The Institute of Paper Chemistry

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Doctor's Dissertation

A Study of the Components of the Lead Subacetate Precipitate of the Leaves of *Populus tremuloides*

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A STUDY OF THE COMPONENTS OF THE LEAD SUBACETATE PRECIPITATE OF THE LEAVES OF POPULUS TREMULOIDES

A thesis submitted by

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SUMMARY

Traditionally, lead subacetate has been added to the water extracts of leaves and barks to precipitate interfering materials from the extracts. In this study the lead subacetate-insoluble fraction of the water extract of quaking aspen leaves was examined. This material was fractionated by extracting with several solvents. The extracts were fractionated by column chromatography. Paper and thin-layer chromatography, melting point data, hydrolysis data, color reactions, ultraviolet spectroscopy and infrared spectroscopy were used to investigate the isolated materials.

A silica gel column eluted with a chloroform-methanol (4:1) mixture was used to fractionate the benzene extract. Pyrocatechol, which accounted for 0.019% of the leaves, was isolated.

The ethyl acetate extract was fractionated by polyamide columns eluted with water. The fractions from the column yielded succinic acid (0.003%), pyrocatechol (0.019%), tremuloidin (0.01%), populin (0.003%), and salireposide (0.008%).

The raffinate was fractionated by polyamide columns. When the columns were eluted with water, myo-inositol (0.075%), pectin (0.80%), rutin (0.012%), and a suspected flavanonol (0.006%) were isolated and studied. When the column was eluted with ethanol-water (1:1) mixture, rhamnetin (0.022%) was isolated.

The raffinate was also fractionated by a cellulose column eluted with water. A mixture of quercetin-3-galactoside and quercetin-3-glucoside (0.11%) was isolated and studied.

The identified compounds represented 17.5% of the total organic material present in the lead subacetate-insoluble fraction. Approximately 13% of the material in this fraction was carbohydrate. The isolated compounds accounted for approximately 90% of the total carbohydrate present.

The rutin and quercetin-3-galactoside were the first two glycosides isolated from quaking aspen that contained a sugar moiety other than glucose. The flavonoid glycosides present in the lead-insoluble fraction of the water extract of quaking aspen leaves are of the same order of abundance as the nonflavonoid glucosides previously isolated from the lead-soluble fraction.

The solubility information from the extraction procedures used in this study demonstrated that the bulk of the material was not soluble in ligroin, or carbon tetrachloride and only partly soluble in benzene or ether. The material was somewhat soluble in ethyl acetate and very soluble in water. This indicated that at least part of the material was macromolecular, possibly containing polyphenols.

An alkaline hydrolysis of the various extracts demonstrated that the unidentified part of the lead-insoluble material was composed of C_6-C_1 and C_6-C_1 units. Vanillic acid, ferulic acid, _p_-hydroxybenzoic acid, _p_-coumaric acid, and salicyl alcohol were identified in the hydrolyzate by paper chromatography. It follows from these observations that part of the unidentified material was noncarbohydrate, macromolecular, and contained _p_-hydroxyphenyl and 4-hydroxy-3-methoxyphenyl moieties.

This study demonstrated that a lead subacetate treatment of a water extract produced a chemical fractionation of the materials in the extract. It can be concluded that in future investigations of plant organ extracts the lead-insoluble components must be studied along with the lead-soluble components. This would prevent the compounds and materials such as those isolated in the present study from being overlooked.

INTRODUCTION

For many years chemists have studied the extracts of leaves and barks. Traditionally, the aqueous extracts have been purified by treatment with lead subacetate (1,2). This treatment was conducted to remove interfering materials from the aqueous extracts. The lead subacetate treatment produced a lead-soluble fraction and a lead-insoluble fraction. The water extract was considered to be purified after the removal of the lead-insoluble materials. The lead-insoluble fraction was thought to contain polyphenols and was usually discarded without further study.

Recent studies on leaves and barks indicated that a large portion of the material in the aqueous extract was precipitated by the addition of lead subacetate (3). Thus, the studies of the extracts of leaves and barks are incomplete unless the nature of the lead subacetate precipitate is studied along with the lead subacetate filtrate. It was the intent of the present study to examine the nature of this previously discarded precipitate.

The present study was limited to the examination of the lead subacetate precipitate of the hot water extract of the leaves of quaking aspen (*Populus tremuloides*). Quaking aspen was chosen for two reasons. First, this species is important commercially. In 1964, 48% of the pulpwood produced in the Lake States (Michigan, Wisconsin, and Minnesota) was aspen (4). Second, there has been considerable work on the extracts of the various organs of this species.

HISTORICAL REVIEW

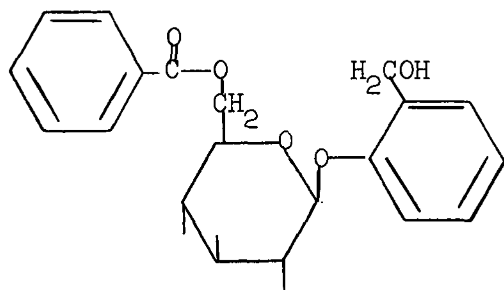
The lead subacetate purification procedure has been used extensively in the study of the glycosides of the family Salicaceae. Of particular interest are the studies of the leaves of quaking aspen and a related species, bigtooth aspen (*P. grandidentata*).

Pearl, Darling, and Justman (5) studied the hot water extract of the leaves of quaking aspen. They treated the extract while hot with lead subacetate to produce a lead subacetate-soluble fraction and a lead subacetate-insoluble fraction. The lead-soluble materials produced crystalline populin after deleading with hydrogen sulfide. The lead-insoluble materials were extracted with ether after deleading with hydrogen sulfide. A paper chromatographic study of the ether extract demonstrated the presence of acetovanillone, vanillin, vanillic acid, ferulic acid, p-hydroxybenzoic acid, and p-coumeric acid. A later study of the hot water extract of quaking aspen leaves showed that the lead-soluble fraction would yield crystalline tremuloidin if the lead subacetate treatment were conducted after cooling the hot water extract (6). This study demonstrated that a hot lead subacetate solution would convert tremuloidin(salicin 2-monobenzoate) into populin(salicin 6-monobenzoate) by migration of the benzoyl group.

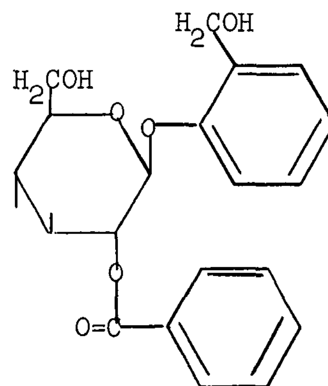
More thorough investigations have been conducted on the nature of the components of the leaves of bigtooth aspen. In one study (5), the hot water extract of bigtooth aspen leaves was divided into a lead subacetate filtrate and a lead subacetate precipitate. The lead-soluble components produced crystalline populin and salicin after deleading with hydrogen sulfide. A paper chromatographic study of the lead subacetate filtrate indicated the presence of sucrose, glucose, and fructose. The lead-insoluble components were extracted with ether after deleading with hydrogen sulfide. The ether extract was then examined by paper chromatography. Vanillin, syringaldehyde, acetovanillone, vanillic acid, syringic acid, p-hydroxybenzoic acid, p-coumaric acid, and ferulic acid were found in the ether extract.

In other investigations of the water extract of bigtooth aspen leaves, the lead-insoluble materials were studied further (7,8). In these works, the lead-

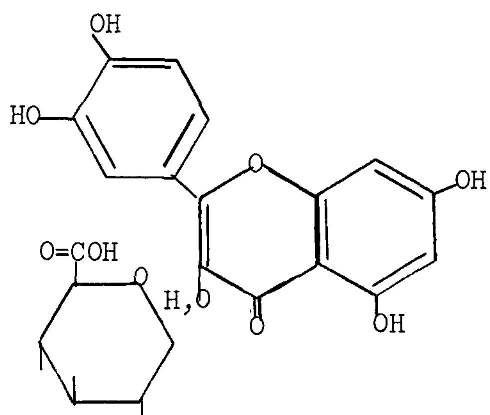
insoluble component, after reconstitution with hydrogen sulfide, yielded crystalline quercetin-3-glucosiduronic acid.



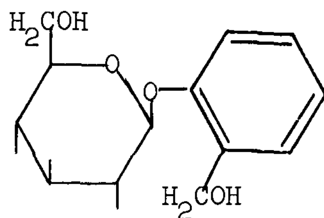
Populin



Tremuloidin



Quercetin-3-glucosiduronic Acid



Salicin

SUMMARY OF THE LITERATURE

Although the hot water extract of the leaves of quaking aspen has been studied, little is known about the composition of the lead subacetate precipitate of this extract.

GENERAL METHODS

COLUMN FRACTIONATION

Partition and absorption column chromatography was used to fractionate mixtures. The object of the fractionation was to obtain pure compounds. Woelm polyamide, silica gel, and magnesium silicate and Whatman cellulose powder were used as packings for the columns. The packed portion of the columns varied in size from 20 to 40 mm. in inside diameter and from 200 to 400 mm. in length. All columns were equipped with a glass capillary tube bent into an "S" shape to prevent the developer level from falling below the surface of the column packing.

The column fractionation was accomplished by placing an aliquot of the material to be investigated on a column. Initially, very small effluent volumes (25 to 50 ml.) were collected from the column. Each effluent volume was examined by either paper or thin-layer chromatography. Each effluent volume was concentrated to a 20-ml. volume under reduced pressure and allowed to evaporate spontaneously at room temperature to promote crystallization. If an individual effluent volume did not produce crystals, then it was combined with other effluent volumes with identical composition (the fractionation produced effluent volumes containing mixtures of unknown materials). These combined effluent volumes constituted the fractions of the column. In several instances the fractions produced crystals upon concentration. As the fractionation of the column continued, it was found that larger effluent volumes could be collected without mixing fractions.

Each polyamide column was prepared by allowing a dilute slurry of polyamide in degassed distilled water to settle onto a glass fiber mat or a porous glass septum located at the bottom of a glass tube. After forming, the column was washed with ethanol, acetone, and distilled water. Glass beads were placed on

the top of the packing to prevent it from being disturbed by the addition of fresh developer. Mixtures to be fractionated were dissolved in a minimum amount of a good solvent and placed on the top of the column. The column was then eluted with water, ethanol – water (1:4)*, ethanol – water (1:1), ethanol – water (4:1), and finally absolute ethanol.

Cellulose columns were prepared by dry packing. Successive addition and tamping of cellulose powder with a glass rod resulted in uniformly packed columns. After packing, the columns were evacuated with a water aspirator to remove trapped air. Degassed distilled water was then introduced slowly into the bottom of the column until the void spaces were filled with water. The columns were ready for use after washing with ethanol and water. Cellulose columns were eluted with the same sequence of developers used for the polyamide columns.

Partition column chromatography utilizing silica gel or magnesium silicate was attempted. No successful sequence of developers could be found for the magnesium silicate. A developer consisting of chloroform – methanol (4:1) was found to be somewhat successful for silica gel.

THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography (9) was used to monitor the column fractionation. The thin-layer plates were developed by the ascending technique in an atmosphere saturated with the developing solvent. The plates were developed with either a chloroform – methanol (4:1) mixture containing 10 ml. of acidic acid per 250 ml. of the mixture or a water – ethanol-benzene (2:11:21) mixture.

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*Ratios indicate volume to volume mixtures.

