PHYTOCHEMICAL INVESTIGATION OF <u>SALIX</u> <u>PETIOLARIS</u> Sm.

(<u>SALIX</u> <u>GRACILIS</u> Anderss. var. <u>TEXTORIS</u> Fern.)

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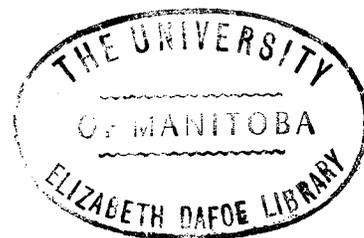
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ABSTRACT

The seasonal variation of total glycoside and individual glycoside content in Salix petiolaris bark, wood and leaf samples was determined. Considerable variation was found throughout the year and the qualitative and quantitative glycoside content must be described with this fact in mind.

The effect of various extraction procedures on the apparent glycosidic content of Salix petiolaris was studied and a suitable, reproducible, non-degradative extraction procedure was developed.

The glycoside content of both total wood and center wood samples of Salix petiolaris was also investigated. Qualitative studies were performed to identify as many components as possible in the Salix petiolaris extracts. Calibration graphs of three phenolic glycosides found in bark samples were prepared and used to determine the amounts of these glycosides present in November Salix petiolaris bark.

Two-dimensional thin-layer chromatographic systems were utilized for detection of phenolic glycosides and these results were compared to GLC results obtained for identical samples.

Glycoside "precursor" material was investigated in Populus and Salix samples. Various aspects, effects of different hydrolytic processes, effects of season and effects of bark moisture, were studied pertaining to this "precursor".

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I.   INTRODUCTION

a) <u>General description</u>

Willow, botanically known as <u>Salix</u>, is a member of the family <u>Salicaceae</u>, of the <u>Salicales</u> order, and is native to all continents except Australia. However, most of the 500-700 species are found in the Northern Hemisphere, with about 20 kinds occurring in temperate Africa and one in South America (1).

Willows are found growing in habitats ranging from 200 feet below sea level to 15,000 feet above sea level on various mountains. They also range from Mexico to the Yukon and withstand temperatures from 100°-130° F. above zero to 75° F. below zero. Tree willows vary in height from 15-120 feet, with one to four trunks, while shrubs vary from a few inches to 15 or 20 feet in height. Prostrate willows rise only 1 or 2 inches and form mats from 1 or 2 feet across (1). An excellent botanical description of <u>Salix</u> <u>petiolaris</u> has previously been presented by Wong (2).

<u>Salix</u> <u>petiolaris</u> Sm. (<u>Salix</u> <u>gracilis</u> Anderss. var. <u>textoris</u> Fern.) is a shrub with slender ascending or olive-brown tough and glabrate branches 1-3 meters 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, mature leaves glabrate or rarely silky, 4-10 cm. long and up to 2 cm. broad, strongly serrate-dentate except at the base, with gland-tipped teeth (3).

<u>Salix</u> <u>petiolaris</u> is found in the southern three-quarters of the Province of Manitoba (4). The majority of the experimental work presented in this thesis has been conducted on <u>Salix</u> <u>petiolaris</u>.

b) <u>Uses</u>

Willows have been useful to man, directly and indirectly through animals, in many ways. They have entered into every phase of his existence and an excellent study of the uses of willows has been presented by Ball (1).

Numerous ceremonial and religious rites of primitive people have been conducted around willows. Superstitions abound with willows; from the curing of diseases to the use of divining rods, willows have a mystical character.

Artists have utilized them as subjects of paintings, and they have been found in ornamental gardens to add beauty.

Numerous wooden products, such as baskets and wickerware, have been produced from willows. The four chief classes of material used are timber (trunks and poles), lumber, withes (slender rods) and roots. The making of containers is a large industry in Europe. Withes and roots and sawed lumber are used in four different types of construction: withes and roots woven; narrow strips of thin lumber woven or plaited into baskets or crates; the same nailed to heavier braces or ends for boxes and all cooperage (barrels). Peeled willow withes and roots are used for hampers and panniers.

In agricultural uses, willow wood has been utilized for fences, tool handles, tools, orchard props, vineyard trellises and tying materials. The blades and even frames of waterwheels have willow construction since it is unaffected by water. Willows have been used as fuel for many years.

Household articles, such as wicker furniture and kitchen utensils, have been produced. Floor mats of woven willow rods have been used. It is believed that the shredded willow twig was the forerunner of the modern toothbrush.

Use for willows has also been applied in travel and transport. Sandals, shoes, snow-shoes and canes have been produced. Boats, kayaks, oars and paddles have willow construction.

Willow wood has found limited use in making paper pulp due to its short fibers.

Numerous chemical and medicinal uses have been associated with willows. As charcoal, dyestuffs, rust preventatives and tanning materials, willows are important. Charcoal, once used for gunpowder, now is used in gas masks.

Willow bark, charcoal, buds, leaves, twigs and flowers have been used throughout historic time, mainly as decoctions and infusions. These have been used for every kind of ailment, disorder and disease of every part of the human body and applied both externally and internally (1). The pain reliever for the American Indian was a brew of willow bark (93). Salicin (I), in the United States Pharmacopeia from 1820 to 1936, was first used as a quinine substitute but now is used as an antiseptic, antiperiodic, febrifuge, antirheumatic, tonic, preservative and skin healer (1).

The bark, inner bark or bast and the silky seed hairs have been used as fibers for cordage and clothing. Livestock and cud-chewing animals utilize willows as forage (1).

Small-sized willow materials have been studied as a source for production of cellulose and hemicellulose (95).

Several pure compounds isolated by Pearl et al. are undergoing evaluation for medicinal and pharmaceutical uses. The production of salicin from Populus species barks has already found limited commercial application. Tremuloidin (II) because of its unique benzoyl substitution on the 2-0-glucose position is particularly suited as a starting material

for the production of 3,4,6-tri-O-methyl-D-glucose, a valuable reference

compound for carbohydrate and sugar research (96).

c) Phenolic glycosides

The phenolic glycosides constitute the major components of

Salix species. Several workers have undertaken the isolation and study

of the glycosides present in these species.

Salicin is found in almost all Salix species, in the bark,

leaves and sap (5). It was first discovered by Buchner in 1828 in the

bark of Salix alba and Salix incana (6).

Populin (III) has been found in the bark of several willow

species (7).

Picein (IV) has also been found in the bark of several Salix

and Populus species (7).

The leaves of Salix purpurea and Salix fragilis have yielded

tremuloidin, but its presence has not yet been detected in the bark (5).

Salireposide (V) and grandidentatin (VI) have been isolated

from the bark of several willow species (5).

Triandrin (VII) has been isolated by Thieme (8) from the early

July bark of Salix triandra. Chemical studies have shown triandrin to

be 3-(4-hydroxyphenyl)-2-propen-1-ol 1-ß-D-glucopyranoside. Triandrin

has also been found in the bark of Salix alba, Salix cinerea, Salix

aurita and Salix viminalis in a yield of 0.45-5.35% (19).

Fragilin (VIII), isolated by Thieme (9) from the leaves and

bark of Salix fragilis, has also been found in Salix alba, Salix purpurea,

Salix triandra and Salix viminalis. Extracted by continuous ethyl acetate

extraction, it was determined to be a monoacetyl derivative of salicin

(6-acetylsalicin) (10).

Salicortin has been isolated from the bark of Salix purpurea

by extraction with 2,5-pentanedione (11). A salicyloylsalicin (IX) derivative, it has been found with salicin in other Salix and Populus species.

Thieme (12) has isolated salidroside (X) from the bark of Salix triandra.

Vimalin (XI) was isolated by Thieme (13) in 0.15% yield from the bark of Salix viminalis by ethyl acetate extraction. It has been characterized as 3-(4-methoxyphenyl)-2-propen-ol 1-ß-D-glucopyranoside.

Isosalicin (XII), a phenolic glycoside which has not yet been detected in Salix species, was isolated by Thieme from the flowers of Filipendula ulmaria (14).

Dytkowska (64) studied the salicin content of several Polish Salix species. It was found that Salix alba contained 0.106%; Salix viminalis 0.129%; Salix caprea 0.106%; Salix fragilis 0.233% and Salix aurita 0.287%.

In earlier work, Thieme (5, 15) stated that salicin was the major glycoside found in Salicaceae. However, more recent work under-taken has shown this statement to be false. Thieme (16, 17) now states that the acetylated salicin derivatives occur in larger concentrations than other phenolic glycosides in all Salix species. Thieme has pre-sented the idea that due to the lead treatment (utilized for purification), the concentration of salicin was increased so that the amount of salicin obtained from extraction was not the true amount present in the bark (15).

The leaves of Salix myrsinifolia, Salix purpurea and Salix repens show a high concentration of salicortin (acetyl derivative) and moderate amounts of salicin (16). Especially high concentrations of salicortin or triandrin were found in the barks of Salix purpurea and Salix viminalis (17). Thieme (15) states that salicortin is the major

phenolic glycoside in many _Salix_ species and by alkaline treatment, from the purification step, salicin is produced.

In laboratory work performed by this author on the monthly samples of _Salix petiolaris_, salicin was never the major phenolic glycoside. Picein was present in large amounts, while salicin was present only in moderate quantities. These results are in agreement with those of Steele et al. (132). In their study of _Salix petiolaris_, picein was the dominant glycoside present while salicin was present in very small amounts.

Salicin and salireposide have been isolated from the bark of _Salix rosmarinifolia_ after extraction with dilute ethanol or ethyl acetate and separation by column chromatography (20).

Extraction of _Salix americana_ bark with boiling methanol produced salicin, salicortin and a new acylphenol glycoside which melted at 203°-205° C. (21). The presence of hyperoside has been detected in the bark of _Salix americana_ (31).

Kompantsev, the Russian worker, has studied the phenolic glycosides present in _Salix pentandroides_ (22).

The phenolic glycosides found in _Salix_ species, with notes on their history, isolation, sources and structure, has been reviewed by Thieme (5). He has also outlined his methods and results from studies on phenolic glycosides in willows (18). A review of the phenolic glycosides recently isolated from _Salicaceae_ has been presented (15). It deals with the elucidation of structure, the determination and spectroscopic detection of trichocarpin (XIII), salicyloyltremuloidin (XIV), vimalin, salidroside, salicortin, salireposide and fragilin.

In his thesis, Wong has presented an outline for several phenolic glycosides found in _Salix_ species (2).

Thieme has also performed many experiments on <u>Populus</u> species. A close phytochemical relationship is seen between <u>Salix</u> and <u>Populus</u> species since they are the only major members of the <u>Salicaceae</u>.

From <u>Populus</u> <u>nigra</u> bark and leaves, Thieme (32) has isolated salicin, salicortin and a new phenolic glycoside, nigracin (XV).

Two water-soluble glycosides, salicin and salicortin; and two difficultly water-soluble glycosides, salireposide and tremulacin were isolated from the bark of <u>Populus</u> <u>tremula</u> by Thieme (33). Tremulacin is a salicyloyltremuloidin derivative (34) and has also been found in the leaves of <u>Populus</u> <u>tremula</u>, bark of <u>Populus</u> <u>tremuloides</u> and the bark and leaves of <u>Populus</u> <u>alba</u> and <u>Populus</u> <u>trichocarpa</u> (33). The presence of 1-0-<u>p</u>-coumaroyl-β-D-glucose (XVI) in the leaves and bark of <u>Populus</u> <u>candicans</u> and <u>Populus</u> <u>trichocarpa</u> has been demonstrated by Thieme (35).

However, the most prolific workers of the <u>Populus</u> species are Pearl <u>et</u> <u>al</u>. and several glycosides have been isolated by these workers from various sources. These results are shown in Table 1.

Various aspects of <u>Populus</u> have also been studied by these workers including the components of the lead subacetate-insoluble fraction of <u>Populus</u> <u>tremuloides</u> leaves (40) and the difference in the glycosidic pattern in the diploid and triploid varieties of <u>Populus</u> (43,49).

Several observations and results of the <u>Populus</u> studies of Pearl <u>et</u> <u>al</u>. will be assessed under other headings in this thesis. Although their studies were carried out on <u>Populus</u> species, due to analogous features between <u>Salix</u> and <u>Populus</u>, the findings of Pearl <u>et</u> <u>al</u>. can be applied to <u>Salix</u> research.

d) <u>Flavonoid components</u>

<u>Salix</u> species have also yielded several flavonoid compounds.

TABLE 1

GLYCOSIDES FOUND IN POPULUS SPECIES

SOURCE	GLYCOSIDES	REFERENCE
P.tremuloides bark	Tremuloidin	36
	Salireposide	37
	Salireposide, tremuloidin, salicin, 1-0-p-coumaroyl-ß-D-glucose	43
P.tremuloides leaves	Populin, salireposide, tremuloidin	39,40
	Triploside	44
P.grandidentata leaves	Quercetin-3-glucosiduronic acid	41
	Populin	39
P.grandidentata bark	Grandidentatin	38
P.balsamifera bark	Salicin, trichoside	42,46,47
	Trichocarpin, salireposide	46,47
P.balsamifera leaves	Salicin, trichocarpin	48
P.trichocarpa bark	Salicin, trichocarpin, salireposide, trichocarposide	45

The purification steps used by many workers, lead subacetate treatment and polyamide chromatography, removes these flavonoids to allow the phenolic glycosides to be studied. The most detailed work in Salix studies has been performed on the phenolic glycosides, although some work has been performed on flavonoid components.

Kompantsev has studied the flavonoid components of Salix purpurea (23) and Salix elbursensis (24). The alcoholic extracts of the leaves of Salix purpurea contained six flavonoid compounds. Four of them isolated were luteolin 7-0-ß-D-glucopyranoside (XVII), quercetin (XVIII), luteolin (XX), and quercetin 7-0-ß-D-glucopyranoside (quercimeritin) (XIX) (23).

A flavonoid (25 mg.) and a phenolcarbonic acid (12 mg.) were isolated from 1 Kg. of willow shoots (Salix rubra) by ethanolic extraction. The flavonoid was identified as a flavanol-3-glucoside which contained the phenolcarbonic acid (25).

The leaves of Salix alba produced alboside (XXI) which was analyzed as rhamnozine 3-ß-D-glucoside by chemical analysis (26).

Thieme (27) indicated that the extracts of the leaves of Salix repens possessed four flavone glycosides. Two were separated as crystalline products after column chromatography and determined to be luteolin 7-ß-D-(6-ß-D-xylosido)glucoside (XXII), which is identical with caesioside and luteolin 7-ß-D-glucoside.

Isosalipurposide (XXIII), the 2'-glucoside of chalconaringenin, was found to be the major flavonoid of the young bark of Salix purpurea. The older bark also contains isomeric forms of naringenin 5-glucoside, salipurposide (XXVI). The leaf of Salix purpurea has a different flavonoid content. Mainly luteolin 7-glucoside is present, with smaller amounts of the 7-glucosides of eriodictyol (XXIV) and naringenin (XXV) present (28).

The flavonoid composition of staminate inflorescences of Salix caprea growing in Azerbaidzhan have been studied (29).

The bark of Salix acutifolia contained up to 5% of diastereo-isomeric forms of naringenin 5-glucoside. The younger bark contained 2-3% chalconaringenin 2-glucoside (30).

e) Other chemical components

Several other chemical compounds are present in Salix species and various workers have studied the makeup of numerous willows. The hemicelluloses from the twigs of the white willow (Salix alba) have been studied (51).

4-0-methylglucuronoxylan has been isolated from the wood of the white willow. By chemical analysis, the 4-0-methylglucuronoxylan backbone was found to consist of 120 units of ß-D-xylopyranose linked together by 1 → 4 glycosidic bonds. On the average, every eleventh D-xylopyranose unit was carrying one 4-0-methyl-D-glucuronic acid or D-glucuronic acid, attached to the backbone through the C-2 of xylose, as the simple terminal branching unit (52).

Polysaccharides, calculated as 16.9% on the weight of the wood, were extracted. Mainly water-soluble, the polysaccharide mixture contained mostly xylose and uronic acids but some galactose, mannose, arabinose and traces of rhamnose (52).

4-0-methylglucuronoxylan has also been isolated from the wood of Populus monilifera (53).

The chemical composition of the bark of several willow trees were analyzed (54). Salix viminalis, Salix cinerea, Salix triandra and Salix purpurea were found to contain moisture from 8.7-10.2%; total dry residue from 17.2-23.5%; water-soluble materials from 17.0-22.9%; water-insoluble materials from 0.2-1.7%; tannides from 4.4-14.5% and non-tannides

from 8.4-13.3%.

The aqueous extract from the bark was extracted with benzene for 5-6 hours and the residue from this was extracted with 95% ethanol for 6-10 hours. This ethanolic extract contained low molecular weight tannins, monosaccharides and oligosaccharides and organic acids (54).

The benzene residue was extracted with water for 8-10 hours and this benzene residue was found to contain lignin, cellulose, pentosans, suherene and ash. The aqueous extract was composed of highly condensed tannins, polyuronides, tannides, pentosans and hexosans (54).

The water content of the fresh bark of male willow trees (determined by drying at 105° C.) was found to be 54.2%; while that from female trees was 55.1%. Bark collected at the end of July contained 0.14% salicin and 0.9% triandrin in male trees; 0.15% salicin and 1.12% triandrin in female trees, all based on dry weights. Bark collected in mid-summer showed no significant differences in salicin and triandrin content between the two sexes (55).

In another study, Skrigan (56) analyzed one-year old shoots of 10 Salix species for their various components. Ash was found to be 1.1-2.19%; fat and resin to be 1.3-3.7%; hot water solubles 5.6-14.8%, mainly glucose with some fructose and traces of arabinose, partly as monosaccharides. The easily hydrolyzable (2% HCl) fraction (21-8%) contained polysaccharides principally composed of xylose and glucose, with some arabinose, galactose and mannose. The difficultly hydrolyzable (80% H_2SO_4) fraction (33-51%) contained polysaccharides composed of glucose (cellulose) with some xylose. Cellulose content of the wood was 25-33%; methoxy 4.5-6.2%; non-hydrolyzable residue 18.8-24.9%. This residue contained 0.6-1.0 Meq./g. of carboxy and 3.5-5.2 Meq./g. of phenolic hydroxyl groups (56).

Several willow species have been known to root very easily and so numerous workers have studied this phenomena with respect to growth factors present in willow shoots.

The growth substances isolated from Salix atrocinerea have been studied by Peña (57) and Vieitez (58). The cuttings were extracted with methanol and the acidic fraction contained indole-3-acetic acid (15 µg./Kg.) and p-hydroxybenzoic acid (850 µg./Kg.). The water-soluble residue, insoluble in ether, showed growth stimulating activity, which probably is due to a bound auxin (IAA-sugar); alkaline hydrolysis of this aqueous residue giving p-hydroxybenzoic acid, vanillic and p-coumaric acids, suggesting the presence (in the cuttings of Salix atrocinerea) of glycosides with aglycones similar to rhamnaceine (XXVII) and scutellareine (XXVIII) (58).

The growth substances isolated from the woody cuttings of Salix viminalis have also been studied. Analysis of the components were carried out with paper chromatography, thin-layer chromatography, ultra-violet analysis, infra-red analysis and fluorescence spectroscopy and Avena coleoptile straight growth test. p-Hydroxybenzoic acid (4 mg./Kg.) protocatechuic acid, two hydroxycinnamic acids and catechol (10 mg./Kg.) were isolated. Syringic, vanillic, and p-coumaric acids (1.3 mg./Kg.) were isolated from the hydrolysate. m-Hydroxybenzoic acid also appeared in the hydrolysate (59).

Free phenols appear to be abundant in the Salicaceae. This is understandable since the aglycones for all the phenolic glycosides are present in the plant and bind with the glycone to form the glycoside. Therefore, by hydrolysis, possibly by extraction procedures, it is possible to obtain phenols from these species.

The phenols from twenty species of Salix leaves have been

studied (60). All species indicated the presence of flavonol glycosides (mostly quercetin derivatives) and esters of various hydroxycinnamic acids. Leucoanthocyanin formation was not found in Salix daphnoides but Salix caprea, cinerea, aurita, alba, viminalis, viminalis x cinerea, caerulia, phylicifolia and caprea x lanata contained high concentrations of leucoanthocyanins (mainly composed to cyanidin (XXXIV), accompanied with delphinidin (XXXV), on acid treatment) and (+)-catechin acid and (+)-gallocatechin (60).

Binns and Blunden (61) found phenolic glycosides in all the species studied by Jaggi et al. (60) but Thieme (15, 17) was in agreement with Jaggi et al. who could not detect glycosides in some of these species.

Pearl et al. in their studies of Populus species have isolated numerous phenols from both bark and leaf tissues. Their results are found in Table 2.

Acid hydrolysis of the purified yellow dye-stuff of the purple willow tumor yielded two aldohexoses, glucose and rhamnose. 5,7,4′-trihydroxy-flavan-4-one was obtained as the aglycone and the disaccharide was attached to the C-7 hydroxyl of the aglycone (62).

Analysis was performed on 2-3 year old willow stem for ATP. The ATP concentrations found were quite high indicating a sizeable energy source in the sieve tube sap. It was proposed that this energy may be utilized by the plant for the translocation mechanism (63).

f) Recent methods of instrumental analysis

As instruments became more refined, the analysis of phenolic glycosides changed from chemical methods to instrumental methods and Kripiakevich (65) studied the various analytical methods utilized by workers of Salicaceae.

A colorimetric determination of salicin has been developed

TABLE 2

PHENOLS FOUND IN POPULUS SPECIES

SUBSTANCE	SOURCE	REFERENCE
Pyrocatechol	P.tremuloides leaves	40,44
	P.trichocarpa bark	45,92
	P.balsamifera bark	46,47
	P.balsamifera leaves	48
Salicyl alcohol	P.trichocarpa bark	45,92
	P.balsamifera bark	42,47
	P.balsamifera leaves	48
p-Coumaric acid	P.balsamifera bark	42,46
	P.balsamifera leaves	48
	P.trichocarpa bark	45
	P.tremuloides leaves	39
	P.grandidentata leaves	39
Cinnamic acid	P.balsamifera bark	47
	P.balsamifera leaves	48
Gentisyl alcohol	P.balsamifera bark	42,46,47
Vanillin	P.grandidentata leaves	39
	P.tremuloides leaves	39
p-Hydroxybenzoic acid	P.grandidentata leaves	39
	P.tremuloides leaves	39
3-Hydroxy-5-phenylvaleric acid	P.balsamifera leaves	48
Syringic acid	P.tremuloides bark	96
Ferulic acid	P.tremuloides bark	96
Vanillic acid	P.tremuloides bark	96

by Dobrowolska et al. (66). After extraction of the bark, the extract
was separated by paper chromatography; the salicin band was eluted with
water and reacted with Millon's reagent. The absorbance was measured
at 540 mμ and compared with a calibration graph. Satisfactory results
were obtained for samples containing up to 1 mg. of salicin.

Thieme also developed a simple and rapid photometric method
for quantitative analysis of phenolic glycosides of Salicaceae. In all
eight glycosides tested, the absorbance showed a linear relationship to
the concentration present. Plant extracts were run on paper chromatograms,
the glycosides were eluted and the solution reacted with Millon's reagent.
Individual glycosides could be determined in amounts up to 300 δ (micrograms)
with the precision of ± 5% (55).

Optical rotatory dispersion studies of phenyl glycosides of
saccharides indicated that the relationship between the configuration
at C-1 and the sign of a Multiple Cotton effect is shown in the 260 mμ
region and is characteristic for the absorption of the phenoxy group (67).

Nuclear magnetic resonance, which has only just recently been
utilized by workers on Salicaceae, has been useful in the elucidation
of the structure of some phenolic plant pigments (68). NMR spectroscopy
has also been utilized by Pearl et al. in their studies on Populus species (48).

However, the most extensively utilized instrumental tool has
been gas-liquid chromatography (GLC). Since all the experimental analyses
presented in this thesis have been obtained by gas chromatography, it
would be of value to mention several studies performed by other workers
and their significant findings.

The analysis of pharmaceutical products containing alkaloids
and glycosides has been performed by gas chromatography (69). Sugars
(70,71,72) and carbohydrates and their derivatives (73) have been studied

by gas chromatography. A trimethylsilylation method for analysis of sugars and related substances was developed to allow quick and efficient analysis (72). This procedure has been utilized in gas chromatographic analysis of phenolic glycosides (103). Trimethylsilylation allows the formation of a more volatile and less polar derivative in a short time and, thus, analysis can be performed on the gas chromatograph.

Gas chromatographic analysis has been linked with infra-red analysis for the detection of the components of a chromatographic trace (75). The brewing industry has adopted gas chromatographic analysis for the detection of phenolic compounds in its work (76).

Méndez has qualitatively separated five naturally occurring phenols found in plants by gas chromatography, using a methanolic solution of the phenols and an SE-30 silicone gum rubber on 60-80 mesh Diatoport S column (77).

The gas chromatographic separation and quantitative analysis of simple phenols important in human metabolism has been undertaken by Bakke and Scheline (78). Some general trends can be distinguished in the elution pattern of simple phenols which can give important suggestions about the chemical structure of unknown metabolites. The methods utilized by these authors allow for rapid, specific and sensitive results to be obtained. Quantitative studies were performed by injecting a standard solution and measurement of peak area by planimetry (78).

Gas chromatography has also been used for the study of methylated and partially methylated methyl glycosides. It provided a highly sensitive method for the analysis of individual methylated sugars and the cleavage products from methylated oligo- and poly- saccharides (79).

The determination of abscisic acid in the xylem sap of willow (Salix viminalis) has been conducted by gas chromatographic analysis (80).

Furuya has studied the gas chromatographic separation of plant glycosides, including several phenolic glycosides. Utilizing an SE-30 silicone rubber (0.75%) on Chromosorb W (80-100 mesh) column, the trimethylsilyl derivatives of these compounds gave sharp and single peaks indicating little or no decomposition. He worked with a representative variety of simple phenolic, coumarin, isocoumarin, isoflavone, anthraquinone, cyanogenetic, isothiocyanate and monoterpene glycosides (81).

A gas chromatographic method of analysis of phenolic glycosides has been developed by Bolan and Steele (74) and this method was followed for the experimental studies in this thesis. Using temperature programming and four different columns, excellent separation could be obtained for the ten phenolic glycosides studied. The relative retention time of the glycoside was independant of the quantity injected provided that this did not exceed 10 µg. per component. Quantities as low as 0.02 µg. could be detected. A trimethylsilylation process was utilized for analysis of the phenolic glycosides and no interconversion between populin and tremuloidin occurred during silylation (74).

By use of temperature programming, Bolan and Steele were able to elute all the phenolic glycosides studied within a reasonable time. Temperature programming also gave superior resolution of the higher molecular weight glycosides. By this method, a screening procedure could be carried out within 50 minutes (74).

The application of gas chromatography and mass spectroscopy is starting to receive widespread attention since the molecular weight of an unknown trimethylsilyl derivative in a mixture can be determined. Pellizzari et al. have presented the results obtained with mixtures of the trimethylsilyl ethers of 28 phenolic compounds and 4 phenolic glycosides. The effluent from gas chromatographic analysis of each component

of a mixture was passed directly into the mass spectrometer and the mass spectrum was obtained (82). Thus, the simultaneous use of gas chromatography and mass spectroscopy provides a useful tool for the separation and identification of components of extracts in biological systems.

Mass spectroscopy has been utilized by numerous workers with satisfactory results. This method of analysis requires only minor quantities of a compound and, in conjunction with gas chromatography, should simplify the structural analysis of phenolic glycosides. Mass spectroscopy has been utilized by early workers for the identification of methylated monosaccharides (83) and carbohydrate derivatives (84,85,86).

Several aroyl aryl glucosides were analyzed by Haslam by mass spectroscopy in order to study the position of acyl substitution. The mode of fragmentation of these glycosides has been presented and acetylated aryl glucosides, independent of the nature of the aglycone, all give very similar fragmentation patterns. Thus, the structures of unknown glucosides could be elucidated by study of known glucoside fragmentation schemes. A difference in fragmentation between a 6- and a 2- aroyl substituted compound (populin and tremuloidin) was indicated (87).

Pearl et al. have studied the fragmentation patterns of the acetates of several Salicaceae glycosides of known structure. The presence or absence of ester substitution in the glucose moiety of the parent glucosides could be determined with certainty, and possible location of substitution was indicated by relative intensities of fragment peaks (88).

Mass spectroscopy is particularly useful when the yield of a glucoside from the biological source is extremely small.

Pearl et al. have analyzed salicin, salirepin (XXIX),

salicyloylsalicin, tremuloidin, populin, salicyloylsalicin-2-0-benzoate
(XXX) and salicyloylsalicin-6-0-benzoate (XXXI). All four glucosides
with benzoyl substitution produced a different spectrum than those with-
out benzoyl substitution. The mass spectra also indicated a difference
in spectrum between the 6- and 2- benzoylated glucosides (88). This
result agrees with that proposed by Haslam (87).

1-0-p-coumaroyl-ß-D-glucose, grandidentatin, and trichocarpin
were analyzed as their acetates by mass spectroscopy (88).

Fragilin, vimalin and picein (low melting Salix glucosides)
were analyzed without prior acetylation. They produced fewer peaks but
indicated that they still followed a systematic fragmentation pattern
rather than pyrolysis (88).

Due to a difference in results obtained from methylation
experiments, Pearl et al. decided to analyze salireposide by mass spectro-
scopy. Thieme (89) obtained 2,3,4-tri-0-methylglucose from methylation
while Pearl et al. obtained 2,3,4,6-tetra-0-methylglucose (91). Thieme
(90) has recently revised his structure of salireposide since by more
efficient methylation procedures he obtained 2,3,4,6-tetra-0-methylglucose.
Pearl et al. have confirmed the structure of salireposide as 2-benzoyl-
oxymethyl-4-hydroxyphenyl-ß-D-glucose from their mass spectroscopy studies
(91).

Trichoside (XXXIII), a new phenolic glucoside, isolated from
the bark of Populus trichocarpa was analyzed by mass spectroscopy. The
mass spectrum of trichoside was very similar to the spectrum of tricho-
carpin (88). This indicated that trichoside is also a derivative of a
glucoside of the benzyl ether of gentisic acid. The principal difference
between the two compounds was in the size of the molecular ion, correspond-
ing to the difference between an acetyl and a methyl group. This result

indicated that one hydroxyl in trichoside is methylated and, thus, cannot be acetylated. The structure of trichoside was elucidated by means of mass spectroscopy (92).

From this short description, it can be seen that the use of instruments are playing a more important role in the analysis of glycosides from Salicaceae. The most useful to date has been gas-liquid chromatography but mass spectroscopy and even NMR seem to be becoming more useful.

g) Seasonal variation

The importance of seasonal studies of biological systems cannot be overemphasized. They correlate the chemical composition of the plant to the seasons. Besides gaining a fuller understanding of plant processes, this type of study allows workers to collect specimens when the chemical content is at a high concentration.

The seasonal development of the secondary phloem in Populus tremuloides has been studied. Cambial activity (division) begins in late March or early April, giving rise to sieve elements, companion cells and parenchyma cells. Approximately one-half of the new phloem is produced by mid-May and sieve element differentiation is completed by July to early August. Cessation of function begins in late September or early October with the formation of callose in sieve elements on the outer margin of the conducting phloem. All sieve elements are devoid of contents and their companion cells have collapsed by late November. Phloem differentiation precedes xylem differentiation by one to one and one-half months, but both cease simultaneously (97).

Several seasonal variation studies on phenolic compounds have been carried out. The annual variations of the phenolic compounds of the leaves of the beech, Fagus sylvatica, were studied (98). The seasonal variations of aliphatic and phenolic acids in the McIntosh apple clone

were studied (99).

Analysis has also been performed on the seasonal fluctuations of the composition of xylem sap in intact and ring-barbed branches of Salix alba (100).

Vieitez and Peña studied the seasonal rhythm of rooting in Salix atrocinerea cuttings. Salix atrocinerea has three rooting phases: one very active in January, February, March and April with plentiful roots, not ramified, originating at the base of the cutting; a second lesser phase from May to August with numerous small and very ramified roots formed at a more ample area on the stalk. Both peaks are separated by a sharp fall in June. From September to December, the third phase of rooting takes place. Response is practically nil and the few small roots formed are originated at the base of the stalk, again, as in the first stage (101).

The rooting capacity and indoleacetic acid content showed some correlation but not enough to warrant definite conclusions. In Salix atrocinerea cuttings, inhibitors were absent during the whole year which supports the theory that root formation may be influenced by the presence or absence of inhibitory growth substances (101).

In seasonal variation studies of phenolic glycosides, only Thieme and Pearl et al. have conducted experiments on this aspect.

Thieme (16,102) found that the highest glycoside concentration of eleven Salix species occurred in the bark in February and March and the lowest concentration occurred in August to October. The maximum gluco-side concentration in the leaves occurred 4-6 weeks later (about the first of May) and decreased until the leaves dropped. In the leaves the glyco-side concentration increased during the night and decreased during the day; the reverse phenomena was seen in the bark.

Especially high concentrations of salicortin or of triandrin were found in the barks of Salix purpurea or Salix viminalis collected in mid-March. In contrast to the barks, the leaves and inflorescences did not always contain phenolic glycosides (17).

The content of salicin and triandrin or the ratio of the two glycosides in the bark of Salix triandra depends on the time of year. In the beginning of July, triandrin is mainly present, while in the middle of October, salicin is mainly present (8).

Pearl et al. have also studied the effects of season on the glucoside content of Populus species and have stated that the content of the hot water extractives of Populus bark is a function of season and that certain glucosides might be present in quantity in March and be essentially absent in September (48).

In a study of May and September leaves from Populus balsamifera considerable ash-containing material separated from the concentrated original hot water extracts of the September (not the May) leaves (48). The total ethyl acetate soluble material from the hot water extractives of May and September leaves was approximately equal; the distribution of identified crystalline components being completely different. Thin-layer chromatography indicated that analogous eluate fractions contained essentially the same components qualitatively but in cases where the May leaves contained large amounts of crystalline components, the September leaves contained small amounts and where the May leaves contained small amounts, the September leaves contained only traces. Thus, the identifiable glucoside and related phenolic crystalline components appeared to decrease during the growing season (48).

Some of the comparative studies of bark at different seasons have indicated some interesting oxidation-reduction systems extant in

Populus species barks. Thus, early spring barks of _Populus_ _balsamifera_ contain high ratios of trichocarpin to its isomer salireposide, whereas late summer and fall barks contain high ratios of salireposide to tricho-carpin. It is apparent that a sort of intramolecular disproportionation took place in which the aglycone was converted from a benzyl ester of gentisic acid to the gentisyl ester of benzoic acid (103):
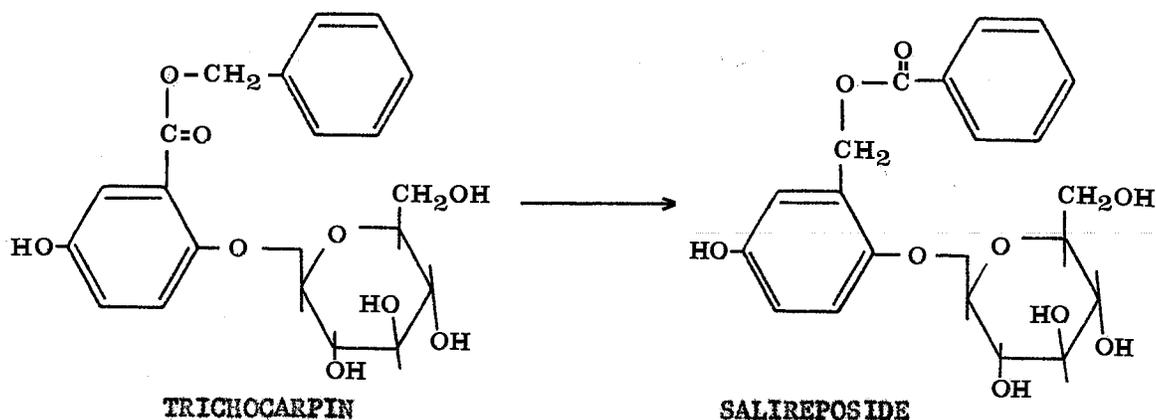
FIGURE 1

INTRAMOLECULAR DISPROPORTIONATION OF TRICHOCARPIN TO SALIREPOSIDE

Leaves from the same clone of _Populus_ _tremuloides_ were collected in September and October and their hot water extractives gave essentially the same results as the earlier July leaf extracts (39).

From these studies, it is clearly apparent that the level of glycosidic material present in plants is related to the season.

h) "Precursor" studies

The idea of a "precursor" substance occurring in _Salicaceae_ has been presented by Pearl et al. from the results of numerous studies on _Populus_. The "precursor" was thought to be some form of oligomeric or polymeric material in the plant which, upon hydrolysis, would yield glycosidic material. Several experiments were conducted by this author into the validity of the existence of a "precursor" in _Salix_ species.

The hot water extractives of <u>Populus trichocarpa</u> bark were extracted with chloroform, ethyl ether and ethyl acetate. These extracts were processed through polyamide columns both before and after lead subacetate treatment. The chloroform extract before treatment contained low molecular weight fatty material. Lead subacetate treatment was effective in modifying these materials so individual components could be isolated. A large amount of salicin, small amount of tremuloidin and trace amounts of salireposide and trichocarpin were obtained after lead treatment. Since these compounds were not present before lead treatment, it appears that some polymeric material has been hydrolyzed to produce them.

The ethyl ether extract contained a high proportion of glycosides before treatment and their yield was not altered after lead subacetate treatment. Therefore, there was no polymeric material present in the ether extract.

The ethyl acetate extract contained salicin, trichocarpin, salireposide and trichocarposide before lead treatment. After lead subacetate treatment, only the yield of salicin increased, from 2% to 9%. This result, again, indicated the presence of polymeric material (45).

The bulk of the hot water extractives of <u>Populus trichocarpa</u> bark was present in the aqueous raffinate from the ethyl acetate extraction. Studies by Pearl <u>et al</u>. suggest these compounds to be composed of pectins, soluble organic acids and salts and polymeric carbohydrate material (45).

In past studies (104,105,106) salicin was shown to exist in a polymeric form in <u>Populus</u> barks and required alkaline (lead subacetate) treatment for its liberation. However, salicin has been obtained in quantity from <u>Populus</u> barks without lead subacetate treatment (42,107).

The study on Populus trichocarpa bark indicated some salicin existed per se in the bark, while after lead subacetate treatment much more is obtained (45). Thus, some salicin exists in a free state in the bark while much more is present in a combined (complex) form.

Pearl et al. state that trichoside was obtained from the bark of triploid Populus tremuloides without lead subacetate treatment and, thus, no oligomeric material was hydrolyzed for its production. Many fractions obtained from column chromatography of the extract can be hydrolyzed by acid or alkaline reagents to produce glycosides which had been previously noted as components of the hot water extractives of the barks and leaves of Populus (44).

In the study of triploid Populus tremuloides bark, a large amount of syrupy material was obtained by ethyl acetate extraction of the hot water extractives of the bark. Because large yields of salicin and tremuloidin were obtained from both diploid and triploid Populus tremuloides bark after treatment of the hot water extractives with lead subacetate (36,49), it is probable that these syrupy products are oligomeric materials which comprise "precursors" for these two glycosides (43). Later column fractions of the ethyl acetate fraction produced syrupy residues which gave salicyloylsalicin-2-benzoate when hydrolyzed by acid. These syrupy residues were converted to hard glassy amorphous solids by evaporation under vacuum and are similar to the "chloroform complex" obtained by Charaux and Rabaté (106) from the chloroform extraction of the water extractives of Populus tremula leaves. Similar chloroform extracts from the leaves and barks of Populus tremuloides and Populus grandidentata have always produced syrups (49,104,105,107,108,109).

The chloroform extract of the hot water extractives of Populus balsamifera bark, when treated with lead subacetate, failed to yield any

glycosidic material (42). This result is surprising since similar treatment of Populus tremuloides and Populus grandidentata bark gave substantial amounts of tremuloidin (105,109). The difference in results with the chloroform extracts of various Populus species may be due to a difference in the character or even an absence of the "precursor" material.

A large yield (1.46%) of salicyloylsalicin-2-benzoate was obtained by hydrolysis of the chloroform extract of the hot water extractives of triploid Populus tremuloides (49).

