PHYTOCHEMICAL INVESTIGATION OF MANITOBA WILLOW SPECIES

A Thesis

Presented to

the Faculty of Graduate Studies

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

CHARLES S. C. WONG

September 1966

A C K N O W L E D G E M E N T S

A B S T R A C T

The author wishes to express his sincere appreciation to the following: Prof. R.C.S. Audette, Dr. J.W. Steele and Dr. J.R. Murray for supervision and encouragement during the course of this work, his colleagues in the School of Pharmacy for many useful discussions and in particular to J.R. Bend and G. Krip for their friendly help, Mrs. D.E. Ash for typing the manuscript.

The author also gratefully acknowledges the receipt of the Warner-Lambert Research Fellowship (Warner-Lambert (Canada) Ltd.) and for summer grants from the Canadian Foundation for the Advancement of Pharmacy.

A complete TLC system on silica gel G and polyamide-cellulose layer was developed for the separation and identification of phenolic glycosides of the Salicaceae. Relative humidity was found to be a very important factor in obtaining reproducibility of the silica gel TLC separation system.

A screening process for Salicaceae phenolic glycosides has been established.

For large scale extraction of phenolic glycosides, a blender extraction in conjunction with a soxhlet extraction process was developed.

The ultra-violet absorption characteristics of phenolic glycosides have been determined and the ultra-violet absorption spectra of Salix phenolic glycosides were recorded.

The bark and leaves of S. petiolaris (S. gracilis var. textoris) collected in the middle of June 1965, were shown to contain salicin, salireposide, tremuloidin, grandidentatin, picein, triandrin and fragilin, and trace amounts of populin and vimalin. Salireposide, salicin and picein were isolated from bark extract, and tremuloidin and salicin were isolated from leaf extracts. The melting points, ultra-violet and infrared absorption spectra for the isolated glycosides have been recorded.

The bark and leaves of S. interior (S. fluviatilis var. sericans) collected at the end of June 1965, were shown to contain salicin, triandrin and salidroside, and trace amounts of vimalin and fragilin.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

TEXT FIGURE PAGE

I N T R O D U C T I O N

History and Clinical Use of Willow

Ancient medicine men made a concoction from the bark of willow trees and gave it to patients to reduce fever. The botanical name for willow is *Salix* and from this we derive the name for salicylate drugs, of which the best known is acetylsalicylic acid (Aspirin). Willow bark or brew made from the bark was the American Indian's aspirin or pain killer. (1)

The leaves have been used to cure malignant boils and scalds, and the decoction of the willow twig or bark has been used for the relief of pain, lowering of fever, rheumatism, and as a tonic and astringent. (2,3) Willow bark has been employed as a bitter and as an astringent and was included in the British Pharmaceutical Codex (1907 to 1934). (4) The main phenolic glycoside of willow, salicin, has been used as an antirheumatic and was included in the United States Pharmacopeia (1882 to 1936) and the National Formulary (1936 to 1955). (5) In the British Pharmacopoeia (1885 to 1932) and the British Pharmaceutical Codex (1907 to 1958) salicin was also suggested for the treatment of acute rheumatism and influenza. (4)

Botanical Description

Willow is a member of the natural order *Salicaceae*

which is the only family in the Salicales. This family consists of two genera, which are Salix (Willow) and Populus (Poplar). There are about 500 species of Salix and about 150 species of Populus. (6)

Salix is composed of trees or shrubs, aments ascending or divergent, rarely drooping; bracts entire or merely toothed, each flower with 1 to 4 basal glands; stamens 1-12; style 1 (or none) with 2 simple or bifid stigmas; and buds with 1 scale. (7) Willows are mainly found in the northern moderate and subarctic zone of Europe, North America and Asia. They have mainly terete branches. The leaves vary from narrowly linear to orbicular. The buds are covered by a single scale, with an inner usually adherent membrane. The flowers are dioecious catkins and usually develop before or with the leaves. Willow has erect catkins and is adapted for pollination by insects. In relation to this, nectar is secreted by small scales at the base of the flower. Male flowers are scented and the pollen is sticky. (8)

Some species are among the more abundant plants of high northern latitudes. They have subterranean creeping stems with only the young shoots projecting from the soil. (8)

In North America fifty-four species of willow have been reported. (7) Besides the introduced species and varieties, 28 species and 3 varieties of willow have been found native to Manitoba. (9) Table 1 of the Appendix lists the species and distribution of willow species indigenous to Manitoba. (9)

Salix petiolaris Sm. (S. gracilis Anderss. var. textoris Fern.) (9)

S. petiolaris is a shrub with slender ascending or olive-brown tough and glabrate branches 1-3 metres high; aments appearing with the leaves on leafy peduncles, flowering from near middle to base and apex; staminate aments ellipsoid-obovoid, 1-2 cm. long, 1-1.3 cm. thick; capsules up to 9 mm. long; the mature leaves glabrate or rarely silky, 4-10 cm. long and up to 2 cm. broad, strongly serrate-dentate except at base, with gland-tipped teeth. (10)

This species grows on thickets, meadows and shores in the southern three-quarters of Manitoba. (9)

Plate 1 is the photograph of S. petiolaris growing in the month of June 1965.

Salix interior Rowlee (S. longifolia Muhl; S. fluviatilis Nutt. var. sericans (Nees) Boivin) (9)

S. interior is also named Sandbar Willow. It is an extensively colonial stoloniferous shrub or small tree up to 5.5 metres (usually only 1-4 m. high, with many grayish stems; branchlets glabrous, brown; leaves glabrous, firm, veiny, linear to narrowly lanceolate, acuminate, remotely dentate with

Plate 1. The photograph of Salix petiolaris Sm. growing in the month of June 1965

sharp teeth; the blades in maturity mostly 5-14 cm. long and 5-10 mm. broad, tapering to short petioles; leafy lateral shoots abundant, often continuing for some time to bear lower secondary aments; aments slender, with pale deciduous yellowish bracts; staminate aments 2-4 cm. long, the secondary later ones smaller; stamens 2, the filaments crispubescent at base; pistillate aments lengthening to 8 cm.; capsules slender, scattered, 7-9 mm. long, thinly silky, often glabrate, with nearly obsolete styles. (11)

They grow in the wild state on wet ground and shores throughout the southern three-quarters of Manitoba. (9)

Plate 2 is the photograph of S. interior growing in the month of June 1965.

Phytochemical Investigation

The study of the phytochemistry of willows began in earnest at the beginning of this century. At that time, workers thought that because of the bitter taste of willow bark it contained alkaloids. (6)

The glycosides which are present in willow species have been found mainly to be phenolic glycosides. They are simple phenols with the sugar molecules linked through the phenolic hydroxyl groups. Some are flavonoid glycosides which possess

Plate 2. The photograph of Salix interior Rowlee growing in the month of June, 1965

a structure containing the flavonoid skeleton (C_6-C_3-C_6).

Salicin was the first phenolic glycoside isolated from willow species and also the first glycoside to be found in nature. (6) It was discovered in 1828 by Buchner in the bark of Salix alba and S. incana. (21) It was also isolated a year later by Leroux from S. helix in the form of white bitter tasting crystals. (23)

The glycosidic character of salicin, which was originally thought to be an alkaloid, was clarified by classic work of Piria in 1845. (6)

The definite structure of salicin, as shown in Figure 1, was worked out in 1906 by Irvine and Rose. (12) Chemically, salicin is a β-D-glucoside of 2-hydroxybenzyl alcohol; the sugar is bonded to the phenolic hydroxyl group of the aglycone.

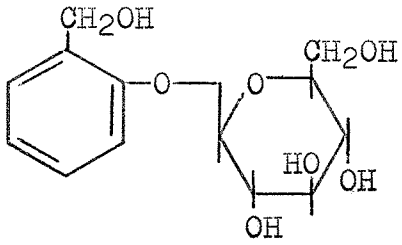


Figure 1. Structure of Salicin

Salicin has been found in 28 different Salix species. (13-27) It also appears to be the major glycoside component of the barks of several Populus species investigated by Pearl et al. (28-32) Therefore salicin is regarded as the chief glycoside of the Salicaceae. Recently it was also identified

by Thieme (33) in all barks of 11 European willow species of Central Germany, but it was present only in small concentration and never was the main glycoside. (33)

Salicin is also found in the leaves and inflorescences of some willow and poplar species. (31,33) Besides the Salicaceae family, it was also isolated from other plants of different families. (34,35)

An iso-salicin has been isolated by Thieme in 1966 from the flowers of Filipendula ulmaria. (36)

Chemically, isosalicin (Figure 2) is an isomer of salicin; the sugar moiety is bonded to the alcoholic group of 2-hydroxybenzyl alcohol.

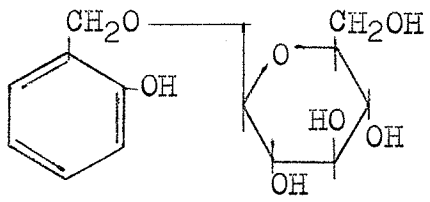


Figure 2. Structure of Isosalicin

A number of acylated salicin derivatives were found in Salix species. They are Fragilin, Salicortin, Populin and Tremuloidin.

Fragilin (Figure 3) is a partially acetylated salicin isolated from the bark of S. fragilis in 1964 by Thieme. (37) Chemically, fragilin is a mono-acetylated salicin; the acetyl group is bonded to the 6-position of the glucose molecule of salicin.

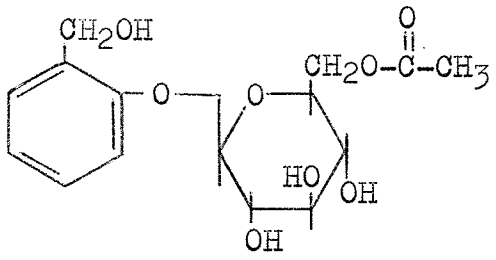


Figure 3. Structure of Fragilin

Salicortin was isolated from the bark of S. purpurea in 1964 by Thieme. (38) This glycoside was also found in the barks and leaves of other Salix and Populus species. (48) The chemical structure of salicortin has not been clarified. It is known that it is a derivative of salicylsalicin as shown in Figure 4.

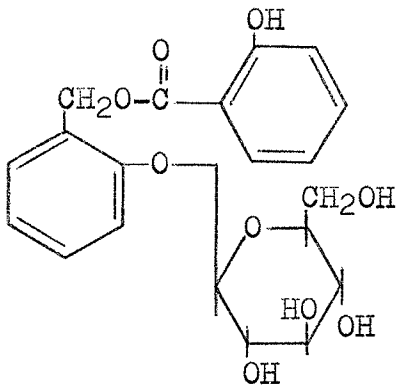


Figure 4. Structure of Salicylsalicin

Populin (Figure 5) is monobenzoyl salicin which was discovered in 1830 by Braconnot (39) in the bark of Populus tremula. The presence of populin in leaves and branches of

several willow species was reported in 1935 by Rabate. (40) Chemically, populin is a 6-benzoyl salicin; the benzoyl group is attached to the 6-position of the glucose molecule of salicin.

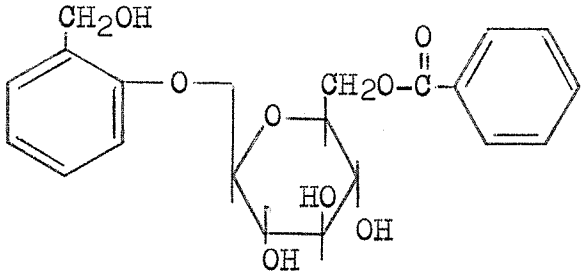


Figure 5. Structure of Populin

Tremuloidin (Figure 6) is an isomer of populin which was isolated from the bark of Populus tremuloides in 1958 by Pearl and Darling. (28,29) It has also been found in the leaves of several willow species by Thieme (6), but no tremuloidin had been detected in the bark of investigated willow species. (6,33) Chemically, tremuloidin is a 2-benzoylsalicin; the benzoyl group is attached to the 2-position of the glucose molecule of salicin.

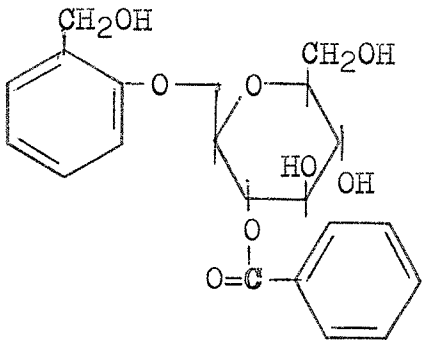


Figure 6. Structure of Tremuloidin

Other phenolic glycosides including Picein, Salireposide, Grandidentatin, Triandrin, Vimalin and Salidroside have been isolated from Salix species.

Picein (Figure 7) is a 4-hydroxyacetophenone glucoside which was first isolated in 1894 by Tanret from the needles of Picea exelsa (Pinaceae). (41) It was found in several willow species by Rabate. (25,40) Chemically, picein is a β-D-glucoside of 4-hydroxylacetophenone.

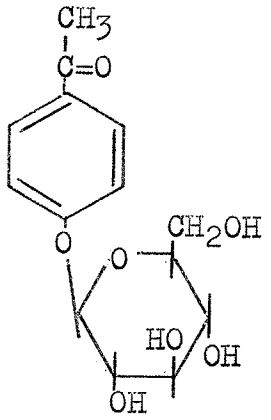


Figure 7. Structure of Picein

Salireposide (Figure 8) was isolated by Wattiez (42) in 1931 from the bark of Salix repens. It was also found in the bark of several other willow and poplar species, but has not been found in the leaves of Salix species. (6,33) Chemically, salireposide is a benzoylated 2,5-dihydroxybenzylalcohol-2-β-D-glucopyranoside, the benzoyl group bonded to the 6-position of the glucose molecule.

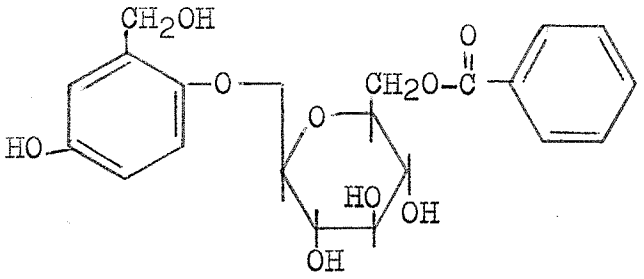


Figure 8. Structure of Salireposide

Grandidentatin (Figure 9) was isolated by Pearl and Darling in 1961 from the bark of Populus grandidentata. (43) It was also found in the bark of several willow species by Thieme, but none was reported in the leaves. (6,33) Chemically, grandidentatin is a cis-cyclohexanediol(1,2)-1-β-D-(2-p-coumaroyl glucopyranoside.

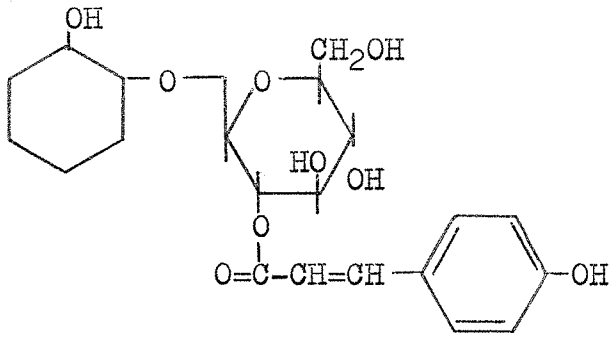


Figure 9. Structure of Grandidentatin

Triandrin (Figure 10) was isolated by Thieme in 1962 from the bark of Salix triandra. (44) It has also been identified in the bark of several other willow species. (6,33) Chemically, triandrin is 3(4-hydroxyphenyl)-2-propene-1-ol-1-β-D-glucopyranoside.

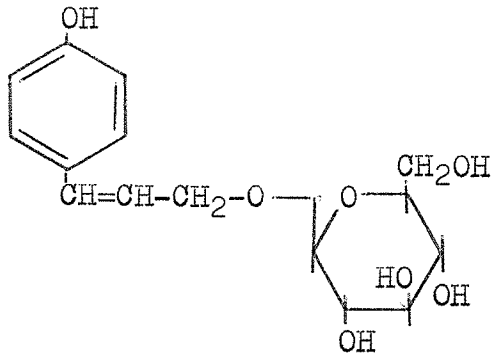


Figure 10. Structure of Triandrin

Vimalin (Figure 11) is a methylated triandrin derivative which was isolated by Thieme (27) in 1964 from the bark of Salix viminalis. It was also identified in the bark of several other Salix species. (33,48) Chemically, vimalin is 3(4-methoxyphenyl)-2-propene-1-ol-1-β-D-glucopyranoside.

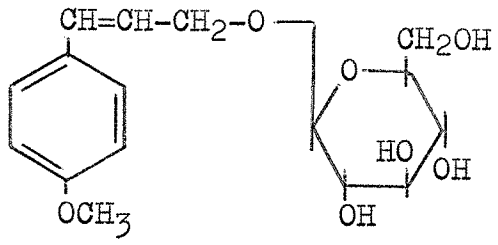


Figure 11. Structure of Vimalin

Salidroside (Figure 12) was first found by Bridel and Beguin (45) in 1926 in the bark of Salix triandra. It was also isolated by Thieme from the leaves and bark of S. triandra (46,48) in 1964, and from the bark of mountain cranberry (Vaccinium vitis-idaea) in 1966. (47) Chemically,

salidroside is a glucoside of 4-hydroxyphenylethanol; the sugar moiety is bound to the alcoholic group of the aglycone.

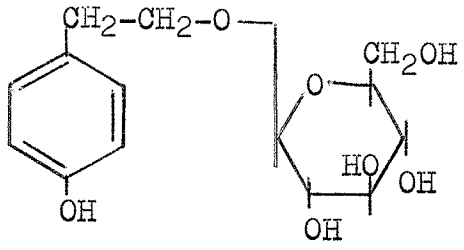


Figure 12. Structure of Salidroside

All of the above phenolic glycosides are found in Salix species. In most cases these have also been isolated or identified in Populus species. In other words, the phenolic glycosides found in poplars could also be isolated from willows. Other phenolic glycosides that have been found in poplar are as follows: Salicylpopulin (6), Trichocarpin (48), Salicyltremuloidin (48) and Tremulacin (49).

Recently Thieme has reviewed all phenolic glycosides of the Salicaceae (6), and has suggested a method of isolation and determination of phenolic glycosides (48,50). Characterization of the individual glycoside is made possible by determination of melting points, specific rotation of these compounds and their acetylated derivatives, infrared absorption spectra and by identification of hydrolytic products. Table 2 of the Appendix lists the melting points and specific rotation of Salicaceae glycosides and their acetylated

derivatives. In Figures 1 to 11 of the Appendix, the infrared absorption spectra of Salix phenolic glycosides are recorded. Tables 3 and 4 of the Appendix record the hydrolysis products of fermentable glycosides and acetylated glycosides.

For identification of the glycosides present in various Salix (or Populus) species, a paper chromatography method is described. The solvent system contains n-Butanol/Xylene/Acetic acid/Water (6:4:2:8) and Millon's Reagent is used, followed by heating at 95°C. The R_f values and different colour spots of the glycosides are sufficient for their differentiation. (48,50) The R_f values and different colour reactions of the Salicaceae glycosides are recorded in Table 6 of the Appendix.

Thieme also suggested a quantitative analysis method for the phenolic glycosides. (17) It is a colourimetric analysis based on the red colour which appears when an aqueous solution of the glycoside is heated with Millon's reagent. Individual glycosides can be determined in the plant extract in amounts up to 300 µgm. with a precision of ±5% after paper chromatographic separation and elution. By this method, the seasonal and conditioned variations of the phenolic glycoside concentration in the leaves and bark of the European (Central Germany) Salix species have been investigated and reported. (51)

Willow barks and leaves are rich in tannins. The highest content has been reported in Salix caprea which contains an average of 13.5 per cent in the bark (52) and

8.0 per cent in the leaves (53). Therefore in the leather industry, willow bark extracts are commonly used as tanning agents. Willow is also used as raw material for the pulp and paper industry.

Thieme, who has been investigating the European Salix species for the last few years in Germany, has isolated several new phenolic glycosides (6,33,48), and in the United States Pearl and Darling and their co-workers have also found many phenolic glycosides in Populus species (28-32). There are many Salix species indigenous to North America, but of these very few have received any serious chemical investigation, especially those of the Canadian prairie region. Therefore the Phytochemical Investigation of Manitoba Willow Species represents an attempt to isolate and characterize the phenolic glycosides present and to study the tannins contained in several Salix species native to Manitoba.

Previous to this study, the method of choice for the identification of the phenolic glycosides was paper chromatography. The disadvantages of this separation were the time involved and the incompleteness of the separation of some glycoside mixtures. Therefore a complete system for the thin layer separation of phenolic glycosides was developed.

Simultaneously a rapid screening procedure for the phenolic glycosides present in Salix (or Populus) species was also investigated.

E X P E R I M E N T A L

I. MATERIALS

Solvents

Acetone, A.S.C. (Fisher Scientific Co.)

Acetic Acid, 99.8%, C.P. (C.I.L.)

Benzene, A.S.C. (Fisher Scientific Co.)

1-Butanol, A.S.C. (Fisher Scientific Co.)

Ethanol, U.S.P. (C.I.L.)

Ether, A.S.C. (Fisher Scientific Co.)

Ethyl Acetate, A.S.C. (Fisher Scientific Co.)

Formic Acid, 90.8%, Reagent (J.T. Baker Chem. Co.)

Isopropanol, Reagent (British Drug Houses)

Methanol, A.S.C. (Fisher Scientific Co.)

Methyl Ethyl Ketone, purified (J.T. Baker Chem. Co.)

Xylene, Reagent (J.T. Baker Chem. Co.)

Chemicals

3,5-Dinitrobenzoyl Chloride (Matheson, Coleman & Bell)

Lead Subacetate, A.S.C. (Fisher Scientific Co.)

Mercury, AnalaR (British Drug Houses)

Nitric Acid, Fuming (May and Baker Ltd.)

Silver Nitrate B.P. (British Drug Houses)

Sodium Bromide B.P. (British Drug Houses)

Sodium Hydroxide U.S.P. (Mallinckrodt Chemical Works)

Sodium Thiosulphate, A.C.S. (Fisher Scientific Co.)

Sulfuric Acid, Reagent (General Chemicals)

Adsorbents

Cellulose Powder, TLC (Gerard Pleuger, Belgium)

Cellulose Powder, standard grade (Whatman)

Magnesium Silicate, Woelm (M. Woelm, Eschwege, Germany)

Polyamide, Woelm (M. Woelm, Eschwege, Germany)

Polyamide, Woelm, TLC (M. Woelm, Eschwege, Germany)

Silica Gel, Adsorption Chromatography (British Drug Houses)

Silica Gel G, TLC (R.S. Co. - Warner-Chilcott Lab. Inst.)

Schleicher & Schull 2043b, MgI, Chromatography Paper (Schleicher & Schull Co.)

Whatman No. 3MM Chromatography Paper (Whatman Co.)

Instruments

Beckman IR-8 Infrared Spectrophotometer (Beckman Instruments Inc., U.S.A.)

Ultrascan Recording Spectrophotometer (Hilger & Watts, England)

Equipment

Chromatographic Pyrex Glass Jars, 30 x 60 cm., fitted with stainless steel rack, glass rods and trough (Canadian Laboratory Supplies)

TLC Developing Tank (Desaga, Germany)

Freeze Dryer (Lyophilizer) (Virtis Research Equipment, U.S.A.)

Long Wave UVL-22 (Black light lamp) (Ultra Violet Products Inc., U.S.A.)

Rinco Rotary Evaporator (Rinco Instrument Co., Inc., U.S.A.)

Short Wave SL-2537 (Ultra violet lamp) (Ultra Violet Products Inc., U.S.A.)

TLC Spreader, model 200-11 (Warner-Chilcott Laboratory Instrument Division - formerly Research Specialties Co., U.S.A.)

Steam Heated Soxhlet Extractor (larger scale) (Quickfit, England)

Steam Heated Flash Evaporator (Quickfit, England)

Syringe Driver (B. Braun Apparatebau, Melsungen, Germany)

Thomas Hoover Capillary Melting Point Apparatus (A.H. Thomas Co., U.S.A.)

Towers Automatic Fraction Collector (J.W. Towers & Co. Ltd., England)

Waring High Speed Blendor (Waring Products Corp., U.S.A.)

II. PAPER CHROMATOGRAPHY

Pearl and Darling Systems

Pearl and Darling separated salicin, populin, tremuloidin and salireposide with n-Butanol/Pyridine/Water (BPW) (10:3:3, v/v), Ethylacetate/Pyridine/Water (EPW) (8:2:1, v/v) and Ethylacetate/Acetic acid/Water (EAW) (9:2:2, v/v) on Whatman No. 3MM paper. They used a modified silver spray and diazotized p-nitroaniline spray for the detection of the glycosides. (28,29)

The author carried out descending paper chromatography with the above solvent systems using the presaturated method (i.e. allowing the paper to saturate with solvent vapour in the chromatographic tank overnight prior to development). The modified silver spray was used as a detection reagent.

Results and Discussion

Table 1. R_f Value of Phenolic Glycosides

	BPW (10:3:3)		EPW (8:2:1)		EAW (9:2:2)	
Glycoside	Reported R_f(28,29)	Determined R_f	Reported R_f(28,29)	Determined R_f	Reported R_f(28,29)	Determined R_f
Salicin	0.52	0.58	0.50	0.45	0.60	0.37
Salireposide	0.68	0.80	0.73	0.67	-	0.70
Tremuloidin	0.77	0.85	0.80	0.78	0.85	0.88
Populin	0.69	0.82	0.79	0.78	0.84	0.85

The R_f values for the phenolic glycosides reported by Pearl and Darling (28,29) could not be duplicated in this laboratory, as outlined in Table 1. Thieme also reported that he had similar difficulty trying to duplicate the systems of Pearl et al. (50). The reason for this could possibly be that the developing conditions of Pearl and co-workers (50) were not completely followed, since in Pearl and co-workers' report the conditions were not clearly defined.

According to the R_f values reported by Pearl et al. (28,29) it was difficult to separate populin and tremuloidin by these systems. Also all of the glycoside spots were distributed over the high R_f region of the chromatogram, the R_f of salicin being greater than 0.5. Therefore if one depended upon the polarity of phenolic glycosides or referred to the resolution power of the glycosides on the Thieme system, it can be presumed that all known phenolic glycosides will be distributed between R_f 0.5-1.0. In other words, many phenolic glycosides will overlap on the chromatograms of the solvent systems developed by Pearl and co-workers (50).

Another disadvantage of the Pearl systems found in this laboratory was that the separated spots tailed on the chromatogram. This was especially noted with glycosides having higher R_f values.

Upon modification two systems with better resolution have been found. These are Ethyl acetate/Xylene/Acetic Acid/Water (EXAW) (10:2:1:5) and Ethyl acetate/Acetic Acid/Water

(EAW) (14:1:5) using descending paper chromatography on Whatman No. 3MM paper with presaturation technique. The results obtained are shown in Table 2.

Table 2

R_f Value of Phenolic Glycosides
Separated on Whatman 3MM Paper

Glycoside	EXAW (10:2:1:5)	EAW (14:1:5)
Salicin	0.08	0.13
Picein	0.14	0.20
Salireposide	0.45	0.58
Tremuloidin	0.70	0.85

Unfortunately the glycosides having a higher R_f value showed some tailing.

Thieme Systems

According to Thieme, ascending chromatography was used on Schleicher & Schull paper 2043b, MgI. Solvent A [n-Butanol/Xylene/Acetic acid/Water (6:4:2:8)] is reported to be suitable for simple glycosides, and solvent B [n-Butanol/Xylene/Acetic acid/Water (2:8:2:8)] is used for the acetylated glycosides. Millon's reagent was found to be the best to bring out the spots. The glycoside formed a yellow or red spot after being sprayed with Millon's reagent and being heated to 95°C.

Solvent system A took 11 to 12 hours to run 30 cm. from the origin, and system B required about half of this time.

Results and Discussion

Table 3. R_f Value of Phenolic Glycosides

Glycoside	BXAW (6:4:2:8)		BXAW (2:8:2:8)	
	Reported R_f	Determined R_f	Reported R_f	Determined R_f
Salicin	0.26	0.22	0	0
Picein	0.33	0.30	0	0
Salireposide	0.78	0.74	0.11	0.09
Populin	0.92	0.86	0.54	0.51
Tremuloidin	0.90	0.88	0.44	0.46
Grandidentatin	0.88	0.84	0.28	0.26

The R_f values are given by Thieme (48,50) (Appendix Table 6) and represent the average of at least ten determinations.

The author's results as shown in Table 3 were collected from a single chromatogram only, but the results were acceptable and the R_f was a little lower than was reported. The R_f value of populin in system A was found to be slightly higher than tremuloidin.

Millon's reagent was prepared according to B.P. 1963. Thieme used a different method of preparation. (48) Millon's reagent B.P. contains more nitric acid, which gave the spots

a brighter colour than Thieme's Millon's reagent. Picein showed up much better with the B.P. reagent. However it was also much easier to burn or char the paper if it was heated higher than 95°C. or longer than 10 minutes when employing the B.P. reagent.

The paper chromatograms developed by Millon's reagent could not be used as a permanent record, since the colour of the spots faded after 1 to 2 days. Even spots developed at room temperature showed a reduction in colour after heating. Also the whole paper chromatogram turned grey and became fragile after two months' storage.

According to Thieme (50), the advantage of Millon's reagent was that the red or yellow colour of the spot depends upon the substitution position of the aglycone. A yellow or brown-yellow colour is produced with all 2-substituted aglycones after heating. All glycosides that are substituted in the 4-position of the aglycone or acid substituted glycoside give a red colour. Glycosides with a free phenol group colour at room temperature. These colours give valuable information for identification.

Glycosides with free phenolic groups can also be detected with diazotized sulfanilamide solution. Picein, which contains 4-hydroxyacetophenone as its aglycone can also be detected with 2,4-dinitrophenylhydrazine.

The R_f values of the cleavage products of phenolic glycosides detected with solvent systems A and B on paper

chromatography have also been reported by Thieme (48,50) (Appendix Table 5).

III. THIN-LAYER CHROMATOGRAPHY

Introduction

Prior to this study, the best method for the identification of the phenolic glycosides was the paper chromatographic method which was developed by Thieme. (50)

However, Thieme's method had several disadvantages. It can be seen from the R_f values in solvent systems A and B (Appendix Table 6) that several of the glycosides could not be separated completely from each other. For example, salidroside (Solvent A, R_f=0.24; Solvent B, R_f=0), salicin (Solvent A, R_f=0.26; Solvent B, R_f=0), and picein (Solvent A, R_f=0.33; Solvent B, R_f=0) have R_f values so close that separation cannot be obtained. This is also true for salicortin (Solvent A, R_f=0.50; Solvent B, R_f=0.05) and fragilin (Solvent A, R_f=0.55; Solvent B, R_f=0.06). Grandidentatin (Solvent A, R_f=0.88; Solvent B, R_f=0.28), tremuloidin (Solvent A, R_f=0.90; Solvent B, R_f=0.44), and populin (Solvent A, R_f=0.92; Solvent B, R_f=0.54) separation could not be obtained by solvent system A, although these three glycosides could be separated in system B. It is especially difficult to differentiate between salicin, salicortin, fragilin, populin and tremuloidin, since all five glycosides gave the same yellow spot when treated with Millon's reagent.

Another disadvantage of Thieme's method is the time involved which requires 11 to 12 hours to develop 30 cm. of chromatogram.

Prior to this study, no data had been reported regarding the TLC separation and identification of phenolic glycosides. However, recently Pearl et al. (67,68) report the use of TLC in the identification of salicin, populin, tremuloidin and salireposide employing silica gel layers, and using 50% sulfuric acid to locate the spots of chromatogram.

TLC is commonly employed as a rapid and sensitive analytical method, therefore a complete system for the thin-layer chromatographic separation was developed.

Since the phenolic glycosides are very similar in polarity, and because of the humidity effects and other influences of the silica gel layer, the separation on a silica gel G layer was not satisfactory at first. Therefore the polyamide system for TLC was also developed simultaneously.

Eleven Salix phenolic glycosides* were available as reference compounds: salicin, populin, salireposide, tremuloidin, grandidentatin, picein, triandrin, fragilin, vimalin, salicortin and salidroside.

* Thieme kindly supplied the following: picein, triandrin, fragilin, vimalin, salicortin and salidroside.
Pearl kindly supplied the following: salicin, populin, tremuloidin, salireposide and grandidentatin.