The movements of the materials spotted on the plates were detected by spraying the plates with 50% sulfuric acid and placing them in a 110°C. oven for 3-5 minutes. The plates were then examined with visual and ultraviolet light.

PAPER CHROMATOGRAPHY

Paper chromatography was used to monitor the column chromatographic fractionation and to provide tentative identification of compounds. Whatman no. 1 paper was used for all chromatograms.

The developers used for paper chromatography were as follows:

(a) the upper phase of _n_-butanol – acetic acid – water (4:1:5) mixture (10,11);

(b) the upper phase of _n_-heptane – _n_-butyl ether – water (6:1:1) (12);

(c) _n_-butanol saturated with 2% aqueous ammonia (13);

(d) _n_-butanol – pyridine – water (10:3:3) (13);

(e) phenol – water (4:1) (weight-to-weight ratio) (14);

(f) ethyl acetate – pyridine-water (8:2:1) (15);

(g) pyridine – ethyl acetate – acetic acid – water (5:5:1:3) (16);

(h) acetic acid – water (1:99).

The spray reagents used for paper chromatography were as follows:

(a) aniline hydrogen phthalate reagent (17);

(b) _p_-anisidine hydrochloride reagent (18);

(c) silver nitrate reagent (19);

(d) urea phosphate reagent (20);

(e) diazotized _p_-nitroaniline reagent (21);

(f) 5% ethanolic aluminum chloride reagent (11);

(g) 5% aqueous sodium carbonate reagent (11);

(h) ferric chloride-potassium ferricyanide reagent (11);

(i) 2,4-dinitrophenylhydrazine reagent (13);

(j) 0.04% aqueous Brom Cresol Green.

WEIGHTS AND YIELDS

All weights reported for sirups, liquids, and leaves were on an ovendry basis except where noted. The yields of the various isolated and identified compounds were calculated with the airdry weight of the isolated material, except for pectin which was dried under vacuum in a vacuum desiccator over phosphorus pentoxide, and the ovendry weight of the leaves.

EXPERIMENTAL PROGRAM

INTRODUCTION

There were two sources of starting material for this study. The first source was a water extract prepared from an ethanol extract of quaking aspen leaves. The second source was a direct hot-water extract of quaking aspen leaves. Each sample was processed in a slightly different manner. Because of this, the experimental program will be divided into two segments, one for each starting material.

The first segment (Processing of Ethanol Extract) deals with the fractionation and identification of the compounds found in the reconstituted lead subacetate precipitate from the water extract of the ethanol extract of quaking aspen leaves. The second segment (Processing of Water Extract) deals with the fractionation and identification of the compounds in the reconstituted lead subacetate precipitate of the hot-water extract of quaking aspen leaves.

PROCESSING OF ETHANOL EXTRACT

ETHANOL EXTRACTION OF QUAKING ASPEN LEAVES

On June 17, 1963, 3070 g. of leaves, 9190 g. fresh, were collected from a single quaking aspen tree. The tree was growing in a mixed stand of quaking aspen and bigtooth aspen. The tree was approximately 40 years old. The tree was growing in Forest County, Wisconsin, civil town of Wabeno.

The leaves were packed in five-gallon pickle jars and batch-extracted with ethanol. Four extractions were conducted over a sixty-day period. The combined extract contained 793 g. solids. A 1000-g. portion of the extract containing 150 g. solids was concentrated to a thick sirup under reduced pressure and used as the starting material in the first part of the experimental program.

WATER EXTRACTION AND LEAD SUBACETATE PRECIPITATION

The concentrated ethanol extract of quaking aspen leaves was used as the starting material for this segment of the experimental program. The sirupy ethanol extract was shaken with boiling water for ten minutes. After cooling, the water-soluble material was separated from the water-insoluble material by filtering though a Celite mat. A second batch of water-soluble material was collected by repeating the procedure. The water extracts were combined and concentrated under reduced pressure to a 2690-ml. volume, 124 g. solids.

The lead subacetate precipitate was formed by adding 97.5 g. of lead subacetate as an aqueous slurry to the concentrated water extract. This resulted in the formation of a bright orange precipitate that separated from a clear, slightly yellow supernatant liquid. The supernatent liquid was discarded and the precipitate was collected.

Before the lead subacetate precipitate could be studied, it was necessary to remove the ionic lead. The lead salt was decomposed by treating with hydrogen sulfide. The treatment was conducted by bubbling hydrogen sulfide gas through a highly agitated slurry of the lead subacetate precipitate in three liters of water. The resulting lead sulfide precipitate was removed and discarded. The remaining dark red filtrate was heated to boiling to remove dissolved hydrogen sulfide gas. The filtrate was concentrated under reduced pressure to yield the reconstituted lead subacetate precipitate (Fraction E) which contained 31.6 g. solids (see Fig. 1).

FRACTIONATION BY EXTRACTION

Fraction E was divided into five parts by extracting with ligroin, carbon tetrachloride, benzene, and ethyl acetate. All extractions were accomplished

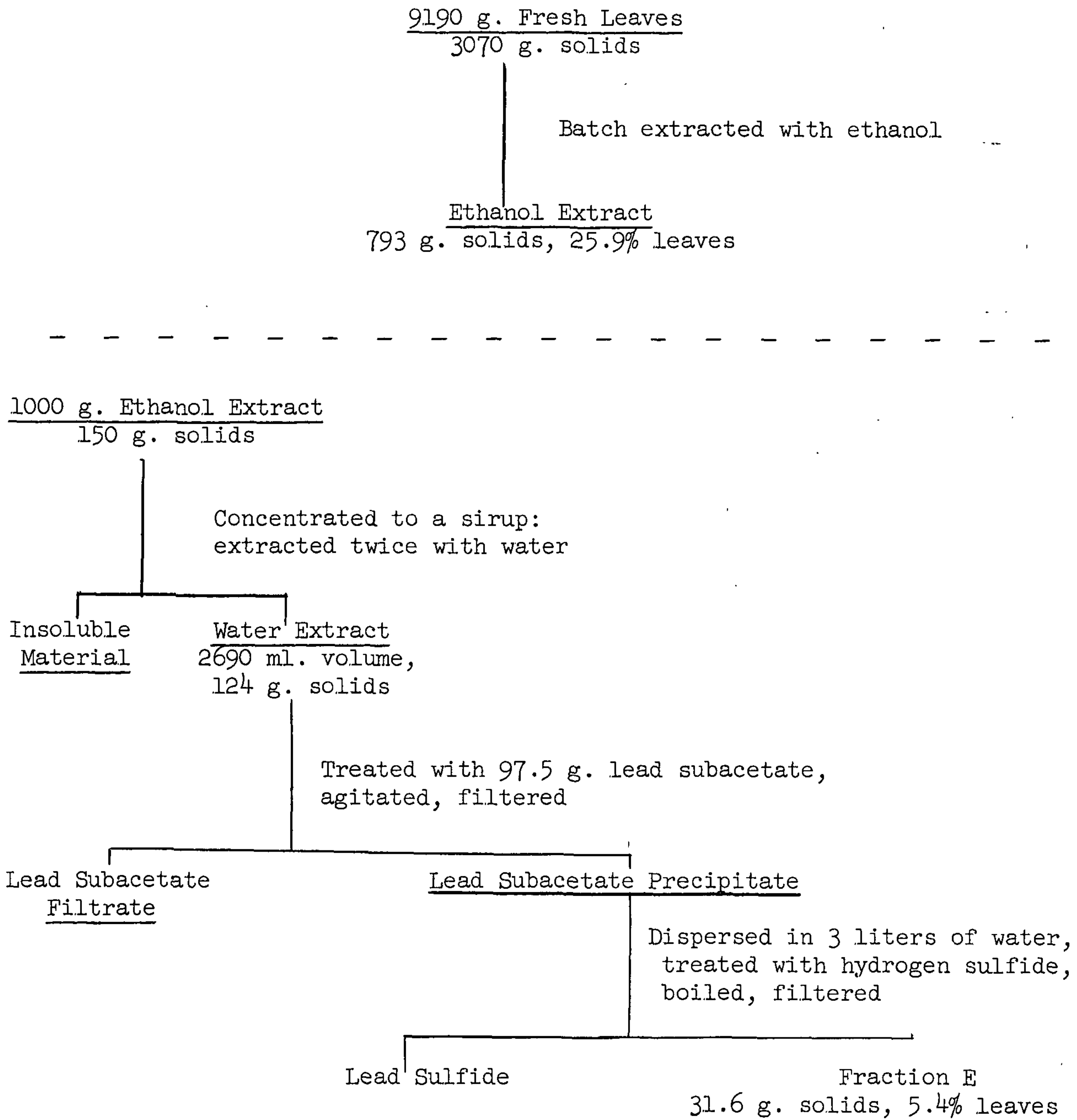


Figure 1. Steps Leading to the Formation of Fraction E

by shaking the entire Fraction E, approximately one liter volume, with several aliquots of each solvent in a 2-liter separatory funnel.

The processes leading to the formation of the four extracts and the raffinate are summarized in Fig. 1 and 2.

The four extracts and the raffinate were examined by paper chromatography. All extracts and the raffinate were found to be complex mixtures. The ligroin extract E-1, the carbon tetrachloride extract E-2, and the benzene extract E-3 were not further studied since together they contained less than 0.75% of Fraction E. The ethyl acetate extract E-4 and the raffinate E-5 were fractionated by polyamide column chromatography.

ETHYL ACETATE EXTRACT E-4

Polyamide column chromatography of the ethyl acetate extract E-4 produced twenty-seven different fractions. Each fraction contained a different mixture of compounds. Three of the fractions yielded colorless crystals that were collected and identified.

A thin-layer chromatogram of the first compound (m.p. 205 to 207°C.) produced a red-brown spot $\underline{R}_{\underline{f}}$ 0.52 when developed with chloroform-methanol (4:1). A thin-layer chromatogram of authentic tremuloidin (m.p. 205 to 207°C.) also produced a red-brown spot $\underline{R}_{\underline{f}}$ 0.48. The material was identified as tremuloidin after comparing its infrared spectrum with that of authentic tremuloidin.

The second crystalline material (m.p. 178.5 to 179.5°C.) was identified as populin (m.p. 178.5 to 179.5°C.). A mixture of the isolated material and authentic populin did not produce a depressed mixed melting point. A thin-layer chromatogram of the isolated material and authentic populin produced red-brown spots, $\underline{R}_{\underline{f}}$ 0.65,

Fraction E

31.6 g. solids

Extracted with 3 200-ml. volumes of ligroin
Extracted with 1 100-ml. volume of ligroin

Ligroin Extract E-1
0.024 g. solids
0.1% of Fraction E

Extracted with 4 150-ml. volumes of carbon tetrachloride

Carbon Tetrachloride Extract E-2
0.12 g. solids
0.4% of Fraction E

Extracted with 4 150-ml. volumes of benzene

Benzene Extract E-3
0.068 g. solids
0.2% of Fraction E

Extracted with 4 200-ml. volumes of ethyl acetate
Extracted with 4 150-ml. volumes of ethyl acetate

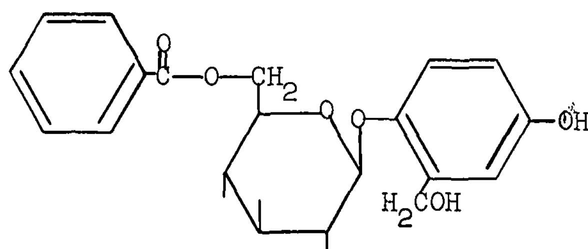
Ethyl Acetate Extract E-4
6.5 g. solids
20% of Fraction E

Raffinate E-5
25 g. solids
79% of Fraction E

Figure 2. Separation of the Materials in Fraction E by Extraction

when the plates were developed with chloroform – methanol (4:1). The identity of the isolated material was established after comparing its infrared spectrum with that of authentic populin.

