diet for chicks. Pyrocatechol and pyrogallol were growth depressing for chicks even at a dietary level of 0.1%. Many phenolic acids have been shown to be toxic to germination and growth (42). The physiological mechanism by which each phenolic acid influences plant growth has not been completely resolved. However, Gortner and Kent (43) found the ferulic acid in pineapple tissue, either as the free acid or as an ester, inhibited the activated indoleacetic acid oxidase system. The same investigators found p-coumaric to be a coenzyme for pineapple indoleacetic acid oxidase. Other research by Rabin and Klein (44) showed that caffeic acid also inhibits indoleacetic acid oxidase activity. The inhibitory effect of different phenolic compounds on endopolygalacturonase from *A. niger* was studied by Bhata, Sharma, and Bajaj (45). These authors indicated phenolic acids and ellagitannins have an inhibitory effect on endopolygalacturonase from *A. niger*. They also suggested an increase in activity of the enzyme was observed in the case of caffeic acid, ferulic acid, and resorcinol.

#### Determination of Total Polyphenol in Plant

The quantitative estimation of total polyphenol in natural extracts has been attempted in a number of different ways. Swain and Hillis (46) employed the Folin-Denis reagent for determining total polyphenol in plant tissues. The method is listed as a standard method by the A. O. A. C. (47) for determination of tannins. The method is based on the oxidation of the



phenolic group with Folin-Denis reagent. The resulting color is estimated spectrophotometrically and compared to that of a standard tannin. Lees and Nelson (48) studied hot-water extracts of Eucalyptus siebei. The polyphenols were determined by the Folin-Denis method. They reduced the solid content of a hot-water extract to 4-5% and found that 65% of the solids were tannins. Alexa and Garaculaca (49) employed this colorimetric method for determination of tannins. They treated an extract of tannins obtained from fir bark with 10% lead acetate. The lead salt of the tannins was separated by decantation and dissolved in 1 ml. of 2% sulfuric acid. The tannin content of the filtrate, obtained after filtration, was determined by the Folin-Denis method using pyrogallol as a standard tannin. Smith, Maynard and Lukton (50) also employed the Folin-Denis method for determination of tannins and related polyphenols in fruits and fruit products.

Peri and Pompi (51) used the Folin-Ciocalteu method to determine quantitatively the amount of tannins present in vegetable extracts. The most commonly used method for the quantitative estimation of polyphenols in vegetable extracts is based on their ability to reduce the phosphomolybdic-phosphotungstic reagent of Folin-Ciocalteu with formation of a blue complex, that can be determined colorimetrically. Tattar and Rich (26) employed the Folin-Ciocalteu method for determination of extractable phenols in clear tissue of both red maple and sugar maple. Seider and Datunashvili (52) also determined phenolic substances in wines by the Folin-Ciocalteu method.

The vanillin-hydrochloric acid method developed for forage crops (53) was modified for determination of tannin in grain sorghum (55). This method, based on the work of Bate-Smith and Lerner (55) is based on leuco-anthocyanins and catechins in extracts of plant tissue. Maxson and

Rooney (56) also suggested this method was the best available method for use with sorghum grain.

Owades, Rubin and Brenner (57) employed an ultraviolet spectrophotometric method for determining tannins in food. This method depended on measurement of the absorbance of tannins at 270 mμ. This absorption is based on a structural feature common to tannins, the presence of at least one oxygenated benzene ring. Owades, Rubin and Brenner (58) also employed this method for determination of tannins in beer and brewing. Johnson, Foreman and Mayer (59) used ultraviolet spectrophotometry to determine polyphenolic substances in various fruit. They determined the UV absorption of phenolic substances extracted from various fruits. Two maxima were obtained, one at 280 mμ and the other at approximately 322 to 324 mμ. Sogawa Hairoschi also used the UV method for determination of polyphenols in beer (60).

Other methods (61, 62, 63) have been used for determination of tannins in natural extracts.

#### Isolation of Polyphenols from Plant Material

A literature survey indicates that there are three general methods for the isolation of polyphenols: 1) extraction with ethyl acetate, 2) absorption on polyvinyl pyrrolidone (PVP), and 3) precipitation with lead acetate.

Vuataz, Brandenberger and Egli (64) isolated polyphenols of the tea leaf with ethyl acetate. And Miskov, Obrad et al. (65) isolated polyphenols in wines and related products with ethyl acetate. Ediz (66) evaluated diethyl ether, chloroform and ethyl acetate for isolation of polyphenols from RWE (HCE). He found solvents such as diethyl ether and chloroform did not

appear to extract as much material from RWE as ethyl acetate.

The presence of phenolic compounds is a hindrance during the isolation of plant enzymes since the two materials complex through hydrogen bonding. To overcome this problem, an insoluble polymer, polyvinyl pyrrolidone (PVP), which is very similar in structure to protein has been used to complex the phenolic compounds (67). Anderson et al. (68) dissociated a PVP-caffeic acid complex with 8 M urea. This indicated that some phenolic compounds are reversibly bound to PVP. Chintam (69) also employed PVP to isolate polyphenols from HCE.

Lead acetate has long been known to precipitate polyphenols (70). Lead acetate precipitates not only polyphenols, but proteins and carbohydrates as well (71). Ediz (66) used lead acetate to isolate polyphenols in HCE and tested the lead acetate precipitate for the presence of carbohydrates. He indicated that lead acetate precipitation did not appear to precipitate carbohydrates in his system. Isolation was made possible by precipitating polyphenolic compounds with saturated lead acetate solution. By adding hydrogen sulfide to an aqueous suspension of lead precipitate, the complexing compounds were released. This technique has been used widely (72, 73, 74).

#### Identification of Polyphenols

Paper partition chromatography was first employed for the separation of phenolic pigments by Bate-Smith in 1948 (75) and has been widely applied to phenolics by others (21, 24, 76, 77). Hergert (21) studied monomeric and polymeric polyphenols in wood, bark and needles from coniferous trees. When quantities of some compounds were small, the original methanol extract was further extracted with organic solvents. Two-dimensional paper chromatography was used with butanol-acetic acid-water (4:1:5) in one direction and

2% acetic acid in the second direction. General identification was accomplished with chromogenic reagents. A number of spray reagents for the detection of phenolic compounds are known (78). Diazotized sulfanilic acid has been used for general purposes (79). Ferric chloride can be used to distinguish between pyrocatechol and pyrogallol derivatives. A mixture of ferric chloride and potassium ferricyanide solution was reported a useful reagent for detecting all phenolic constituents (80). A mixture of 5% sodium nitrite and 10% sodium tungstate solution containing 5% trichloroacetic acid was a sensitive chromogenic reagent for naturally occurring phenolic compounds (81).

Of the newer techniques, thin-layer chromatography is undoubtedly the most versatile and has proved especially valuable for separating the classes of phenols (e.g., phenolic acid and hydroxyquinones) that do not lend themselves readily to separation on paper. Three absorbents have been of value in the separation of plant phenolic derivatives: cellulose, silica gel and polyamide.

Tattar and Rich (26) identified polyphenols of extracts from sugar maple and red maple. Extracts were chromatographed in two dimensions on cellulose thin-layer chromatography plates with butanol-acetic acid-water (6:1:2) and 7% acetic acid-0.03% sodium acetate.

Silica gel thin-layer chromatography was used for identification of polyphenols by Torrent *et al.* (82). Best separation was with  $\text{CHCl}_3$ -EtoAC- $\text{HCO}_2\text{H}$  (5:4:1). Various solvents have been described for identification of polyphenols on silica gel thin-layer chromatography (78). Polyamide thin-layer chromatography plates have proved useful in the separation of phenolic constituents of extractives of some conifers (83, 84, 85).

Gas chromatography of phenols has not been widely explored, but it provides the only chromatographic technique which will resolve mixtures of simple isomeric phenols. Its use with less volatile phenolic compounds is being actively studied (86). The low volatility of some phenols can be overcome by treating the support with trimethyl silyl ethers before hand. Furuya (87) used the latter procedure with plant glycosides, such as arbutin and aesculin, which he then placed on a column of 0.75% SE-30 on chromosorb W. Alternatively, a preliminary methylation will aid volatility, and has been used with mono- and dichlorophenols; similarly phthalic and hydroxybenzoic acids have been separated as their dimethyl esters.

The separation and identification of phenolic acid has been investigated by high pressure liquid chromatography. The best results have been obtained by W. Hovermann (88) et al. using partition chromatography with Merckogel Si 150-columns. Ternary two-phase compositions served as the mobile phase. Elution is accelerated by acidifying the mobile phase. The cis-trans isomers of p-coumaric acid and ferulic acid have been separated with the system described.

Gel filtration using Sephadex-type polymers has been useful in fractionating phenolic constituents from different sources (89, 90). Eluted components were located by monitoring the effluent with UV detector or by using chromogenic reagents and were identified by paper chromatography.

## MATERIALS AND METHODS

Commercial, aqueous hemicellulose extract of wood (HCE) was supplied by the Masonite Corporation (Chicago, Illinois). HCE is a by-product of the Masonite process of producing hardboard and was obtained in a concentrated form from the Masonite production unit located at Ukiah, California.

### Total Polyphenol Content in Hemicellulose Extract

Several methods of tannin analysis have been reported in the literature. The following methods were investigated for use with HCE because they did not require expensive equipment and suggested tests which could be accomplished in most laboratories.

#### (A) Folin-Denis Method

Fifty grams of a 65% viscous liquid HCE was introduced into a 500 ml erlenmeyer flask. Approximately 350 ml water was added and the solution was refluxed for 5 hours. The solution was then cooled, diluted to 500 ml, mixed, and let stand. A 10 ml proportion of the supernatant was diluted to 10,000 ml with  $H_2O$  to give a 1/10,000 dilution of the original sample. One milli-liter of this solution was then combined with 8 ml of  $H_2O$  in a 10 ml volumetric flask. Folin-Denis reagent, 0.5 ml (47) and one ml of saturated, aqueous sodium carbonate solution were added and diluted to 10 ml with  $H_2O$ . The solution was thoroughly mixed and allowed to stand at room temperature for 30 min. The absorbance was measured at 725 nm. in a Beckman DB-G. A standard curve (absorbance vs. ug. tannic acid) was prepared using tannic acid. The amount of polyphenol, as tannic acid, was determined directly from this standard curve.

### (B) Folin-Ciocalteu Method

One gram of a 65% viscous liquid HCE was diluted to a volume of 1000 ml with  $H_2O$ . A 0.5 ml proportion of the 1/1000 dilution was pipetted into a test tube to which 0.1 ml of  $CHCl_3$  was added. The solution was mixed and covered with paper towels to prevent dust contamination. The test tube was then placed in a water bath and warmed to 37-39°C and incubated at 34-37°C for 18-24 hours. After incubation 4.5 ml of diluted Folin-Ciocalteu reagent (51) was added, the solution was mixed, and allowed to stand for 3 min. The solution was then filtered and 5 ml of the filtrate was transferred to a clean test tube to which 1 ml of  $Na_2CO_3$  solution was added. The test tube was placed in a boiling water bath for 5 min. and then the contents filtered. The filtrate was allowed to cool and color was read on a Beckman DB-G at 650  $\mu m$ . A standard curve (absorbance vs. mg. tannic acid) was prepared using tannic acid. The amount of polyphenol, as tannic acid, was determined directly from this standard curve.

### (C) Vanillin-HCL Method

Five grams of 65% viscous liquid HCE was diluted to 50 ml with methanol in a flask. The flask was stoppered and swirled occasionally for 24 hours. After the 24 hours swirling period solids in the sample were permitted to settle. One ml of the supernatant was pipetted into a test tube to which 5 ml of Vanillin-HCL solution (53) was added. Color was allowed to develop for 20 min. and was then read at 500  $\mu m$ . on a Beckman DB-G spectrophotometer. A standard curve (absorbance vs. catechin) was prepared using catechin. The amount of polyphenol, as catechin, was determined directly from this standard curve.

#### (D) Ultraviolet Method

Fifty grams of a viscous liquid HCE was diluted approximately to 400 ml with water in a 500 ml erlenmeyer flask and refluxed for 5 hours. The solution was allowed to cool and was further diluted to 500 ml. The solution was then permitted to stand. A 10 ml aliquot of the supernatant was diluted to 5,000 ml with H<sub>2</sub>O to give a 1/50,000 dilution of the original sample. A 5 ml aliquot of the diluted solution was then measured at 270 mμ on a Beckman DB-G. A standard curve of absorbance vs. concentration, was constructed, using solutions containing known weights of tannic acid.

#### Extraction of Polyphenols from Hemicellulose Extract

##### I. Extraction of Polyphenols in HCE

After a number of experiments to determine the best procedure for achieving a relatively pure extraction the following procedure was developed: (a scheme diagram of this method is shown in Fig. 1.)

Two hundred grams of HCE (65% solid) and 40 ml of H<sub>2</sub>O were combined to bring final solids content to 40-50% and heated to 65°C. Four hundred ml of ethyl acetate was placed in a blender and agitation started followed by slow addition of the heated HCE. Agitation continued for one min. and was then stopped. The separation of the bulk of the ethyl acetate fraction took place almost immediately.

The dark colored ethyl acetate fraction was decanted and the remainder of the material was centrifuged. A three part separation (ethyl acetate fraction, emulsion fraction, and sugar fraction) was obtained. The ethyl acetate fraction was concentrated using reduced pressure and was then dissolved in 95% ethanol. The polyphenol material was identified as ethyl acetate extract (EAE).



## 2. Dialysis of polyphenols (ethyl acetate extracts).

Twenty grams of the polyphenol complex (82% solids) were diluted to 100 ml with H<sub>2</sub>O, heated to 60°C and adjusted to pH 8 with 10% NaOH. The solution was then decanted. Eighty ml of a soluble fraction (A) and an insoluble fraction (B) were obtained. Both fractions were dialyzed. The dialysis procedure was as follows:

Soluble fraction (A): Eight ml of the soluble fraction was added to dialysis tubing and immersed in the diffusion medium (700 ml of H<sub>2</sub>O was adjusted to pH 10 with 10% NaOH) using a magnetic stirrer to agitate the latter for one hour and 35 minutes. There was an increase in volume of liquid in the dialysis tube and some precipitate formed. The contents of the tube were emptied, filtered, and the insoluble portion added to fraction (B). The diffusate identified as 1 (material which passed through the membrane) and the dialysate identified as 1 (material which does not pass through the membrane) were obtained.

Diffusate 1: The color of the solution was very dark. The solution was adjusted to pH 5.4 with 10% HCL and the color of the solution changed to dark red. The solution was then extracted with ethyl acetate three times. Three hundred and fifty ml of ethyl acetate was added to the diffusate and was blended for one minute. This procedure was repeated twice more and the ethyl acetate extracts were combined, vacuum concentrated and dissolved in 95% ethanol to give sample D-1A. The extracted H<sub>2</sub>O fraction was light yellow in color. It was vacuum concentrated and dissolved in 100% ethanol to give sample D-1B.

Dialysate 1: Ninety ml of the dialysate (the dialysate increased in volume from 80 to 90 ml) was divided into two portions (a) 50 ml and (b) 40

ml. Each was dialyzed for one hour and 35 minutes in fresh diffusion solution.

Diffusate 2: Both diffusates from (a) the 50 ml and (b) the 40 ml of dialysate 1 which were adjusted to pH 5.4 with 10% HCL were separately extracted three times with ethyl acetate (350 ml of ethyl acetate was added for each extraction). The extracts were combined, vacuum concentrated and dissolved in 95% ethanol to give sample D-2A. The H<sub>2</sub>O fractions were combined, vacuum concentrated and dissolved in 95% ethanol to give sample D-2B.

Dialysate 2: Each of the solutions obtained from (a) the 50 ml and (b) the 40 ml of dialysate 1 through dialysis contained some insoluble material which was removed by filtration and the residue was returned to fraction (B). The combined filtrates (120 ml) were adjusted to pH 8 with 10% NaOH. The solution was divided into two equal parts and each was dialyzed in 700 ml of fresh diffusion medium for 2 hours and 45 minutes.

Diffusate 3: Both diffusates were adjusted to pH 5.4 with 10% HCL prior to extraction of each portion with 350 ml of ethyl acetate. The ethyl acetate fractions were combined, vacuum concentrated and dissolved in 95% ethanol to give sample D-3A. The H<sub>2</sub>O fractions were combined, vacuum concentrated and dissolved in 95% ethanol to give sample D-3B.

Dialysate 3: There was no appreciable insoluble material in either sample. The combined samples (140 ml) had a pH of 8. The material was divided into equal parts and each was dialyzed in 700 ml fresh diffusion medium for two hours and 45 minutes.

Diffusate 4: Both diffusates had a pH of 8 which was adjusted to pH 5.4 prior to extraction with ethyl acetate. The ethyl acetate fractions were combined, vacuum concentrated and dissolved in 95% ethanol to give

sample D-4A. The  $H_2O$  fractions were combined, vacuum concentrated and dissolved in 95% ethanol to give sample D-4B.

Dialysate 4: There was no apparent insoluble material formed, the combined volume increased to 150 ml. The pH of the solution remained at 8 and the solution was divided into two equal parts and dialysis of each sample was continued for fifteen hours and forty five minutes.