Silica Gel G TLC System

Silica Gel G Layer Preparation

Initially, the silica gel G layer was coated by the standard method (according to Stahl). (61) This layer did not give satisfactory results with solvent systems having a high water concentration. Therefore the slurry method of Fike and Sunshine (62) was substituted for the preparation of plates.

The silica gel G slurry and the chromatoplates were prepared in the following manner: 200 g. of the desired silica gel G (Research Specialties Co.) was stirred into 500 ml. of water. The mixture was stirred (or shaken) mechanically for 0.5 hour and then allowed to stand until needed. Just prior to the coating operation the slurry was shaken vigorously to resuspend the silica gel. Fifty-five ml. of the slurry was used to coat five 20 x 20 cm. glass plates with 300 μ silica gel G. The coated plates were allowed to stand until the slurry set (about 10 minutes) and were dried in the air overnight.

The dried plates were marked with a probe at 15 mm. from the front edge as the starting point, and a channel was cut 15 cm. above the starting point, as shown in Figure 13.

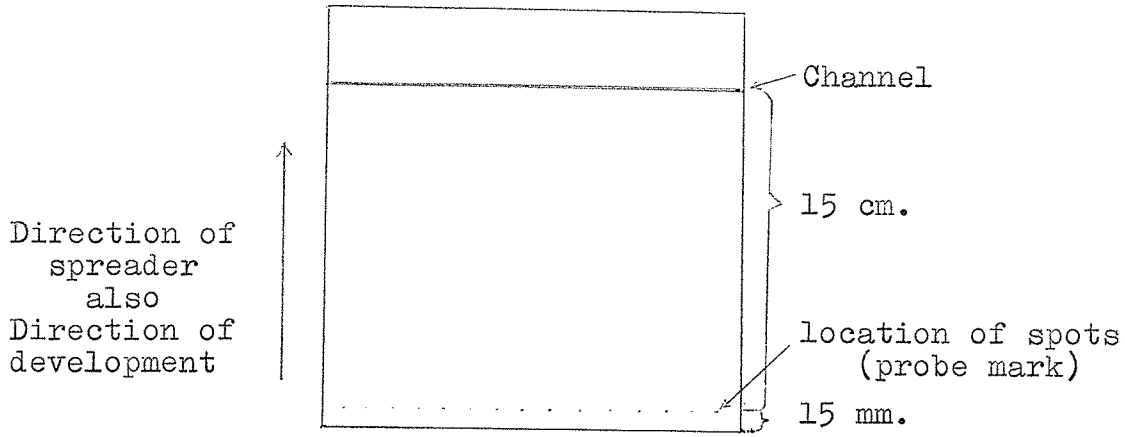


Figure 13

The plates for two-dimensional separation were marked differently. The spot upon which the sample was to be placed was located in the lower right hand corner 15 mm. from the front edge and 15 mm. from the right side of the plate. Two channels were then cut onto the plate using a template. One channel was 15 cm. above the application point and the other was 15 cm. left of the application point, as shown in Figure 14.

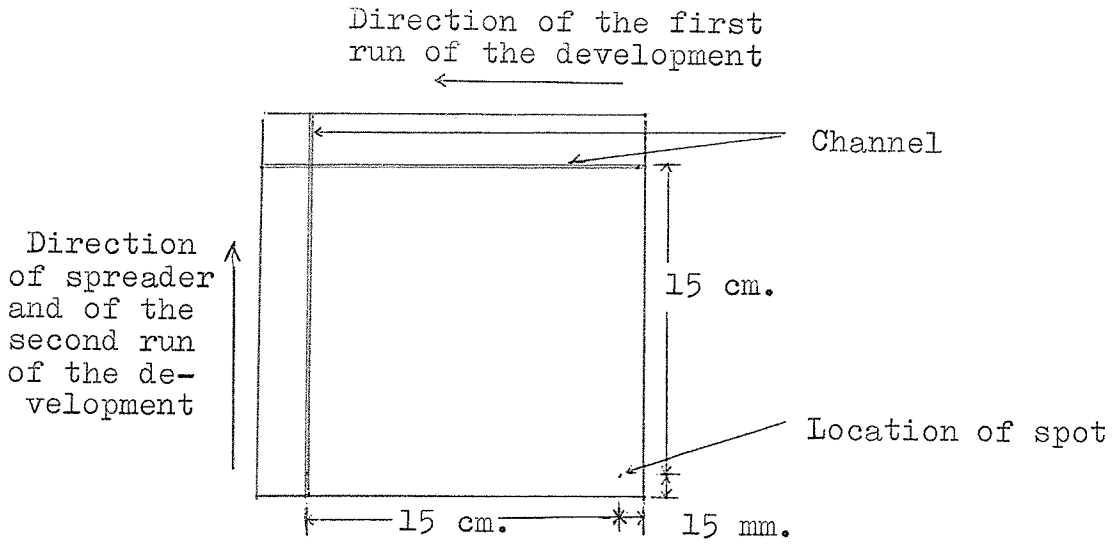


Figure 14

These channels were cut deeply to prevent the solvent front moving during the over running time. The plates were now ready for use.

Results and Discussion

The silica gel G TLC plates were prepared by Stahl's standard method using an air dried layer, however, grandidentatin and picein could only be separated using high water containing (about 4%) solvent systems. When this kind of solvent system was used, the silica gel G layer would flake or fly off when sprayed with reagent or when removed from the developing chamber.

This difficulty was overcome by using the slurry method

for the preparation of the silica gel G plates. This kind of layer gives very good adhesion and more resistance to flaking in high water containing systems. It was also very convenient to make chromatoplates at any time after the slurry was prepared. That is, the slurry could be used for up to three months after its preparation for the manufacture of silica gel G chromatoplates simply by shaking to cause resuspension.

Development of Chromatogram

An alcoholic solution of the sample was applied to the starting point of the prepared chromatoplate. The spot was dried and the plate subjected to humidity control. After the humidification, the plate was transferred directly to the developing tank which was prepared by the super-saturated method. (61)

The chromatogram was immediately developed in the solvent concerned (preferably at 22±1°C.). The solvent front was allowed to migrate 10 cm. or 15 cm. from the origin. The time required for the solvent to reach the top of the layer was noted and the plate was left for a further 15 minutes in the solvent (over running time). (63) After development in this way, the plate was removed, the solvent allowed to evaporate, and the glycoside spot developed with 4% sulfuric acid in anhydrous ethanol.

For the two-dimensional chromatograms, an alcoholic solution containing sample was applied to the pre-marked

starting point. If an extract or unknown sample was applied, the reference sample was applied on the parallel free space near the edge to make the comparable chromatogram. The development distance from the origin to the solvent front was 15 cm. in each direction.

After the plate was developed in the first direction, it was dried, and re-humidified before development in the second direction. Following this it was treated as described previously.

Results and Discussion

For spotting the sample solution on the chromatoplate a melting point capillary which had been pulled out to a fine point in a Bunsen flame was used. It was found to be easier to control and more convenient to work with than a micropipette.

The amount of solution applied can be controlled by the size of the spot on the dried layer. For example one λ solution on 300 μ thickness of silica gel G layer gave one spot of 3.0 mm. diameter.

The humidity control and over running time were suggested by Dallas (63) in the "General Procedure for Obtaining Reproducible R_f Values" of TLC separation.

According to Dallas, this procedure should result in reproducible R_f values and "Absolute" R_f values. Unfortunately from this work no "Absolute" R_f was obtained, but agreeable

results were obtained.

The advantages of "over running" technique are: (63) the distance from origin to solvent front is accurately predetermined, and the Rf values across the plate become more constant. This makes comparisons with control substances more reliable, but unfortunately does not correct the strong "edge effect" of the solvent system.

In this work, it was found that the humidity effect is a most important factor for the separation of the phenolic glycosides on silica gel layers. The results are shown in Figures 15 to 18. Humidity is also important for the reproducibility of the Rf values. This will be discussed later.

The saturation of the atmosphere in the developing chamber with solvent vapour, and room temperature were also found to have moderate effects upon the Rf values. The room temperature variation especially affected the running time and the solvent front stability. If the temperature differed very much there was a direct effect upon the separation.

When chromatoplates were pre-saturated in the solvent vapours before development, it was found that better separation of phenolic glycosides resulted.

Multiple development in a single solvent system also produced better separation.

Humidity Control

The plate was spotted and then placed for 16 hours in an enclosed, filter paper lined normal developing chamber which contained 100 ml. of either a saturated aqueous sodium bromide solution (which gave a relative humidity of 58%), or a saturated solution of sodium thiosulphate (which produced a relative humidity of 78%) according to the procedure of Dallas. (63)

Results and Discussion

Under regular laboratory working conditions, the R_f values of the glycosides showed a large variation using the same solvent system. This resulted because the air drying time and room relative humidity differed, which caused different activity on the thin layer. In order to get comparable results, the layer was standardized under constant relative humidity conditions.

First the relative humidity was controlled at 58% by using a saturated sodium bromide aqueous solution, but this relative humidity produced activity of the layers which was not quite suitable for the phenolic glycoside separation. With a wide variety of differing solvents almost the same resolution effect in separating the glycosides was obtained. This gave a fairly acceptable separation, but the glycosides were not as widely separated as was desired. The results are shown in Figure 15. No single system completely separated

each of the reference glycosides.

When the relative humidity was changed from 58% to 78% good separation was found on the chromatograms of the phenolic glycosides. The results are shown in Figure 16.

The only disadvantage of this system is the long time required to control the humidity.

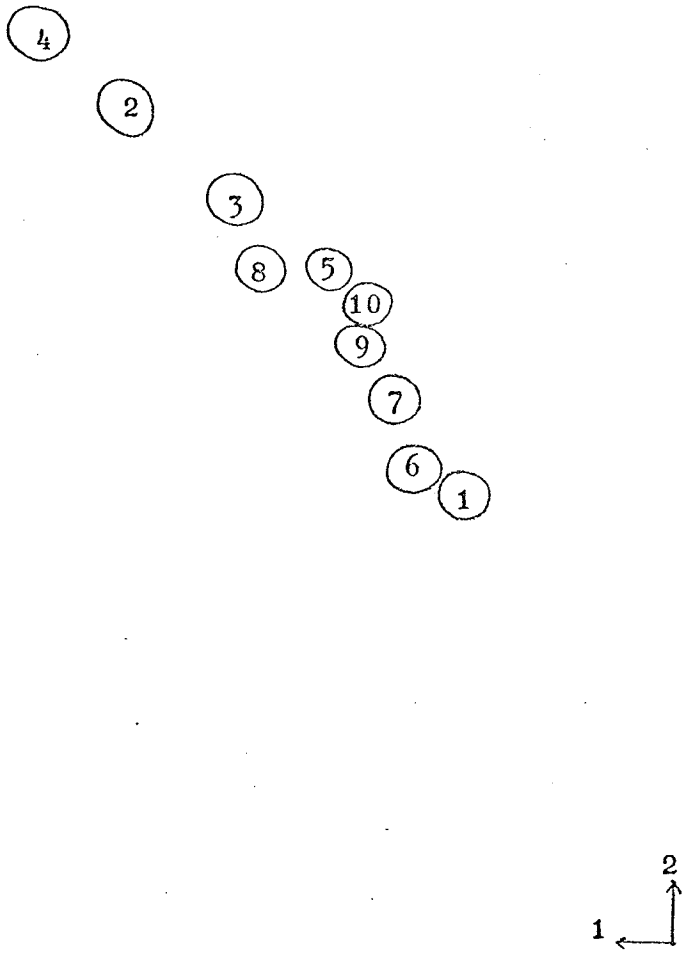
The chromatograms of phenolic glycosides are quite sensitive to the relative humidity. It was found that, when a plate was exposed to the air for too long a period of time before being developed in the high humidity system, tailing resulted.

Due to this fact and some other unknown factors, even under the complete humidity control conditions, it was impossible to get "Absolute" R_f values for the phenolic glycosides.

The above might explain why silica gel TLC separation gives variable results. When used only for identification purposes, especially when reference compounds are available, the rigid humidity control is not necessary.

Since humidity control required a great deal of time, the conditions were modified in the following method to speed up the procedure. The relative humidity of the laboratory ranged from 60-80%, and no special treatment is necessary in this range. The separation results are shown in Figure 17. Instead of the 78% relative humidity control for 16 hours, one can leave the spotted chromatoplate in a water

Figure 15. Two-dimensional Chromatogram of Salix Phenolic Glycosides on silica gel G Layer



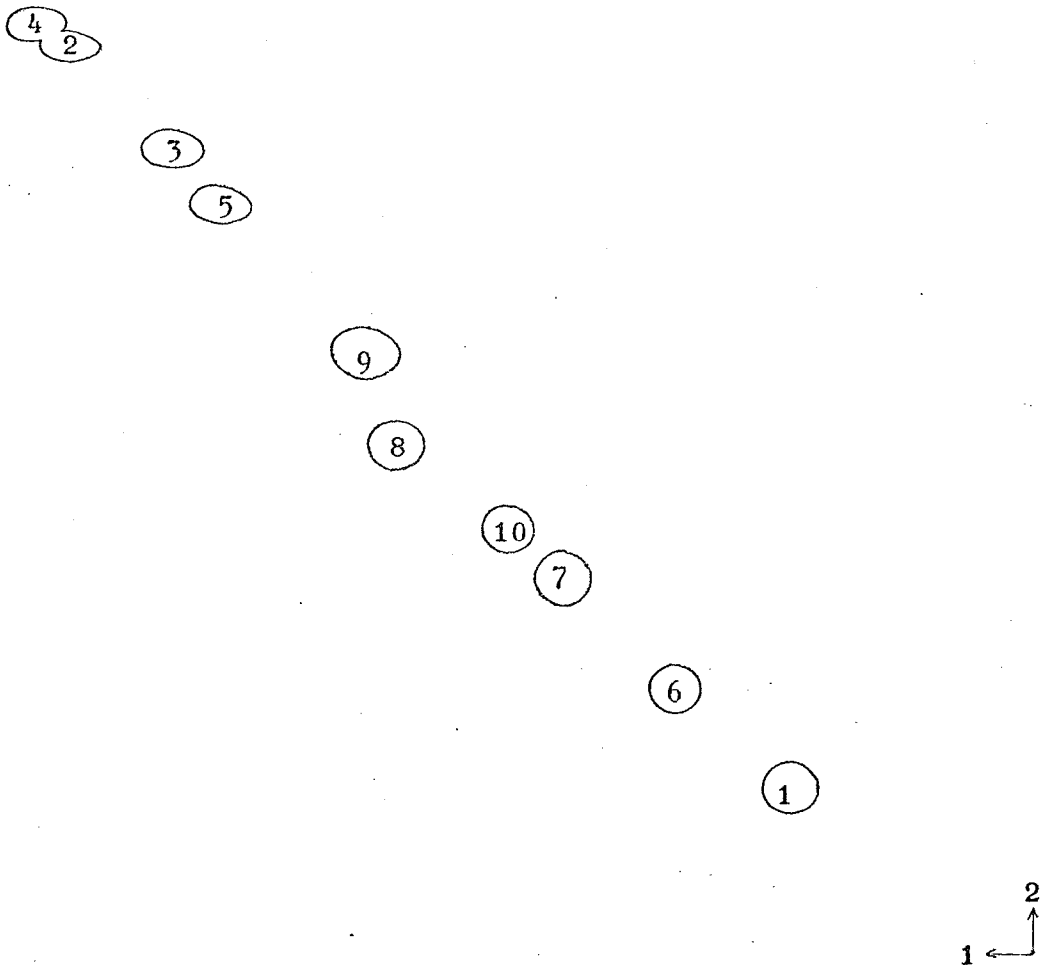
Solvents: (1)Ethylacetate/Ether/Formic Acid/Water (67:4:4:5)
(2)Ether/Methylethylketone/Formic Acid/Water (7:1:1:1)

Conditions: R.T. 22 °c, H. 58%, 15 cm. for each direction.

Reagent: 4% Sulfuric acid in Anhyd. Ethanol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin.

Figure 16. Two-dimensional Chromatogram of Salix Phenolic Glycosides on Silica gel g Layer



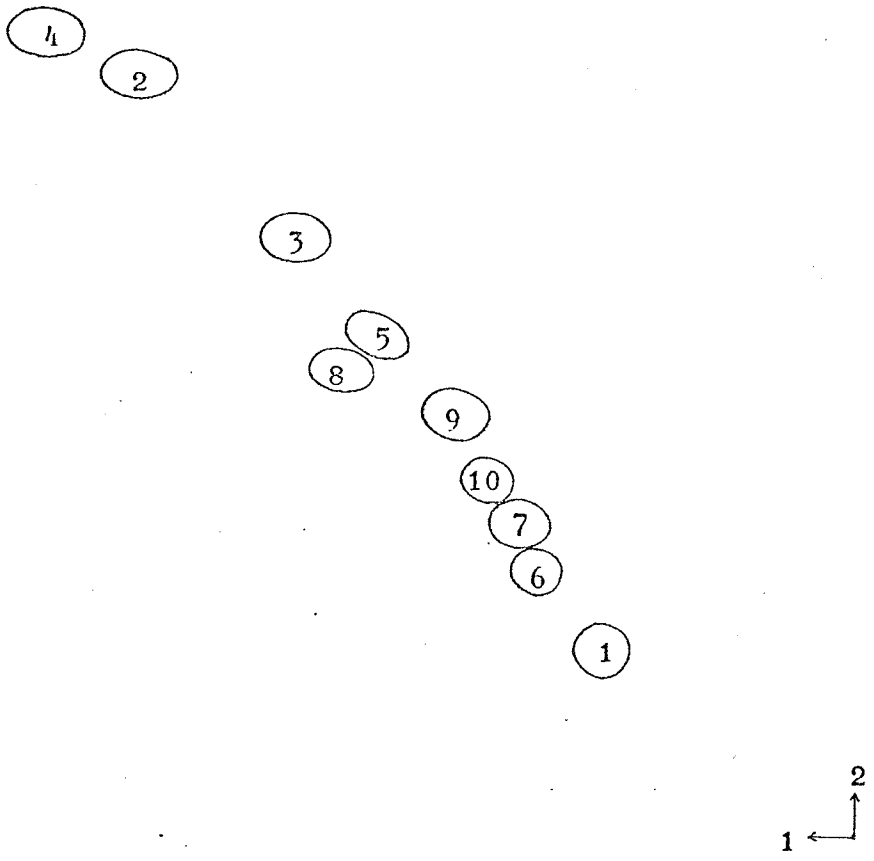
Solvents: (1)Ethylacetate/Methylethylketone/Formic Acid/Water (15:3:1:1)
(2)Ethylacetate/Xylene/Formic Acid/Water (35:1:2:2)

Conditions: R.T. 22 °c, H. 78%, 15 cm. for each direction.

Reagent: 4% Sulfuric acid in Anhyd. Ethanol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin.

Figure 17. Two-dimensional Chromatogram of Salix Phenolic Glycosides on Silica gel G Layer



Solvents: (1)Ethylacetate/Methylethylketone/Formic Acid/Water (15:3:1:1)
(2)Ethylacetate/Xylene/Formic Acid/Water (35:1:2:2)

Conditions: R.T. 22 °c, R.H. 72%, 15 cm. for each direction.

Reagent: 4% Sulfuric acid in Anhyd. Ethamol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin.

vapour saturated tank (which was prepared as mentioned and contained only water). The chromatoplate then was allowed to stand in the saturated tank for 40 minutes.

This resulted in a separation of the phenolic glycosides which was almost comparable with the separation which was received under 78% relative humidity control conditions (Figures 16 and 18).

The water content of the silica gel layer on the plate can be controlled by the duration of time which the plate spends in the saturated water vapour chamber.

The room relative humidity was determined by a dry and wet bulb thermometer.

Development of a Suitable Solvent System

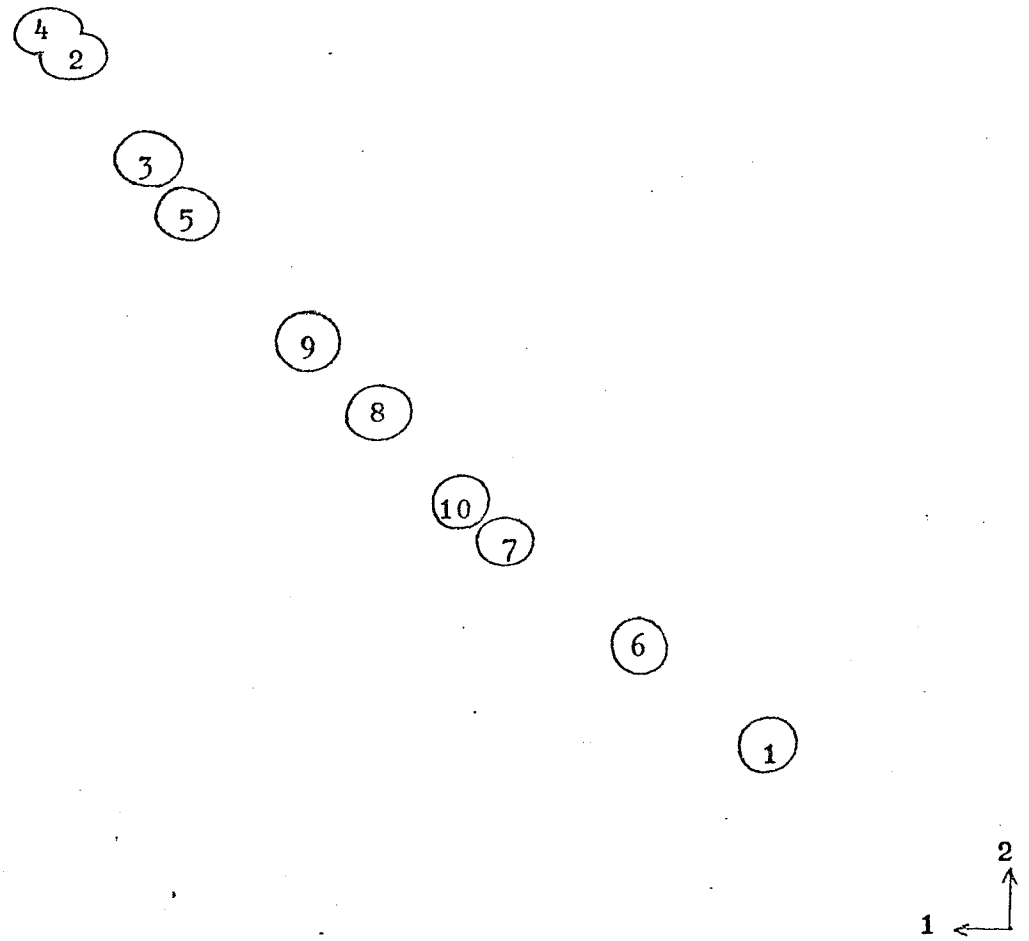
All solvent systems were screened by running reference compound chromatograms on small chromatoplates (5 x 20 cm.) which were prepared as described above.

In each case, the simplest solvent or composition was first tried. The R_f values and resolution powers were compared and then modified to improve the separation characteristics of the solvent system as desired.

Solvent systems were investigated under regular laboratory working conditions. The humidity was controlled at a specific temperature (22± 1°C.).

Before humidity control was employed, 83 different solvents or solvent mixtures had been investigated and the

Figure 18. Two-dimensional Chromatogram of Salix Phenolic Glycosides on Silica gel G Layer



Solvents: (1)Ethylacetate/Methylethylketone/Formic Acid/Water (15:3:1:1)
(2)Ethylacetate/Xylene/ Formic Acid/Water (35:1:2:2)

Conditions: R.T. 22 °c, 40 min. in water vapour satd. chamber, 15 cm. run.

Reagent: 4% Sulfuric acid in Anhyd. Ethanol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Ficein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin.

chromatograms were recorded.

Under 58% relative humidity conditions, 75 different solvent systems chromatograms were developed and recorded.

The best solvent systems which were obtained from the above experiments were tried and further modified under 78% relative humidity conditions, until excellent separation of the phenolic glycosides was received.

Results and Discussion

Under 58% relative humidity control the acceptable solvent systems were found to be:

(i) Ethyl acetate/Xylene/Formic acid/Water (35:1:2:2)

(ii) Ethyl acetate/Ether/Formic acid/Water (67:4:4:5)

(iii) Methyl ethyl ketone/Xylene/Water (37:1:2)

(iv) Ether/Methyl ethyl ketone/Formic acid/Water (7:1:1:1)

(v) Ethyl acetate/Ether/Formic acid/Water (17:1:1;1)

All ten phenolic glycosides cannot be completely separated with these single systems.

Two-dimensional chromatograms using solvents (ii) and (iv) gave good resolution of all glycosides, but the separation of low R_f glycosides such as salicin, picein, triandrin, grandidentatin, vimalin and salicortin remained too near each other (Figure 15).

The chief advantage of the 58% relative humidity condition was that populin and tremuloidin separated in each solvent system and were easy to identify from each other.

Under 78% relative humidity conditions the following solvent systems were used:

(i) Ethyl acetate/Xylene/Formic acid/Water (35:1:2:2)

(vi) Ethyl acetate/Methyl ethyl ketone/Formic acid/Water (15:3:1:1)

(vii) Ethyl acetate/Methyl ethyl ketone/Formic acid/Water (13:5:1:1)

(viii) Methyl ethyl ketone/Benzene/Water (18:1:1)

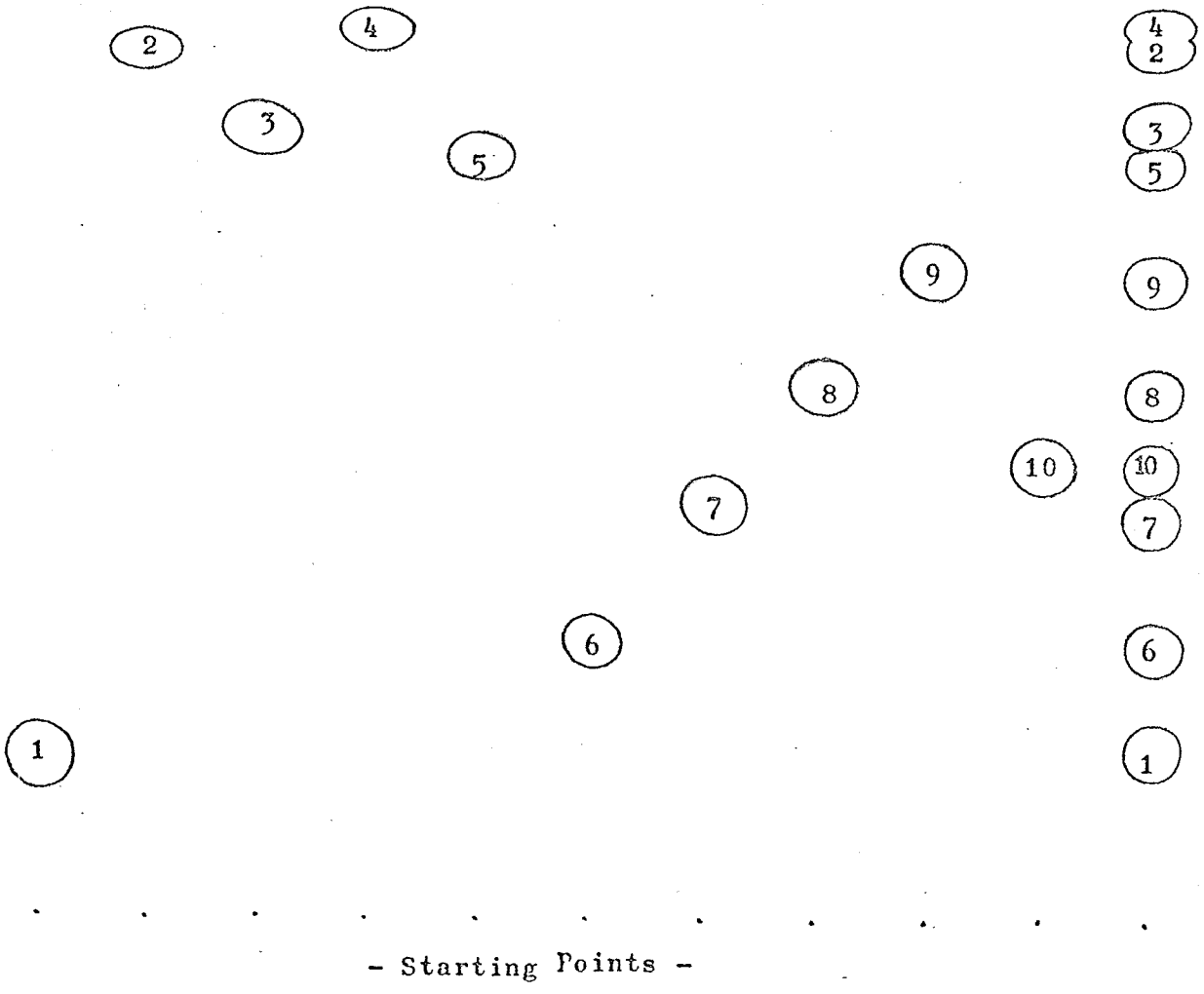
Under the 78% relative humidity control system, the ten reference compounds were separated by a single system, although some glycoside spots touch each other, but all of the glycosides were easily identified by the developed spot colours.

On two-dimensional chromatograms, the reference glycosides which were completely separated are: salicin, salireposide, grandidentatin, picein, triandrin, fragilin, vimalin, salicortin and salidroside. Only populin and tremuloidin were not completely separated from each other by using EXFW (35:1:2:2) and EMFW (15:3:1:1) systems. The separation chromatograms are shown in Figure 16. All ten phenolic glycosides were separated by EXFW (35:1:2:2) and EMFW (13:5:1:1). The chromatograms are shown in Figure 21.

The best solvent system was found to be EXFW (35:1:2:2) which possessed the strongest mobility. The chromatogram spots were always very compact.

The disadvantage of methyl ethyl ketone containing systems was that longer spots were formed (Figure 20).

Figure 19. Chromatogram of Salix Phenolic Glycosides on Silica gel G Layer



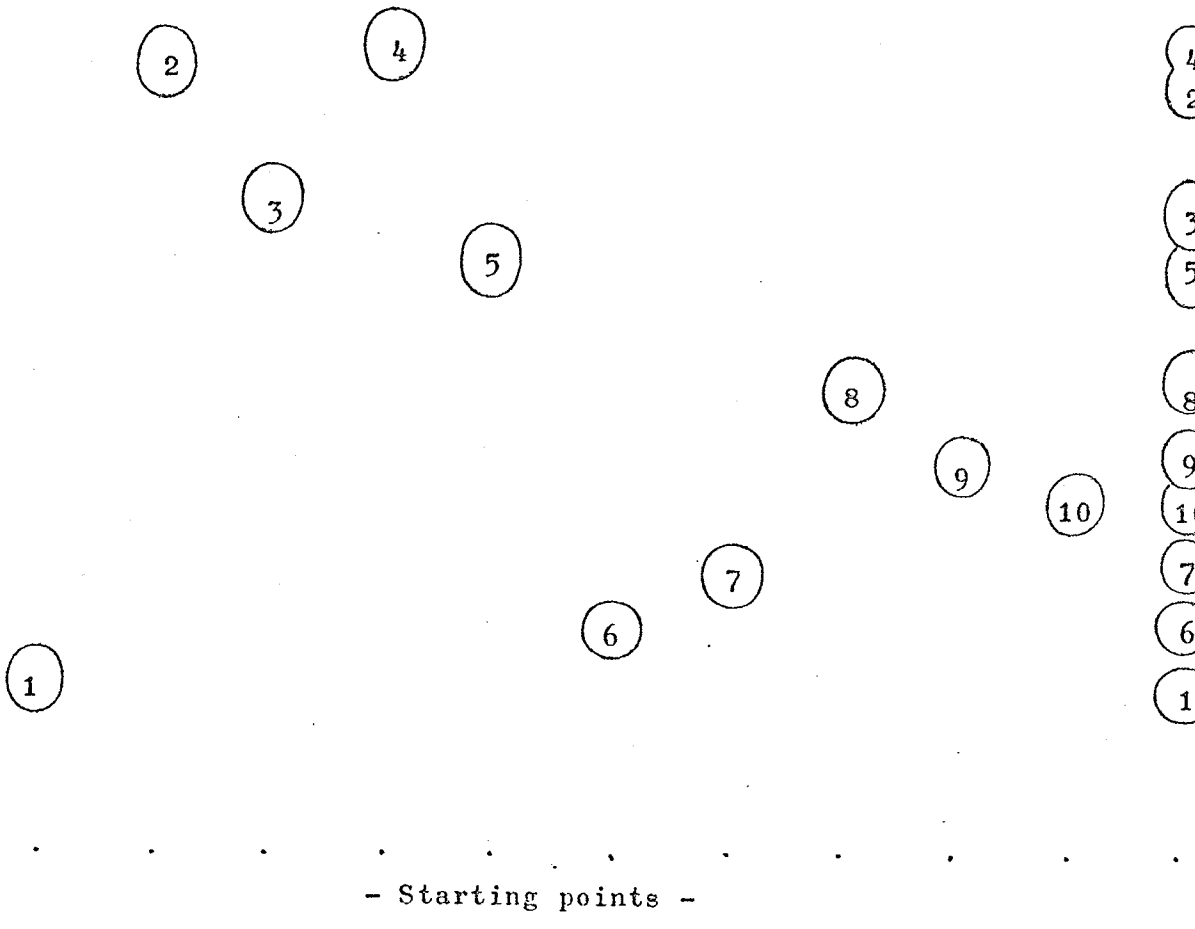
Solvent: Ethylacetate/Xylene/Formic Acid/Water (35:1:2:2)

Conditions: R.T. 22 c, 40 min. in water vapour satd. chamber, 15 cm. run.