The third group of crystals which shrank at 159°C. and melted at 203 to 204°C. was identified as salireposide. A sample of authentic salireposide shrank at 155°C. and melted at 201 to 202°C. A mixture of the isolated material and authentic salireposide melted at 201 to 202°C. When examined by thin-layer chromatography using the chloroform – methanol (4:1) developer, the isolated material and authentic salireposide both produced orange spots with $\underline{R}_f$ 0.47 and 0.42, respectively. As before, the identity of the material was established by comparing the infrared spectra of the isolated and authentic material.



Salireposide

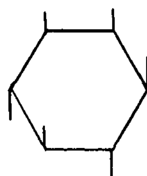
RAFFINATE E-5

The raffinate E-5 from the solvent extraction of Fraction E was divided into two parts. The first part was fractionated by polyamide column chromatography. This resulted in the formation of twenty-one different fractions. A paper chromatographic study of the first two fractions, which yielded sirups upon concentration, indicated the presence of glucose, fructose, and glucuronic

acid. The tentative identification was accomplished by developing the chromatograms with n-butanol – acetic acid – water (4:1:5) and employing the silver nitrate, urea phosphate, and p-anisidine hydrochloride reagents to detect the materials.

The second part of the raffinate E-5 was fractionated chemically by adding lead acetate to the raffinate. This treatment resulted in the formation of a lead acetate precipitate and a lead acetate filtrate. The lead acetate filtrate was treated with hydrogen sulfide, boiled, and filtered to produce the lead acetate-soluble fraction E-5-A. The lead acetate precipitate from the lead acetate treatment of the aqueous raffinate E-5 was not studied further.

The polyamide column fractionation of the lead acetate-soluble fraction E-S-A yielded nineteen different fractions. All fractions were mixtures of several different components. Only one fraction produced pure crystals (m.p. 219°C.). The isolated material did not produce a spot when examined with thin-layer chromatography but did give a spot, R_f 0.03, when a paper chromatogram of the material was developed with n-butanol – acetic acid – water (4:1:5). The material was detected on paper chromatograms with silver nitrate reagent. Authentic myo-inositol (m.p. 217 to 219°C.) behaved in an identical fashion when examined by paper and thin-layer chromatography. A mixture of the isolated material and authentic myo-inositol melted at 217.5 to 219.5°C. Paper chromatograms of the isolated and authentic material were developed with phenol – water (4:1), a developer which is capable of separating all eight inositol isomers (14). It was found that both of the materials moved with R_f 0.22 (literature value for myo-inositol R_f 0.21). The infrared spectra of the isolated and authentic myo-inositol were identical.



myo-Inositol

No other pure materials were isolated from Fraction E. Several yellow solids believed to be mixtures of flavonoids were collected, but the individual components of these mixtures could not be separated. The compounds isolated and identified in Fraction E are summarized in Table III (page 46).

PROCESSING OF WATER EXTRACT

HOT-WATER EXTRACTION OF QUAKING ASPEN LEAVES

On June 14, 1965, 2990 g. of leaves, 7770 g. fresh, were collected from a clone of male quaking aspen trees. Since all the trees were harvested from the same clone, the trees possessed an identical heredity. The 20-year old trees were growing on a tree farm operated by The Institute of Paper Chemistry. The farm is located near Greenville in Outagamie County, Wisconsin.

The hot-water extract was formed by placing 1000-g. portions of the fresh leaves in 10-liter stainless steel buckets and covering them with preheated, distilled water. The buckets were then heated until the water began to boil, a time interval of about 15 minutes. The heating was continued for an additional 15 minutes after the start of boiling. The resulting water extract was collected by filtering through cloth. The once-extracted leaf pulp was re-extracted with one half the amount of hot water, following the same procedure. All extracts were combined to yield 78 liters of a yellow-green turbid solution. The time lapse between the collection and the extraction was less than six hours.

LEAD SUBACETATE PRECIPITATION

The lead subacetate precipitate was formed by adding a slurry of lead subacetate to the hot-water extract of fresh quaking aspen leaves. A total of 583 g. of lead subacetate was required to complete the precipitation. As before, the bright orange precipitate settled rapidly leaving a clear, slightly yellow supernatant liquid. The precipitate was collected by filtration and the supernatant liquid was discarded.

The lead subacetate precipitate was dispersed in ten liters of water and treated with hydrogen sulfide while being stirred violently. The resulting dark red-brown solution of the reconstituted lead subacetate precipitate was boiled to remove dissolved hydrogen sulfide and filtered to remove the lead sulfide precipitate. The filtered solution was concentrated under reduced pressure to yield the lead-insoluble fraction (Fraction W), 186 g. The steps leading to the formation of the Fraction W are summarized in Fig. 3.

A more carefully determined material balance for the processes of hot-water extraction and lead subacetate precipitation was obtained by repeating the procedure on a small scale. The leaves for the extraction were collected from the same clone of quaking aspen, frozen in tightly sealed plastic bags, and stored in the frozen state until needed. The conditions used for this process were scaled to the conditions used previously. The results of this study are presented in Fig. 4.

LEAD SULFIDE PRECIPITATE

The lead sulfide precipitate formed during the treatment of the lead subacetate precipitate was examined for the presence of organic material. The precipitate was extracted with ethanol and ethanol – benzene mixtures. A thin-layer chromatographic study of the extracts demonstrated the absence of organic material.