Diffusate 5: Diffusate 5 was much lighter in color than diffusate 4. The pH of the solution was adjusted from 8 to 5.4 and the solution was extracted with ethyl acetate. The ethyl acetate fractions were combined, vacuum concentrated and dissolved in 95% ethanol to give sample D-5A. The  $H_2O$  fraction was treated in a like manner to give sample D-5B.

Dialysate 5: The volume of the dialysate 5 increased from 150 ml to 162 ml. The pH of the solution was adjusted from 7.5 to 8.0 and the solution was divided into two equal parts and each was dialyzed for seventeen hours.

Diffusate 6: The color of the solution was greatly reduced and the pH was adjusted from 7.5 to 5.4 prior to extraction with ethyl acetate. The ethyl acetate fractions were combined, vacuum concentrated and dissolved in 100% ethanol to give sample D-6A. The  $H_2O$  fraction was treated in like manner to give sample D-6B.

Dialysate 6: The volume of the dialysate 6 increased from 162 to 200 ml and the pH of the solution was adjusted to 5.4. The solution was then extracted with ethyl acetate. Both the ethyl acetate and  $H_2O$  fractions were vacuum concentrated and dissolved in 95% ethanol. The sample dialysate 6A (ethyl acetate fraction) and dialysate 6B ( $H_2O$  fraction) were obtained.

Fraction (B): The insoluble portion of the original sample at pH 8

was suspended in  $H_2O$  and diluted to 100 ml. The sample was heated to  $60^{\circ}C$  and the pH of the sample was adjusted to 10. The soluble portion was decanted off for dialysis and the insoluble portion was washed with  $H_2O$  and dissolved in 95% ethanol and made to 60 ml to give sample B-1. One hundred ml of the soluble portion was treated with 10% HCL to reduce it to pH and was dialyzed in diffusion medium (700 ml of  $H_2O$  was adjusted to pH 10 with 10% NaOH) for seven hours and forty five minutes. Both a diffusate DB-1 and a dialysate DB-1 were obtained.

Diffusate DB-1: The very dark colored solution had a pH of 8.9 which was adjusted to pH 5.4 with 10% HCL prior to extraction with ethyl acetate. Both the ethyl acetate fraction and the  $H_2O$  fraction were vacuum concentrated and dissolved in 95% ethanol. Sample DB-1A (ethyl acetate fraction) and DB-1B ( $H_2O$  fraction) were obtained.

Dialysate DB-1: The pH of the solution was 9.0 and the volume of the solution had increased from 100 to 130 ml. The sample was divided into two equal parts and each was dialyzed for thirteen hours and thirty minutes.

Diffusate DB-2: The sample had a pH of 8.2 which was adjusted to pH 5.4 prior to extraction with ethyl acetate. Both the ethyl acetate fraction and the  $H_2O$  fraction were vacuum concentrated and dissolved in 95% ethanol. Samples DB-2A (ethyl acetate fraction) and DB-2B ( $H_2O$  fraction) were obtained.

Dialysate DB-2: The volume of the sample increased from 130 to 172 ml and the pH of the sample was adjusted from 8.2 to 8.9 prior to dialyzing for twelve hours and thirty minutes.

Diffusate DB-3: The color of the solution was greatly reduced and the pH of the solution was adjusted from 9 to 5.4 prior to extraction with

350 ml of ethyl acetate. Both the ethyl acetate and the H<sub>2</sub>O fractions were vacuum concentrated and dissolved in 95% ethanol. Samples DB-3A (ethyl acetate fraction) and DB-3B (H<sub>2</sub>O fraction) were obtained.

Dialysate DB-3: The volume of the solution had increased from 176 to 214 ml. The solution was filtered. The pH of the filtrate was adjusted to 5.4 with 10% HCL before extraction with ethyl acetate. Both the ethyl acetate and the H<sub>2</sub>O fractions were vacuum concentrated and dissolved in 95% ethanol. Samples DB-3A (ethyl acetate fraction) and DB-3B (H<sub>2</sub>O fraction) were obtained.

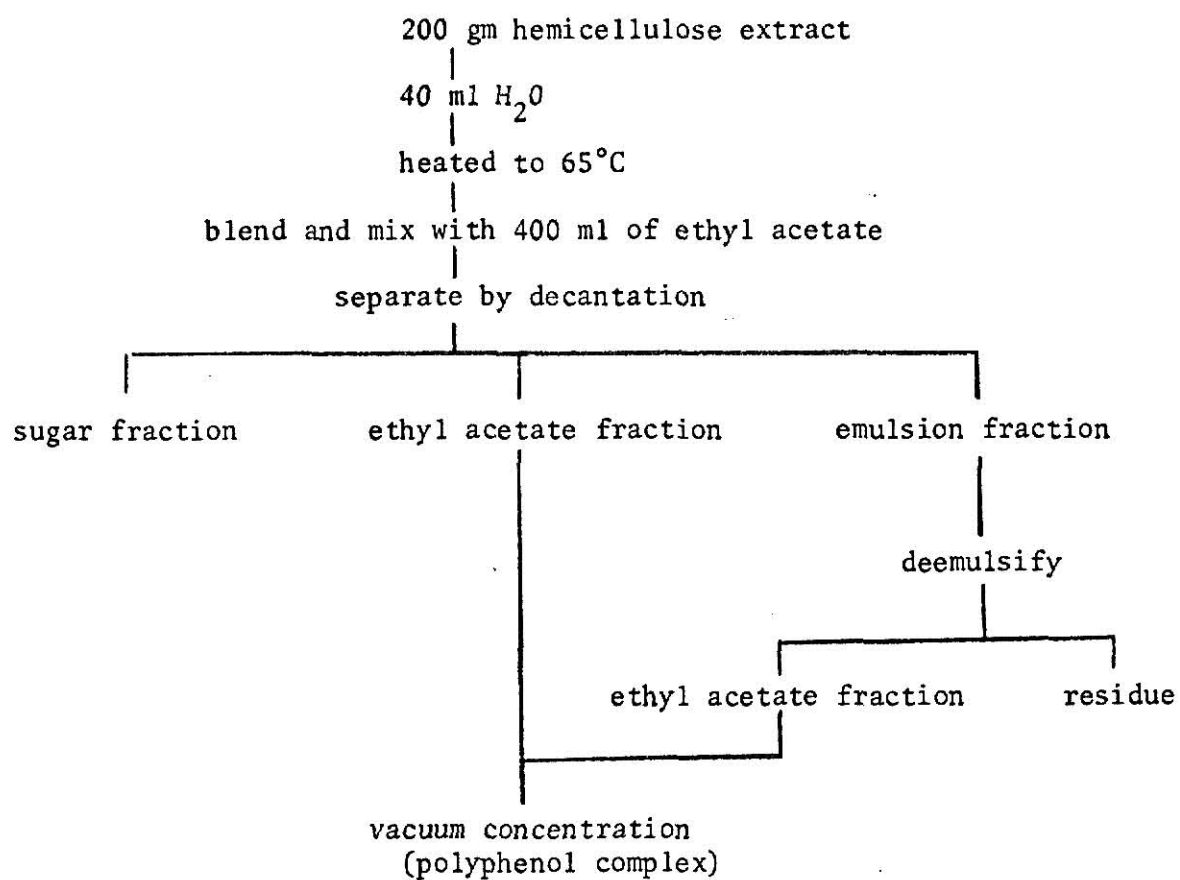
The twenty-four samples obtained in this manner were studied by thin-layer chromatography.

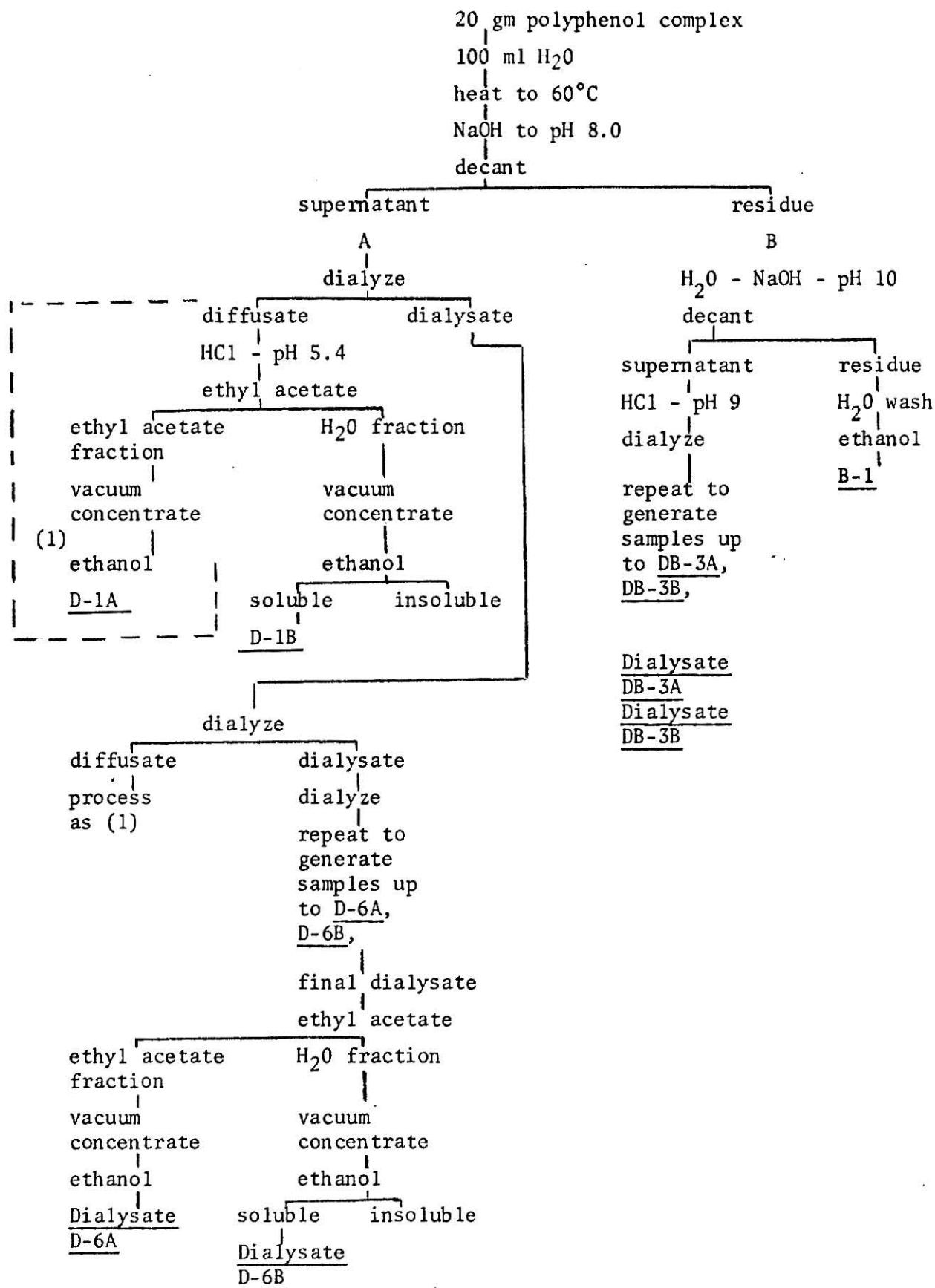
#### Testing for the Presence of Polyphenols

The ferric chloride test is probably the most widely used for phenol identification. This test was conducted by adding 0.5 ml of freshly prepared 5% ferric chloride solution and 2 ml of water to the polyphenol complexes (EAE). Samples obtained from the dialysis of EAE (D-1A, DB-1A, and D-1B) and tannic acid were studied in this manner. Initially all solutions became dark green. After standing at room temperature for 2 hours, the polyphenol complex solution (EAE) formed a dark brown precipitate leaving the supernatant slightly brown. The color of a tannic acid solution was unchanged. Three other solutions (D-1A, DB-1A, D-1B) became dark brown.

In another test, 0.5 ml of freshly prepared mixture of 5% sodium nitrite and 5% sodium tungstate containing 5% trichloroacetic acid (81) were added to EAE, D-1A, DB-1A, D-1B and tannic acid. The tannic acid solution became light brown and the other four solutions became greenish brown.

Fig. 1. Scheme for isolation of polyphenols in hemicellulose  
extract







The maximum UV absorption of EAE, D-1A, DB-1A, B-1 was also investigated.

#### Column Chromatographic Separation of Ethyl Acetate Extracts

##### Ascending Technique:

A column (2.5 cm x 46 cm) with glass beads and glass wool placed at one end was packed with a slurry of activated silica gel G in a solvent mixture of chloroform-ethyl acetate-formic acid (5:4:1). Above it, a filter paper disc, a sample of ethyl acetate extract of HCE in dry silica gel, another filter paper disc, glass wool and glass beads were placed in that order.

The column was inverted and attached to a solvent reservoir. The solvent supply (in ascending position) was stopped after 48 hours. The excess solvent was removed from the column and the core was removed and air dried. After visual examination the dried column was cut into 13 segments.

The samples of the core were slurried with 40 ml 95% ethanol, vacuum filtered (Watman No. 50 paper) and the residue washed with 10 ml ethanol (sample code: 8-1 through 8-13). The filtrates were concentrated to smaller volume and examined by TLC. The solids content was determined by placing 2 ml of each sample in a tared petri dish which was then oven dried at 110°C for 30 minutes. The dried sample was redissolved in ethanol and returned to the original sample.

#### Paper Chromatographic Studies of Ethyl Acetate Extracts of Hemicellulose Extract and Fractions Separated by Dialysis (D-1A, D-2A)

Aliquots of ethyl acetate extract, D-1A and D-2A were spotted on Whatman #1 chromatography paper (46 x 12 cm) and were chromatographed in a

descending system. Butanol-acetic acid-water (4:1:5), benzene-propionic acid-water (2:2:1) and acetic acid-ethyl acetate-water (3:6:2) were used as developing solvents.

The chromatograms were removed when the solvent front was approximately 10 cm from the bottom of the paper and were air dried. The spray reagent was a mixture containing 6 ml of a 10% solution of sodium tungstate, 6 ml of a 5% solution of trichloroacetic acid and 3 ml of 0.5 N hydrochloric acid with 6 ml of freshly prepared 5% sodium nitrite solution (81). Two dimensional chromatography with butanol-acetic acid-water (4:1:5) in the first direction and 7% acetic acid in the second direction was also used.

Thin-Layer Chromatographic Studies of Ethyl Acetate Extract and Fractions by Dialysis (D-1A and D-2A) and Column Separation (8-3, 8-7, and 8-9)

To 30 gm of silica gel G, 60 ml of water was added and mixed. The resulting slurry was used to prepare five 20 x 20 cm plates with 250 u thickness. Plates made by using 0.3 M sodium acetate solution, instead of water, were also used for further investigation. Plates were air dried, activated at 110°C for one hour and placed in a desiccator containing calcium chloride.

The developing chamber was lined with Whatman #1 filter paper and was saturated with solvent for 12 hours prior to use. The solvent was freshly prepared for each run. To achieve optimum separations, a spectrum of solvents and solvent mixtures of different polarities were used: benzene-chloroform (1:1), benzene-acetone (9:1), toluene-ethyl formate-formic acid (5:4:1), Toluene-chloroform-acetone (40:25:35), toluene-chloroform-acetone (30:15:55), ethyl acetate-butanone-formic acid-water (5:3:1:1), chloroform-ethyl acetate-formic acid (5:4:1), benzene-methanol-acetic acid (48:8:4), and

benzene-dioxane-acetic acid (90:25:4). The plates were spotted 1.5 cm from the bottom using a capillary tube and the solvent front was allowed to migrate 12 cm above the point of sample application. Developed plates were air-dried, sprayed with spray reagent (a mixture containing 6 ml of a 10% solution of sodium tungstate, 6 ml of a 5% solution of trichloroacetic acid, 3 ml of 0.5 N hydrochloric acid and 6 ml of freshly prepared 5% sodium nitrite solution (81)).

Identification was made by comparing migration values of unknown samples with those of known compounds. Twenty four commercial compounds were used as standard reference compounds.

High Pressure Liquid Chromatographic Studies of Ethyl Acetate  
Extract and Fractions by Dialysis (D-1A) and Column Separation  
(8-3, 8-7, and 8-9)

Suitable volumes of unknown solutions and of standard polyphenolic solutions were injected into a Water Associates ALC 202 high pressure liquid chromatography equipped with a UV photometric detection measured at 250 nm. The chromatography was equipped with a commercial column (1/8 o.d. x 100 cm) packed with porasil A. The elutes were dichloromethane-ethanol-water (400:5:1) and dichloromethane-ethanol-water (400:35:8). The column flow rate was 2.0 cm/min. and chart rate was 0.5 cm/min. The peaks were identified by retention time of unknown samples compared to retention time of known standard polyphenols and cochromatography with known standard polyphenols.

## RESULTS AND DISCUSSION

### Total Polyphenol Content in HCE

Several methods have been used to estimate total polyphenol content of natural plant materials. None appears well established and reliable. It may not be possible to develop a practical method for analyzing large numbers of polyphenol samples for absolute polyphenol content, but tests based on relative values should be standardized. Further research is needed to develop a generally acceptable, reproducible method for analyzing polyphenols in natural plants.