Reagent: 4% Sulfuric Acid in Anhyd. Ethanol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin.

Figure 20. Chromatogram of Salix Phenolic Glycosides on Silica gel G Layer



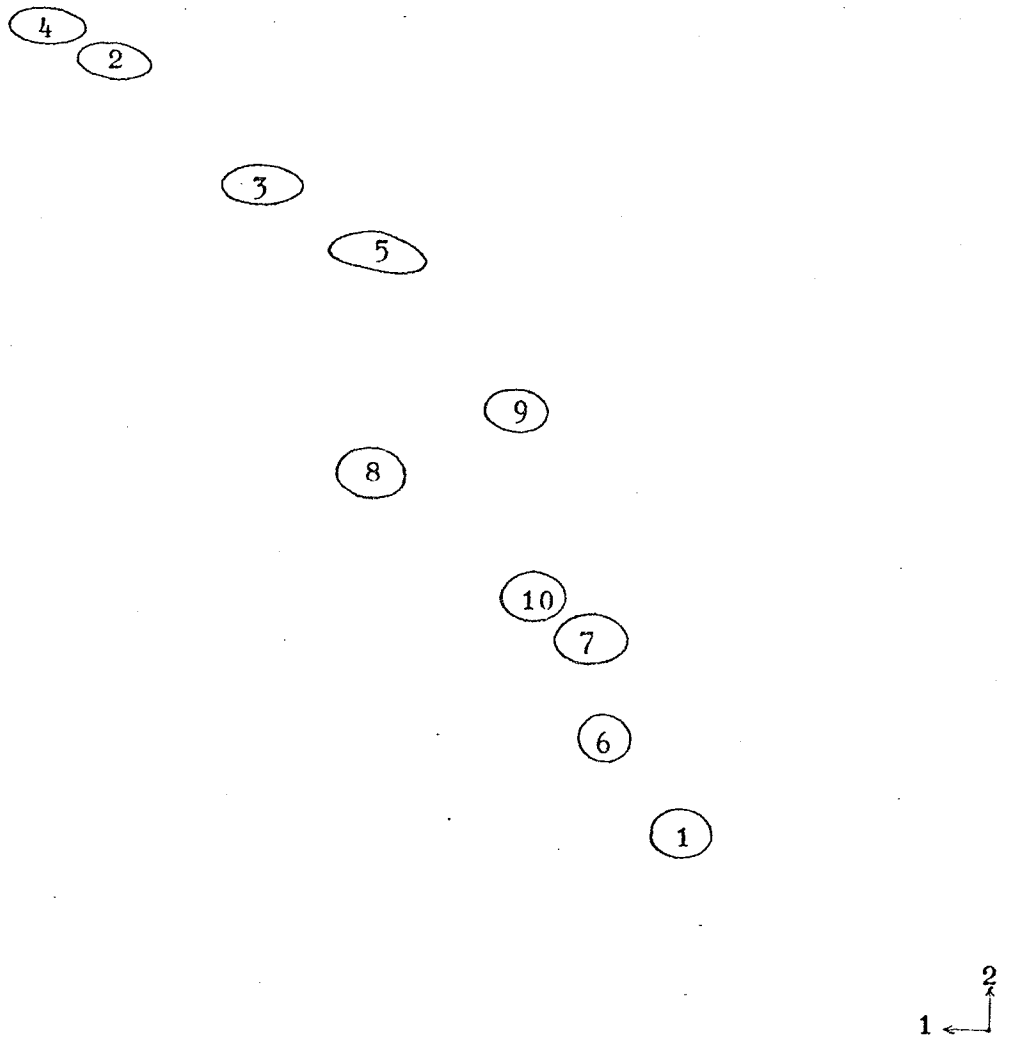
Solvent: Methylethylketone/Benzene/Water (18:1:1)

Conditions: R.T. 22 c, 40 min. in water vapour satd. chamber, 15 cm. run.

Reagent: 4% Sulfuric acid in Anhyd. Ethanol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin.

Figure 21. Two-dimensional Chromatogram of Salix Phenolic Glycosides on Silica gel G Layer



Solvents: (1) Ethylacetate/Methylethylketone/Formic Acid/Water (13:5:1:1)

(2) Ethylacetate/Xylene/Formic Acid/Water (35:1:2:2)

Conditions: R.T. 22 °c, H. 78%, 15 cm. for each direction.

Reagent: 4% Sulfuric acid in Anhyd. Ethanol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin.

Also the spots developed with sulfuric acid reagent displayed a dull yellowish colour.

The EMFW (13:5:1:1) system had similar resolution power to EMFW (15:3:1:1) but the R_f value of glycosides was higher and some tailing was noted.

The only disadvantage of the whole silica gel separation system was that the solvent systems were a little too complicated. This was due to the fact that the phenolic glycosides are very similar in polarity.

In general, a two-dimensional chromatogram should be developed in two completely different solvent systems. However, the author found that if the same solvent systems were used changing only the humidity conditions (58% and 78%), better resolution and distribution of the phenolic glycosides were given than if two different solvent systems in the same humidity condition were used. This was not only simpler but also solved the problem of completely separating populin and tremuloidin (isomers of each other).

Detection Reagents

Several reagents have been tried:

(i) Millon's reagent (50,55)

(ii) Kedde reagent (64)

(iii) 2,3,5-Triphenyltetrazolium chloride (TTC) (64)

(iv) Antimony trichloride in chloroform (61,64)

(v) Ammoniacal silver nitrate reagent (65)

(vi) Silver nitrate (Trevelyan) reagent (66)

(vii) 4% Sulfuric acid in anhydrous ethanol.

Long and short wave ultra-violet light absorption was also used to detect spots on developed chromatograms.

Results and Discussion

Millon's reagent was used by Thieme (48,50) as a locating reagent for paper chromatography and was used in the author's laboratory. It was found to be significantly less sensitive than the sulfuric acid reagent on silica gel G layers.

The other disadvantage of this reagent is that the coloured spots fade after 1 to 2 days, and it is a strongly corrosive reagent, consequently it is not convenient to work with.

Kedde reagent was found to be an excellent aid in the identification of some glycosides. For example, picein gave a purple colour, grandidentatin yellow, triandrin and salicortin brown-orange immediately after being sprayed with this reagent. Other glycosides could not be detected on silica gel layers.

After spraying with 2,3,5-triphenyltetrazolium chloride (TTC), grandidentatin gave a yellow spot, and salicortin a red rose colour, but other glycosides could not be detected on the silica gel layers.

After spraying with antimony trichloride in chloroform and heating to 110°C. for 20 minutes, salireposide gave an

orange spot, triandrin and vimalin purple, fragilin brownish yellow and salicortin showed a yellow spot. The other glycoside spots which were colourless after spraying and heating all showed purple colour under long-wave ultra-violet light.

Ammoniacal silver nitrate reagent must be freshly prepared. After this reagent was sprayed on the plate it stood at room temperature for 10 minutes. Salireposide and triandrin gave grey spots, salicortin gave a brown spot. The plate was then sprayed with N/2 alcoholic sodium hydroxide solution and stored at room temperature for 20 to 30 minutes. All glycosides gave dark brown spots against a light brown background. The grey developed spots gave black spots.

Silver nitrate (Trevelyan) reagent was used by Pearl et al. (28,29) as a locating reagent on their paper chromatograms. After spraying the chromatoplates, no glycoside spots appeared. After 5 to 10 minutes the plates were sprayed with N/2 alcoholic sodium hydroxide solution and left at room temperature for 10 to 15 minutes. All glycosides showed dark brown spots (salireposide and triandrin were darker) against the brownish background. For ammoniacal silver nitrate and silver nitrate reagent, more alcoholic alkaline solution can be sprayed if necessary. Later the plate can be sprayed with 10% aqueous sodium thiosulfate solution to give clearer spots against a light brown background.

4% Sulfuric acid in anhydrous ethanol was found by the author to be the best reagent for phenolic glycosides on

silica gel layers. After the chromatogram was developed, the plate was heated at 110°C. for 10 minutes. Then the hot plate was sprayed with the reagent directly. Triandrin and vimalin produced blue colour spots, salireposide orange, grandidentatin yellow (but the colour faded after a few minutes) and salicortin brownish yellow immediately. After the plate had been heated a further 15 minutes at 110°C. all eleven reference glycosides gave a specific colour: salicin, populin, tremuloidin and fragilin gave red colours, salireposide orange, grandidentatin yellow-brown, picein and salidroside orange-brown, triandrin brown-blue, vimalin grey-blue and salicortin orange-red.

This pre-heated treatment was found to be superior to spraying followed by heating. Using the pre-heated treatment it was easy to locate triandrin, vimalin, salireposide and grandidentatin.

Picein and grandidentatin required longer heating to obtain a clear spot. After heating for 30 minutes or longer all coloured spots became dark.

Methanol and 95% ethanol could also be used as solvents for this reagent, but anhydrous ethanol gave spots of much brighter colour.

The sensitivity of the sulfuric acid reagent was determined by using 0.1% ethanolic solution of the ten reference glycosides and running a chromatogram on the plate. The detection limit of all of the glycosides except grandidentatin

and picein was 0.2 µg in daylight. These two glycosides could be detected in concentrations of 0.4 µg. If the glycosides were detected with the help of a long wave ultra-violet light after heating, salireposide, triandrin and vimalin could be detected in concentrations of 0.1 µg. The other glycosides were detectable in concentrations of 0.2 µg.

The advantages of this reagent included the simplicity, convenience and high sensitivity; as well as these, all of the glycosides developed a specific colour which was easily located and identified.

Under long-wave ultra-violet light only grandidentatin was detectable as a fluorescent spot. All glycosides could be detected with short-wave ultra-violet light, either by their fluorescence or absorption of ultra-violet light, but this method was not sensitive enough to locate the phenolic glycoside chromatograms.

Polyamide-Cellulose TLC System

Pure Polyamide Layer

The initial experiments were carried out on a pure polyamide layer which was prepared by suspending 5 g. Polyamide Woelm TLC in 45 ml. ethanol. This suspension was then coated onto TLC plates to a depth of 300 µ using a Research Specialties Co. spreader. The layers were air dried.

Polyamide-Cellulose (2:1 w/w) Layer

Five grams of Polyamide Woelm TLC was mixed with 2.5 g. of cellulose TLC powder (Gerard Pleuger). The mixture was suspended in 50 ml. ethanol in a 125 ml. glass stoppered Erlenmeyer flask. It was shaken vigorously for at least 5 minutes. This suspension was then spread over five 20 x 20 cm. glass plates to a depth of 300 u. The plates were left to dry in the air. The dried plates were marked and prepared the same as the silica gel G TLC plates.

Results and Discussion

Separation of the phenolic glycosides with high resolution was obtained on pure polyamide layers. The results are shown in Figure 23.

Unfortunately this pure polyamide layer had several disadvantages. The first of these is that the layers were very weak and cracked (no flaking), but it was still satisfactory for one-dimensional separation. However, when applied to two-dimensional separations, the dried developed layer easily flaked off on spraying with the colouring reagents.

The author found that combined polyamide TLC and cellulose TLC powder (2:1 w/w) gave a much better adhesive layer than did pure polyamide. There was no cracking of the layer. Also this polyamide-cellulose layer gave about the same

resolution power as did the pure polyamide layer (Figures 24 and 25).

The Woelm Company suggested that 45 ml. of chloroform-methanol (2:3) should be used for preparing the pure polyamide slurry. However, the author found that ethanol gave a better layer, because the mixture solvent evaporated too quickly which made the layer crack more. Also the chloroform dissolved and consequently would have ruined the operating template.

The disadvantage of both of these types of layers was restricted to the fact that acid spraying reagents could not be used.

Development of Chromatograms

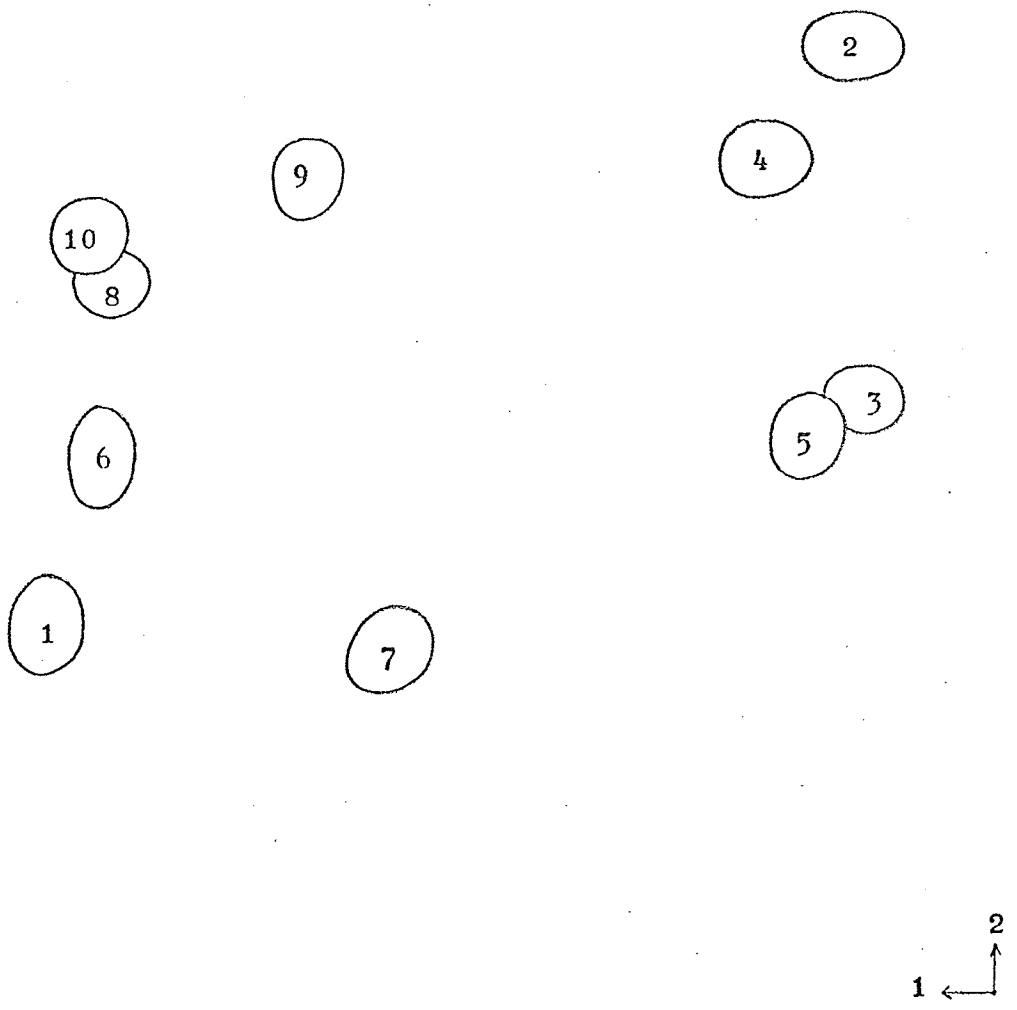
The chromatoplate was prepared and developed the same as was described for silica gel layers, but no humidification was necessary for these plates.

After development, the plates were sprayed and the spots located with reagents.

Development of a Suitable Solvent System

The solvent system was developed under the same conditions and in the same manner as were the silica gel G solvent systems except that humidity control procedures were found to be unnecessary.

Figure 22. Two-dimensional Chromatogram of Salix Phenolic Glycosides on Polyamide-cellulose Layer



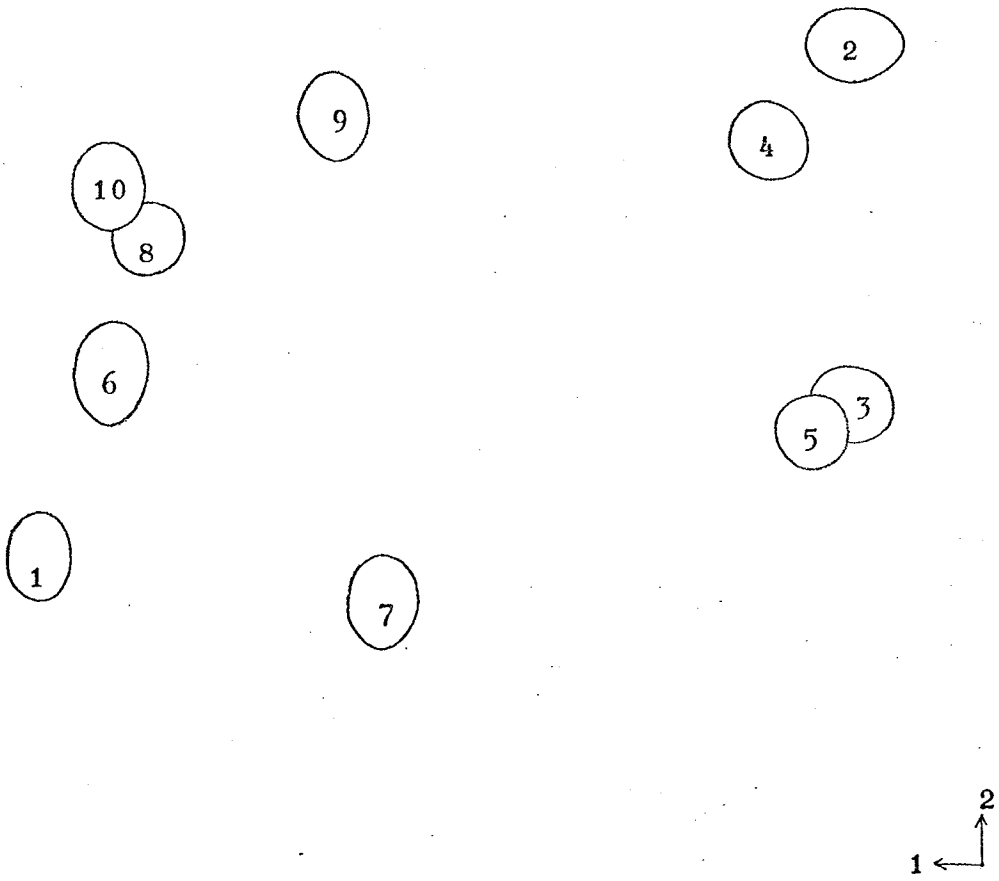
Solvents: (1) Water/Ether (24:1)
(2) Ethylacetate/Acetic Acid/Water (23:1:1)

Conditions: R.T. 22 c, 15 cm. for each direction.

Reagents: Kedde reagent-Millon's reagent.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin.

Figure 23. Two-dimensional Chromatogram of Salix Phenolic Glycosides on Polyamide-cellulose Layer



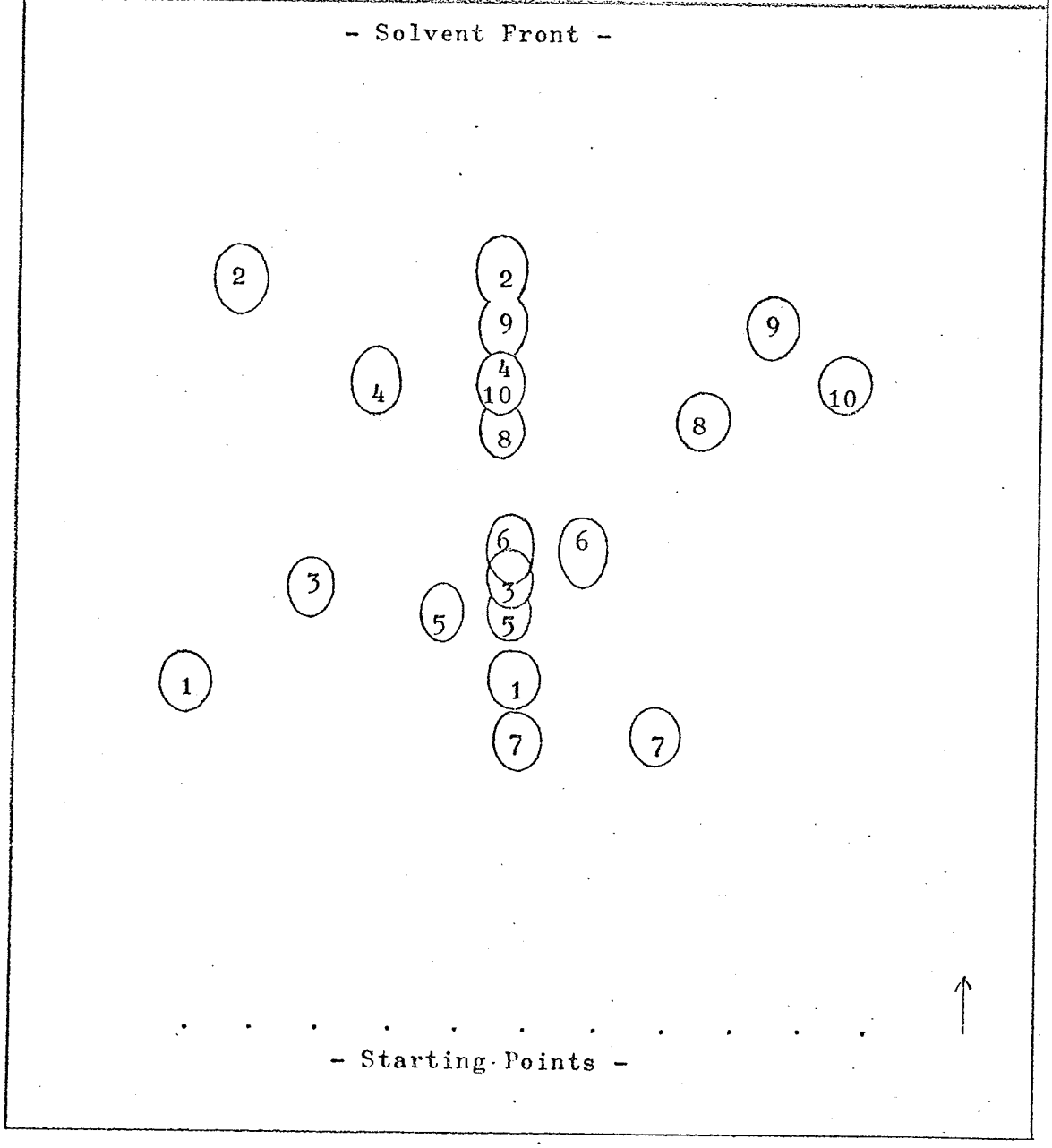
Solvents: (1) Water
(2) Ethylacetate/Acetic Acid/Water (23:1:1)

Conditions: R.T. 22 °c, 15 cm. for each direction.

Reagents: Kedde reagent-Silver nitrate reagent.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin.

Figure 24. Chromatogram of Salix Phenolic Glycosides on Polyamide-cellulose Layer



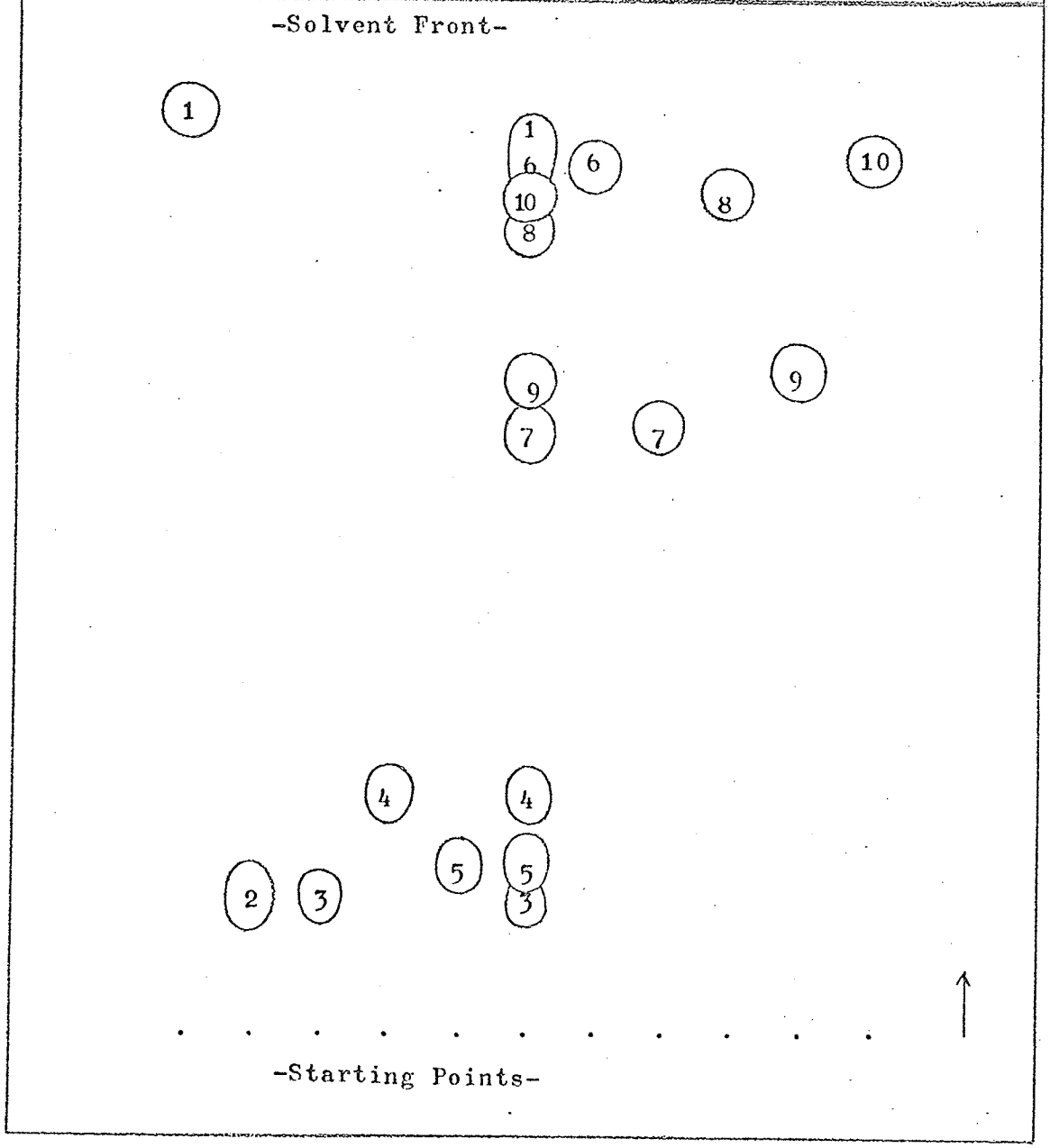
Solvent: Ethylacetate/Formic Acid/Water (23:1:1)

Conditions: R.T. 22 °C, 34 minutes for 15 cm. run.

Reagents: Kedde reagent-Silver nitrate reagent.

Glcosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin.

Figure 25. Chromatogram of Salix Phenolic Glycosides on Polyamide-cellulose Layer



Solvent: Water/Ether (24:1)

Conditions: R.T. 22 °C, 60 minutes for 15 cm. run.

Reagents: Kedde reagent-Silver nitrate reagent.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin.

First, plates coated with a pure polyamide layer were used. These were later modified by using a polyamide-cellulose (2:1) layer in order to improve the adhesion of the layer to the plates.

Water and ethylacetate were chosen as the fundamental solvents of two different solvent systems. Methyl ethyl ketone also was tried.

During the course of development of the best solvent separation, 27 different solvent systems were investigated and the chromatograms were recorded in each.

Results and Discussion

The best solvent systems were found to be the following:

(i) Water

(ii) Ethylacetate/Acetic acid/Water (23:1:1)

(iii) Ethylacetate/Acetic acid/Water (91:5:4)

The resolution powers for the separation of the phenolic glycosides of solvents (ii) and (iii) were found to be close to each other. The glycosides' R_f values were found to be slightly higher in system (iii) than in system (ii).

At first tailing was noted on the plates run in solvent (i), therefore ether was combined with the water to compact the spots. However, tailing still occurred. The tailing on the plates was finally found to be due to the fact that the silver nitrate reagent was not sensitive enough, and the tailing on

the plates was due only to overloading the chromatogram of ten phenolic glycosides as shown in Figures 22 and 23.

Both of these aqueous and organic solvent systems did not completely separate the ten reference glycosides using one-dimensional development on polyamide-cellulose layer. Every glycoside was identified by using these two solvent systems (aqueous and organic). This is because in two of the solvent systems they gave a completely reverse and different separation result as shown in Figures 24 and 25.

This gave especially good results on the two-dimensional separation map as shown in Figures 22 and 23.

Detection Reagents

The following reagents were employed:

(i) Millon's reagent (50,55)

(ii) Potassium permanganate T.S. (55)

(iii) Antimony trichloride (61,64)

(iv) 2,3,5-Triphenyltetrazolium chloride (64)

(v) Kedde reagent (64)

(vi) Ammoniacal silver nitrate reagent (65)

(vii) Silver nitrate (Trevelyan) reagent (66)

Results and Discussion

The detection of phenolic substances on polyamide layers is usually done by examining the chromatograms under ultra-

violet light. The chromatograms are then sprayed with fluorescent reagent and the colour of the fluorescence is again determined under ultra-violet light. (66)

The reagents used for the detection of phenolic glycosides on polyamide-cellulose layers were restricted by the acid sensitivity of the polyamide. The usual diazotized reagents, and acid glycosidic reagents including antimony trichloride could not be used due to spoilage and reaction with the layers.

Millon's reagent on polyamide-cellulose layers caused phenolic glycosides to produce colour spots of the same, as described under silica gel G detection reagents. However, after heating, the layer was spoiled, but the spot could still be located. Most of the reference glycosides were located by heating the plates to 95°C. for 2 to 3 minutes. Under these conditions the layer was damaged less. However, picein required a longer heating time to develop on the polyamide-cellulose layer.

Potassium permanganate T.S. could be used for the detection of salicin, salireposide, grandidentatin, triandrin, vimalin and salicortin. All of the spots showed a yellow colour, but the spots were difficult to locate on the chromatogram.

Antimony trichloride caused the layer to become transparent after heating and the spots were difficult to locate.

2,3,5-Triphenyltetrazolium chloride-sodium hydroxide reagent, when sprayed onto the chromatoplate, caused salicortin

to produce a red rose coloured spot, and grandidentatin a yellow spot. After heating, the other glycosides became red in colour. It was difficult to distinguish these glycosides on the chromatogram due to the reddish colour of the background and to the lack of sensitivity of the reagent.

The Kedde reagent gave similar results on polyamide-cellulose layers to those obtained on silica gel G layers, that is, grandidentatin gave a yellow colour, picein purple, triandrin and salicortin orange-brown. After heating to 110°C. for 10 minutes, the background became brownish purple. The other reference glycosides gave purple spots after heating and previously developed spots were also darker. It was difficult to locate this reagent's spots on the chromatogram.

Ammoniacal silver nitrate-sodium hydroxide reagent and silver nitrate (Trevelyan)-sodium hydroxide reagent also gave similar results to those obtained on silica gel G layers. However, the background became reddish-brown in colour after the plate was sprayed with 10% sodium thiosulphate solution.

The Kedde reagent can be used in conjunction with the silver nitrate-sodium hydroxide reagent. The Kedde reagent was used first, followed by the silver nitrate reagent. This combined treatment gave much better results for the identification of grandidentatin, picein, triandrin and salicortin. All glycosides gave the same colour spots with the combined reagent that they did with the silver nitrate-sodium hydroxide reagent, but the background after being sprayed with sodium

thiosulphate became much lighter and the spots were clearer.

Unfortunately this combined treatment was still not sensitive enough for phenolic glycoside identification. The detection limit of salireposide, grandidentatin, triandrin and salicortin with this reagent was 2 μg. With picein and vimalin it was 10 μg. Salicin, populin, tremuloidin and fragilin could only be detected in a concentration of 20 μg.