Mild acid hydrolysis of the ethyl acetate extract of the hot water extractives of Populus trichocarpa bark gave an unidentified product which when hydrolyzed by alkaline reagents produced salicin and salicylic acid (45). This indicated the presence of oligomeric material which was similar in nature to that responsible for the production of salicyloylsalicin and salicyloylsalicin-2-benzoate from Populus tremuloides and Populus grandidentata bark and leaves (104,108,109).

It is interesting to note that Thieme who has worked extensively with Salix species glycosides, has not mentioned obtaining syrups from the eluate fractions of his columns (110).

When the hot water extractives of Populus trichocarpa bark were treated with lead subacetate only salicin was produced. In addition, neither salicyloylsalicin nor salicyloylsalicin-2-benzoate were found after mild acid hydrolysis (45). It is apparent that the polymeric material present in Populus trichocarpa is decidedly different than that found in Populus tremuloides or Populus grandidentata. With this observation, it would seem reasonable to assume that different Populus species would have different polymeric complexes which upon hydrolysis would produce different compounds.

From these observations, it is clear that the use of lead

subacetate or other alkaline agents affect the yield and character of glucosides when extracted from a biological source. Thus, Pearl et al. have commenced utilizing Thieme's procedure of polyamide column chromatography without lead subacetate treatment, for their recent experiments.

To summarize, from the studies of Pearl et al., it appears that there is some sort of "precursor" or oligomeric material present in Populus species.

i) Extraction studies

The method of extraction of biological materials is very important, since depending on the method utilized, varying results may be obtained from experiments. An ideal extraction procedure should be simple, fast, efficient in its extraction and have no effect on the biological system so that the components obtained by extraction exist per se in the plant.

The effect of different extraction methods on the glycoside content of Salix petiolaris was investigated by gas chromatographic analysis. Analysis of lead subacetate treated and untreated Soxhlet extracts of bark samples indicated that this lead processing was effective in increasing the concentration of salicin and altering the nature of other glycosides (132).

Purification of bark extracts by continuous liquid-liquid extraction with ethyl acetate proved to be ineffective (132).

Tests with various solvents were performed on pure glycosides to establish their effect on these glycosides. Tremuloidin was refluxed with ethanol, methanol, water, pyridine, dimethylformamide, acetone and ethyl acetate. All these solvents except acetone and ethyl acetate produced some decomposition of tremuloidin into salicin, populin or unidentified compounds. When populin was refluxed with water, salicin and other compounds

were produced. Almost complete decomposition of fragilin and grandidentatin occurred when they were refluxed with water. Thus, it appears that the extraction of Salix species with water, especially at elevated temperatures, causes some form of decomposition of glycosides and would not be practical for an extraction method (132).

Due to varying results being obtained by other workers, the extraction procedures utilized on Salicaceae were questionable in their efficiency. Therefore, a rapid, small scale screening procedure, utilizing gas chromatographic analysis, was developed by Steele and Bolan (130).

This method proved to be a milder and more reliable extraction procedure than previous methods. High grade acetone was utilized for the extraction solvent since it was shown not to decompose glycosides. Extraction was performed by refluxing rather than by Soxhlet since a shorter time was required for glycoside extraction and, therefore, the chance of decomposition was decreased. The acetone residue was extracted with water to reduce the possibility of any non-glycosidic material being present in the final solution. Contact with water was minimized to prevent hydrolysis or decomposition of any material and, thus, contact with water was approximately 5-10 minutes. Total screening time, on one gas chromatographic column, was about three hours. This time includes the extraction process, preparation of the trimethylsilyl derivative and actual analysis on the gas chromatograph (130). An application of this extraction procedure was used by this author in his experimental work.

Since 1830, when Braconnot (127) initiated the lead subacetate purification process, workers have used it without question. The lead subacetate precipitates have been removed except in a few cases where they were reconstituted and submitted to hydrolysis or filtration of crystalline products (41,105,128,129).

Pearl et al. have studied this lead subacetate precipitate since a large portion of the water extractives of Populus have been discarded in the precipitate in previous work. In their studies, they boiled the solution after treatment with hydrogen sulfide. This is quite drastic and may be responsible for the liberation of some material, by hydrolysis, into their final solution (40).

The reconstituted lead precipitate was extracted with various solvents and column chromatographed. Numerous compounds determined were tremuloidin, populin, salireposide, myo-inositol, pectin, pyrocatechol, succinic acid, quercetin-3-galactoside, quercetin-3-glucoside, rutin and rhamnetin. It was thought that the phenolic glycosides were present due to occlusion on the gelatinous precipitate (40).

Pearl et al. have studied the migration of acyl groups during the isolation of glycosides from Populus species. The water extractives of Populus grandidentata leaves will yield either populin or tremuloidin when purified by the lead subacetate method, depending upon the experimental conditions. If the purification of the aqueous extract is performed at elevated temperatures with an excess of lead subacetate, the labile tremuloidin will isomerize to give stable populin. Thus, purification of Populus grandidentata leaves with lead subacetate at elevated temperatures gives populin; whereas, similar purification at room temperatures gives only tremuloidin. Magnesium oxide also causes rearrangement of benzoyl groups in tremuloidin and in the benzoate group of salicyloyl-salicin (104).

These results agree with previous data that the acyl group on sugar moieties usually migrates away from the carbonyl group (131).

Binns and Blunden did not report any interconversion of populin and tremuloidin with their extractions when lead subacetate was used at

room temperature (122).

j) Experiments relating to Salicaceae

Several workers have undertaken the task of determining the
biosynthetic processes of phenolic glycosides in plants.

Phenolic-ß-glucosides have been prepared in vitro by incubating
various phenols with uridine diphosphoglucose in the presence of plant
protein preparations (111,112).

Pridham and Saltmarsh have analyzed the nature of the gluco-
sides formed by feeding simple phenols to the broad bean (Vicia faba) and
comparing these products with those obtained from in vitro experiments.
Their findings indicated that the primary products formed when feeding
mono-, di-, and tri-hydric phenols were the corresponding mono-ß-gluco-
sides. However, the formation of oligoglucosides or glucosylation of
more than one hydroxyl group could not be excluded. The results from
the in vitro experiments were closely related to the in vivo observations.
It was proposed that uridine diphosphoglucose was the donor molecule for
the glucosylation of phenols in the bean and, indeed, in all higher plant
tissues (113). Pridham has studied the importance of uridine diphospho-
glucose in the glucosylation of phenols in plant tissues under different
conditions (114).

Saligenin was shown not to be the "precursor" for salicin. From
studies with maize and Salix daphnoides, it was found that O-hydroxyphenyl-
ß-glucoside, the isomeric glycoside, was the major glucoside formed from
saligenin feedings (113).

The intramolecular transfer of glucose from the phenolic hydroxyl
of saligenin to the alcoholic group has been analyzed. Isosalicin was
found after infiltration of salicin into germinating seedlings of Geum
urbanum and other phenolic products were probably di-,tri- and tetra-

glucosyl derivatives of saligenin. Glucose, saligenin, isosalicin and saligenin gentiobioside were produced one by one from the salicin feedings and radioactivity from glucose-U-^{14}C was incorporated only into the molecule of saligenin gentiobioside (115).

An excellent review of the biochemistry and chemistry, under the topics of occurrence and distribution of phenolic glycosides in the Plant Kingdom, properties, functions and biosynthesis of phenolic glycosides, has been presented by Hopkinson (116). Pridham has also studied the function of phenolic glucosides in plants (94).

Peel has studied the phenomena of movement of compounds in willow stems. The movement of ^{14}C-labelled assimilates (117) and of phosphates and sugars into the sieve elements of bark strips of willows (118,119) were determined. Peel has discussed his findings on the transport mechanism in Salix (120). These findings may be useful in establishing whether or not glycosides are transported in willows and if they are, by what means?

Argus has found five new hybrids in the Salix of Alaska and Yukon: Salix rotundifolia ssp. dodgeana, Salix ovalifolia var. arctolitoralis, Salix ovalifolia var. gracilis, Salix planifolia ssp. pulchra and Salix planifolia ssp. pulchra var. yukonesis. These taxa are examined and the reasons for the new combinations are explained (121).

The distribution of leucoanthocyanidin, phenolic glycosides and piperidine-based imino-acids were studied in the leaves of Salix species and hybrids. When both parents contained either leucocyanidin or leucocyanidin and leucodelphinidin, the hybrids produced were found to contain the same compounds. When species containing leucocyanidin were crossed with species containing leucocyanidin and leucodelphinidin, the hybrids did not give any uniform result. This result would be of

importance in the determination of the parents of hybrids. Phenolic glycosides were detected in all species and hybrids but no obvious taxonomic pattern could be seen (122).

Gel filtration has been utilized for the separation of phenolic glucosides and carbohydrates from the bark extract of Populus tremula (123) and for the separation of phenolic glycosides (124).

Glucosides (salicin and tremuloidin) produced adsorption effects on Sephadex G-25 columns which did not conform with the theory of gel filtration. Salicin has an adsorbing aromatic ring and the distribution coefficient was higher than carbohydrates. Tremuloidin was more strongly adsorbed due to its two aromatic rings. Thus, from gel filtration, glucose was collected, then salicin and finally tremuloidin (123).

The glucoside content of a triploid variety of Populus species barks and leaves was seen to be far greater than the content of the corresponding diploid variety, both before and after lead subacetate treatment (49). The results from extractions on both varieties can be seen in Table 3.

TABLE 3

GLYCOSIDE CONTENT OF DIPLOID AND TRIPLOID VARIETIES OF POPULUS TREMULOIDES

DIPLOID	TRIPLOID
0.057% Populin from lead subacetate treated leaf extract	1% Populin from lead subacetate treated leaf extract
Absence of Populin upon direct concentration of leaf extract	0.57% Populin upon direct concentration of leaf extract

It was found that the 20% yield of ethyl acetate extractives from triploid bark was far greater than the yield from diploid bark (43). This result is quite interesting since the new variety permits greater yields of glycosidic material to be obtained.

Different types of bark from the same tree contained a different glycoside content. Populus balsamifera twig bark contained more ethyl acetate soluble material than the trunk bark and, in general, the yields of individual crystalline components were higher in the twig bark than in the trunk bark. Trichocarpin, the dominant glycoside, had a concentration in the twig bark double that found in the trunk bark. Salicin was found in a concentration in the twig bark of seven times that found in the trunk bark. The nature of the individual components of the ethyl acetate-soluble fractions vary much more in the twig bark fractions than in the trunk bark fractions (46). These results are rather significant and probably apply also to Salix species.

Detection of phenolic glycosides and aglycones has also been performed by paper chromatography (125) and thin-layer chromatography (126).

The paper chromatographic system was sensitive for arbutin (XXXII) over a range of 0 to 100 γ and for saligenin from 0 to 70 γ with an error of ±4% (125).

Silica gel G thin-layer plates without humidity control were not as effective as plates run under 78% relative humidity for the separation of ten reference phenolic glycosides. Polyamide plates were also tested but proved unsatisfactory unless mixed in the ratio of two parts polyamide to one part cellulose. A two-dimensional thin-layer system was developed, proving to be quite useful in the separation of phenolic glycosides. For the detection of phenolic glycosides on the

plates, 4% sulphuric acid in absolute ethanol proved most effective (126).

An interesting side-light was the isolation of a phenolic glycoside from _Psilotum nudum_, which is similar in nature to the glycosides in _Salicaceae_. Found in the shoots of the plant, its structure has been elucidated as 6(4-ß-D-glucopyranosyloxyphenyl)-5,6-dihydro-2-oxo-2H-pyran (50):

Psilotin

β-D-glucopyranosyl
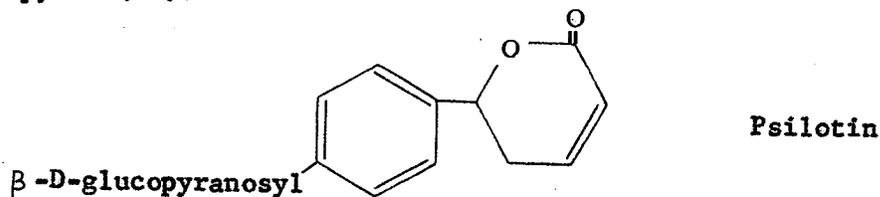
k) Object of the proposed work

By the use of gas chromatography and a reliable extraction procedure, a study of the seasonal variations of Manitoba _Salix petiolaris_ var. _gracilis_ was undertaken.

The glycosides present in the monthly samples were qualitatively and quantitatively determined.

Several studies on extraction procedures and for the detection of a "precursor" in _Salix_ species were performed.

II.   EXPERIMENTAL

a) <u>Materials and equipment</u>

All solvents used were reagent grade and chemicals were of the purest grade available. The silylating reagent used was Tri-Sil (Pierce Chemical Co.) and the solutions were injected by microsyringe (Hamilton Co. Ltd.). Materials utilized for thin-layer chromatography were Silica Gel G (R. S. Co. - Warner-Chilcott Lab. Inst.) and a thin-layer spreader (Quickfit, England). Micro-columns used for extract purification were prepared with cellulose powder, standard grade (Whatman) and polyamide, Woelm (M. Woelm, Eschwege, Germany).

All gas chromatography was done with a Beckman GC-4 gas chromatograph, equipped with a flame ionization detector and a temperature programmer. The signal was recorded on a Beckman 10 inch linear, potentiometric recorder set to a range of 1 mv. Helium was used as the carrier gas throughout the work.

Botanical samples utilized in experimental studies were <u>Salix purpurea gracilis</u>, <u>Salix purpurea nana</u>, <u>Salix interior (fluviatilis)</u>, <u>Populus grandidentata</u>, <u>Populus tremuloides</u> and <u>Populus songarica</u>. The majority of the experimental work in this thesis was performed on <u>Salix petiolaris (gracilis)</u>.

b) <u>Authentication of Salix samples</u>

<u>Salix petiolaris</u> Sm. (<u>Salix gracilis</u> Anderss. var. <u>textoris</u> Fern.) was authenticated by Dr. B. Boivin of the Plant Research Institute, Ottawa.

c) <u>Collection</u>

Monthly samples (except February) of <u>Salix petiolaris (gracilis)</u> were collected near Stonewall, Manitoba, approximately 30 miles north-west of Winnipeg. Twigs, one to three years old, usually with greenish outer bark, were collected. The bark was removed from the branches and, where

possible, leaves, flowers and seeds were collected and separated. All samples were dried in an oven at 50-60° C. until thoroughly dried. A Thomas comminuter was utilized for powdering the bark and wood. The leaves were crumbled to a small size by hand and all samples were stored in polyethylene air-tight bags for analysis.

TABLE 4

COLLECTION OF SALIX PETIOLARIS (GRACILIS) SAMPLES

DATE	SAMPLE COLLECTED
January 21, 1969	Bark, wood
March 3, 1969	Bark, wood
April 4, 1969	Bark, wood
May 10, 1969	Bark, wood, leaves, flowers
June 9, 1969	Bark, wood, leaves, flowers, seeds
July 9, 1969	Bark, wood, leaves
August 8, 1968	Bark, wood, leaves
September 11, 1968	Bark, wood, leaves
October 7, 1968	Bark, wood, leaves
November 12, 1968	Bark, wood
December 12, 1968	Bark, wood

d) Extraction methods

Various extraction studies were performed in order to establish a satisfactory extraction procedure and, also, to study the effects of various methods on the apparent glycosidic content of Salix species.

1) Extraction of Salix petiolaris with various solvents

i) Five samples of 100 mg. of Salix petiolaris bark were refluxed for two hours with 20 ml. of ethanol (95%), methanol, acetone, ethyl acetate and water. The extracts were filtered and, except for the water extract, were vacuum evaporated to dryness at room temperature. The

residue was extracted with hot water (4-5 ml.) and suction filtered.
Each solution was treated with 2-3 ml. of strong lead subacetate solution
(see Appendix B). The solution was suction filtered and hydrogen sulfide
gas was bubbled through the filtrate until precipitation of lead was
complete. The solutions were again suction filtered and the filtrates
were vacuum evaporated to dryness on a 40-50° C. water bath. A portion
of each residue was trimethylsilylated and analyzed on the OV-17 column,
utilizing temperature program 2.[*] Another portion of each residue was
taken up in ethanol and spotted on thin-layer plates (silica gel G, 250 μ),
developed in ethyl acetate-methanol, 9:1 v/v and sprayed with 4% sulfuric
acid in absolute ethanol.

ii) Salix petiolaris bark (20 mg.) was refluxed with 5 ml. of
pyridine for one-half hour. The extract was filtered and the filtrate
vacuum evaporated to a small volume. This solution was spotted on thin-
layer plates (silica gel G, 250 μ), developed in ethyl acetate-methanol,
9:1 v/v and sprayed with 4% sulfuric acid in absolute ethanol.

iii) Salix petiolaris bark (10 mg.) was refluxed with 2 ml.
of pyridine and 4-5 drops of water for 45 minutes. The extract was
filtered and the filtrate was vacuum evaporated to a small volume. This
solution was spotted on thin-layer plates (silica gel G, 250 μ), developed
in ethyl acetate-methanol, 9:1 v/v and and sprayed with 4% sulfuric acid
in absolute ethanol.

2) Effect of refluxing on glycosides

Tremuloidin and salireposide were refluxed in water for two
hours. The solution was vacuum evaporated and the residue was analyzed
by gas and thin-layer chromatography. A portion of the residue was tri-
methylsilylated and analyzed on OV-17 column, with temperature program 2.[*]
Part of the residue was dissolved in ethanol and spotted on thin-layer

* Description of Program 2 on page 42.

plates (silica gel G, 250 μ), developed in ethyl acetate-xylene-formic acid-water,35:1:2:2 v/v and sprayed with 4% sulfuric acid in absolute ethanol.

3) Purification of bark extracts

i) Five samples of 10 mg. of Salix interior (fluviatilis) bark were refluxed with 2 ml. of acetone for two hours. Pretreated polyamide and cellulose columns were prepared on a microscale (0.5 x 5 cm.). The extracts were filtered and the filtrates were vacuum evaporated to a small volume and then placed on a column. Five columns were run:

a) Polyamide column eluted with 5 ml. of water.

b) Polyamide column eluted with 25 ml. of water.

c) Cellulose column eluted with 5 ml. of acetone.

d) Cellulose column eluted with 25 ml. of acetone.

e) Cellulose column eluted with 10 ml. of acetone, eluate dried and then applied to a polyamide column and eluted with 10 ml. of water.

The acetone eluate fractions were vacuum evaporated to dryness at room temperature and the water eluate fractions were freeze-dried. All samples were analyzed by gas chromatography utilizing the OV-17 column and temperature program 2.

ii) Salix interior (fluviatilis) bark (10 mg.) was refluxed with 2 ml. of acetone for two hours. The extract was filtered and the filtrate was vacuum evaporated to dryness at room temperature. The residue was extracted with 4-5 ml. of water, the extract placed on a pretreated polyamide column and the column eluted with 25 ml. of water. The eluate was freeze-dried and the residue was analyzed by gas chromatography, utilizing the OV-17 column and temperature program 2.

4) Effect of water extraction on Salix petiolaris extracts

i) Two samples of 10 mg. of July <u>Salix</u> <u>petiolaris</u> bark were refluxed with 2 ml. of acetone for two hours. Both extracts were filtered and the flask washed with more acetone. The washings were filtered and added to the other filtrate.

One extract was vacuum evaporated to dryness at room temperature. The residue was trimethylsilylated and analyzed by gas chromatography on the OV-17 column using temperature program 2.

The other extract was vacuum evaporated to dryness at room temperature. The residue was extracted with 4-5 ml. of water and filtered. This solution was vacuum evaporated to dryness on a 40-50° C. water bath. The residue was trimethylsilylated and analyzed on the gas chromatograph, using the OV-17 column and temperature program 2.

ii) Two samples of 50 mg. of July <u>Salix</u> <u>petiolaris</u> bark were refluxed with 10 ml. of acetone for two hours. Both extracts were filtered and the flask was washed with more acetone. The washings were filtered and added to the other filtrate. The two solutions were treated exactly as outlined in Extraction methods, experiment 4 i. The dry residues remaining after treatment for both samples were scraped out and a portion was trimethylsilylated and analyzed on the OV-17 column using temperature program 2.

iii) Two samples of 50 mg. of July <u>Salix</u> <u>petiolaris</u> leaf and wood were refluxed with 10 ml. of acetone for two hours. Both extracts were filtered, the flask was washed with more acetone and the washings were filtered and added to the other filtrate. The two solutions of each sample were treated exactly as outlined in Extraction methods, experiment 4 i. A portion of the residue remaining was scraped out and trimethylsilylated. Analysis was performed on the OV-17 column using temperature program 2.

5) <u>Extraction of the monthly samples of Salix petiolaris</u>

Bark and leaf samples (50 mg.) and wood samples (100 mg.) were accurately weighed into 25 and 50 ml. flasks respectively. Acetone (10 ml.) was added to the bark and leaf samples and 20 ml. of acetone were added to the wood samples. All samples were refluxed for two hours and then filtered. Each marc was washed with fresh acetone, then filtered and the combined filtrates were vacuum evaporated to dryness at room temperature.

The acetone residues were shaken with 4-5 ml. of water and very slightly warmed. The solutions were filtered into tared flasks and vacuum evaporated to dryness on a 40-50° C. water bath. The flasks were placed in a vacuum desiccator and allowed to dry completely.

Each total residue was weighed and then scraped into a powder. One-fifth of the bark and leaf residue and one-tenth of the wood residue was transferred to a vial and accurately weighed for analysis on the OV-17 column. A second portion of the residue, equal in weight to the first portion removed, was taken for analysis on a second column (OV-1).

This extraction procedure was also followed on flower and seed samples. For flower samples 50 mg. were used and for the seed sample 626 mg. were used for the extraction.

e) <u>Analysis procedures</u>

1) <u>Thin-layer chromatography</u>

Silica gel G (22 g.) was weighed and triturated with 44 ml. of water in a mortar. This slurry was applied to a thin-layer chromatograph spreader and five 20 x 20 cm. plates at a thickness of 250 μ were prepared. The plates were allowed to air-dry overnight and were activated before use by heating in an oven at 105° C. for 10-15 minutes.

The dried plates were marked with a probe 15 mm. from the front

edge as the starting point and a channel was cut 15 cm. above the starting point. Side channels were cut in the plate to ensure even development.

For two-dimensional plates, a spot was located in the lower right-hand corner 15 mm. from the front edge and 15 mm. from the right side of the plate as the starting point. Two channels were then cut in the plate. One was 15 cm. above the application point and the other was 15 cm. left of the application point. Diagrams of these plates are found in Wong's thesis (2).

The sample was applied to the starting point and allowed to air-dry. The plate was then placed in the developing solvent and allowed to develop. After completion, the plate was removed and air-dried. If two-dimensional, the plate was placed in the second solvent system and allowed to develop in the other direction. The plate was removed after completion and allowed to air-dry.

Solvent systems utilized were:

i) Ethyl acetate-methanol, 9:1 v/v.

ii) Benzene-methanol, 7:3 v/v.

iii) Ethyl acetate-xylene-formic acid-water, 35:1:2:2 v/v.

After drying, the plate was sprayed with 4% sulfuric acid in absolute ethanol and placed in an oven at 105° C. for 10-15 minutes. The colored spots were then located and circled with a probe.

2) <u>Gas chromatography</u>

The 0.3% OV-17 on Chromosorb G AW/DMCS 60/80 mesh column and the 0.3% OV-1 on Chromosorb G AW/DMCS 60/80 mesh column were prepared and conditioned by Bolan, as outlined in his paper (74).

The following temperature programs were used:

For the OV-1 column, start at 190° and hold for 10 min, then 2° per min rise to 208° and hold for 5 min. Finally 5.25° per min rise

to 250° and hold until the last component is eluted. This is referred
to as temperature program 1.

For the OV-17 column, start at 190°, hold for 10 min, then 6°
per min rise to 250° and hold until the last component is eluted. This
is referred to as temperature program 2.

Tri-Sil (100 µl.) was added to the extract by microsyringe and,
after shaking, the mixture was allowed to stand in a stoppered vial for
at least 10 minutes. A Hamilton 0-25 µl. syringe was used to inject
10 µl. of this solution into the gas chromatograph and the various peaks
eluted were timed. Retention times of the extract peaks were recorded
relative to trimethylsilylarbutin, injected after every 3-4 extract runs,
to compensate for possible gas flow variations.

TABLE 5

OPERATING CONDITIONS FOR GLC

	OV-1	OV-17
Retention time of TMS arbutin (min.)	10.93	7.90
Carrier gas flow at outlet (ml./min.)	100	100
Column temp. (°C.)	190-250	190-250
Inlet temp. (°C.)	250	250
Detector temp. (°C.)	300	300
Column length (ft.)	6	6
Sensitivity (amps, f.s.d.)*	25×10^{-10}	25×10^{-10}

f) Qualitative studies

Several micrograms of the reference glycosides were placed in
a vial and Tri-Sil was added. The vial was shaken and allowed to stand
for 10 minutes. A portion of the solution was injected into the gas
chromatograph and the retention time noted. The retention time was cal-
culated relative to trimethylsilylarbutin, which was injected at inter-

* amps, f.s.d. = amps, full scale deflection.

vals during analysis.

December <u>Salix petiolaris</u> bark (100 mg.) was extracted as out-
lined in Extraction methods, experiment 5. Several fractions, each re-
presenting one-tenth of the residue weight, were weighed into vials for
analysis. Tri-Sil (100 μl.) was added and the solution allowed to stand
10 minutes. 10 μl. of this solution were injected into the gas chromato-
graph and the times of the eluted peaks were noted. The times were con-
verted to the relative arbutin ratio with trimethylsilylarbutin which
was run immediately before each extract analysis.

The reference glycosides were injected in varying volumes
until the recorder deflections of their eluted peaks were within ± 5
recorder units of the December extract peaks suspected to be identical.
Analysis was performed utilizing the OV-1 and OV-17 columns and their
temperature programs.

If any doubt occurred in identification, the reference glyco-
side was added to the December bark extract and the mixture was co-
chromatographed. An increase in peak height of the extract peak or the
appearance of two separate peaks was noted.

Reference glycosides utilized for qualitative studies were
picein, salicin, isosalicin, vimalin, salireposide, tremuloidin, populin,
salicortin, triandrin, fragilin and salidroside.

g) <u>Quantitative studies</u>

1) <u>Preparation of calibration graphs</u>

Three phenolic glycosides, salicin, picein and salireposide,
were quantitatively analyzed. Exactly 10 mg. of the reference glycoside
were weighed into a 1 ml. volumetric flask on a Mettler semi-micro balance.
Salicin and picein were dissolved in ethanol (95%) and salireposide in
acetone and accurately made up to volume.

For salicin, 1, 2, 4, 6, 8, 10, 12 μl. (representing 10, 20, 40, 60, 80, 100, 120 μg. of salicin) were removed from the standard solution by microsyringe and transferred to fine taper tubes (133). This is a specially designed 15 ml. standard taper test-tube with a capillary base having a capacity of 100 μl.

For picein, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 μl. (representing 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 μg. of picein) were removed from the standard solution by microsyringe and transferred to fine taper tubes.

For salireposide, 1, 2, 4, 6, 8, 10, 12, 14 μl. (representing 10, 20, 40, 60, 80, 100, 120, 140 μg. of salireposide) were removed from the standard solution by microsyringe and transferred to fine taper tubes.

These manipulations were repeated two more times for each reference glycoside so that a total of three samples were obtained for each weight of reference glycoside. The tubes were placed in an oven at 70° C. until the solvent had evaporated.

Tri-Sil (100 μl.) was added to each tube by microsyringe and the solution was thoroughly mixed and allowed to stand for 3-4 minutes. A portion (10 μl.) of each solution was injected into the gas chromatograph using the OV-17 column and temperature program 2 for picein and salireposide. Salicin was analyzed isothermally at 190° C. on the OV-17 column. By utilizing one-tenth of the Tri-Sil solution, analysis was ultimately performed on 1, 2, 4, 6, 8, 10, 12 μg. of salicin; 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 μg. of picein and 1, 2, 4, 6, 8, 10, 12, 14 μg. of salireposide.

The sensitivity for salicin and salireposide was 25×10^{-10} amps, f.s.d. throughout the analysis but for picein had to be changed to 0.5×10^{-8} amps, f.s.d. at 12 μg. since the peak was off scale for weights

greater than 10 µg.

Two injections (10 µl.) were performed from two tubes and 1 injection (10 µl.) from the other tube for a total of five injections for each weight of reference glycoside. The area was calculated by triangulation and the average area from the five tests was calculated. A calibration graph of weight (µg.) vs. peak area (cm^2) was then constructed for the three phenolic glycosides.

2) Application of quantitative studies

November Salix petiolaris bark (50 mg.) was extracted as outlined in Extraction methods, experiment 5. One-fifth of the residue was weighed into a vial and 100 µl. of Tri-Sil were added. The vial was shaken and allowed to stand for 10 minutes. A portion (10 µl.) of this solution was injected into the gas chromatograph, utilizing the OV-17 column and temperature program 2. The times of the eluted peaks were calculated relative to trimethylsilylarbutin and the peak areas of salicin, picein and salireposide were determined. The weight of these phenolic glycosides present in the bark extract were determined by applying their peak area to the calibration graph. The areas of these three glycosides obtained in this experiment were also compared to the area obtained for these glycosides from earlier analysis of the November bark sample.

h) Comparison of glycoside content in total wood to center wood of Salix petiolaris

Salix petiolaris samples were collected on October 9, 1969 from the area previously described. The bark was removed and the wood was dried in an oven at 40-50° C. A few pieces of wood (taken as total wood sample) were powdered by use of a Thomas comminuter.

Approximately 4 to 6 mm. were shaved off other wood samples to

obtain center wood which was also powdered with a Thomas comminuter.

Equal quantities (100 mg.) of both samples were weighed into 50 ml. flasks and extracted as outlined in Extraction methods, experiment 5. The residue from each extraction was weighed and two samples from each extraction, representing one-tenth of the total residue, were weighed into vials for gas chromatographic analysis.

Tri-Sil (100 μl.) was added by microsyringe, the vial shaken and allowed to stand for 10 minutes. A portion (10 μl.) of this solution was injected into the gas chromatograph using the OV-1 column and temperature program 1. The second sample was treated similarly and injected into the OV-17 column using temperature program 2.

i) Two-dimensional thin-layer chromatographic studies

October and November Salix petiolaris bark samples (50 mg.) were extracted as outlined in Extraction methods, experiment 5. Thin-layer chromatographic plates were prepared with silica gel G for two-dimensional studies as outlined in Analysis procedures, experiment 1.

One-fifth of the residue remaining after extraction was removed and dissolved in acetone. The solutions were spotted on the plates and the plates developed as outlined in Analysis procedures, experiment 1.

Solvent systems utilized for two-dimensional thin-layer chromatogram development were:

i) Ethyl acetate-methanol, 9:1 v/v.

ii) Benzene-methanol, 7:3 v/v.

iii) Ethyl acetate-xylene-formic acid-water, 35:1:2:2 v/v.

j) "Precursor" studies

1) Analysis of Populus bark

The bark (100 mg.) of three Populus species (P. tremuloides, P. grandidentata, P. songarica) were weighed into a piece of filter paper.

The samples were extracted with benzene for 24 hours by Soxhlet.

After extraction, the solution was filtered into a tared flask and the benzene removed by vacuum evaporation at room temperature. Residue weight was determined and one-tenth of the residue was accurately removed for gas chromatographic analysis.

Hot water was added to the remaining residue and the solution was shaken for several minutes. The solution was filtered and cooled. Excess of strong lead subacetate solution was added, the solution suction filtered with the aid of celite and the filter pad was thoroughly washed with water. Hydrogen sulfide was passed through the combined filtrates until no further precipitation occurred. This solution was suction filtered with the aid of celite, with subsequent washing of the filter pad, into a tared flask. The filtrate was vacuum evaporated to dryness on a 40-50° C. water bath, the residue was weighed and a weight of the residue, equal to the weight of benzene residue previously removed was again removed for GLC analysis.

The samples were treated and analyzed as outlined in the GLC Analysis procedures, on OV-17 column and using temperature program 2.

2) Analysis of Salix bark

i) The bark (100 mg.) of three Salix species (S. petiolaris, S. purpurea gracilis, S. purpurea nana) was weighed out and extracted and processed identically as outlined for the Populus samples studied in "Precursor" studies, experiment 1. The Salix petiolaris sample used was the June sample.

ii) The bark (100 mg.) of three Salix species (S. petiolaris (June sample), S. purpurea gracilis, S. purpurea nana) was weighed into a piece of filter paper. The samples were extracted by Soxhlet extraction with benzene for 24 hours.

The extracts were filtered into a tared flask and vacuum evaporated to dryness at room temperature. The residue weight was obtained and one-tenth of the residue was accurately removed into a vial for GLC analysis.

Hot aqueous ethanol (60-70%) was added to the remaining residue and the solution was shaken. Excess of strong lead subacetate solution was added and the flask was shaken for a few minutes. The solution was suction filtered with the aid of celite with subsequent washing of the filter pad. Hydrogen sulfide was passed through the filtrate until no further precipitation occurred. The solution was suction filtered with the aid of celite, with washing of the filter pad. The filtrate was vacuum evaporated to dryness on a 40-50° C. water bath and the weight of the residue was determined. A portion of this residue, equal in weight to the weight of benzene residue previously removed, was removed for GLC analysis.

The samples were analyzed as outlined in the GLC Analysis procedures, using OV-17 column and temperature program 2.

iii) May _Salix_ _petiolaris_ bark (1.41 Kg.) was extracted with benzene for 18 hours by Soxhlet. After extraction, the benzene was removed by vacuum evaporation at room temperature.

Two samples of the benzene residue (2 mg.) were accurately removed. One sample was placed in a vial for GLC analysis. The second sample was shaken with boiling aqueous ethanol (60-70%) and the solution was also boiled for a few minutes. Alcoholic-aqueous lead subacetate solution (4-5 ml.) (see Appendix C) was added to the solution and the solution was again boiled for several minutes. The solution was suction filtered with the aid of celite with subsequent washing of the filter pad. Hydrogen sulfide was passed through the filtrate until no further

precipitation occurred. The solution was suction filtered with the aid of celite, with washing of the filter pad. The filtrate was vacuum evaporated to dryness on a 40-50° C. water bath. The entire residue was taken for GLC analysis.

Both samples were analyzed as outlined in GLC Analysis procedures, using OV-17 column and temperature program 2.

iv) November and March Salix petiolaris bark (100 mg.) were extracted with benzene for 24 hours by Soxhlet extraction.

The benzene was removed by vacuum evaporation at room temperature and the weight of the residue was obtained. One-tenth of these residues were accurately removed into vials for GLC analysis.

A few milliliters (5-6 ml.) of hot ethanol (95%) were added to the remaining residue and the solution was shaken. Hot water (2-3 ml.) was added and the solution was boiled for a few minutes. Excess of strong lead subacetate solution was added and the solution was processed exactly as in previous experiments. The final residue was weighed and a portion of the residue, equal to the weight of benzene residue removed for GLC analysis, was removed for analysis.

All samples were analyzed as outlined in GLC Analysis procedures, using OV-17 column and temperature program 2.

v) A sample of November Salix petiolaris bark was thoroughly dried in a vacuum oven at 40° C. for four days.

Samples (100 mg.) of dried and undried November Salix petiolaris bark were then extracted with benzene for 24 hours by Soxhlet extraction. The benzene was removed by vacuum evaporation at room temperature and the residue was weighed. One-tenth of the residues were accurately removed into vials for GLC analysis.

The remaining residues were treated with 7-8 ml. of boiling

aqueous ethanol (60-70%). This solution was boiled for several minutes.
Alcoholic-aqueous lead subacetate solution (4-5 ml.) was added and the
solution was processed in the usual way. The final residues obtained
were not suitable for scraping and so the total residue (representing
nine-tenths of the original benzene residue) was analyzed.

Analysis was performed as outlined in GLC Analysis procedures,
using OV-17 column and temperature program 2.

III. RESULTS AND DISCUSSION

Collection

Monthly samples*were collected except for the month of February when weather conditions prevented access to the area. The samples were thoroughly dried in an oven at 50-60° C. to remove moisture and stored in air-tight bags to prevent any alteration of the glycoside content. The bark, wood and leaf samples were reduced to a moderately fine powder to facilitate extraction.

Extraction methods

1) Extraction of Salix petiolaris with various solvents

The effectiveness of ethanol (95%), methanol, acetone, ethyl acetate and water as extraction solvents for Salix petiolaris was determined. Thin-layer chromatographic results of these experiments are found in Table 6.

Each solvent produced a different glycoside pattern. This may be due to the extent of extraction performed by the solvent and/or decomposition of certain glycosides by the solvent.

The samples were refluxed since it has been found that refluxing for two hours is adequate for complete extraction of glycosides from the bark and also appears to be less destructive on the glycosides present (132). Lead subacetate was tried for purification of the extracts but this step has been found to be destructive and alters the glycoside pattern from that which occurs per se in the plant (132). This step should therefore be omitted from any future analysis of Salix samples and an alternative non-destructive purification process utilized.

Water and ethyl acetate appeared to be poor extraction solvents since, by analysis, the TLC spots were very faint and the gas chromatographic peaks obtained were of small area. Water has been found to cause decomposition of certain phenolic glycosides and the production of other

* Samples were collected from numerous trees in the area previously described.

TABLE 6

*TLC RESULTS OF SALIX PETIOLARIS EXTRACTION WITH VARIOUS SOLVENTS

(R_f values and color reactions)

ETHANOL	METHANOL	WATER	ETHYL ACETATE	ACETONE
0.16 red	0.16 red	0.18 red	0.17 red	0.18 red
-	-	-	0.22 yellow	0.22 yellow
0.30 yellow	0.30 yellow	-	0.31 brown	0.31 brown
0.40 orange	0.41 orange	0.41 yellow	0.42 orange	0.41 orange
0.47 red	-	-	-	0.44 red
0.60 yellow	0.58 yellow	0.63 yellow	0.62 yellow	0.62 yellow
0.65 blue	-	0.66 blue	0.65 blue	0.66 blue

* Silica Gel G, 250 μ run in Ethyl acetate-methanol, 9:1 v/v.

*REFERENCE GLYCOSIDES

(R_f values and color reactions)

Salicin	0.18 red	Tremuloidin	0.41 red-orange
Picein	0.72 blue	Salireposide	0.31 yellow

glycosides when refluxed with pure glycosides (132). This confirms previous findings that ethyl acetate is a poor extraction solvent since it requires an extended period of time for complete extraction of the bark (132).

Methanol and ethanol (95%) had a greater extractive effect on the bark although they produced fewer spots on thin-layer chromatograms and fewer peaks by gas chromatographic analysis. However, the colors of the spots produced by TLC analysis were quite bright and the peaks found by GLC analysis were of relatively large peak area. On the

other hand, ethanol and methanol have been reported to cause some decomposition of certain glycosides (132). Therefore, these solvents would not be entirely satisfactory for glycoside extraction of the bark.

The acetone extract on TLC plates produced numerous spots which appeared to be fairly deep in color. GLC analysis also produced numerous peaks, several of which were present in quite considerable concentration. Acetone has also been found not to cause decomposition of certain phenolic glycosides when it was refluxed with pure glycosides (132). It appears therefore that acetone is the solvent of choice for extraction of phenolic glycosides from Salix and that extraction by refluxing for two hours would be the most useful process, from the results of this experiment and other studies (130,132).

The usefulness of pyridine as an extraction solvent for Salix phenolic glycosides was also investigated. Thin-layer chromatographic results are shown in Table 7. Only three spots were identified by TLC analysis. This result may be due to the short time allowed for extraction or may be due to poor extractive properties of pyridine for phenolic glycosides. Pyridine, however, has been reported to cause a substantial amount of decomposition (132). Pyridine, therefore, is not recommended as an extraction solvent for Salix glycosides.

The effect of water contamination of pyridine was studied pertaining to its effect on phenolic glycoside extraction and thin-layer chromatographic results are shown in Table 7.

The results from this experiment were identical with the results obtained from pyridine extraction of the bark and the presence of water did not therefore appear to alter the glycosidic pattern which is obtained by extraction with pyridine alone.