Four methods were investigated for determination of polyphenols in HCE. Each of the four methods appeared to give markedly different results. The difference in the relative values for HCE by these methods may be due to the measurement of different phenolic compounds. The results of the methods are discussed below.

**Folin-Denis Method:** The Folin-Denis reagent is a mixture of compounds which produce a blue color with reducing substances. As such it is non-specific for any group of polyphenols. A standard curve was prepared with tannic acid (1 ug to 20 ug, Fig. 2). Based on tannic acid, 1.89% of phenolic compounds was found in HCE (Table I). This method is easy and reproducible. However, it would be questionable because a positive phenol reaction is produced by ascorbic acid (91) and several other non-polyphenol compounds (92). Heat favors the breakdown of protein into phenols or compounds that give a positive reaction with this method (93).

**Folin-Ciocalteu Method:** This method is based on the ability of reducing the phosphomolybdic-phosphotungstic reagent of Folin-Ciocalteu,

Fig. 2. Standard curve using tannic acid for the determination of phenolic compounds by the Folin-Denis method.

**THIS BOOK  
CONTAINS  
NUMEROUS PAGES  
WITH DIAGRAMS  
THAT ARE CROOKED  
COMPARED TO THE  
REST OF THE  
INFORMATION ON  
THE PAGE.**

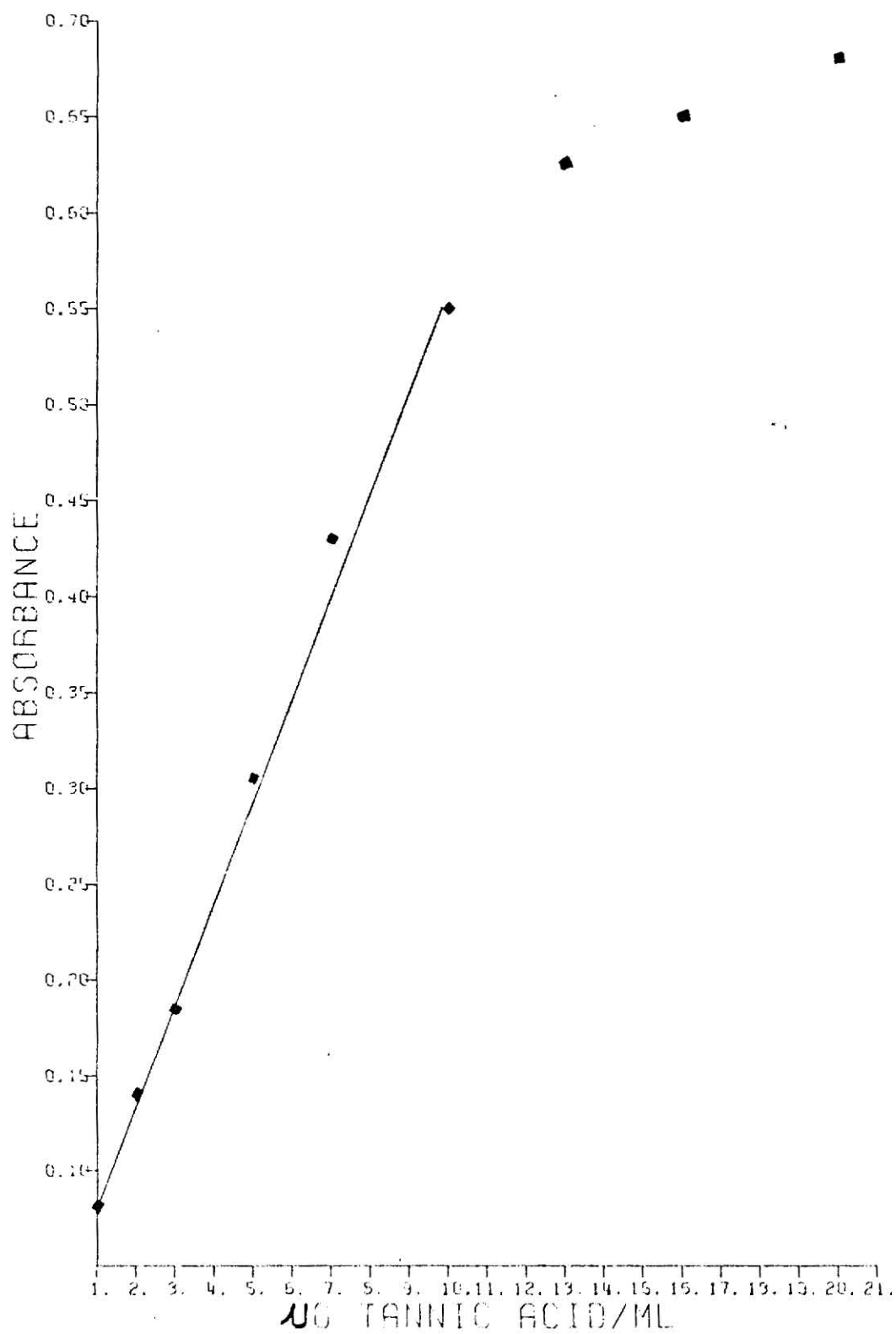
**THIS IS AS  
RECEIVED FROM  
CUSTOMER.**

# **ILLEGIBLE DOCUMENT**

**THE FOLLOWING  
DOCUMENT(S) IS OF  
POOR LEGIBILITY IN  
THE ORIGINAL**

**THIS IS THE BEST  
COPY AVAILABLE**

## FOLIN-DENIS METHOD





with formation of a blue complex. The Folin-Ciocalteu procedure gives a measure of the total phenolic hydroxyl groups. A standard curve was prepared with tannic acid (10 ug to 150 ug, Fig. 3). Based on tannic acid, 17.675% of phenolic compounds was found in HCE (Table I). This method was reproducible, but the procedure for it is complex and laborious. The high values obtained by this method are questionable and probably relative rather than actual levels.

**Vanillin-HCL Method:** This method is based on the production of a red color when hydrochloric acid and vanillin are reacted with catechin and/or leucoanthocyanins. A standard curve was prepared with catechin (2 mg to 60 mg, Fig. 4). Based on catechin, 1.22% of phenolic compounds was found in HCE (Table I). The reproducibility of this method is less than the first two. In this investigation, the red extract of HCE may cause increased absorbance because the vanillin-HCL reagent produces a red color in the presence of polyphenols. However, the simplicity, speed, and specificity make it a valuable means of estimating polyphenol content of natural plants.

**Ultraviolet Method:** This method is based on the absorbance of ultraviolet light (210 to 300 nm.) by phenolic compounds present in natural plant materials. The choice of 270 nm was indicated by a maxima obtained from polyphenols extracted from HCE (Fig. 5). Many workers (57, 58, 59) have reported similar maxima for several tannins isolated from natural plants. A standard curve was prepared with tannic acid (2 ug to 15 ug, Fig. 6). Based on tannic acid, 11.402% of phenolic compounds was found in HCE (Table I). The high value obtained by this may be due to the fact that non-polyphenol material may be extracted which absorb in the ultraviolet region and which would be soluble in water. The ultraviolet method is reproducible and rapid.

Fig. 3. Standard curve using tannic acid for the determination of phenolic compounds by the Folin-Ciocalteu method.

## FOLIN-CIOCALTEU METHOD

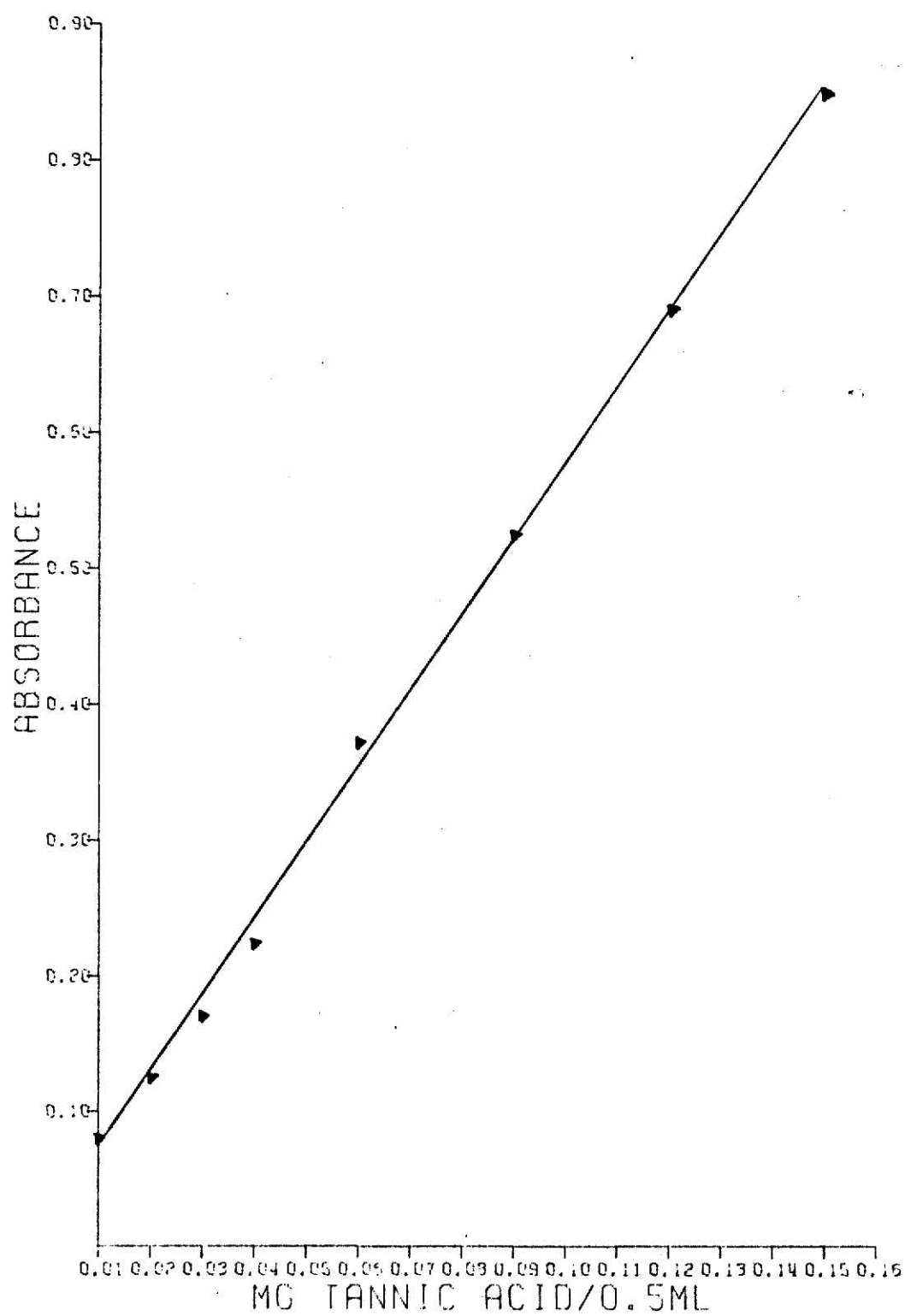


Fig. 4. Standard curve using catechin for the determination of phenolic compounds by the Vanillin-HCL method.

## VANILLIN-HCL METHOD

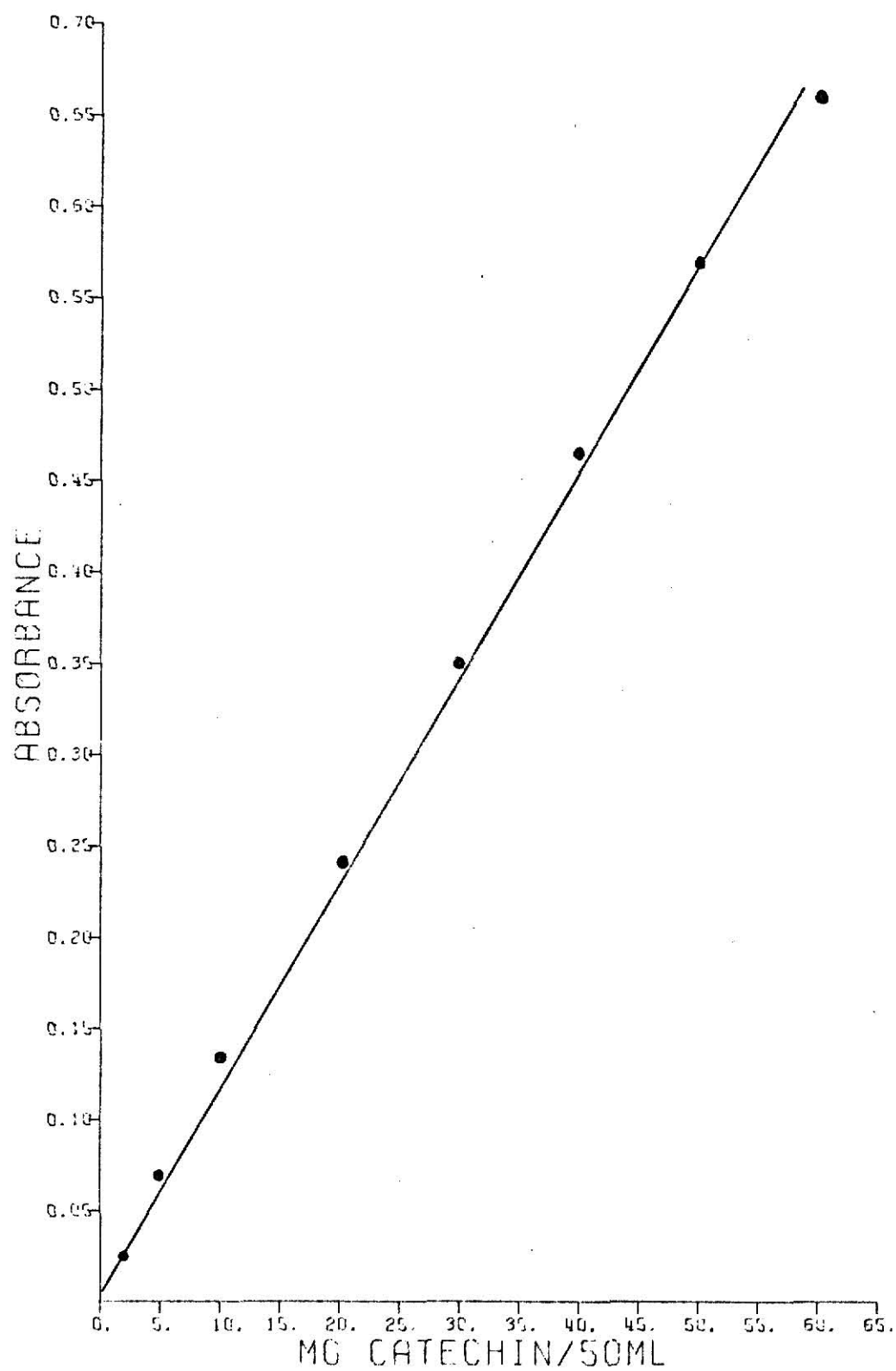


Fig. 5. Absorption curve of polyphenols in Hemicellulose Extract.

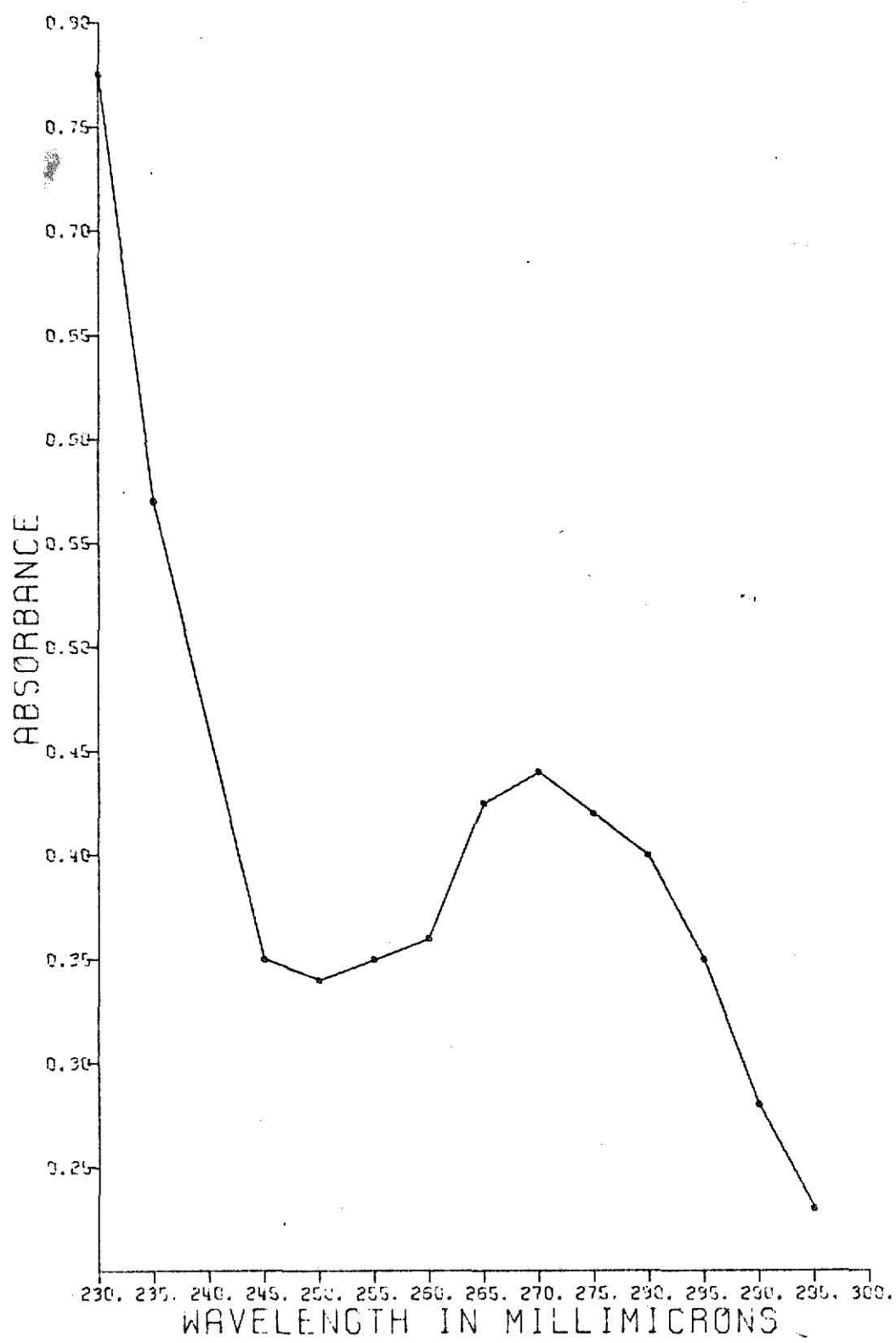


Fig. 6. Standard curve using tannic acid for the determination of phenolic compounds by the ultraviolet method.