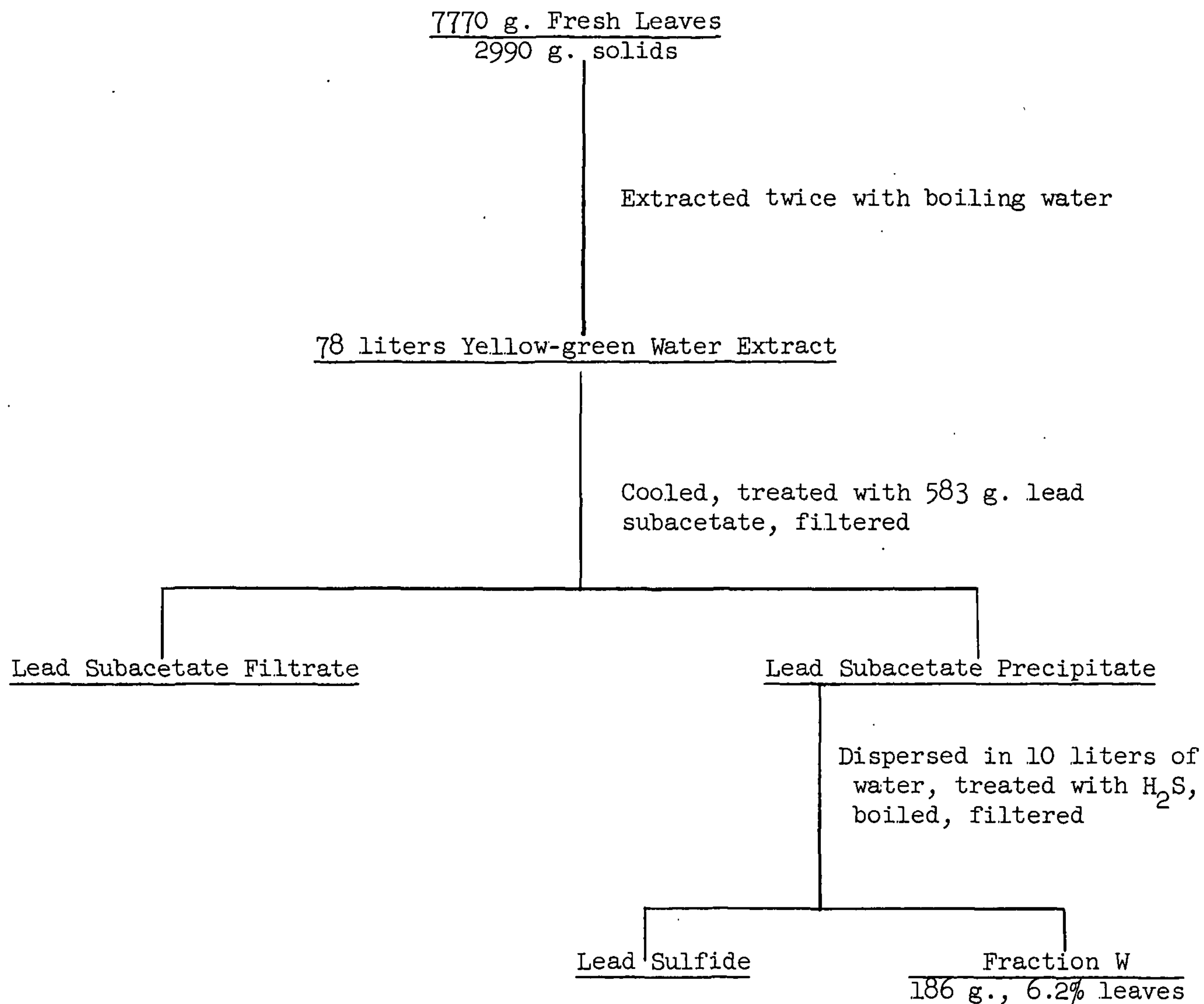


Figure 3. Steps Leading to the Formation of Fraction W

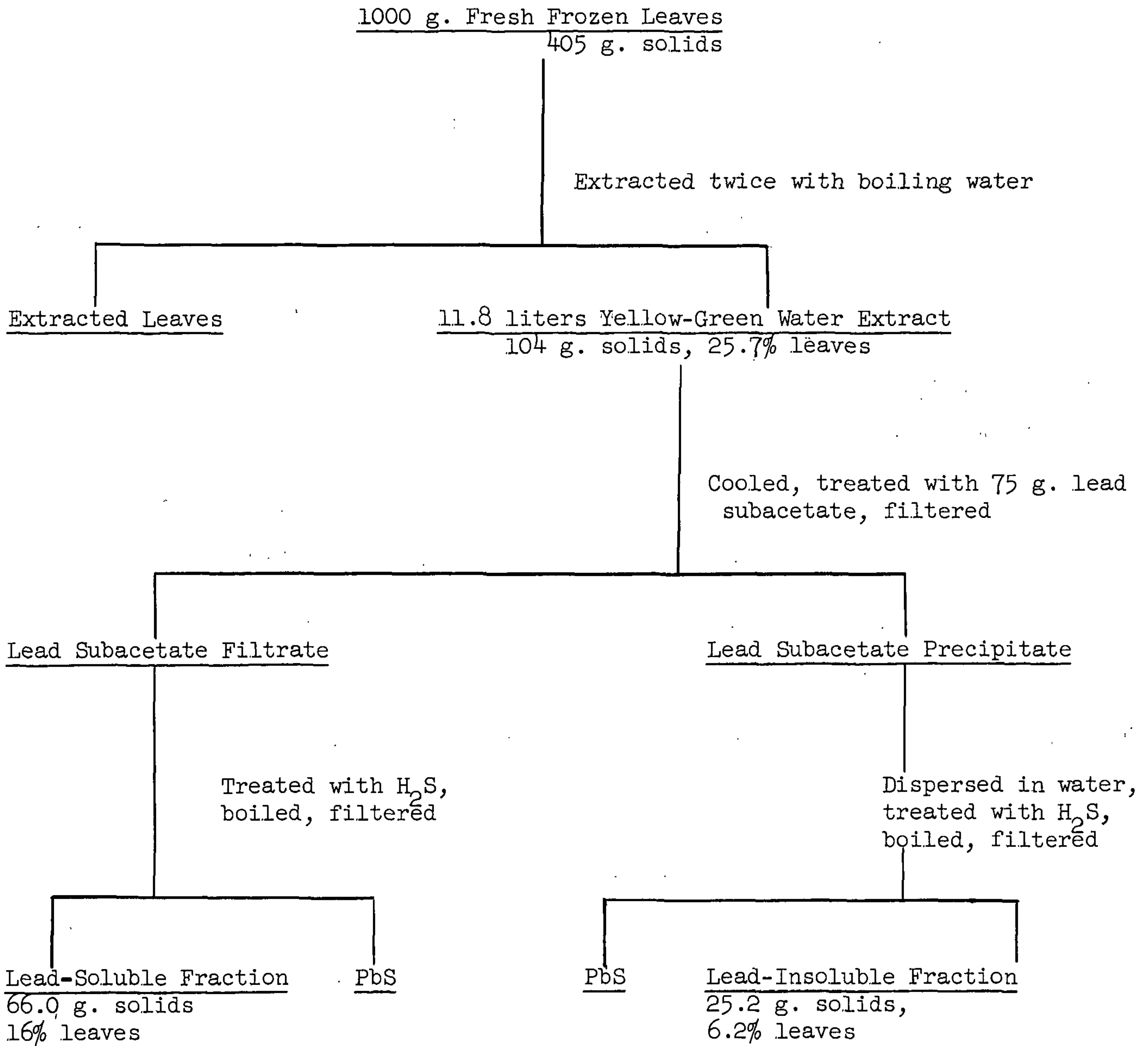


Figure 4. Flow Diagram of Small-Scale Extraction and Lead Subacetate Precipitation Procedure

FRACTIONATION BY EXTRACTION

Fraction W was divided into three parts by extracting with benzene and ethyl acetate. As before, the extractions were accomplished by shaking the entire Fraction W (approximately 2.5 liter volume) with several aliquots of each solvent.

The benzene extract W-1 was produced by shaking Fraction W with five 250-ml. volumes of benzene. The raffinate was then shaken with six 500-ml. volumes of ethyl acetate to produce the ethyl acetate extract W-2 and the raffinate W-3.

The raffinate W-3 was examined for the presence of inorganic material by conducting a sulfated ash determination. Tared crucibles containing aliquots of the raffinate and excess sulfuric acid were heated until all organic material was decomposed. Final ignition was carried out at 800 C. in an electric muffle furnace. The raffinate W-3 was found to contain 2.2% ash.

A material balance for the extraction is presented in Fig. 5. The sum of the ovendry weights of the three fractions was more than 186 g. It seemed likely that incomplete removal of water or solvent from one or more of the fractions was responsible for the increase in weight. The percent values given are based on the total ovendry weight of the fractions and are therefore only approximate.

BENZENE EXTRACT W-1

The entire benzene extract W-1 was concentrated and placed on a silica gel column. The column was eluted with chloroform – methanol (4:1). Most of the material moved rapidly through the column. Only three different fractions, each a complex mixture, were obtained. The second fraction produced colorless needle-like crystals from a dark oil. It was found that the crystals could be separated from the oil by extracting with petroleum ether.

Fraction W
186 g. solids

Extracted with 5 250-ml. portions of benzene

Benzene Extract W-1
3%

Extracted with 6 500-ml. portions of ethyl acetate

Ethyl Acetate Extract W-2
18%

Raffinate W-3
79%

Figure 5. Separation of the Material in Fraction W by Extraction

The crystalline material, m.p. 102 to 104°C., was identified as pyrocatechol. Tentative identification was made by paper and thin-layer chromatography. Color reactions with silver nitrate reagent and diazotized paranitroaniline reagent were used to detect the material on paper chromatograms. When the isolated material and authentic pyrocatechol, m.p. 104°C., were mixed, the mixed melting point was not depressed. The identity of the material was established by comparing the infrared spectrum of the isolated material with the spectrum of authentic material.

The benzene extract W-1 was examined for the presence of fatty acids by thin-layer chromatography. No spots corresponding to fatty acids could be detected when the plates were developed with hexane.

ETHYL ACETATE EXTRACT W-2

The ethyl acetate extract W-2 was fractionated by polyamide column chromatography. Two fractions yielded colorless crystals and several fractions yielded yellow powders. A total of twenty-three different fractions were collected. The colorless crystalline compounds were identified as pyrocatechol, also found to be a component of the benzene extract, and succinic acid.

The isolated succinic acid, m.p. 182 to 183°C., was collected from a dark tar by spreading the tar-crystal mixture over a porous clay plate. The tar flowed into the plate, leaving the crystals on the surface. A paper chromatogram of the isolated material was developed with *n*-butane – pyridine – water (10:3:3) and sprayed with Brom Cresol Green Reagent. Authentic oxalic acid and succinic acid, m.p. 182 to 184°C., were also spotted on the chromatogram. The isolated and authentic succinic acid moved the same distance from the starting

line, while the oxalic acid moved only 20% as far. The infrared spectrum of the isolated material and authentic succinic acid were identical.

The yellow powders from the polyamide column fractionation were also studied. All of these powders produced several spots when examined with either paper or thin-layer chromatography. Several of the powders were suspected to be mixtures of flavone or flavonol glycosides. Paper chromatograms of these powders developed with acetic acid – water (1:99) produced yellow-colored spots. When exposed to ammonia or sprayed with aluminum chloride reagent, the yellow colors became more vivid. Examination of these spots under ultraviolet light revealed a dark brown color when no spray was used and a bright yellow color when the spot was exposed to ammonia or sprayed with aluminum chloride reagent. These color properties are consistent with the behavior of flavone or flavonol glycosides under the same conditions (<u>11</u>).

RAFFINATE W-3

The raffinate W-3 was fractionated by polyamide and cellulose column chromatography. The raffinate was found to contain a polymeric material composed of sugars and uronic acids, several flavonoid glycosides and flavonoids.

The first fraction eluted from the polyamide column yielded a sirup upon concentration. Attempts to recover the solid by concentration failed. The solid did precipitate, however, upon the addition of ethanol. After collection, the precipitate dried into a horny solid that could not be redissolved in water.

The solid could be collected as a white powder by a freeze exchange technique.* An aqueous solution of the material was frozen and placed in a large beaker of 0°C.

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*James Manning, Personal communication, 1965.

acetone. The water was replaced by the acetone to yield the solid as a white powder. After washing with fresh acetone, the powder was dried in a vacuum desiccator over phosphorus pentoxide. The resulting material was found to be water soluble.

Paper chromatograms of the material were developed with n-butanol – acetic acid – water (4:1:5) and treated with p-anisidine hydrochloride reagent, urea-phosphate reagent, and silver nitrate reagent. All sprays showed one spot, R_f 0.00. A naphthoresorcinol test for uronic acids was negative for the material.

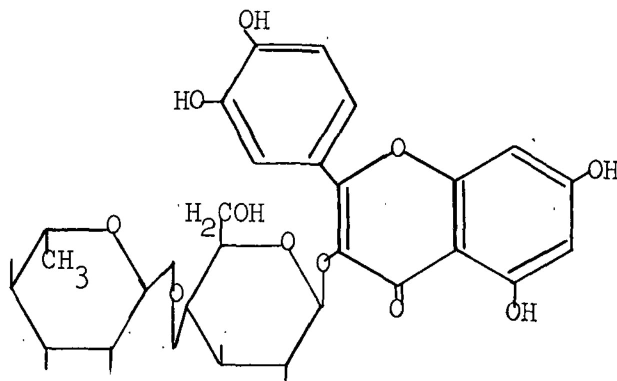
The material was hydrolyzed enzymatically and with acid. The enzymatic hydrolysis was conducted by adding a 50-mg. quantity of Pectinol 10-M (Rohm & Haas) to 100 mg. of the material dissolved in 20 ml. of water (pH adjusted to 5.5 with sodium hydroxide and acetic acid solutions). After two hours of digestion at room temperature, the mixture was filtered to yield the hydrolyzed sugars and uronic acids. The naphthoresorcinol test for uronic acids was then found to be positive for the hydrolyzate.

The acid hydrolysis was conducted by dissolving 340 mg. of the polymeric material in 1 ml. of 72% sulfuric acid. The dissolved material was diluted to a 24-ml. volume with distilled water and boiled for three hours to complete the hydrolysis. The sulfuric acid was then neutralized by adding an equimolar quantity of calcium carbonate. The hydrolyzed sugars and uronic acids were separated from the calcium sulfate precipitate by filtering.

The hydrolyzates were examined by paper chromatography. The paper chromatograms were developed with ethyl acetate – pyridine – water (8:2:1) and ethyl acetate – pyridine – acetic acid – water (5:5:1:3) and sprayed with p-anisidine-hydrochloride reagent and aniline phthalate reagent. Authentic glucose, galactose,

mannose, arabinose, xylose, rhamnose, glucuronic acid, and galacturonic acid were spotted on the chromatograms as known compounds. Chromatographic evidence was found for the presence of galactose, glucose, mannose, arabinose, glucuronic acid, and galacturonic acid. As determined by the intensity of the corresponding spot, galacturonic acid was the major component, while arabinose and mannose were the most abundant and the least abundant sugars, respectively. Additional spots for unknown materials were also present.

The raffinate W-3 was found to contain several flavonoid glycosides. The first glycoside identified was rutin. Rutin is quercetin-3-β-rutinoside (22). Quercetin is a 3,5,7,3',4'-pentahydroxyflavone, and rutinose is a disaccharide composed of glucose and rhamnose (23). The structure of rutinose has been studied and has been demonstrated to be 6-O-α-L-rhamnopyranosyl-D-glucopyranose (22).



Rutin

When heated, the isolated compound sintered at 184°C. and melted at 196°C. Authentic rutin sinters at 185°C. and melts from 190 to 192°C. (24). When the isolated material was examined by paper chromatography, one yellow spot was detected. The material yielded R_f values of 0.28 and 0.38 when paper chromato-

grams were developed with acetic acid – water (1:99) and *n*-butanol – acetic acid – water (4:1:5), respectively. According to Harborne (11) the R_f values for rutin are 0.26 and 0.38 when paper chromatograms are developed with water and with *n*-butanol – acetic acid – water (4:1:5), respectively.

When a solution of the isolated rutin was dried on paper, it could be detected as a yellow spot. If the area was exposed to ammonia fumes or sprayed with aluminum chloride reagent, the yellow color became more intense. When examined under ultraviolet light, the area appeared brown. If exposed to ammonia fumes or sprayed with aluminum chloride reagent and examined under ultraviolet light, it appeared as a bright yellow fluorescent spot. These color reactions are characteristic of the flavone and flavonol glycosides (11). The identity of the isolated rutin was tentatively established by melting point data, paper chromatographic R_f values, color reactions, and by the use of ultraviolet absorption spectra.

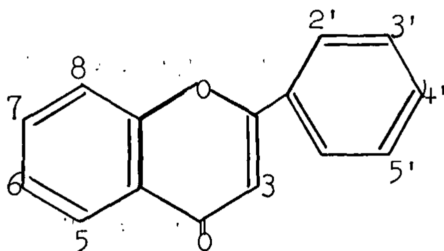
The experimental techniques used to determine the ultraviolet spectra were patterned on the experimental procedure developed by Jurd and Horowitz (25). Spectral curves were obtained from a solution of the unknown material in absolute ethanol. Beckman fused quartz cells were used to hold the solutions. The spectra were determined at room temperature by using either a Cary Recording Spectrophotometer or a Beckman Ratio Recording Spectrophotometer Model DK-2.