Alternatively, the author found that when the Kedde reagent was used in conjunction with Millon's reagent, fairly good results for the identification of phenolic glycosides on polyamide-cellulose layers were obtained. The treatment consisted of using the Kedde reagent first to locate picein, grandidentatin, triandrin and salicortin. Then the chromatogram was sprayed with Millon's reagent.

Under this combined treatment, the layer was damaged less by the strong acid and no further heating was necessary to detect picein. The detection sensitivity was also acceptable.

The Stability and Change of the Reference Phenolic Glycoside Solution (in Alcohol)

During the period of TLC system development the alcoholic solution of the reference glycoside was found to have several extra spots on the chromatogram of the single glycoside solution, after the solution had been stored at room temperature

for a period of a few months.

The salicin derivatives such as populin, tremuloidin, fragilin and salicortin showed salicin and other spots on this chromatogram. It was especially interesting to note that tremuloidin (isomer of populin) also contained a fairly high concentration of populin.

The freshly prepared populin, tremuloidin, fragilin and salicortin solutions were heated at 60-64°C. for 24 hours. They were then subjected to TLC. It was found that tremuloidin, fragilin and salicortin gave a salicin spot, but populin was not detected in the tremuloidin that had been placed on the chromatogram. Therefore most of the phenolic glycosides are not very stable in alcoholic solution.

Column Chromatography

Thieme (50) successfully used a polyamide product called Miramid (VEB Leuna-werke) for the separation of phenolic glycosides from willow extracts by column chromatography. Water was used to elute the column. The combined fractions were further separated with a cellulose column to isolate glycosides having close solubility characteristics. n-Butanol/Xylene/Water (4:6:8) was used as the mobile phase for the cellulose column. (50)

Pearl and Darling (29) also isolated a mixture of salicin, populin and tremuloidin from a poplar extract by column

chromatography using a dry packed column of cellulose powder (Whatman standard grade) and eluting with ethylacetate/acetic acid/water (9:2:2) as a developer.

The author used polyamide (Woelm) and employed the procedure which was suggested by Thieme. (50)

The polyamide column was prepared using 10 gm. polyamide (Woelm) which had been extracted twice with boiling methanol and had been rinsed with hot water. The purified polyamide was resuspended in water and poured into a 2 x 40 cm. column. Four hundred mg. of crude phenolic glycoside were dissolved in methanol and mixed with 0.5 g. of polyamide powder. The mixture was dried in a vacuum desiccator. The dried mixture was poured onto the top of the prepared column and the column was eluted with water.

The author also tried a cellulose (Whatman standard grade) column. The sample was treated as described above. Ethylacetate/acetic/water (9:2:2) was used as a developer according to Pearl and Darling. (29)

Several other solvent systems and adsorbents were tested.

(i) Polyamide (Woelm) column eluted with ethylacetate.

(ii) Polyamide (Woelm) column eluted with water saturated ethylacetate.

(iii) Polyamide (Woelm) column eluted with petroleum ether/methanol/n-butanol (8:1:1).

(iv) Magnesium silicate (Woelm) column eluted with water saturated ethylacetate.

(v) Magnesium silicate (Woelm) column eluted with benzene/methanol (85:15).

(vi) Silica gel (BDH) column eluted with ethylacetate/xylene/isopropanol (10:2:3).

A gradient elution technique was also applied to a magnesium silicate (Woelm) column which consisted of 40 g. adsorbent in a 2.5 x 50 cm. column. The developers that were used in sequence were as follows: (i) Benzene/methanol (BM) (90:10) 400 ml., (ii) BM (85:15) 400 ml., and (iii) BM (80:20) 800 ml.

The eluted fractions were collected by our automatic fraction collector. Twenty-five ml. of eluate were collected in each fraction.

The fraction eluates from each column were determined by the TLC identification system.

The aqueous eluates from the polyamide column were first dried in a lyophilizer and were applied to TLC.

Results and Discussion

According to Thieme (33,50) the best way to purify and separate phenolic glycosides is column chromatography carried out in a polyamide column. Besides the preliminary fractionation of phenolic glycosides the clarification of the plant extract and the removal of the tanning principles and flavonoid glycosides occurred simultaneously by this procedure.

The polyamide (Woelm) gave similar resolution results

as Miramid (VEB Leuna-werke), but the two products have slightly different retention powers.

The disadvantages of polyamide chromatography for the separation of phenolic glycosides were that the early fractions contained too many glycosides, such as salicin, salidroside, picein, fragilin and salicortin which came off simultaneously. Triandrin and vimalin were also very near to this initial crowded area and in most cases were mixed with them. Therefore it was difficult to isolate these phenolic glycosides. Meanwhile the less soluble glycoside, especially salireposide, was distributed in too many fractions. Another disadvantage was that the fractionated aqueous eluate could not be left for too long a time at room temperature. This caused hydrolysis of the glycosides and was often accompanied by mould growth.

Therefore several organic solvents and different adsorbents were tried, but the polyamide with water system had the best resolution of all those tried.

The cellulose column system of Pearl and Darling (29) also did not give good separation.

The gradient elution in a magnesium silicate column gave acceptable results. The glycosides which had the higher R_f on silica gel TLC were eluted through the column first.

The silica gel column gave resolution effect which was similar to the magnesium silicate column.

For the further separation of the glycoside mixtures obtained from the polyamide aqueous fractions it was found

that a polyamide column with water saturated ethylacetate as a mobile phase gave much better separation than did a cellulose column. This gave almost the same results as the two-dimensional separation on polyamide TLC. The used polyamide can be regenerated by boiling with methanol and water.

The magnesium silicate and silica gel systems were also usable for the further separation of phenolic glycoside mixtures which had been obtained from polyamide aqueous fractions.

Screening Procedures

Both leaves and bark of *Salix petiolaris* (*S. gracilis* var. *textoris*) were used for screening procedures. These were collected in the Fall of 1964 from trees growing near Stonewall, Manitoba.

After harvesting, the leaves and bark were separated, oven dried (under 70°C.), and the bark powdered using the Fitzpatrick Hammer Mill. The samples were stored in separate plastic bags at room temperature until ready for investigation.

I. Screening for Alkaloids

Methods

Ten grams of bark powder (or leaves) were macerated with 50 ml. of ammoniacal alcohol (10 ml. strong ammonia in 90

ml. of 95% ethanol) (55) approximately 5 hours in a percolator. After the extract was collected and repercolated with 50 ml. of 95% ethanol, the percolates were acidified with dilute hydrochloric acid (55), and the solvent was removed under vacuum. To the resultant syrupy residue, 10 ml. of water was added. This acidic mixture was extracted with 3 x 10 ml. benzene, the benzene layer being discarded. The aqueous portion was made alkaline with ammonia, and then extracted with 3 x 5 ml. of chloroform. The chloroform extract was transferred to a distilling flask to which was added 1 ml. of 1% hydrochloric acid. The organic solvent was removed on a Rinco rotary evaporator until the organic and aqueous volumes were approximately equal. The normal alkaloidal tests were performed on the acidic aqueous extract as follows: Two drops were placed on a clean microscopic slide and the appropriate reagents were added. The resultant precipitate was examined for the presence of alkaloids.

The aqueous extract of leaves and bark were tested with the following five alkaloidal reagents: Dragendorff's (54), Hager's (55), Meyer's (55), silicotungstic acid solution (55) and Wagner's (54).

Results and Discussion

There are no reports of alkaloids having been isolated from willow species. The results of these tests tend to confirm the absence of alkaloidal material in willow species.

Table 4. Reaction of Alkaloidal Reagents

Extract	Dragen-dorff's	Hager's	Silicotung-stic acid	Meyer's	Wagner's
Bark	-	-	-	-	+
Leaves	-	-	-	-	+

Wagner's reagent gave a brown-red precipitate. Meyer's reagent became cloudy at first but cleared on being mixed well.

Arthur (56) has reported that plant extracts of Salix babylonia have given positive tests for the presence of alkaloid, but no further information about this has appeared.

The contradictory results of Arthur (56) might be due to interference by some other plant material.

II. Screening for Saponins and Tannins

Methods

Preparation of Extract - Half a gram of dry samples were mixed with 5 ml. water and heated at 100°C. for 10 minutes. This mixture was filtered by negative pressure and washed with water to give a filtrate of approximately 5 ml. This filtrate was examined for saponins and tannins as follows:

Foam Test for Saponins (57) - One ml. of the filtrate

was vigorously shaken in a test tube (20 x 100 mm.) and then allowed to stand. The height of the foam layer was measured after 15 minutes.

Microchemical Tests for Tannins (60) - Separate drops of the aqueous extract were distributed on a microslide and treated with 1 drop each of the following reagents: Ferric chloride T.S. (55), Basic Lead Acetate solution (55), Potassium dichromate T.S. (55) and 1% Gelatin solution.

Results and Discussion

The Foam Test for the detection of saponins developed by Reichstein and Abisch (57), which requires 1 ml. digitonin solution (1 mg. digitonin in 5 ml. water) as a reference standard, under these conditions, produces a foam layer 27 cm. high in the reference solution.

Bark and leaves extracts each produced a foam layer less than 0.5 cm. high. These results indicated little or no saponins in the plant material examined.

Only a triterpenoid (epifriedelinol) has been reported from the bark of S. japonica. (58)

Tannins in willow are reported to be soluble in hot water. (59) Therefore, water extracts were used in tests for tannins.

Many colour reactions have been suggested by Hass and Hill (60) as selective for the detection of tannins.

Table 5. Colour Reaction of Extracts

Reagent	Colour Reaction	
	Bark Extract	Leaves Extract
Ferric Chloride T.S.	green	green
Basic Lead Acetate Solution	yellow	yellow
Potassium dichromate T.S.	brown	brown
1% Gelatin Solution	grey	grey

The results for both bark and leaves indicate the presence of tannins in the species examined as indicated by the colour reactions as presented in Table 5.

III. Screening for Glycosides

Methods

Preparation of Extract - Ten grams of powdered bark (or leaves) were extracted in a soxhlet apparatus with 95% ethanol for 10 hours. The ethanol was recovered, and the syrupy residue taken up with 80 ml. water. The resulting mixture was extracted with three 10 ml. portions of benzene and the benzene layer discarded. The aqueous solution was treated with 10 ml. of strong lead subacetate solution (55), the precipitate was filtered off and washed with a small volume of

water. The clear filtrate was saturated with hydrogen sulfide, and the resulting precipitate filtered off. The filtrate was concentrated to about 10 ml. and extracted with five 10 ml. portions of ethylacetate in a separatory funnel. The final ethylacetate solution was concentrated to about 2 ml. which was used for the determination of phenolic glycosides.

Determination of Phenolic Glycosides - One dimensional TLC was used for the determination of phenolic glycosides. The concentrated ethylacetate extracts and reference glycosides were spotted on the same silica gel G or polyamide-cellulose, and then the plate was developed as described in the TLC section. The compounds were located on the silica gel G layer with 4% sulfuric acid in anhydrous ethanol. Kedde's reagent and silver nitrate-sodium hydroxide reagent were used on polyamide-cellulose layers. Millon's reagent was used to confirm the phenolic glycosides on both types of layers. The glycosides were identified by comparison of the characteristic colour produced by the reagents and by the R_f values of chromatograms with reference compounds.

Results and Discussion

Utilizing the colour development and the chromatographic behaviour of the spots as criteria and relating to reference phenolic glycosides run on the same plate, it was demonstrated that S. petiolaris contained salicin, salireposide,

tremuloidin, grandidentatin and picein. In addition, several unknown spots were detected on the chromatograms.

The leaves of S. petiolaris contained salicin, grandidentatin and tremuloidin, and several unknown spots were also found on the chromatograms.

Extraction Procedures

Preliminary Experiments

Several methods for extraction of crude phenolic glycosides were tried, using 95% ethanol in every case.

The bark and leaves of S. petiolaris, collected in the Fall of 1964, were used in these trials, as described under screening procedures.

Method I (50)

Alcohol 95% (1000 ml.) was added to powdered bark (200 g.) in a high speed Waring Blendor. The blender was operated for 5 minutes, then the alcohol extract was decanted and the residue was extracted with a second volume of alcohol (500 ml.). The extracts were combined, the alcohol was removed under vacuum in a Rinco rotary evaporator and the syrupy residue was taken up with three 100 ml. portions of hot water.

After standing overnight, the aqueous mixture was filtered through cotton wool, the filtrate was concentrated under

vacuum to about 150 ml., and the concentrated solution was extracted with ethylacetate in a separatory funnel (five 150 ml. portions).

Results and Discussion

This method was suggested by Thieme (50) for the extraction and separation of phenolic glycosides. Unfortunately, this method was not carried out to a conclusion because of emulsification during the attempted extraction with ethylacetate.

Method II (29)

Bark powder (200 g.) was covered with 95% ethanol, and after standing at room temperature for one week, the supernatant liquid was decanted, and the bark was covered with fresh alcohol. After another week the solvent was changed again. The combined alcoholic extracts were filtered and the alcohol was removed under vacuum. The residue was stirred with 300 ml. water at room temperature and allowed to stand overnight. The aqueous extract was filtered through filter paper. The slightly turbid pale brown solution was diluted to 500 ml. and treated with an excess of strong solution of lead subacetate (55) at room temperature and the resulting precipitate was filtered off. The filtrate was saturated with hydrogen sulfide and filtered to give a colourless solution. This solution was evaporated under vacuum to about 150 ml. then extracted with

ethylacetate (5 x 150 ml.). The combined ethylacetate extracts were concentrated under reduced pressure to about 20 ml. This brown coloured concentrated extract was kept in a freezer overnight and the crude glycosides were deposited.

Results and Discussion

The deposited white crystals were separated to yield 0.533 g. of crude glycoside, shown to contain salicin, salireposide, picein and tremuloidin by TLC (silica gel G) and a number of unidentified substances.

This procedure was suggested by Pearl and Darling (29) for the extraction of tremuloidin, populin and salicin. The disadvantage of this method is the long time required in the maceration process.

Both Method I and Method II are cold extraction processes.

Method III

Bark powder (200 g.) was extracted with benzene in a soxhlet apparatus for 10 hours, the benzene extract was discarded and then the powdered drug was extracted with 95% ethanol for 12 hours. The solvent was removed under reduced pressure and the syrupy residue was taken up in 500 ml. water. The aqueous mixture was treated at room temperature with lead subacetate and hydrogen sulfide as described in Method II.

The clear filtrate was evaporated under reduced pressure to about 150 ml. and then extracted with ethylacetate (5 x 150 ml.). The combined extracts were reduced to about 15 ml., then placed in a freezer.

Results and Discussion

The crystals deposited from the extract were separated to yield 0.584 g. of crude glycosides.

TLC (silica gel G) showed the presence of the same glycosides as described in Method II.

The advantages of this procedure are its simplicity and the removal of much of the non-glycosidic material by a preliminary benzene extraction. The benzene extraction makes the later ethylacetate extraction step easier by removal of materials causing emulsification.

The disadvantage of this method is that it is not suitable for use with fresh plant material, because of the high water content of the fresh plant material.

Method IV

Bark powder (200 g.) was extracted with 95% ethanol in a soxhlet apparatus for 10 hours and the alcoholic extract was evaporated under reduced pressure. The residue was taken up in 300 ml. water at room temperature and then extracted with benzene in a liquid-liquid continuous extractor apparatus

(see Plate 3) designed by the author. After 10 hours' extraction, the benzene layer was discarded, the separated aqueous portion was diluted to about 500 ml., then treated with lead subacetate and hydrogen sulfide as before. The colourless filtrate was evaporated under reduced pressure to about 150 ml. The concentrated aqueous solution was extracted with ethylacetate in the continuous extractor for 24 hours.

The ethylacetate extract was concentrated under vacuum and crude glycosides deposited after cooling.

Results and Discussion

The crude glycoside precipitate (2.2 g.) was shown to contain the same glycosides as before by TLC (silica gel G).

Methods III and IV are hot extraction processes. From the two-dimensional TLC chromatogram, on silica gel and polyamide-cellulose layers, almost identical results were obtained from the cold and hot process extracts. The soxhlet method (hot extraction) was found more convenient than the cold extraction process, and the extraction of phenolic glycosides was more complete.

The extraction steps were made more convenient and more complete by the use of a liquid-liquid continuous extractor. The author designed apparatus was constructed from standard quickfit units (distilling head with adapters, condenser and two distilling flasks) as shown in Plate 3. A long, narrow

Plate 3. Liquid-liquid continuous extraction apparatus

funnel, made from 8 mm. diameter glass tubing, was used in the column for receiving the solvent drops from the condenser and delivering the solvent to the bottom of the vessel. Besides the usual advantages of a continuous extractor, the apparatus was especially convenient and adaptable to any volume of extraction fluid as desired. A magnetic stirrer was used to assist the extraction.

Method V

Bark powder (200 g.) was extracted and treated in the same manner as in Method IV until the benzene extraction step. After benzene extraction, the aqueous layer (about 300 ml.) was extracted with ethylacetate in the continuous extractor for 12 hours. The ethylacetate extract was evaporated under vacuum to remove solvent, the residue was stirred and dissolved in sufficient water then treated with lead subacetate and hydrogen sulfide as before. The aqueous portion remaining after extraction with ethylacetate was also treated with lead subacetate and hydrogen sulfide. The two colourless filtrates were each evaporated under reduced pressure in a quickfit flash evaporator to about 40 ml. The concentrated aqueous solutions were cooled in the refrigerator and any crystals deposited were filtered off and rinsed with small amounts of water. Finally both concentrated aqueous solutions were combined and continuously extracted with ethylacetate as described in Method IV.

Results and Discussion

The author found this was the best procedure of those attempted for the extraction and separation of phenolic glycosides. An ethylacetate extraction was added to this process before lead treatment because some glycosides such as salireposide, populin, tremuloidin and grandidentatin (higher molecular weight, more highly substituted molecule) are not very soluble in water, but are quite soluble in ethylacetate. This additional ethylacetate extraction avoided possible loss of glycosides during filtration steps and also provided a preliminary fractionation into glycosides soluble and less soluble in water.

The additional advantage of a speedy extraction process, comparable with that of Thieme (50) was achieved in the following manner: The willow bark or leaves were harvested and separated, then macerated in 95% ethanol for 3-4 hours. The mixture was ground in a high speed blender (Waring). The alcoholic extract was decanted and the residue was extracted with fresh 95% alcohol in soxhlet apparatus for 10 hours then the alcoholic extracts were combined and treated as described above.

Because tannin is also soluble in alcohol, this process is suitable for tannin extraction, and Rosenthaler (69) has suggested a lead method for the purification of tannin. The lead precipitate (tannate) from this extraction method was

therefore used for tannin estimation.

The scheme for the large scale extraction of phenolic glycosides and tannins is shown in Figures 26 and 27.

Figure 26. Extraction of Phenolic Glycosides

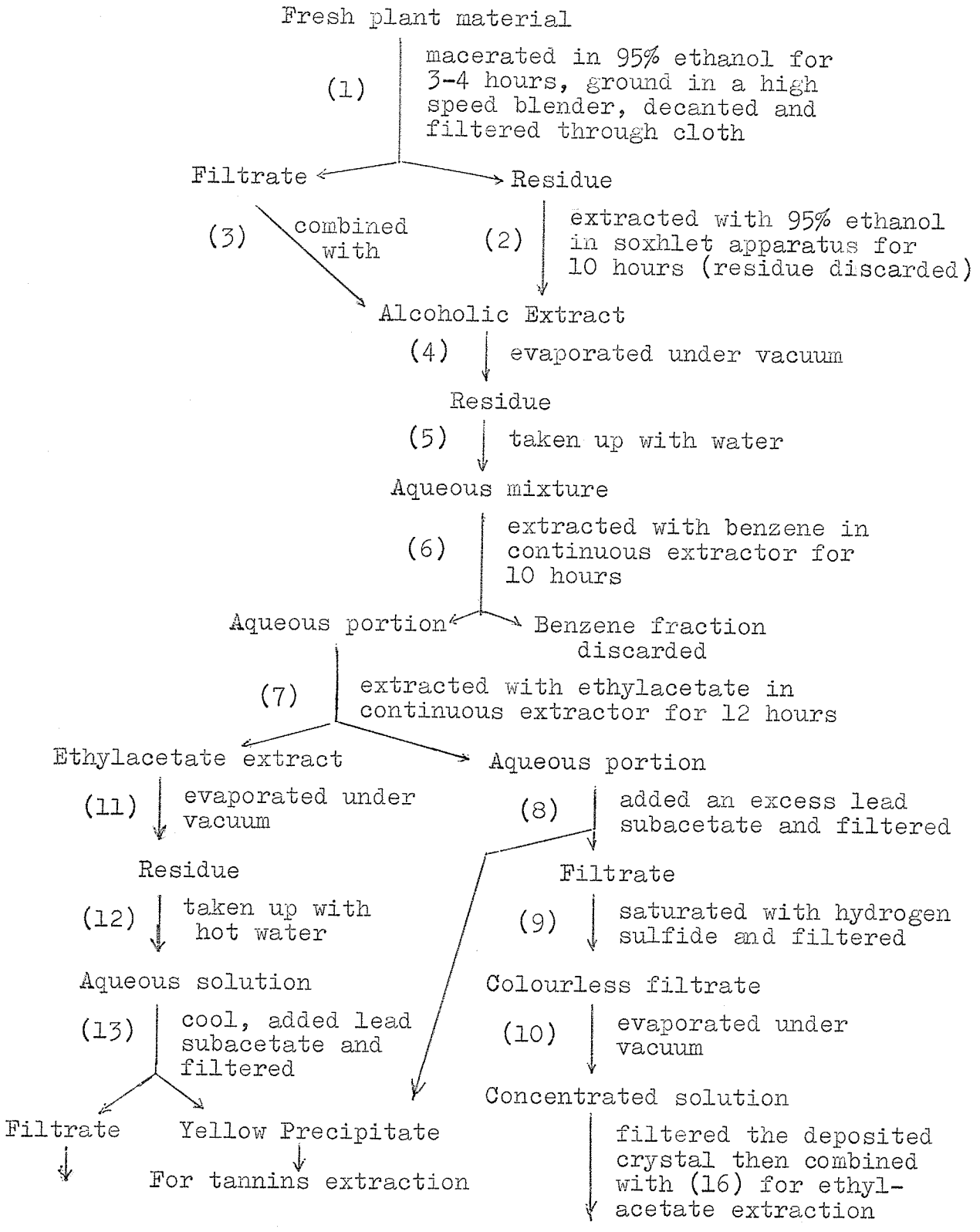


Figure 26. Extraction of Phenolic Glycosides (cont'd)

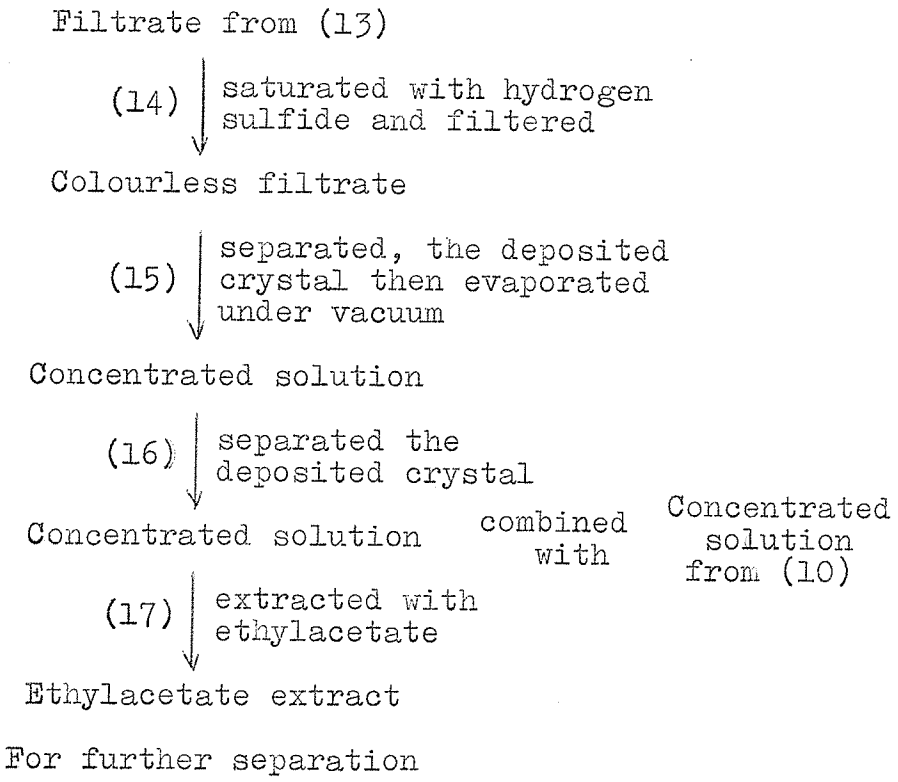
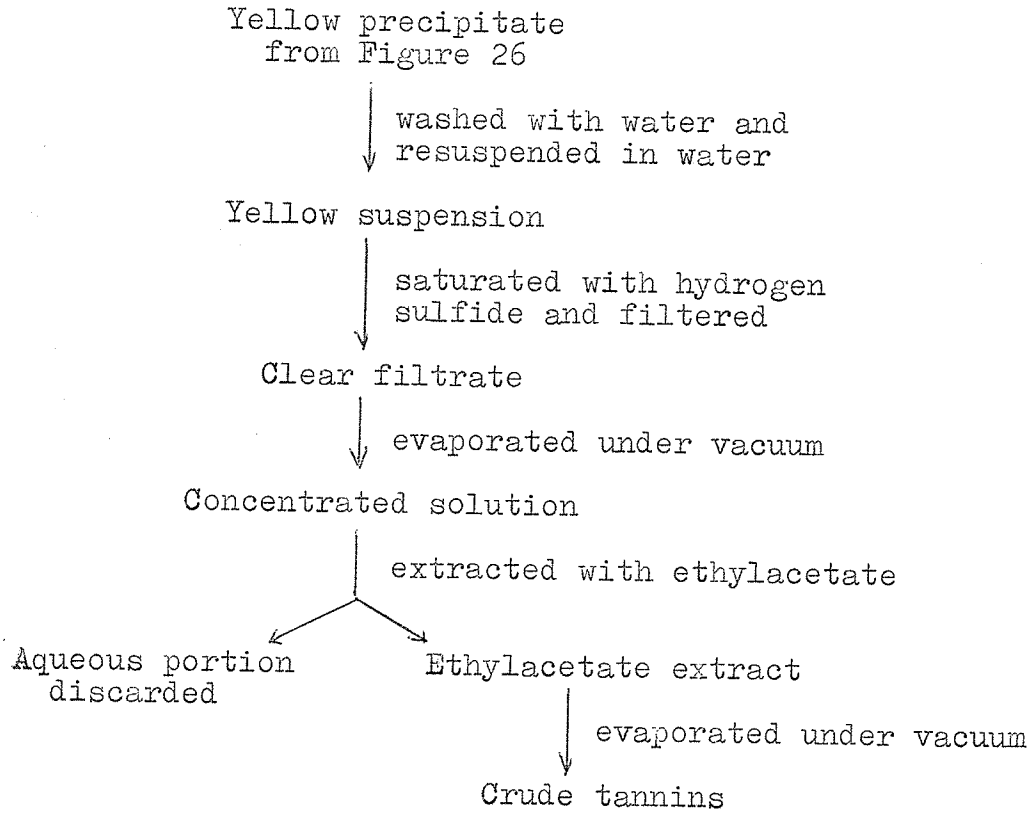


Figure 27. Extraction of Tannins



The Large Scale Extraction of Phenolic Glycosides and Tannins

I. Salix petiolaris (S. gracilis var. textoris)

The samples were collected on June 17, 1965 from the 4-7 year-old trees growing near Stonewall, a small township about 20 miles north-west of Winnipeg, Manitoba.

Fresh bark (1200 g.) and fresh leaves (2100 g.) were macerated separately in 95% ethanol overnight. The samples were then cut with a high speed blender (Waring), and processed as described in Method V (Figure 26).

After a period of extraction (Figure 26, step number 17), the concentrated aqueous solution was further concentrated and then extracted with ethylacetate again.

The approximate water content of samples was determined by oven drying at 90-95°C. for 2 days.

Results and Discussion

Crude glycosides from bark (73.1 g.) and from leaves (43.8 g.) were deposited and separated from the ethylacetate extracts. Salireposide (4.2 g.) was separated from the purified aqueous solution in step 15 of bark extraction.

The mother liquor yielded 13.5 g. of residue from the bark extract and 36.3 g. from the leaf extract.

The total yield of crude glycosides (including

separated crystals and mother liquor residue) amounted to 8.7% in bark and 5.6% in leaves, both on the basis of the original dried materials. On the same basis, the bark yielded 6.1 g. crude tannin (0.6%) and the leaf yielded 2.3 g. crude tannin (0.2%).

The water content was 12.8% in the bark and 31.7% in the leaves.

II. S. interior (S. fluviatilis var. sericans)

The samples were collected on June 30, 1965 from 4-7 year-old trees which were growing on the Red River bank at D'Arcy Drive, Winnipeg 19, Manitoba.

The bark and leaves were processed exactly as described above for S. petiolaris.

Results and Discussion

No glycoside crystals were obtained from the final ethylacetate extract but only a brown syrupy residue at the bottom of the receiver.

The mother liquor yielded 15.4 g. from the bark extract and 7.8 g. from the leaf extract.

The total yield of crude glycosides amounted to 6.0% in bark and 3.2% in leaves, based on the weight of the dried sample. The crude tannin yields were 1.75 g. from bark (0.7%) and 0.8 g. (0.4%) from leaves. The water content was 49.0%

in the bark and 62.0% in the leaves.

The tannin results from both Salix species were much lower than those normally reported in other Salix species with other extraction methods. (52,53)

The method employed in this laboratory may not be suitable for tannin extraction and separation and further work is necessary in this area.

III. S. interior (S. fluviatilis var. sericans)

The sample was collected on August 6, 1965 from 4-6 year-old trees growing on Patricia Beach on the south-east shore of Lake Winnipeg, Manitoba.

Five hundred grams of fresh bark were processed in the same manner as described above, but only phenolic glycosides were extracted. The yield of crude glycosides (3.3%) was again obtained as a syrupy residue.

Separation and Identification of Phenolic Glycosides

Thin-Layer Chromatography Separation and Identification

The mother liquors of glycoside ethylacetate extract were applied on two-dimensional TLC by using different solvent systems on silica gel G layers, and the polyamide-cellulose layer system was used for confirmation.