## UV METHOD

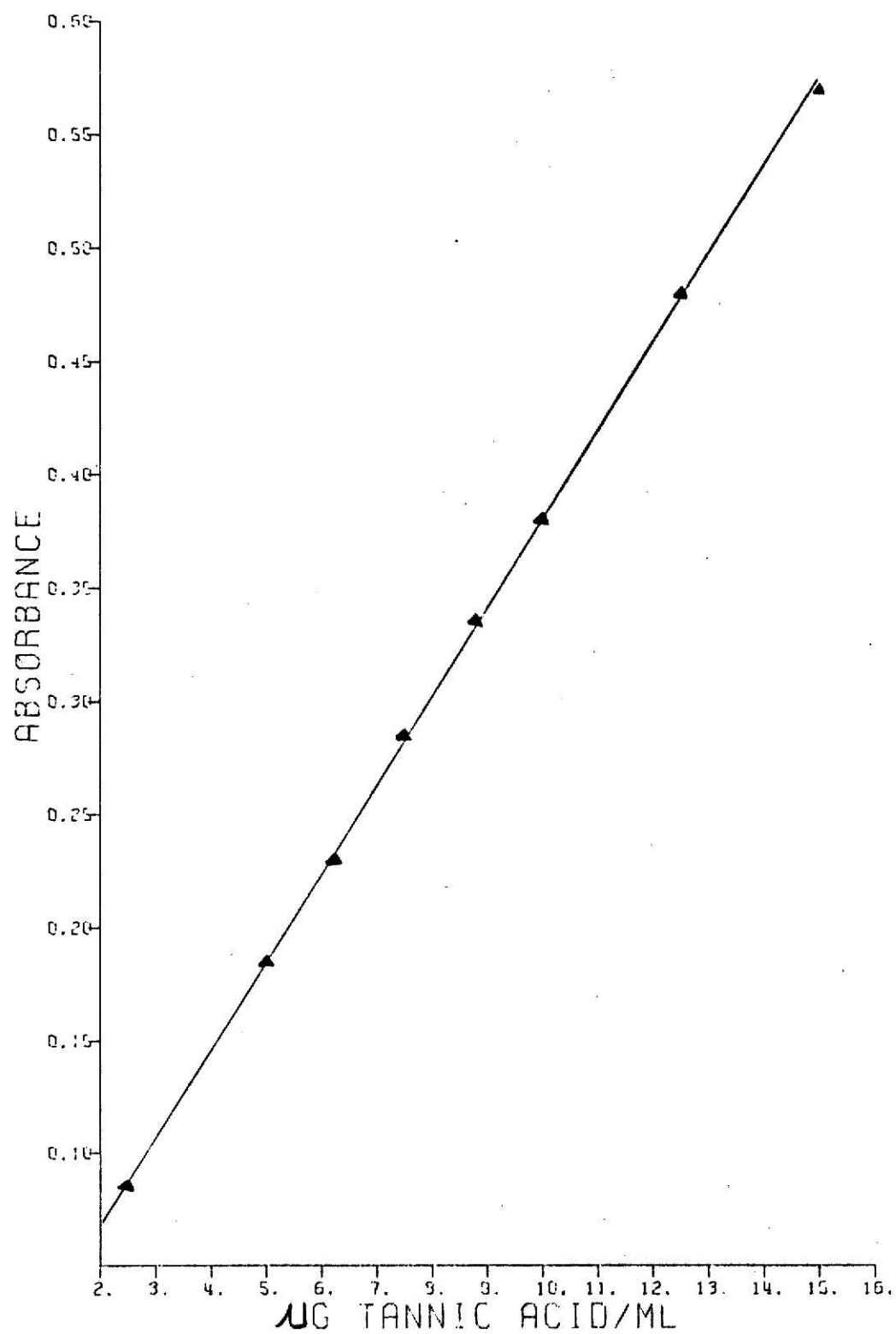


Table I

Tannin Content of HCE by Various Methods and Reproducibility

	Tannin, mg/g (Dry Basis)				A. V. Tannin mg/g	% Reproducibility
	1	2	3	4		
Folin-Denis	19.02	18.67	18.56	19.60	18.96	95
Folin-Ciocalteu	183	176	178	170	176.75	93
Vanillin-HCL	11.63	13.15	12.31	11.69	12.20	89
Ultraviolet	114.77	113.24	113.58	114.50	114.02	98

### Isolation of Polyphenols from HCE

There are several potential ways of removing the polyphenol complex from HCE. Extraction with ethyl acetate, however, offers promise for a continuous operation. The limited solubility of ethyl acetate in water lends itself to phase separation after processing and the low boiling point allows it to be removed from the water phase by distillation. For the most efficient solubilization of the polyphenol complex in ethyl acetate, effective mixing for intimate contact of HCE and ethyl acetate must be achieved. A 93.65% recovery of three fractions (ethyl acetate fraction, emulsion fraction, and sugar fraction) shown in Table II.

Dialysis of Polyphenol Complex (EAE): The polyphenol complex included some compounds other than polyphenols. Dialysis was used to attempt to separate and purify this complex. The solution containing the polyphenol complex was adjusted to pH 8 with NaOH and decanted. An insoluble fraction was adjusted to pH 10 with NaOH and again, decanted. With this treatment, two samples (DB-1A, B-1) separated under alkaline conditions were obtained. These two samples contained the bulk of polyphenols as evidenced by the numbers of compounds (Table III). The supernatant at pH 8 was dialyzed then the diffusate solution was adjusted to pH 5.4 prior to treatment with ethyl acetate. This was necessary to insure complete extraction of the undissociated tannin molecules. The first three diffusates D-1A, D-2A, and D-3A of the supernatant at pH 8 contains the bulk of the polyphenols as evidenced by the numbers of compounds shown by thin-layer chromatography (Table III). No polyphenols on TLC of the final dialysates (Dialysate 6A, Dialysate 6B, Dialysate DB-3A, Dialysate DB-3B) indicated this dialysis process was satisfactory. Samples obtained from water fractions showed

Table II  
Recovery Studies of Three Fractions Obtained  
from Hemicellulose Extract

	gm (Dry Basis)	%
Sugar Fraction	95.4	73.4
Emulsion Fraction	16.9	13.0
Polyphenol Complex	9.5	7.3
Total	121.8	93.7

\*130 gm of hemicellulose extract was used in this study.

Table III

Rf Values from Thin-Layer Chromatography of Ethyl Acetate Extract and Fractions from Dialysis of Ethyl Acetate Extract of Hemicellulose Extract.

Spot No.	Dialysate		Dialysate		Dialysate		Dialysate		Dialysate		B-1
	6A	6B	DB-1A	DB-1B	DB-2A	DB-2B	DB-3A	DB-3B	DB-3A	DB-3B	
1.			0.06	0.06							0.06
2.			0.10	0.10	0.10						0.10
3.			0.16	0.16	0.16	0.16	0.16				0.10
4.			0.19								0.19
5.			0.26	0.26	0.26	0.26	0.26				0.26
6.			0.31								0.31
7.			0.35								0.35
8.			0.41								0.41
9.			0.47								0.47
10.			0.53								0.53
11.			0.56								0.56

\*Solvent system: Toluene:Chloroform: Acetone (40:25:35).

\*\*A blank indicates absence of a spot on thin-layer chromatography plate.

Table III

Rf Values from Thin-Layer Chromatography of Ethyl Acetate Extract and Fractions from Dialysis of Ethyl Acetate Extract of Hemicellulose Extract

Spot No.	EAE	D-1A	D-1B	D-2A	D-2B	D-3A	D-3B	D-4A	D-4B	D-5A	D-5B	D-6A	D-6B
1.	0.06	0.06		0.06		0.06		0.06		0.06			
2.	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10		0.10			
3.	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16		0.16			
4.	0.19	0.19		0.19		0.19							
5.	0.26	0.26	0.26	0.26	0.26	0.26		0.26		0.26		0.26	
6.	0.31	0.31		0.31		0.31							
7.	0.35	0.35		0.35		0.35							
8.	0.41	0.41		0.41		0.41							
9.	0.47	0.47		0.47		0.47							
10.	0.53	0.53		0.53		0.53							
11.	0.56	0.56		0.56		0.56							

\*Solvent system: Toluene: Chloroform: Acetone (40:25:35)

\*\*A blank indicates absence of a spot on Thin-layer Chromatography plate.

only two or three spots on TLC. It also indicated ethyl acetate extracted most of the polyphenols. It was also concluded that dialysis at different pH levels provided a means of separation and purification of the polyphenol complex from HCE.

#### Evidence for the Presence of Polyphenols

The color reactions of aqueous solutions of polyphenol complex (EAE), and fractions by dialysis of ethyl acetate extracts of HCE (D-1A, DB-1A, and B-1) with 0.5 ml of 5% ferric chloride and another color reagent containing 5% sodium nitrite, 10% sodium tungstate, and 5% trichloride acetic acid parallels the responses of an aqueous solution of tannic acid with the same chromogenic reagents.

An aqueous solution of ethyl acetate extract gave an absorption maximum at 267-270 nm and the other three aqueous solutions (D-1A, DB-1A, and B-1) formed an absorption maximum at 273-275 nm. Phenolic materials are known to have absorption maxima at these wavelengths.

Table III also shows a number of polyphenols following thin-layer chromatography of these samples (EAE, D-1A, DB-1A, and B-1).

All evidence indicates EAE, D-1A, DB-1A, and B-1 contained predominantly polyphenols.

#### Column Separation of Ethyl Acetate Extract of Hemicellulose Extract

Column treatment of ethyl acetate extracts of HCE was used to help separate polyphenols. The color of the dried and extracted silica gel samples varied from a light gray to a light cream color as the chromatography column was separated from 1 to 13. There was no apparent reaction with ferric chloride, indicating the absence of available phenolic groups. Sample

color varied from brown to light tan (8-1 to 8-13). Sample (fraction 8-13) was more yellow than tan and there was a trace of insoluble material in samples 8-2, 8-3, and 8-4. Rf values of the 13 fractions on thin-layer chromatography are shown in Table IV. The combination of three fractions (8-3, 8-7, and 8-9) would indicate all polyphenols found in original extract of hemicellulose extract. Therefore, they were further studied in two other solvent systems using thin-layer chromatography and high pressure liquid chromatography. A 98.1% recovery of polyphenols from the 13 segments is shown in Table V.

Paper Chromatographic Studies of Ethyl Acetate Extracts of Hemicellulose Extract and Fraction Separated by Dialysis (D-1A, D-2A)

Paper chromatography studies of ethyl acetate extracts, D-1A and D-2A were not successful. The samples migrated as a streak from the point of sample application to the solvent front and resolution was not achieved.

Thin-Layer Chromatographic Studies of Ethyl Acetate Extracts of Hemicellulose Extract and Fractions by Dialysis (D-1A, and D-2A) and Column Separation (8-3, 8-7, 8-9)

Thin-layer chromatographic studies of ethyl acetate extract, D-1A, D-2A, and fractions 8-3, 8-7, and 8-9 using silica gel-coated plates showed that toluene-ethyl formate-formic acid (5:4:1), toluene-chloroform-acetone (40:25:35), and chloroform-ethyl acetate-formic acid (5:4:1) were the best solvent systems. A mixture of 6 ml of a 10% sodium tungstate, 6 ml of a 5% trichloroacetic acid, 3 ml of 0.5 N hydrochloric acid and 6 ml of freshly prepared 5% sodium nitrite (81) proved to be the most generally useful reagent since it gave different colors with phenolic compounds. Rf values were recorded for ethyl acetate extract, D-1A, D-2A, fractions 8-3, 8-7,



Table IV

Rf Values from Thin-Layer Chromatography of Fractions Obtained from Column Chromatography of Ethyl

Acetate Extract of Hemicellulose Extract

Spot No.	8-1	8-2	8-3	8-4	8-5	8-6	8-7	8-8	8-9	8-10	8-11	8-12	8-13
1.		0.11	0.11										
2.		0.20	0.20	0.20	0.20	0.20							
3.		0.26	0.26	0.26									
4.		0.30	0.30	0.30	0.30	0.30	0.30	0.30					
5.			0.36					0.36					
6.				0.43	0.43	0.43	0.43	0.43					
7.				0.45	0.45	0.45	0.45	0.45	0.45				
8.				0.54	0.54	0.54	0.54	0.54		0.54			
9.						0.62	0.62	0.62	0.62	0.62			
10.						0.67	0.67	0.67	0.67	0.67			
11.						0.71	0.71	0.71	0.71	0.71			

\*Solvent system: Chloroform:Ethyl Acetate:Formic Acid (5:4:1).

\*\*A blank indicates absence of a spot on thin-layer chromatography plate.

Table V  
Recovery Studies of 13 Fractions Obtained from Column Chromatography  
of the Ethyl Acetate Extract of Hemicellulose Extract

Fraction	Volume (ml)	gm/ml	Total (gm)	%
8-1	7	0.0086	0.0602	5.90
8-2	7	0.0184	0.1284	12.62
8-3	7	0.0197	0.1379	13.51
8-4	8	0.0219	0.1752	17.16
8-5	8	0.0116	0.0928	9.09
8-6	8	0.0107	0.0856	8.39
8-7	7	0.0109	0.0763	7.48
8-8	7	0.0069	0.0483	4.73
8-9	7.5	0.0090	0.0675	6.61
8-10	7	0.0114	0.0798	7.82
8-11	7	0.0032	0.0224	2.19
8-12	7	0.0015	0.0105	1.03
8-13	7.5	0.0022	0.0165	1.62
Total				98.15%

\*1.0208 gm of polyphenol complex was used in this study.

8-9, and reference compounds with all three solvent systems. (Tables VI, VII, VIII, and IX).

The polyphenols present in ethyl acetate extract were tentatively identified by comparing their migration values with those of known compounds. They were catechol, vanillin, hydroquinone, syringic acid, quercetin, gentisic acid, caffeic acid, gallic acid, and catechin. The identification of polyphenols in ethyl acetate extract was made using any of the three solvents (I, II, III). O-coumaric acid and p-coumaric acid were observed in ethyl acetate extract using solvents I and III only but were not separated by solvent II.

High Pressure Liquid Chromatographic Studies of Ethyl Acetate Extracts of Hemicellulose Extract and Fractions by Dialysis (D-1A) and Column Chromatography (8-3, 8-7, and 8-9)

Ethyl Acetate Extract, fractions D-1A, 8-3, 8-7, and 8-9 were analyzed for polyphenol composition (Fig. 7 to 11). Stepwise elution developed by Mauro (94) was employed in this study. Retention times for reference compounds, EAE, fractions D-1A, 8-3, 8-7, and 8-9 are shown in Table X.

Polyphenols in ethyl acetate extract were tentatively identified by HPLC as catechol, vanillin, hydroquinone, p-coumaric acid, syringic acid, quercetin, gentisic acid and caffeic acid. These were identified by retention time of unknown samples compared to retention time of known standard polyphenols and cochromatography with known polyphenols. Polyphenols present in ethyl acetate extracts were commonly identified by both HPLC and TLC with three different solvents as: hydroquinone, vanillin, catechin, syringic acid, quercetin, caffeic acid, and gentisic acid. P-coumaric acid could be present in ethyl acetate extract because it was identified by both HPLC and TLC with

Table VI

Rf Values from Thin-Layer Chromatography of Ethyl Acetate Extract  
and Various Fractions of Ethyl Acetate Extract  
of Hemicellulose Extract

Spot No.	EAE	D-1A	D-2A	8-3	8-7	8-9
1.	0.07*	0.07*	0.07*	0.07*		
2.	0.11	0.11	0.11	0.11		
3.	0.13*	0.13*	0.13*			
4.	0.18	0.18	0.18	0.18	0.18	
5.	0.22*	0.22*	0.22*	0.22*	0.22*	
6.	0.25*	0.25*	0.25*		0.25*	
7.	0.31*	0.31*	0.31*		0.31*	0.31*
8.	0.37*	0.37*	0.37*		0.37*	0.37*
9.	0.43	0.43	0.43		0.43	0.43
10.	0.51*	0.51*	0.51*		0.51*	0.51*
11.	0.56	0.56	0.56		0.56	0.56

\*The spot was tentatively identified by comparing its value with that of known compound.