Three different spectral curves were obtained for the isolated rutin. A stock solution was made by dissolving 0.5 mg. of the rutin in 10 ml. of absolute ethanol. The solution for the first curve was made by diluting 2 ml. of the stock solution to 10 ml. with absolute ethanol. For the second curve, a mixture of 4 ml. of the stock solution and 10 drops of a saturated solution of fused sodium acetate in absolute ethanol was diluted to 10 ml. with absolute ethanol.

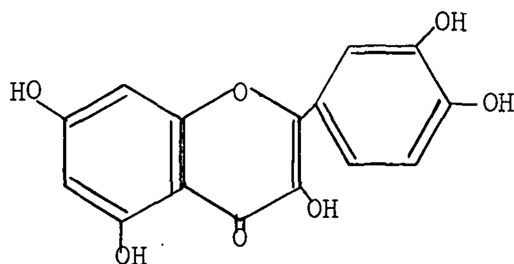
The solution for the third curve was made by adding 4 ml. of the stock solution to 10 drops of a 5% alcoholic aluminum chloride solution and diluting the mixture to 10 ml. with absolute ethanol.

The spectrum of the isolated rutin in ethanol was very similar to the spectra of known quercetin-3-glycosides. The spectrum of the isolated rutin yielded peaks of maximum absorption ($\gamma_{max.}$) at 258 and 360 nm. Quercetin-3-methyl ether ($\gamma_{max.}$ 258, 360 nm.), quercetin-3-rhamnoside ($\gamma_{max.}$ 258, 350 nm.), quercetin-3-L-arabinoside ($\gamma_{max.}$ 260, 360 nm.), quercetin-3-glucoside ($\gamma_{max.}$ 258, 360 nm.), and rutin ($\gamma_{max.}$ 258, 361 nm.) have very similar ultraviolet spectra (25,26).

The ultraviolet spectrum of the isolated material in alcoholic sodium acetate solution contained two peaks with maximum absorption at 268.5 and 384 nm. The 10.5 nm. shift of the lower wavelength band found for the isolated material indicated that the 7-position contained a free hydroxyl group (26).



The ultraviolet spectrum of the isolated material in alcoholic aluminum chloride solution contained two peaks with the wavelengths of maximum absorption occurring at 275.5 and 434 nm. The 74 nm. shift of the higher wavelength band indicated the presence of a free hydroxyl group in the 3-position (26). Because of this, it is believed that the glycoside was contaminated with its aglycone, quercetin. Paper chromatography, however, did not indicate the presence of quercetin.

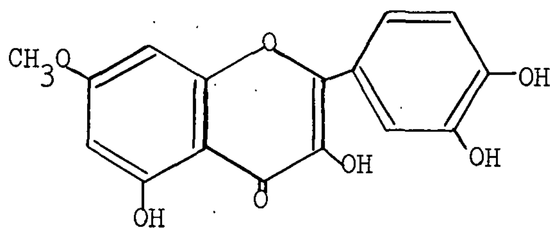


Quercetin

An acid hydrolysis was used to examine the nature of the carbohydrate portion of the isolated material. The hydrolysis was conducted by dissolving 4 mg. of the material in 12 drops of 3% hydrochloric acid solution and heating on a steam bath under reflux for one hour. The hydrolysis products were collected by concentrating the hydrolyzate under reduced pressure. The resulting residue was dissolved in tetrahydrofuran-water (1:1) and examined by paper chromatography. The chromatogram was developed with ethyl acetate – pyridine – water (8:2:1) and the sugars were detected with silver nitrate reagent. The chromatogram indicated the presence of glucose and rhamnose.

The identity of the isolated rutin was established by comparing its infrared spectrum with the spectrum of commercial rutin. The spectra were identical.

The raffinate W-3 was also found to contain rhamnetin (3,5,3',4'-tetrahydroxy-7-methoxyflavone). This flavonoid aglycone was obtained as a yellow powder from the polyamide column fractionation of the raffinate. It was necessary to elute the column with ethanol – water (1:1) to remove this material.



Rhamnetin

The isolated material darkened when heated to 259°C. and melted sharply at 295°C. According to Gripenberg (23), rhamnetin melts from 294 to 296°C. When the isolated material was spotted on paper, the treated area appeared yellow under both ultraviolet light and ordinary light. The treated area appeared yellow-green in color under both ultraviolet light and ordinary light when exposed to ammonia fumes or sprayed with aluminum chloride reagent. A paper chromatographic examination of the isolated material showed that only one flavonoid material was present. When paper chromatograms were developed with acetic acid – water (1:99) and n-butanol – acetic acid – water (4:1:5), the material gave spots at R_f values of 0.00 and 0.75, respectively. According to Harborne (11), rhamnetin gives spots at R_f values of 0.00 and 0.72 in water and n-butanol – acetic acid – water (4:1:5) developers, respectively.

Various ultraviolet spectra were obtained for the isolated rhamnetin. As before, a stock solution was made by dissolving 0.5 mg. of the material in 10 ml. of absolute ethanol. The spectrum for the material in ethanol was determined with the solution resulting from the dilution of 2 ml. of the stock solution to 10 ml. with absolute ethanol. The resulting curve was very similar to a literature spectrum for authentic rhamnetin ($\gamma_{max.}$ 257, 371 nm. for the isolated material and $\gamma_{max.}$ 256, 371 nm. for authentic material) (25).

The ultraviolet spectrum of the isolated rhamnetin in alcoholic sodium acetate solution was determined with a solution made by adding 10 drops of a saturated sodium acetate solution to 2 ml. of the stock solution and diluting the mixture to 10 ml. with absolute ethanol. The wavelengths of maximum absorption were located at 257 and 376 nm. Since no shift of the lower wavelength peak was detected in the alcoholic sodium acetate solution, the compound did not contain a free hydroxyl group on the 7-position (26). Authentic rhamnetin in sodium acetate solution ($\gamma_{max.}$ 257 nm.) behaves in the same fashion (25).

The spectrum of the isolated rhamnetin in alcoholic aluminum chloride was determined with a solution made by adding 10 drops of 5% aluminum chloride in ethanol to 2 ml. of the stock solution and diluting the mixture to 10 ml. with absolute ethanol. The wavelengths of maximum absorption occurred at 268 and 429 nm. [268 and 429 nm. for authentic rhamnetin in alcoholic aluminum chloride solution (26)]. This shift of the higher wavelength band indicated the presence of a hydroxyl group in the 3-position (26).

The infrared spectrum of the isolated rhamnetin is presented in Fig. 6.

A second material believed to be a flavonoid was obtained by the polyamide column fractionation of the raffinate W-3. The isolated material was a yellow-gray solid that melted from 239 to 240°C. Color reactions of the material spotted on paper were not specific for any one class of flavonoid.

The isolated material was examined by paper chromatography. Paper chromatograms developed with n-butanol – acetic acid – water (4:1:5) and acetic acid – water (1:99) yielded one spot with R_f values 0.65 and 0.23, respectively.

The isolated material was subjected to an acid hydrolysis. The hydrolysis was conducted by dissolving 3 mg. of the material in 15 drops of 3% hydrochloric acid solution and heating on a steam bath under reflux for one hour. The hydrolyzate was examined for the presence of sugars by paper chromatography. No sugars were found.

The ultraviolet spectrum of the material in ethanol was determined. A stock solution was made by dissolving 1 mg. of the material in 10 ml. of absolute ethanol. The spectrum of the material was determined with the solution resulting from the dilution of 2 ml. of the stock solution to 10 ml. with absolute ethanol.

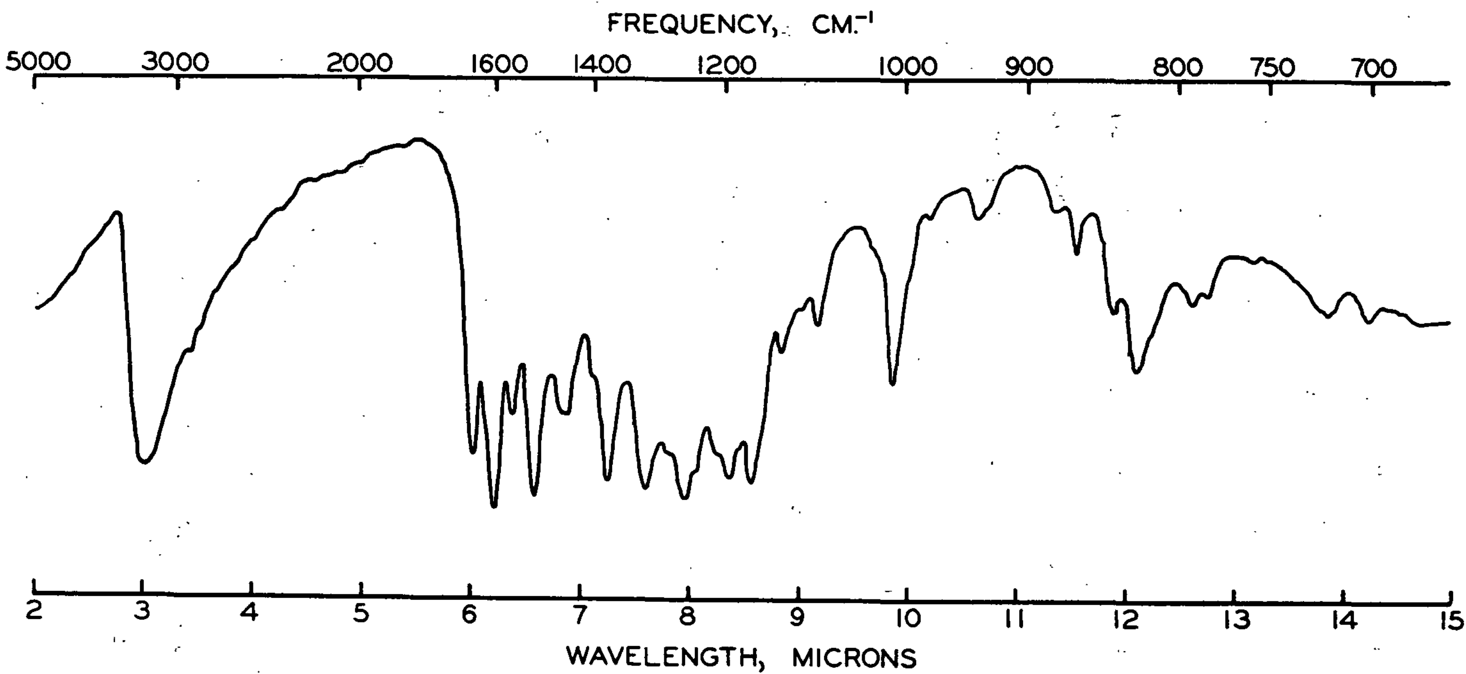


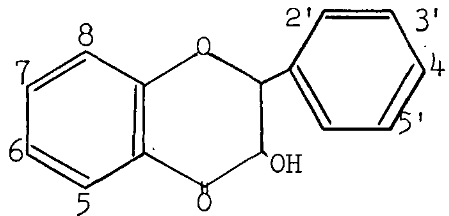
Figure 6. Infrared Spectrum of Isolated Rhamnetin

The resulting spectrum contained a peak at 292 nm. and an inflection at 330 nm. The spectrum was very similar in shape to the spectra of several flavanones and flavanonols (26).