Sulfuric acid (4%) in anhydrous ethanol was used as the

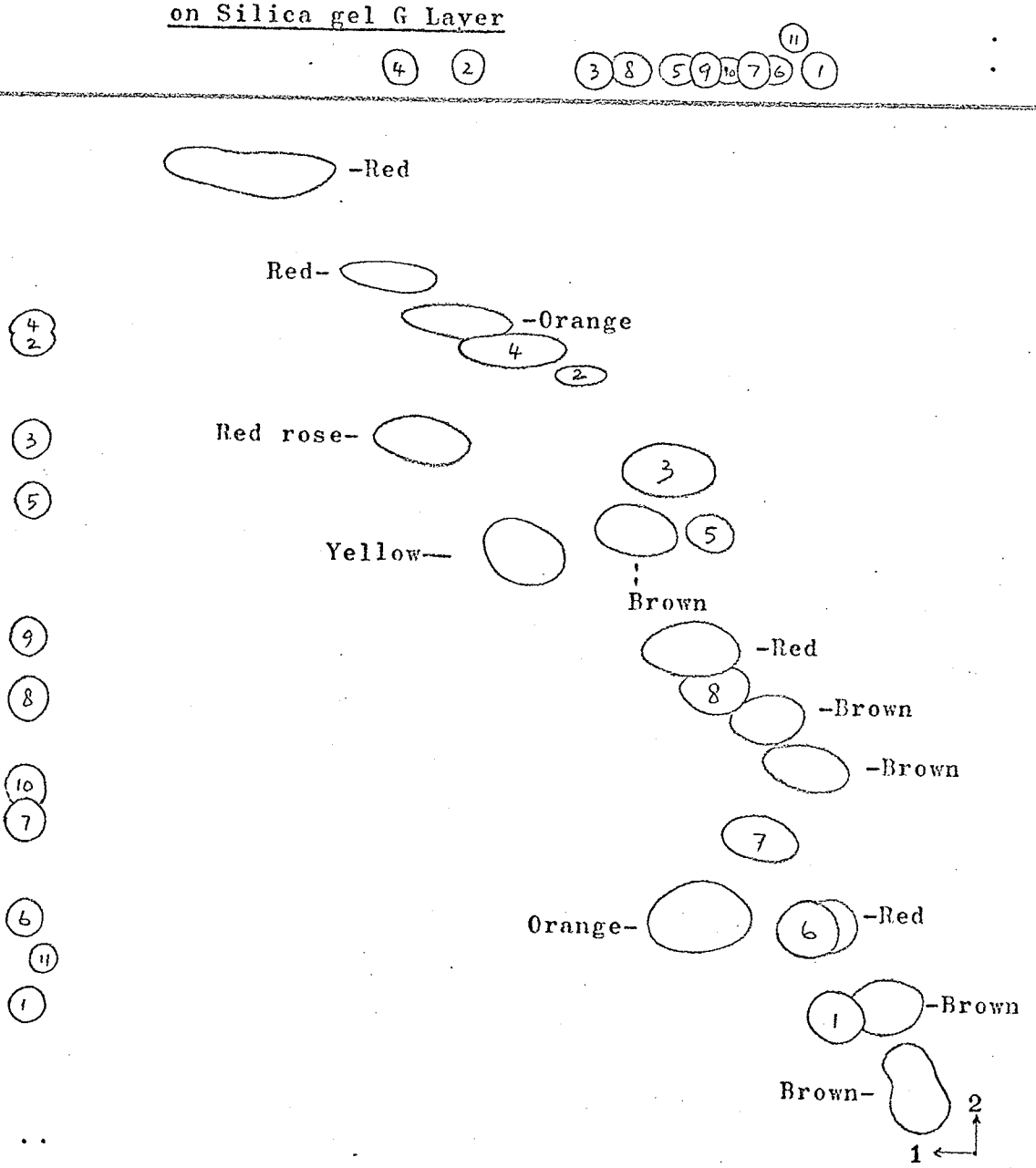
primary detection reagent and Kedde reagent and Millon's reagent were used for confirmation. The chromatograms were prepared and developed as described in thin-layer chromatogram development section, page 29. The identity of individual phenolic glycosides was determined by the characteristic colour produced with the reagents and their R_f values as described before (TLC section).

Results and Discussion

On the basis of the colours produced with the reagents and the chromatographic behaviour in relationship to the reference phenolic glycosides, both bark and leaves of S. petiolaris (S. gracilis var. textoris) were shown to contain salicin, picein, triandrin, fragilin, grandidentatin, salireposide, tremuloidin and traces of populin. The chromatograms are reproduced in Figures 28 and 29, and almost the same unknown spots were found on the chromatogram of leaves and bark, but no salicortin and salidroside has been detected in these samples.

Both bark (including the bark which was collected from Patricia Beach) and leaves of S. interior (S. fluviatilis var. sericans) were shown to contain salicin, triandrin, fragilin, vimalin and salidroside, and almost the same unknown spots were also found on the chromatograms of the leaf and bark samples, but no salireposide, grandidentatin, populin, tremuloidin, picein and salicortin could be found in these samples.

Figure 28. Two-D-chromatogram of Bark Extract of S. petiolaris on Silica gel G Layer



Solvents: (1) & (2) EtOAc/Xylene/HCOOH/H_2O (35:1:2:2)

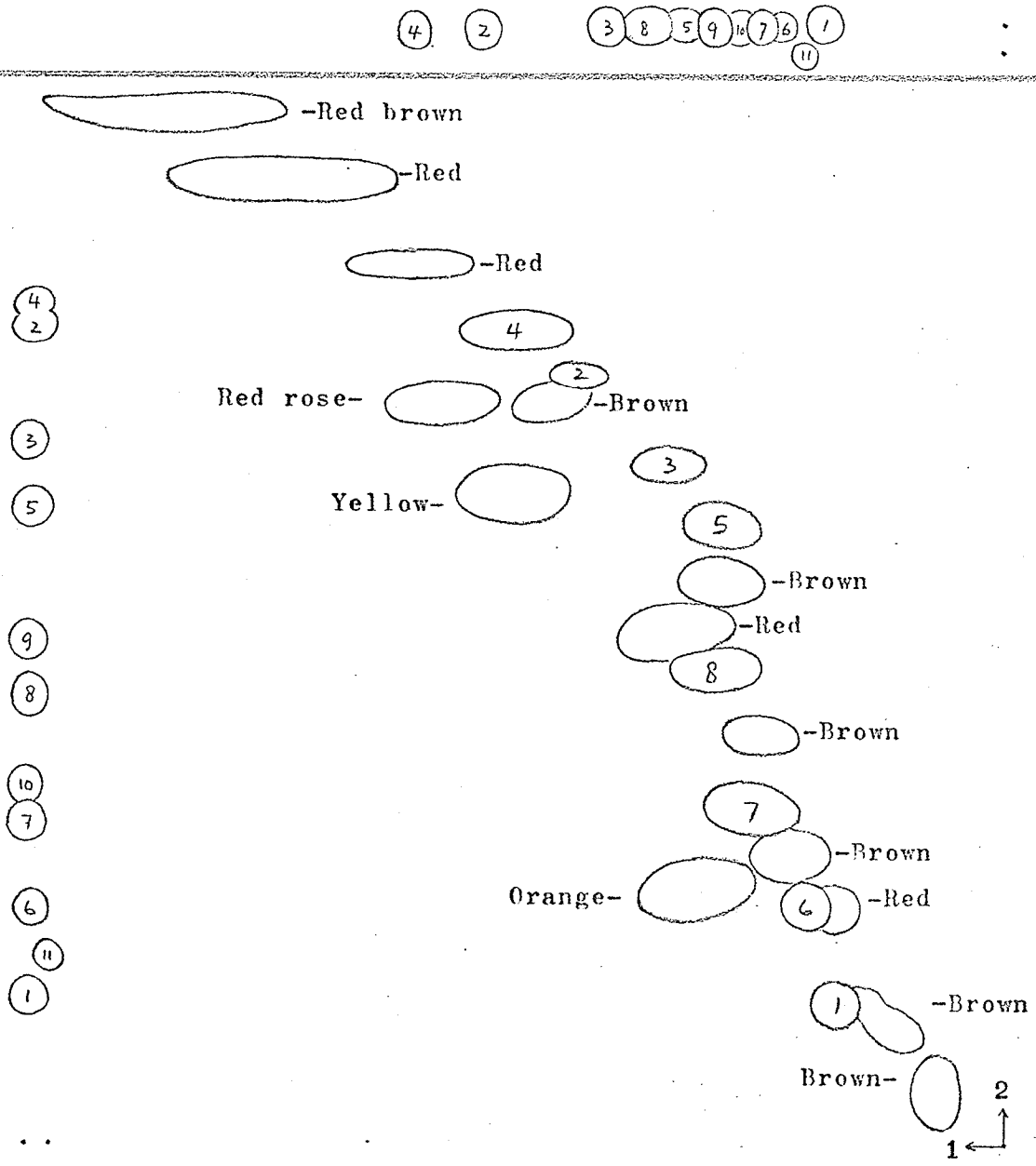
Conditions: (1) R.H. 56%, (2) 40 min. in water vapour satd. chamber, R.T. 22 °c, 15 cm. for each direction.

Reagent: 4% Sulfuric acid in Anhyd. Ethanol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin, (11)Salidroside.

Unknown spots: The unnumbered spots.

Figure 29. Two-D-chromatogram of Leaf Extract of S. petiolaris on Silica gel G Layer



Solvents: (1) & (2) EtOAc/Xylene/HCOOH/H_2O (35:1:2:2)

Conditions: (1) R.H. 54%, (2) 40 min. in water vapour satd. chamber, R.T. 22 °c, 15 cm. for each direction.

Reagent: 4% Sulfuric acid in Anhyd, Ethanol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin, (11)Salidroside.

Unknown spots: The unnumbered spots.

The chromatograms are shown in Figures 30 to 32.

The thin-layer chromatographic characteristics of the unknown spots present in the leaf extract were reasonably similar to those of bark extract in each of the two species. However, there appeared to be variation in the concentration of some of the unknown spots in leaf extract when compared to the unknown spots detected in bark extract.

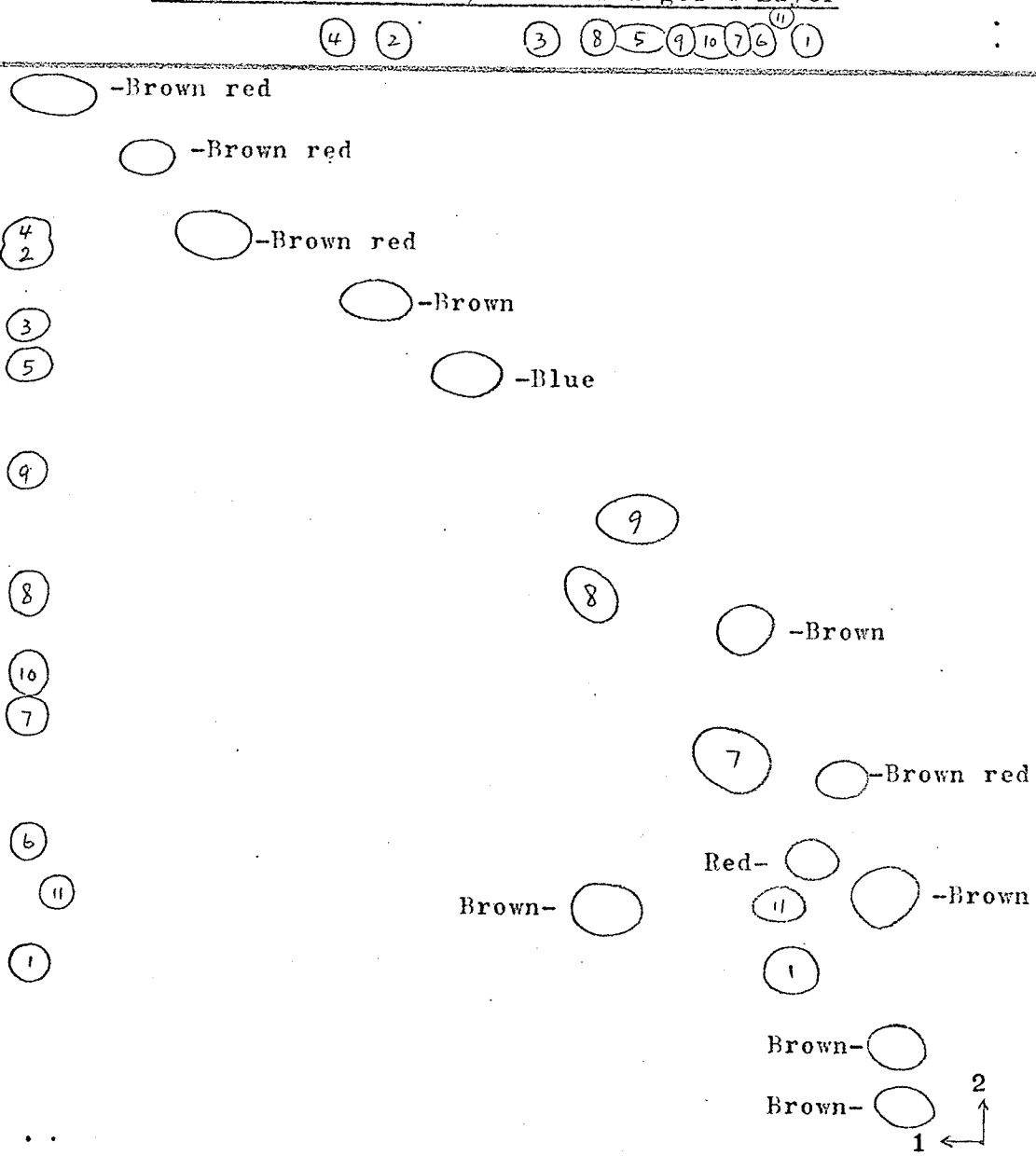
Some of the unknown spots were stained by the glycoside detecting silver reagent, and it is likely that these spots represent glycosides which cannot be identified at present.

Preliminary Separation of Phenolic Glycosides

During the extraction process, the highly concentrated less water soluble glycosides could be deposited in the lead free aqueous solutions. For example, salireposide was deposited in the bark extract of S. petiolaris.

The more water soluble glycosides such as salicin also crystallized out of the ethylacetate extract from S. petiolaris leaves. These glycoside crystals were separated with a Buchner funnel under reduced pressure and washed with a small amount of solvent then dried separately. The constituent glycosides were determined by TLC and the fractions later purified further.

Figure 30. Two-D-chromatogram of Bark Extract of S. interior (from Red River bank) on Silica gel G Layer



Solvents: (1) & (2) EtOAc/Xylene/HCOOH/H_2O (35:1:2:2)

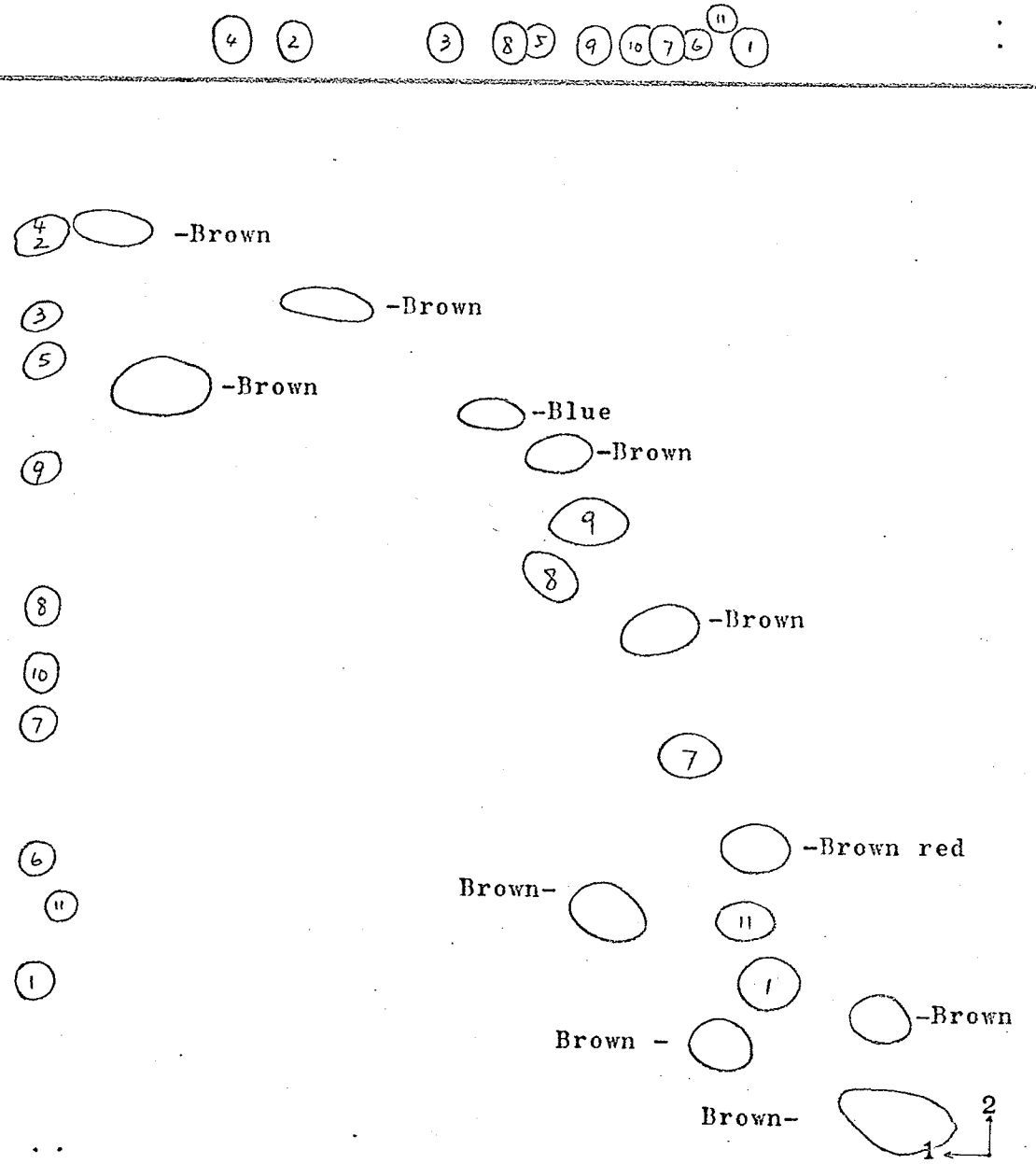
Conditions: (1) R.H. 64%, (2) 40 min. in water vapour satd. chamber, R.T. 22 °c, 15 cm. for each direction.

Reagent: 4% Sulfuric acid in Anhyd. Ethanol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin, (11)Salidroside.

Unknown spots: Unnumbered spots.

Figure 31. Two-D-chromatogram of Leaf Extract of S. interior (from Red River bank) on Silica gel G Layer



Solvents: (1) & (2) EtOAc/Xylene/HCOOH/H_2O (35:1:2:2)

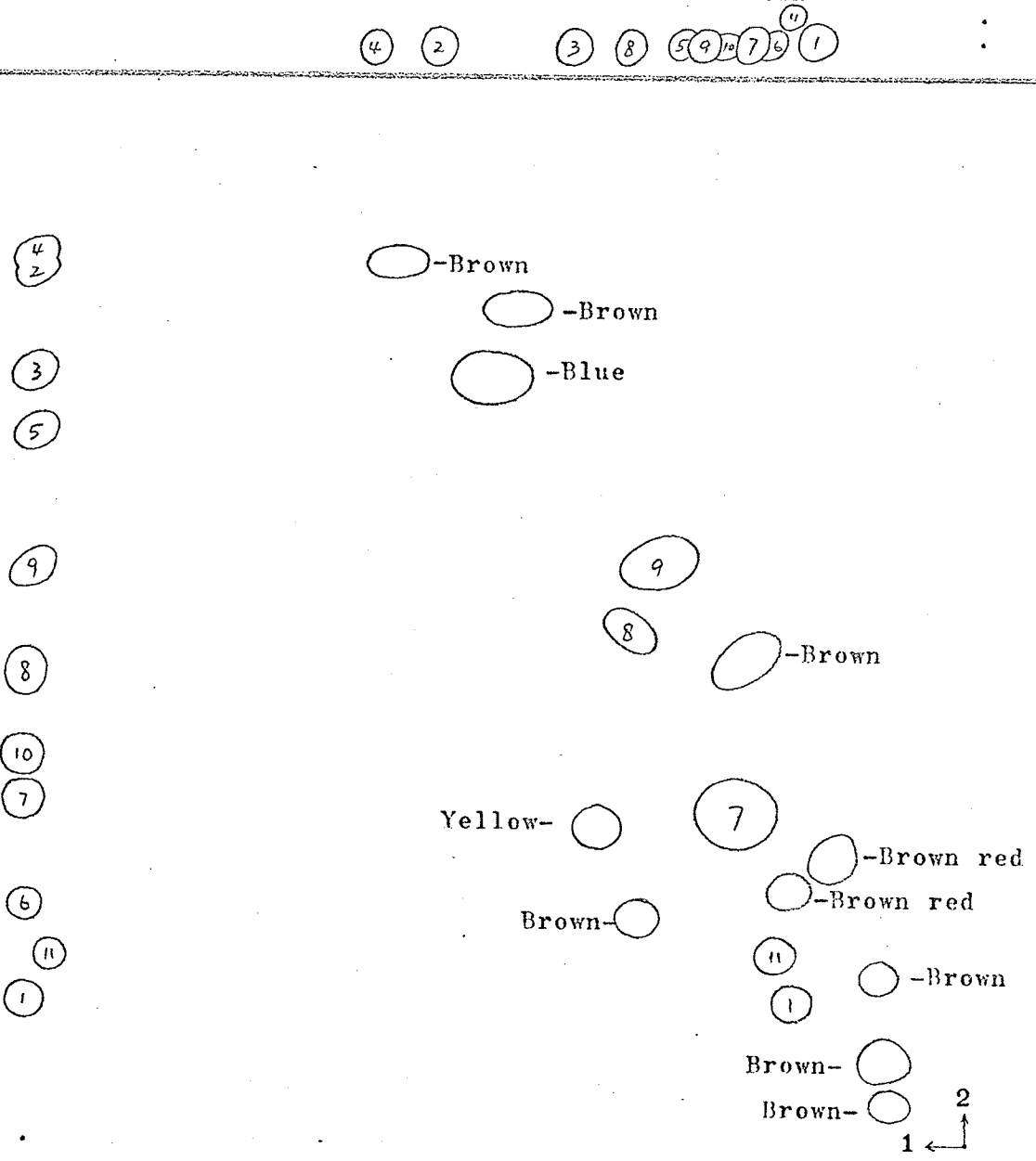
Conditions: (1) R.H. 68%, (2) 40 min. in water vapour satd. chamber, R.T. 22 c, 15 cm. for each direction.

Reagent: 4% Sulfuric acid in Anhyd. Ethanol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin, (11)Salidroside.

Unknown spots: Unnumbered spots.

Figure 32. Two-D-chromatogram of Bark Extract of S. interior (from Patricia Beach) on Silica gel G Layer



Solvents: (1) & (2) EtOAc/Xylene/HCOOH/H_2O (35:1:2:2)

Conditions: (1) R.H. 61%, (2) 40 min. in water vapour satd. chamber, R.T. 22 °c, 15 cm. for each direction.

Reagent: 4% Sulfuric acid in Anhyd. Ethanol.

Glycosides: (1)Salicin, (2)Populin, (3)Salireposide, (4)Tremuloidin, (5)Grandidentatin, (6)Picein, (7)Triandrin, (8)Fragilin, (9)Vimalin, (10)Salicortin, (11)Salidroside.

Unknown spots: Unnumbered spots.

Results and Discussion

In most cases the crude glycosides separated in this manner were almost pure. (For example, salireposide [B_{cl}] and salicin [L_{cl}] separated from S. petiolaris). They showed only one spot with TLC (silica gel G). However, the crude glycosides separated from the ethylacetate extract of S. petiolaris bark was a mixture of salicin and picein presumably because both glycosides have similar solubilities and are present in about equal concentration in the sample.

Column Chromatography

An attempt was made to separate pure phenolic glycosides by column chromatography.

Crude glycoside (including separated glycosides and mother liquor residue, 2 g.) from bark extract of S. petiolaris was dissolved in 5 ml. methanol and mixed with 0.5 g. polyamide powder (Woelm) then dried in a vacuum desiccator. The dried mixture was poured onto the top of a polyamide column prepared from 40 g. polyamide powder (Woelm) in a 3.5 x 50 column, then developed in the same manner as described in the polyamide column chromatography section. Ninety-five fractions of 25 ml. aqueous eluate were collected, lyophilized to dryness in a freeze dryer (Virtis) and examined by TLC (silica gel G).

The crude glycosides (2 g.) from leaf extract of S.

petiolaris were separated and determined exactly as described above for the bark.

The mother liquor residues, 2.7 g. from bark and 3.6 g. from leaves of S. petiolaris were also applied separately on a polyamide column prepared from 20 g. polyamide (Woelm) powder in a 3.5 x 30 cm. column, as described above.

The glycoside mixture (salicin, picein, etc., 1 g.) obtained from fractions 11 to 13 of the polyamide water column system of S. petiolaris bark was applied to a second polyamide column and eluted with water saturated ethylacetate, and 60 fractions of 25 ml. were collected and examined by TLC. An attempt to separate the glycoside mixture (same source as above) was also made on 1 mm. layer (thick layer) of silica gel G and polyamide-cellulose.

Results and Discussion

In the bark extract of S. petiolaris, salicin and picein were found to be the main glycosides, and were distributed in fractions 9 to 14. These two glycosides are present in about the same concentration in the bark and the mixture represents about 75% (1.50 g.) of the total crude glycoside. A small amount of fragilin was also found in fractions 9 to 14, traces of vimalin were detected in the mixture from fractions 14 to 18, and small amounts of triandrin in fractions 14 to 19. Tremuloidin was present (about 0.1% of the total crude glycoside) in fractions 32 to 40 and traces of populin were found

in fractions 40 to 50. The populin may possible form from tremuloidin during the process of extraction. (70) Small amounts of grandidentatin were found in fractions 44 to 58 and salireposide was present (about 2% of the total crude glycoside) in fractions 56 to 95.

In the extract of leaves of S. petiolaris, salicin was found to be the main glycoside (about 65% [1.30 g.] of the total crude glycoside) and was found distributed in the fractions 8 to 15 (the greater part in fractions 9 and 10). Approximately 2% of the crude glycosides was tremuloidin, isolated from fractions 30 to 44 and small amounts of grandidentatin were detected in fractions 44 to 56. Picein in fractions 9 to 12, fragilin in fractions 9 to 13, vimalin in fractions 12 to 18, triandrin in fractions 13 to 19, populin in fractions 38 to 50 and salireposide in fractions 67 to 80 were found in trace amounts only. The same resolution results were found in both long (40 g. polyamide) and short (20 g. polyamide) columns when eluted by the water system. Only the phenolic glycosides were fractionated in fewer fractions (about half of the number) than the long column. For the preliminary fractionation and purification of phenolic glycosides, the short column was found to be quite acceptable.

Salicin and picein mixtures were separated by polyamide column eluted by water saturated ethylacetate. Salicin (0.15 g.) was isolated from fractions 14 to 24 and picein (0.12 g.) was separated in fractions 30 to 42, but it was impossible

to separate these two glycosides completely with this column and elution system, because their solubilities are too similar.

Thick layer chromatography (silica gel G and polyamide-cellulose) was also tried in attempts to separate these two glycosides. Silica gel G layer was found to possess higher loading capacity and gave better resolution results than the polyamide-cellulose layer, but the separation band of silica gel G thick layer tailed and did not separate when more than 50 mg. of the crude glycosides were applied on a 20 x 20 cm. chromatoplate. This is a useful technique for the quantitative examination.

Purification of Isolated Phenolic Glycosides

The crude glycosides after separation by the preliminary processes were examined by TLC (silica gel G).

The following almost pure glucosides were recrystallized using water and water saturated ethylacetate as solvents: B_{c1} (salireposide, separated from aqueous solution of S. petiolaris bark extract), L_{p1} (tremuloidin, separated from S. petiolaris leaves by polyamide column with water system), L_{c1} (salicin, separated from ethylacetate extract of S. petiolaris leaves), L_{p2} (salicin, from same source as L_{p1}), B_{p1} (salicin, separated from S. petiolaris bark by polyamide column with water saturated ethylacetate system), B_{p2} (picein, from same source as B_{p1}).

The glycoside fraction was dissolved in sufficient boiling solvent then cooled in a refrigerator. The deposited crystals were separated by Buchner funnel and dried. The purified glycoside was then subjected to further TLC (silica gel G) and melting point determinations as checks of purity.

Results and Discussion

The isolated glycosides were recrystallized at least three times from water and water saturated ethylacetate until the melting point and TLC examinations were acceptable.

First recrystallization from water then water saturated ethylacetate was found to give the best results for recrystallization of phenolic glycosides, but aqueous alcohol mixture was also found to be useful.

The determined melting points and reported melting points of the glycosides were as shown in Table 6.

Table 6. Melting Points of Isolated Glycosides

	Determined m.p. °C.	Reported m.p. (50) °C.
B_{c1} (Salireposide)	205.5-206.0	206.0-207.0
L_{p1} (Tremuloidin)	208.0-209.0	207.0-208.0
L_{p2} (Salicin)	199.5-200.0	201.0
L_{c1} (Salicin)	199.5-200.5	201.0
B_{p1} (Salicin)	199.7-200.2	201.0
B_{p2} (Picein)	194.5-195.0	195.0

Characterization of Phenolic Glycosides

The separated and purified glycosides were submitted to mixed melting point determination and ultra-violet and infra-red absorption determination.

The ultra-violet absorption spectra of isolated glycosides were recorded, using aqueous solutions.

The infra-red absorption spectra of isolated glycosides were recorded. Potassium bromide discs were prepared using 400 mg. potassium bromide to dilute 1 mg. of sample.

Results and Discussion

The ultra-violet absorption spectra of *Salix* phenolic glycosides are shown in Figures 33 to 43.

Salicin (Figure 34) and fragilin (acetylated salicin, Figure 39) showed the same absorptional behaviour and gave maximum absorbances at 212 mμ and 268 mμ. Salicortin (salicin derivative, Figure 41) also has a similar spectrum with maximum absorbances at 212 mμ and 270 mμ.

Populin (Figure 33) and tremuloidin (isomer of populin, Figure 35) have maxima at 220 mμ, 232 mμ and a broad maximum between 268 to 274 mμ. In the populin spectrum, the 220 mμ peak is higher than the peak at 232 mμ, and vice versa in the tremuloidin spectrum.

Triandrin (Figure 37) and vimalin (methylated triandrin, Figure 40) also have similar absorption characteristics with

Figure 33.

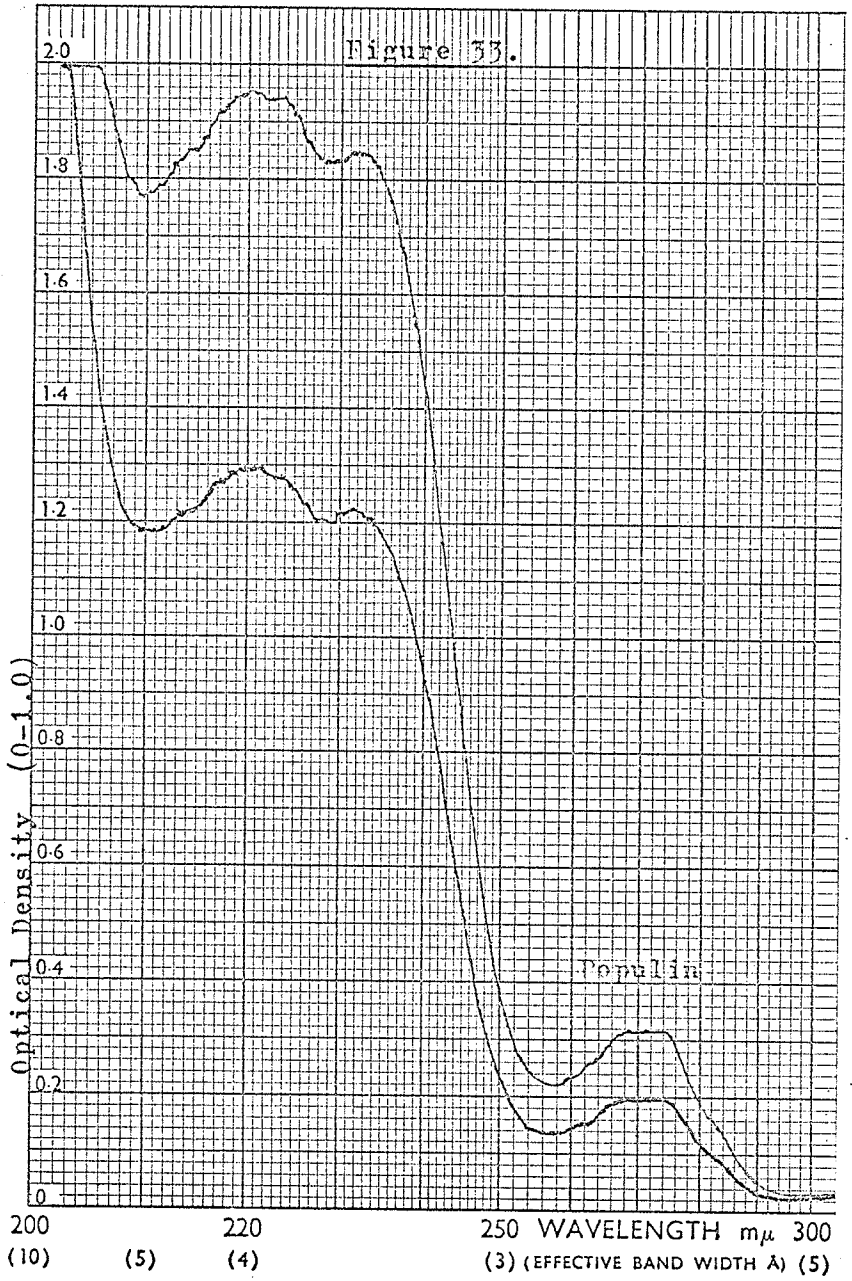


Figure 34.