2) Effect of refluxing on glycosides

TABLE 7

*TLC RESULTS OF PYRIDINE EXTRACTIONS ON SALIX PETIOLARIS

(R_f values and color reactions)

PYRIDINE	PYRIDINE AND WATER	REFERENCE GLYCOSIDES
0.15 yellow	0.16 yellow	0.16 red (salicin)
0.20 red	0.22 red	-
0.28 yellow	0.29 yellow	0.31 yellow (salireposide)
		0.38 pink (tremuloidin)

* Silica Gel G, 250 μ run in Ethyl acetate-methanol, 9:1 v/v.

The effect of refluxing pure glycosides in water was investigated. Thin-layer chromatographic results are shown in Table 8.

TABLE 8

**TLC RESULTS OF THE EFFECTS OF REFLUXING GLYCOSIDES IN WATER

(R_f values and color reactions)

SALIREPOSIDE RESIDUE	TREMULOIDIN RESIDUE	REFERENCE GLYCOSIDES
-	0.18 red	0.17 red (salicin)
0.34 yellow	-	0.36 yellow (salireposide)
-	0.52 red	0.52 red (tremuloidin)
	0.75 red	

** Silica Gel G, 250 μ run in Ethyl acetate-xylene-formic acid-water, 35:1:2:2 v/v.

Results indicated that salireposide was not altered by refluxing with water. TLC results of the salireposide residue indicated only one spot which corresponded to salireposide by R_f value and color reaction. GLC analysis also indicated one peak which corresponded with salireposide.

Tremuloidin, however, was substantially decomposed by refluxing with water. Three spots appeared from TLC analysis; two corresponded to salicin and tremuloidin and the third was suspected to be populin from

the high R_f value and the red color reaction. Other TLC analyses of populin have indicated a high R_f value and red color reaction with 4% sulfuric acid in absolute ethanol (2,126). GLC analysis also indicated three peaks which corresponded with salicin, populin and tremuloidin. The tremuloidin peak possessed a broad shoulder indicating further decomposition of the phenolic glycoside.

Steele et al. (132) have discovered similar results when pure glycosides were refluxed with ethanol, methanol, water and pyridine and Wong has reported that heating of tremuloidin, fragilin and salicortin alcoholic solutions at 60-64° C. for 24 hours produced decomposition (2).

Therefore, extraction of Salix bark with boiling water, at any step, would tend to alter the glycoside content of the extract and give misleading results. It is distinctly possible that the large yield of salicin obtained by workers from previous Salix studies (5,15) was due to the degradative effects of boiling solvents.

3) Purification of bark extracts

A method was sought for the purification of bark extracts to replace the lead subacetate-hydrogen sulfide procedure. The acetone extraction was satisfactory for Salix samples but a step was required in the extraction process which would eliminate any waxy, fatty or high molecular weight material from the final solution. Passage of the extract through polyamide and cellulose columns was investigated for this purpose since Thieme has utilized this method for the purification of his Salix extracts.

The eluates from the polyamide columns produced several peaks when analyzed by gas chromatography. Eight peaks were found when both eluates from the polyamide column were analyzed. The areas of the peaks obtained by elution of the column with 25 ml. of water were greater

indicating that the larger volume of water was more effective for eluting the bark extract components.

The cellulose columns were found unsatisfactory since the eluate from either column did not produce any major peaks when analyzed but only trace peaks. It appears that the cellulose column adsorbs the extract components to a greater degree and, therefore, requires further elution.

In the case of the polyamide column, the acetone extract was not taken to dryness but was applied to the column as a concentrated acetone extract. It was therefore possible that some of the fats, waxes or other materials present in the extract would be washed through the column with the large water elution if the column was not prepared properly.

This experiment was repeated utilizing polyamide columns only and the acetone extract was completely dried. The residue was extracted with water since it was thought the water would dissolve any phenolic glycosides present and leave fats, waxes and high molecular weight material in the flask. This was, in fact, achieved since after water extraction, the flask contained a residue which tended to bead water indicating a waxy or fatty nature. The column was eluted with 25 ml. of water to ensure complete removal of the extract from the column. At no time was the water extract heated (since it has already been proven that hot water causes a degradative effect on phenolic glycosides). The gas chromatographic analysis of the eluate residue produced a very good chromatogram, with several large peaks which corresponded to known glycosides. It was significant that the chromatogram obtained from this second experiment differed greatly from the chromatogram obtained from the previous experiment in the number and total area of peaks obtained.

The chromatogram obtained from this experiment possessed a greater number of peaks which had greater area than found in the previous experiment where the acetone extract was not completely dried.

However, since water was used as a purification step to prevent unwanted material from being present in the final solution, it was concluded that passage of this water extract through a polyamide column was probably unnecessary since the water step itself acts as a purification process. Since passage of the extract through the column required a considerable length of time, this extended period of contact of the extract with water could lead to some decomposition. Therefore, for purification of _Salix_ extracts, a water extraction step is recommended without the subsequent use of elution through a polyamide column.

4) Effect of water extraction on Salix petiolaris extracts

The effect of the water extraction (purification) step on the glycoside content of _Salix_ _petiolaris_ bark was investigated. Both acetone and acetone/water extracts contained identical components since the gas chromatographic traces were identical in respect to the number of peaks. The peaks found in the analysis of the water-extracted residue were a little higher than the corresponding peaks found in the analysis of the residue not utilizing water extraction. Since the salicin peak did not increase substantially, it was thought that any effect was not a degradative one since the salicin peak would otherwise be greatly increased. It is more likely that the increased height was due to a minor variation in injection technique, since _all_ the peaks were higher. Vacuum evaporation of water (5-7 ml.) on a 40-50° C. water bath requires 10-15 minutes and it was thought that this short exposure to heat would have very little effect on the glycosides present.

No significant difference in the glycoside content was therefore

noticeable when the water extraction step was utilized in the processing of *Salix* *petiolaris* bark and it seemed possible to omit this step in analysis of bark samples. However, the water extraction procedure proved to be essential in the processing of leaf and wood samples and since it did not greatly affect bark content, it was decided to retain the water extraction step for bark extraction as well, to keep the extraction procedure uniform for all *Salix* *petiolaris* monthly samples.

Utilizing 50 mg. of sample, the nature of the residue obtained from water extraction of the acetone extraction residue of *Salix* *petiolaris* bark was investigated. This experiment was performed under the same conditions as the previous experiment to confirm results. It was hoped to obtain a residue which was suitable for scraping since two identical samples would then be obtainable from one extraction. These two samples could then be analyzed on different GLC columns. This procedure eliminated the need for a second extraction of that month's sample and, also, the extraction procedure and conditions would be uniform for the two samples; thus, no error due to a difference in extraction technique would enter the results obtained.

After thorough drying of the acetone/water-extracted residue, the product obtained was a hard, glassy substance which was easily powdered by scraping. With the extraction of 50 mg. of bark sample, it was possible to obtain an adequate residue which could easily be divided into two equal portions.

With regard to the effect of water extraction on the glycoside content of the residue, the results obtained from this experiment were identical with the results obtained from the previous experiment.

The effect of water extraction on the glycoside content of *Salix* *petiolaris* leaf and wood samples was also investigated. It was

thought that the water purification procedure would be necessary for the
wood and especially the leaf sample since they contain numerous other
components. Leaves, in fact, have a high content of fats, waxes and
pigments.

In another study performed in the School of Pharmacy, University
of Manitoba, a sample of Salix leaf was extracted with ether and when
analyzed by GLC indicated several peaks. When the ether residue was
extracted with water and the water extracted residue analyzed by GLC,
no peaks were seen indicating that the water step acted as a purification
process by not dissolving the ether soluble components found in leaves.

Gas chromatographic analysis of the acetone extract of Salix
petiolaris leaf indicated several peaks with two large peaks after
approximately 20 minutes. After incorporation of the water purification
step, the number of peaks found by GLC analysis had decreased by two
and one of the large peaks at 20 minutes had decreased in concentration
by approximately three-quarters its content found in the acetone residue.
It was possible that the water extraction removed a component which was
masking this other component.

Analysis of the acetone extract of the wood sample indicated
several glycosides in low concentration. After water extraction the gas
chromatographic trace showed the number of peaks had decreased and that
the heights of the remaining peaks were smaller than those produced from
the acetone extract directly. With wood analysis, the water extraction
procedure appears to eliminate certain non-glycosidic components from
the final solution. The water extraction (purification) step is there-
fore necessary for the analysis of leaf and wood samples of Salix
petiolaris.

The dry residue remaining after processing 50 mg. of leaf

sample was adequate for division into two samples but the residue remaining after processing 50 mg. of wood sample was not sufficient for this division. A minimum of 100 mg. of wood sample was therefore utilized for extraction when a solid residue was required.

It was also found that after vacuum evaporation of the water, it was advisable to store the residues in a vacuum desiccator over-night to facilitate thorough drying.

5) Extraction of monthly samples of Salix petiolaris

This experiment was performed to obtain the weight of glycoside components of the monthly samples of Salix petiolaris. The extraction method utilized was developed from the results of extraction studies performed in this thesis and from the results of the extraction studies conducted by Steele et al (130,132).

Weights of the acetone/water-extracted residues of Salix petiolaris monthly samples are presented in Table 9 and Figure 2.

The residue from the bark samples was slightly brown in color; the leaf residues were slightly green; the wood residues were colorless. This indicated that some coloring material was carried over in the extraction of the bark and leaf samples but this did not appear to affect the final gas chromatographic analysis of the residues.

By using one-fifth of the bark and leaf residue and one-tenth of the wood residue and injecting one-tenth of the trimethylsilylation reaction solution, gas chromatographic analysis was effectively performed on 1 mg. of plant material. These conditions were used for all samples and results obtained were therefore comparable for all gas chromatographic analyses.

The extracts from the bark samples had the highest content in the months of June (9.0 mg.) and November (9.4 mg.) and the lowest content

TABLE 9

WEIGHT OF EXTRACTS FROM SALIX PETIOLARIS MONTHLY SAMPLES *

(wt. in mg.)

	BARK	WOOD	LEAF	FLOWER	SEED
January	7.1	2.1			
March	5.7	1.4			
April	8.5	1.8			
May	8.3	0.8	1.9	0.9	0.2
June	9.0	0.9	3.5	0.1	
July	7.5	0.9	3.5		
August	6.6	0.7	3.1		
September	7.7	1.6	4.5		
October	7.8	2.0	3.8		
November	9.4	1.8			
December	7.6	1.7			

* All residue weights are based on 50 mg. of sample material.

in the months of March (5.7 mg.) and August (6.6 mg.). The general

pattern of extract weight in the bark was as follows:

- starting off at moderate levels in January, falling to

a low level in March, rising from April to June, falling off in July to

an August low and then rising through September and October to a

November high and then a fall off in content in December.

For the wood (total wood) extracts, the highest content

was found in January (2.1 mg.) and October (2.0 mg.). Lowest content

was found in May (0.8 mg.), June (0.9 mg.), July (0.9 mg.) and August

(0.7 mg.). The extract weights were all less than those obtained from

the bark extract from the corresponding month. This was expected since

FIGURE 2.   SEASONAL VARIATION IN TOTAL EXTRACT WEIGHTS

Bark extract

Leaf extract

Wood extract

Weight in mg.

10.0

8.0

6.0

4.0

2.0

0

JAN.   MARCH   APRIL   MAY.   JUNE   JULY   AUG.   SEPT.   OCT.   NOV.   DEC.
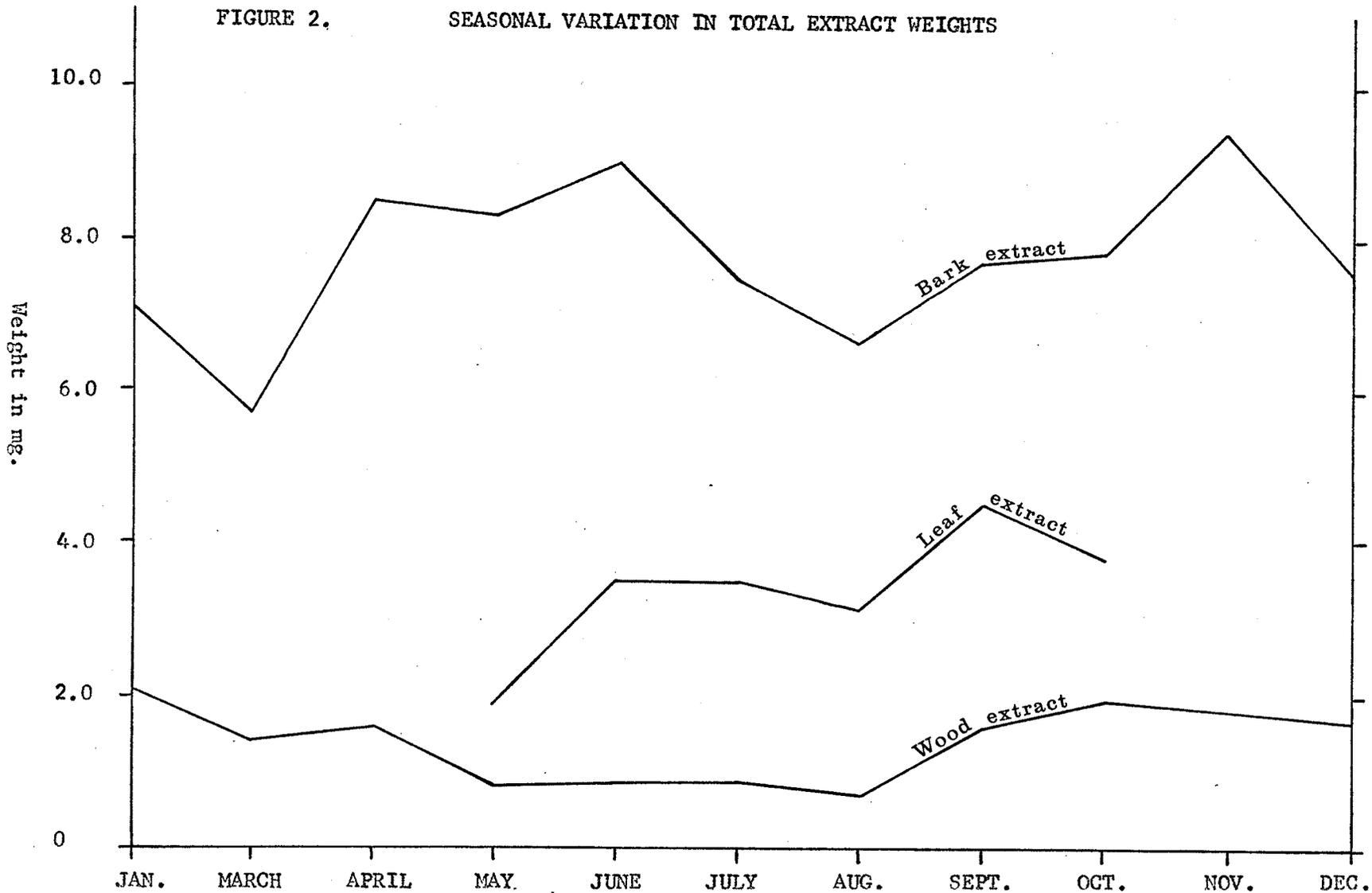
the major glycoside content is normally found in the bark. The general

pattern of extract weight in the wood was as follows:

- starting off at a high level in January, falling in March

and rising in April. A low content was found from May to August, rising

in content in September to a high level in October and a slight decrease

to the end of the year.

Leaf residues started at a low content in May (1.9 mg.) and

built up during the year until the leaves fell in November. Highest

extract weight was found in September (4.5 mg.)

Analysis procedures

1) Thin-layer chromatography

The method of preparation for thin-layer plates was developed

from previous thin-layer chromatographic studies of phenolic glycosides

(2,126). The spray reagent used was 4% sulfuric acid in absolute ethanol

since this solution gave the best results for detection. Thin-layer

chromatography was used alone or with gas chromatographic analysis in

several experiments.

2) Gas chromatographic analysis

Columns, temperature programs, procedures and conditions

utilized for gas chromatographic analysis were developed by Bolan and

Steele (74). Peak retention times were calculated as a ratio to tri-

methylsilylarbutin, which was injected after every 3-4 extract injections.

This was done in order to overcome day to day variations in the gas flow

and to allow comparison and identification of similar peaks throughout

the year. Peak areas were determined by triangulation. Typical gas

chromatographic traces of Salix petiolaris bark, leaf and wood extracts

are presented in Figures 3-8. The peak for trimethylsilylarbutin has

been superimposed, on all traces, in dotted lines.

FIGURE 3   GLC CHROMATOGRAM OF JULY S. petiolaris BARK ON OV-17 COLUMN
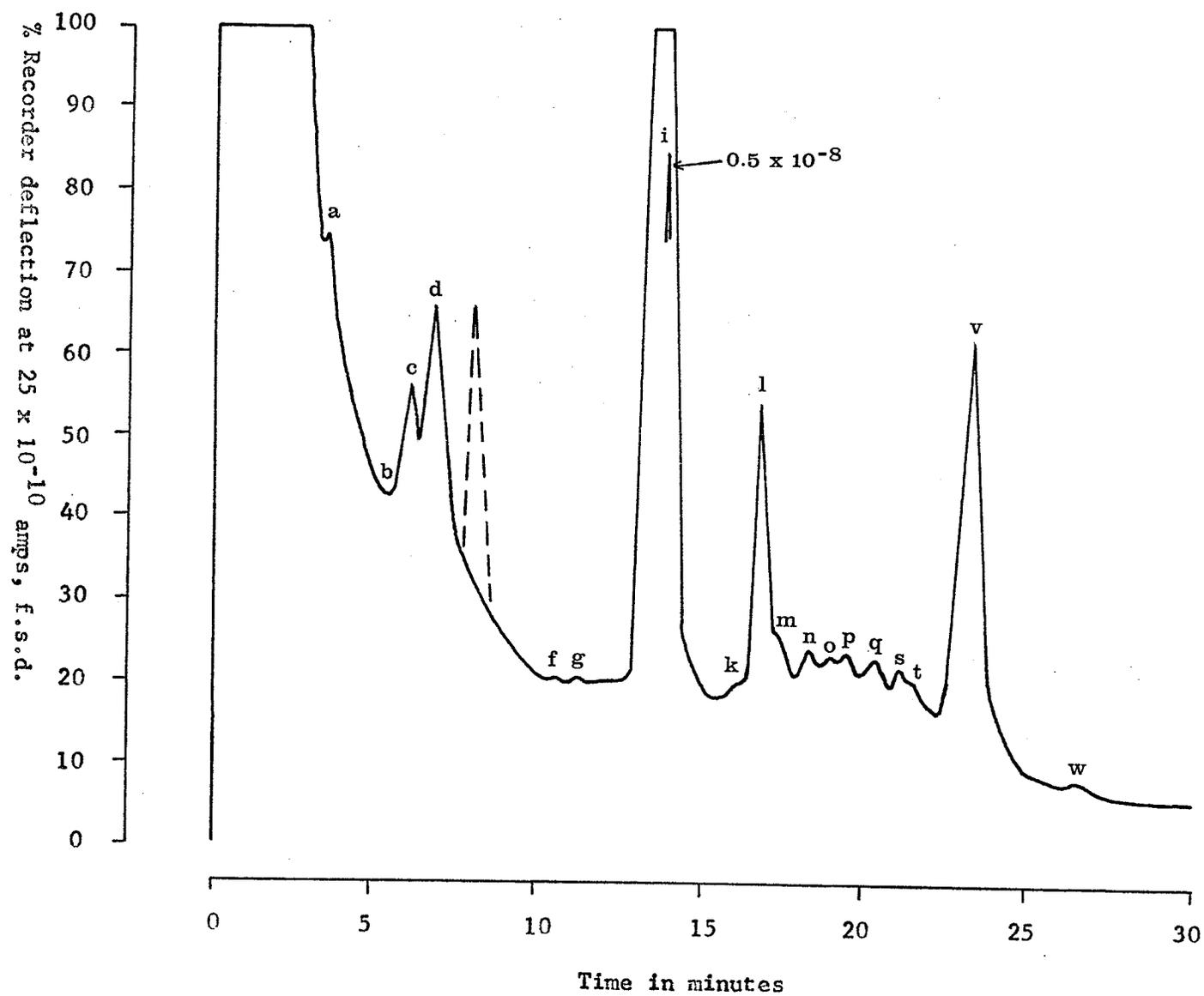
FIGURE 4   GLC CHROMATOGRAM OF JULY S. petiolaris BARK ON OV-1 COLUMN
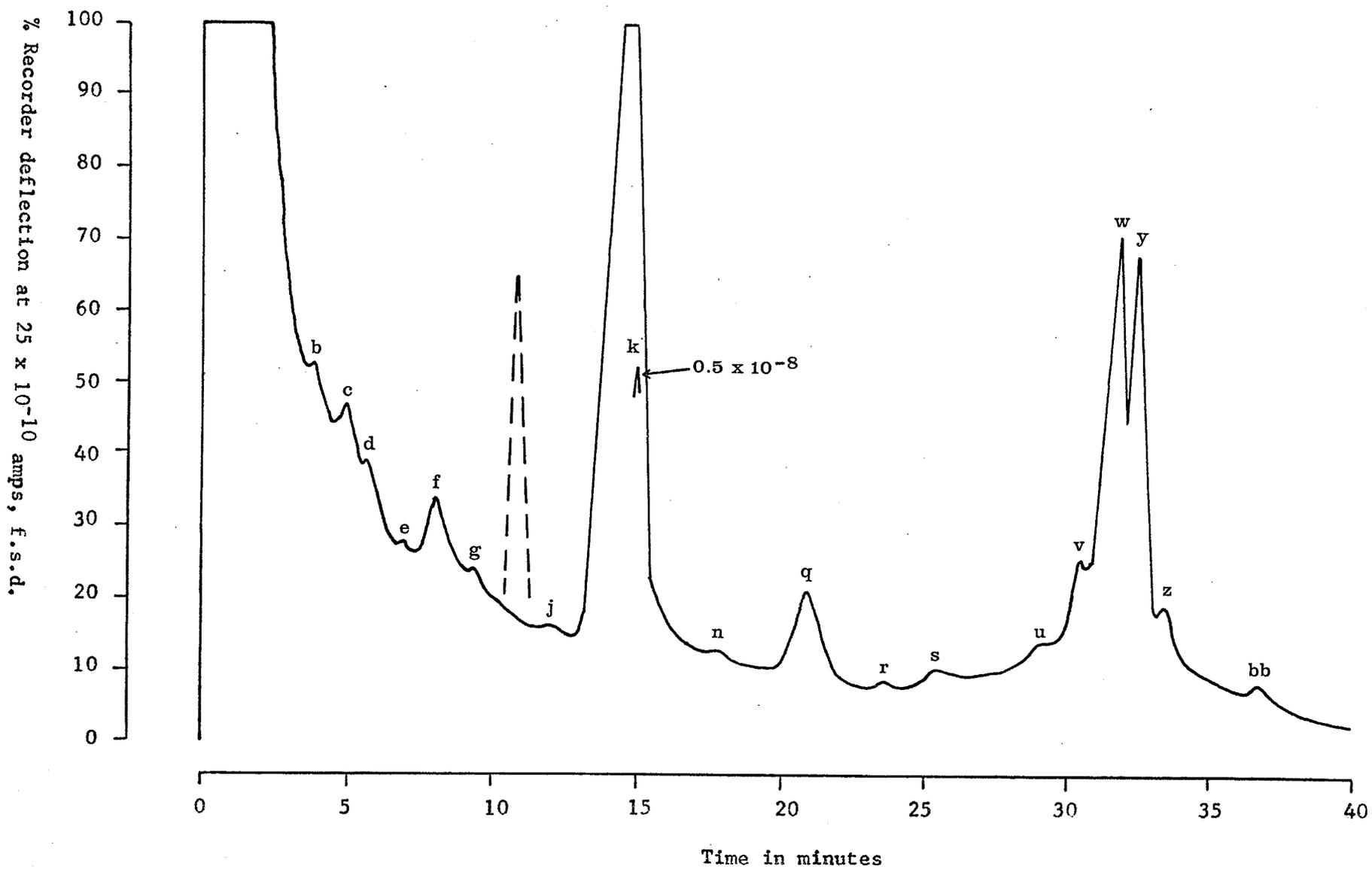
FIGURE 5   GLC CHROMATOGRAM OF SEPTEMBER S. petiolaris LEAF ON OV-17 COLUMN
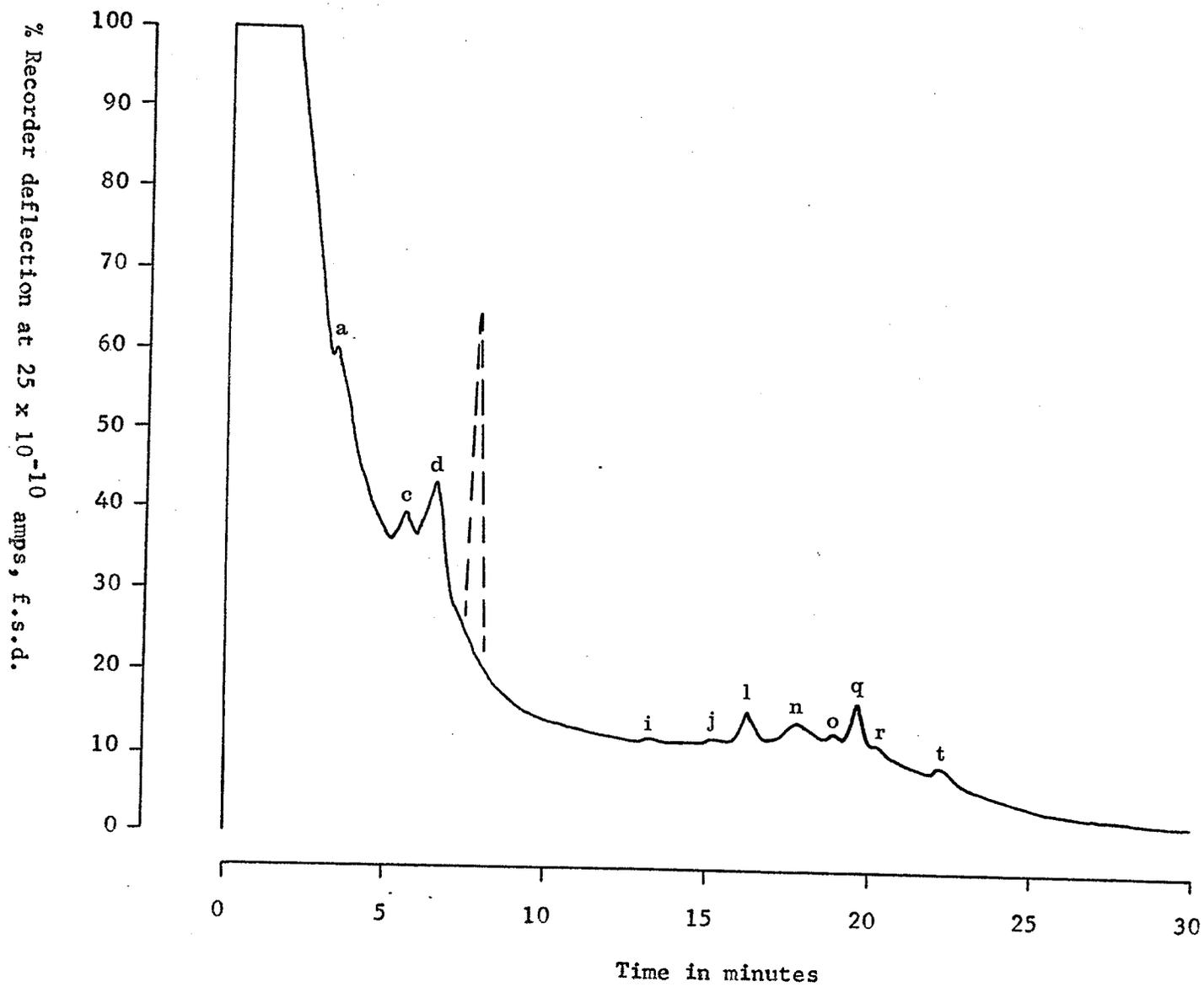
FIGURE 6   GLC CHROMATOGRAM OF SEPTEMBER S. petiolaris LEAF ON OV-1 COLUMN
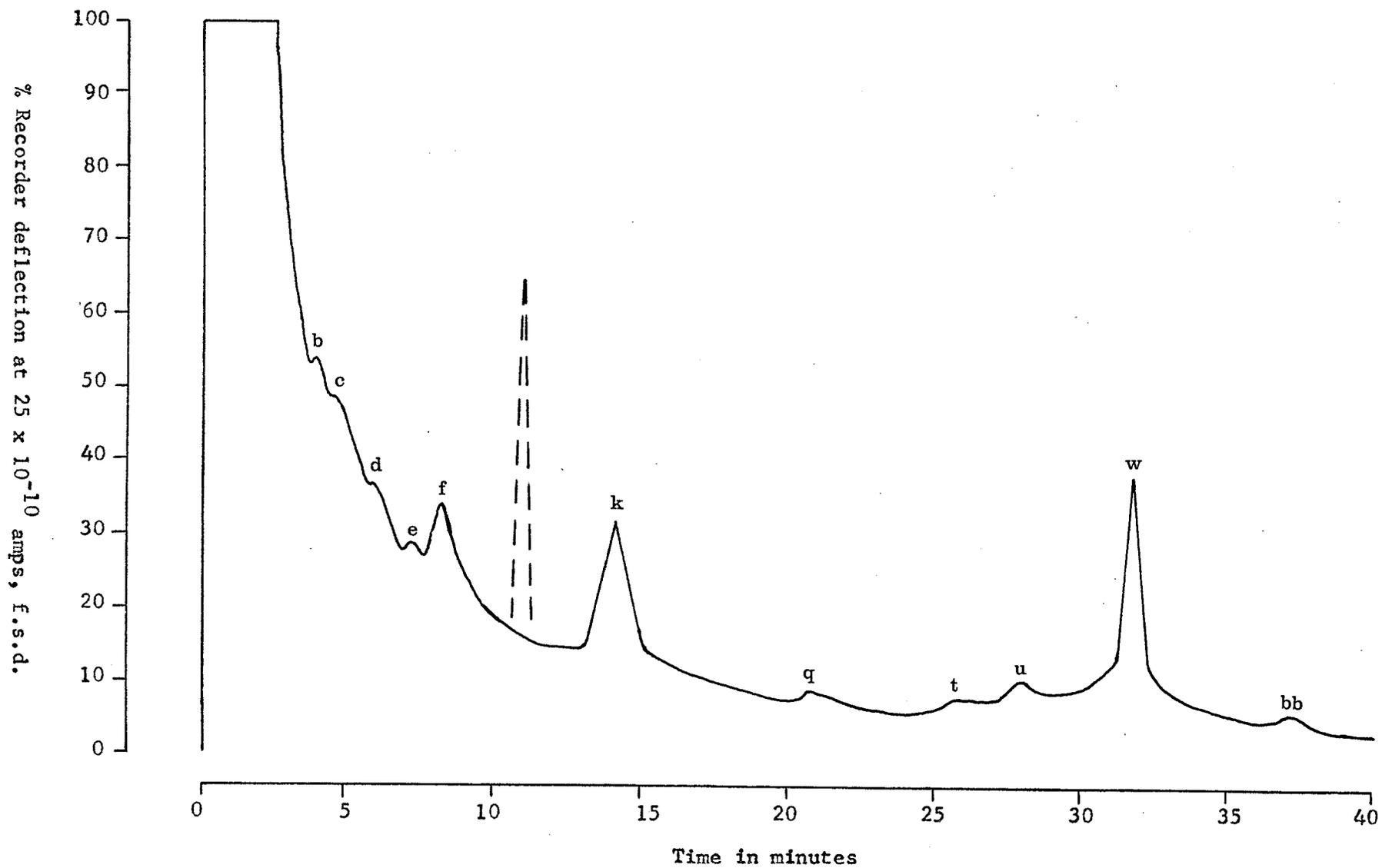
FIGURE 7   GLC CHROMATOGRAM OF JANUARY S. petiolaris WOOD ON OV-17 COLUMN
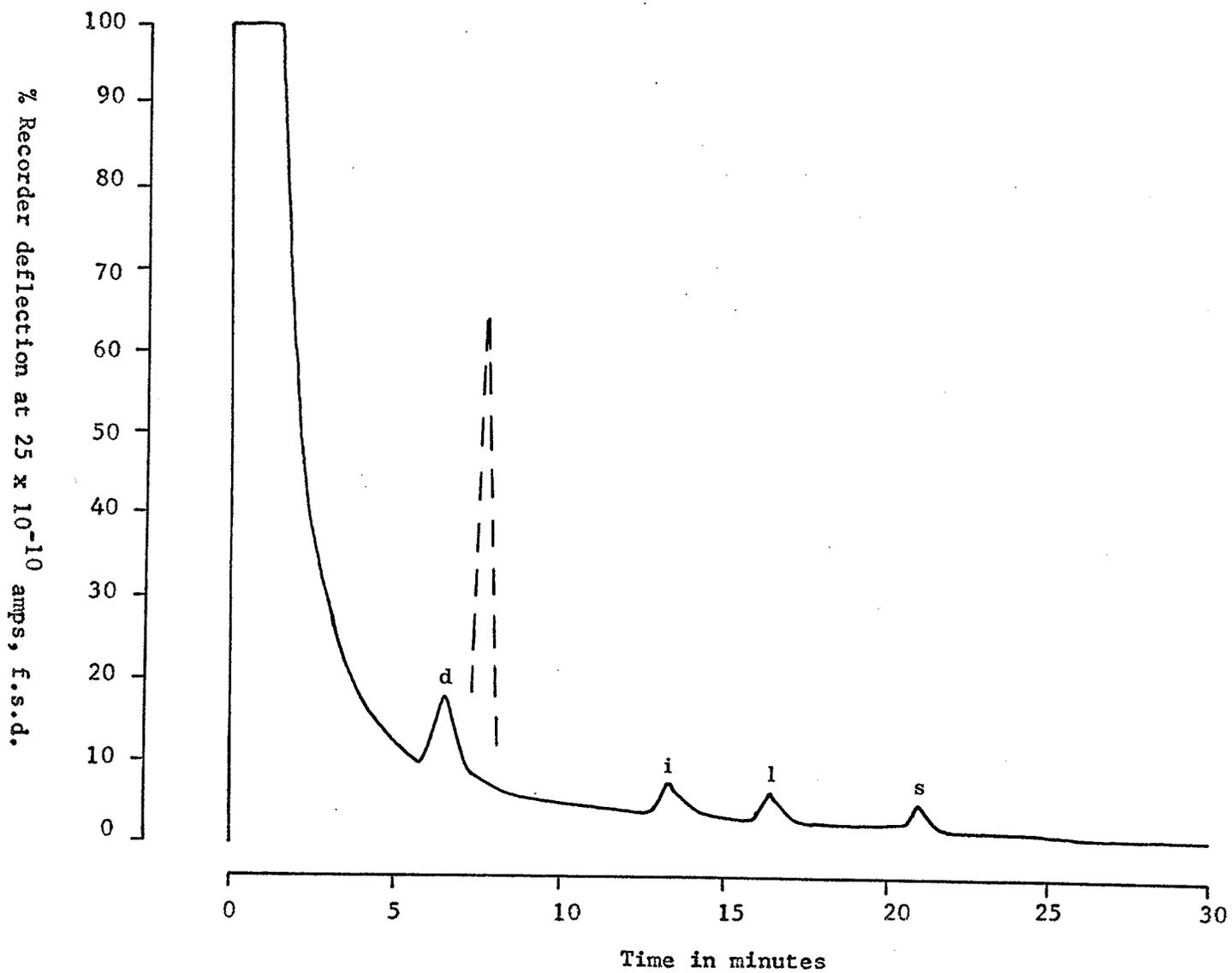
FIGURE 8 GLC CHROMATOGRAM OF JANUARY S. petiolaris WOOD ON OV-1 COLUMN
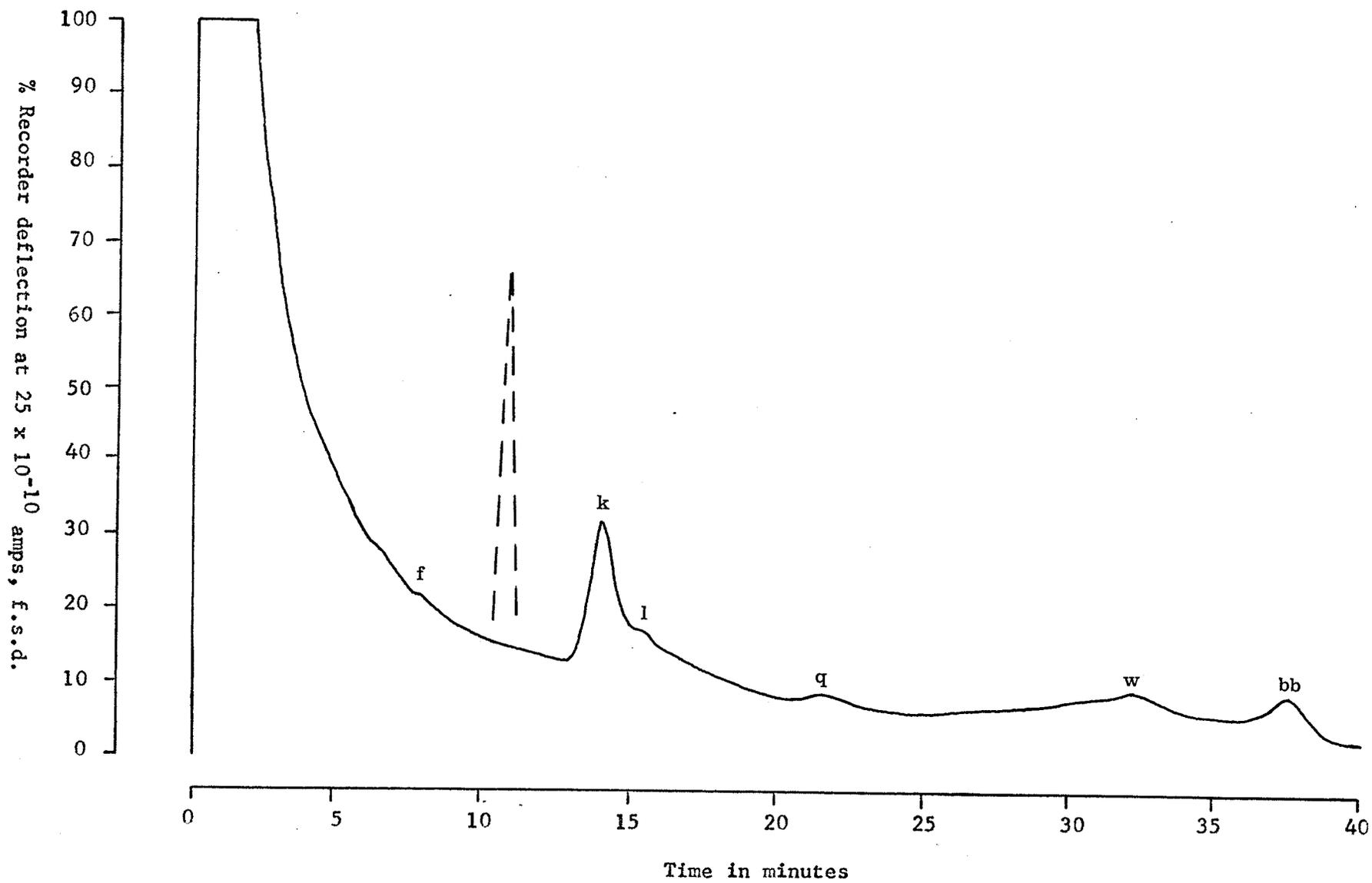
The relative arbutin times for all components found by analysis from any sample on OV-1 column are presented in Table 10 and the relative arbutin times for the components found by analysis on the OV-17 column are presented in Table 11.

The lettered component peaks (a, b, c, etc.) from the OV-1 column are not identical to the similarly lettered component peaks from the OV-17 column since it was virtually impossible to relate the peaks without possessing pure samples of all components. Therefore, when interpreting the peak area tables (Tables 12 and 13) of components from either column, this fact should be kept in mind. That is, peak a from OV-1 and peak a from OV-17 columns may well be different compounds.

The OV-1 column results indicate the presence of five additional peaks compared with results from the OV-17 column. However, four of these peaks have very low relative arbutin times and are almost certainly non-glycosidic in nature. All peaks of low relative arbutin times (0.26-0.68) were considered to be non-glycosidic in nature.