The ultraviolet spectrum of the material was also determined in alcoholic sodium acetate solution and alcoholic aluminum chloride solution. The procedures used previously for making similar spectral solutions were followed. The spectrum in alcoholic sodium acetate contained two peaks at 297 and 330 nm. The spectrum in alcoholic aluminum chloride contained two peaks at 313 and 380 nm. The magnitude of the shift of the lower wavelength peak in alcoholic sodium acetate solution was 5 nm. According to Jurd (26), a 10-nm. shift of the lower wavelength peak indicates the presence of a hydroxyl group in the 7-position of a flavanone or a flavanonol while the absence of a shift of the lower wavelength peak indicates the 7-position does not contain a hydroxyl group. The 5-nm. shift detected for the isolated material revealed little about the structure of the material except that the material was probably not pure. The 50-nm. shift of the higher wavelength peak that was detected after the addition of alcoholic aluminum chloride solution indicated the presence of a hydroxyl group in the 3-position.

The physical data for the isolated material are conflicting. No known flavanone aglycones possess the $\underline{R}_f$ values found for the isolated material. Several flavanones do possess similar $\underline{R}_f$ values as do several flavanonol aglycones. The isolated material, however, does not contain a sugar moiety so that it cannot be a glycoside. The ultraviolet spectrum of the isolated material in ethanol and alcoholic aluminum chloride are similar to the ultraviolet spectra of flavanonols.

The isolated material is probably an impure flavonoid aglycone. It is most likely a member of the flavanonol class of flavonoids. An infrared spectrum of the suspected flavanonol compound contained peaks of maximum absorption at 3380, 1640, 1470, 1160, 1080, 1020, and 985 $cm.^{-1}$.



Flavanonol Structure

The raffinate W-3 was also fractionated by cellulose column chromatography. One fraction yielded yellow needlelike crystals that melted from 220 to 221°C. Paper chromatograms of the material developed with acetic acid – water (1:99) yielded a single yellow spot with $\underline{R}_f$ 0.10. When the material was spotted on paper, it appeared yellow when viewed under ordinary light and dark when viewed under ultraviolet light. If a spot of the material on paper was exposed to ammonia fumes or sprayed with aluminum chloride reagent, it yielded a yellow color when examined with either ordinary light or ultraviolet light. These color reactions are characteristic of flavonol glycosides (11).

The ultraviolet spectra of the material were determined following the procedure previously described. A stock solution was made by dissolving 1 mg. of the material in 10 ml. of absolute ethanol. The spectrum in ethanol was determined with the solution resulting from the dilution of 2 ml. of the stock solution to 10 ml. with absolute ethanol. This spectrum was very similar to the spectra of quercetin-3-glycosides. The wavelengths of maximum absorption occurred at 258 and 363 nm.

The spectrum of the isolated material was also determined in alcoholic sodium acetate solution. The solution for the spectral determination was made by adding 10 drops of a saturated solution of sodium acetate in absolute ethanol to 2 ml. of the stock solution and diluting the mixture to 10 ml. with absolute ethanol. The resulting spectrum contained two peaks at 269 and 375 nm. The 11-nm.

shift of the lower wavelength peak indicated the presence of a free hydroxyl group in the 7-position (26).

A third spectrum was determined for the material in alcoholic aluminum chloride solution. As before, the solution for the spectral determination was made by diluting to 10 ml. a mixture of 10 drops of 5% alcoholic aluminum chloride solution and 2 ml. of the stock solution. This spectrum contained two peaks at 272 and 408 nm. The 45-nm. shift of the higher wavelength band indicated the presence of a 5-position hydroxyl group and the absence of a 3-position hydroxyl group (26).

An acid hydrolysis of 9.6 mg. of the crystalline material yielded 4.6 mg. of a yellow solid. The solid melted from 320 to 323°C. [literature melting point for authentic quercetin 316 to 318°C. (23)] and yielded two spots on a paper chromatogram developed with n-butanol – acetic acid – water (4:1:5). The spots possessed R_f values of 0.49 (unhydrolyzed glycoside) and 0.76 (same R_f value as authentic quercetin run on the same paper). An ultraviolet spectrum of the aglycone in ethanol contained two peaks at 256 and 370 nm. The spectrum was very similar to a literature spectrum of authentic quercetin, $\gamma_{max.}$ 257, 370 nm. (26). The ultraviolet spectrum of the aglycone in alcoholic aluminum chloride contained two peaks at 271 and 445 nm. The literature value for the upper band of quercetin in alcoholic aluminum chloride is 431 nm. (26).

The hydrolyzate from the acid hydrolysis was examined by paper chromatography for the presence of sugars. The chromatograms were developed with ethyl acetate – pyridine – water (8:2:1) and n-butanol – acetic acid – water (4:1:5). The sugars were detected with silver nitrate reagent. The chromatograms indicated the presence of glucose and galactose.

The data indicated the material was a quercetin glycoside. The ultraviolet data indicated that the linkage was probably through the 3-position because no free 3-position hydroxyl group could be detected in the unhydrolyzed material. Since acid hydrolysis yielded two sugars, there were several possibilities. The isolated material could be a quercetin-3-bioside, a quercetin-3-?-diglycoside, or a mixture of two different quercetin-3-glycosides. The chromatographic data suggested that the isolated material was not a bioside or a diglycoside inasmuch as known quercetin biosides (quercetin-3-rhamnoglucoside) and diglycosides (quercetin-3-rhamnoglucoside-7?-glucoside) have $\underline{R}_{\underline{f}}$ values considerably greater than the isolated material (11). Because of this, it was suspected that the isolated material was a mixture of two different quercetin-3-glycosides. When a paper chromatogram of the isolated material was developed with acetic acid – water (1:99) for 46 hours, the material separated into two different spots. The isolated material was then believed to be a mixture of quercetin-3-glucoside and quercetin-3-galactoside. Literature $\underline{R}_{\underline{f}}$ values for these glycosides are 0.08 and 0.09, respectively, when paper chromatograms are developed with water (11).

In order to demonstrate that the isolated material was a mixture of quercetin-3-glucoside and quercetin-3-galactoside, it was necessary to separate the two compounds and hydrolyze them. Several attempts were made to separate the compounds by thick-paper chromatography. The best results were obtained when 4 mg. of the mixture was applied to Whatman No. 17 chromatographic paper. When the paper was developed for three days with acetic acid – water (1:99), the material separated into two bands. The bands consisted of yellow streaks across the width of the sheet, while the area between the bands was a lighter yellow color. The bands were cut from the sheet and the glycosides were eluted with methanol – water (1:1).

The eluted material was concentrated to a 10-drop volume and 1 drop of concentrated hydrochloric acid was added. The material eluted from each band

was then hydrolyzed for one hour. The hydrolyzates were examined by paper chromatography. The hydrolyzate from the faster moving band contained only galactose, while the hydrolyzate from the slower moving band contained galactose and glucose. The presence of galactose in the hydrolyzate from the slower moving band is believed to be due to incomplete separation of the mixture on the thick-paper chromatogram.

ALKALINE HYDROLYSIS

Aliquots of the ethyl acetate extract W-2 and the raffinate W-3 were subjected to an alkaline hydrolysis by following the procedure of Pearl, Beyer, Lee, and Laskowski (27). The hydrolysis was conducted by refluxing 2.0-g. portions of the ethyl acetate extract W-2 and the raffinate W-3 in 100 ml. of a 4% sodium hydroxide solution for 8 hours. The "neutrals" fraction was collected by continuously extracting the hydrolyzate with ethyl ether for 12 hours. The "acids and phenols" fraction was collected by repeating the extraction after the acidification of the hydrolyzate with dilute sulfuric acid. During the second extraction, a water-insoluble, ether-insoluble tar formed. This tar was collected by filtering the hydrolyzate after the extraction had been completed. The yields of the various fractions and tars are expressed as a weight percent in Fig. 7. The weights of the various fractions were determined after evaporating to dryness under reduced pressure in tared, round-bottomed flasks.

All fractions were examined by paper chromatography. Paper chromatograms were developed with _n_-butanol – pyridine – water (10:3:3), _n_-butanol saturated with 2% aqueous ammonia, acetic acid – water (1:99), and the upper phase of _n_-heptane – _n_-butyl ether – water (6:1:1). The phenols were detected with diazotized _p_-nitroaniline reagent. The chromatograms were also sprayed with 2,4-dinitrophenylhydrazine reagent to detect aldehydes.

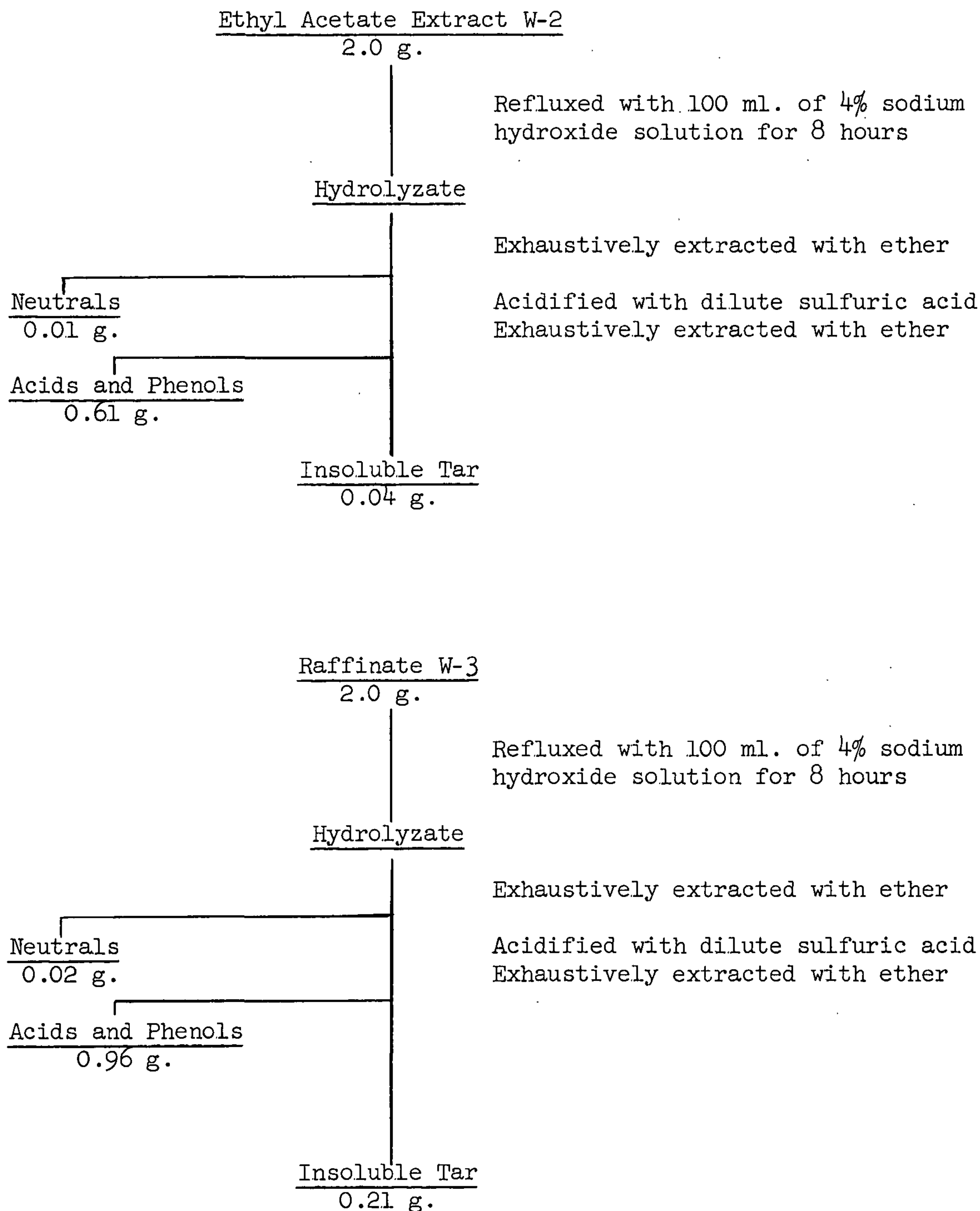


Figure 7. Flow Diagram of the Alkaline Hydrolysis of the Ethyl Acetate Extract W-2 and the Raffinate W-3

The "acids and phenols" fractions of both the ethyl acetate extract W-2 and the raffinate W-3 were found to contain ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, vanillic acid, and salicyl alcohol. The "neutrals" fractions were examined for the presence of aldehydes. No aldehydes could be detected with the 2,4-dinitrophenylhydrazine reagent. A collection of the pertinent paper chromatographic data is presented in Table I.