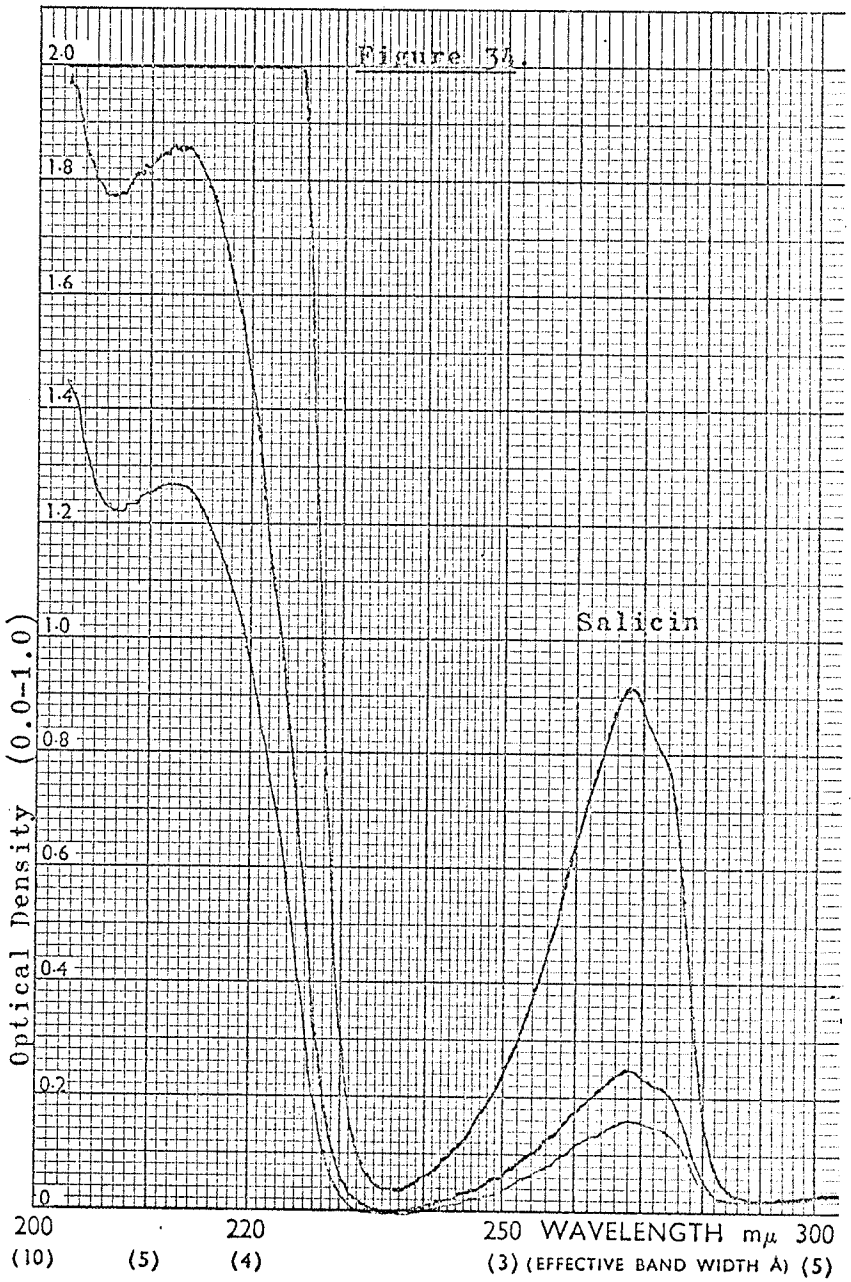


Figure 35.

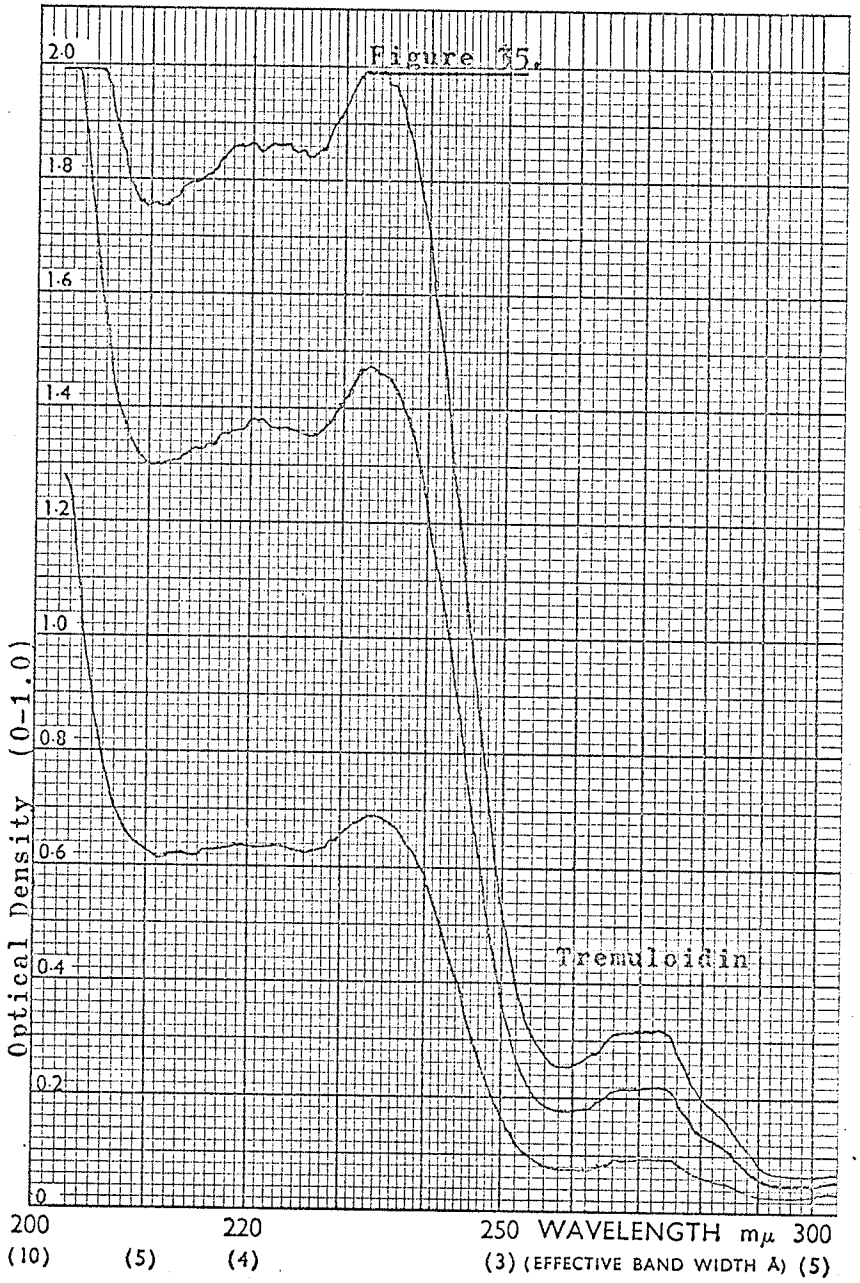
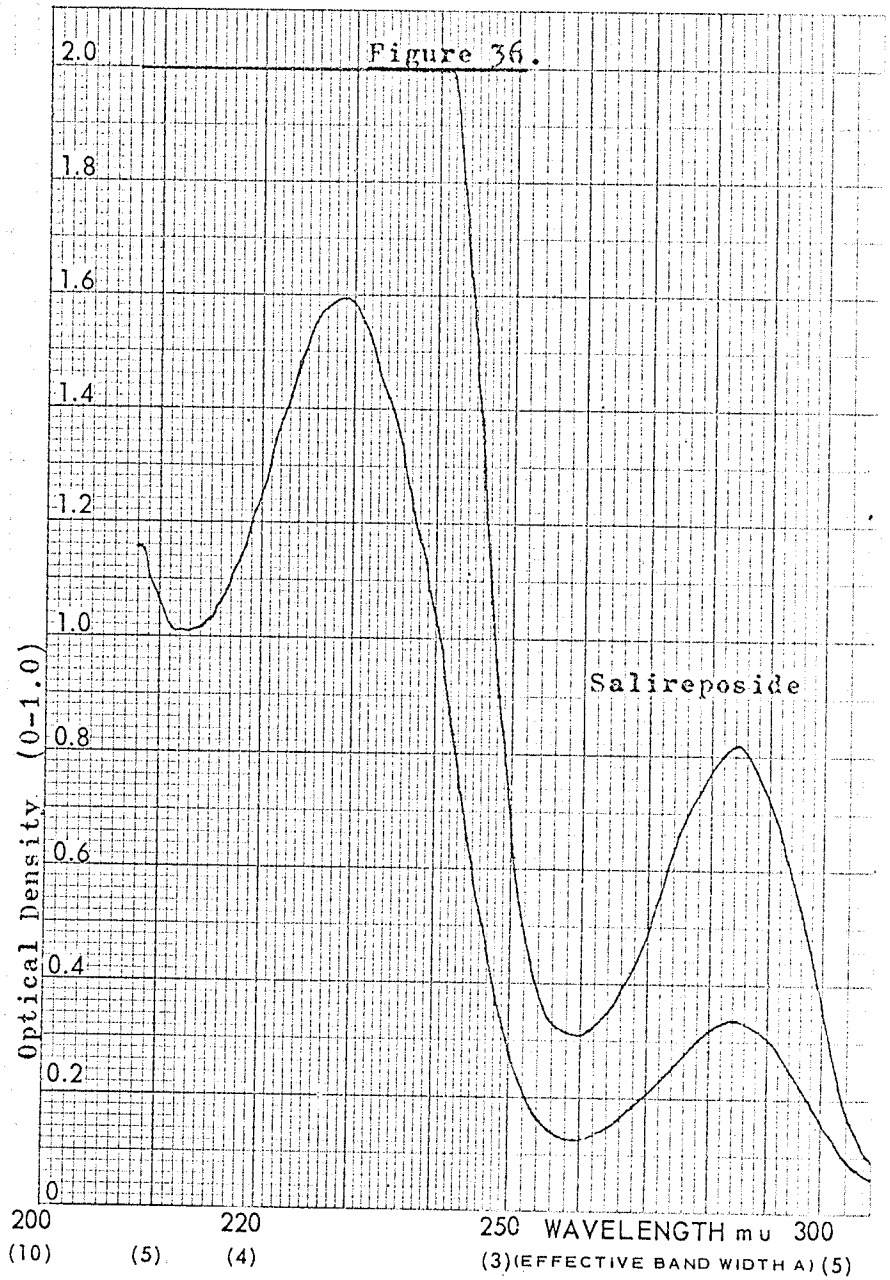


Figure 36.



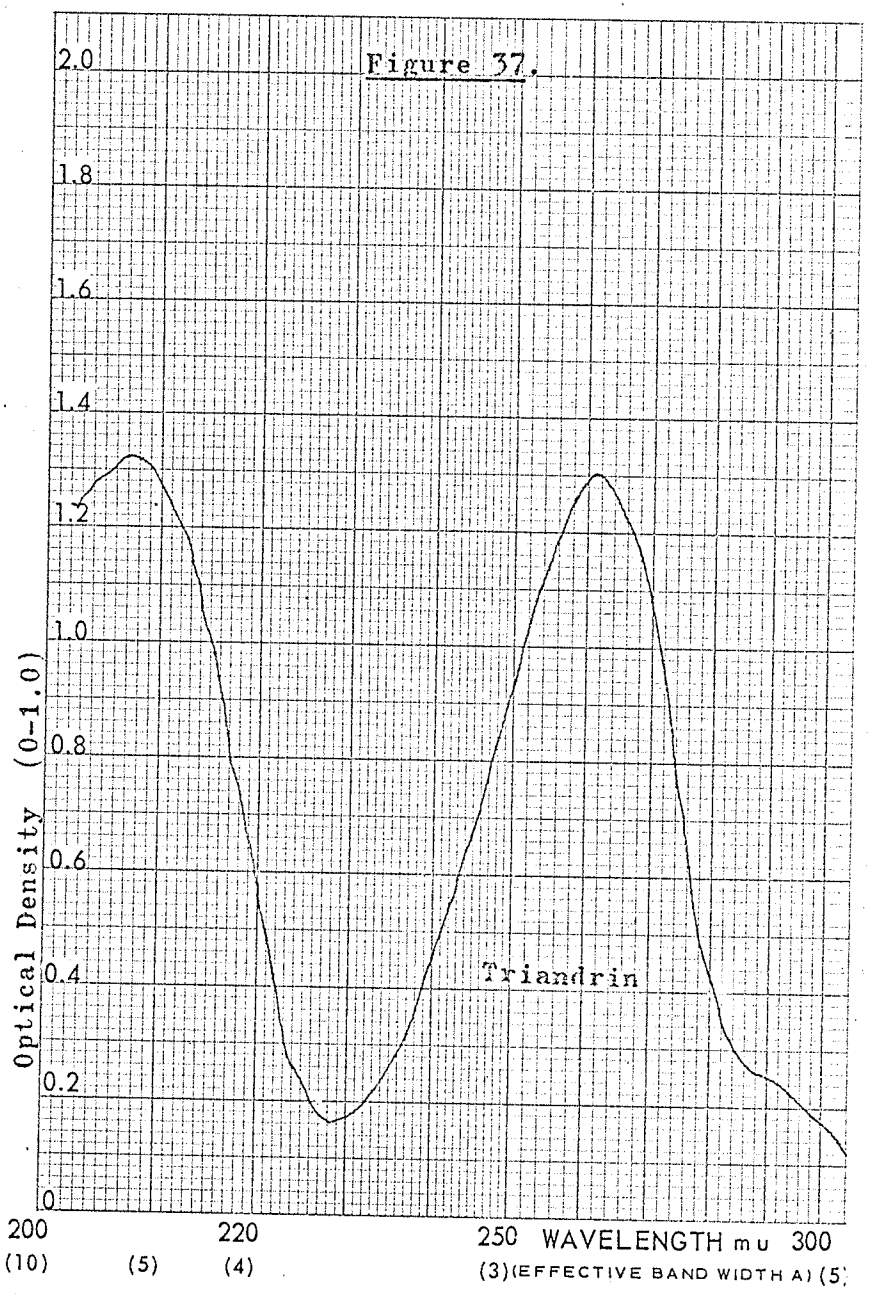


Figure 37.
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Optical Density (0-1.0)
Triandrin
200
(10)
220
(5)
(4)
250 WAVELENGTH mu 300
(3)(EFFECTIVE BAND WIDTH A) (5)

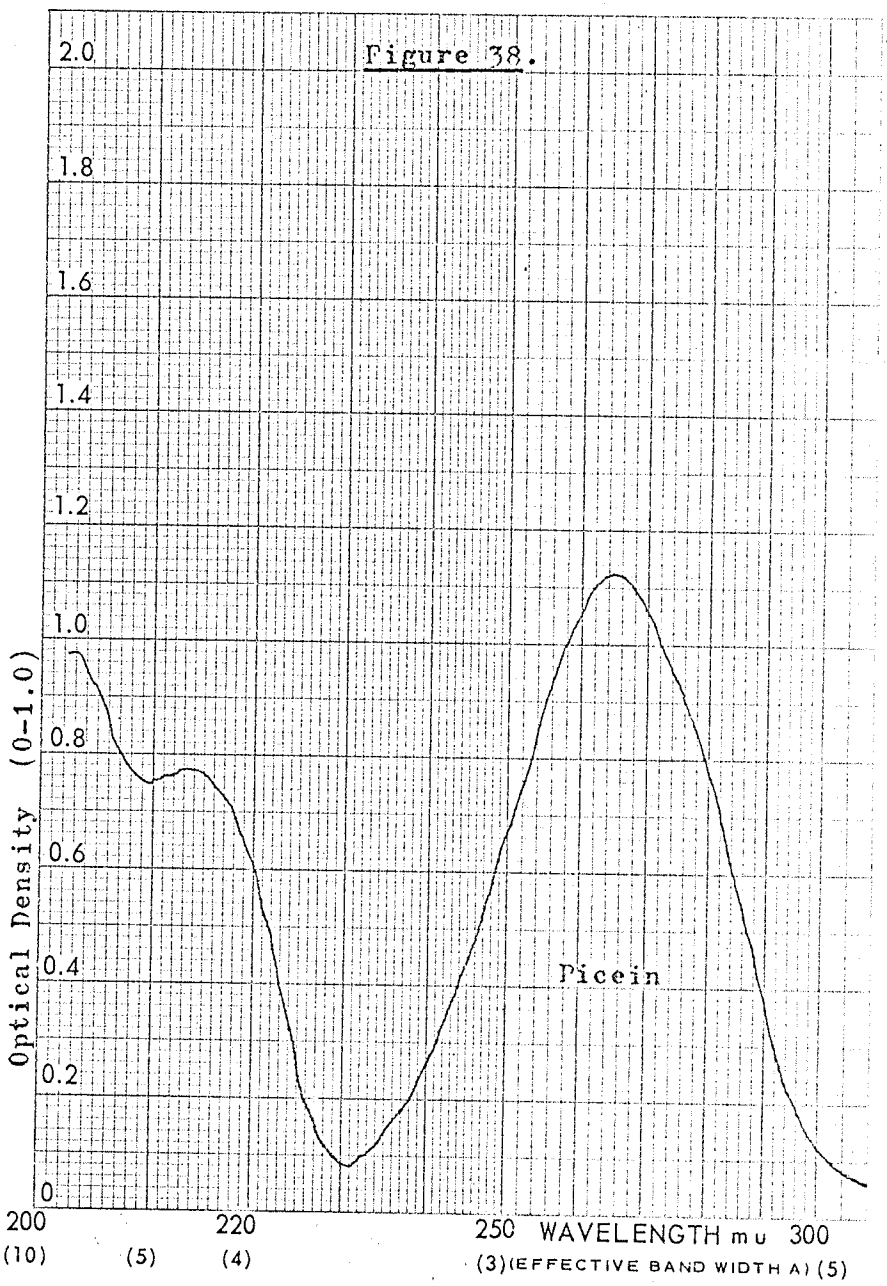


Figure 38.
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Optical Density (0-1.0)
Picein
200
(10)
220
(5)
(4)
250 WAVELENGTH mu 300
(3)(EFFECTIVE BAND WIDTH A) (5)

Figure 39.

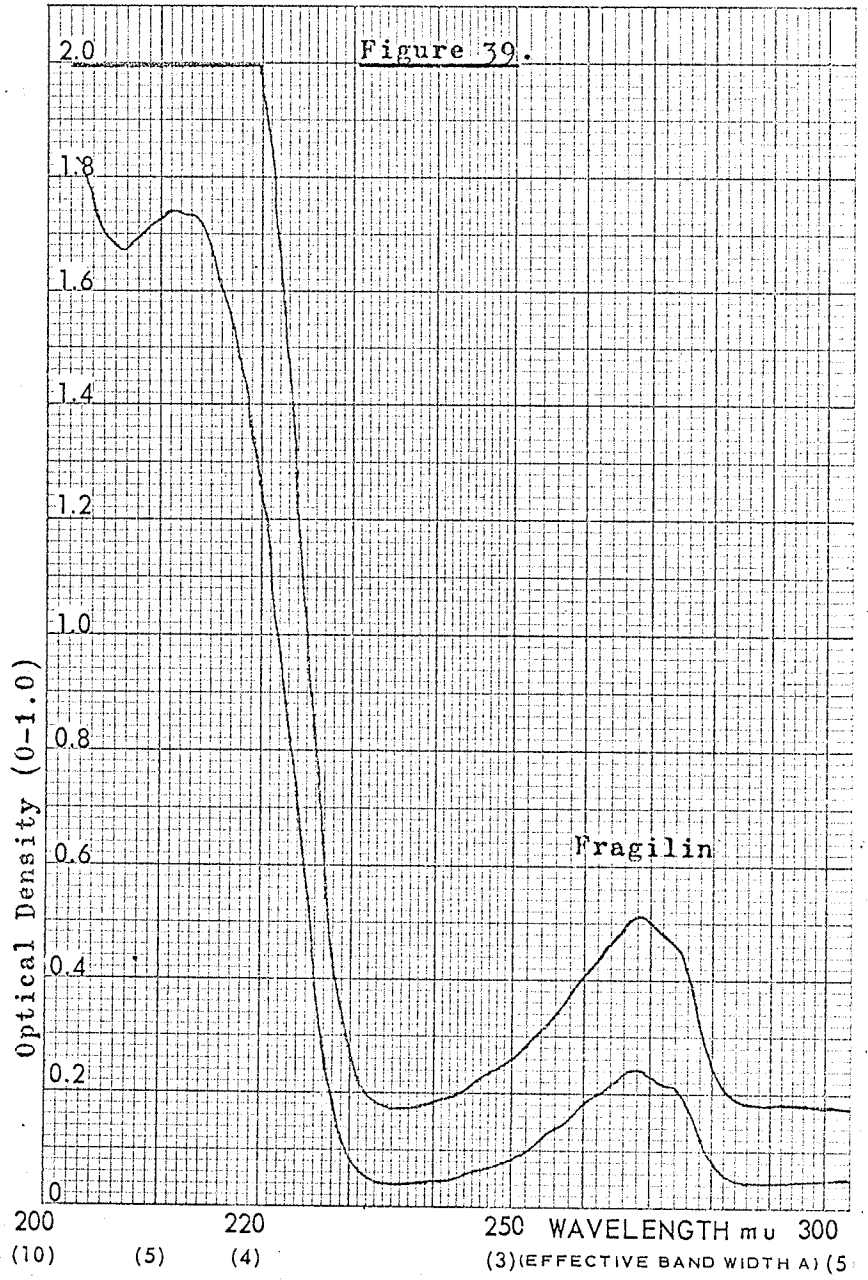
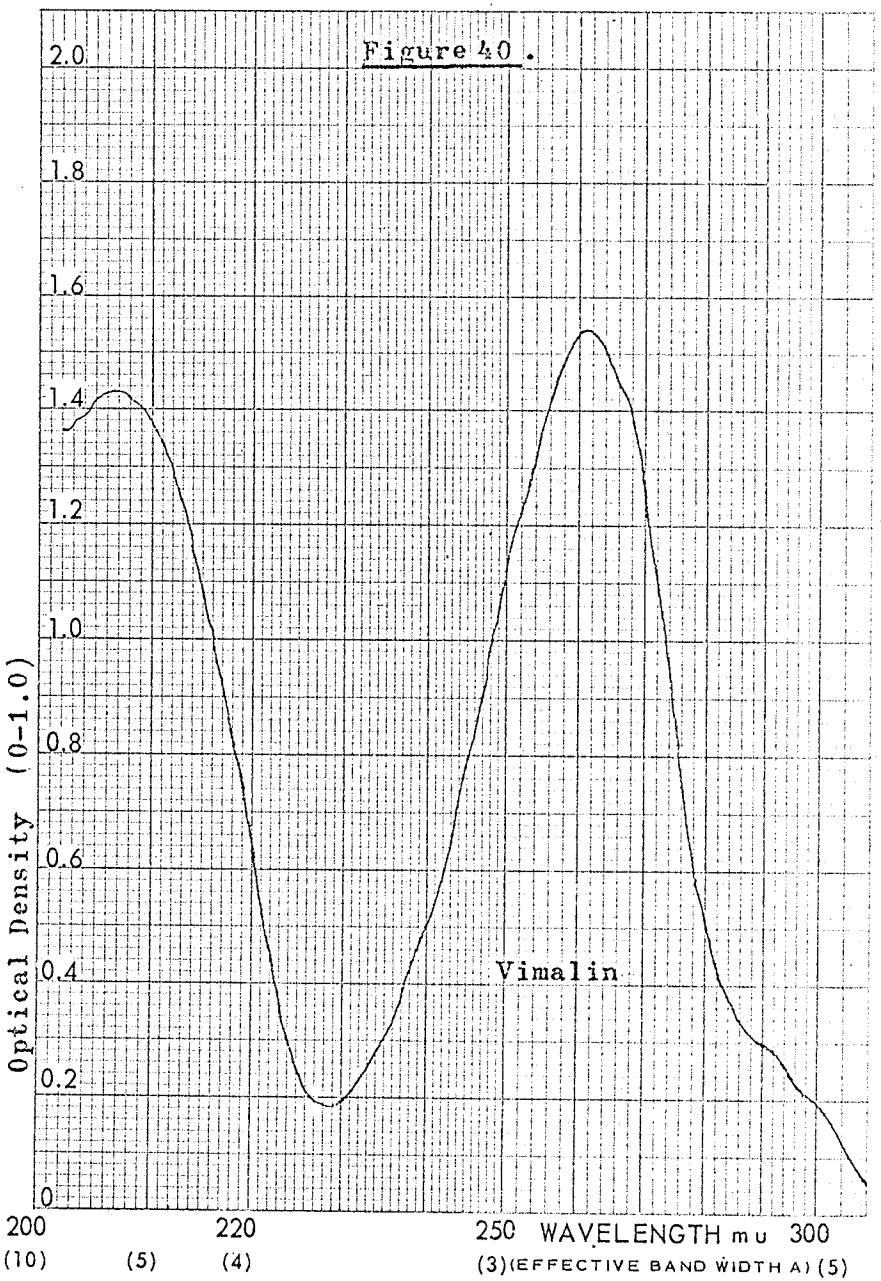


Figure 40.



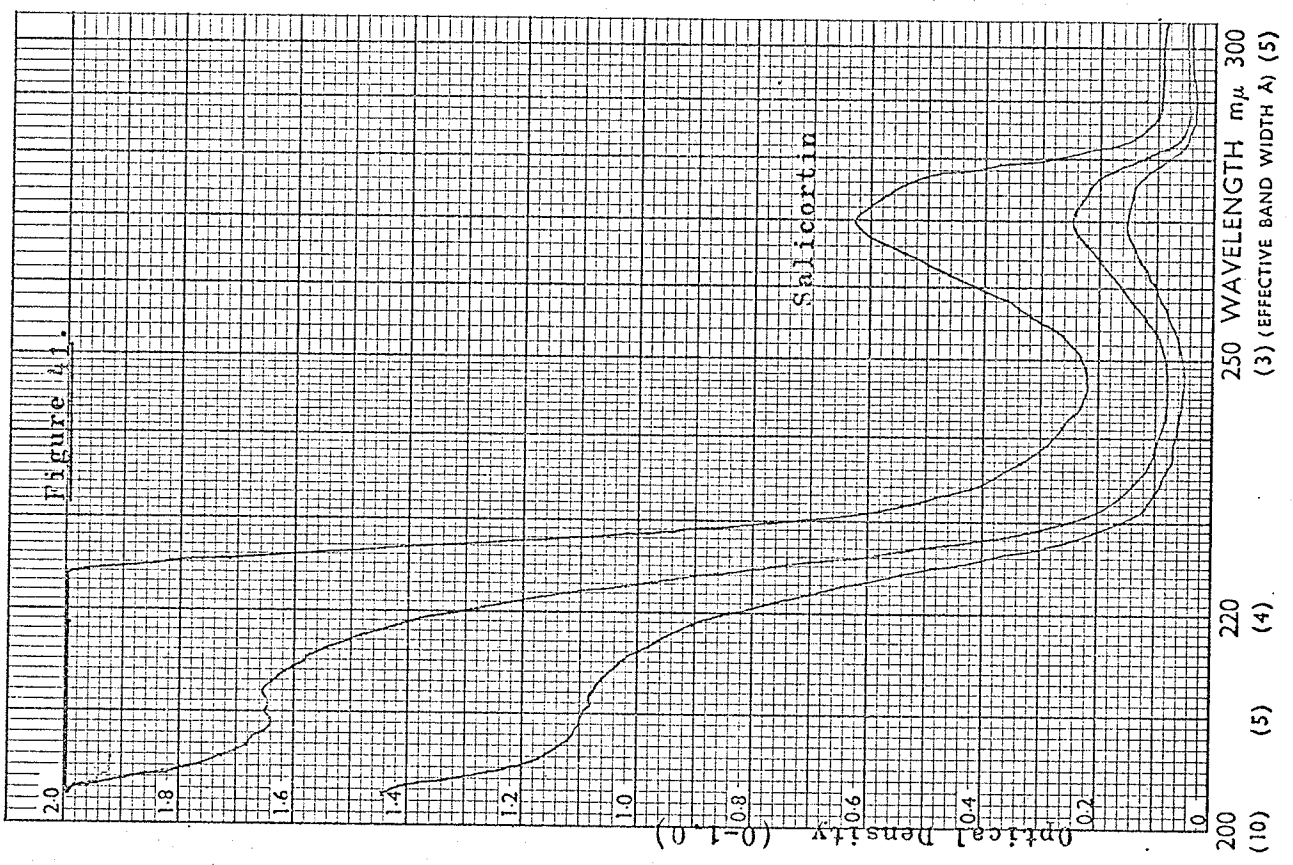


Figure 41.
Salicortin
Optical Density (0-1.0)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
200 (10) (5) 220 (4) 250 WAVELENGTH mμ 300 (3) (EFFECTIVE BAND WIDTH A) (5)

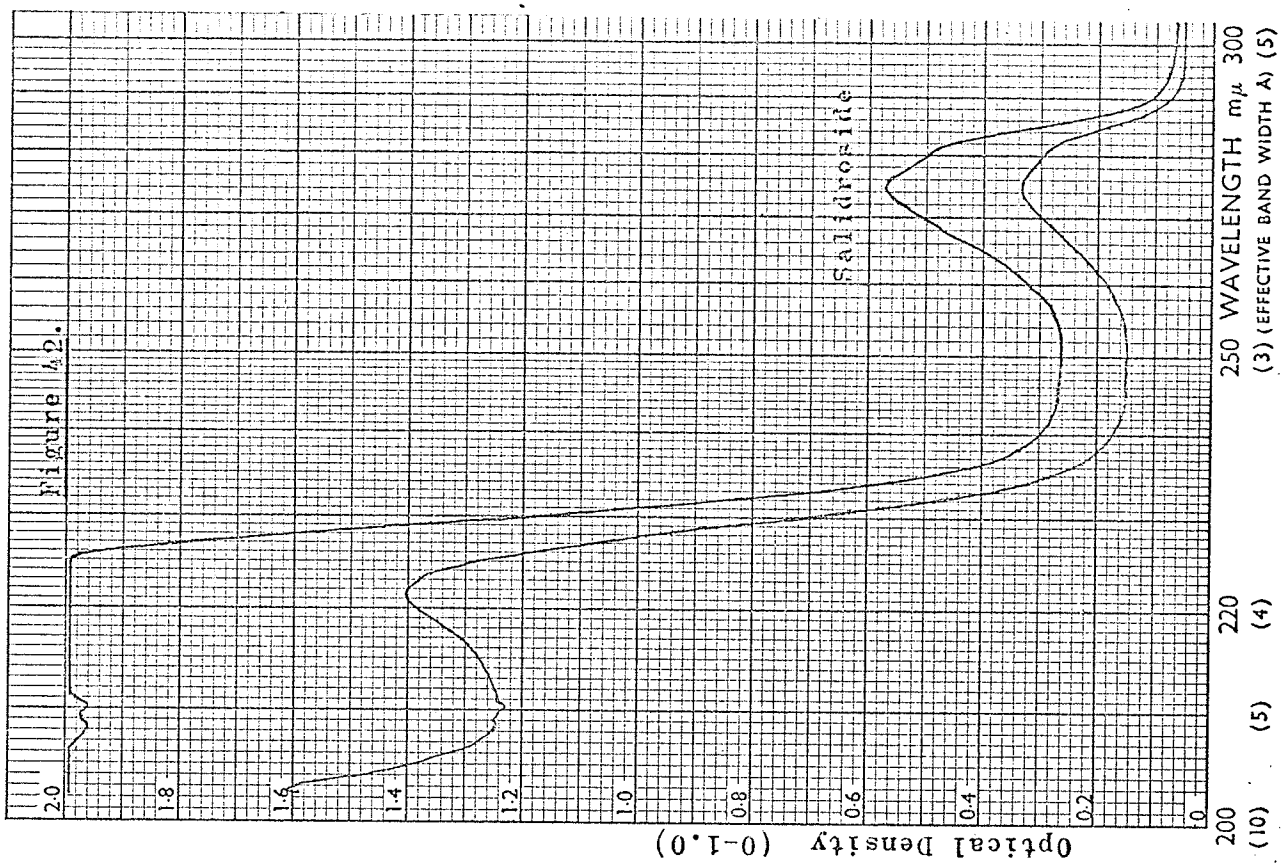
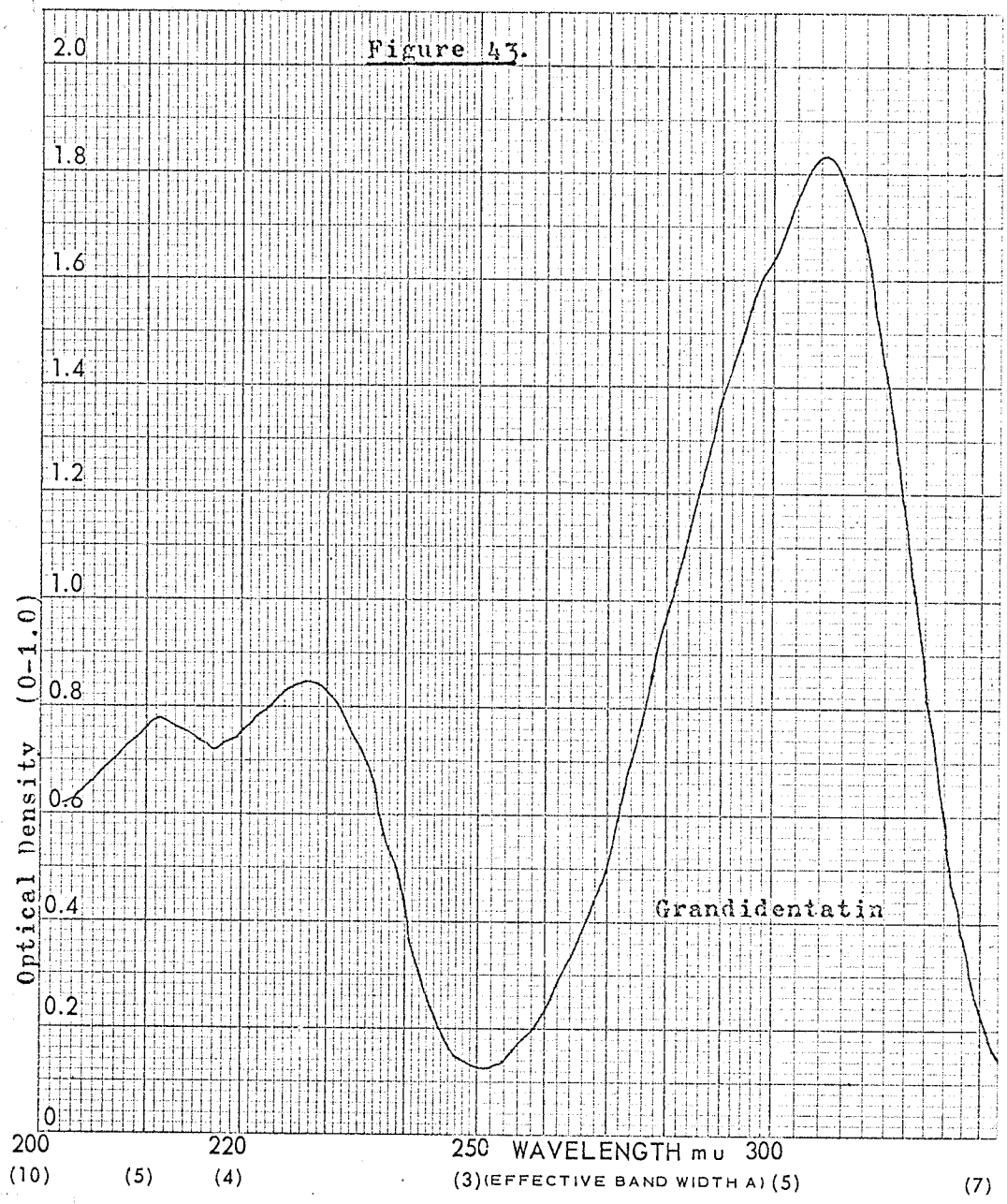


Figure 42.
Salidroside
Optical Density (0-1.0)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
200 (10) (5) 220 (4) 250 WAVELENGTH mμ 300 (3) (EFFECTIVE BAND WIDTH A) (5)



Ultra-violet Absorption Spectra of Salix Phenolic Glycosides

Instrument: Ultrascan (Hilger & Watts)

Solvent: Water

Cell thickness: 1 cm.