With the OV-1 column analyses, the relative arbutin time for picein is given in two ranges. One range (1.25-1.29), for leaf and wood samples, was obtained when the concentration of picein was small. The other range, for bark samples (1.32-1.43), was obtained when the concentration of picein was very high, causing typical over-loading effects on the column.

Tables 10 and 11 also contain a list of reference glycosides and their relative arbutin times. These were matched with relative arbutin times for peaks found in OV-1 and OV-17 analysis. Salicin, picein, salireposide, tremuloidin, triandrin and fragilin were matched with peaks in both tables.

The close relationship between the OV-1 results and the OV-17

TABLE 10

RELATIVE ARBUTIN TIMES FOR COMPONENTS FROM OV-1 COLUMN

PEAK	RELATIVE ARBUTIN TIME	REFERENCE GLYCOSIDE	RELATIVE ARBUTIN TIME
a	0.26		
b	0.37-0.38		
c	0.42-0.47		
d	0.53-0.58		
e	0.64-0.68		
f	0.74-0.78	Salicin	0.76
g	0.86-0.89		
h	0.97-0.99		
i	1.02-1.08		
j	1.07-1.13	Fragilin	1.14
k	1.25-1.29; 1.32-1.43	Picein	1.31
l	1.39		
m	1.56-1.59		
n	1.64		
o	1.70		
p	1.78-1.79		
q	1.90-1.97		
r	2.15-2.19		
s	2.33-2.38	Triandrin	2.38
t	2.39-2.42		
u	2.59-2.69	Tremuloidin	2.67
v	2.77-2.81		
w	2.89-2.94		
x	2.92		
y	2.94-2.99	Salireposide	2.93
z	3.03-3.09		
aa	3.16-3.18		
bb	3.34-3.40		

TABLE 11

RELATIVE ARBUTIN TIMES FOR COMPONENTS FROM OV-17 COLUMN

PEAK	RELATIVE ARBUTIN TIME	REFERENCE GLYCOSIDE	RELATIVE ARBUTIN TIME
a	0.48		
b	0.72		
c	0.73-0.79	Salicin	0.79
d	0.81-0.89		
e	0.95-0.97		
f	1.29-1.34		
g	1.46-1.52	Fragilin	1.48
h	1.56-1.60		
i	1.69-1.79	Picein	1.78
j	1.93-1.97		
k	1.99-2.07		
l	2.08-2.18		
m	2.15-2.23	Triandrin	2.23
n	2.28-2.36		
o	2.35-2.46		
p	2.40-2.49		
q	2.51-2.58		
r	2.54-2.62	Tremuloidin	2.59
s	2.61-2.74		
t	2.68-2.78		
u	2.75		
v	2.88-2.99	Salireposide	2.97
w	3.29-3.38		

results can be seen by examining the occurrence of the _identified_ phenolic glycosides in the _Salix_ samples. In some cases the results from the two columns were virtually identical while in other cases there was only a slight variation. In OV-1 results, salicin showed a high level in the leaf, moderate level in the bark and a low level in the wood sample. This was identical with OV-17 results. Salireposide indicated a high level of content in the bark with an absence of this glycoside in wood and leaf samples by both OV-1 and OV-17 analysis. OV-1 analysis indicated fragilin was present in a low level in bark samples and at very low levels in wood and leaf samples. This was again identical with OV-17 results. Picein showed a high content in bark samples, moderate in wood and a moderate to low level in leaf samples by OV-1 analysis. OV-17 analysis indicated a high picein concentration in bark samples, moderate levels in wood and very low levels in leaf samples. Triandrin showed a moderate level in bark samples while being absent in wood and leaf samples by OV-1 analysis. OV-17 results indicated a low level of triandrin in bark and wood samples and none in leaf samples. OV-1 results indicated a high tremuloidin content in leaf samples, low content in bark and an absence in wood samples. OV-17 results indicated a high tremuloidin level in leaf samples with only traces in wood and bark samples.

Table 12 presents the peak areas of components found in bark, wood and leaf samples by OV-1 analysis and Table 13 presents the peak areas of components found in the same samples by OV-17 analysis. From these tables, the occurrence and relative level of components in bark, wood and leaf samples can be followed throughout the year.

When analyzed by OV-1, several components (peaks a, g, h, i, j, l, m, n, o, p, t, x, aa) were present only in very minor concentration in the bark and/or wood and/or leaf sample. Some components were found

o	p	q	r	s	t	u	v	w	x	y	z	aa	bb
-	-	2.420	0.059	0.211	-	s.	1.885	4.498	-	4.040	1.230	s.	0.952
-	-	2.120	sp.	sp.	-	sp.	1.343	2.671	-	2.834	0.748	s.	0.553
-	-	3.197	0.084	0.227	-	-	5.424	4.541	-	3.734	1.284	sp.	0.773
-	sp.	2.307	0.130	0.247	-	-	4.205	3.623	-	3.441	1.286	-	0.208
-	sp.	2.835	0.045	0.400	-	sp.	6.297	6.840	-	5.722	1.450	-	0.200
-	-	1.722	sp.	0.297	-	sp.	2.487	5.458	-	5.729	1.494	-	0.170
-	-	1.355	sp.	0.181	-	sp.	2.220	4.940	-	4.272	0.618	sp.	0.151
-	sp.	2.716	sp.	0.281	-	sp.	2.919	6.048	-	5.175	1.166	-	0.163
-	-	2.580	sp.	0.296	sp.	sp.	4.695	8.595	-	5.414	1.973	sp.	0.576
-	-	2.652	0.070	-	0.124	-	4.457	4.563	-	3.925	1.324	sp.	0.982
-	-	2.268	0.115	0.205	-	sp.	2.574	2.894	-	3.067	0.748	sp.	0.720
-	-	sp.	-	-	-	-	-	sp.	-	-	-	-	0.399
-	-	0.119	-	-	-	-	-	sp.	-	-	-	-	0.616
-	-	0.759	-	-	-	-	-	sp.	sp.	-	sp.	-	0.468
-	-	sp.	-	-	-	-	-	sp.	-	-	-	-	sp.
-	-	sp.	-	-	-	-	-	sp.	-	-	-	-	-
-	-	sp.	-	-	-	-	-	sp.	-	-	-	-	-
-	-	-	-	-	-	-	sp.	sp.	-	sp.	-	-	-
-	-	0.070	-	-	-	-	-	sp.	-	sp.	sp.	-	-
-	-	0.975	-	-	-	-	-	sp.	-	sp.	-	0.585	0.585
-	-	-	-	-	-	-	-	sp.	-	-	-	-	0.360
-	-	-	-	-	-	-	-	sp.	-	-	-	-	0.558
-	-	-	-	-	-	2.279	-	sp.	-	-	-	-	-
-	-	-	-	-	sp.	4.398	-	0.968	-	-	-	-	-
-	-	-	-	-	-	2.028	-	0.538	-	-	-	-	-
-	-	-	-	-	0.213	0.487	-	0.228	-	-	-	-	-
-	-	0.290	-	-	sp.	0.435	-	3.395	-	-	-	-	sp.
-	-	-	-	-	sp.	1.048	-	1.322	-	-	-	-	sp.

* s. - shoulder

sp. - small peak

TABLE 12

AREA OF ELUTED PEAKS IN CM^2 FROM OV-

BARK EXTRACTS

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
January	-	sp.	0.476	s.	0.154	0.670	s.	-	-	sp.	13.280	-	-	sp.
March	-	sp.	0.386	s.	sp.	0.561	s.	sp.	-	sp.	9.665	-	-	sp.
April	-	sp.	0.941	s.	0.227	0.841	s.	-	-	sp.	14.961	-	-	sp.
May	-	sp.	0.942	s.	0.086	0.734	s.	-	-	-	18.152	-	-	sp.
June	-	sp.	0.750	s.	0.210	1.237	s.	-	-	-	16.225	-	-	sp.
July	-	sp.	0.706	sp.	0.107	1.116	sp.	-	-	sp.	15.147	-	-	sp.
August	-	0.135	0.519	0.083	0.456	1.355	-	sp.	-	sp.	11.540	-	-	sp.
September	-	0.130	0.633	0.120	0.422	1.408	-	-	-	0.010	16.930	-	-	sp.
October	-	0.215	0.890	0.082	0.759	2.499	-	-	-	sp.	17.672	-	-	sp.
November	-	sp.	0.928	s.	0.187	0.967	-	-	-	-	20.611	-	-	sp.
December	-	0.010	0.579	s.	0.176	0.948	s.	sp.	-	sp.	16.192	-	sp.	sp.

WOOD EXTRACTS

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
January	-	-	-	-	-	sp.	-	-	-	-	2.743	s.	-	-
March	-	sp.	-	sp.	-	0.079	-	-	-	-	2.893	-	-	-
April	-	sp.	-	sp.	-	0.225	-	-	-	-	4.906	-	-	-
May	-	0.097	-	0.124	-	sp.	-	-	-	-	2.537	-	-	-
June	-	0.098	-	0.125	-	sp.	-	-	-	-	1.028	-	-	-
July	-	sp.	-	sp.	-	sp.	-	-	-	-	0.874	-	-	-
August	sp.	0.135	-	0.105	-	sp.	-	-	sp.	-	0.620	-	-	-
September	-	0.140	-	0.080	-	sp.	-	-	-	sp.	2.880	-	-	sp.
October	-	0.100	-	0.105	-	0.080	s.	-	sp.	sp.	4.230	-	sp.	-	s
November	-	-	-	-	-	sp.	-	-	-	-	3.480	-	-	-
December	-	-	-	-	-	sp.	-	-	-	-	3.686	-	-	-

LEAF EXTRACTS

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
May	-	0.271	-	0.203	-	5.801	-	-	-	sp.	sp.	-	-	-
June	-	0.086	-	0.129	sp.	8.086	-	0.193	-	-	-	-	-	-
July	-	sp.	-	sp.	-	2.863	-	-	-	sp.	sp.	-	-	-
August	-	0.101	-	0.076	0.121	3.576	-	0.137	-	-	0.914	-	-	-
September	-	0.145	sp.	sp.	0.239	1.632	-	-	-	-	3.721	-	-	-
October	-	0.121	0.684	sp.	sp.	2.287	-	-	-	-	0.456	-	-	-

TABLE 13

OF ELUTED PEAKS IN CM^2 FROM OV-17 COLUMN *

BARK EXTRACTS

j	k	l	m	n	o	p	q	r	s	t	u	v	w
-	-	2.679	-	0.107	sp.	sp.	sp.	-	s.	0.946	-	3.880	sp.
-	sp.	2.140	-	0.110	sp.	sp.	sp.	-	s.	0.480	-	2.091	sp.
-	sp.	3.683	-	0.198	s.	sp.	0.109	-	sp.	0.874	-	5.109	sp.
-	sp.	1.684	-	0.050	s.	sp.	sp.	sp.	s.	0.235	sp.	2.138	-
-	sp.	2.960	-	0.287	s.	0.112	0.110	-	0.160	0.180	-	5.460	-
-	s.	1.879	s.	0.290	0.126	0.179	0.168	-	0.168	s.	-	4.290	sp.
-	-	0.933	s.	0.334	0.266	sp.	sp.	-	0.342	sp.	-	1.154	sp.
-	sp.	2.231	-	0.312	0.077	sp.	sp.	-	0.167	0.253	-	3.011	-
-	sp.	1.830	s.	sp.	sp.	0.314	0.282	-	sp.	0.543	-	4.119	-
-	sp.	2.591	s.	0.125	0.152	sp.	-	-	sp.	1.378	-	2.519	-
-	sp.	2.633	s.	0.086	sp.	sp.	sp.	-	s.	0.676	-	3.420	-

WOOD EXTRACTS

j	k	l	m	n	o	p	q	r	s	t	u	v	w
-	-	0.307	-	-	-	-	-	-	0.215	-	-	-	-
-	-	0.194	-	-	-	-	-	-	0.239	-	-	-	-
-	-	0.203	-	-	-	-	-	-	0.079	-	-	-	-
-	-	0.077	-	-	-	-	-	-	0.044	-	-	-	-
-	-	0.094	-	-	-	-	sp.	-	-	-	-	-	-
-	-	-	sp.	sp.	sp.	-	sp.	-	-	-	-	-	-
-	-	-	-	-	sp.	-	sp.	sp.	sp.	sp.	-	-	-
sp.	sp.	0.302	-	-	-	-	sp.	sp.	sp.	sp.	-	-	-
sp.	sp.	0.900	s.	-	-	-	sp.	sp.	sp.	0.400	-	-	-
s.	-	0.228	s.	-	sp.	-	sp.	-	0.516	-	-	-	-
-	-	0.151	-	-	-	-	-	-	0.398	-	-	-	-

LEAF EXTRACTS

j	k	l	m	n	o	p	q	r	s	t	u	v	w
-	-	-	-	0.305	-	-	1.492	-	-	-	-	-	-
-	-	-	-	0.090	sp.	-	1.506	-	-	-	-	-	-
sp.	-	sp.	-	0.131	-	-	sp.	1.552	-	-	-	-	-
-	-	0.031	-	0.144	0.472	-	0.740	0.779	-	-	-	-	-
sp.	-	0.391	-	0.551	0.032	-	0.406	sp.	-	0.050	-	-	-
-	-	0.159	-	0.425	sp.	-	1.915	sp.	-	sp.	-	-	-

* s. - shoulder

sp. - small peak

TABLE 13

AREA OF ELUTED PEAKS IN CM^2 FROM OV-

BARK EXTRACTS

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
January	-	-	0.241	1.625	-	sp.	-	-	10.720	-	-	2.679	-	0.1
March	-	-	0.451	0.738	-	sp.	sp.	-	7.343	-	sp.	2.140	-	0.1
April	-	-	0.504	2.724	-	sp.	sp.	-	11.277	-	sp.	3.683	-	0.1
May	-	-	0.294	4.222	-	sp.	-	-	9.847	-	sp.	1.684	-	0.0
June	-	-	0.810	3.690	-	sp.	sp.	-	10.470	-	sp.	2.960	-	0.2
July	sp.	sp.	1.552	2.661	-	sp.	sp.	-	12.730	-	s.	1.879	s.	0.2
August	-	-	0.624	2.892	-	sp.	-	-	5.892	-	-	0.933	s.	0.3
September	-	-	0.573	5.020	-	sp.	-	-	11.505	-	sp.	2.231	-	0.3
October	-	-	2.331	4.874	-	-	0.181	-	13.323	-	sp.	1.830	s.	sp
November	-	-	0.510	2.035	-	-	sp.	sp.	14.921	-	sp.	2.591	s.	0.1
December	-	-	0.864	1.512	-	-	sp.	-	14.994	-	sp.	2.633	s.	0.0

WOOD EXTRACTS

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
January	-	-	-	0.830	-	-	-	-	0.322	-	-	0.307	-	-
March	-	-	sp.	0.666	-	-	-	-	0.233	-	-	0.194	-	-
April	-	-	-	0.524	-	-	-	-	0.241	-	-	0.203	-	-
May	-	-	-	0.654	-	-	-	-	sp.	-	-	0.077	-	-
June	-	-	-	0.354	-	-	-	-	0.075	-	-	0.094	-	-
July	-	-	-	0.784	s.	sp.	-	sp.	-	-	-	-	sp.	sp
August	-	-	-	-	-	sp.	sp.	-	-	-	-	-	-	-
September	-	-	s.	2.475	-	sp.	sp.	-	0.495	sp.	sp.	0.302	-	-
October	-	-	s.	2.530	-	sp.	sp.	sp.	0.960	sp.	sp.	0.900	s.	-
November	-	-	sp.	1.620	-	-	-	-	0.870	s.	-	0.228	s.	-
December	-	-	-	0.990	-	-	-	-	0.374	-	-	0.151	-	-

LEAF EXTRACTS

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
May	-	-	1.424	-	-	-	-	-	-	-	-	-	-	0.3
June	-	-	2.291	0.489	-	sp.	-	-	-	-	-	-	-	0.09
July	-	-	2.055	-	-	sp.	sp.	sp.	sp.	sp.	-	sp.	-	0.1
August	-	-	1.764	s.	-	-	sp.	-	sp.	-	-	0.031	-	0.1
September	sp.	-	0.689	1.697	-	-	-	-	sp.	sp.	-	0.391	-	0.5
October	-	-	2.416	s.	-	sp.	-	-	sp.	-	-	0.159	-	0.4

in a greater concentration in certain parts of the plant. Peaks c, e, k, q, r, s, v, w, y, z and bb were found to have their largest concentration in bark samples. Peaks b, f and u had their largest concentration in leaf samples.

OV-17 results also indicated several components (peaks a, b, e, f, g, h, j, k, m, u and w) were present only in minor concentration in the bark and/or wood and/or leaf sample. Peaks d, i, l, n, o, p, q, t and v had their largest concentration in bark samples. Peaks c, q and r had their largest concentration in leaf samples. Analysis on both columns indicated that wood samples possessed a low glycoside content.

Seasonal variations of the components of Salix petiolaris

With bark extract analysis, the OV-1 column gave better resolution of the glycoside peaks than did the OV-17 column. Peaks from the OV-1 chromatogram were well-spaced allowing easier identification and matching of peaks while the OV-17 column gave several peaks close together, having close relative arbutin times, presenting some difficulty in identifying peaks.

The total areas of peaks from the bark extracts on both columns was calculated and are presented in Figure 9. The OV-17 column indicated that high levels of glycoside content occurred in the bark in the months of April and June. The highest level occurred in October. A low glycoside content was found in March and again in August. The OV-1 column indicated high glycoside content in the bark in the months of June and November with the highest level occurring in October. Low levels of glycoside content were found in August and March.

Thieme (16,102) found that the highest glycoside content occurs in Salix species bark in February and March and the lowest in August to

FIGURE 9   TOTAL AREA OF GLC PEAKS FROM MONTHLY BARK EXTRACTS
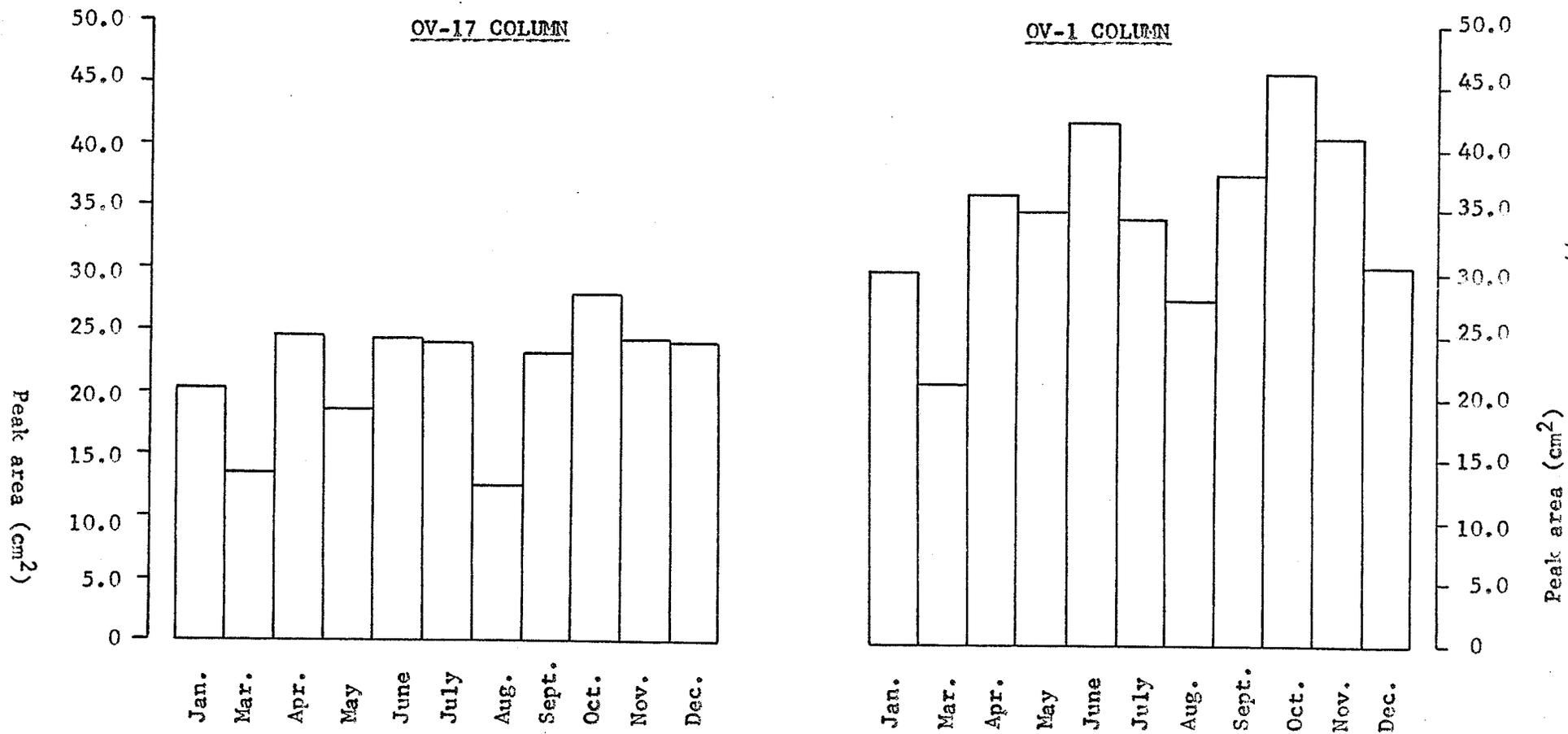
October. These results differ from those obtained by this author since

the highest content was found in October and low content was found in

March and August. The difference in results obtained is probably due

to the different climatic conditions under which the Salix species were

grown. Spring occurs earlier in Germany and, thus, a high glycoside

content in March bark would be expected for Thieme's results.

Total area levels calculated from the OV-1 column were greater

than those calculated from the OV-17 column for every month indicating

that the OV-1 column is more sensitive to Salix extract components.

There is one discrepancy to be found in Figure 9. In all months ex-

cept May, the larger areas obtained from OV-1 results were in proportion

to the areas obtained from OV-17 results. However, for May, there was

a substantial increase in the OV-1 total area which was out of proportion

to the OV-17 results. This arose from the difference in results obtained

for picein content for that month on the two columns. With OV-17, picein

content was found to be slightly lower than previous months. However,

with OV-1, the picein content was somewhat increased over previous months

and was, in fact, the second highest level for the year.

The results from the OV-1 column pertaining to total glycoside

content closely follow the results obtained from extract weights, which

indicated high extract weight in the months of June and November and low

levels in March and August.

Graphs (Figures 10 part a), b), c) and 11 part a), b), c)) of

individual glycoside variation throughout the year also closely follow

the pattern of total glycoside content. Since a great difficulty arose

in matching peaks found from OV-1 and OV-17 analysis, it is imperative

to remember that the lettered peaks of individual components obtained

from OV-1 analysis do not correspond with similarly lettered peaks

FIGURE 10 part a)   INDIVIDUAL GLYCOSIDE SEASONAL

VARIATION ON OV-1 COLUMN
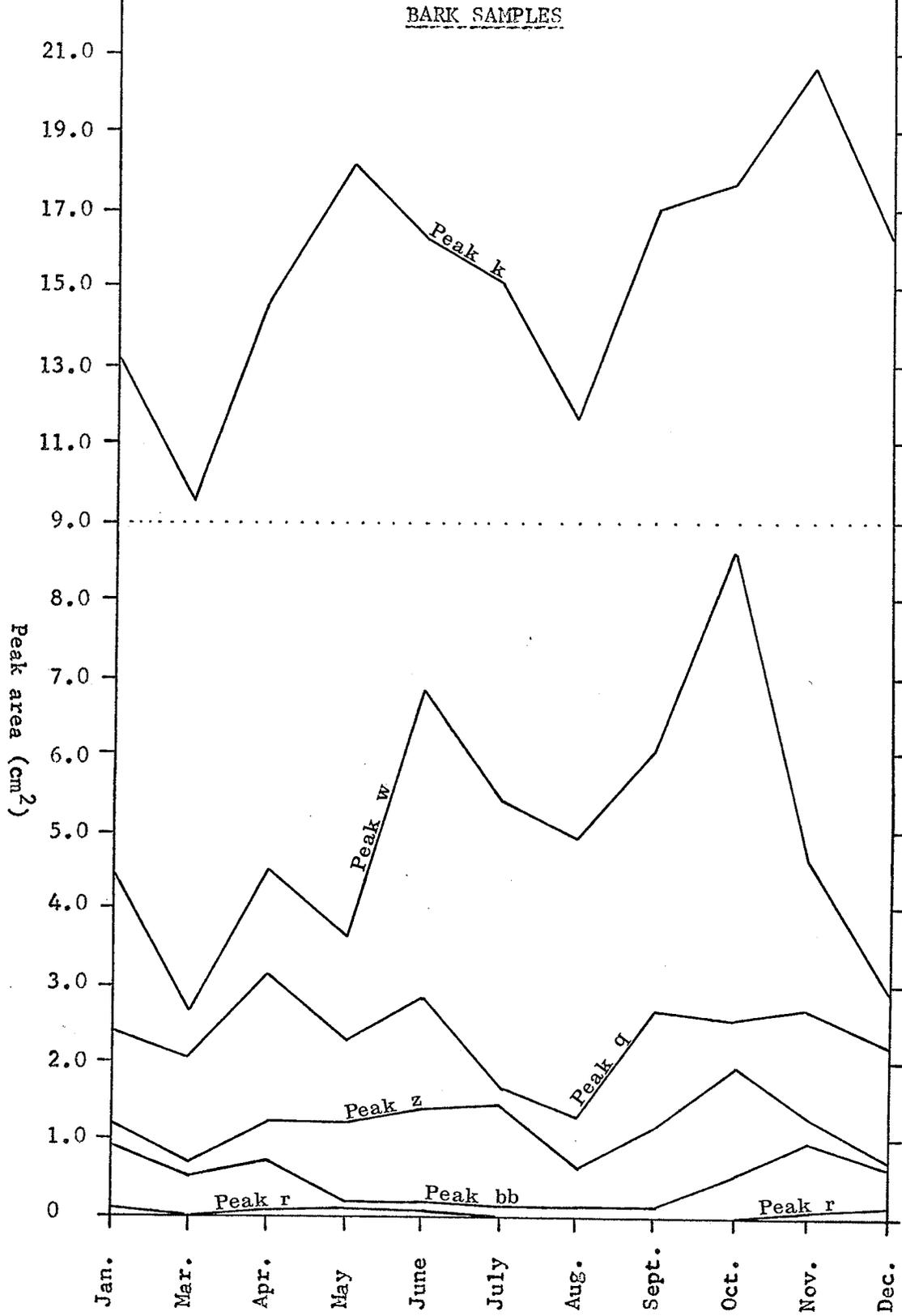
BARK SAMPLES

FIGURE 10 part b)   INDIVIDUAL GLYCOSIDE SEASONAL

VARIATION ON OV-1 COLUMN
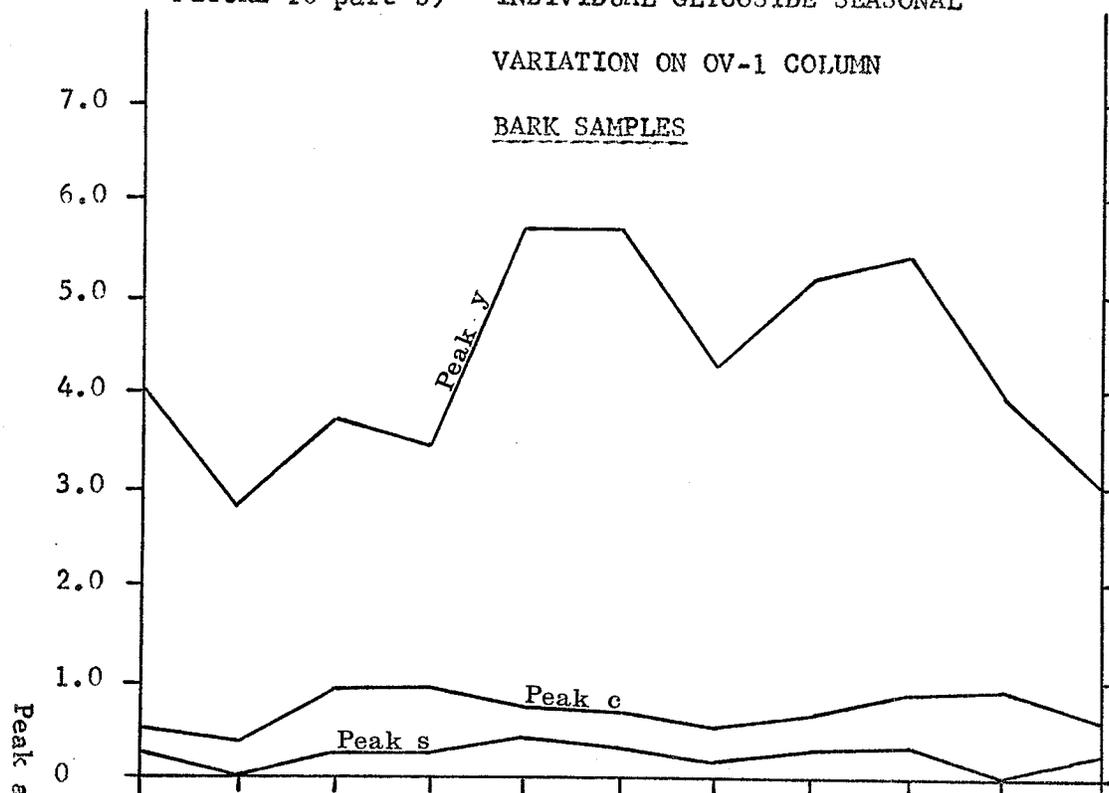
<u>BARK SAMPLES</u>

FIGURE 10 part c)

<u>BARK SAMPLES</u>
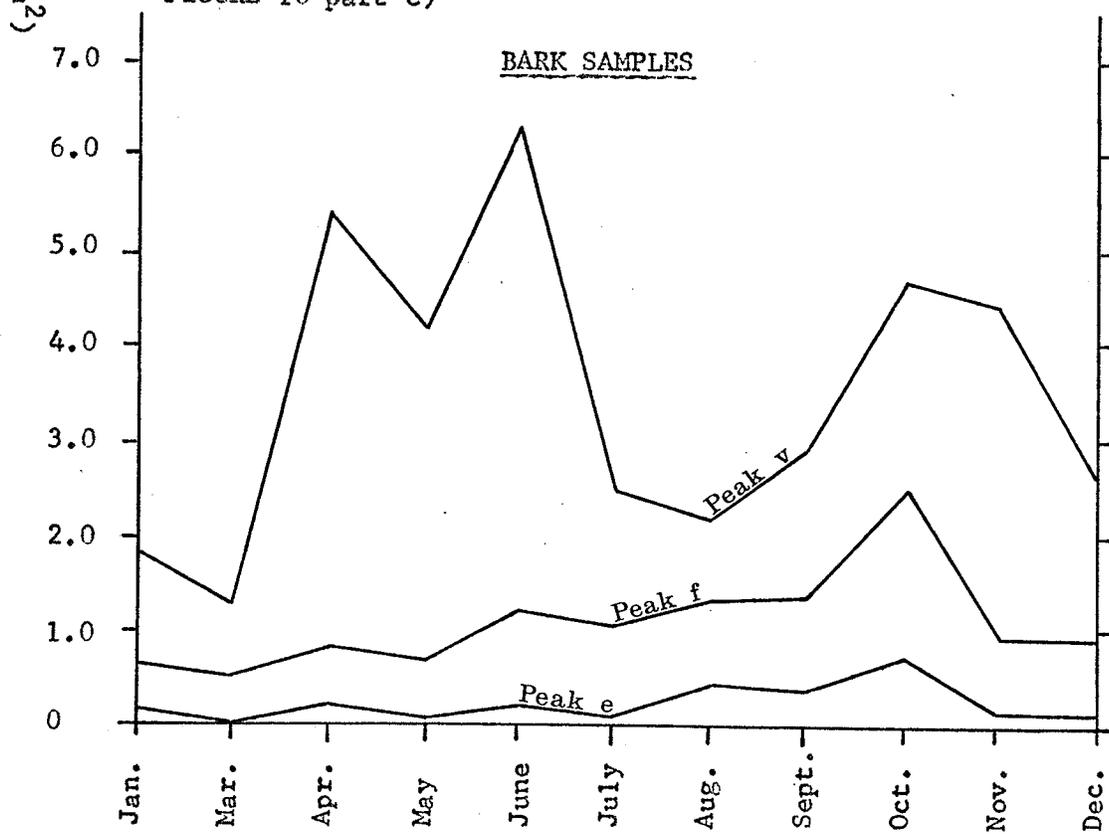
FIGURE 11 part a)   INDIVIDUAL GLYCOSIDE SEASONAL

VARIATION ON OV-17 COLUMN

BARK SAMPLES
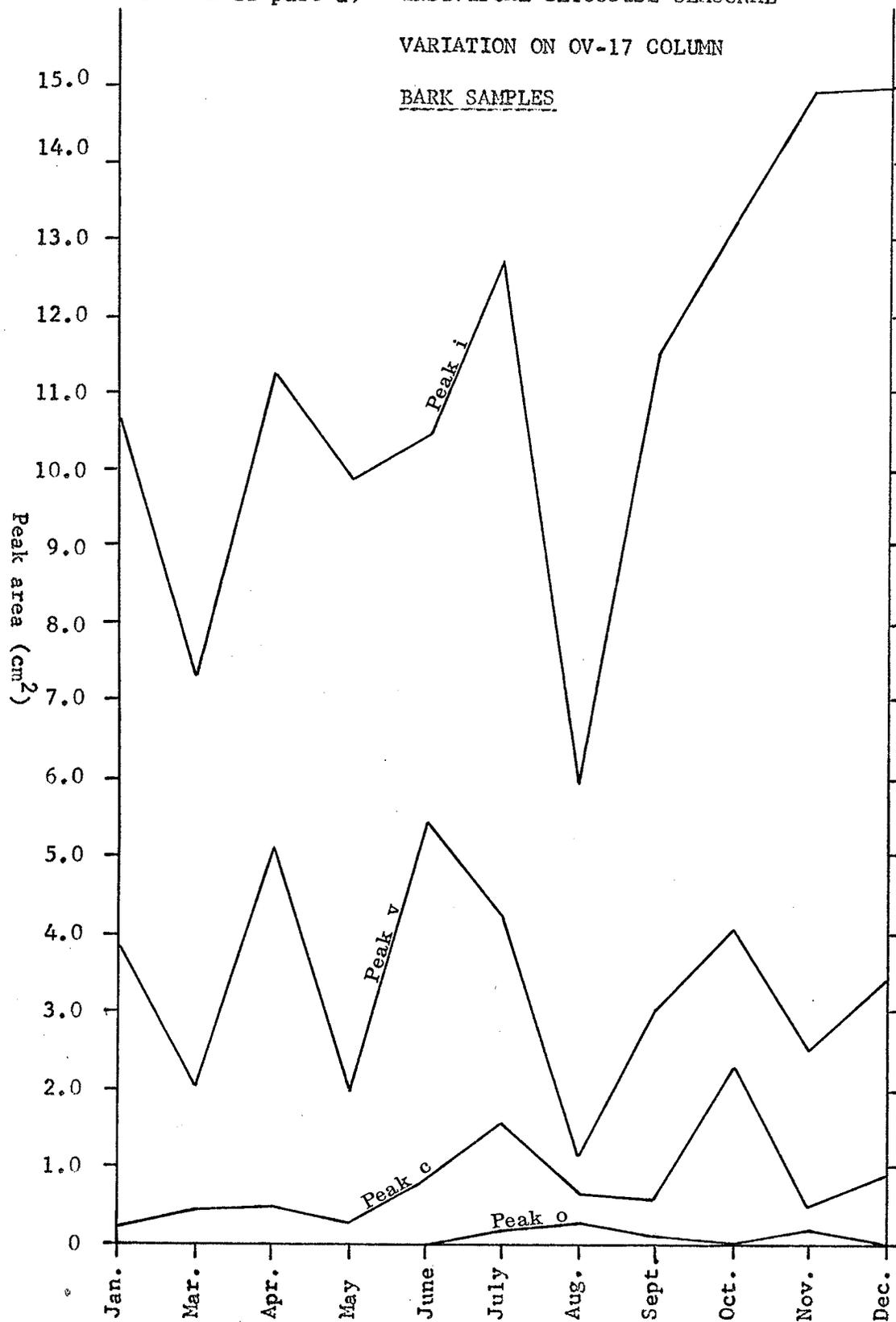
FIGURE 11 part b)   INDIVIDUAL GLYCOSIDE SEASONAL

VARIATION ON OV-17 COLUMN

BARK SAMPLES

FIGURE 11 part c)

BARK SAMPLES
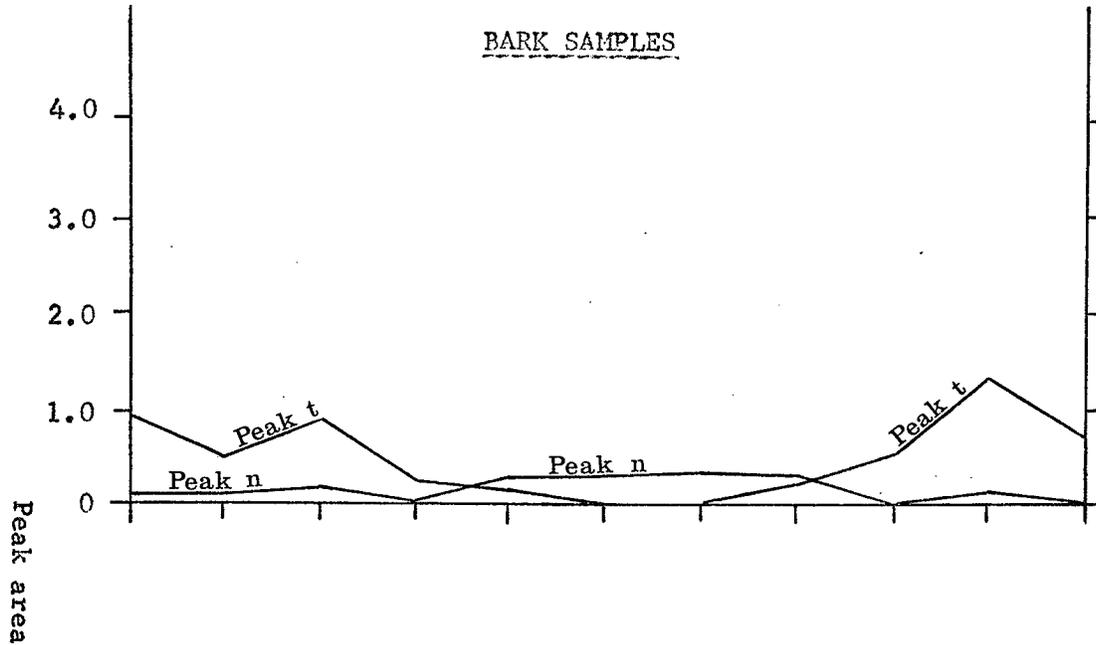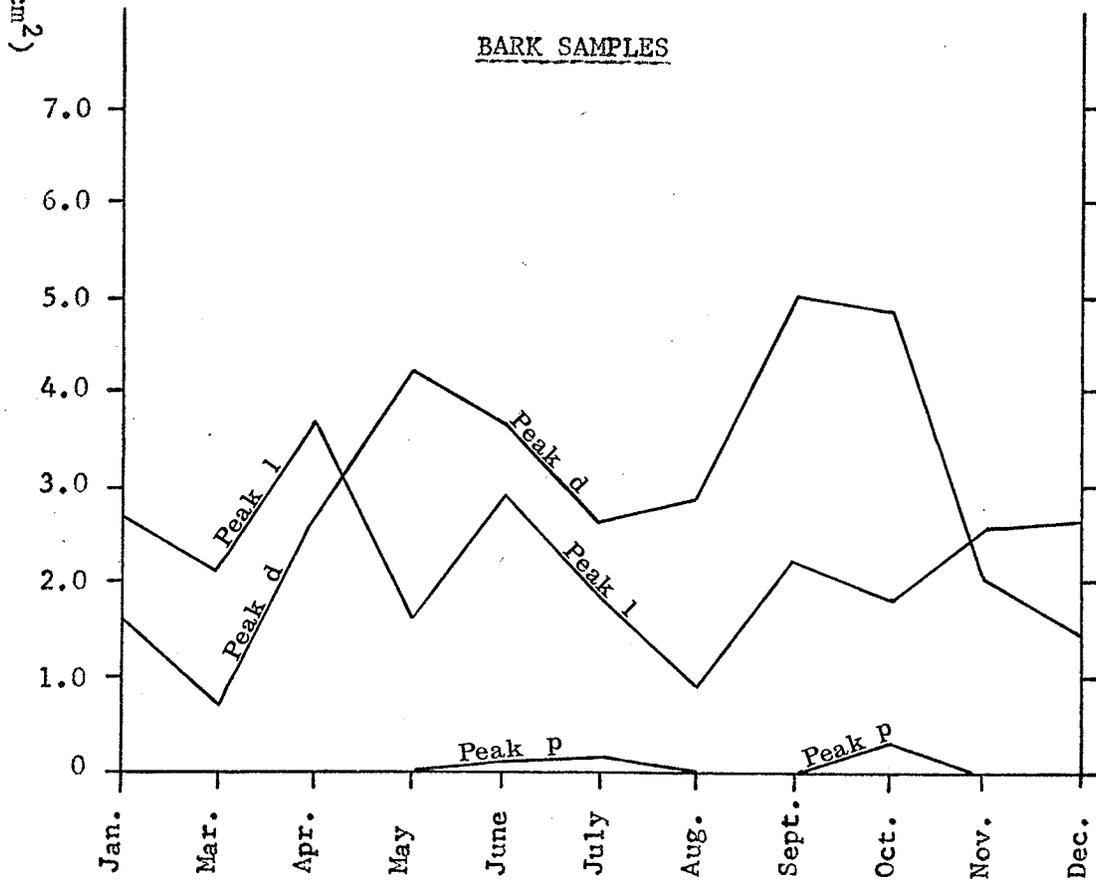
obtained from OV-17 analysis when interpreting these graphs and other
graphs in this thesis. The concentration of glycosides present in the
bark varies sharply from month to month. The principal glycoside in
the bark was eluted from both columns at approximately 14 minutes and
was proved to be picein from qualitative studies.