\*\*Solvent system: Toluene: Ethyl Formate: Formic Acid (5:4:1).

Table VII

Rf Values from Thin-Layer Chromatography of Ethyl Acetate Extract  
and Various Fractions of Ethyl Acetate Extract  
of Hemicellulose Extract

Spot No.	EAE	D-1A	D-2A	8-3	8-7	8-9
1.	0.04	0.04	0.04	0.04		
2.	0.07	0.07	0.07	0.07		
3.	0.13*	0.13*	0.13*	0.13*	0.13*	
4.	0.19	0.19	0.19	0.19		0.19
5.	0.21*	0.21*	0.21*	0.21*	0.21*	0.21*
6.	0.25	0.25	0.25	0.25	0.25	0.25
7.	0.29	0.29	0.29		0.29	
8.	0.34*	0.34*	0.34*		0.34*	
9.	0.37*	0.37*	0.37*		0.37*	0.37*
10.	0.45*	0.45*	0.45*		0.45*	0.45*
11.	0.51*	0.51*	0.51*		0.51*	0.51*

\*The spot tentatively identified by comparing its migration value with that of known compound.

\*\*Solvent system: Toluene:Chloroform:Acetone (40:25:35).

Table VIII

Rf Values from Thin-Layer Chromatography of Ethyl Acetate Extracts  
and Various Fractions of Ethyl Acetate Extract  
of Hemicellulose Extract

Spot No.	EAE	D-1A	D-2A	8-3	8-7	8-9
1.	0.13	0.13	0.13	0.13		
2.	0.21	0.21	0.21	0.21		
3.	0.28*	0.28*	0.28*	0.28*		
4.	0.30	0.30	0.30	0.30	0.30	
5.	0.37	0.37	0.37	0.37	0.37	
6.	0.41*	0.41*	0.41*		0.41*	
7.	0.45	0.45	0.45		0.45	0.45
8.	0.55*	0.55*	0.55*		0.55*	
9.	0.62*	0.62*	0.62*		0.62*	0.62*
10.	0.67*	0.67*	0.67*		0.67*	0.67*
11.	0.70*	0.70*	0.70*		0.70*	0.70*

\*The spot was tentatively identified by comparing its migration value with that of known compound.

\*\*III. Solvent system: Chloroform:Ethyl Acetate:Formic Acid (5:4:1).

Table IX

Rf Values from Thin-Layer Chromatography of Standard Polyphenols

Substance	Solvents		
	I	II	III
Catechol	0.37	0.51	0.67
Vanillin	0.51	0.51	0.70
Hydroquinone	0.31	0.45	0.62
Vanillic Acid	0.41	0.40	0.65
O-Coumaric Acid	0.37	0.41	0.67
Pyrogallol	0.25	0.39	0.58
P-Coumaric Acid	0.37	0.41	0.62
Ferulic Acid	0.41	0.38	0.64
Syringic Acid	0.31	0.37	0.62
Quercetin	0.25	0.37	0.62
Gentisic Acid	0.31	0.34	0.55
Caffeic Acid	0.22	0.34	0.55
Gallic Acid	0.13	0.21	0.41
Catechin	0.07	0.13	0.28

Solvent systems:

- I. Toluene:Ethyl Formate:Formic Acid (5:4:1).
- II. Toluene:Chloroform:Acetone (40:25:35).
- III. Chloroform:Ethyl Acetate:Formic Acid (5:4:1).

Table X

Retention Times by High Pressure Liquid Chromatography of Known  
Polyphenols and Fractions Separated from Hemicellulose

Peak	Standard Polyphenol	Retention Time (minutes)	Retention Time					
			EAE	D-1A	D-2A	8-3	8-7	8-9
a.								
1.	Hydroquinone	2.0	2.0	2.0	2.0	2.0	2.0	2.0
2.			2.5	2.5	2.5			
3.			3.0				3.0	
4.	Vanillin	3.5	3.5	3.5	3.5	3.5	3.5	3.5
5.			4.5	4.5	4.5	4.5	4.5	4.5
6.			7.2	7.2	7.2			
7.	Catechol	8.4	8.4	8.4	8.4		8.4	8.4
8.			12.0	12.0	12.0			
9.	Syringic Acid	14.0	14.0	14.0	14.0		14.0	14.0
10.			16.4					
11.			25.5	25.5	25.5			
12.	P-Coumaric Acid	46.0	46.0	46.0	46.0			
b.								
13.			5.0	5.0	5.0	5.0	5.0	5.0
14.	Quercetin	6.6	6.6	6.6	6.6	6.6		
15.	Caffeic Acid	8.6	8.6	8.6	8.6	8.6		
16.	Gentisic Acid	11.6	11.6	11.6	11.6	11.6	11.6	
17.			15.8	15.8	15.8	15.8	15.8	
18.			26.2	26.2	26.2			
19.			33.4	33.4	33.4	33.4		

Solvent systems:

a. Dichromethane:Ethanol:Water (400:5:1).

b. Dichromethane:Ethanol:Water (400:35:8).



two different solvents. Other components were present which were not identified. Catechin and gallic acid could also be present in ethyl acetate extract but was not identified by HPLC because the peaks for standard catechin and gallic acid did not elute in a period of 48 minutes by HPLC with solvents (a and b). Fractions D-1A, 8-3, 8-7, and 8-9 contained components which were tentatively identified in ethyl acetate extracts.

It is concluded that some of the polyphenols of HCE are catechol, vanillin, hydroquinone, p-coumaric acid, syringic acid, quercetin, gentisic acid, caffeic acid, gallic acid, and catechin.

Fig. 7. High pressure liquid chromatogram of ethyl acetate extract using stepwise elution.

Operating Conditions:

Sample: Ethyl acetate extract

Instrument: Waters Associates ALC 202

Column: Porasil A (1/8 o.d. x 100 cm)

Solvent: A. Dichloromethane: Ethanol: Water  
(400:5:1)

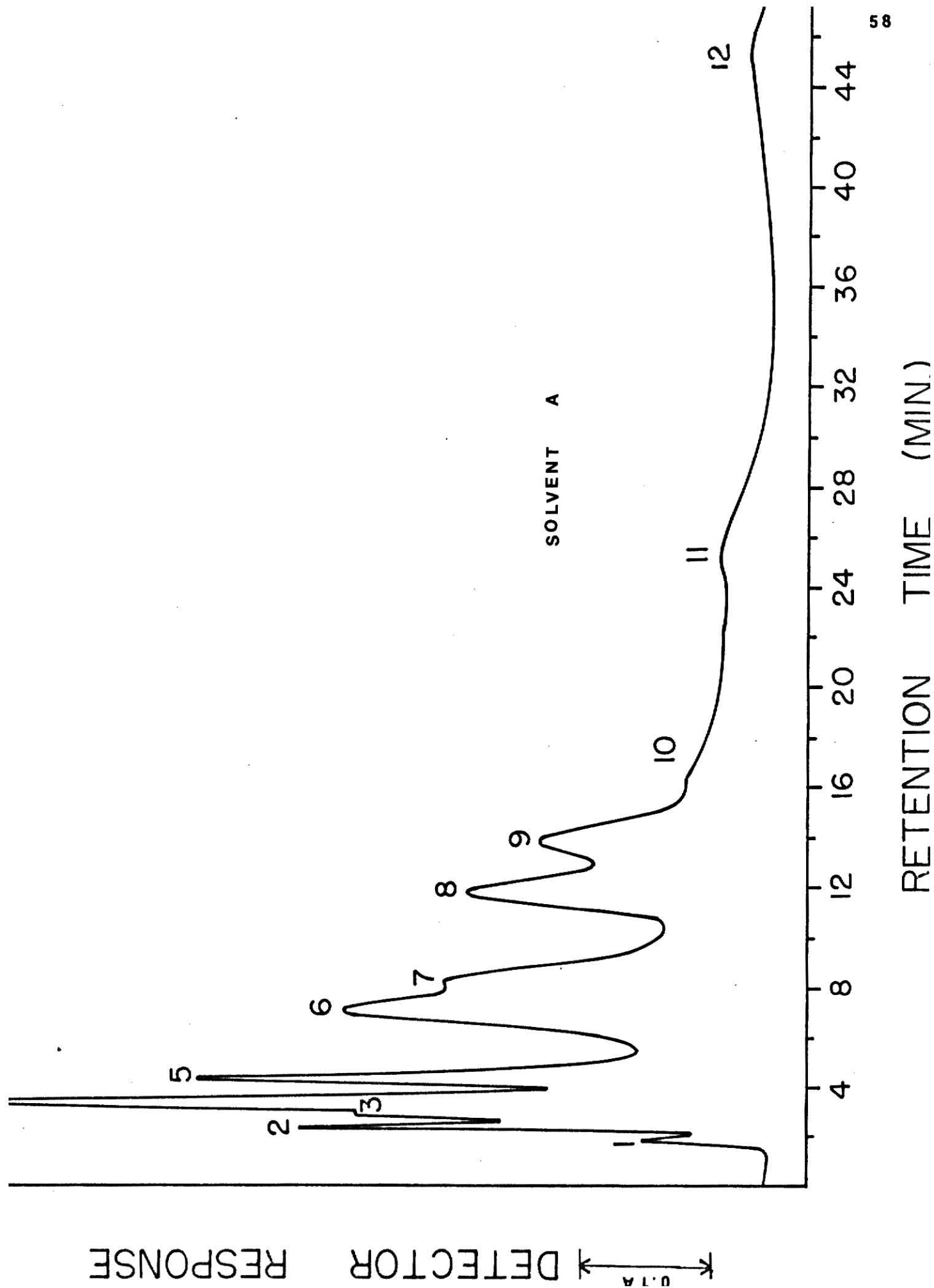
B. Dichloromethane: Ethanol: Water  
(400:35:8)

Flow rate: 2.0 cm/min.

Chart rate: 0.5 cm/min.

Detector: UV photometer at 254 nm.

Temperature: room temperature.



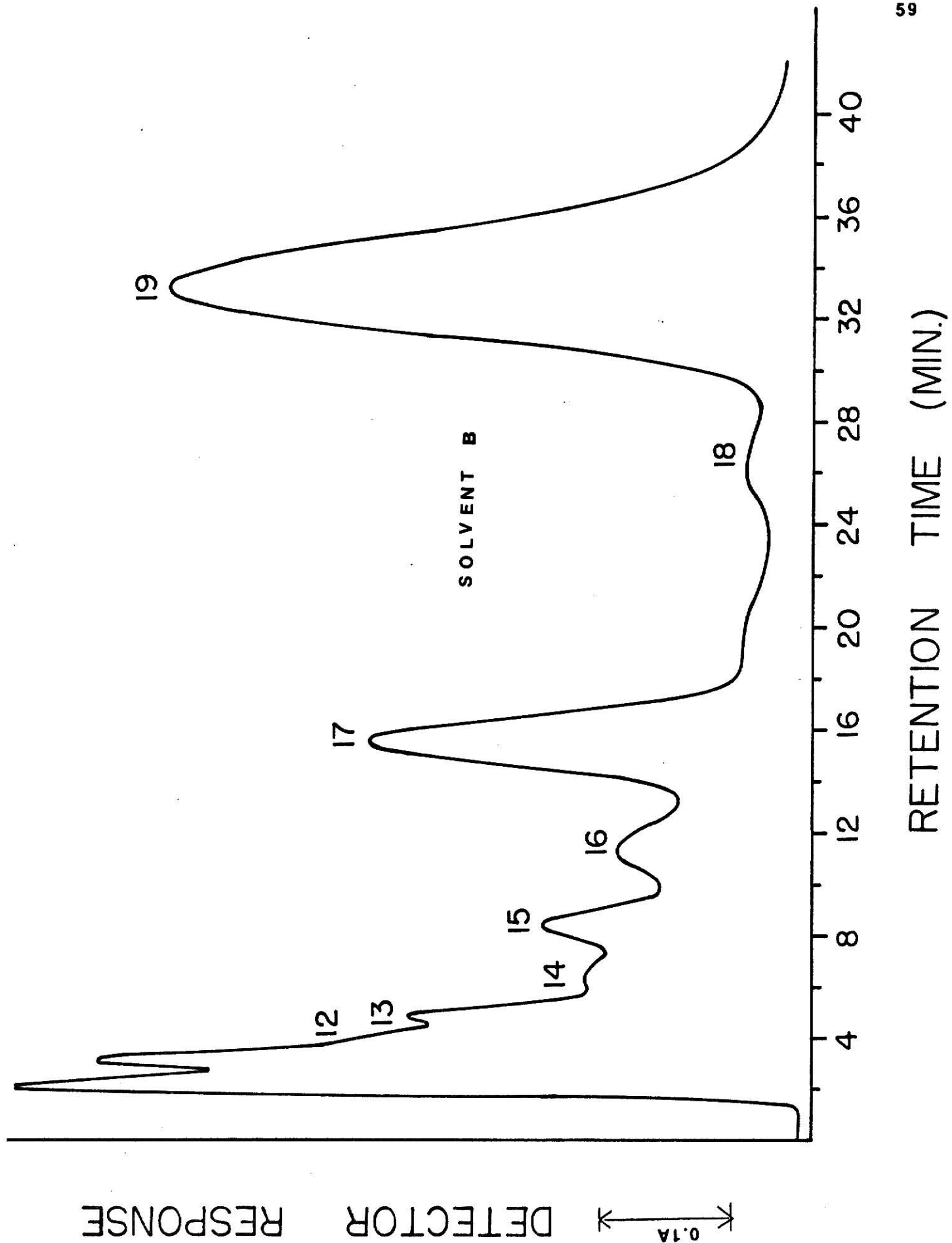


Fig. 8. High pressure liquid chromatogram of a fraction by dialysis of ethyl acetate extract of hemicellulose extract (D-1A) using stepwise elution.

Operating Conditions:

Sample: D-1A

Instrument: Waters Associates ALC 202

Column: Porasil A (1/8 o.d. x 100 cm)

Solvent: A. Dichloromethane: Ethanol: Water  
(400:5:1)

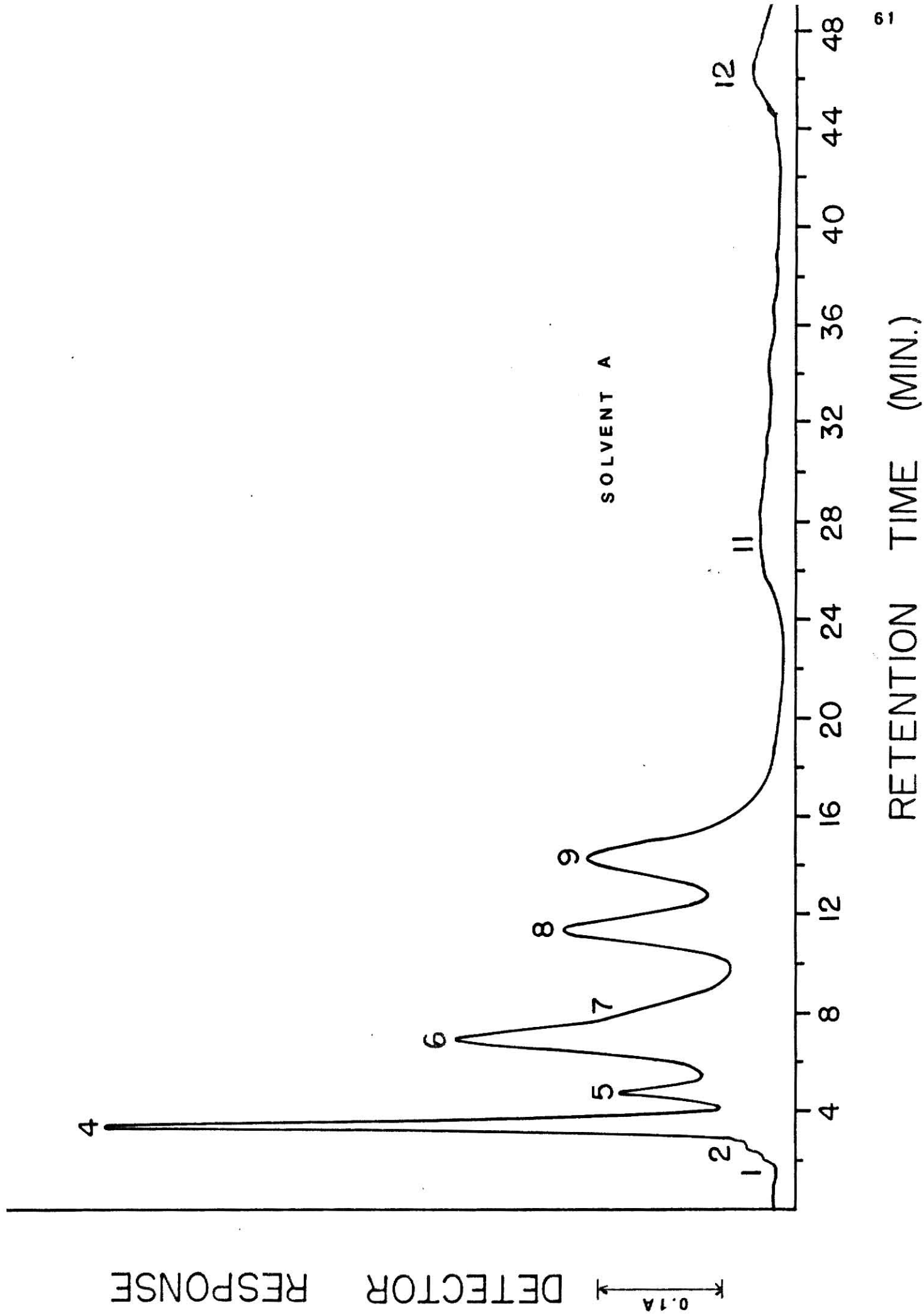
B. Dichloromethane: Ethanol: Water  
(400:35:8)

Flow rate: 2.0 cm/min.