Density: 1

Scan time (minutes): 4

Bandwidth: 1

Figure 33. UV-Spectrum of Populin

Figure 34. UV-Spectrum of Salicin

Figure 35. UV-Spectrum of Tremuloidin

Figure 36. UV-Spectrum of Salireposide

Figure 37. UV-Spectrum of Triandrin

Figure 38. UV-Spectrum of Picein

Figure 39. UV-Spectrum of Fragilin

Figure 40. UV-Spectrum of Vimalin

Figure 41. UV-Spectrum of Salicortin

Figure 42. UV-Spectrum of Salidroside

Figure 43. UV-Spectrum of Grandidentatin

maximum at 261 mu and what might be a false maximum at 207 mu, and only slightly different intensity of the peaks. In the triandrin spectrum the peak at 207 mu is higher than the peak at 261 mu, and vice versa in the vimalin spectrum.

Salireposide (Figure 36) gave maximum absorbances at 228 mu and 284 mu, the first absorption peak being higher than the second.

Grandidentatin (Figure 43) showed maximum absorbances at 212 mu, 227 mu and 311 mu, the latter being higher than the others.

Picein (Figure 38) showed maxima at 214 mu and 264 mu, the second peak being higher than the first.

Salidroside (Figure 42) showed maximum absorbances at 221 mu and 275 mu, and the spectrum was quite similar to salicin.

The ultra-violet absorption spectra and the infra-red absorption spectra of the isolated glycosides are shown in the Figure numbers of Table 7.

Table 7

Isolated Glycosides	UV Absorption Spectra	IR Absorption Spectra
B_{c1} (Salireposide)	Figure 45	Figure 52
L_{p1} (Tremuloidin)	Figure 44	Figure 53
L_{p2} (Salicin)	Figure 46	Figure 49
B_{p1} (Salicin)	Figure 47	Figure 51
B_{p2} (Picein)	Figure 48	Figure 50

The mixed melting points of B_{c1} + salireposide, L_{p1} + tremuloidin, L_{p2} + salicin, B_{p1} + salicin and B_{p2} + picein were not depressed. Also according to the TLC chromatographic behaviour, ultra-violet absorption spectra and infra-red absorption spectra as compared with those of the reference phenolic glycosides, B_{c1} isolated crystals were identical with salireposide, L_{p1} was identical with tremuloidin, L_{p2} and B_{p1} were identical with salicin and B_{p2} was identical with picein.

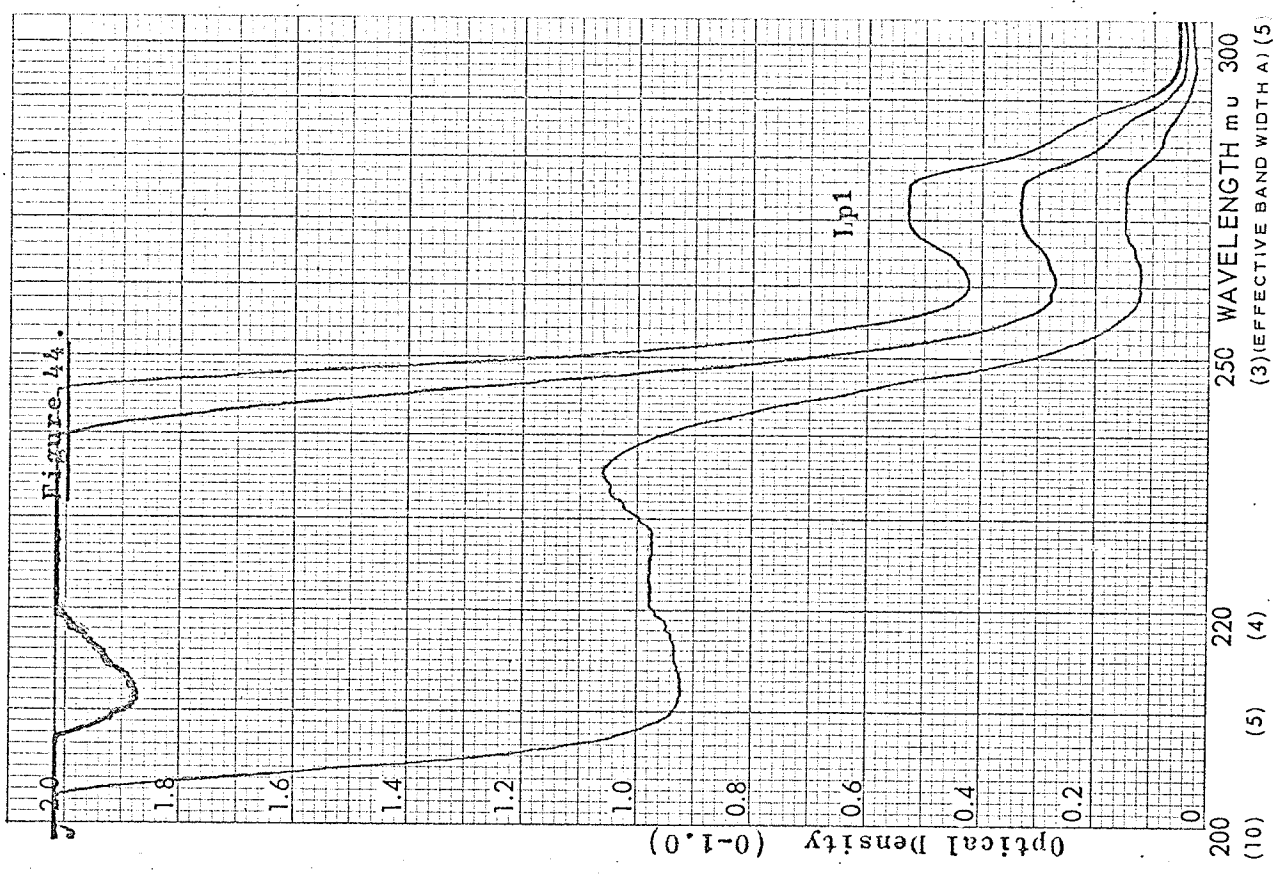


Figure 44.
Optical Density (0-1.0)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
200
220
250
300
WAVELENGTH mu
(10)
(5)
(4)
(3)(EFFECTIVE BAND WIDTH A) (5)
Lp1

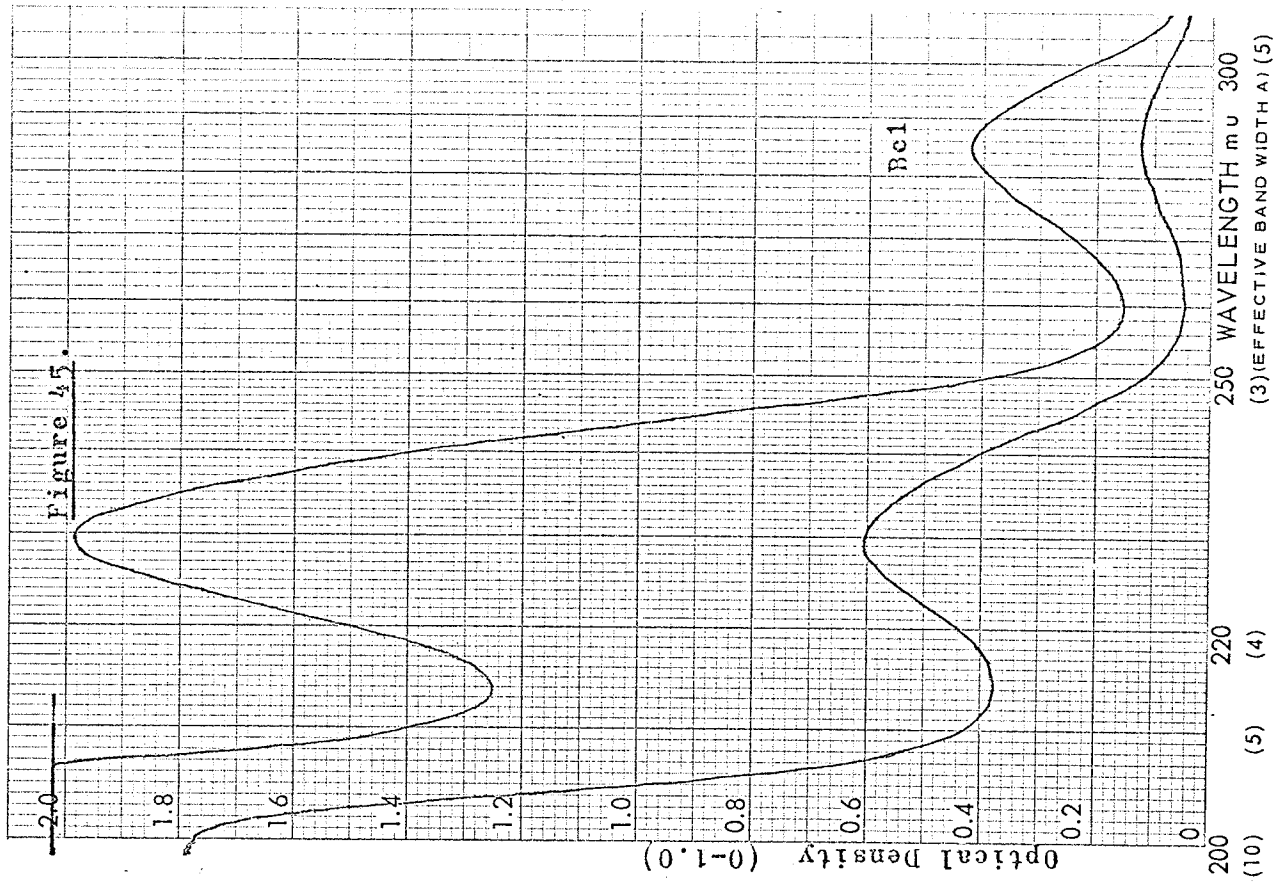


Figure 45.
Optical Density (0-1.0)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
200
220
250
300
WAVELENGTH mu
(10)
(5)
(4)
(3)(EFFECTIVE BAND WIDTH A) (5)
Bc1

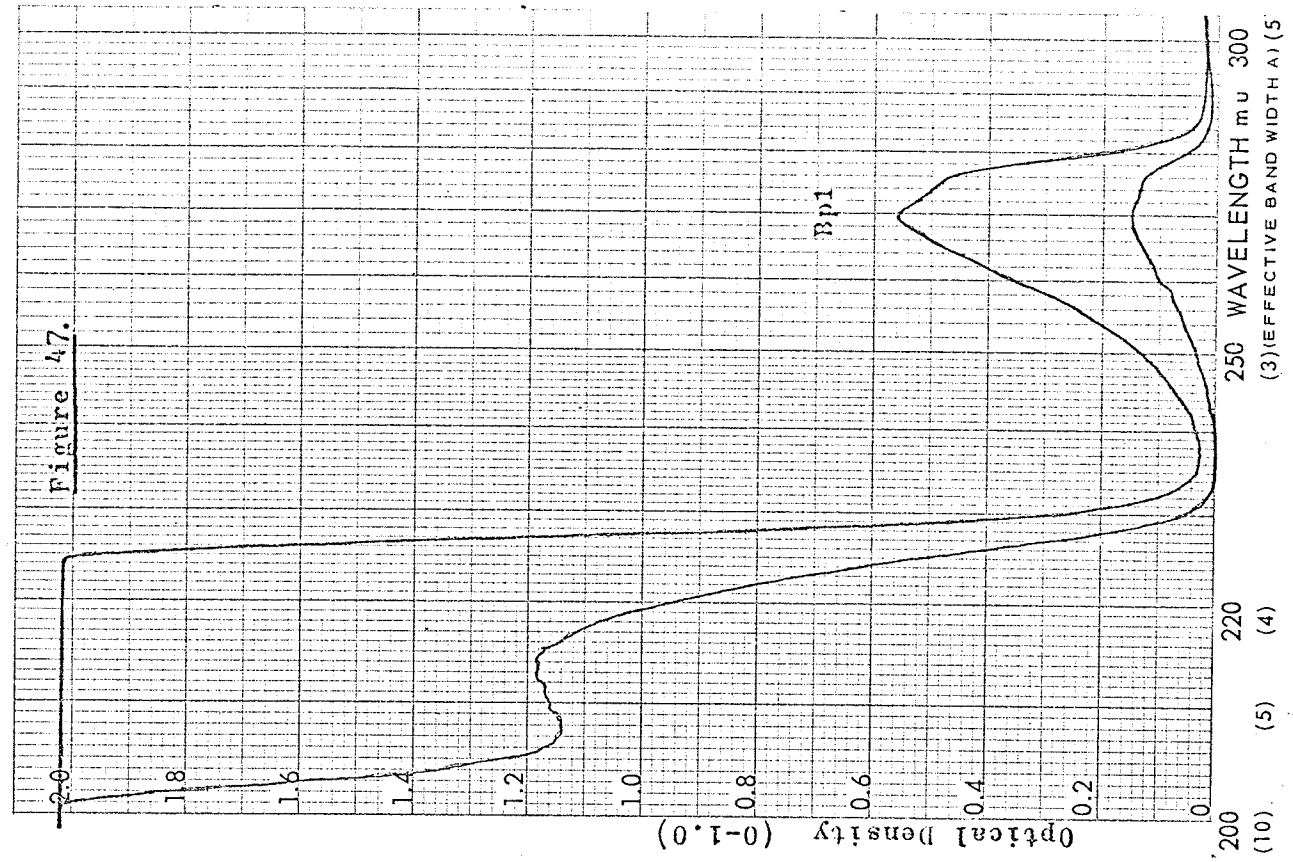


Figure 47.
Bp1
Optical Density (0-1.0)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
200
220
250
300
WAVELENGTH mu
(10)
(5)
(4)
(3)(EFFECTIVE BAND WIDTH A) (5

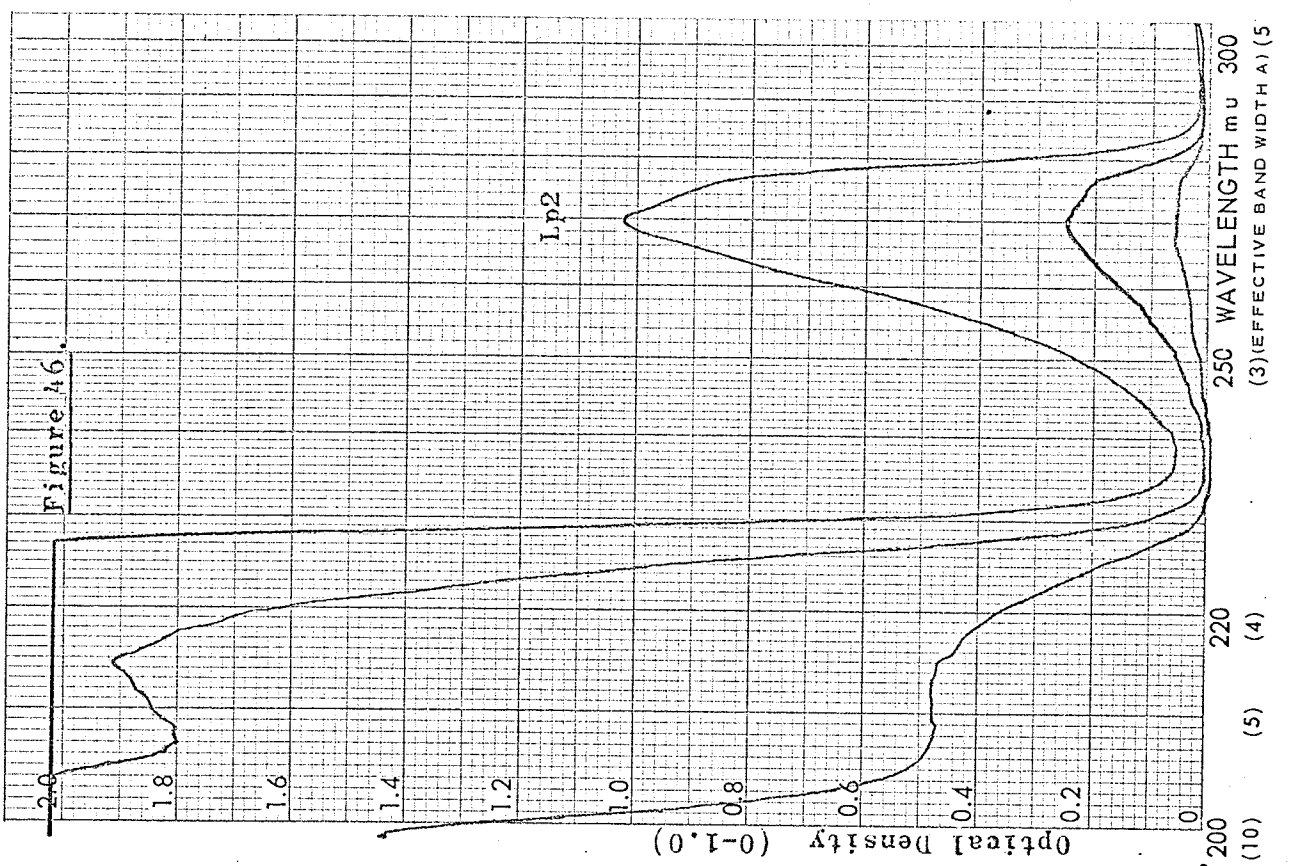
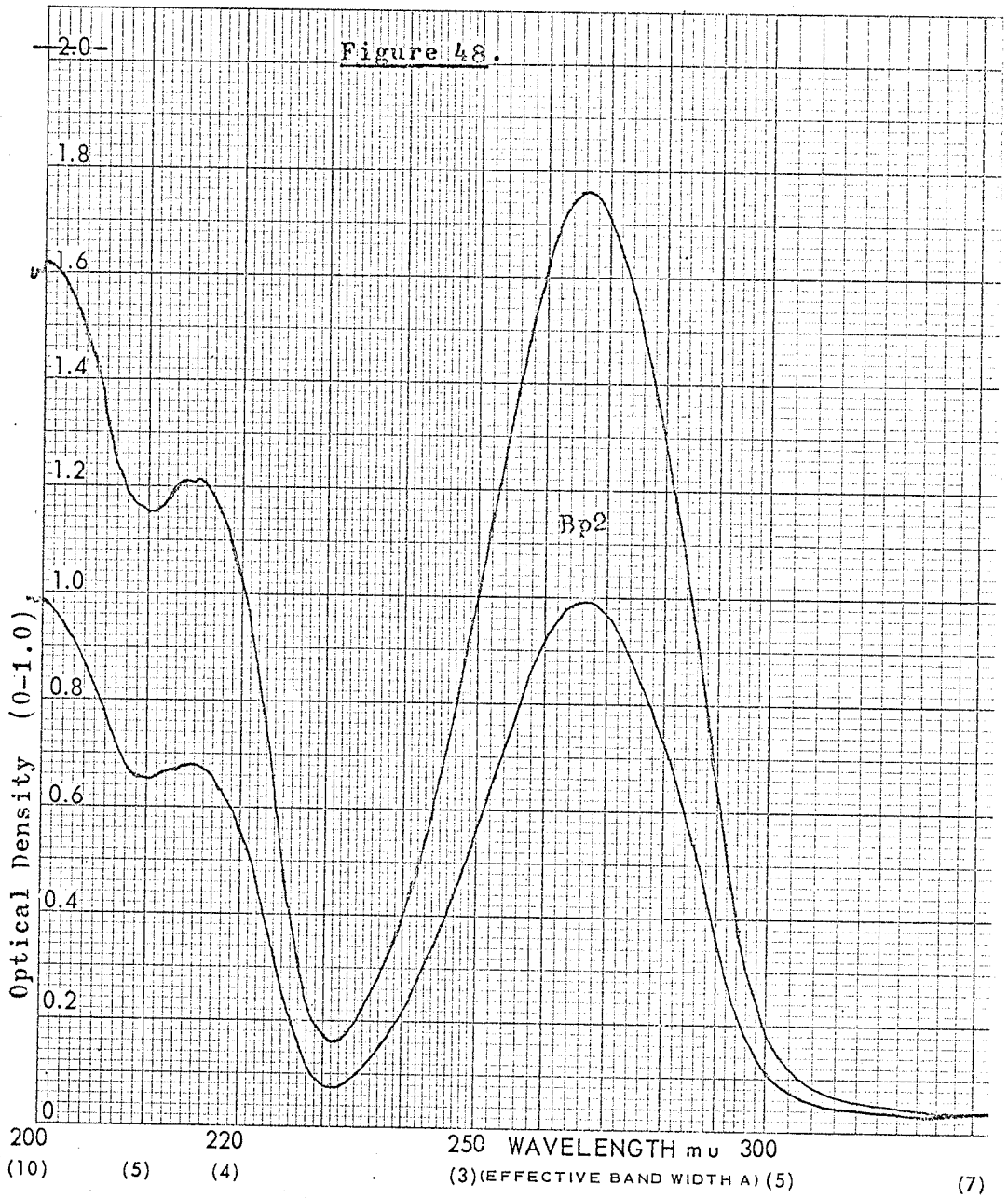


Figure 46.
Lp2
Optical Density (0-1.0)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
200
220
250
300
WAVELENGTH mu
(10)
(5)
(4)
(3)(EFFECTIVE BAND WIDTH A) (5



Ultra-violet Absorption Spectra of Isolated Phenolic Glycosides

Instrument: Ultrascan (Hilger & Watts)

Solvent: Water

Cell thickness: 1 cm.

Density: 1

Scan time (minutes): 4

Bandwidth: 1

Figure 44. UV-Spectrum of Lp1(Tremuloidin)

Figure 45. UV-Spectrum of Bc1(Salireposide)

Figure 46. UV-Spectrum of Lp2(Salicin)

Figure 47. UV-Spectrum of Bp1(Salicin)

Figure 48. UV-Spectrum of Bp2(Picein)

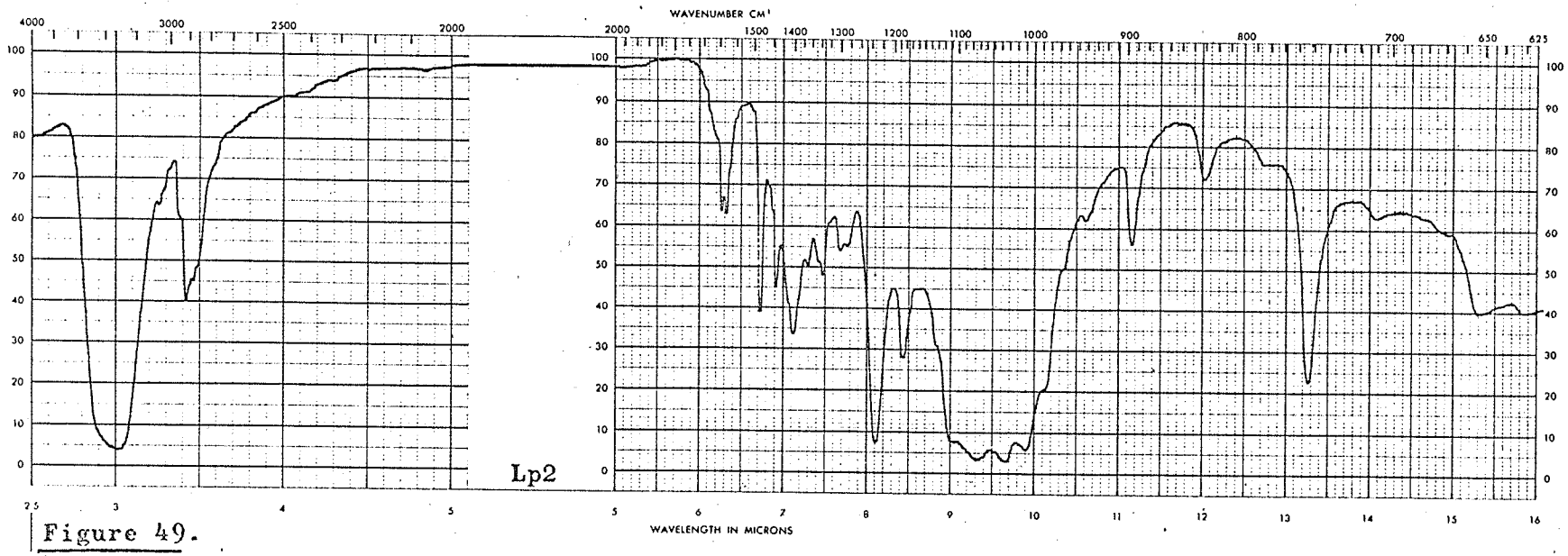


Figure 49.

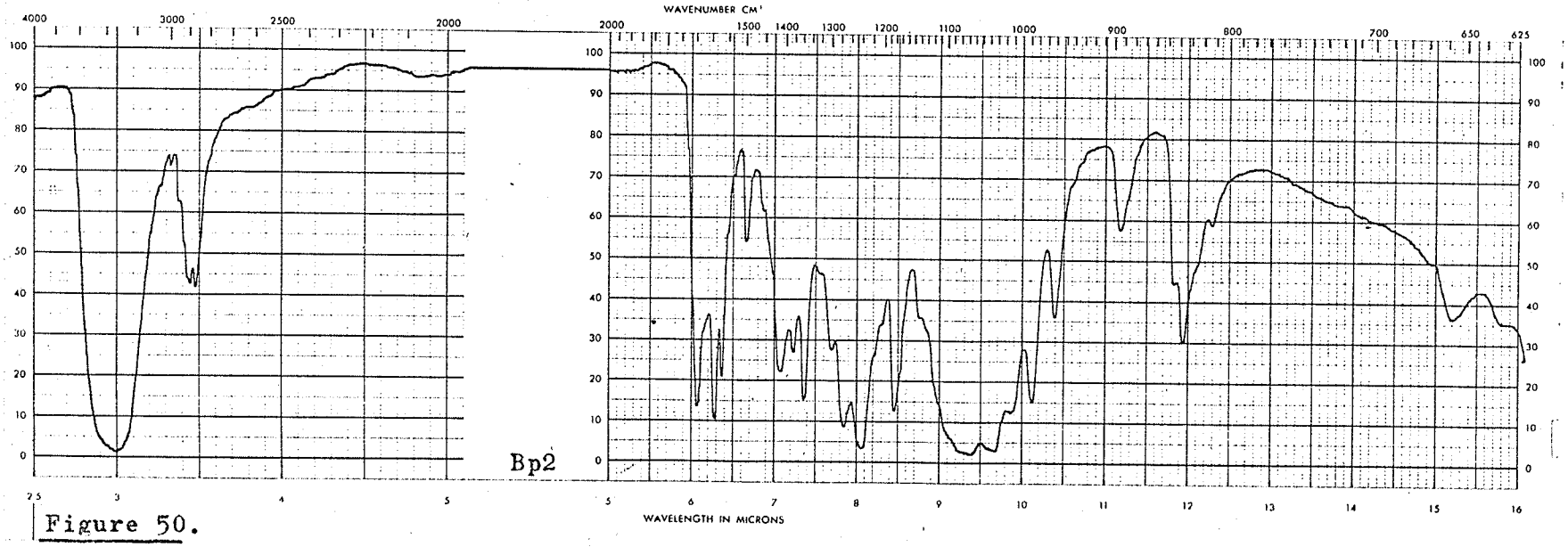


Figure 50.

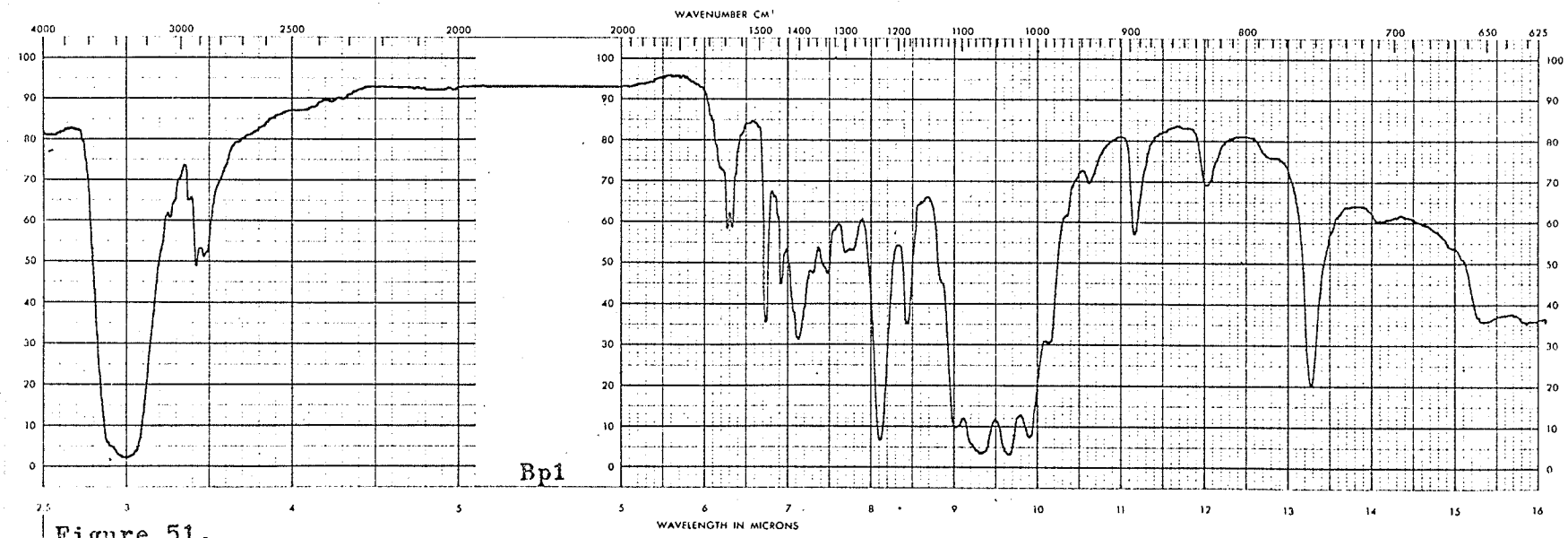


Figure 51.