Identification and matching of GLC peaks from wood extracts
was easier because of the relatively few peaks compared with bark ex-
tracts for the corresponding month. The total area of the eluted peaks
from wood analysis on OV-1 and OV-17 columns is presented in Figure 12.
Results by OV-1 analysis showed changes in glycoside content in wood
throughout the year. The pattern of glycoside content obtained by OV-1
analysis differed greatly from the pattern of glycoside content found
by OV-17 analysis and from the wood extract weights. Analysis with OV-1
indicated there was an increase in glycoside content from January to
April with a fall off in content to August and then a sharp increase in
levels to October with high content found in November and December. In-
dividual high levels of glycoside content in wood were found in March,
April, October, November and December. A low glycoside content was
found in July and again in August.

With OV-17 analysis, glycoside content in January was high with
a steady decrease in level to August where the glycoside level was nil.
The content started to increase after August to the highest level in
October and then fell off in the months of November and December. Results
on OV-17 closely followed the results of the wood extract weights which
had a high weight in January and October with low levels from May to
August.

Graphs (Figures 13 part a), b), c), d), e) and 14 part a), b), c))
have been constructed to show the individual glycoside level variation in

FIGURE 12   TOTAL AREA OF GLC PEAKS FROM MONTHLY WOOD EXTRACTS

OV-17 COLUMN

OV-1 COLUMN

Peak area (cm^2)

Peak area (cm^2)
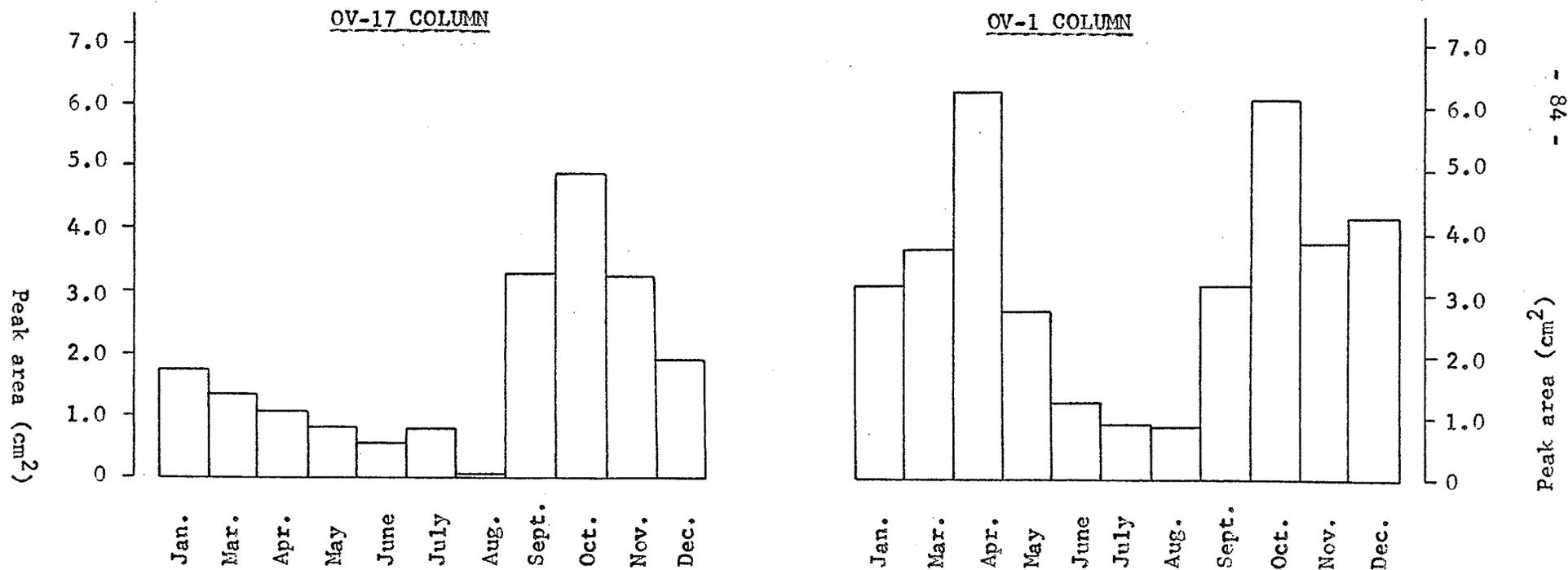
FIGURE 13 part a)   INDIVIDUAL GLYCOSIDE SEASONAL

VARIATION ON OV-1 COLUMN

WOOD SAMPLES
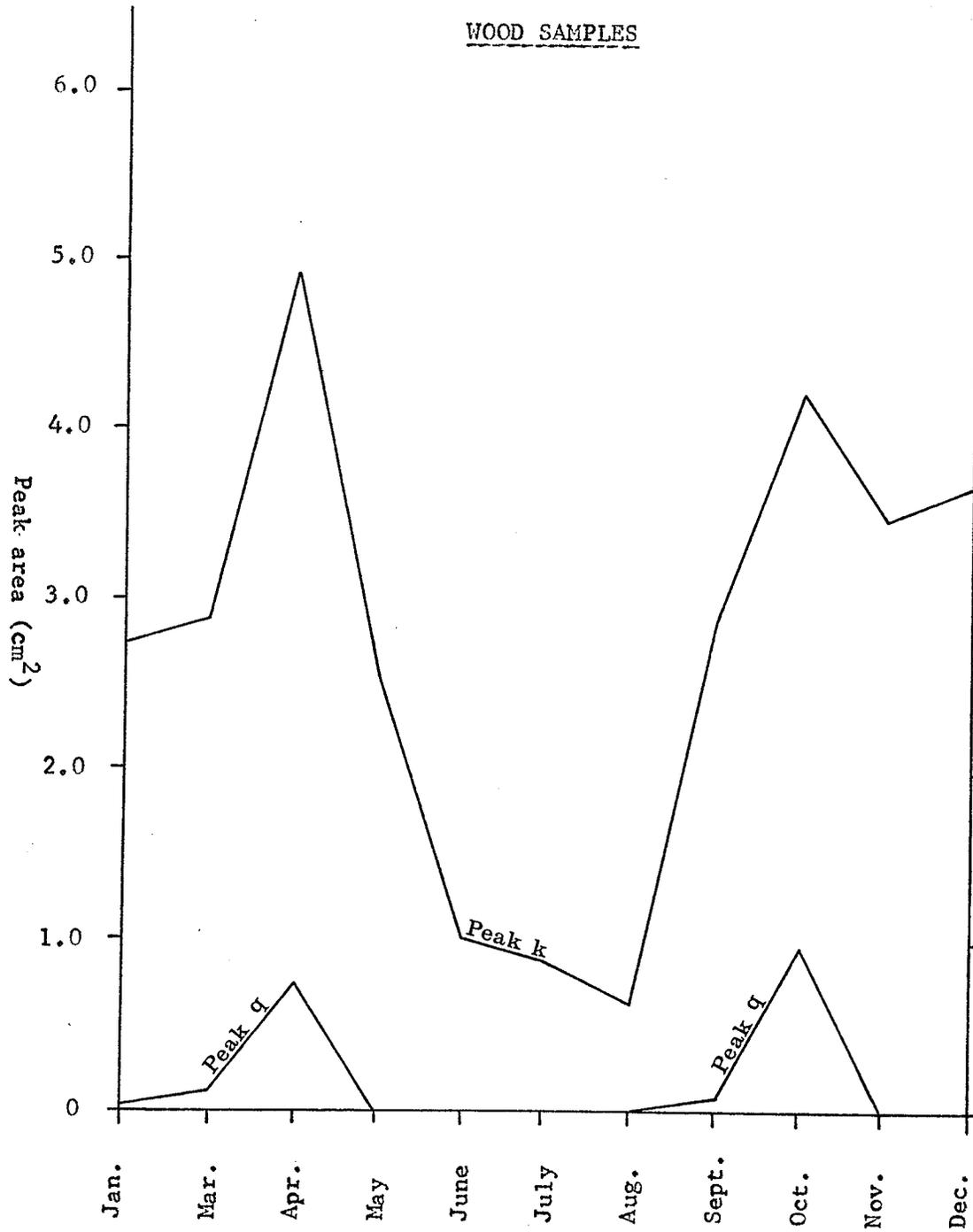
FIGURE 13 part b)   INDIVIDUAL GLYCOSIDE SEASONAL

VARIATION ON OV-1 COLUMN

WOOD SAMPLES

Peak b

FIGURE 13 part c)

WOOD SAMPLES

Peak d

FIGURE 13 part d)

WOOD SAMPLES

Peak f

FIGURE 13 part e)

WOOD SAMPLES

Peak bb

Peak bb
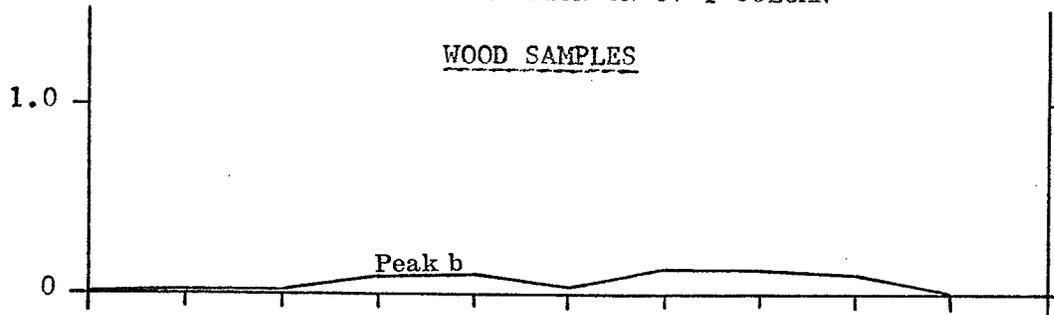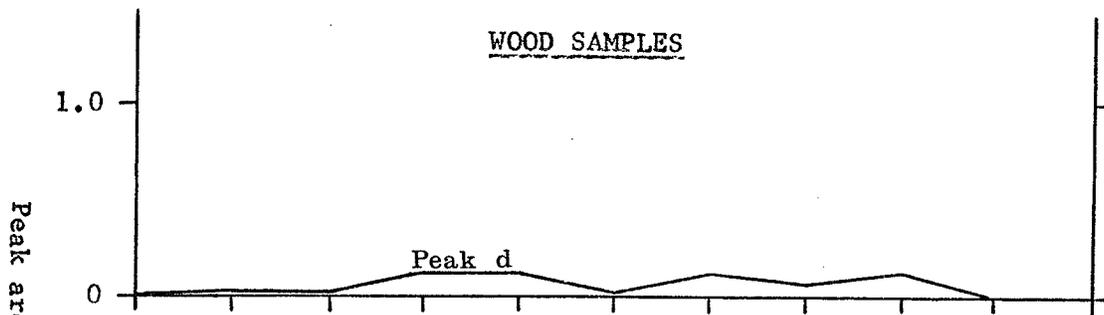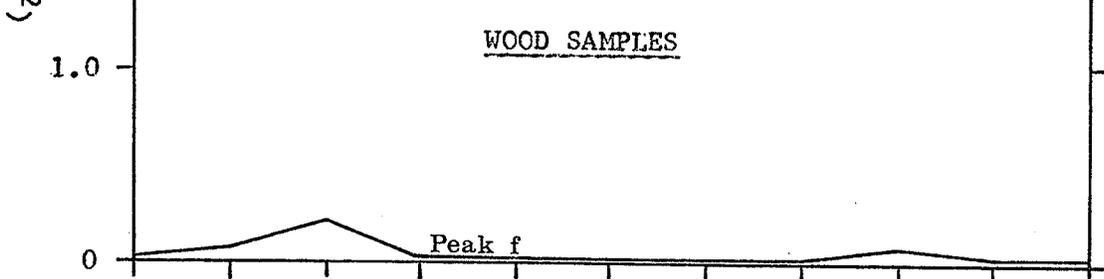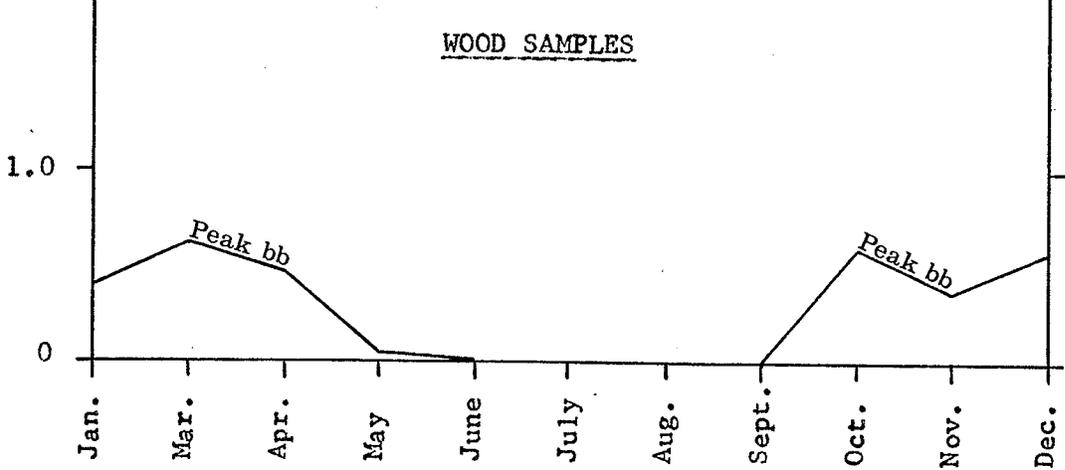
FIGURE 14 part a)   INDIVIDUAL GLYCOSIDE SEASONAL

VARIATION ON OV-17 COLUMN

WOOD SAMPLES
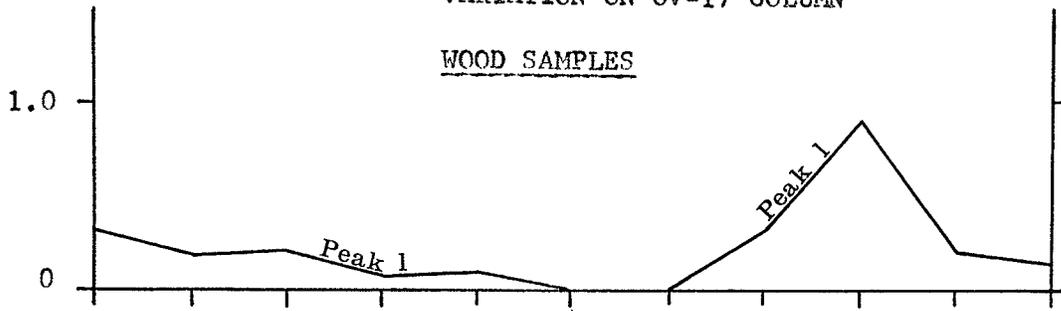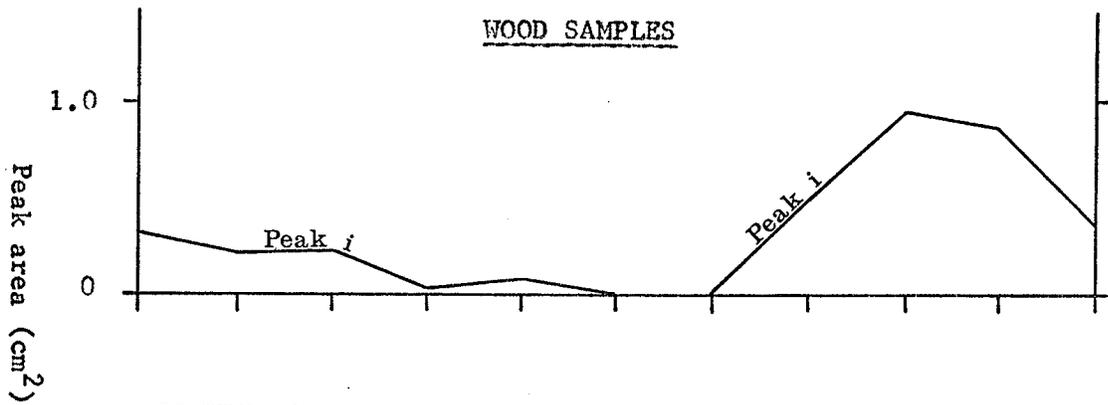
FIGURE 14 part b)

FIGURE 14 part c)
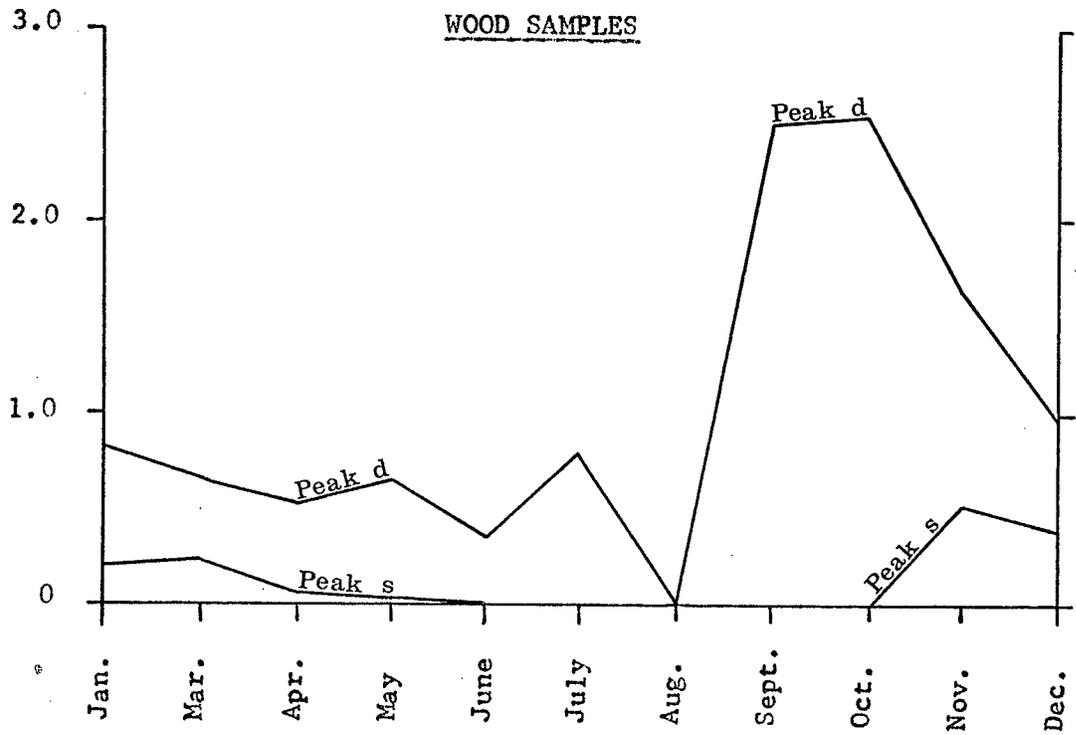
wood throughout the year. It is important to remember the warning presented after bark analysis pertaining to interpreting these graphs. Figure 14 part a), b), c) (OV-17 column results) indicate high individual glycoside levels in September, October and November with August possessing the lowest levels. These results correlate well with those obtained from extract weight and total peak area results. Figure 13 part a), b), c), d), e) (OV-1 column results) indicate high individual glycoside levels in April, September and October. Low levels of individual glycosides were found in August.

The glycoside levels in the wood extracts appeared to follow the pattern of glycoside levels in bark extracts for corresponding months. For wood and bark extracts, low glycoside levels were found in August and high levels in September to November. Since no wood was present in bark samples extracted, the results obtained from analysis of the bark samples were considered to be acceptable. However for wood samples, the bark was stripped off and the inner bark was not scraped off the wood. Therefore, it is possible that some bark material remained on the wood and was responsible for the components obtained from gas chromatographic analysis of wood samples.

Investigations were carried out on wood samples to determine whether or not glycosides do occur in Salix wood. Samples of total wood and center wood were prepared for extraction. The total wood sample was prepared exactly as for the monthly wood studies where the bark was carefully peeled off but no attempt was made to remove any inner bark adhering to the wood. Center wood was prepared by peeling off the bark and then scraping the outer wood (removing approximately 4-6 mm. of sample) to ensure removal of any bark or inner bark that was present. GLC chromatograms of total and center wood are presented in Figures 15-18 and

FIGURE 15   GLC CHROMATOGRAM OF OCTOBER S. petiolaris

ON OV-1 COLUMN

TOTAL WOOD SAMPLE

FIGURE 16   GLC CHROMATOGRAM OF OCTOBER S. petiolaris

ON OV-1 COLUMN

CENTER WOOD SAMPLE

FIGURE 17   GLC CHROMATOGRAM OF OCTOBER S. petiolaris
ON OV-17 COLUMN
TOTAL WOOD SAMPLE

FIGURE 18   GLC CHROMATOGRAM OF OCTOBER S. petiolaris

ON OV-17 COLUMN

CENTER WOOD SAMPLE
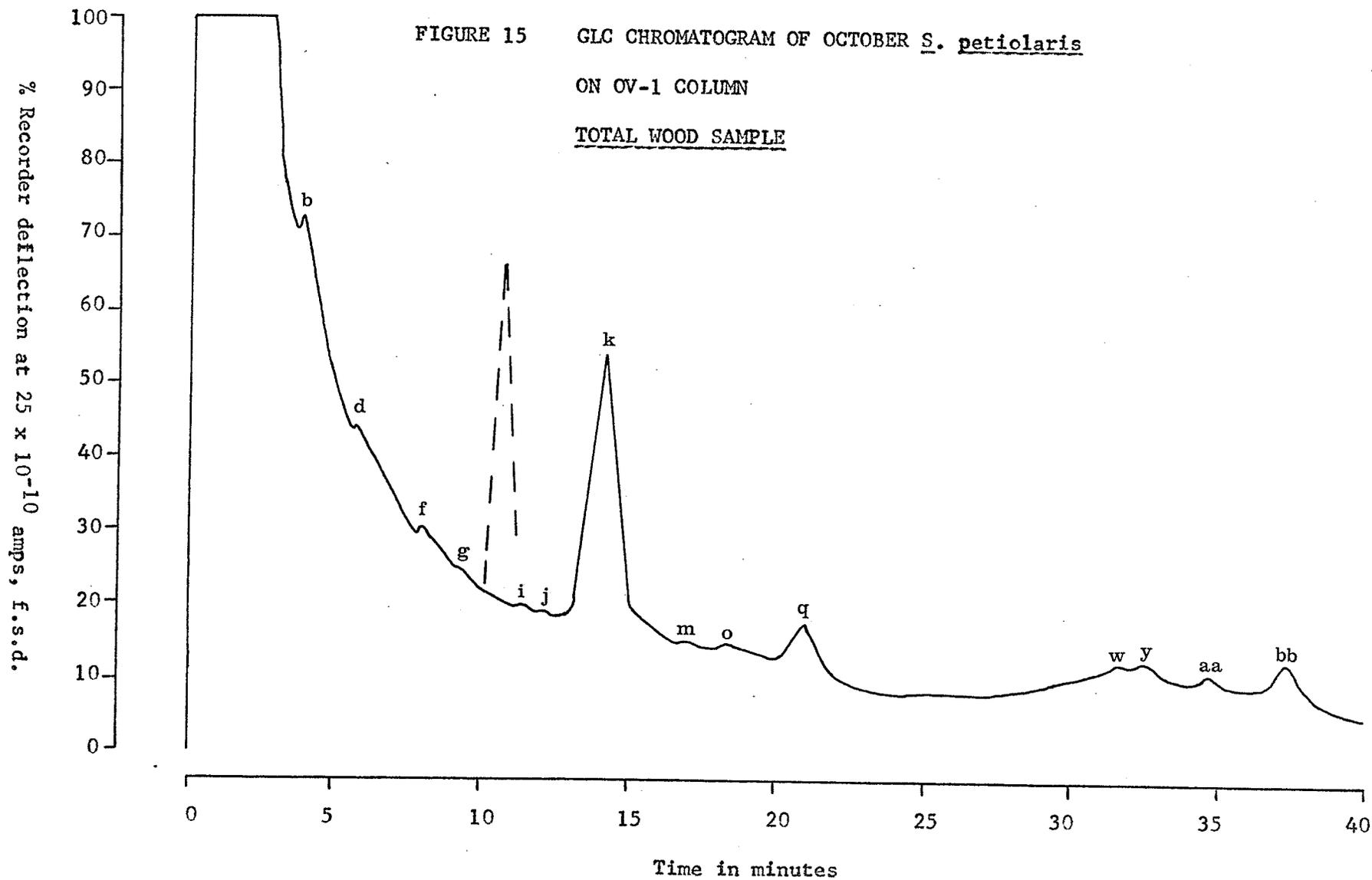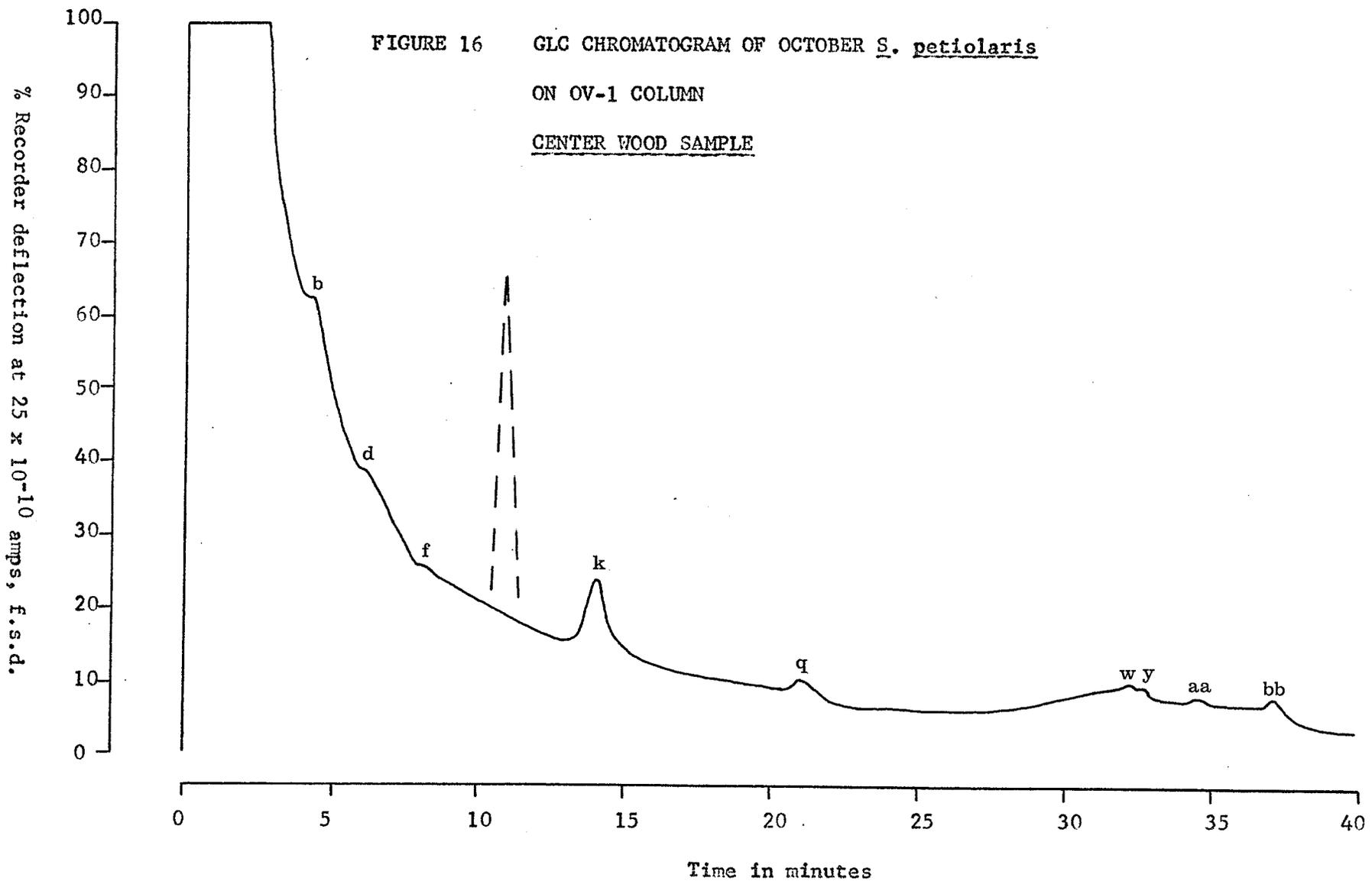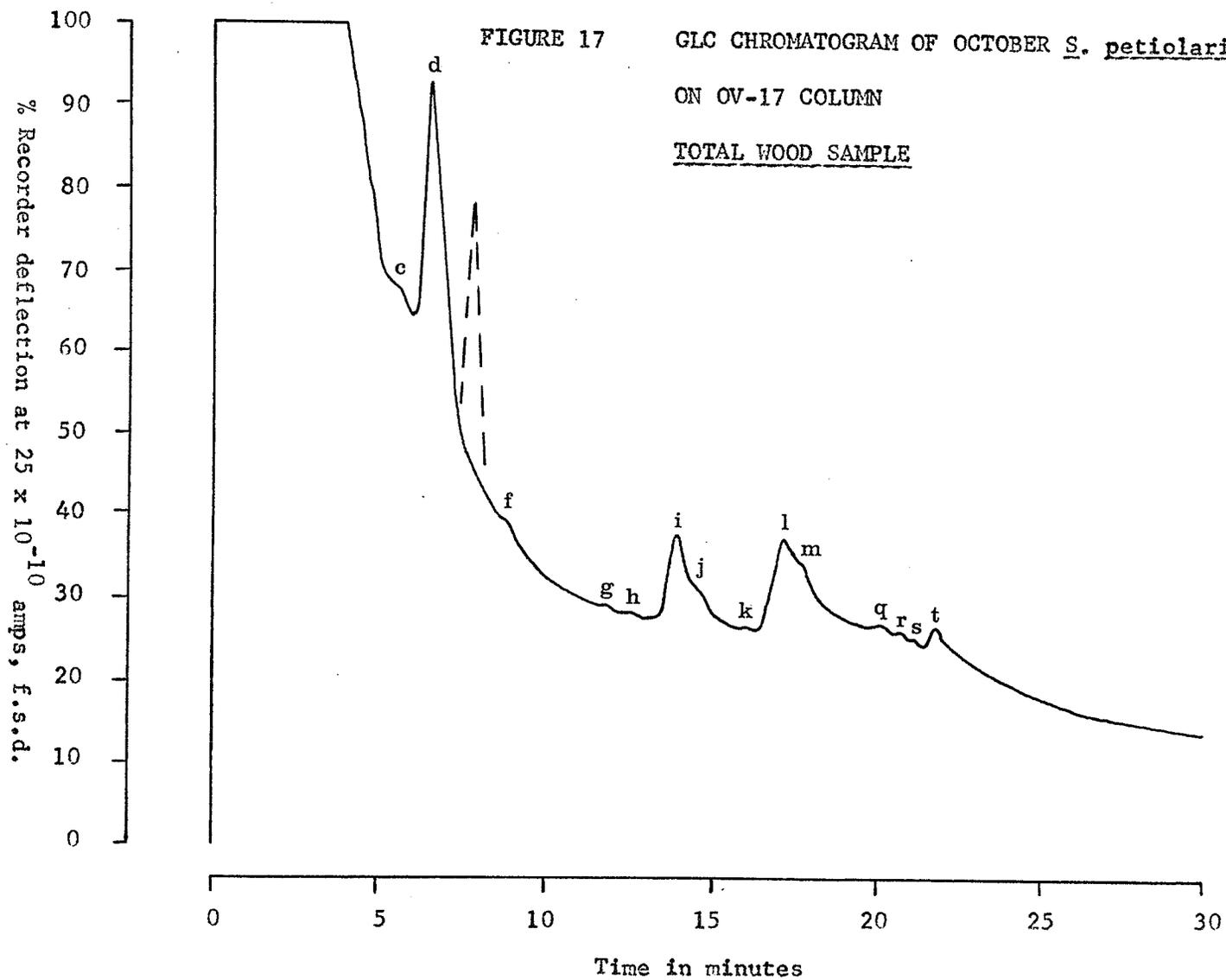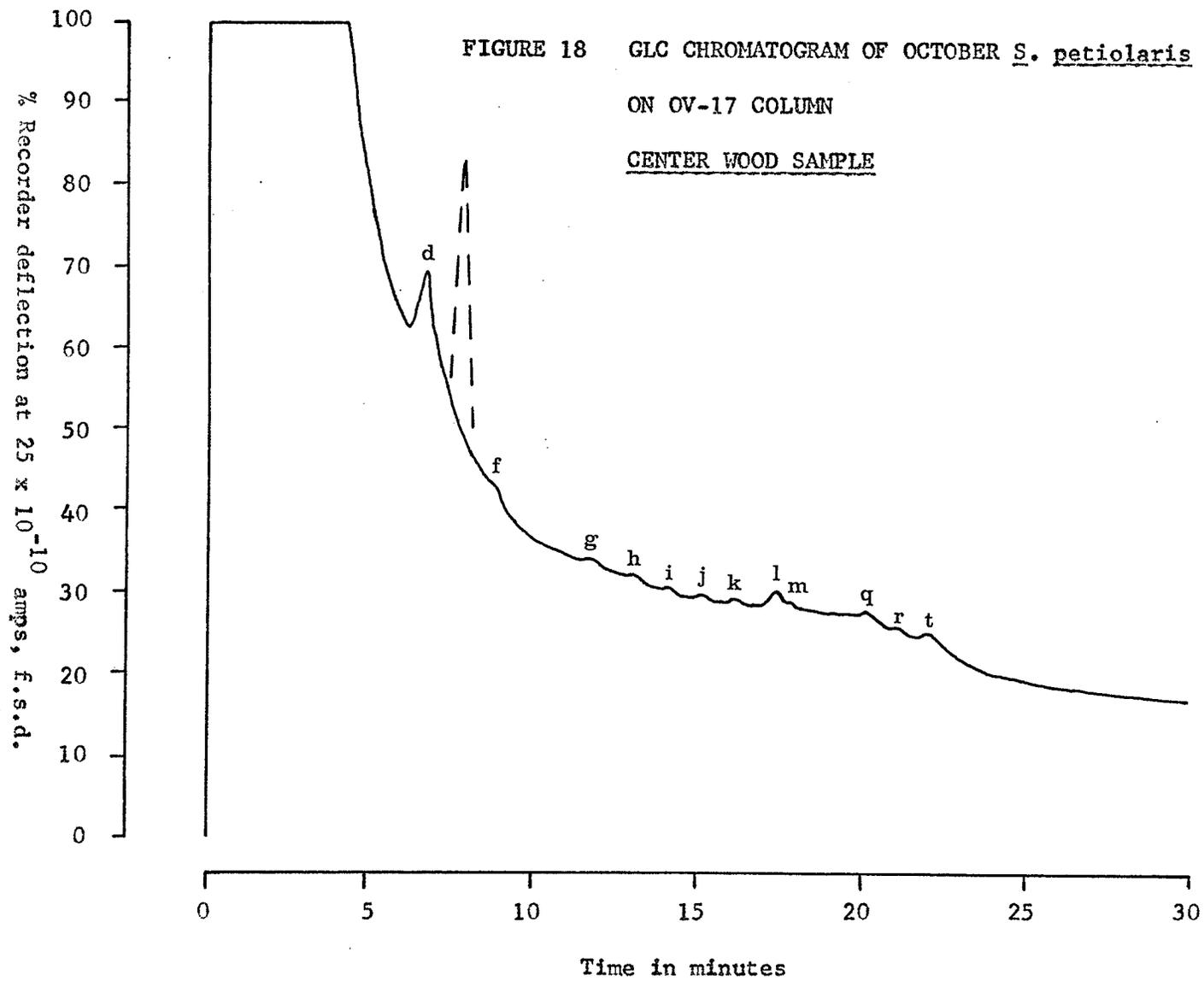
calculations from the GLC analyses are presented in Tables 14 and 15.

TABLE 14

GLC PEAK AREAS OBTAINED FROM OV-1 ANALYSIS OF TOTAL AND CENTER OCTOBER

SALIX PETIOLARIS WOOD

RELATIVE ARBUTIN TIME	TOTAL WOOD Peak area (cm^2)	CENTER WOOD Peak area (cm^2)
0.38	0.100	0.100
0.56	0.105	small peak
0.77	0.082	small peak
0.86	shoulder	-
1.08	small peak	-
1.13	small peak	-
1.29	4.230	1.040
1.56	small peak	-
1.70	small peak	-
1.92	0.975	0.120
2.92	small peak	small peak
2.99	small peak	small peak
3.17	0.585	small peak
3.40	0.585	0.070

The weights of the acetone/water residues of these wood samples differed markedly. The total wood sample gave a residue of 4.0 mg. while the center wood gave a residue of 2.4 mg. This indicated that there are less extractives in the center wood and, thus, the glycoside content would also be expected to be low.

Analysis on OV-1 and OV-17 columns gave similar results. The center wood contained glycoside levels which were considerably lower than

TABLE 15

GLC PEAK AREAS OBTAINED FROM OV-17 ANALYSIS OF TOTAL AND CENTER OCTOBER

SALIX PETIOLARIS WOOD

RELATIVE ARBUTIN TIME	TOTAL WOOD Peak area (cm^2)	CENTER WOOD Peak area (cm^2)
0.75	shoulder	-
0.88	2.530	0.520
1.34	small peak	small peak
1.52	small peak	small peak
1.60	small peak	small peak
1.78	0.960	small peak
1.92	small peak	small peak
2.06	small peak	small peak
2.18	0.900	0.150
2.23	shoulder	small peak
2.57	small peak	small peak
2.62	small peak	small peak
2.68	small peak	-
2.77	0.400	0.055

the glycoside levels obtained from total wood. However, glycosidic compounds are still definitely present in center wood samples. It appears that a large proportion of the glycoside content found in previous wood studies may have been due to inner bark adhering to wood samples.

Both columns produced excellent resolution and separation of the components present in the leaf extracts. However, there were several trace peaks produced on gas chromatographic chromatograms of the leaf extract on both columns and the peak area could not be

calculated for these.