SUGARS AND URONIC ACID CONTENT OF THE ETHYL ACETATE EXTRACT W-2 AND THE RAFFINATE W-3

The ethyl acetate extract W-2 and the raffinate W-3 were analyzed to determine the quantitative sugar composition and the uronic acid content. The analysis was conducted by the Analytical Group of The Institute of Paper Chemistry. Analytical Group Method Number 53 was used to determine quantitatively the amount of glucose, galactose, mannose, arabinose, and xylose. This method involves an acid hydrolysis of the carbohydrate-containing sample, a paper chromatographic separation of the resulting monosaccharides, an elution of the separated sugars from the paper chromatogram, and a colorimetric determination of the individual sugars. The results were expressed as percent glucose, galactose, mannose, arabinose, and xylose (Table II). The weight percent was calculated on the basis of a $C_6H_{10}O_5$ unit for hexoses and $C_5H_8O_4$ for pentoses.

The uronic acid content of the ethyl acetate extract W-2 and the raffinate W-3 were also determined by the Analytical Group of The Institute of Paper Chemistry. Institute Method Number 25 describes the procedure used. The sample was heated with 12% hydrochloric acid to decompose the uronic acids into furfural and carbon dioxide. The evolved carbon dioxide was determined gravimetrically after being absorbed by Ascarite. The results are presented in Table II. The weight percent was calculated on the basis of a $C_6H_8O_6$ unit.

TABLE I

RESULTS OF ALKALINE HYDROLYSIS

Identified cpds.	Developers for Paper Chromatograms: BA[a] R_f	BA[a] Fraction[d]	BA[a] Color[e]	BPW[b] R_f	BPW[b] Fraction[d]	BPW[b] Color[e]	AW[c] R_f	AW[c] Fraction[d]	AW[c] Color[e]
Vanillic acid	0.10	W-2 (A-P) W-3 (A-P)	V	0.55	W-2 (A-P) W-3 (A-P)	V	0.51	W-2 (A-P)	V
p-Hydroxybenzoic acid	0.13	W-2 (A-P) W-3 (A-P)	R	0.67	W-2 (A-P)	R	0.58	W-2 (A-P)	R
Ferulic acid	0.14	W-2 (A-P) W-3 (A-P)	G-B	0.49	W-2 (A-P)	G-Gy	0.26	W-2 (A-P) W-3 (A-P)	G-Gy
p-Coumaric acid	0.20	W-2 (A-P) W-3 (A-P)	P	0.62	W-2 (A-P)	P	0.38	W-2 (A-P) W-3 (A-P)	P
Salicyl alcohol	0.84	W-2 (N) W-3 (N)	R	0.82	W-2 (N)	R			
Unidentified cpds.	0.08	W-2 (A-P) W-3	O	0.19	W-2 (A-P)	Br	0.00	W-3 (N)	O
	0.24	W-2 (A-P) W-3 (A-P)	O-Gy	0.92	W-2 (N) W-2 (A-P) W-3 (N) W-3 (A-P)	Gy	0.00	W-2 (N)	Pk
							0.28	W-3 (A-P)	O
	0.92	W-2 (N) W-2 (A-P) W-3 (N)	O O				0.44	W-2 (A-P) W-3 (A-P)	O
							0.68	W-2 (A-P)	B-Gy
							0.74	W-2 (N)	Pk

[a] BA = n-butanol-concd. ammonium hydroxide-water (25:14:1).
[b] BPW = n-butanol-pyridine-water (10:3:3).
[c] AW = acetic acid-water (1:99).
[d] W-2 = ethyl acetate extract W-2; W-3 = raffinate W-3; (A-P) = acids and phenols fraction; (N) = neutrals fraction.
[e] Colors: V = violet; R = red; G = green; B = blue; P = purple; O = orange, Gy = gray; Pk = pink.

TABLE II

SUGARS AND URONIC ACID CONTENT OF ETHYL ACETATE EXTRACT W-2 AND RAFFINATE W-3

Sample, %	Glucose	Galactose	Mannose	Arabinose	Xylose	Uronic Acid
Ethyl acetate extract W-2						
Test 1	3.18	0.72	none[a]	none[a]	none[a]	6.68
Test 2	3.13	0.67				6.79
Average	3.16	0.70				6.7
Raffinate W-3						
Test 1	5.63	0.81	none[a]	none[a]	none[a]	7.69
Test 2	5.43	0.86				7.83
Average	5.52	0.84				7.8

[a] Limit of detection is 0.5%.

EVALUATION OF COLUMN FRACTIONATION

In this study polyamide, silica gel, and cellulose columns were used to fractionate the various extracts. An aliquot of the ethyl acetate extract E-4 containing 6.5 g. was fractionated by a 35 by 510-mm. polyamide column. When the column was developed with water, the first 1500 ml. yielded mixtures of unidentified material. The fraction collected between 1500 and 1700 ml. volume yielded tremuloidin upon concentration. The fractions collected between 1700 and 2100 ml. volume were complex mixtures. The fraction collected between 2100 and 2400 ml. volume yielded crystalline populin upon concentration. The fraction collected between 2400 and 2700 ml. volume was an unknown mixture. The fraction collected between 2700 and 3100 ml. volume yielded crystalline salireposide upon concentration. Mixtures of unknown composition continued to be removed from the column until 7650 ml. volume. No further development of the column was attempted.

The raffinate E-5 was fractionated by a polyamide column. An aliquot of the raffinate containing 2.5 g. was placed on a 8 by 470 mm. column. When the column was developed with water, the first 100 ml. contained glucose, fructose, and glucuronic acid as determined by paper chromatography. The next 25-ml. fraction yielded crystalline *myo*-inositol. Unknown mixtures of materials continued to be removed from the column with water. After 1620 ml. of water, the developer was changed to ethanol – water (1:4). Only complex mixtures of unknowns were removed by 3250 ml. of this mixture, 9500 ml. of an ethanol – water (1:1) mixture, and 3500 ml. of absolute ethanol.

A silica gel column was used to fractionate the benzene extract W-1. The entire extract (5.9 g.) was placed on a 35 by 420 mm. column. The column was developed with chloroform – methanol (4:1). The first fraction (0-160 ml.) was a mixture of unknown materials. The second fraction (160-1240 ml.) yielded

pyrocatechol upon concentration. The column was then developed with ethanol. This solvent moved a dark band through the column. The material removed by ethanol was a complex mixture. Following the dark band, no more material could be removed from the column.

The ethyl acetate extract W-2 was fractionated by polyamide column chromatography. An aliquot containing 12.0 g. was placed on a 32 by 365 mm. column. When the column was developed with water, the first 200 ml. removed a complex mixture. The fraction collected between 200 and 300 ml. yielded succinic acid upon concentration. The fraction collected between 300 and 500 ml. contained an unknown mixture. The fraction collected between 500 and 600 ml. yielded pyrocatechol upon concentration. The column was further developed with ethanol – water (1:3), ethanol – water (1:1), and absolute ethanol. Mixtures of unknown materials were removed by these developers.

The raffinate W-3 was fractionated by polyamide column chromatography. An aliquot containing 18.8 g. was placed on a 48 by 350 mm. column. Pectin, rutin, and a flavanonol were removed from the column by development with water. Pectin was found in the 200 to 400-ml. fraction, rutin in the 4700 to 5300-ml. fraction, and the suspected flavanonol in the 5300 to 5600-ml. fraction. The fractions between these materials contained mixtures of unknown materials. The column was exhaustively developed with water (7400 ml.), ethanol – water (1:3) (2800 ml.), ethanol – water (1:1) (1300 ml.), and ethanol – water (3:1) (1000 ml.). The fraction collected between 3900 and 4400-ml. effluent volume of ethanol – water (1:1) yielded crystalline rhamnetin upon concentration. No other pure compounds were recovered from the water and ethanol – water fractions.

A cellulose column was used to fractionate the raffinate W-3. An aliquot containing 3.1 g. was placed on a 50 by 350-mm. column. The raffinate was

fractionated into six 50-ml. fractions by developing the column with water. The third fraction contained a mixture of quercetin-3-galactoside and quercetin-3-glucoside. All other fractions contained unknown mixtures.

A summary of the column fractionation data is presented in Table III.

TABLE III

COLUMN FRACTIONATION DATA

Extract	Weight, g.	Column Packing	Column Dimension, mm.	Developer	Fraction, ml.	Compound	Yield, g.
Ethyl acetate extract E-4	6.5	Polyamide	33 by 510	Water	1500-1700	Tremuloidin	0.057
Ethyl acetate extract E-4	6.5	Polyamide	33 by 510	Water	2100-2400	Populin	0.016
Ethyl acetate extract E-4	6.5	Polyamide	33 by 510	Water	2700-3100	Salireposide	0.047
Raffinate E-5	2.5	Polyamide	8 by 470	Water	100-125	<u>myo</u>-Inositol	0.093
Benzene extract W-1	5.9	Silica gel	35 by 420	Chloroform-methanol (4:1)	40-60	Pyrocatechol	0.011
Ethyl acetate extract W-2	12.0	Polyamide	32 by 365	Water	200-300	Succinic acid	0.032
Ethyl acetate extract W-2	12.0	Polyamide	32 by 365	Water	500-600	Pyrocatechol	0.191
Raffinate W-3	18.8	Polyamide	48 by 350	Water	200-400	Pectin	2.80
Raffinate W-3	18.8	Polyamide	48 by 350	Water	4700-5300	Rutin	0.040
Raffinate W-3	18.8	Polyamide	48 by 350	Water	5300-5600	Flavanonol	0.020
Raffinate W-3	18.8	Polyamide	48 by 350	Water	7400		
				Ethanol – water (1:3)	2800		
				Ethanol – water (1:1)	3900-4400	Rhamnetin	0.024
Raffinate W-3	3.1	Cellulose	50 by 350	Water	100-150	Quercetin-3-galactoside and Quercetin-3-glucoside	0.062

SUMMARY OF EXPERIMENTAL PROGRAM

During the study of the ethanol extract, salireposide, tremuloidin, populin, and myo-inositol were isolated and identified as components of Fraction E. Paper chromatographic evidence was obtained for the presence of fructose, glucose, and glucuronic acid. Mixtures of materials believed to be flavonoids were present, but the individual components of the mixtures could not be isolated and identified. The yields of the materials identified are presented in Table IV.