Chart rate: 0.5 cm/min.

Detector: UV photometer at 254 nm.

Temperature: room temperature



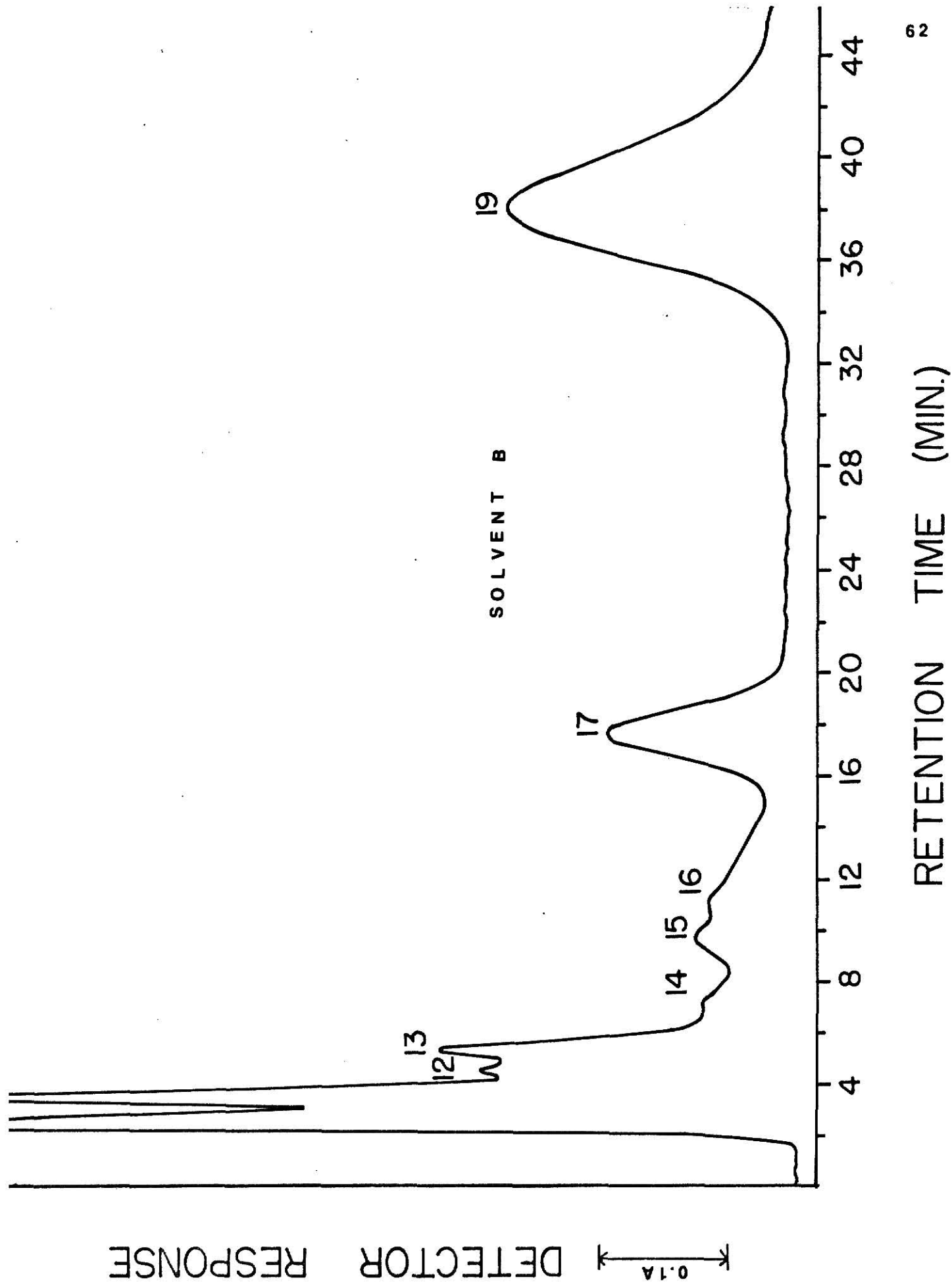


Fig. 9. High pressure liquid chromatogram of a fraction by column separation of ethyl acetate extract of hemicellulose extract (8-3) using stepwise elution.

Operating Conditions:

Sample: 8-3

Instrument: Waters Associates ALC 202

Column: Porasil A (1/8 o.d. x 100 cm)

Solvent: A. Dichloromethane: Ethanol: Water  
(400:5:1)

B. Dichloromethane: Ethanol: Water  
(400:35:8)

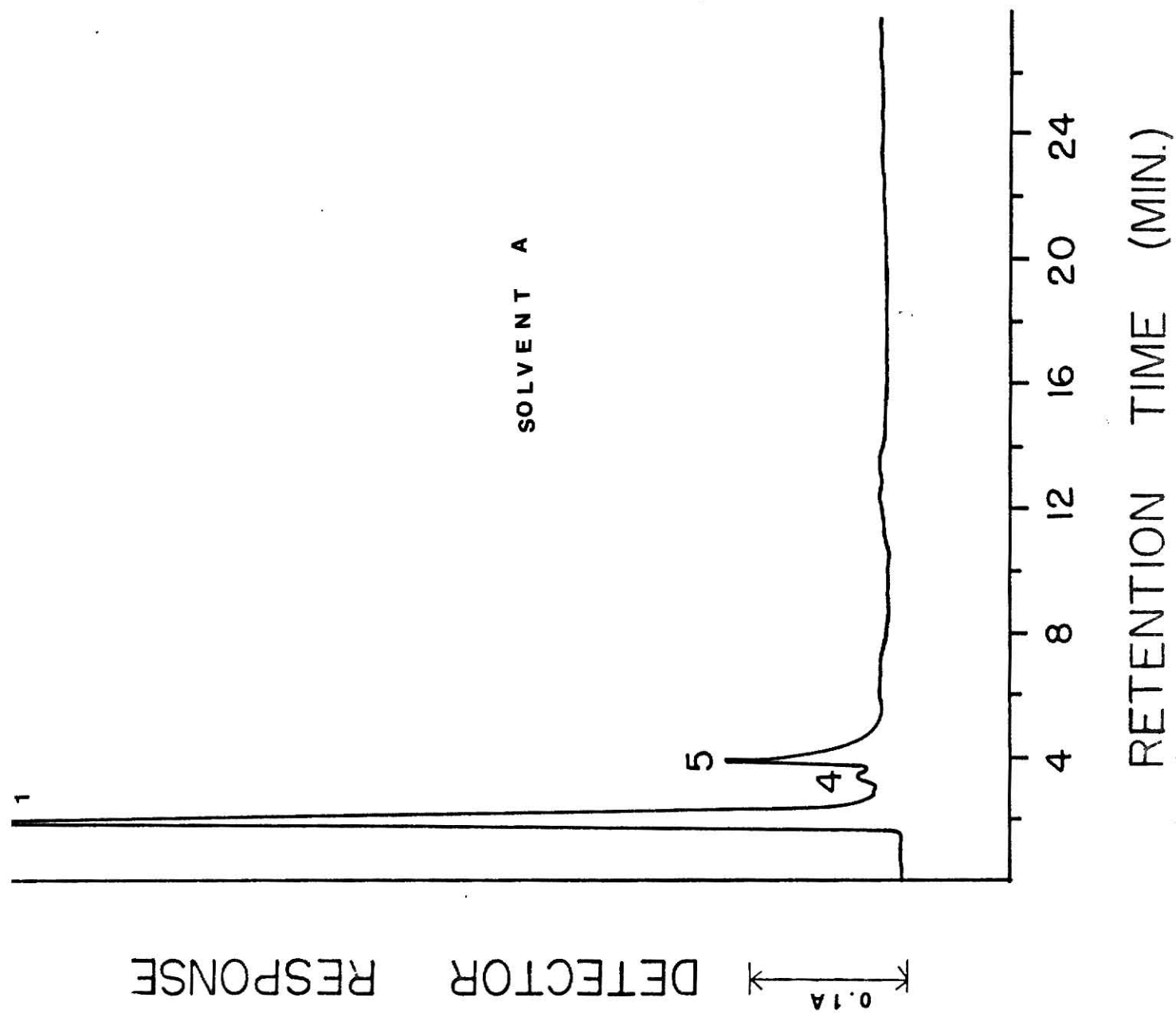
Flow rate: 2.0 cm/min.

Chart rate: 0.5 cm/min.

Detector: UV photometer at 254 nm.

Temperature: room temperature





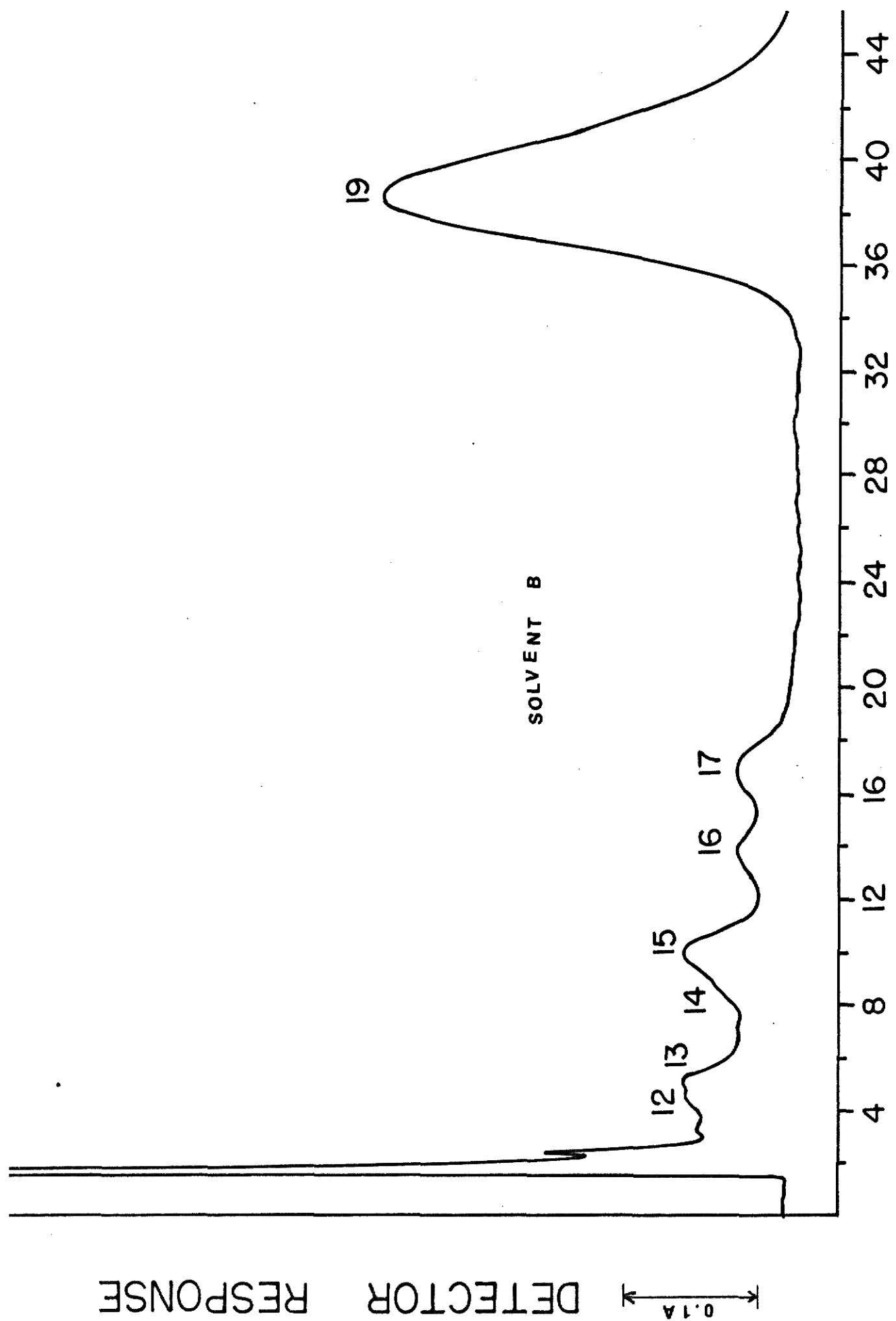


Fig. 10. High pressure liquid chromatogram of a fraction by column separation of ethyl acetate extract of hemicellulose extract (8-7) using stepwise elution.

Operating Conditions:

Sample: 8-7

Instrument: Waters Associates ALC 202

Column: Porasil A (1/8 o.d. x 100 cm)

Solvent: A. Dichloromethane: Ethanol: Water  
(400:5:1)

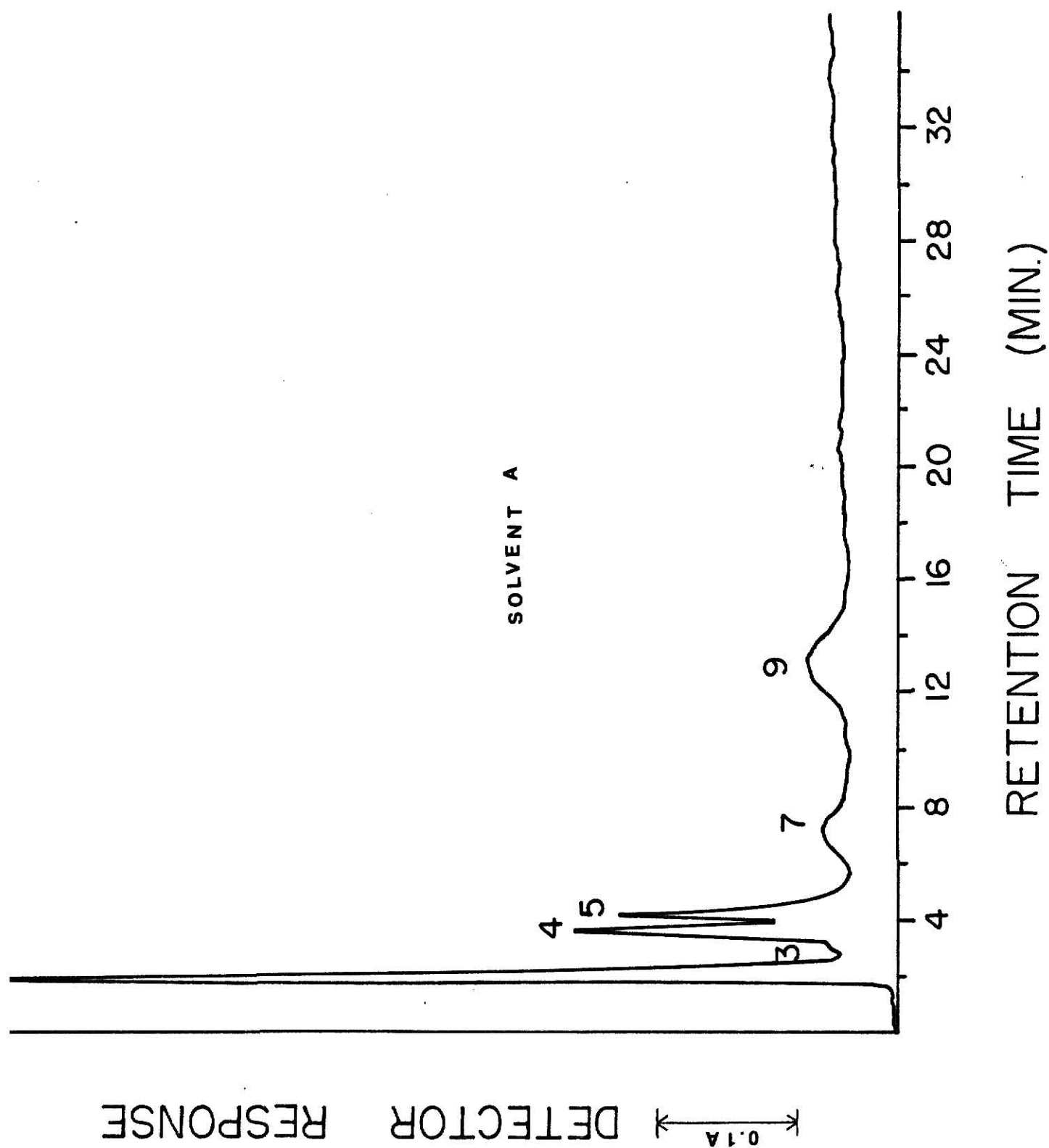
B. Dichloromethane: Ethanol: Water  
(400:35:8)