The total area of eluted peaks from the monthly leaf extracts was calculated and is presented in Figure 19. The OV-17 column indicated almost identical glycoside levels for all leaf samples with a slightly higher level in June and October. OV-1 analysis indicated similar results. All months contained almost identical glycoside levels with slightly higher levels found in June and September.

The OV-1 column indicated larger glycoside content in the leaf samples than shown by OV-17 analysis. The difference in results were probably due to the difference in response of the two columns to salicin, as seen in Tables 12 and 13. The OV-1 column produced a large response for salicin while OV-17 indicated only a moderate level present. Picein and tremuloidin also indicated a different pattern in leaf samples depending on the column used for analysis. Thus, the difference in the total glycoside content found in leaf studies, and to a lesser extent in bark and wood samples, may be due to a different responsiveness or selectivity of each column to certain phenolic glycosides. The total peak area results from the OV-17 column correlate quite well with the results of the weights of leaf extracts. The extract weight was lowest in May and highest in September. Thieme (16,102) presented an entirely different picture for leaf glycoside content. He stated that the content was highest in May and decreased throughout the season until the leaves fell.

Graphs (Figures 20 part a), b), c), and 21 part a), b), c), d), e)) have been constructed to show the level of individual glycosides present in the leaf throughout their growth period. Results from these graphs indicate that the level of individual glycosides in the leaves is quite variable. Several glycosides appear in moderate concentration in May and then their level decreases during the season. Other glycosides

FIGURE 19   TOTAL AREA OF GLC PEAKS FROM MONTHLY
LEAF EXTRACTS
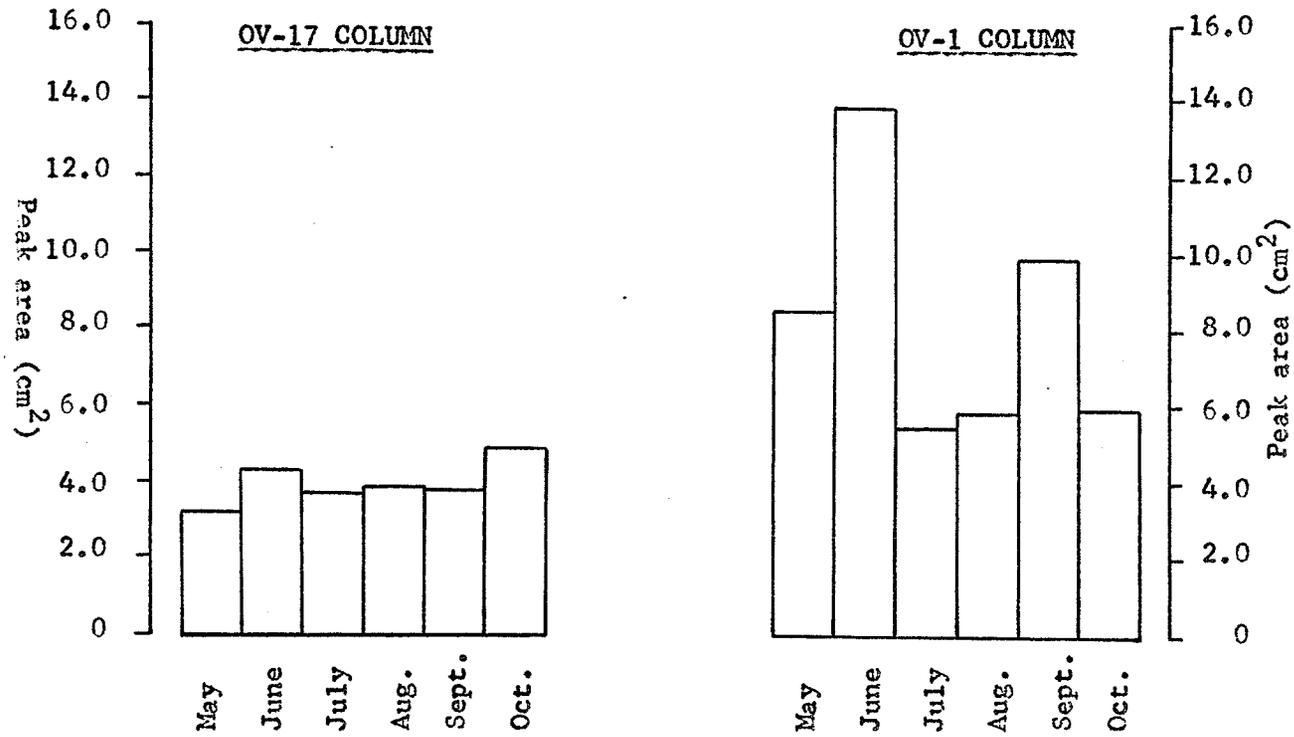
FIGURE 20 part a)   INDIVIDUAL GLYCOSIDE SEASONAL VARIATION ON OV-1 COLUMN LEAF SAMPLES

FIGURE 20 part b)

INDIVIDUAL GLYCOSIDE

SEASONAL VARIATION ON

OV-1 COLUMN

LEAF SAMPLES

Peak k

Peak b

Peak b

FIGURE 20 part c)

LEAF SAMPLES

Peak w

Peak d

Peak area (cm^2)

May   June   July   Aug.   Sept.   Oct.

FIGURE 21 part a)

INDIVIDUAL GLYCOSIDE SEASONAL VARIATION ON

OV-17 COLUMN

LEAF SAMPLES

FIGURE 21 part b)

LEAF SAMPLES

FIGURE 21 part c)

INDIVIDUAL GLYCOSIDE SEASONAL VARIATION ON

OV-17 COLUMN

LEAF SAMPLES

Peak d

FIGURE 21 part d)

LEAF SAMPLES

Peak o

FIGURE 21 part e)

LEAF SAMPLES

Peak l

Peak area (cm^2)

May   June   July   Aug.   Sept.   Oct.

start off at low concentrations in May but their level increases during the season until the leaves fall where their level again returns to a low value.

Gas chromatographic analysis of flower and seed extracts showed only two or three trace peaks indicating that there is no significant glycoside content present in these parts of willow.

The level of glycoside content was therefore greatest in the bark samples, as expected, and lowest in wood samples. Leaf samples had only moderate levels of glycosidic components.

Qualitative studies

Experiments were performed to identify as many components as possible in the extracts of Salix petiolaris.

December Salix petiolaris bark was chosen as a representative sample since by GLC analysis it possessed the usual components, found in moderate concentration, in bark samples. Peaks obtained from bark analyses were recorded relative to trimethylsilylarbutin and these ratios were compared and in some instances matched with the trimethylsilylarbutin ratios obtained with the reference glycosides analyzed.

Sufficient of each silylated reference glycoside was injected so that the recorder response was within ± 5% of the deflection of the bark extract peak suspected. This was done so that comparison of the elution times could be made on an equal concentration basis, since lower or higher concentrations can give different retention times due to poor resolution, overloading of the column or other factors.

Two columns were utilized for detection of components so that results could be cross-checked. It would be desirable to cross-check results on three columns but time did not allow this to be done. Results of the qualitative studies are given in Tables 16 and 17.

TABLE 16

RESULTS FROM QUALITATIVE STUDIES ON OV-17 COLUMN

REFERENCE GLYCOSIDE	RETENTION TIME (min.)	RELATIVE ARBUTIN TIME *	DECEMBER EXTRACT PEAK TIME (min.)	RELATIVE ARBUTIN TIME *
Salicin	6.24	0.78	6.30	0.79
Isosalicin	7.37	0.92	7.11	0.89
Fragilin	11.80	1.48	12.07	1.51
Salidroside	13.68	1.73	--	
Picein	14.14	1.77	14.16	1.78
Triandrin	17.70	2.25	17.62	2.23
Vimalin	17.91	2.26	--	
Salicortin	18.90	2.42	19.00	2.39
Tremuloidin	20.31	2.59	20.30	2.59
Populin	20.51	2.61	20.76	2.64
Salireposide	23.47	2.97	23.46	2.97

* Trimethylsilylarbutin Retention Time = 7.90 min.

By gas chromatographic analysis, salicin, fragilin, picein, triandrin, tremuloidin and salireposide were detected in December Salix petiolaris bark. These reference glycosides corresponded to peaks found by analysis of the bark extract on both columns. Grandidentatin, a phenolic glycoside found in some Salix species, was shown to be absent from Salix petiolaris. It is eluted at approximately 29-30 minutes (relative arbutin time 3.78) on the OV-17 column and at approximately 38-39 minutes (relative arbutin time 3.50) on the OV-1 column (74). At no time during the analysis of the monthly Salix petiolaris samples was a peak detected at such a high retention time on either column.

Isosalicin was thought to be present by the close relation-

TABLE 17

RESULTS FROM QUALITATIVE STUDIES ON OV-1 COLUMN

REFERENCE GLYCOSIDE	RETENTION TIME (min.)	RELATIVE ARBUTIN TIME*	DECEMBER EXTRACT PEAK TIME (min.)	RELATIVE ARBUTIN TIME*
Salicin	8.34	0.76	8.28	0.76
Isosalicin	9.35	0.86	9.75	0.91
Fragilin	12.43	1.14	12.36	1.13
Picein	14.48	1.31	14.55	1.31
Salidroside	18.08	1.66	--	
Vimalin	22.01	2.02	--	
Triandrin	26.04	2.38	25.80	2.36
Populin	28.37	2.62	--	
Tremuloidin	28.99	2.67	29.20	2.69
Salicortin	31.76	2.90	--	
Salireposide	32.46	2.93	32.48	2.93

* Trimethylsilylarbutin Retention Time = 10.93 min.

ship of an extract peak to the reference glycoside on both columns. However, when isosalicin and the December bark extract were co-chromatographed, two distinct peaks were obtained. Isosalicin is therefore absent from Salix petiolaris bark and, indeed, this glycoside has not yet been identified in any Salix species.

Quantitative studies

1) Preparation of calibration graphs

Calibration graphs (weight of glycoside vs. peak area) were obtained for three principal phenolic glycosides - salicin, picein and salireposide. A standard solution of each glycoside, containing 10 µg./µl. was prepared by accurate procedures and from this solution the weights

of glycosides injected could be calculated. The short exposure of the glycosides to heat, when evaporating the stock solutions, did not cause any degradative effects since only one peak was obtained for each glycoside and results were reproducible. The trimethylsilylation reaction was performed in the fine taper tube since it allowed trimethylsilylation to proceed and retarded the rate of evaporation of the Tri-Sil. Tri-Sil reagent was allowed to react with the glycosides for only 3-4 minutes, since after this time the Tri-Sil tended to evaporate and condense on the sides of the tube causing a change in the concentration of the solution. The peak areas plotted were a result of the average of five determinations for that specific weight of glycoside. It was hoped in this way to obtain a more statistically acceptable graph.

Salicin and salireposide produced straight line calibration graphs throughout the range of concentrations studied. For analysis of picein, the sensitivity of the detector had to be decreased by a factor of two since the peaks were going off the recorder scale. This decrease in sensitivity also caused a change in the response of the detectors and two calibration graphs were prepared for picein; one at one sensitivity (from 0-10 µg.) and another at the other sensitivity (12-20 µg.). The calibration graphs obtained for salicin, picein and salireposide are presented in Figures 22-25.

2) Application of quantitative studies

Gas chromatographic analysis of a November Salix petiolaris bark sample was performed immediately upon completion of the experiments carried out for preparation of the calibration graphs. In this way, the instrumental conditions in force for the preparation of the calibration graphs would be identical for the analysis of the November bark extract.

The peak areas for salicin, picein and salireposide for the

FIGURE 22   CALIBRATION GRAPH FOR SALICIN

ON OV-17 COLUMN

FIGURE 23   CALIBRATION GRAPH FOR PICEIN ON OV-17 COLUMN
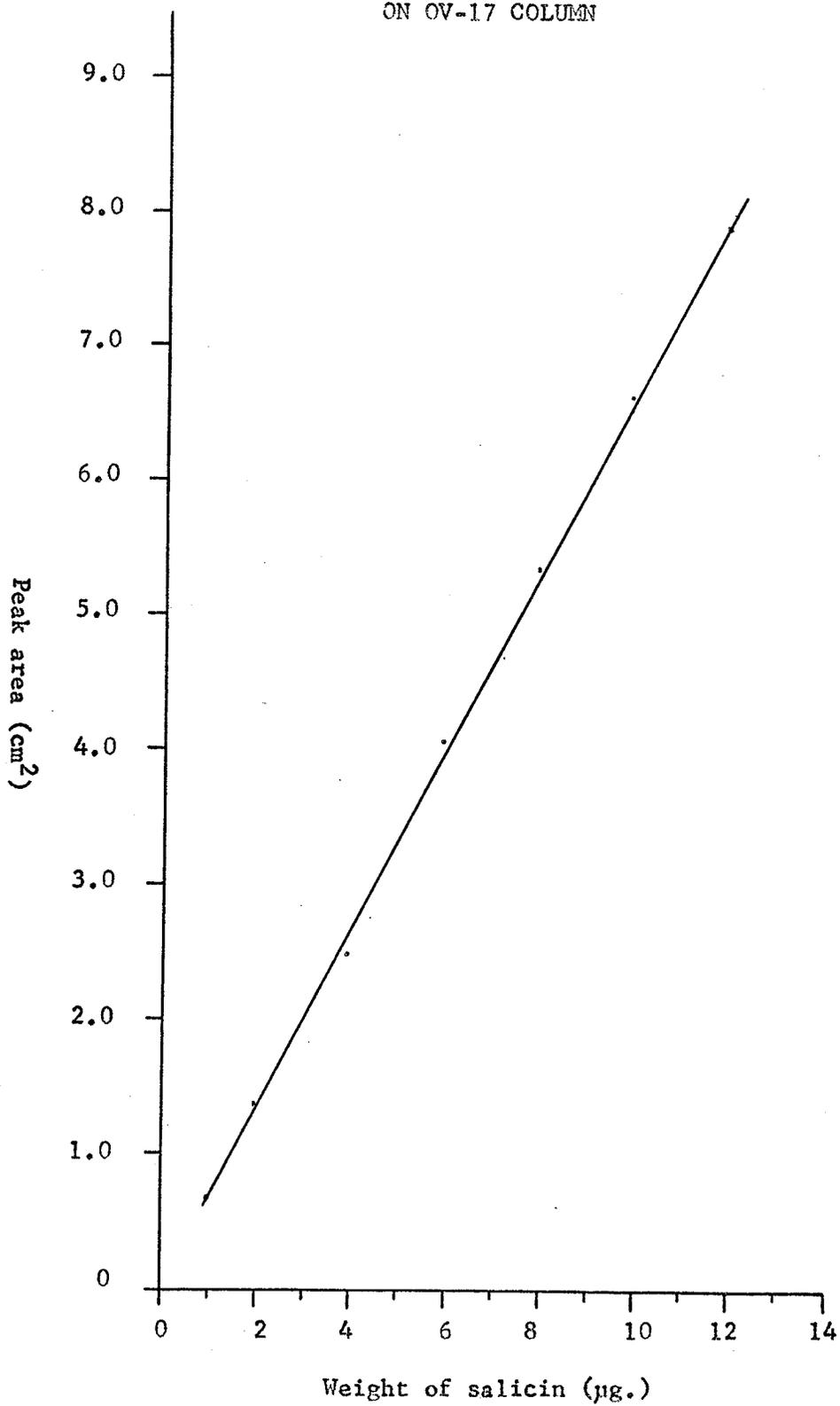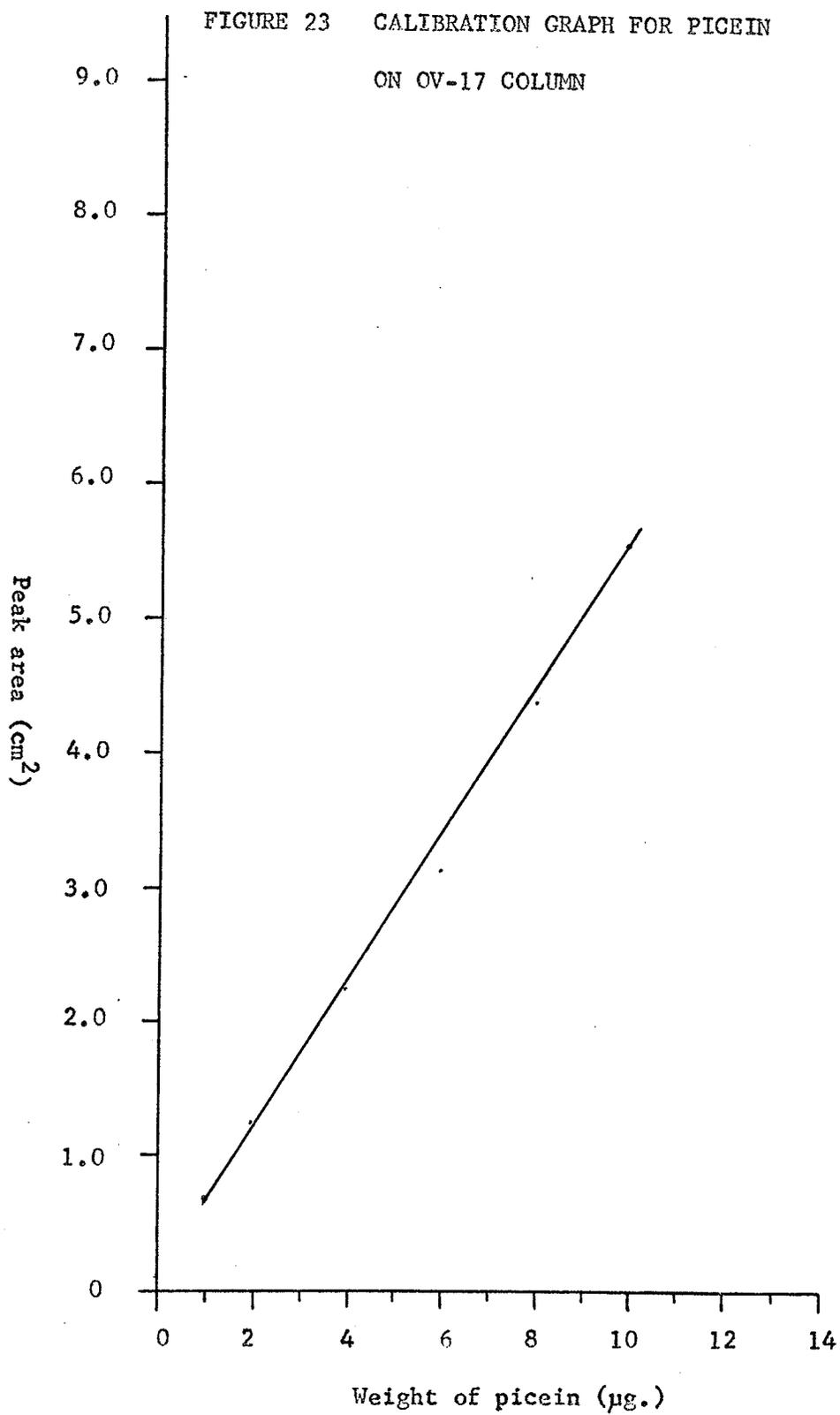
FIGURE 24   CALIBRATION GRAPH FOR PICEIN
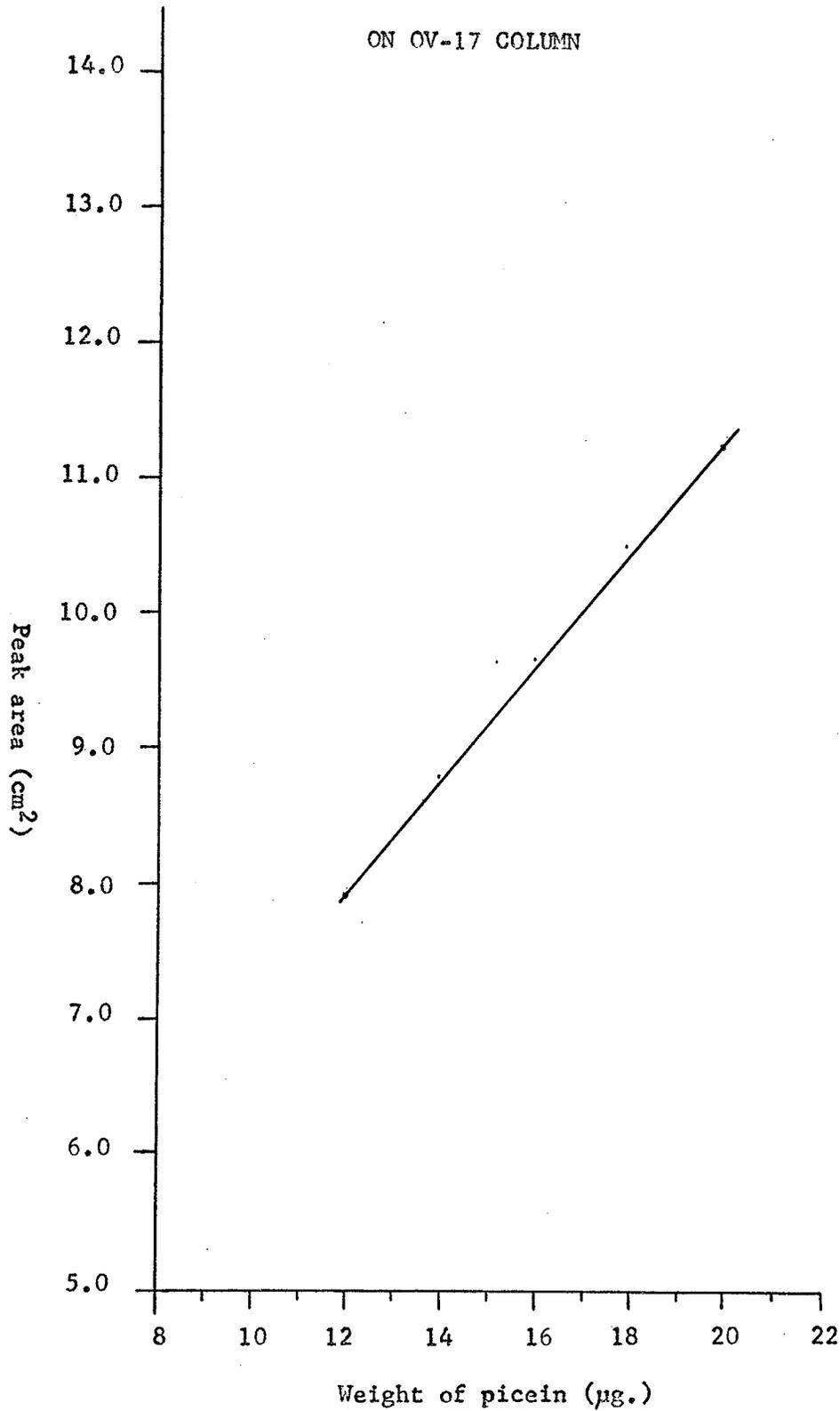
ON OV-17 COLUMN

FIGURE 25   CALIBRATION GRAPH FOR SALIREPOSIDE ON OV-17 COLUMN
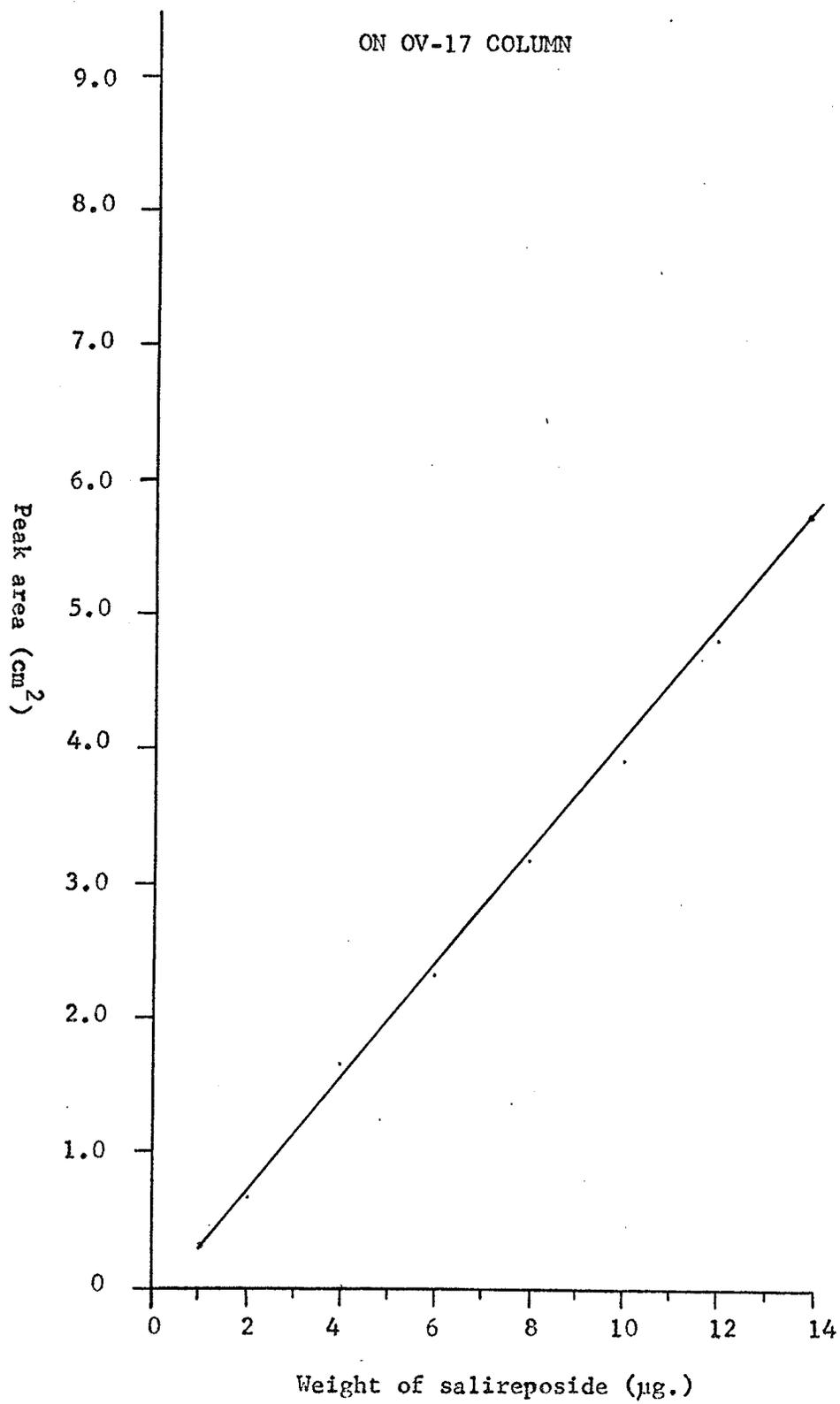
November bark extract analyzed at this time were determined and cor-
related with the calibration graphs to obtain the weight of each glyco-
side (in µg.) present in the bark sample. These results are presented in
Table 18. By the use of 50 mg. of bark sample and removal of one-fifth
of the extract residue and one-tenth of the Tri-Sil solution, GLC analysis
was performed on the extractives from 1 mg. of bark sample.

TABLE 18

AMOUNT OF THREE PHENOLIC GLYCOSIDES FOUND IN 1 mg. OF NOVEMBER SALIX

PETIOLARIS BARK

GLYCOSIDE	PEAK AREA (cm^2)	WEIGHT (µg.)
Salicin	1.214	1.8
Picein	17.547	27.8
Salireposide	5.793	13.9

The greatest glycoside proportion of the bark extract was
composed of picein (2.78%) while salireposide (1.39%) and salicin (0.18%)
were present in smaller amounts.

The peak areas obtained for these three glycosides in this
study were greater than the areas obtained in the previous analysis of
November Salix petiolaris bark, as seen in Table 19.

It was thought that the conditions of the gas chromatograph
including detector responsiveness (due to many factors) to the glyco-
sides had altered sufficiently during the time interval between the
studies to produce this difference.

Two-dimensional thin-layer chromatographic studies

Phenolic glycosides present in October and November Salix
petiolaris bark were also detected by two-dimensional thin-layer

TABLE 19

COMPARISON OF PEAK AREAS OBTAINED FROM NOVEMBER SALIX PETIOLARIS BARK

(area in cm^2)

GLYCOSIDE	FROM SEASONAL VARIATION STUDY	FROM QUANTITATIVE STUDY
Salicin	0.510	1.214
Picein	14.921	17.547
Salireposide	2.519	5.793

chromatography. Procedures and solvent systems were obtained from the work of Wong (2) who has extensively studied thin-layer chromatography of phenolic glycosides.

Results of this study are presented in Figures 26-29. The solvent systems which produced the best resolution and separation of the phenolic glycosides in the bark extracts were ethyl acetate-methanol, 9:1 v/v, and ethyl acetate-xylene-formic acid-water, 35:1:2:2 v/v. With these systems, the spots were well resolved over the plate, allowing easy detection of the spots. With the use of ethyl acetate-methanol, 9:1 v/v and benzene-methanol, 7:3 v/v the spots were not so well distributed over the plate and there were indications that several components were not being separated but developed together.

At least 10 to 12 spots were detected with the thin-layer study on October and November bark with the ethyl acetate-methanol, 9:1 v/v and ethyl acetate-xylene-formic acid-water, 35:1:2:2 v/v systems. Gas chromatographic analysis of these bark extracts indicated 14 to 15 peaks so it can be seen that TLC analysis is not as effective in detecting glycoside components of Salix bark. Ethyl acetate appears to affect the adhesive properties of silica gel G. Upon spraying the plates, the lower corner where the sample was spotted tended to flake off in some
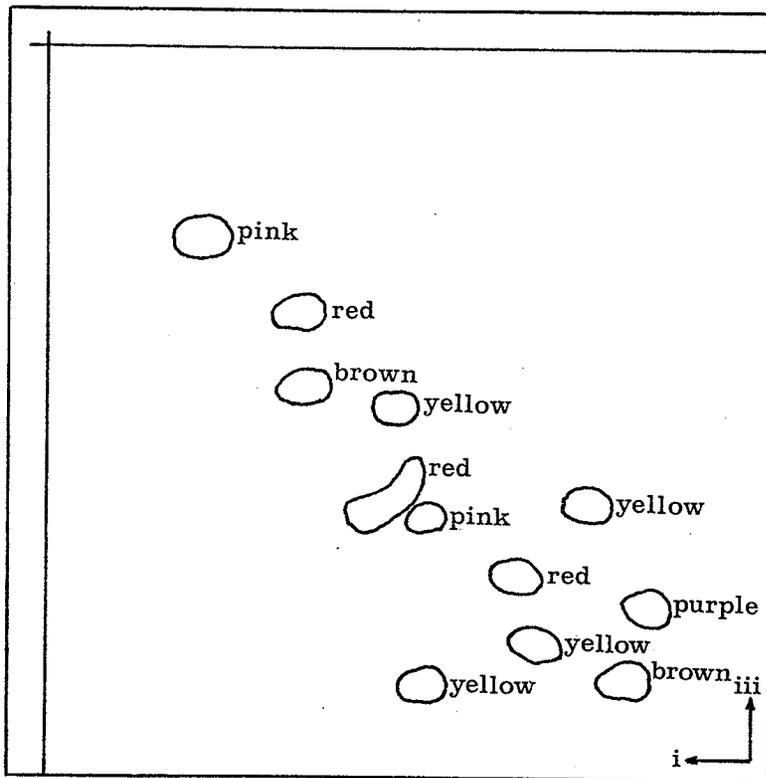
FIGURE 26   TWO-DIMENSIONAL CHROMATOGRAM OF OCTOBER

S. petiolaris BARK EXTRACT

SYSTEMS   i) Ethyl acetate-methanol, 9:1 v/v

iii) Ethyl acetate-xylene-formic acid-

water, 35:1:2:2 v/v
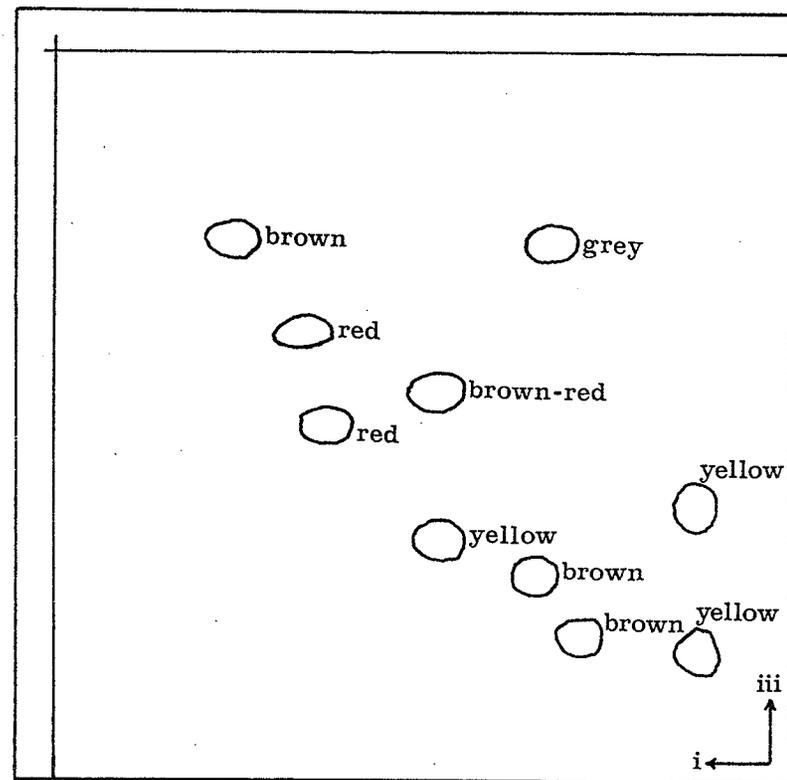

FIGURE 27   TWO-DIMENSIONAL CHROMATOGRAM OF NOVEMBER

S. petiolaris BARK EXTRACT

SYSTEMS   i) Ethyl acetate-methanol, 9:1 v/v

iii) Ethyl acetate-xylene-formic acid-

water, 35:1:2:2 v/v
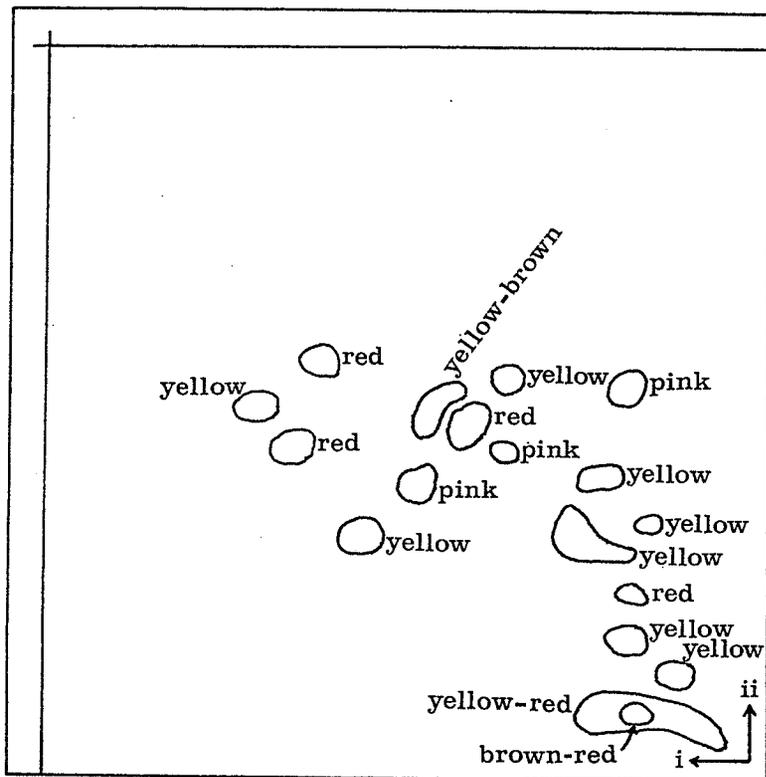
FIGURE 28   TWO-DIMENSIONAL CHROMATOGRAM OF OCTOBER

S. petiolaris BARK EXTRACT

SYSTEMS   i) Ethyl acetate-methanol, 9:1 v/v

ii) Benzene-methanol, 7:3 v/v
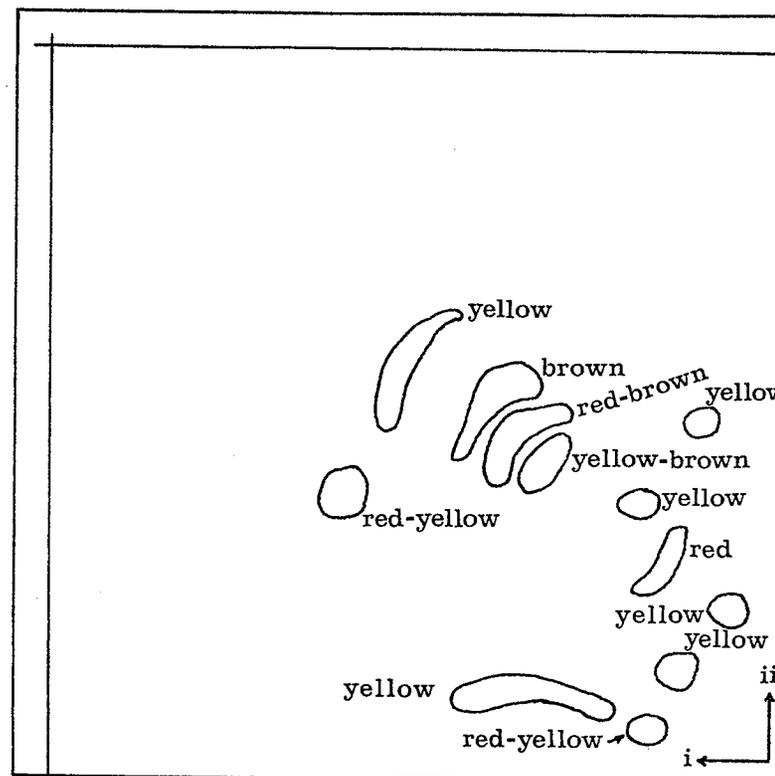
FIGURE 29   TWO-DIMENSIONAL CHROMATOGRAM OF NOVEMBER

S. petiolaris BARK EXTRACT

SYSTEMS   i) Ethyl acetate-methanol, 9:1 v/v

ii) Benzene-methanol, 7:3 v/v

instances causing a loss of a small portion of the chromatogram. However, with extreme care in spraying and by utilization of solvent systems i) and iii) excellent separation of the components of the bark extract was obtained.

"Precursor" studies

Populus species bark was investigated in an effort to detect the presence of any glycoside "precursor" material.

Since the bark samples had to be extracted for an extended period of time, Soxhlet extraction was utilized. Extraction was considered to be complete after 24 hours since no further color was then removed from the bark sample. Benzene was used as the extraction solvent since it does not affect the glycoside components during extraction (132). It was hoped to extract any polymeric components from the bark without subsequent decomposition of this material. Benzene is a poor extraction solvent for phenolic glycosides. Several peaks in the benzene residue could be attributed to glycosidic material but these components were present in very minimal quantities. Any water present in the benzene solvent would produce a hydrolytic effect on polymeric material present and the benzene was therefore dried over sodium for several days before use.

The residues from all benzene extracts appeared waxy in nature and tended to bead water. The residues also possessed an odor resembling that of rancid fats. When processing the benzene residue, the bulk of the benzene residue remains undissolved by water extraction and is filtered off. By removing one-tenth of the benzene residue and by suitable Tri-Sil dilutions, gas chromatographic analysis was ultimately performed on an extract equivalent to 1 mg. of bark sample. The weight of the corresponding treated (hydrolyzed) residue taken for GLC analysis was equal to the weight

of the benzene residue. In this way GLC analysis was being performed on equal quantities and, therefore, the traces could be approximately compared in respect to the peaks present and their concentrations. Accurate comparison was impossible since the benzene residue would contain many non-glycosidic and non-polymeric materials which the lead subacetate treatment would remove.

Hot water was utilized to extract the benzene residue. It was thought that this solvent would dissolve any "precursor" material present and also, if it caused any "precursor" hydrolysis, the glycosides produced would be soluble in the water. Various workers have utilized boiling water for the extraction of phenolic glycosides from plant samples (40,42,44,46-48) and it was desired to observe the effect of hot water on the benzene residue. Lead subacetate and hydrogen sulfide were utilized as hydrolytic agents since this was the process of purification of Salix extracts previously in common use (36,38,39) and it was desired to observe the effect of this process on the glycoside content of the extracts.