During the study of the water extract, rhamnetin, pyrocatechol, succinic acid, and rutin were isolated and identified as the components of Fraction W. A pectin material was found to be the major isolated component of Fraction W. A mixture of two flavonoid glycosides, believed to be quercetin-3-glucoside and quercetin-3-galactoside, was isolated and studied. Some evidence was obtained for the presence of a flavanonol in Fraction W. The yields of the materials found are presented in Table V.

Two extracts of Fraction W were studied by hydrolysis. The ethyl acetate extract W-2 and the raffinate W-3 were subjected to an alkaline hydrolysis. Vanillic acid, p-coumaric acid (major alkaline hydrolysis product), p-hydroxybenzoic acid, ferulic acid, and salicyl alcohol were identified as the alkaline hydrolysis products by paper chromatography.

A quantitative analysis of the sugars and uronic acids indicated that glucose was the major sugar obtained by hydrolysis (3.2% of the ethyl acetate extract W-2 and 5.5% of the raffinate W-3), while galactose was less abundant (0.70% of the ethyl acetate extract W-2 and 0.84% of the raffinate W-3). Mannose, arabinose, and xylose were either absent or accounted for less than 0.5% of the two extracts. The ethyl acetate extract W-2 was found to contain 6.7% uronic acids and the raffinate W-3 was found to contain 7.8% uronic acids.

TABLE IV

ISOLATED COMPONENTS OF FRACTION E

Material Isolated	Source of Material	Percent of Fraction E	Percent of the Leaves
myo-Inositol	Raffinate E-5	1.4	0.075
Populin	Ethyl Acetate Extract E-4	0.050	0.0027
Salireposide	Ethyl Acetate Extract E-4	0.15	0.0082
Tremuloidin	Ethyl Acetate Extract E-4	0.18	0.0098

TABLE V

ISOLATED COMPONENTS OF FRACTION W

Material Isolated	Source of Material	Percent of Fraction W	Percent of the Leaves
Flavanonol	Raffinate W-3	0.097	0.0057
Pectin	Raffinate W-3	13	0.80
Pyrocatechol	Benzene Extract W-1 Ethyl Acetate Extract W-2	0.31	0.019
Mixture of Quercetin-3-glucoside and Quercetin-3-galactoside	Raffinate W-3	1.7	0.11
Rhamnetin	Raffinate W-3	0.35	0.022
Rutin	Raffinate W-3	0.19	0.012
Succinic acid	Ethyl Acetate Extract W-2	0.053	0.0033

DISCUSSION

In this study a total of 17.5% of the material in Fraction E and Fraction W was identified. The quantitative sugar analysis revealed that part of Fraction W contained combined sugars and uronic acids (free sugars and uronic acids were not detected in Fraction W by paper chromatography). The ethyl acetate extract W-2 and the raffinate W-3 were found to contain a total of 11 and 14% sugars and uronic acids, respectively (these figures are approximate since the limit of detection of sugars was 0.5%). Since the ethyl acetate extract W-2 was 18% of Fraction W and the raffinate was 79 of Fraction W, then approximately 13% of Fraction W was composed of uronic acids and sugars. This is interesting since the glycosides and the pectin isolated from the raffinate W-3 can account for over 90% of the sugars and uronic acids in Fraction W. This means that the major carbohydrate containing components of Fraction W were isolated. Obviously, a large portion of the unidentified part of Fraction W cannot be glycosidic.

The quantity of glycosides isolated from the lead-insoluble fraction in this study was of the same order of magnitude as the quantity of glycosides isolated in previous studies from the lead-soluble fraction of a quaking aspen lead extract (5). Clearly, the lead subacetate treatment of the hot-water extract was a chemical fractionation that produced two very different groups of materials, the lead-soluble fraction and the lead-insoluble fraction. It is interesting that the isolated quercetin glycosides were not glucosides. Rutin and quercetin-3-galactoside are believed to be the first glycosides isolated from quaking aspen that contained a sugar moiety other than glucose. The presence of flavonols in the extracts of higher plants is not unusual, and quercetin glycosides have been isolated frequently from the leaves of woody plants (28).

In addition to the flavonol glycosides, rhamnetin and an impure material believed to be a flavanonol were found in Fraction W in the present study.

Rhamnetin glycosides had been isolated previously from the berries of several Rhamnus species (24). Usually, the aglycones of flavonoid glycosides are isolated only from dead tissue such as heartwood or storage tissue such as seeds (28). The isolation of rhamnetin, a rather infrequently isolated flavonol, may have taxonomic significance.

One obvious result of this work was that the nonflavonoid glycosides populin, salireposide, and tremuloidin were present only in Fraction E. The absence of these compounds in Fraction W was believed to be due to the conditions of the lead subacetate treatment. The aqueous solution which was treated with lead subacetate and hydrogen sulfide to yield Fraction E was six times as concentrated as the solution, which after the same treatment, yielded Fraction W. The aqueous solution from which Fraction E was obtained was probably supersaturated with populin, salireposide, and tremuloidin. This indicates that the conditions of the lead subacetate treatment were important in determining the nature of the compounds found in the lead-insoluble fraction. It was also possible that the gelatinous lead subacetate precipitate entrained small quantities of these compounds.

Populin and tremuloidin have been found previously in a lead subacetate-treated hot-water extract of quaking aspen leaves (6). Salireposide has been isolated from a lead subacetate-treated hot-water extract of quaking aspen bark (29). The isolation of very small quantities of these glycosides from the Fraction E and the absence of these compounds in Fraction W supports the hypothesis that these compounds do not belong in the lead subacetate-insoluble fraction and were present only because of their low water solubility.

The water-soluble pectin was found only in the raffinate W-3 of Fraction W. The absence of pectin in Fraction E was due to the fact that Fraction E was

obtained from an ethanol extract of quaking aspen leaves. Since pectin is not soluble in ethanol, it was not found in Fraction E. The isolated pectin yielded galacturonic acid, arabinose, and galactose upon hydrolysis. Pectins with similar compositions have been removed from numerous plant tissues by extraction with water (30, 31).

Pyrocatechol has been found in the barks of quaking and bigtooth aspen (15, 32). In the present study, it was found only in Fraction W. The failure to find pyrocatechol in Fraction E was probably due to the experimental techniques used to investigate this fraction. To review briefly, Fraction E was extracted with four solvents to yield five fractions. Only two of these fractions, the ethyl acetate extract E-4 and the raffinate E-5 (which together contained 99% of Fraction E) were investigated. The ligroin extract E-1, the carbon tetrachloride extract E-2, and the benzene extract E-5 were only examined qualitatively by paper chromatography. Since pyrocatechol is soluble in all three of these solvents, it was probably present in the extracts not examined.

Even though myo-inositol has been found in small quantities in many plant and animal tissues (33), this cyclitol was the major identified component of Fraction E. Recent studies on the in vitro cultivation of quaking aspen callus tissue established that myo-inositol was a required nutrient (34). This means that cambial tissue is not capable of synthesizing the myo-inositol it requires for life. This suggests that myo-inositol must be supplied to the cambial area by some external organ during normal tree growth. Since a large quantity of myo-inositol was found in the extract of quaking aspen leaves in the present study, it is possible that the leaves function as the organ of synthesis of this cyclitol. Perhaps myo-inositol is synthesized in the leaves and is then transported to the cambial area where it is required for growth.

The alkaline hydrolysis of Fraction W revealed considerable information about the nature of the unidentified 82.5% of the lead-insoluble material. In a previous study of the hot-water extract of quaking aspen leaves, Pearl and co-workers (5) found that an extraction of the lead-insoluble material with ether would remove 0.25% (based on the ovendry weight of the leaves). In the present study, 3% (based on the ovendry weight of the leaves) or 12 times as much material was soluble in ether after alkaline hydrolysis. A paper chromatographic study of the alkaline hydrolyzate revealed the presence of p-hydroxybenzoic acid, ferulic acid, p-coumaric acid, vanillic acid, and salicyl alcohol. Several unidentified materials which produced colors with diazotized p-nitroaniline were also observed. Inasmuch as no attempt was made to identify nonphenols that may have been present in the alkaline hydrolyzate, large amounts of material may have been overlooked.

The flavonols isolated from the lead-insoluble fraction could yield phenols upon alkaline hydrolysis. None of the identified hydrolysis products, however, had hydroxylation patterns consistent with the structures of the isolated materials. The alkaline hydrolyzate did not contain detectable quantities of caffeic acid, protocatechuic acid, or phloroglucinol. These phenols would be the expected alkaline hydrolysis products of quercetin.

This indicated that the phenolic acids produced by the alkaline hydrolysis were degradation products of the unidentified portion of the lead-insoluble fraction. The unidentified material probably contained molecules constructed of C6-C1 and C6-C3 units. Since p-coumaric acid was the major alkaline hydrolysis product, a large portion of the unidentified material may have contained C6-C3 units with hydroxyl groups in the para position. Evidently, 4-hydroxy-3-methoxyphenyl moieties were also present.

The solubility information from the extraction procedures used in this study demonstrated that the bulk of the lead-insoluble material was not soluble

in ligroin or carbon tetrachloride and only slightly soluble in benzene or ether. The material was partly soluble in ethyl acetate and very soluble in water. This indicated that at least part of the lead-insoluble material was macromolecular, possibly containing polyphenols.

The fraction-by-fraction monitoring of the column fractionation by paper chromatography revealed many unidentified materials in each fraction. Each fraction contained numerous materials which produced colors with diazotized p-nitroaniline. Frequently, several fluorescent compounds were present in a single fraction. Judging from these observations, the lead-insoluble fraction appeared to contain many phenols.

The nonglycosidic water-soluble material that comprises the major portion of the lead-insoluble fraction would be an interesting material for future study.

CONCLUSION

The types of compounds extracted from leaf tissue can be controlled to some extent by the solvent used for the extraction. In this study the compounds isolated from the ethanol extract of quaking aspen leaves were different from the compounds isolated from the water extract of quaking aspen leaves. Part of these differences were attributed to the type of solvent used for the initial extraction.

Some nonphenolic compounds, which are extractable from quaking aspen leaves by water or ethanol, form lead subacetate precipitates. In this study the isolated nonphenolic compounds were a cyclitol, a dibasic acid, and a polyuronide.

The sugar moiety of the glycosides present in the water extract of quaking aspen leaves is not exclusively glucose. The flavonol glycosides isolated in this study contained galactose and rhamnose as well as glucose.

The flavonoid glycosides present in the water extract of quaking aspen leaves are of the same order of abundance as the nonflavonoid glycosides found in the same extracts. The flavonoid glycosides isolated in the present study represent approximately 0.12% of the leaves.

A large portion of the lead subacetate-insoluble material found in the water extract of quaking aspen leaves is macromolecular. Part of the macromolecular material was a pectin and part of the material was believed to be polyphenolic.

The lead subacetate-insoluble polyphenolic material which is water extractable from quaking aspen leaves is composed of C_6-C_1 and C_6-C_3 units. The results of an alkaline hydrolysis revealed that this polymeric material contained *p*-hydroxyphenyl and 4-hydroxy-3-methoxyphenyl moieties.

Finally, this study demonstrated that the material which is precipitated from a water extract of plant tissue by lead subacetate is partly phenolic and partly nonphenolic. The phenolic portion was composed of glycosides, flavonoids, and phenols. The nonphenolic portion was composed of polyuronic acids, a dibasic acid, and a cyclitol. Thus, future investigations of the components of plant organ extracts must include the lead-insoluble material along with the lead-soluble material if the compounds such as those identified in this study are not to be overlooked.

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