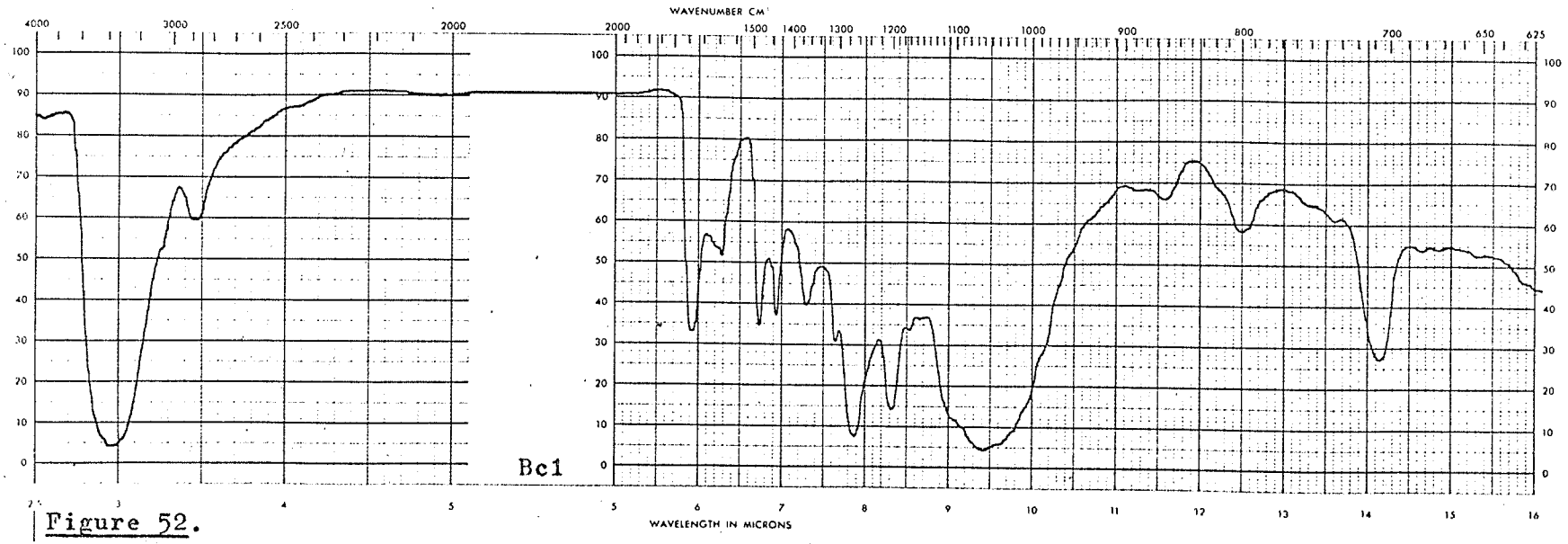


Figure 52.

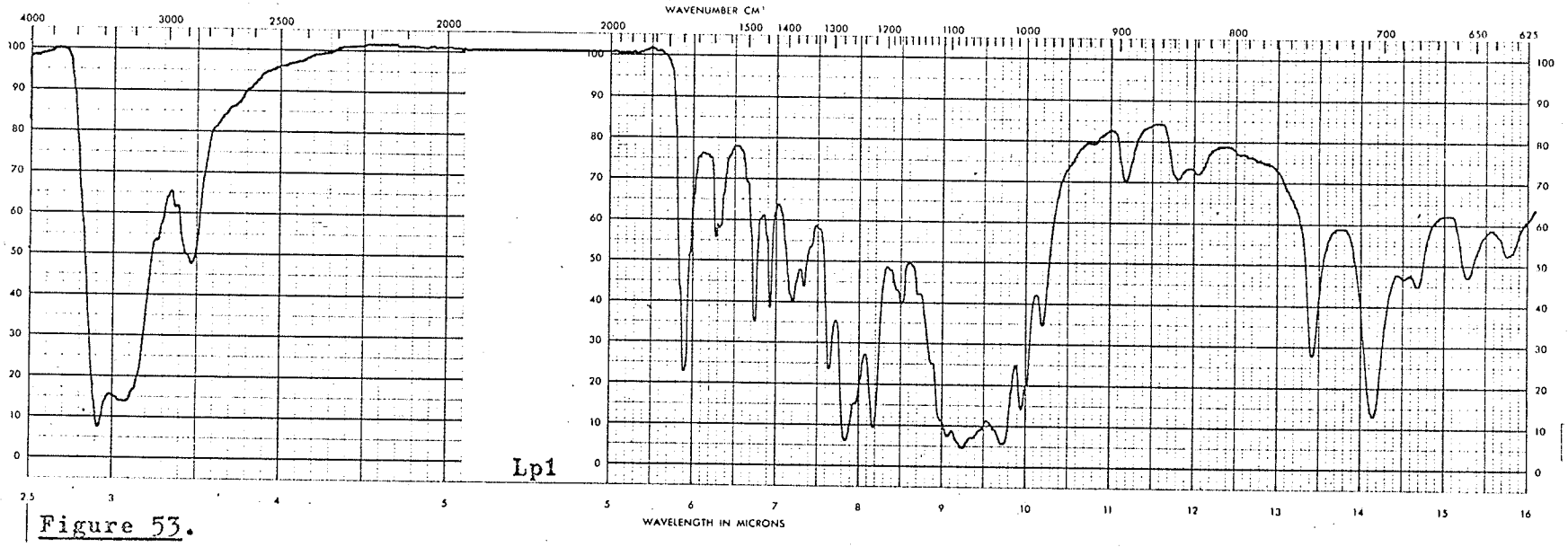


Figure 53.

Infrared Absorption Spectra of Isolated Phenolic Glycosides

Instrument: Beckman IR-8 Infrared Spectrophotometer (Beckman)

Solvent: Potassium bromide

Concentration: 1 mg/400 mg

Figure 49. IR-Spectrum of Lp2 (Salicin)

Figure 50. IR-Spectrum of Bp2 (Picein)

Figure 51. IR-Spectrum of Bp1 (Salicin)

Figure 52. IR-Spectrum of Bc1 (Salireposide)

Figure 53. IR-Spectrum of Lp1 (Tremuloidin)

SUMMARY

1. A complete TLC system on silica gel G and polyamide-cellulose layer was developed for the separation and identification of phenolic glycosides of the Salicaceae. Relative humidity was found to be a very important factor in obtaining reproducibility of the silica gel TLC separation system.

2. A screening process for Salicaceae phenolic glycosides has been established.

3. For large scale extraction of phenolic glycosides, a blender extraction in conjunction with a soxhlet extraction process was developed.

4. The ultra-violet absorption characteristics of phenolic glycosides have been determined and the ultra-violet absorption spectra of Salix phenolic glycosides were recorded.

5. The bark and leaves of S. petiolaris (S. gracilis var. textoris) collected in the middle of June 1965, were shown to contain salicin, salireposide, tremuloidin, grandidentatin, picein, triandrin and fragilin, and trace amounts of populin and vimalin. A number of unknown spots were also detected. Salireposide (B_{c1}), salicin (B_{p1}) and picein (B_{p2}) were isolated from bark extract, and tremuloidin (L_{p1}) and salicin (L_{p2}) were isolated from leaf extracts. The melting points, ultra-violet and

infra-red absorption spectra for the isolated glycosides have been recorded.

6. The bark and leaves of S. interior (S. fluviatilis var. sericans) collected at the end of June 1965 from the bank of the Red River at Fort Garry, Winnipeg, and from Patricia Beach (Lake Winnipeg) were shown to contain salicin, triandrin and salidroside, and trace amounts of vimalin and fragilin. A number of unknown spots were also detected, but these could not be identified with reference compounds.

B I B L I O G R A P H Y

1. Cooley, D., "News & Views of Health Medicine Pharmacy," Pharmaceutical Manufacturers Association of Canada, Vol. 3, No. 1 (1966).

2. Gan, W.S., "Manual of Medicinal Plants in Taiwan," Vol. 1, National Research Institute of Chinese Medicine, Taipei, 1958, p. 69.

3. Chopra, R.N., Nayar, S.I., and Chopra, I.C., "Glossary of Indian Medicinal Plants," Council of Scientific and Industrial Research, New Delhi, India, 1956, p. 218.

4. "The British Pharmaceutical Codex," The Pharmaceutical Press, London, 1934, p. 918.

5. Claus, E.P., and Tyler, V.E. Jr., "Pharmacognosy," Lea & Febiger, Philadelphia, 1965, p. 148.

6. Thieme, H., Die Pharmazie, 18, 770 (1963).

7. Fernald, M.L., "Gray's Manual of Botany," American Book Co., N.Y., 1950, 8th edition, p. 487.

8. Strasburger, E., Jost, L., Schenck, H., and Karsten, G., "A Text Book of Botany," 4th English Edition revised with the 10th German edition, by W.H. Lang, McMillan & Co. Ltd., London, 1912, p. 553.

9. Scoggan, H.J., "Flora of Manitoba," National Museum of Canada, Bulletin No. 140, The Minister of Northern Affairs and National Resources, Ottawa, 1957, p. 227.

10. Fernald, M.L., "Gray's Manual of Botany," American Book Co., N.Y., 1950, 8th edition, p. 516.

11. Fernald, M.L., "Gray's Manual of Botany, American Book

Co., N.Y., 1950, 8th edition, p. 506.

12. Irvine, J.C., and Ross, R.E., J.Chem.Soc. 89, 814 (1906).

13. Mori, A.D., Riv.Ital.Essezec Profumi 14, 238 (1932).

14. Thieme, H., Die Naturwissenschaften 50, 571 (1963).

15. I.R.F. and I.A.A. (Cairo), J.Am.Pharm.Assoc., Sci.Ed., 37, 276 (1948).

16. Fujikawa, F., and Satoh, N., J.Pharm.Soc.Japan 67, 85 (1947).

17. Thieme, H., Die Pharmazie 19, 535 (1964).

18. Rabate, J., J.Pharm.Chim. 28, 443 (1938).

19. Rabate, J., J.Pharm.Chim. 24, 311 (1936).

20. Univ.Kyoto, J.Pharm.Soc.Japan 62, 514 (1942).

21. Buchner, A., Repert. fur die Pharmazie 29, 411 (1828).

22. Chioriotti, C., Rev.Facultad Cienc.Quin. (Univ.Nael. La Plata) 16, 171 (1941).

23. Leroux, M., Ann.Chim.Phys. (2) 43, 440 (1830).

24. Clark, R.H., and Gilhie, K.B., Am.J.Pharm. 93, 618 (1921).

25. Rabate, J., Bull.Soc.Chim.Biol. 17, 319 (1935).

26. Wattiez, N., Bull.Soc.Chim.Biol. 13, 658 (1931).

27. Thieme, H., Die Naturwissenschaften 51(9), 217 (1964).

28. Pearl, I.A., et al., Tappi 44, 475 (1961).

29. Pearl, I.A. and Darling, S.F., J.Org.Chem. 24, 731 (1959).

30. Pearl, I.A. and Darling, S.F., J.Org.Chem. 24, 1616 (1959).

31. Pearl, I.A., et al., J.Org.Chem. 27, 2685 (1962).

32. Pearl, I.A., et al., Tappi 45, 663 (1962).

33. Thieme, H., Die Pharmazie 20(9), 570 (1965).

34. Evans, Iwamoto and Krantz, J.Am.Pharm.Assoc. 34 207 (1945).

35. Thieme, H., Die Pharmazie 21(2), 122 (1966).

36. Thieme, H., Die Pharmazie 21(2), 123 (1966).

37. Thieme, H., Die Naturwissenschaften 51(13), 310 (1964).

38. Thieme, H., Die Pharmazie 19, 725 (1964).

39. Braconnot, H., Ann.Chim.Phys. (2)44, 296 (1830).

40. Rabate, J., Bull.Soc.Chim.Biol. 17, 439 (1935).

41. Tanret, C., Bull.Soc.Chim.France 11, 944 (1894).

42. Wattiez, M.N., Bull.Soc.Chim.Biol. 13, 658 (1931), Bull. Acad.roy.Med.Belgique (5)12, 433 (1932).

43. Pearl, I.A., and Darling, S.F., J.Org.Chem. 27, 1806 (1962).

44. Thieme, H., Z.Chemie 2, 372 (1962).

45. Bridel, M., and Beguin, C., C.R.hebd.Seances Acad.Sci. 183, 231 (1926).

46. Thieme, H., Die Naturwissenschaften 51(15), 360 (1964).

47. Thieme, H., Die Pharmazie, 21(3), 182 (1966).

48. Thieme, H., Die Pharmazie 20(7), 436 (1965).

49. Thieme, H., Die Pharmazie, 21(4), 251 (1966).

50. Thieme, H., Die Pharmazie, 19(7), 471 (1964).

51. Thieme, H., Die Pharmazie, 20(11), 688 (1965).

52. Koev, D., Gorskostopan Nauka (Sofia) 1(2), 71 (1964).

53. Bryansk., T., Lesokhoz.Inst. 7, 204 (1956).

54. Shellard, E.J., "Practical Plant Chemistry for Pharmacy Students," Pitman Medical Publishing Co. Ltd., London, 1957, p. 125.

55. "British Pharmacopoeia," The Pharmaceutical Press, London, 1963.

56. Arthur, H.R., Proc.8th Pacif.Sci.Congr. 4A, 52 (1953).

57. Reichstein, T., and Abisch, E., Helv.Chim.Acta 43, 1844 (1960).

58. J.Pharm.Soc.Japan 75, 80 (1955).

59. Tsiskarishvili, T.P., Trudy Inst.Vinogradarstva i Vinodeliya, Akad.Nauk Gruzin. S.S.R. 9, 193 (1956).

60. Haas, P., and Hill, T.G., "An Introduction to the Chemistry of Plant Products," Vol. 1, 3rd edition, Longmans, Green and Co., London, 1921, p. 196.

61. Stahl, E., "Thin Layer Chromatography," Academic Press Inc., N.Y., 1965, p. 27.

62. Fike, W.W., and Sunshine, I., J.Chromatog. 18, 405 (1965).

63. Dallas, M.S.J., J.Chromatog. 17, 267 (1965).

64. Babbitt, J.M., "Thin Layer Chromatography," Reinhold Publishing Co., 1963, p. 88.

65. Beer, J.Z., J.Chromatog. 11, 247 (1963).

66. Libman, D.D., "Kurt Randerath - Thin Layer Chromatography," Academic Press, N.Y., 1963, p. 200.

67. Pearl, I.A., and Darling, S.F., Tappi 48(9), 506 (1965).

68. Pearl, I.A., and Estes, T.K., Tappi 48(9), 532 (1965).

69. Rosenthaler, L., "The Chemical Investigation of Plants," G. Bell and Sons Ltd., London, 1930, p. 112.

70. Pearl, I.A., and Darling, F.S., Archives of Biochem. and Biophys. 102, 33 (1963).

A P P E N D I X

Table 1. Willow Species in Manitoba (9)

Botanical Name & Synonyms	Distribution Location
Salix amygdaloides Anderss. Peach-leaved Willow	Moist ground and shores of southern Manitoba
S. serissima (Bailey) Fern. Autumn-Willow	Moist ground and shores in southern three-quarters of Manitoba
S. lucida Muhl. (Inc.var.angustifolia Anders) Shining Willow	Moist ground and shores in southern three-quarters of Manitoba
S. interior Rowlee (S. longifolia Muhl.; S. fluviatilis Nutt.var.sericans (Nees) Boivin) Sandbar Willow	Wet ground and shores throughout southern three-quarters of Manitoba
S. vestita Pursh.	Hayes River, Nelson River, York Factory, Churchill
S. reticulata L.	York Factory, Churchill
S. herbacea L.	Nejanilini Lake, Nueltin Lake, Baralzon Lake
S. arctophila Cockerell	Churchill, Cochrane River, Nejanilini Lake, Baralzon Lake
S. cordifolia Pursh var. callicarpaea (Trautv) Fern.	Nelson River, York Factory, Knife Lake, Churchill
S. brachycarpa Nutt. (Incl.var.mexiae Ball)	Plains of Manitoba, York Factory, Churchill
S. brachycarpa var.antimima (Schneid.) Raup	Gillam, Churchill
S. maccalliana Rowlee	Wet ground, bogs and shores in southern three-quarters of Manitoba
S. lutea Nutt.	Dry to moist ground throughout southern three-quarters of Manitoba

Table 1. Willow Species in Manitoba (cont'd)

S. myrtillifolia Anderss.	Wet ground, bogs, and shores almost throughout the province
S. pseudomonticola Ball (S. barclayi Anderss.)	Moist woods and shores in southern three-quarters of Manitoba
S. pyrifolia Anderss. (S. balsamifera Barratt) Balsam-Willow	Thickets, borders of woods, and shores throughout the province
S. calcicola Fern. & Wieg. (S. richardsonii Hook.var. macouniana Bebb)	York Factory, Churchill, Hay Island
S. alaxensis (Anderss.) Cov.	Known in Manitoba only from Churchill
S. candida Flugge Hoary Willow	Bogs, thickets, and shores throughout the province
S. bebbiana Sarg. (S. rostrata Richards.)	Thickets, swamps, and shores throughout the province
S. pedicellaris Pursh var. hypoglauca Fern. (S. myrtilloides of Am. auth.)	Wet ground and acid bogs throughout the province
S. athabascensis Raup (S. fallar Raup)	Sidney, Carberry, Riverton, MacBride River, York Factory
S. petiolaris Sm. (S. gracilis Anderss. var. textoris Fern.)	Thickets, meadows and shores in southern three-quarters of Manitoba
S. petiolaris var. rosmarinoides (Anderss.) Schneid.	Similar habitats in the southern half of Manitoba
S. humilis Marsh. Gray Willow	Thickets, prairie, and rock outcrops in the southern half of Manitoba
S. humilis var. microphylla (Anderss.) Fern. (S. tristis Ait.)	Dry prairie and rock outcrops of southern Manitoba
S. discolor Muhl. Pussy Willow	Damp thickets and shores in southern two-thirds of the province

Table 1. Willow Species in Manitoba (cont'd)

S. scouleriana Barratt	Nelson River at High Rock, Flin Flon, Reindeer Lake
S. planifolia Pursh (S. shlorophylla Anderss. var. denudata Anderss.)	Thickets and shores almost throughout the province
S. pellita Anderss.	Only the northern half of Manitoba
S. arbusculoides Anderss.	Nelson River, Churchill

Table 2. Melting Point and Specific Rotation of Salicaceae Glycosides and Their Acetylated Derivatives (from Thieme) (48,50)

Glycoside	m.p. (°C.)	$(\alpha)_D^{20}$	Acetylated Derivatives	
			m.p.(°C.)	$(\alpha)_D^{20}(CHCl_3)$
Salicin (Salicoside)	201	-62.5 (water)	132-133	-22.7
Populin	181-183	-29.7 (80% acetone)	128	-5.86
Salireposide	206-207	-35.6 (80% acetone)	128	-10.9
Tremuloidin	207-208	-12.3 (80% acetone)	114-115	+33.9
Grandidentatin	201-202	-66.3 (methanol)	167-169	-8.37
Picein (Piceoside)	195	-89.0 (water)	172-173	-28.6
Triandrin	177-179	-60.5 (water)	108	-37.2
Fragilin	177-179	-38.7 (water)	132-133	-22.7
Vimalin	143-144	-60.6 (methanol)	86-87	-37.3
Salicortin	141-142	-164.2 (water)	90-92	-20.8
Salidroside	159-160	-32.1 (water)	no crystal	-
Salicylpopulin	191-193	-30.8 (acetone)	136-137	+8.85
Salicyltremuloidin	191.5-193.5	-30.9 (acetone)	136-137	+8.8
Tricocarpin	134-136	-46.3 (methanol)	-	-
Tremulacin	127-129	-126.5 (methanol)	-	-

Table 3. Fermentative Hydrolyzable Glycosides and Their Hydrolytic Products (From Thieme) (48,50)

Glycoside	Sugar	Aglycone
Salicin	Glucose	2-Hydroxybenzyl alcohol
Picein	Glucose	4-Hydroxyacetophenone
Triandrin	Glucose	4-Hydroxycinnamic alcohol
Vimalin	Glucose	4-Methoxycinnamic alcohol
Salidroside	Glucose	4-Hydroxyphenyl ethanol (Tyrosol)
Trichocarpin	Glucose	Trichocarpinin (Gentisinic acid benzyl ester)

Table 4. Hydrolytic Products of Acylated Glycosides by Alkaline Hydrolysis (From Thieme) (48,50)

Glycoside	Deacylated Glycoside	Acid
Populin	Salicin	Benzoic acid
Tremuloidin	Salicin	Benzoic acid
Fragilin	Salicin	Acetic acid
Salireposide	Gentisinic alcohol-2-glucoside	Benzoic acid
Grandidentatin	Grandidentin (cis-Cyclohexanediol-(1,2)-1-glucoside	4-Hydroxycinnamic acid (p-Coumaric acid)
Salicortin	Salicin	Salicylic acid + not identified aliphatic hydroxy- acid
Salicylpopulin	Salicin	Benzoic acid + Salicylic acid
Salicyltremuloidin	Salicin	Benzoic acid + Salicylic acid

Table 5. R_f Value and Colour Reaction of Hydrolytic Products of Salicaceae Glycosides (From Thieme) (48,50)

Hydrolytic Products	BXAW* (6:4:2:8) R_f values	BXAW* (2:8:2:8) R_f values	Colour Reaction with Millon's Reagent	Colour Reaction with Diazotized Sulfanilamide Solution
Gentisinic alcohol-2-glucoside	0.09	0	yellow brown	red
Grandidentin	0.22	0	yellow	-
Not identified hydroxyl-acid	0.46	0	-	-
Gentisinic alcohol	0.57	0.09	yellow	yellow brown
cis-Cyclohexanediol-(1,2)	0.78	0.48	yellow	-
4-Hydroxyphenylethanol	0.87	0.55	red	red
2-Hydroxybenzylalcohol	0.87	0.68	yellow brown	yellow brown
4-Hydroxycinnamic alcohol	0.90	0.67	red	red
4-Hydroxycinnamic acid	0.90	0.71	red	red
Salicyl salicin	0.92	0.62	yellow	yellow
4-Hydroxyacetophenone	0.95	0.84	red	-
Salicylic acid	0.96	0.93	yellow	yellow
4-Methoxycinnamic alcohol	0.96	0.94	red	-
Trichocarpinin	0.97	0.94	yellow brown	brown
Benzoic acid	1.00	0.86	-	-

Paper: Schleicher and Schull 2043b MgI
Ascending method.

* n-Butanol-Xylene-Acetic acid-Water.

Table 6. R_f Value and Colour Reaction of Salicaceae Glycosides (From Thieme) (48.50)

Glycosides	BXAW* (6:4:2:8) R_f values	BXAW* (2:8:2:8) R_f values	Colour Reaction with Millon's Reagent	Colour Reaction with Diazotized Sulfanilamide Solution
Salicin	0.26	0	yellow	-
Populin	0.92	0.54	yellow	-
Salireposide	0.78	0.11	yellow	red
Tremuloidin	0.90	0.44	yellow	-
Grandidentatin	0.88	0.28	red	red
Picein	0.33	0	red	-
Triandrin	0.41	0	red	red
Fragilin	0.55	0.06	yellow	-
Vimalin	0.72	0.14	red	-
Salicortin	0.50	0.05	yellow	brown (5-10 min.)
Salidroside	0.24	0	red	red
Salicyltremuloidin	0.94	0.81	yellow	yellow
Trichocarpin	0.80	0.29	yellow	red

Paper: Schleicher and Schull 2043b MgI
Ascending method.

* n-Butanol-Xylene-Acetic acid-Water

Figure 1. IR-Spectrum of Salicin

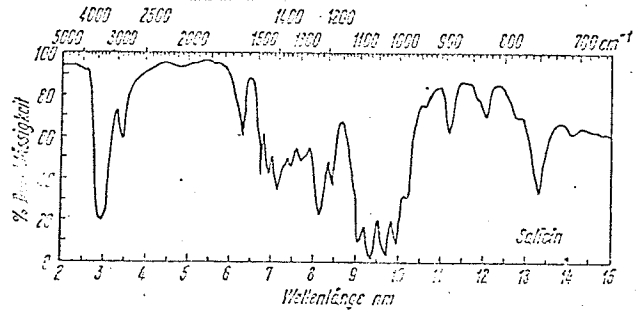


Figure 2. IR-Spectrum of Populin

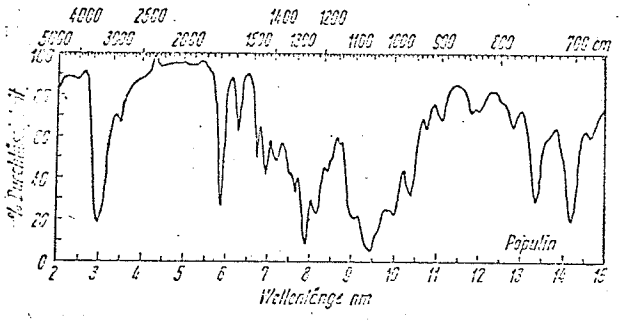


Figure 3. IR-Spectrum of Salireposide

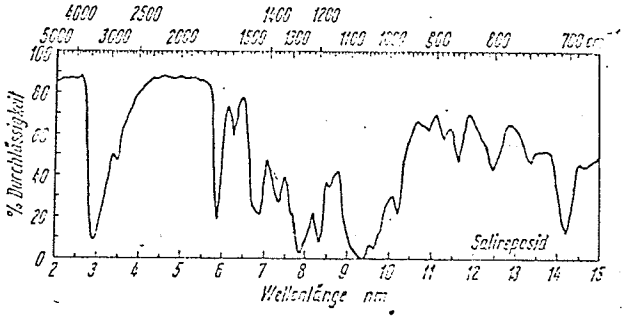


Figure 4. IR-Spectrum of Tremuloidin

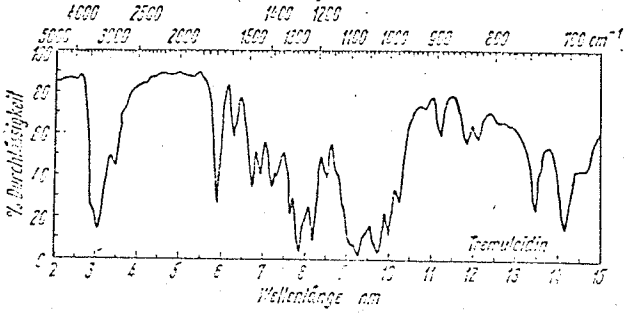


Figure 5. IR-Spectrum of Grandidentatin

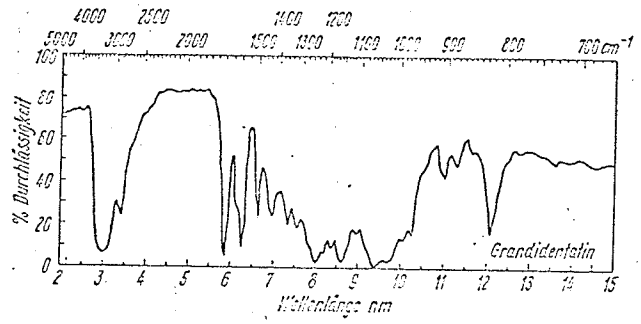


Figure 6. IR-Spectrum of Picein

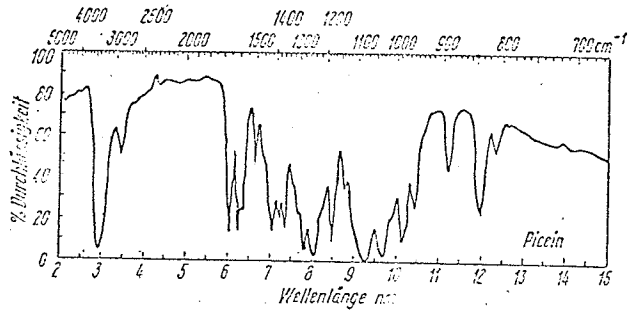


Figure 7. IR-Spectrum of Triandrin

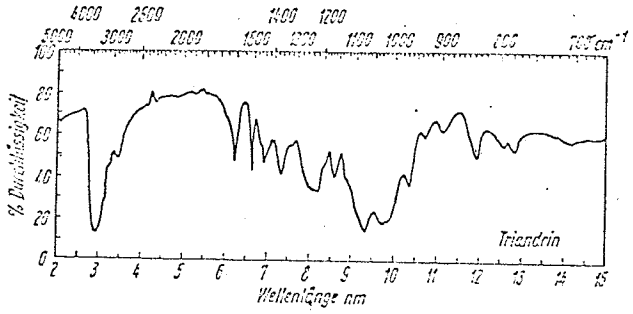


Figure 8. IR-Spectrum of Fragilin

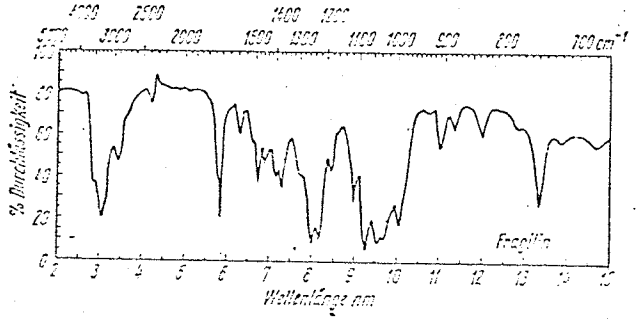


Figure 9. IR-Spectrum of Vimalin

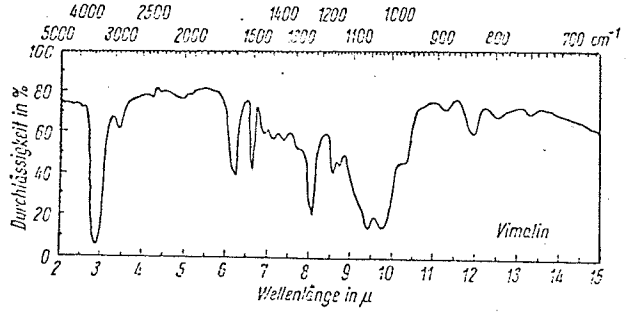


Figure 10. IR-Spectrum of Salicortin

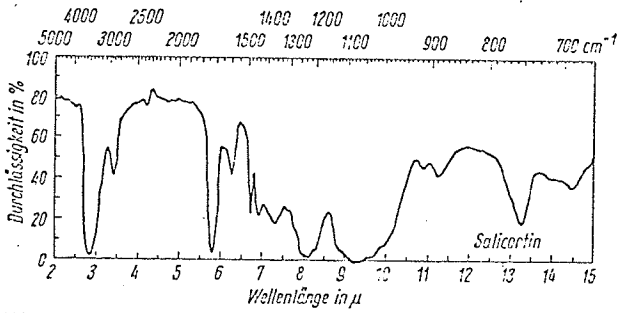
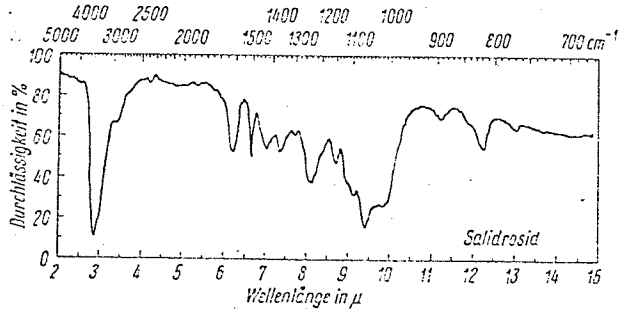


Figure 11. IR-Spectrum of Salidroside



※ From Thieme Articles (48, 50)