The filter pads were thoroughly washed after each filtration to prevent any material from being adsorbed.

Populus samples produced rather large amounts of benzene residue as seen in Table 20. The weights of the corresponding treated (hydrolyzed) residues are also seen in Table 20.

TABLE 20

WEIGHTS OF RESIDUES OBTAINED IN POPULUS "PRECURSOR" STUDIES

	BENZENE RESIDUE	TREATED RESIDUE
P. tremuloides	17.0 mg.	7.2 mg.
P. grandidentata	12.9 mg.	3.4 mg.
P. songarica	6.5 mg.	6.0 mg.

The weight of the treated residue was substantially less than

the benzene residue for P. tremuloides and P. grandidentata while both residues for P. songarica were approximately equal in weight. The difference could be attributed to the elimination of pigments, tannins and other materials. The two GLC chromatograms for P. tremuloides are presented in Figures 30 and 31 as an example of the results obtained from GLC analysis of the benzene and treated benzene residues.

The benzene residue of P. tremuloides indicated the presence of seven moderate peaks and two minor peaks from gas chromatographic analysis. Seven of the nine peaks were found to have decreased in concentration or have disappeared completely in the treated residue. Only three major peaks and three minor peaks were found by analysis of the treated benzene residue. Of the three major peaks, one was found as a moderate peak, one was found as a minor peak and one was found only as a trace peak in the benzene residue. These results are shown in Table 21. Similar peaks are presented on the same line to allow a comparison to be made of the content of the two residues.

Total area of the peaks from the benzene residue of Populus tremuloides was 7.905 cm^2 and for the treated residue was 44.196 cm^2. Therefore, there was approximately a six-fold increase in area of the peaks found in the treated residue. This increase in peak area cannot be accounted for simply by the decomposition of other glycosidic components present in the benzene residue to produce the two major glycosidic components found in the treated residue since there was such a substantial increase in total content. Besides many of the components of the benzene residue are non-glycosidic anyway. Thus, some material must have been hydrolyzed to produce these two glycosides in such a large concentration.

Gas chromatographic analysis of the benzene residue of Populus

FIGURE 30   GLC CHROMATOGRAM OF BENZENE RESIDUE FROM

P. tremuloides BARK ON OV-17 COLUMN

FIGURE 31   GLC CHROMATOGRAM OF TREATED RESIDUE FROM P. tremuloides BARK ON OV-17 COLUMN

- 117 -
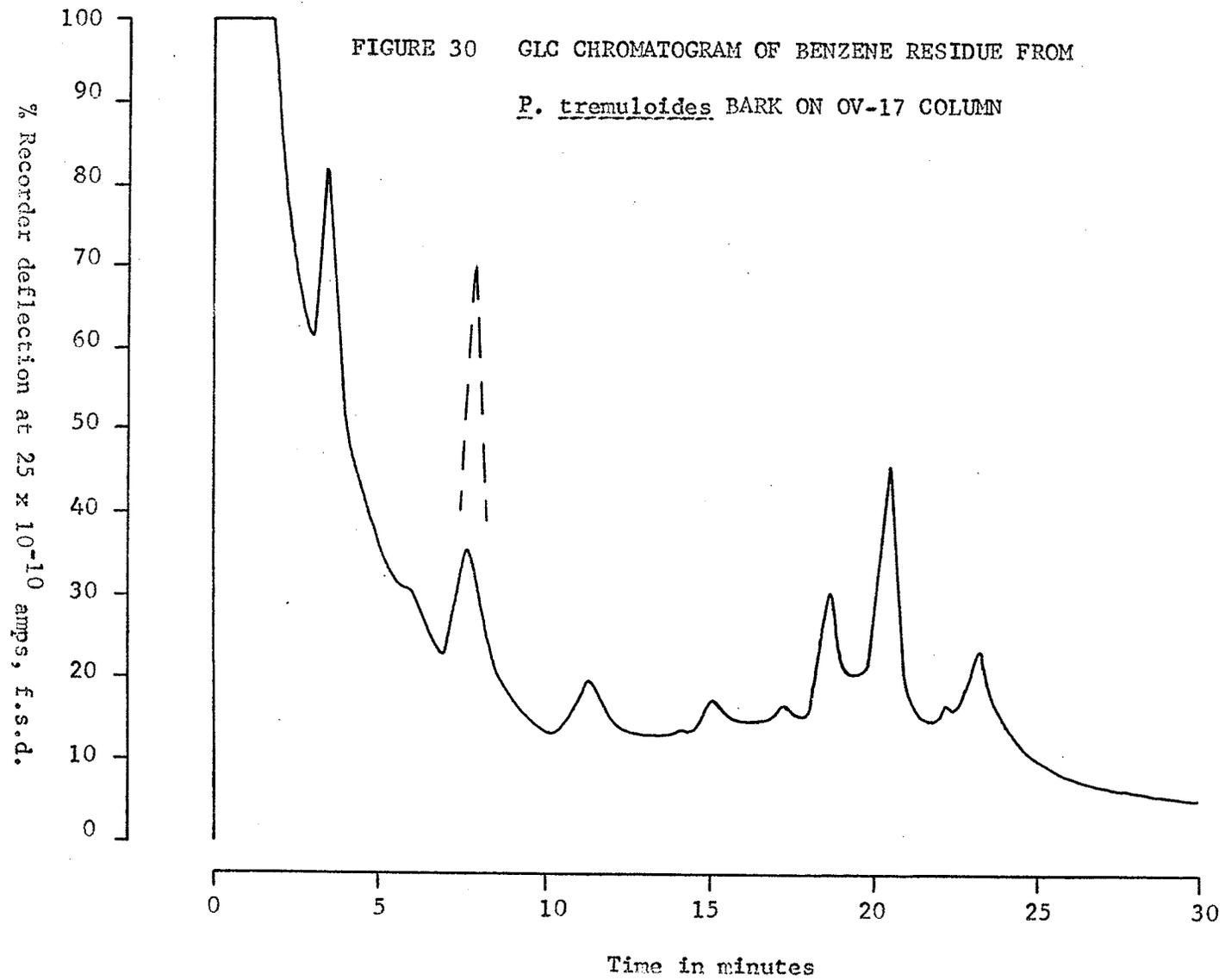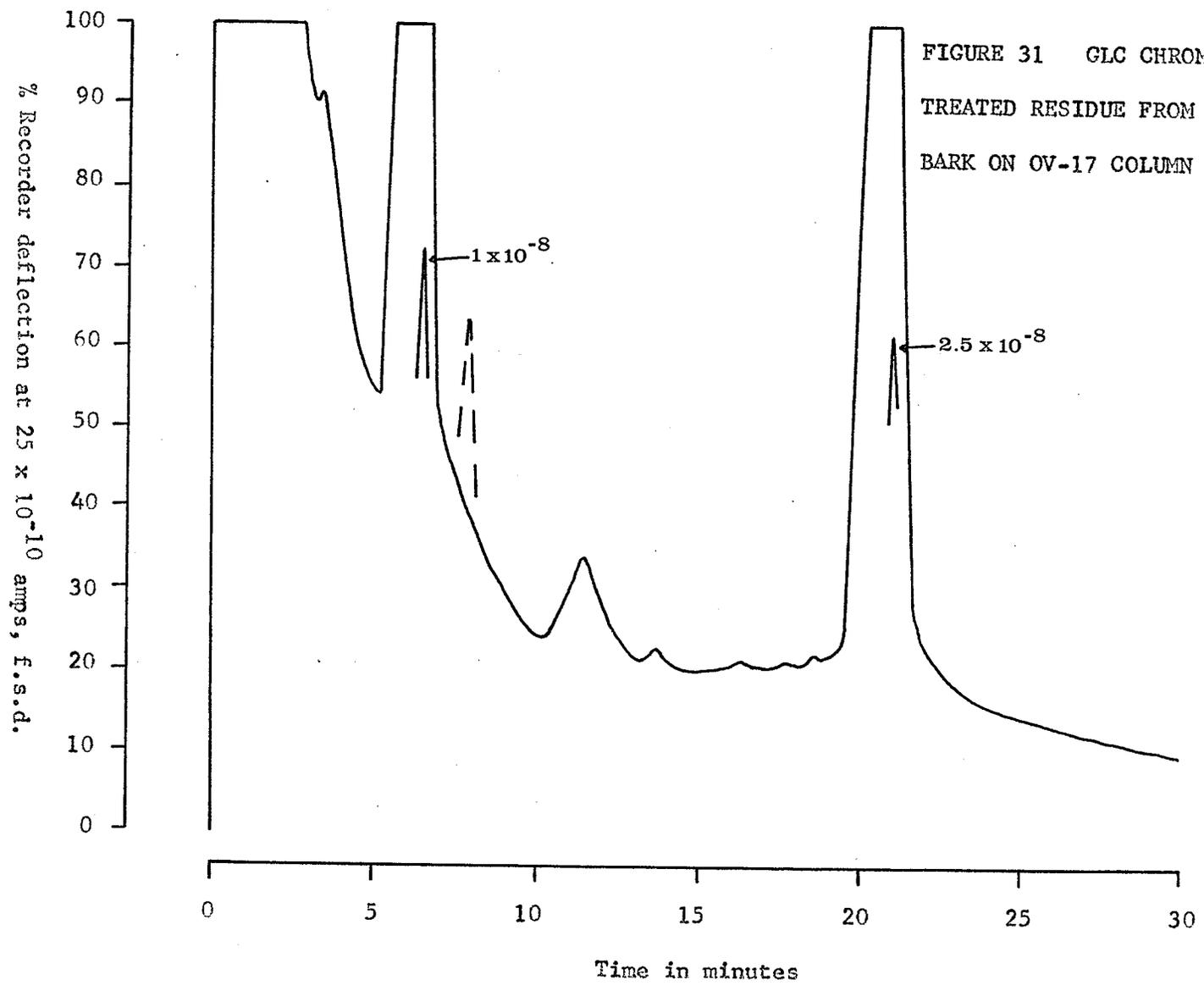
TABLE 21

COMPARISON OF PEAK AREAS FROM GLC OF POPULUS TREMULOIDES BARK

Benzene Residue	Treated Residue	Benzene Residue	Treated Residue
RELATIVE ARBUTIN TIME		PEAK AREA (cm^2)	
0.46	0.46	1.400	0.212
0.77	0.84	small peak	16.240
0.98	-	1.725	-
1.43	1.44	0.720	1.032
1.77	1.74	small peak	0.140
1.92	1.96	0.270	small peak
2.16	2.20	0.150	small peak
2.32	2.32	1.277	0.052
2.57	2.60	1.360	26.520
2.81	-	0.148	-
2.90	-	0.855	-

grandidentata sample indicated six peaks of moderate area and four peaks of minor area. It was seen that after treatment of the benzene residue five of these moderate peaks had either disappeared completely or decreased greatly in concentration. Analysis of the treated residue indicated only two peaks which were present in very large amounts. One of these major peaks was present in the benzene residue as a moderate peak while the other was present only as a minor peak. These results are presented in Table 22.

Total area of the peaks of the benzene residue of Populus grandidentata was 5.082 cm^2 while the total area of the peaks of the treated residue was 15.765 cm^2. Therefore, there was approximately a three-fold increase in area of the glycoside peaks found in the treated

TABLE 22

COMPARISON OF PEAK AREAS FROM GLC OF POPULUS GRANDIDENTATA BARK

Benzene Residue	Treated Residue	Benzene Residue	Treated Residue
RELATIVE ARBUTIN TIME		PEAK AREA (cm^2)	
0.45	0.47	0.405	small peak
0.76	0.79	0.160	8.645
0.97	-	0.330	-
1.45	1.47	0.190	small peak
1.76	1.79	0.180	small peak
1.92	1.96	0.180	small peak
2.16	-	0.210	-
2.33	2.37	1.187	small peak
2.57	2.59	0.960	7.120
2.92	-	1.280	-

residue. It is apparent that this considerable increase was due to the production of glycoside material from some source ("precursor") as stated before.

Analysis of Populus songarica produced slightly different results. Only four moderate peaks were found in the benzene residue analysis. All of these peaks were found to disappear during treatment and only one moderate peak and two trace peaks were detected by analysis of the treated residue. The moderate peak of the treated residue was present only as a trace peak in the GLC chromatogram of the benzene residue. Results of the GLC analysis of Populus songarica are presented in Table 23.

Total area of the peaks of the benzene residue was 1.222 cm^2 and for the treated residue was 0.620 cm^2. These results do not follow

TABLE 23

COMPARISON OF PEAK AREAS FROM GLC OF POPULUS SONGARICA BARK

Benzene Residue	Treated Residue	Benzene Residue	Treated Residue
RELATIVE ARBUTIN TIME		PEAK AREA (cm^2)	
0.45	0.46	0.195	small peak
0.75	0.76	small peak	0.620
1.91	1.93	0.632	small peak
2.35	-	0.270	-
2.92	-	0.125	-

the pattern indicated from the other Populus samples. The difference

in results obtained from Populus songarica could be due to the low glyco-

side content which is normally present in this species as compared to

the other Populus species. Populus songarica could also possess a dif-

ferent type of "precursor" which requires different conditions for

hydrolysis or it may have only a low content of "precursor" material.

The disappearance of many peaks from the treated residue,

previously found in the benzene residue, may indicate "purification"

processes due to the lead subacetate procedure. However, from the sub-

stantial increase in glycoside content found in the treated residue,

it seems clear that some form of "precursor" of glycosidic material

exists in some Populus species. This is in agreement with the work of

Pearl et al. (42,43,45).

2) Analysis of Salix bark

Analysis of the residues obtained from the three Salix species

studied (S. petiolaris, S. purpurea gracilis, S. purpurea nana), utilizing

the same treatment procedures as previously outlined for the Populus

investigation gave completely different results. The benzene residues

contained only minor amounts while the treated residues of all three

Salix species indicated very little or no glycoside content by GLC

analysis. Therefore, these three _Salix_ species were submitted to re-

examination using a different method for processing the benzene residues.

The benzene residue was extracted with hot aqueous ethanol

(60-70%) since it was thought that water alone may not have been effect-

ive in dissolving whatever "precursor" material occurs in the _Salix_

species. The mixed solvents were used at elevated temperatures to

further facilitate the solution of any glycosidic material in the ben-

zene residue. Lead subacetate and hydrogen sulfide gas were used as

before, except that they were added to warm extract solutions to accentu-

ate their hydrolytic effects. The weights of both crude and treated

residues obtained from the two investigations of _Salix_ species are pre-

sented in Table 24.

TABLE 24

WEIGHTS OF RESIDUES OBTAINED FROM SALIX "PRECURSOR" STUDIES

| | PREVIOUS EXPERIMENT (Page 113) | | PRESENT EXPERIMENT (Page 120) | |
	Benzene Residue	Treated Residue	Benzene Residue	Treated Residue
S. petiolaris	4.1 mg.	5.3 mg.	5.7 mg.	2.1 mg.
S. purpurea gracilis	4.4 mg.	5.7 mg.	4.1 mg.	5.1 mg.
S. purpurea nana	1.9 mg.	1.4 mg.	3.6 mg.	4.9 mg.

It can be seen that the weights of the benzene and treated

residue obtained for the same species were quite variable between the two

studies. The nature of the "precursor" material being examined is un-

known and it is not too surprising that these varying results were obtained.

It is possible that slight differences in extraction technique or in the

processing of the benzene residue could account for such differences.

The benzene residue from June Salix petiolaris indicated one
moderate peak and five minor peaks by gas chromatographic analysis.
Several trace peaks were also seen on the chromatogram. After treatment,
five of these peaks had either disappeared completely or had decreased
greatly in concentration. The treated residue indicated the presence of
two moderate and two minor peaks. Of the two moderate peaks, one was
not previously found in the benzene residue while the other was present
in the benzene residue as a minor peak. These results are listed in
Table 25.

TABLE 25

COMPARISON OF PEAK AREAS FROM GLC OF JUNE SALIX PETIOLARIS BARK

Benzene Residue	Treated Residue	Benzene Residue	Treated Residue
RELATIVE ARBUTIN TIME		PEAK AREA (cm^2)	
0.47	0.48	0.210	small peak
-	0.78	-	1.787
-	0.96	-	0.105
1.78	1.78	0.067	0.330
1.82	-	0.187	-
1.86	-	0.210	-
2.41	-	0.402	-
2.57	2.61	0.220	0.160

Total area of the peaks found in the benzene residue was
1.296 cm^2 and for the treated residue was 2.382 cm^2. Therefore, there
was a two-fold increase in the content in the treated residue, although
both total areas are small compared with the corresponding Populus areas.

GLC analysis of the benzene residue of Salix purpurea nana

indicated one moderate, one minor peak and several trace peaks. After treatment, both peaks had disappeared completely and only one moderate peak was found. These results are listed in Table 26.

TABLE 26

COMPARISON OF PEAK AREAS FROM GLC OF SALIX PURPUREA NANA BARK

Benzene Residue	Treated Residue	Benzene Residue	Treated Residue
RELATIVE ARBUTIN TIME		PEAK AREA (cm^2)	
-	0.77	-	0.325
1.00	-	0.800	-
1.85	-	0.127	-

Total area of the peaks found in the benzene residue was 0.927 cm^2 and in the treated residue was 0.325 cm^2. Therefore, there was a decrease in the content of the treated residue but the area of the peak obtained in the treated residue was produced by the treatment procedure since this peak was not previously present in the benzene residue. Both total areas were again very small.

Analysis of the benzene residue of Salix purpurea gracilis indicated two moderate, one minor peak and several trace peaks. After treatment, all these peaks had disappeared with the production of two moderate peaks in the treated residue. One of these peaks in the treated residue had been present as a small peak in the benzene residue while the other was not seen in the benzene residue. These results are listed in Table 27.

Total area of the peaks in the benzene residue was 2.020 cm^2 and of the treated residue was 1.701 cm^2. There was a slight decrease in content in the treated residue but the components of the treated residue were again produced by the treatment procedure, since they were

not present in the crude benzene residue. The GLC chromatograms of
the crude benzene and treated benzene residues of Salix purpurea gracilis
are given in Figures 32 and 33 as an example of the results obtained
from "precursor" studies on Salix species.

TABLE 27

COMPARISON OF PEAK AREAS FROM GLC OF SALIX PURPUREA GRACILIS BARK

Benzene Residue	Treated Residue	Benzene Residue	Treated Residue
RELATIVE ARBUTIN TIME		PEAK AREA (cm^2)	
0.48	0.48	0.120	small peak
-	0.77	-	1.440
1.03	-	0.560	-
1.81	-	1.340	-
2.58	2.61	small peak	0.261

The results obtained from this second study on the three Salix
species differs markedly from the results obtained from the first "precursor"
study performed on the same Salix species. The results of the second
"precursor" study (presented above in detail) were more in line with
the results obtained with the Populus species, although not of the same
magnitude, in that some glycosidic material was produced by the treatment
procedures. The difference in results between Populus and Salix species
may be due to any of a number of possible reasons. The "precursor"
material in Salix species may require longer extraction or stronger
hydrolysis to liberate glycosides or Salix species may only contain a
small amount of this "precursor".

A large scale extraction of May Salix petiolaris bark was per-
formed to obtain an adequate supply of the benzene residue for further
investigations. In the study of this benzene residue, several minor

FIGURE 32   GLC CHROMATOGRAM OF BENZENE RESIDUE FROM S. purpurea gracilis BARK ON OV-17 COLUMN

FIGURE 33   GLC CHROMATOGRAM OF TREATED RESIDUE FROM
S. purpurea gracilis BARK ON OV-17 COLUMN
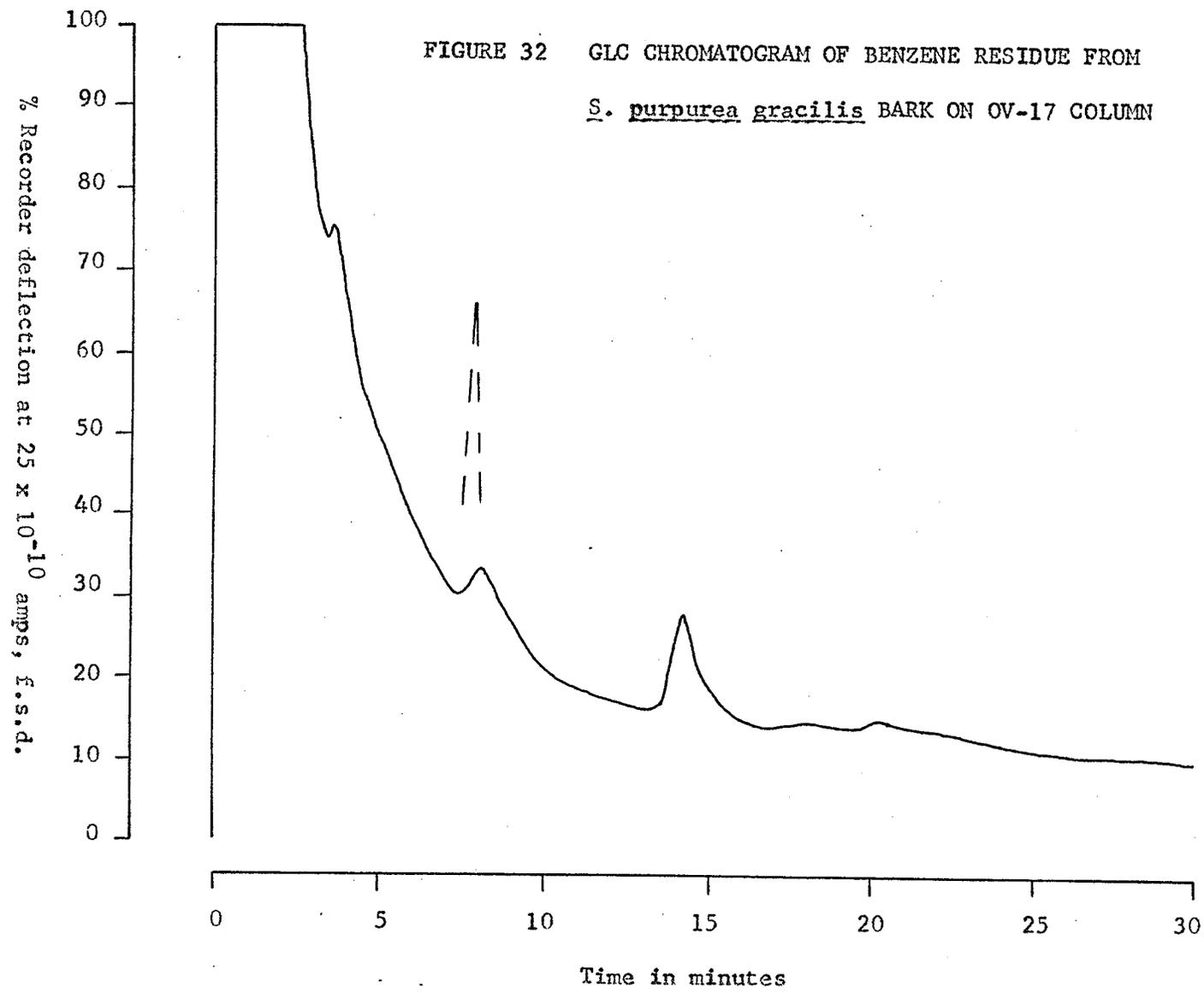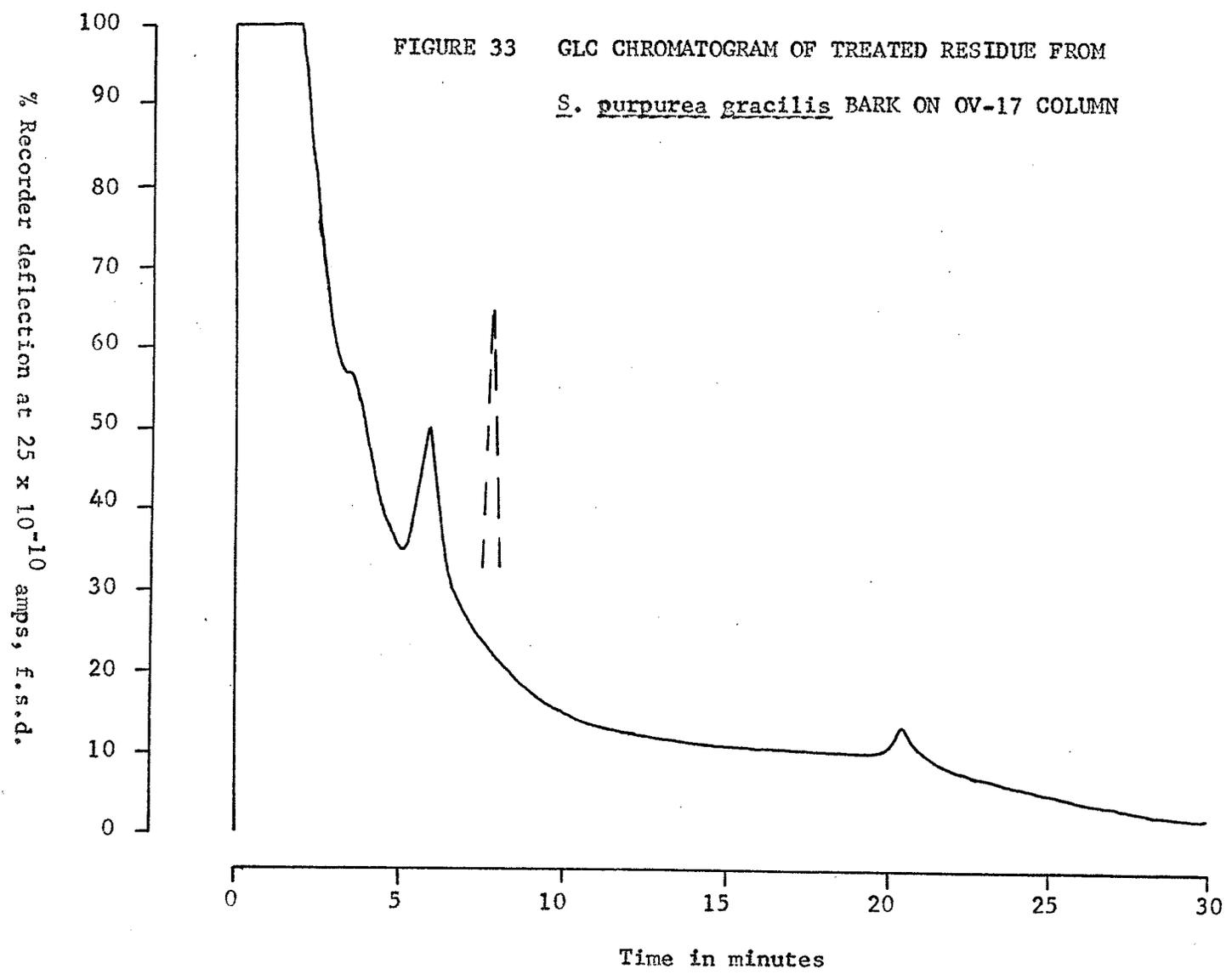
variations in hydrolysis procedures were introduced. In one such variation, boiling aqueous ethanol (60-70%) was added to the benzene residue and the lead subacetate solution was added directly so that the lead subacetate could come into contact with the solution and any undissolved benzene residue. Alcoholic-aqueous lead subacetate solution was used since it was thought that the effect of lead subacetate might be enhanced if it were in solution rather than in suspension. The solution was also boiled after addition of the lead subacetate to further enhance its action. By these manipulations the lead subacetate would have a greater chance of reacting with the components of the benzene residue and it was hoped that a greater hydrolytic effect might be produced.

Gas chromatographic analysis of the benzene residue obtained from the large scale extraction of _Salix petiolaris_ bark showed fourteen peaks present. Eight were of a major or moderate concentration, five were of minor concentration, one was a small peak and several trace peaks were seen. After treatment, nine of the peaks found in the benzene residue either decreased sharply in concentration or disappeared completely. Five of the peaks found in the benzene residue were found to have a greater concentration in the treated residue.

The treated residue indicated eleven peaks from gas chromatographic analysis. Nine were of a major or moderate concentration while one was minor in concentration and one was a small peak. Five of these peaks were present in the benzene residue in smaller concentrations, two were not present in the benzene residue while four were present in the benzene residue in greater amounts. These results are listed in Table 28.

Total area of the peaks of the benzene residue was 15.921 cm^2 and from the treated residue was 13.992 cm^2. A slight decrease in the area of the treated residue was found but several components of the treated residue

TABLE 28

COMPARISON OF PEAK AREAS FROM GLC OF MAY SALIX PETIOLARIS LARGE SCALE

EXTRACTION

Benzene Residue	Treated Residue	Benzene Residue	Treated Residue
RELATIVE ARBUTIN TIME		PEAK AREA (cm^2)	
-	0.46	-	1.818
0.68	-	0.272	-
0.76	0.78	0.999	7.890
1.00	1.00	2.100	0.370
-	1.30	-	0.060
1.50	1.48	0.165	0.304
1.80	1.75	2.180	0.658
1.96	1.93	1.977	0.756
2.19	2.16	0.161	small peak
2.32	2.33	small peak	0.444
2.41	-	3.720	-
2.61	2.60	0.160	1.340
2.71	2.75	0.104	0.352
2.82	-	3.683	-
3.12	-	0.175	-
3.16	-	0.225	-

were produced by the treatment procedure since they were present in

smaller concentrations in the benzene residue. It is apparent that lead

subacetate affected the glycosidic pattern between the two residues. In

this experiment, it was not certain whether this effect was due to hydro-

lysis of a "precursor", freeing glycoside material or due to chemical

changes in the composition of glycosidic material, such as migration

of acyl groups. It appears that the lead subacetate had a hydrolytic effect on some "precursor" material since the benzene extracted glycosides to a minimal extent and a change in the chemical composition of the glycosides found in the crude benzene residue could not account for the large peak areas for glycosides found in the treated benzene residue. The GLC chromatograms of the residues studied from the large scale extraction of May _Salix_ _petiolaris_ bark are presented in Figures 34 and 35.

Since June _Salix_ _petiolaris_ bark had already been studied for "precursor" content, analysis was performed on March and November bark samples to study the seasonal variation of the "precursor" material in _Salix_ _petiolaris_ bark.

The weights of the residues obtained in the study are listed in Table 29.

TABLE 29

WEIGHTS OF RESIDUES OBTAINED FROM "PRECURSOR" STUDIES ON SALIX PETIOLARIS BARK

MONTH	BENZENE RESIDUE	TREATED RESIDUE
March	8.3 mg.	10.7 mg.
June	5.7 mg.	2.1 mg.
November	4.9 mg.	8.1 mg.

Gas chromatographic analysis of the crude benzene and treated residues obtained from the November bark sample indicated no peaks. This result was rather surprising since all previous _Salix_ "precursor" studies have shown the presence of components in the benzene and/or the treated residue, although perhaps in minor concentrations. The November bark sample, however, was shown to contain a very high free glycoside content

FIGURE 34   GLC CHROMATOGRAM OF BENZENE RESIDUE FROM LARGE SCALE EXTRACTION OF MAY S. petiolaris BARK ON OV-17 COLUMN
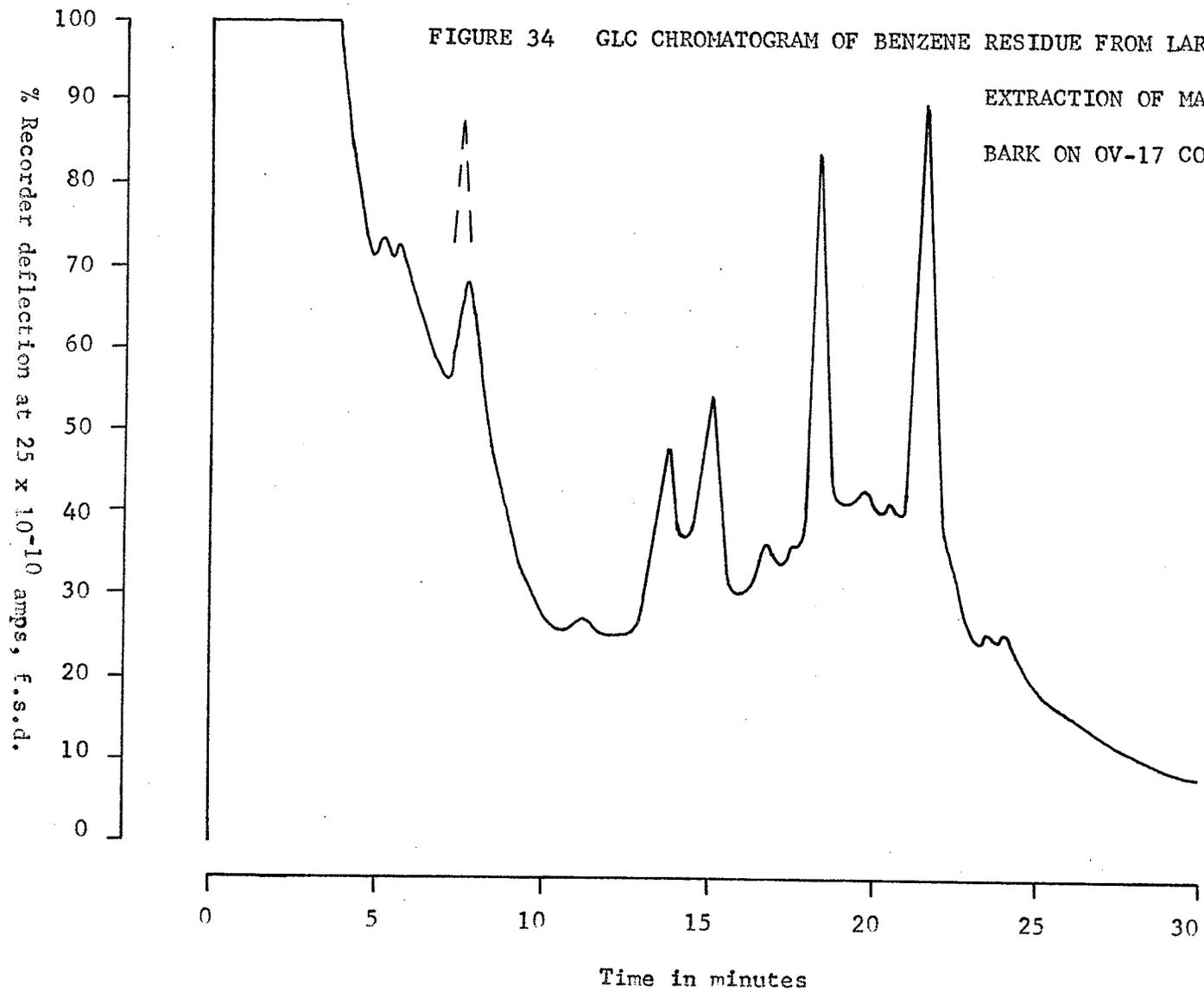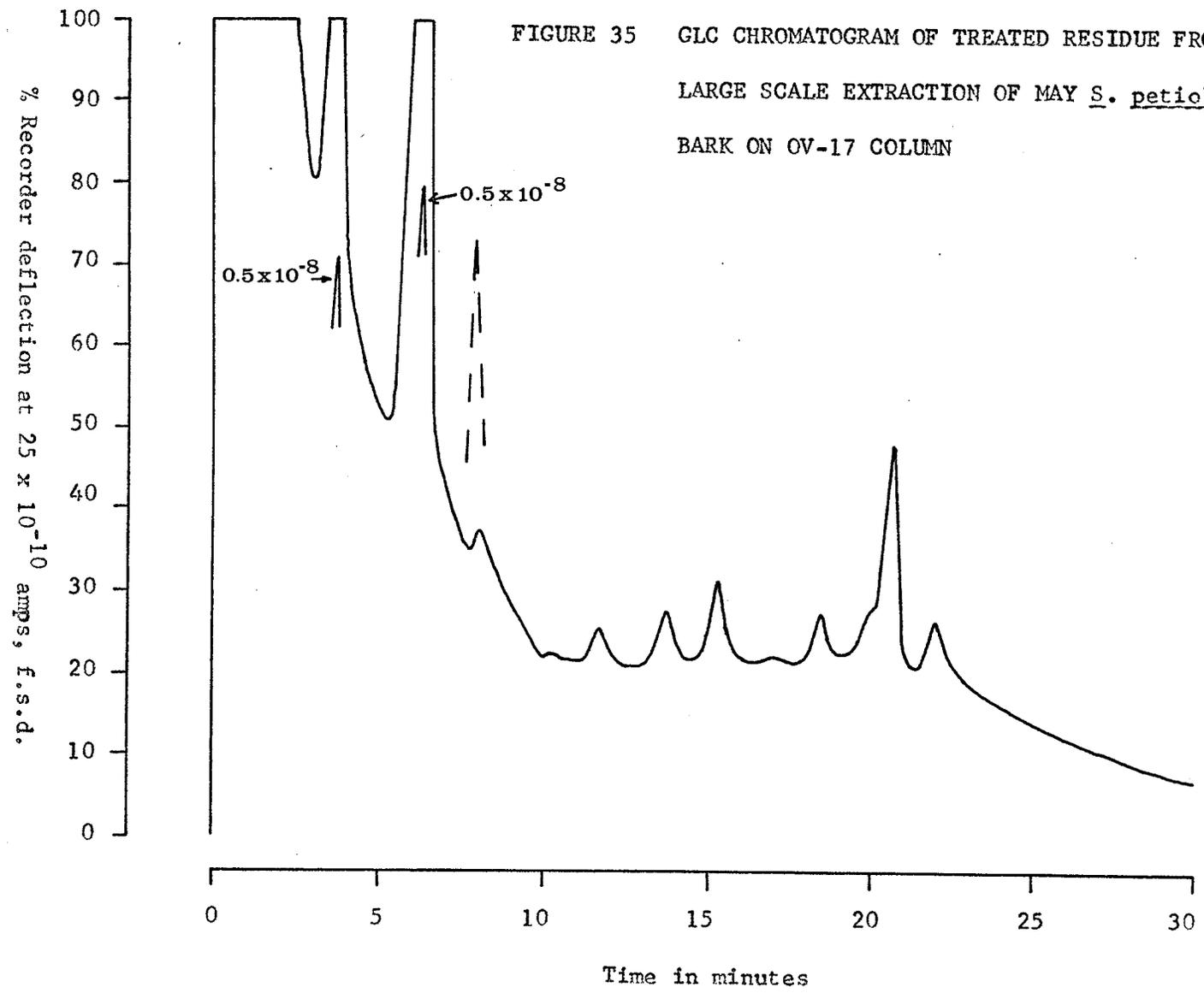
FIGURE 35 GLC CHROMATOGRAM OF TREATED RESIDUE FROM LARGE SCALE EXTRACTION OF MAY S. petiolaris BARK ON OV-17 COLUMN

(Analysis procedures, experiment 2).

Gas chromatographic analysis of the benzene residue of March bark indicated several peaks; four of moderate concentration, two of minor concentration and several trace peaks. After treatment, four of these peaks had disappeared, one had increased greatly in concentration and one had decreased slightly. The treated residue indicated three peaks - two moderate and one minor in concentration. All three of these peaks were found in the benzene residue chromatogram, one was larger, one was a moderate peak and one was a small peak in the benzene residue. These results are listed in Table 30.

TABLE 30

COMPARISON OF PEAK AREAS FROM GLC OF MARCH SALIX PETIOLARIS BARK

Benzene Residue	Treated Residue	Benzene Residue	Treated Residue
RELATIVE ARBUTIN TIME		PEAK AREA (cm^2)	
0.49	-	0.255	-
0.76	0.75	0.200	1.512
1.08	-	0.165	-
1.75	1.75	0.391	0.327
1.84	-	1.248	-
2.54	2.57	small peak	0.175
2.71	-	0.080	-

Total area of the peaks of the benzene residue was 2.339 cm^2 and for the treated residue was 2.014 cm^2. There was a slight decrease in the concentration to be found in the treated residue but since only three peaks existed as compared to six in the benzene residue there must be some production of glycosidic material in the treated residue.

It appears that November bark does not possess any "precursor"

material while the March and June samples do contain some "precursor" material. It is interesting to note the results of the free glycoside studies on these bark samples (Analysis procedures, experiment 2). The November bark sample has a high free glycoside content while June possesses a low content and March has the lowest free glycoside level of the three.