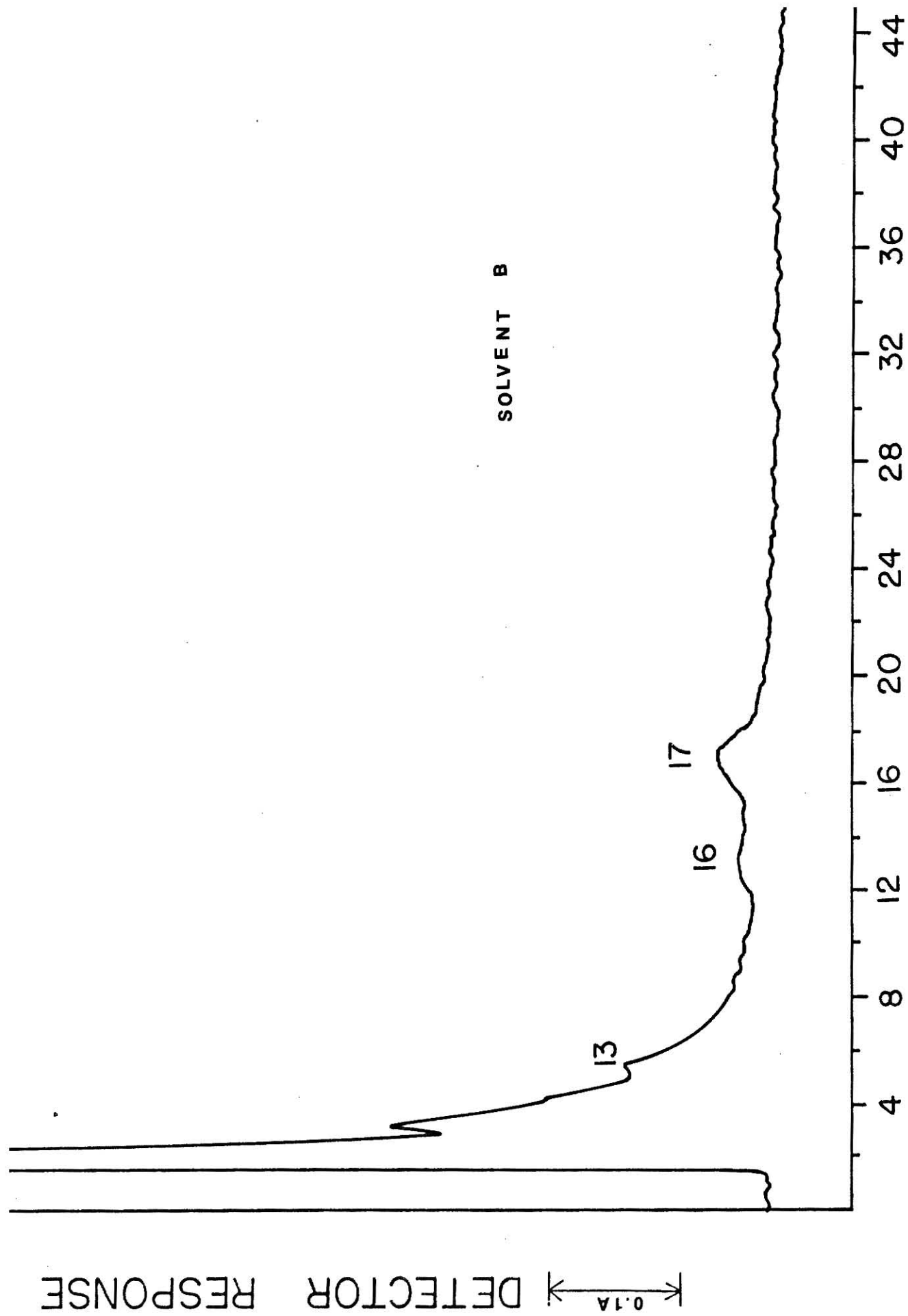
Flow rate: 2.0 cm/min.

Chart rate: 0.5 cm/min.

Detector: UV photometer at 254 nm.

Temperature: room temperature





RETENTION TIME (MIN.)

Fig. 11. High pressure liquid chromatogram of a fraction by column separation of ethyl acetate extract of hemicellulose extract (8-9) using stepwise elution.

Operating Conditions:

Sample: 8-9

Instrument: Waters Associates ALC 202

Column: Porasil A (1/8 o.d. x 100 cm)

Solvent: A. Dichloromethane: Ethanol: Water  
(400:5:1)

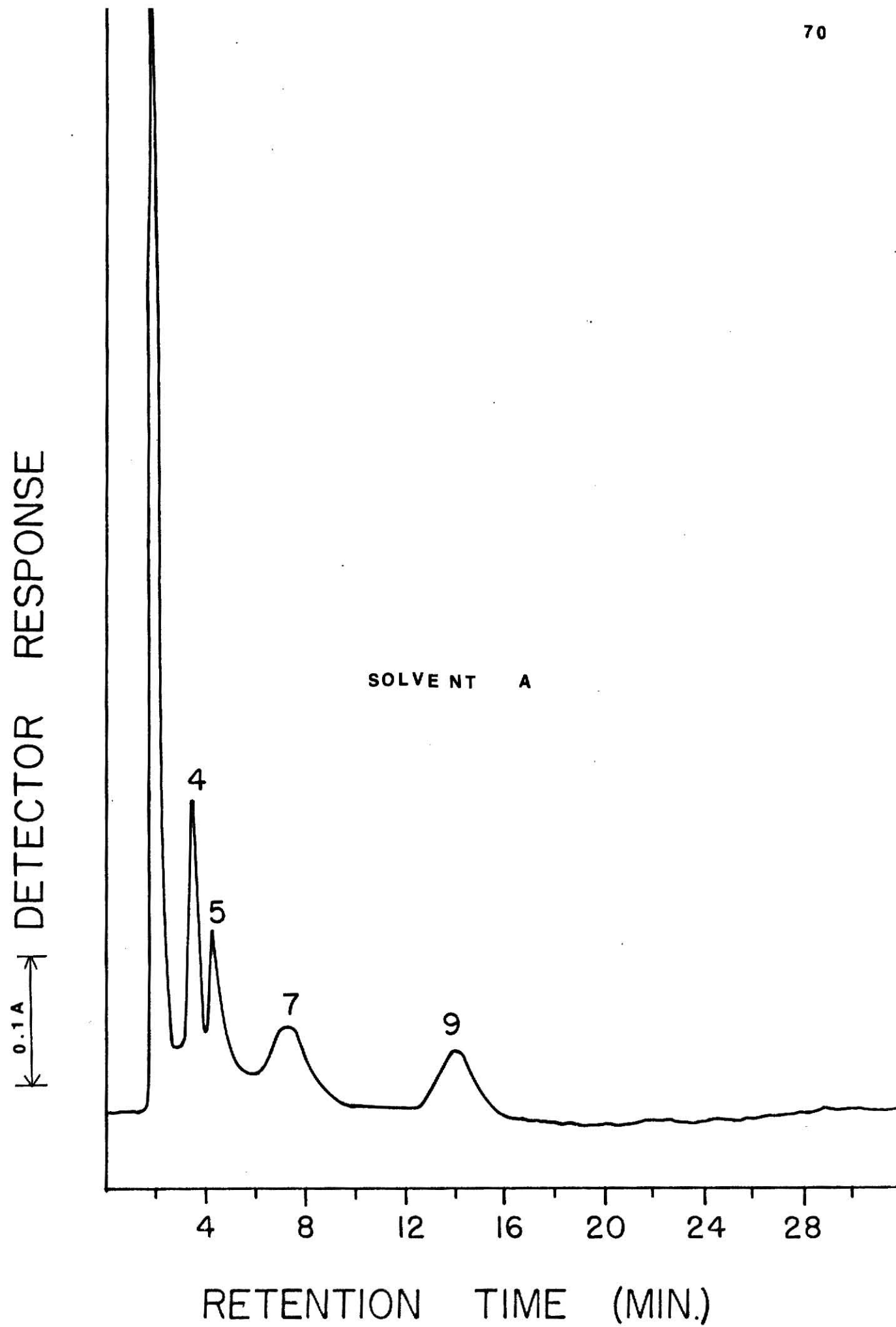
B. Dichloromethane: Ethanol: Water  
(400:35:8)

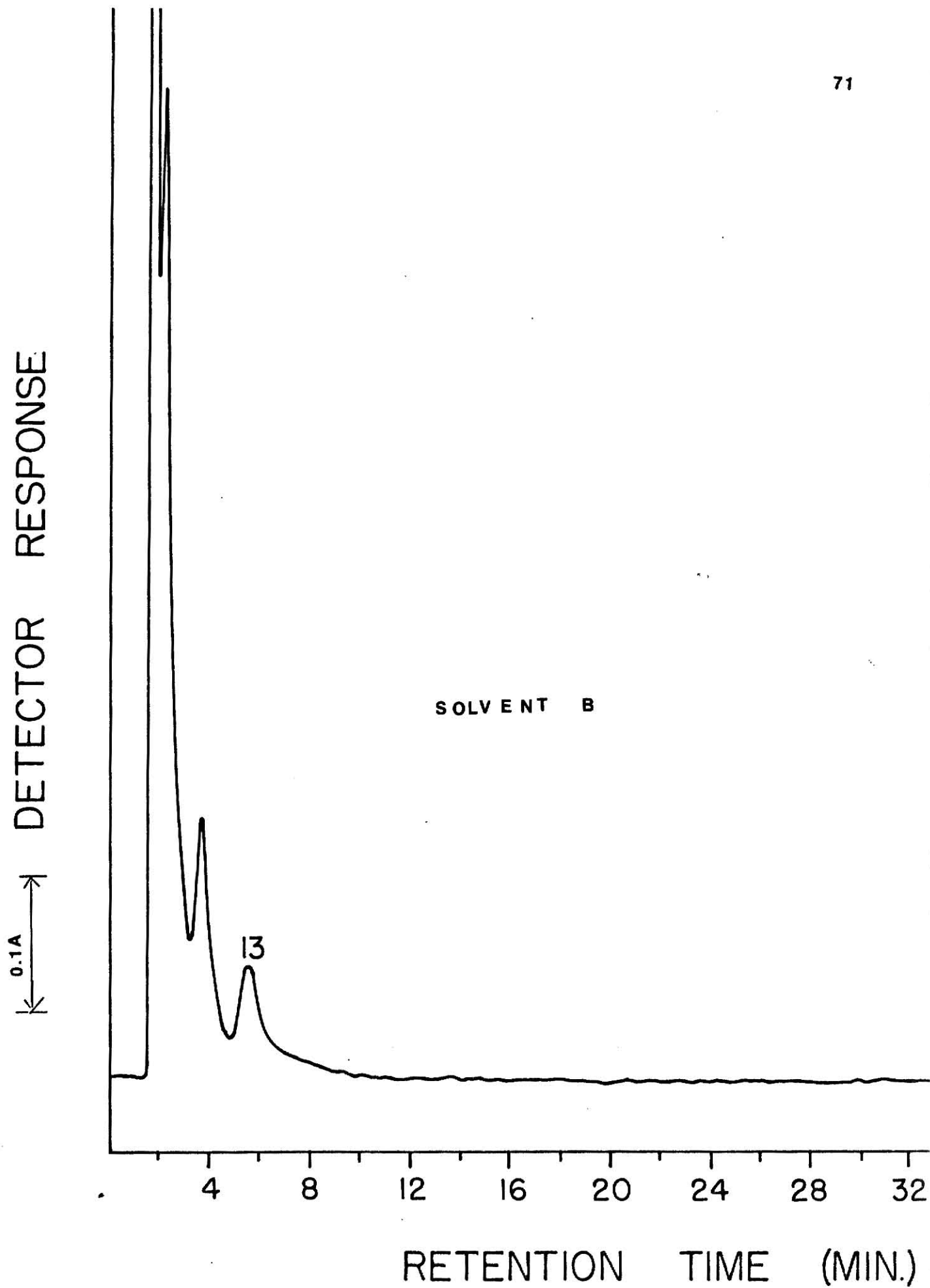
Flow rate: 2.0 cm/min.

Chart rate: 0.5 cm/min.

Detector: UV photometer at 254 nm.

Temperature: room temperature







## SUMMARY

Total polyphenols in hemicellulose extract estimated by four different methods were as follows: Folin-Denis 1.896%, Folin-Ciocalteu 17.675%, Vanillin HCL 1.22%, and ultraviolet 14.40%. Each of the four methods appear to be markedly different in their results. The differences in the relative values for hemicellulose extract by these methods may be due to the measurement of different polyphenolic compounds. None of the methods appear well established and reliable. It may not be possible to develop a practical method for analyzing large numbers of samples for absolute polyphenols content, but tests based on relative values should be standardized. Further research is needed to develop a generally acceptable reproducible method for analyzing polyphenols in natural plants.

There are several methods for removing the polyphenol complex from hemicellulose extract. Extraction with ethyl acetate, however, offers promise because it separates readily from the aqueous medium and traces remaining in solution can be removed by distillation. Dialysis was used to separate ethyl acetate into components. Samples (D-1A, D-2A, D-3A, DB-1A, and B-1) obtained by dialysis indicated predominantly polyphenols by color reactions (ferric chloride test and a mixture of 5% sodium nitrite, 10% sodium tungstate, 5% trichloroacetic acid and 0.5 N HCL) and thin-layer chromatography. They were also evaluated by UV absorption maximum. An aqueous solution of ethyl acetate extract had an absorption maximum at 267-270 nm. and other samples (D-1A, DB-1A, and B-1) gave an absorption maximum at 273-275 nm. Phenolic materials are known to have absorption maximum at these wavelengths.

Ascending column chromatography was employed to separate ethyl acetate extract. Fractions 8-1 to 8-13 obtained column chromatography had no apparent reaction with ferric chloride. This indicated the absence of available phenolic groups for these fractions.

Polyphenols present in ethyl acetate extract identified by both high pressure liquid chromatography and thin-layer chromatography were hydroquinone, vanillin, catechol, syringic acid, quercetin, caffeic acid, and gentisic acid. P-coumaric acid could also be present in ethyl acetate extract because it was identified by both HPLC and TLC with two different solvents. Other components were present which were not identified. Catechin and gallic acid could have been present in ethyl acetate extract, though they were not identified by high pressure liquid chromatography. Polyphenols in fractions D-1A, 8-3, 8-7, and 8-9 were tentatively identified as the same as those in ethyl acetate extract. It is concluded that some polyphenols of hemicellulose extract are catechol, vanillin, hydroquinone, p-coumaric acid, syringic acid, quercetin, gentisic acid, caffeic acid, gallic acid, and catechin.

## LITERATURE CITED

1. Farmer, R. H. Chemistry of wood components. In: Chemistry in the utilization of wood, ed. by Farmer, R. H. Pergamon Press: New York (1967).
2. Browning, B. L. The composition and chemical reactions of wood. In: The chemistry of wood, ed. by Browning, B. L. Interscience Publ.: New York (1963).
3. Wenzl, H. F. J. The chemical technology of wood. Academic Press, Inc.: New York (1970).
4. Scott, R. W., Millett, M. A., and Hajny, G. J. Wood wastes for animal feeding. Forest Products J. 19(4): 14 (1969).
5. Daniels, L. B., Campbell, J. R., Martz, F. A., and Hedrick, H. B. J. Animal Sci. 30: 593 (1970).
6. Dinius, D. A. Wood waste materials as feedstuff for ruminants. Feedstuffs 45(14): 22 (1973).
7. Millett, M. A., Baker, A. J., Satter, L. D., McGovern, J. N., and Dinius, D. A. Pulp and paper making residues as feedstuffs for ruminants. J. Anim. Sci. 37: 599 (1973).
8. Daniels, L. B., Campbell, J. R., Martz, F. A., and Hedrick, H. B. An evaluation of newspaper as feed for ruminants. J. Anim. Sci. 30: 593 (1970).
9. Nishimuta, F., Sherrod, C. B., Furr, R. D. and Hansen, K. R. Nutritive value of sheep ration containing various levels of paper. J. Anim. Sci. 29: 642 (1969).
10. Thomas, J. W., Yu, Y., and Hoffer, J. A. Digestibility of paper and dehydrated feces. J. Animal Sci. 31: 255 (1970).
11. Perry, T. W. New wood molasses (Hemicellulose extract) equivalent to cane molasses for beef cattle. Feedstuff. 36(46): 26 (1964).
12. Boren, F. W., Pfost, H. B., Smith, E. F., and Richardson, D. Cane molasses and hemicellulose extract in rations for finishing steers. Kansas Agric. Expt. Station. Bull-483 (1965).
13. Bartley, E. E., Farmer, E. L., Pfost, H. B., and Dayton, A. D. Comparative value of dry and liquid hemicellulose extract and liquid cane molasses for lactating dairy cows. J. Dairy Sci. 51: 706(1969).