The effect of moisture in bark samples on the results obtained in "precursor" investigation was also studied. It was thought that if any moisture was present in the sample it would produce a hydrolytic effect on the bark components during benzene extraction and, therefore, produce erroneous results (ie. low "precursor" and high free glycoside content).

Gas chromatographic analysis of the benzene extract of dried November Salix petiolaris bark showed no peaks, similar to the findings for the November Salix petiolaris bark in the previous study. The benzene residue of the undried November bark sample indicated several peaks by GLC analysis, all of very small concentration.

Analysis of the treated benzene residues indicated analogous results. The treated residue of the dried bark indicated one peak of moderate concentration and several trace peaks. The treated residue of the undried bark indicated four peaks of moderate concentration and several trace peaks. Therefore, the undried bark sample possessed a greater content than the dried bark sample both in the benzene and treated residues. Possibly due to a hydrolytic effect, the presence of moisture in bark samples affected the results.

From these "precursor" studies, it is seen that there is some form of glycoside production, almost certainly by hydrolysis of a "precursor" substance. Several other workers in this laboratory have attempted to duplicate several of the "precursor" experiments presented in this

thesis and have obtained variable results. It appears that this "precursor" is very sensitive and may be a non-specific or loosely bonded type of material. These findings are similar to those of Pearl et al. and confirm the principle of a constituent which contains glycosides in a bound form. There is no evidence from the present work as to the exact nature of the bound form and Pearl's claim of "polymeric material" remains to be confirmed.

IV.   SUMMARY

1. Water, ethyl acetate, ethanol (95%), methanol and pyridine were found to be unsuitable for extraction of phenolic glycosides from Salix petiolaris samples.

2. In co-operation with other workers, a suitable procedure for the extraction of phenolic glycosides was developed. This method utilized acetone as the extraction solvent and the use of a water extraction step for purification of the extract.

3. Qualitative studies on the phenolic glycosides present in Salix petiolaris samples indicated the presence of salicin, fragilin, picein, triandrin, tremuloidin and salireposide in bark samples.

4. The seasonal variation of the phenolic glycosides in Salix petiolaris bark, wood and leaf samples was determined. Bark samples contained the highest glycoside content of all three samples with high levels of glycoside content in April, June, October and November. Low glycoside levels were found in the bark in March and August. Wood samples contained high glycoside levels in January and October with low levels in August. Leaf samples had a low content in May with the highest levels found in June and October. The levels of glycoside content in all samples were seen to vary considerably from month to month. No glycosides were found in flower or seed samples.

5. The center wood from October Salix petiolaris indicated a considerably lower, but definite presence of glycoside content, when compared to total wood samples for the same month.

6. Calibration graphs for three phenolic glycosides (salicin, picein and salireposide) found in bark samples were prepared. From these graphs, it was found that 1 mg. of November Salix petiolaris bark contained 1.8 μg. of salicin, 27.8 μg. of picein and 13.9 μg. of salireposide.

7. Two-dimensional thin-layer chromatographic studies produced good separation of the components of October and November _Salix_ _petiolaris_ extracts but the TLC results were not as effective as the GLC results in resolving the number of components.

8. The presence of some form of glycoside "precursor" material was determined in several _Populus_ and _Salix_ species. It was found that this "precursor" was quite variable in amount, composition and susceptibility to hydrolysis, from species to species. A seasonal variation was detected in "precursor" material and the presence of moisture in bark samples appeared to affect results.

V.   BIBLIOGRAPHY

1. Ball, C. R., Scientific Monthly 69, 48 (1949).

2. Wong, C. S. C., Univ. of Man. MSc thesis 1966.

3. Scoggan, H. J., Flora of Manitoba, National Museum of Canada,
    Bulletin #140, Minister of Northern Affairs and National Resources,
    p. 227, 1957.

4. Fernald, M. L., Gray's Manual of Botany 8th Ed., Am. Book Co.,
    N. Y. p. 516 (1950).

5. Thieme, H., Pharmazie 18, 770 (1963)
        through Chem. Abstr. 60, 9098e (1964).

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

7. Rabaté, J., Bull. Soc. Chim. Biol. 17, 439 (1935).

8. Thieme, H., Naturwissenschaften 50, 571 (1963).
        through Chem. Abstr. 59, 14225e (1963).

9. Thieme, H., Naturwissenschaften 50, 477 (1963)
        through Chem. Abstr. 59, 7853f (1963).

10. Thieme, H., Naturwissenschaften 51, 310 (1964)
        through Chem. Abstr. 61, 10918a (1964).

11. Thieme, H., Pharmazie 19, 725 (1964)
        through Chem. Abstr. 62, 7587h (1965).

12. Thieme, H., Naturwissenschaften 51, 360 (1964)
        through Chem. Abstr. 61, 12244f (1964).

13. Thieme, H., Naturwissenschaften 51, 217 (1964)
        through Chem. Abstr. 61, 3350h (1964).

14. Thieme, H., Pharmazie 21, 123 (1966)
        through Chem. Abstr. 65, 2521a (1966).

15. Thieme, H., Pharmazie 20, 436 (1965)
        through Chem. Abstr. 63, 11685g (1965).

16. Thieme, H., Naturw. Reihe 14, 748 (1965)
        through Chem. Abstr. 65, 2631d (1966).

17. Thieme, H., Pharmazie 20, 570 (1965)

   through Chem. Abstr. 64, 1013c (1966).

18. Thieme, H., Pharmazie 19, 471 (1964)

   through Chem. Abstr. 61, 8131h (1964).

19. Kytkowska, O., Herba Pol. 13, 177 (1967)

   through Chem. Abstr. 69(14), 54269n (1968).

20. Bukowiecki, H., Acta. Pol. Pharm. 25, 591 (1968)

   through Chem. Abstr. 70(13), 54809j (1969).

21. Kowalewski, Z. and Samula, K., Ann. Pharm. 6, 49 (1967)

   through Chem. Abstr. 71(18), 84492p (1969).

22. Kompantsev, V. A., Khim. Prirod. Soedin. 3, 183 (1969).

23. Kompantsev, V. A., Khim. Prirod. Soedin. 6, 381 (1968).

24. Kompantsev, V. A., Khim. Prirod. Soedin. 4, 323 (1969).

25. Bokarev, K. and Kefeli, V. I., Fiziol. Rast. 13, 274 (1966)

   through Chem. Abstr. 64, 20196h (1966).

26. Thieme, H., Tetrahedron Letters 19, 2301 (1968).

27. Thieme, H., Tetrahedron Letters 23, 2781 (1968).

28. Jarret, J. M. and Williams, A. H., Phytochem. 6, 1585 (1967).

29. Nasudari, A. A., Izv. Akad. Nauk. Azerb. SSR. Ser. Biol. Nauk.
   4, 97 (1964).

   through Chem. Abstr. 64, 13087h (1966).

30. Skrigan, A. I. and Vinokurov, I. I., Vesti Akad. Navuk. Belarus SSR.
   Ser. Khim. Navuk. 3, 72 (1969).

   through Chem. Abstr. 71(16), 73989e (1969).

31. Kowalewski, Z. and Samula, K., Acta Pol. Pharm. 24, 515 (1967)

   through Chem. Abstr. 68(10), 41223x (1968).

32. Thieme, H. and Benecke, R., Pharmazie 22, 59 (1967)

   through Chem. Abstr. 67(25), 117194r (1967).

33. Thieme, H. and Richter, R., Pharmazie 21, 251 (1966)

       through Chem. Abstr. 65, 5875c (1966).

34. Thieme, H., Planta Med. 15, 35 (1967).

35. Thieme, H. and Richter, R., Pharmazie 21, 252 (1966)

       through Chem. Abstr. 65, 5875d (1966).

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

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

38. Pearl, I. A. and Darling, S. F., J. Org. Chem. 27, 1806 (1961).

39. Pearl, I. A. and Darling, S. F., J. Org. Chem. 27, 2685 (1962).

40. Pearl, I. A. and Kinsley, H., Tappi 50, 419 (1967).

41. Pearl, I. A. and Darling, S. F., J. Org. Chem. 28, 1442 (1963).

42. Pearl, I. A. and Pottenger, C. R., Tappi 49, 152 (1966).

43. Pearl, I. A. and Darling, S. F., Tappi 50, 324 (1967).

44. Pearl, I. A. and Darling, S. F., Tappi 50, 193 (1967).

45. Pearl, I. A. and Estes, T. K., Tappi 50, 318 (1967).

46. Pearl, I. A. and Darling, S. F., Phytochem. 7, 1855 (1968).

47. Pearl, I. A. and Darling, S. F., Phytochem. 7, 1851 (1968).

48. Pearl, I. A. and Darling, S. F., Phytochem. 7, 1845 (1968).

49. Pearl, I. A. and Darling, S. F., Tappi 49, 278 (1966).

50. McInnes, A. G. and Yoshida, S., Tetrahedron 21, 2939 (1965).

51. Kováč, P. and Rendoš, F., Chem. Zvesti 22, 198 (1968)

       through Chem. Abstr. 68(22), 96911a (1968).

52. Karácsonyi, Š. and Kubačková, M., Coll. Czech. Chem. Comm. 32, 3597 (1967).

53. Karácsonyi, Š. and Kubačková, M., Coll. Czech. Chem. Comm. 34, 2002 (1969).

54. Pesheva, M. and Blazhei, A., Kozhi Obuvki (Sofia) 5, 1 (1964)

       through Chem. Abstr. 63, 6017e (1965).

55. Thieme, H., Pharmazie 19, 535 (1964)

       through Chem. Abstr. 61, 12329d (1964).

56. Skrigan, A. J., Murashkevich, T. V. and Pershina, G., Khim. Prirod. Soedin. <u>3</u>, 392 (1967).

   through Chem. Abstr. <u>68(22)</u>, 96894x (1968).

57. Peña, J., An. Edafol. Agrobiol. <u>27</u>, 681 (1968)

   through Chem. Abstr. <u>70(15)</u>, 65121e (1969).

58. Vieitez, E. and Seoane, E., Phys. Plant. <u>20</u>, 232 (1967).

59. Vazquez, A. and Méndez, J., Phytochem. <u>7</u>, 161 (1968).

60. Jaggi, J. and Haslam, E., Phytochem. <u>8</u>, 635 (1969).

61. Binns, W. W., Blunden, G. and Woods, D., Phytochem. <u>7</u>, 1577 (1968).

62. Zeitler, H. S. and Sauer, F., Z. Physiol. Chem. <u>348</u>, 1401 (1967).

63. Gardner, D. C. J. and Peel, A. J., Nature <u>222(56253)</u>, 774 (1969).

64. Dytkowska, O., Farm. Polska <u>22</u>, 33 (1966)

   through Chem. Abstr. <u>65</u>, 9341b (1966).

65. Kripiakevich, T. A., Seminar, School of Pharmacy, Univ. of Manitoba 1969.

66. Dobrowolska, B. and Twardowska, K., Dissert. Pharm. <u>17</u>, 537 (1965)

   through Chem. Abstr. <u>64</u>, 17358c (1966).

67. Sticzay, T. and Peciar, C., Tetrahedron <u>25</u>, 3521 (1969).

68. Madhavan Nair, P., Bull. Nat. Inst. Sci. India <u>31</u>, 1 (1965)

   through Chem. Abstr. <u>66(9)</u>, 36974x (1967).

69. Mirolyubova, S. P. and Shemyakin, F. M., Farmatsiya <u>17</u>, 74 (1968)

   through Chem. Abstr. <u>68(22)</u>, 98657w (1968).

70. Gorovets, T. T., Khim. Prirod. Soedin. <u>1</u>, 49 (1969).

71. Davison, P. K. and Young, R., J. Chromatog. <u>41</u>, 12 (1969).

72. Sweeley, C. C. and Bentley, R., J. Am. Chem. Soc. <u>85</u>, 2497 (1963).

73. Bishop, C. T., Adv. Carb. Chem. <u>19</u>, 95 (1964).

74. Bolan, M. and Steele, J. W., J. Chromatog. <u>36</u>, 22 (1968).

75. Daniels, N. W. R., Process Biochem. <u>3</u>, 34 (1968)

   through Chem. Abstr. <u>68(18)</u>, 84161t (1968).

76. Dallos, F. C., Lautenbach, A. F. and West, D., Am. Soc. Brew. Chem. Proc. p. 103 (1967).

   through Chem. Abstr. 69(3), 9669g (1968).

77. Méndez, J., J. Gas Chromatog. 6, 168 (1968).

78. Bakke, O. M. and Scheline, R. R., Analyt. Biochem. 27, 439 (1969).

79. Aspinall, G. O., J. Chem. Soc. 314, 1676 (1963).

80. Lenton, J. R., Nature 220(5162), 86 (1968).

81. Furuya, T., J. Chromatog. 18, 152 (1965).

82. Pellizzari, E. D., J. Chromatog. 40, 285 (1969).

83. Kochetkov, N. K. and Chizhov, O. S., Biochim. Biophys. Acta 83, 134 (1964).

84. Kochetkov, N. K. and Wulfson, N. S., Tetrahedron 19, 2209 (1963).

85. Golovkina, L. S., Izvest. Akad. Nauk. SSR. Serkin 7, 1915 (1966).

86. Heyens, K., Fortschr. Chem. Forsch. 5, 448 (1966).

87. Haslam, E., Carbohyd. Res. 5, 161 (1967).

88. Pearl, I. A. and Darling, S. F., Phytochem. 7, 831 (1968).

89. Thieme, H., Naturwissenschaften 51, 291 (1964)

   through Chem. Abstr. 61, 7090g (1964).

90. Thieme, H., Pharmazie 21, 769 (1966)

   through Chem. Abstr. 67(15), 73840s (1967).

91. Pearl, I. A. and Darling, S. F., Phytochem. 7, 821 (1968).

92. Pearl, I. A. and Darling, S. F., Phytochem. 7, 825 (1968).

93. Cooley, D., "News and Views of Health Medicine Pharmacy", Pharmaceutical Manufacturers Assoc. of Canada 3, (1966).

94. Pridham, J. B., Phenolics Plant Health Disease Proc., Plant Phenolics Group Symp., Bristol (1959) p. 9.

95. Khristov, J. and Draganova, R., Khim. Ind. (Sofia) 37, 370 (1965)

   through Chem. Abstr. 64, 16116c (1966).

96. Pearl, I. A., Tappi 52, 428 (1969).

97. Davis, J. D. and Evert, R. F., Bot. Gaz. $\underline{129}$, 1 (1968).

98. Tissut, M., Physiol. Veg. $\underline{6}$, 351 (1968)

    through Chem. Abstr. $\underline{70(21)}$, 93955y (1969).

99. Dalungu, J. C., Diss. Abstr. B. $\underline{29}$, 2701 (1969)

    through Chem. Abstr. $\underline{71(3)}$, 10245p (1969).

100. Gerola, M. and Grill, M., Ann. Fac. Agraria, Univ. Studi, Milano $\underline{8}$, 83 (1963)

    through Chem. Abstr. $\underline{60}$, 4458d (1964).

101. Vieitez, E. and Peña, J., Physiol. Plant. $\underline{21}$, 544 (1968).

102. Thieme, H., Pharmazie $\underline{20}$, 688 (1965)

    through Chem. Abstr. $\underline{64}$, 11552c (1966).

103. Pearl, I. A. and Darling, S. F., Phytochem. In press

    through Tappi $\underline{52}$, 428 (1969).

104. Pearl, I. A. and Darling, S. F., Arch. Biochem. Biophys. $\underline{102}$, 33 (1963).

105. Pearl, I. A. and Estes, T. K., Tappi $\underline{48}$, 532 (1965).

106. Charaux, C. and Rabaté, J., J. Pharm. Chim. $\underline{(9)2}$, 289 (1947)

    through Chem. Abstr. $\underline{38}$, 2327$_8$ (1944).

107. Pearl, I. A. and Darling, S. F., Tappi $\underline{48}$, 607 (1965).

108. Pearl, I. A. and Darling, S. F., Tappi $\underline{48}$, 506 (1965).

109. Pearl, I. A. and Darling, S. F., Tappi $\underline{47}$, 377 (1964).

110. Thieme, H., Planta Med. $\underline{13}$, 431 (1965).

111. Yamaha, T. and Cardini, C. E., Arch. Biochem. Biophys. $\underline{86}$, 127 (1960).

112. Pridham, J. B. and Saltmarsh, M. J., Biochem. J. $\underline{74}$, 42p (1960).

113. Pridham, J. B. and Saltmarsh, M. J., Biochem. J. $\underline{87}$, 218 (1963).

114. Pridham, J. B., Chem. and Ind. 1172 (1961).

115. Psenak, M. and Kovács, P., 9th Annual Meeting of Am. Soc. of Pharmacog. (1968).

116. Hopkinson, S., Quart. Rev. $\underline{23}$, 98 (1969).

117. Peel, A. J., J. Exptl. Bot. 15, 104 (1964).

118. Peel, A. J., J. Exptl. Bot. 17, 156 (1966).

119. Peel, A. J. and Ford, J., J. Exptl. Bot. 20, 536 (1969).

120. Peel, A. J., Annals of Botany 33, 743 (1969).

121. Argus, G. W., Can. J. Botany 47, 795 (1969).

122. Binns, W. and Blunden, G., Phytochem. 8, 1235 (1969).

123. Repas, A. and Nikolin, B., J. Chromatog. 35, 99 (1968).

124. Repas, A., J. Chromatog. 44, 184 (1969).

125. Pridham, J. B., Analyt. Chem. 29, 1167 (1957).

126. Audette, R. C. S., Blunden, G., Steele, J. W. and Wong, C. S. C.,
    J. Chromatog. 25, 367 (1966).

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

128. Pearl, I. A., Justman, O., Beyer, D. L., and Whitney, D.,
    Tappi 45, 663 (1962).

129. Pearl, I. A. and Larson, K., Tappi 48, 714 (1965).

130. Steele, J. W., Bolan, M., Audette, R. C. S., and Kripiakevich, T. A.,
    Unpublished results.

131. Thompson, A. and Wolfrom, M. L., Carbohydrates - Chemistry, Biochemistry,
    Physiology, Acad. Press, N. Y. p. 147 (1957).

132. Steele, J. W., Bolan, M. and Audette, R. C. S., J. Chromatog.
    40, 370 (1969).

133. Beckett, A. H., Tucker, G. T. and Moffat, A. C., J. Pharm. Pharmacol.
    19, 273 (1967).

PART A

I

II

III

IV

V

VI

CH=CH-CH₂-O structure with sugar ring, labeled **VII**

VII

structure labeled **VIII**

VIII

structure labeled **IX**

IX

structure labeled **X**

X

structure labeled **XI**

XI

XII

XIII

XIV

XV

XVI

XVII

XVIII

XIX

XX

XXI

6-ß-D-XYLOSE-ß-D-GLUCOSE—O

XXII

ß-D-GLUCOSE

XXIII

ß-D-GLUCOSE—O
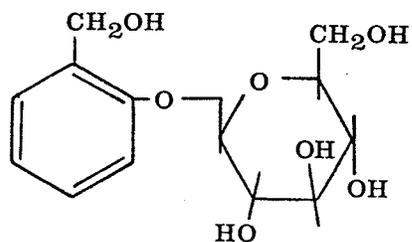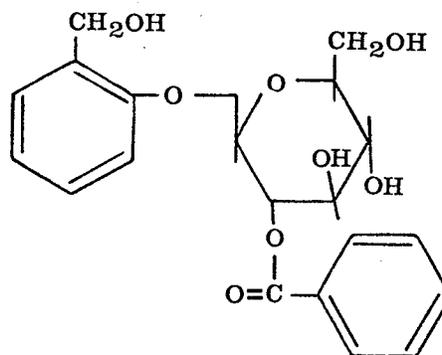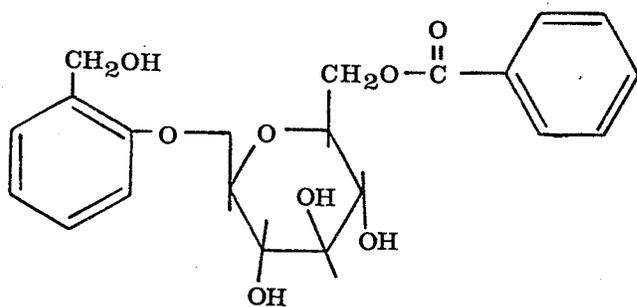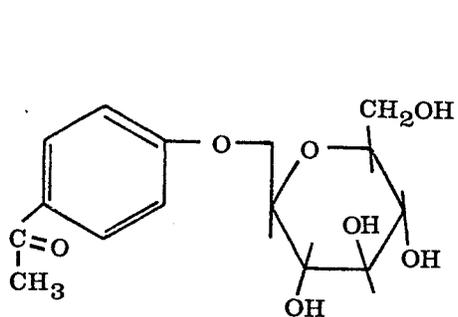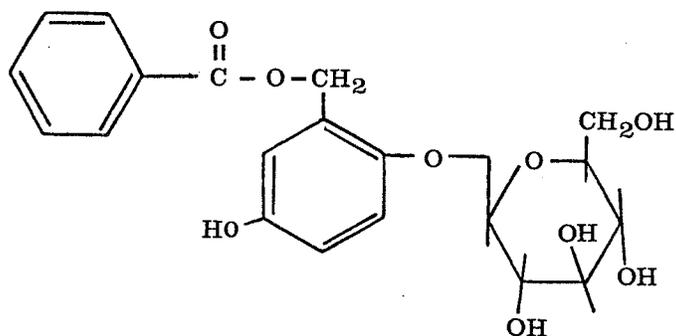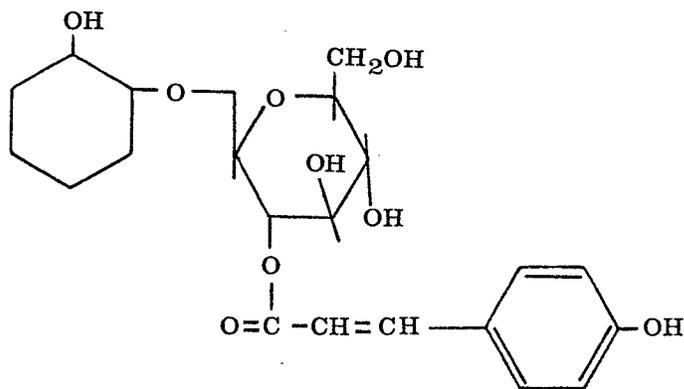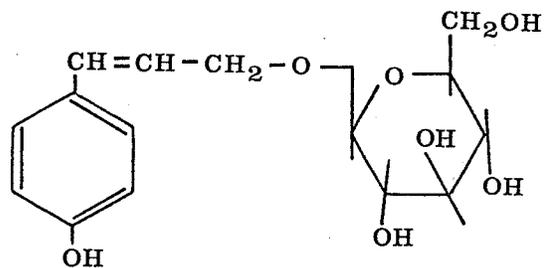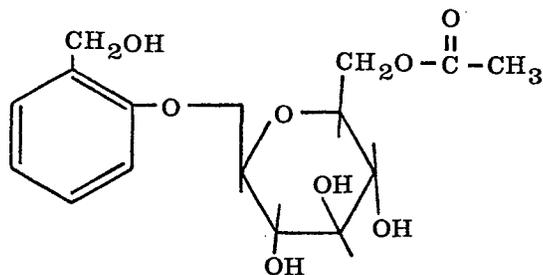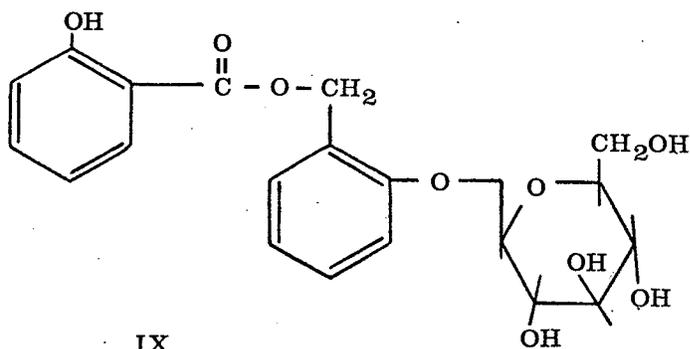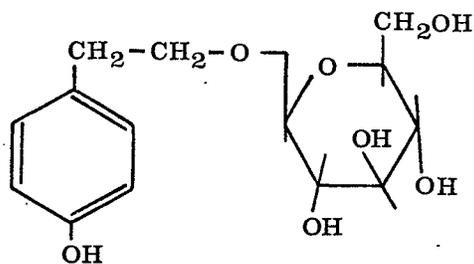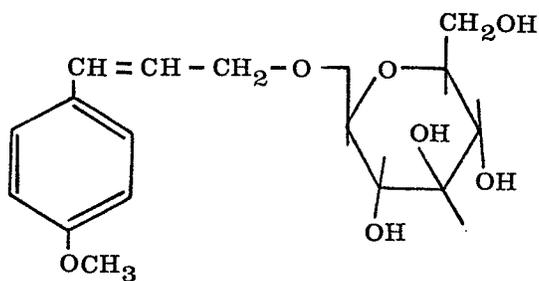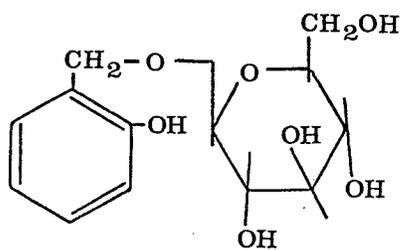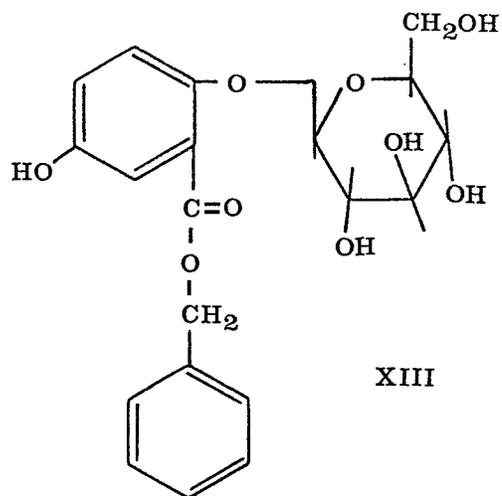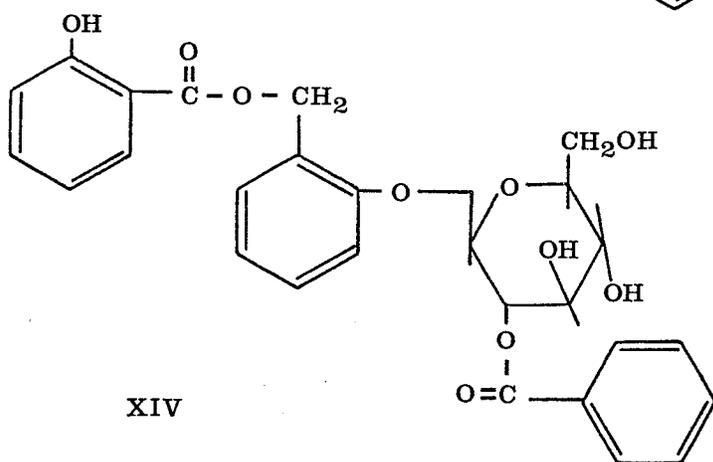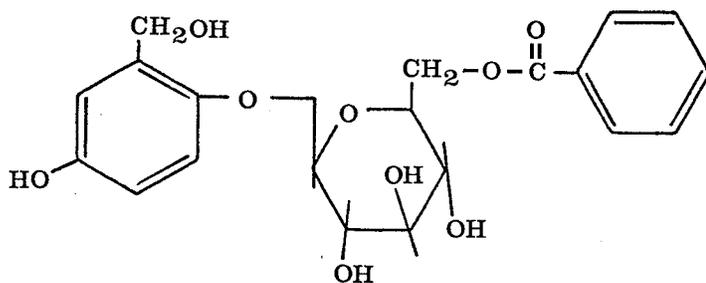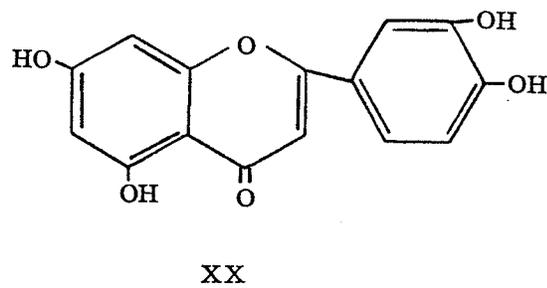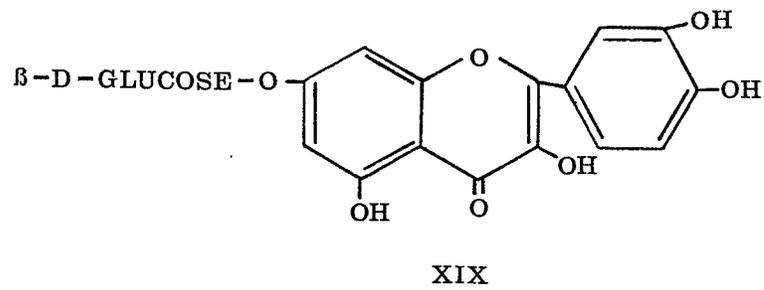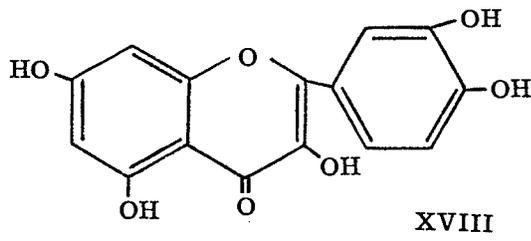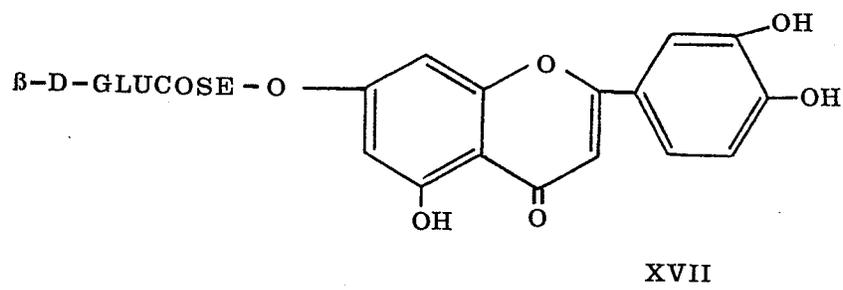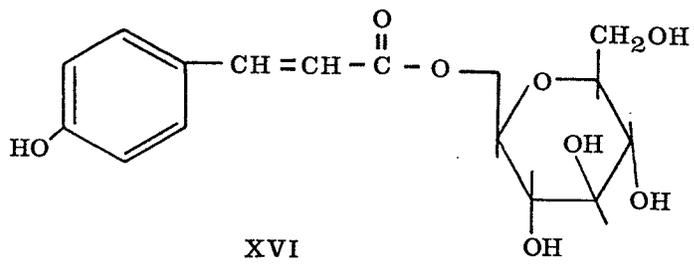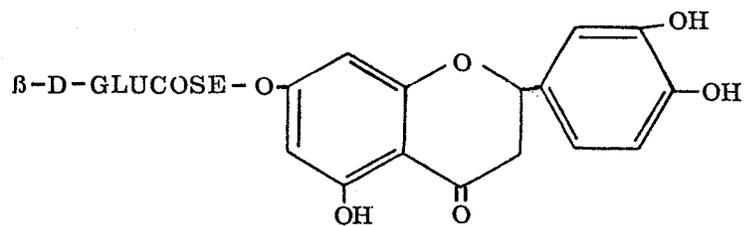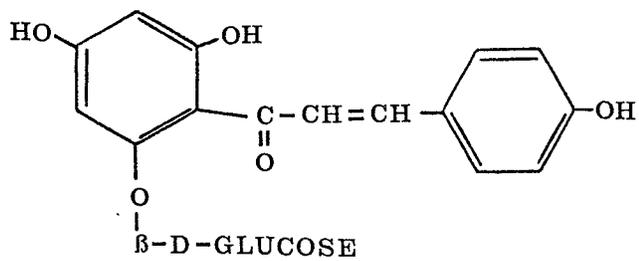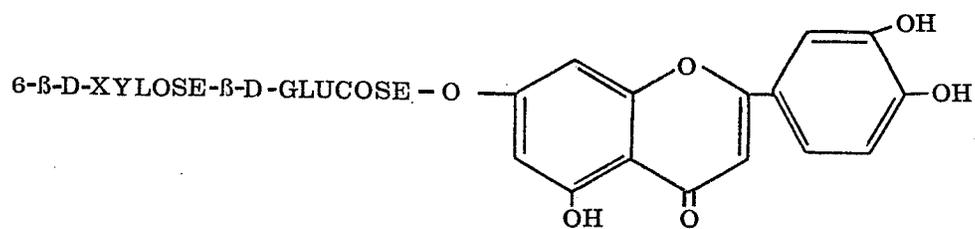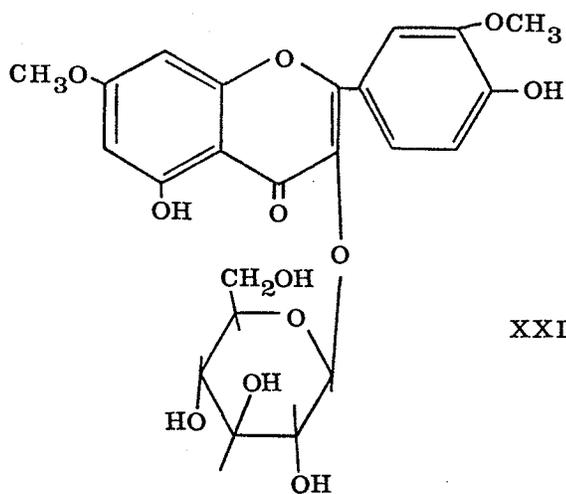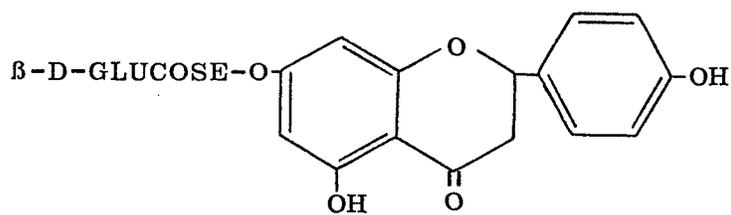
XXIV

XXV
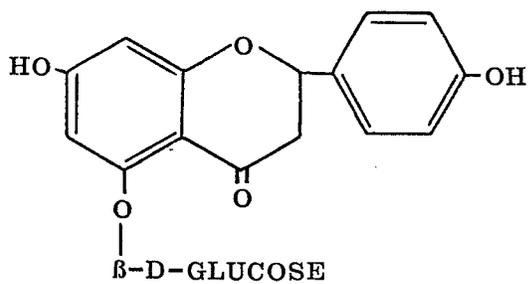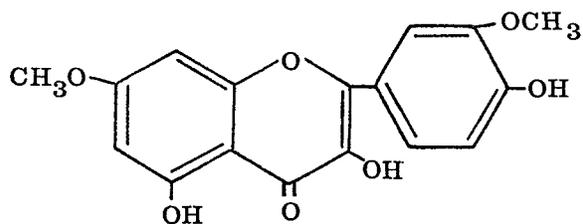


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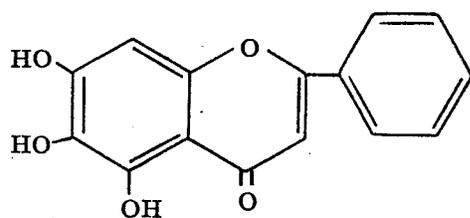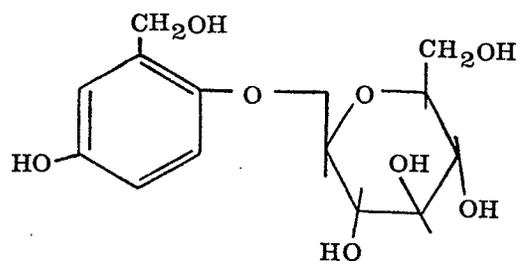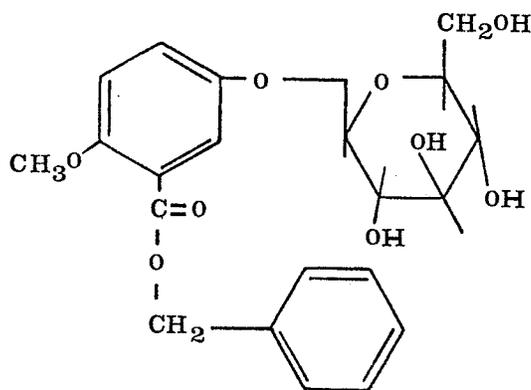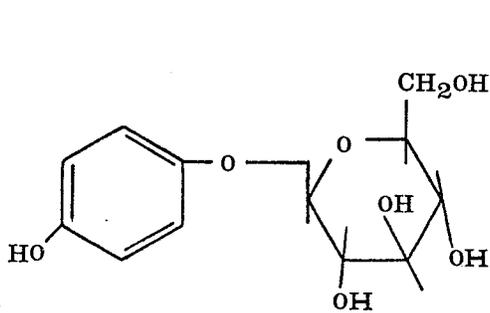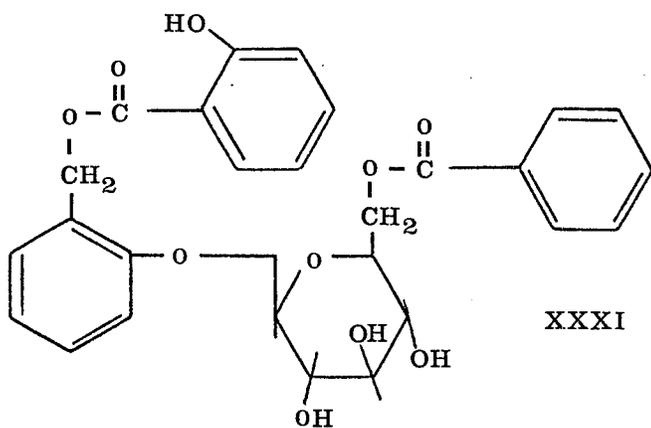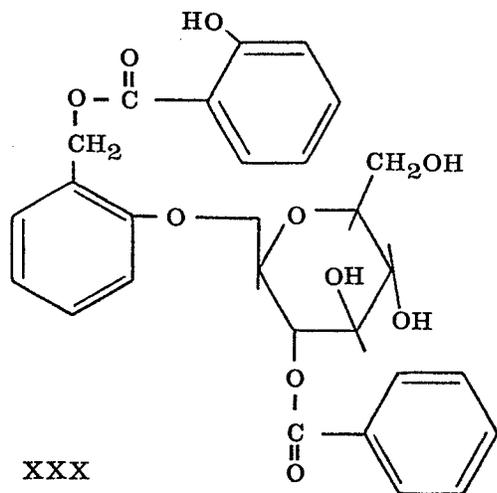
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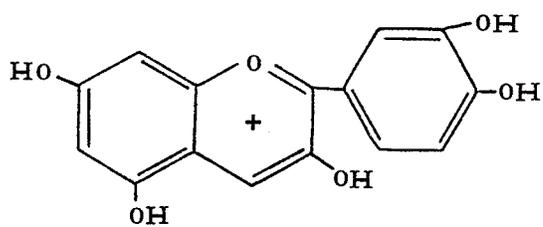
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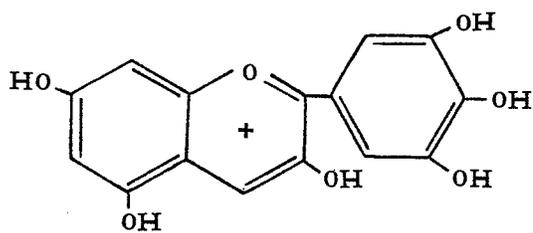
XXXV

PART B

Preparation of strong lead subacetate solution

Strong lead subacetate solution was prepared according to the 1963 British Pharmacopoeia. This is a solution which contains 20% w/w lead.

PART C

Preparation of alcoholic-aqueous lead subacetate solution

Ethanol (95%) was added to strong lead subacetate solution and the solution was boiled until it was clear.