14. Williams, D. L., Moore, J. D., Martin, L. C., and Tillman, A. D. Studies on liquid hemicellulose and cane molasses as carbohydrate sources in urea-containing diets of sheep. *J. Animal Sci.* 28: 667 (1969).
15. Hartnell, G. F., and Satter, L. D. The effect of Masonex (HCE) on protein degradation by rumen microorganisms in vitro: A research report to the Masonite corporation. Dec. (1974).
16. Swain, T., and Bate-Smith, E. C. In comparative biochemistry. Vol. III, ed. by Florkin, A. M., and Mason, H. S. Academic Press, New York (1962).
17. Singleton, V. L., Kratzer, F. H. Plant phenolics. In toxicants occurring naturally in foods. ed. by Committee on Food Protection Food and Nutrition Board, National Research Council 2nd. (1973).
18. Halslam, E. Chemistry of vegetable tannins. Academic Press, New York (1966).
19. Thomson, R. H. Structure and reactivity of phenolic compound, ed. by Harborne, J. B. Academic Press: New York (1964).
20. Erich Heftmann. Chromatography. New York 2nd. p. 677 (1967).
21. Hergert, H. L. Chemical composition of tannins and polyphenols from conifer wood and bark. *Forest Prod. J.* 10: 610 (1960).
22. Jurd, L. Plant polyphenols, 1. The polyphenolic constituents of the pellicle of the walnut. *J. Am. Chem. Soc.* 78: 3445 (1956).
23. Hillis, W. E. and Clarke, A. Formation of phenolic substances in eucalyptus gigantea and eucalyptus sieberiana. *Biochem. J.* 74: 607 (1960).
24. Mayer, W. Tannins from chestnut wood and oak wood. *Leder* 22 (12): 277 (1971); *Chem. Abstracts* 76: 101390b (1972).
25. Seikel, U. K., Hostettler, F. D., and Niemann, G. J. Phenolics of quercus rubra wood. *Phytochem.* 10: 2249 (1971).
26. Tattat, Terry A., and Rich, A. E. Extractable phenols in clear, discolored and decayed woody tissue and bark of sugar maple and red maple. *Photopathology* 63: 167 (1973).
27. Ringrose, R. C., and Morgan, C. C. The nutritive value of lespedeza for poultry feeding. *S. Carolina Agr. Exp. Sta. Annual Report* 53: 91 (1940).
28. Alumot, E., Nachtom, E., and Bornstein, S. Low chloric value of carob as the possible cause of growth depression in chicks. *J. Sci. Food Agr.* 15: 259 (1964).

29. Mueller, W. S. The significance of tannic substances and theobromine in chocolate milk. *J. Dairy Sci.* 25: 221 (1942).
30. Vohra, P., Kratzer, F. H., and Joslyn, M. A. The growth depressing and toxic effects of tannins to chicks. *Poultry Sci.* 45: 135 (1966).
31. Dollahite, J. W., Pigeon, R. F., and Camp, B. T. The toxicity of Gallic acid, pyrogall, tannic acid, and quercus havardi in the rabbit. *Amer. J. Vet. Res.* 23: 1264 (1962).
32. Fenny, P. P. and Bostock, H. Seasonal changes in the tannin content of oak leaves. *Phytochem.* 7: 87 (1968).
33. Cadman, C. H. Phenolics in plants in health and disease. Pridham, J. B. ed. Pergamon, New York, p. 101 (1960).
34. Feeny, P. Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* 51: 565 (1970).
35. McDonald, I. W. The absorption of ammonia from the rumen of the sheep. *Biochem. J.* 42: 584 (1948).
36. Driedger, A., and Hatfield, E. E. Influence of tannin on the nutritive value of soybean meal for ruminants. *J. Animal Sci.* 34: 465 (1972).
37. Driedger, A., Hatfield, E. E. and Garrigus, U. S. Effect of tannic acid treated soybean meal on growth and nitrogen balance. *J. Animal Sci.* 29: 156 (1969).
38. Driedger, A., and Hatfield, E. E. Utilization of tannin treated SBM infused per rumen or abomasum. *J. Animal Sci.* 31: 1038 (Abstr.) (1970).
39. Joslyn, M. A. and Glick, A. Comparative effects of gallotannic acid and related phenolics on the growth of rats. *J. Nutr.* 98: 119 (1969).
40. Dollahite, J. W., Pigeon, R. F. and Camp, B. J. The toxicity of gallic acid, pyrogallol, tannic acid and quercus havardi in the rabbit. *Am. J. Vet. Res.* 23: 1264 (1962).
41. Rayudu, G. V. N., Kadirvel, R., Vohra, D. and Kratzer, F. H. Toxicity of tannic acid and its metabolites for chickens. *Poultry Sci.* 45: 135 (1966).
42. Varga, M. and Koves, E. Phenolic acids as growth and germination inhibitors in dry fruit. *Nature* 183: 401 (1959).

43. Gortner, W. A., and Kent, M. H. The coenzyme requirement and enzyme inhibitors of pineapple indoleacetic acid oxidase. *J. Biol. Chem.* 233: 731 (1958).
44. Rabin, R. S., and Klein, R. M. Chlorogenic acid as a competitive inhibitor of indoleacetic acid oxidase. *Arch. Biochem. Biophys.* 70: 11 (1957).
45. Bhatia, I. S., Sharma, H. K., and Bajaj, K. L. A study of the inhibitory effects of polyphenols on endopolygalactyronase from *aspergillus niger*. In *Zentralblatt. Veb Gustav Fischer Verlag Iena (India)*.
46. Swain, T., and Hillis, W. E. Phenolic constituents of *prunus domestica* L. Quantitative analysis of phenolic constituents. *J. Sci. Agr.* 10: 63 (1959).
47. Association of Official Agricultural Chemists. *Official Methods of Analysis*. 9th ed.: Washington, D. C. (1960).
48. Lees, G. J., and Nelson, P. F. An examination of hot water extracts of *eucalyptus sieberi*. *Appita* 20 (5): 113 (1967); *Chem. Abstracts* 67: 33957d (1967).
49. Alexa, G., and Caraculaca, G. Colorimetric determination of tannins. *Acad. Rep. Populare Romine, Filiala Iasi, Studii Ceretari stiint, Chim.* 13 (2): 263 (1962); *Chem. Abstracts* 59: 12189f (1963).
50. Christian, J. B., Smith, Joshyn, Maynard A., and Lukton, Aaron. Determination of tannins and related polyphenols in foods. *Analytical Chem.* 27: 7 (1955).
51. Peri, C., and Pompei, C. Estimation of different phenolic groups in vegetable extracts. *Phytochem.* 10: 2187 (1971).
52. Seider, A. I., and Datunashvili, E. N. Determination of phenolic substances in wines. *Vinodel vinograd SSSR* 1972, (6), 31-34 (RUSS); *Chem. Abstracts* 78: 41476p.
53. Burns, R. E. Methods of tannin analysis for forage crop evaluation. *Univ. of Ga. Agr. Exp. Sta. Tech. Bull. N. S.* 32 p. 14 (1963).
54. Burns, Robert E. Method for estimation of tannin in grain sorghum. *Agronomy J.* 63: 511 (1972).
55. Bate-Smith, E. C., and Lerner, N. H. Leuco-anthocyanins systematic distribution of Leuco-anthocyanins in leaves. *Biochem. J.* 58: 126 (1954).
56. Maxson, E. D., and Rooney, L. W. Two methods of tannic analysis for *sorghum bicolor* (L.) Moench grain. *Crop Sci.* 12: 253 (1972).

57. Owades, Joseph L., Rubin, George and Brenner, M. W. Determination of food tannins by ultraviolet spectrophotometry agricultural and food chemistry. V. 6 N. 1 (1958).
58. Owades, Joseph L., Rubin, George, and Brenner, M. W. Determination of tannins in beer and brewing materials by ultraviolet spectrophotometry. Amer. Soc. Brew Chem. Proc. p. 66 (1958).
59. Johnson, Gestur, Foreman, E. M., and Mayer, M. M. The ultraviolet absorption of polyphenolic substances from various fruits. Food Technology. p. 237 (1950).
60. Sogawa Hairoschi. Polyphenols in brewing. I. Determination of polyphenols and isolation of polyphenolic constituents. Rep. Res. Lab. Kirin Brew Co. No. 14, 21-9 (Eng.) (1971).
61. Assoc. Offic. Agr. Chemists, Washington, D. C., "Official Methods of Analysis." 8th ed., p. 241 (1955).
62. Mejbaum-Katzenllenbogem, W. and Z. Kudrewicz-Hubica. Appreciation of urea, ferric ammonium sulfate and casein for determination of tannin substances in plants. Acta Biochem. Pol. 13: 57 (1966).
63. Bate-Smith, E. C. and Rasper, V. Tannins of grain sorghum: luteoforal (leucoluteolinidin), 3',4, 4'5, 7-pentahydroxyflavan. J. Food Sci. 34: 203 (1969).
64. Vuataz, H. Brandenberger, and Egli, R. H. Plant phenols 1. speration of the tea leaf polyphenos by cellulose column chromatograph. J. Chromatog. 2: 173 (1959).
65. Miskov, Obrad; Bourzlix, Michel; Heredia, Nicolas. Isolation of individual phenolic acids and catechins and densitometric evaluation of their respective contents in musts, juices of red and white grapes, and white wines. Ind. Aliment Agr. 87: 1515 (1970). Chem. Abstract (75).
66. Ediz, S. H. Isolation of insect repellent constituents from coast redwood (sequoia sempervirens) hemicellulose extract. Ph.D. Thesis, Kansas State University, Kansas (1973).
67. Loomis, W. D. and Battaile, J. Plant phenolic compounds and isolation of plant enzymes. Phytochem. 5: 423 (1966).
68. Anderson, R. A., and Sowers, J. A. Optimum conditions of bonding plant phenols to insoluble pvp. Phytochem. 7: 293 (1968).
69. Subba Reddy Chintam. Wood and fiber extractives protein utilization. Ph.D. Thesis, Kansas State University, Manhattan, Kansas (1974).



70. Gieman, T. A. The chemistry of flavonoid compound. Macmillan, New York (1973).
71. Ng, E. and Mosek, M. A method for estimating total polyphenols in beer. J. Inst. Brew 79: 165 (1973).
72. Anderson, A. B., Riffer, R. and Wong, A. Chemistry of the genus *sequoia sempervirens*. Phytochem. 7: 1867 (1968).
73. Fuleki, T., and Francis, F. J. Quantitative methods for anthocyanins III. Purification of cranberry anthocyanine. J. Food Sci. 33: 266 (1968).
74. Kinsley, H., and Pearl, I. A. Studies on the leaves of the family salicaceae. IX. Compositions of the lead subtanin insoluble fraction of *populus tremuloides* leaves. Tappi. 50: 419 (1967).
75. Bate-Smith, E. C. Nature of anthocyanins and related substances in petal extracts. Chromatography 161: 835 (1948).
76. Seoan, E. and Carriccer, A. Identification of natural phenols by microscale reactions of their functional groups. Microchem. J. 12: 291 (1967).
77. Pridham, J. B. Paper electrophoresis and paper chromatography of phenolic compounds. J. Chromatog. 2: 605 (1959).
78. Stahl, E. Thin-layer Chromatography. A Laboratory Handbook, Springer, New York, p. 705 (1969).
79. Hanke, M. T., and Koessler, K. K. A microchemical colorimetric method for estimating tyrosine, tyranmine and other phenols. J. Biol. Chem. 50: 235 (1922).
80. Barton, G. M., Evans, R. S., and Gardner, J. A. F. Chromatography of phenolic substances. Nature 170: 249 (1952).
81. Bhatia, I. S., Singh, Joginder, and Bajaj, K. L. A new chromogenic reagent for the detection of phenolic compounds on thin-layer plates. J. Chromatog. Note 79: 350 (1973).
82. Torrent, M. T., Adzet, D. T., and Iglesias, A. J. M. (Spain). Physicochemical methods for studying phenolic acids in drugs. Rev. Real Acad. Farm, Barcelona (1972).
83. Wang, K., and Lin, Y. Polyamide thin-layer chromatography. II. A modified method of preparation of thin-layer and its application to some naturally occurring phenolic substances. J. Chinese Chem. Soc. 10: 146 (1963).



84. Wang, K. Polyamide thin-layer chromatography. I. Phenols.  
J. Chinese Chem. Soc. 8: 24 (1961).
85. Lin, Y., Wang, K., and Lin, S. Polyamide thin-layer chromatography.  
II. Application of phenolic constituents of some conifers.  
J. Chinese Chem. Soc. 9: 68 (1962).
86. Norman, R. O. C., Smith, J. R. L., and Radda, G. K. In methods in  
polyphenol chemistry. Pridham, J. B. (ed.) Pergamon, Oxford,  
p. 125 (1964).
87. Furuya, T. Gas-liquid chromatography of plant glycosides. J.  
Chromatography 18: 12 (1964).
88. Horermann, W., Rapp, A., and Ziegler, A. Eine Method zur trennung  
der phenolcarbonsauren durch Hochdruck-Flussigkeits-Chromatographie.  
Chromatographia vol. 6, No. 7 (1973).
89. Anggard, E., Sjoqvist, B., and Sjoström, R. Phenolic acids and  
alcohols. J. Chromatog. 50: 251 (1970).
90. Woof, J. B. Investigation of phenolic components of brewing materials  
by Gel filtration. Nature 195: 184 (1962).
91. Leonard, S., Luh, B. S., and Hinreier, E. Flavor evaluation of  
canned cling peaches. Food Technology. 7: 480 (1953).
92. Levin, V. E. The action of proteins on the phenol reagent of Folin  
and Denis. Science 52: 612 (1920).
93. Deichmann, W., and Schafer, L. J. Phenol Studies. I. Review of  
the literature. Am. J. of Clin. Path. 12: 129 (1942).
94. Mauro, Dave. Unpublished research data. Department of Grain Science  
and Industry, Kansas State University, Manhattan, Kansas.

### ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to Dr. C. W. Deyoe, his major advisor, for his guidance and continuous interest in this study. He is grateful to the other members of supervisory committee: Drs. William J. Hoover, David Wetzél, and Paul Seib for their advice and help during the preparation of this thesis.

The author is grateful to Mr. Glenn W. Kerr, and his colleagues: Dave Mauro, Tom Brandt, M. F. Tu, C. C. Tu, and J. M. Wu for their advice and help.

Grateful acknowledgment is made for the financial support received from the Masonite Corporation.

Last, but certainly not least, he wishes to express his heartfelt thanks to his parents for encouragement and continued support.

ISOLATION AND IDENTIFICATION OF POLYPHENOLS  
OF HEMICELLULOSE EXTRACTS

by

HSIN-CHUAN TSAI

B. S., Taiwan Provincial College of Marine  
and Oceanic Technology (Taiwan), 1971

---

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

in

FOOD SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY  
Manhattan, Kansas

1975

HCE, a by-product of the Masonite Corporation, is a water soluble wood extract resulting from a pressure steam reaction on wood chips. One such commercial product, HCE, has found widespread use in animal feeds.

Phenolic compounds occur extremely widely in wood, though often in rather small amounts. Much evidence indicates phenolic compounds hold the potential for improved animal performance.

The present investigation was undertaken to determine total polyphenols in HCE and to isolate and identify the polyphenols from HCE.

The total polyphenols in HCE were determined by four different methods as follows: Folin-Denis 1.896%, Folin-Ciocalteu 17.675%, Vanillin-HCL 1.220%, and Ultra Violet 14.40%. Each of four methods appeared to be markedly different in results. The difference in the relative value of HCE for these methods may be due to the measurement of different polyphenolic compounds.

There are numerous ways of removing the polyphenol complexes from HCE. Extraction with ethyl acetate, however, offers good promise for a continuous plant operation. Dialysis was used to separate and purify the ethyl acetate extracts. Samples (D-1A, D-2A, D-3A, DB-1A, and B-1) obtained from dialysis indicated they contained predominantly polyphenols by color reagent (ferric chloride and a mixture of 5% sodium nitrite, 10% sodium tungstate, 5% trichloroacetic acid, and 0.5 N HCL) and thin-layer chromatography. Ascending column chromatography was also employed to separate and purify ethyl acetate extracts. The fractions (8-1 through 8-13) obtained from column chromatography had no apparent reaction by ferric chloride test.

Polyphenols present in ethyl acetate extracts were commonly identified by both high pressure liquid chromatography and thin-layer chromatography with three different solvents as: hydroquinone, vanillin, catechol, syringic acid, quercetin, caffeic acid, and gentisic acid. P-coumaric acid could be present in ethyl acetate extracts because it was identified by both HPLC and TLC with two different solvents. Other components were present which could not be identified. Catechin and gallic acid could also be present in ethyl acetate extracts though they could not be proved by high pressure liquid chromatography. Samples (fraction D-1A, 8-3, 8-7, and 8-9) contained compounds which were tentatively identified the same as the ethyl acetate extracts. It is concluded that some of polyphenols of HCE are catechol, vanillin, hydroquinone, p-coumaric acid, syringic acid, quercetin, gentisic acid, caffeic acid, gallic acid, and catechin.