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PHYTOCHEMISTRY OF *SALIX* SPECIES

I. A GAS–LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE SEPARATION AND IDENTIFICATION OF PHENOLIC GLYCOSIDES

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SUMMARY

A method for the gas chromatographic analysis of ten phenolic glycosides commonly found in *Salix* species is described. These compounds can be successfully separated using cyclohexanedimethanol succinate on Chromosorb G, with the exception of fragilin and salidroside. The latter two compounds can be separated using the other columns described. Infrared and ultraviolet data of some fractions collected are also given and the methods are applied to a crystalline fraction of a crude glycoside extract from a species of *Salix*.

INTRODUCTION

Methods for the separation and identification of microquantities of phenolic glycosides are of great importance for work in phytochemistry, pharmacognosy and chemotaxonomy. The thin-layer chromatography procedures normally used[1] are neither readily adaptable to quantitative and preparative work, nor do they possess the high sensitivity which is often required.

Recently, FURUYA[2] developed a method for the gas–liquid chromatography of the trimethylsilyl derivatives of seventeen phenolic glycosides from different plant species, including salicin from *Salix* species, on an SE-30 column of low liquid phase loading.

As part of an investigation of the phytochemistry of *Salix* species native to Manitoba, a gas–liquid chromatographic procedure has been developed for the separation and identification of ten phenolic glycosides commonly found in this genus[3,4]. This paper describes the procedure, including methods for fraction collection of the individual components of a mixture of phenolic glycosides and subsequent infrared and ultraviolet analysis.

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EXPERIMENTAL

A Beckman model GC-4 gas chromatograph, equipped with a flame ionisation detector and a temperature programmer, was used. The signal was recorded on a Beckman linear, potentiometric recorder set to a range of 1 mV. Helium was used as the carrier gas throughout the work.

A preliminary investigation showed that the best results were obtained with supports which had been acid washed and treated with dimethyldichlorosilane.

Column packings were prepared by adaptation of a method recommended by the manufacturer (Johns-Manville). For Chromosorb G packings, one part by weight of the support was soaked, with stirring, in five parts by volume of a solution containing double the required percentage of liquid phase in a suitable solvent. After 15 min the mixture was vacuum filtered, and the wet packing was transferred to a fluid-bed dryer (Applied Science Labs., Inc.) and dried in a stream of dry nitrogen preheated to 50°. The Chromosorb W packing was prepared similarly, except that the support was soaked in a solution containing half the required percentage of liquid phase. After drying, the packings were screened to the given mesh range to remove fines which had formed during the coating procedure.

To check these packing procedures, the volume of filtrate from the mixture was measured, and the weight of liquid phase remaining on the wet packing was calculated. The results showed that the percentage of liquid phase remaining on the support was very close to the expected value.

The columns, 1/8 in. O.D. grade 304 stainless steel tubes, were packed prior to coiling. Each column was then conditioned for 12 h at the maximum usable temperature of the relevant liquid phase, while helium was passed through it at a flow rate of 100 ml per minute.

The packings used in this work were 0.3% OV-1 on Chromosorb G AW/DMCS 60/80 mesh, 0.3% OV-17 on Chromosorb G AW/DMCS 60/80 mesh, 1.5% diethylene glycol succinate (DEGS) on Chromosorb W AW/DMCS 80/100 mesh and 0.5% cyclohexanedimethanol succinate (CHDMS) on Chromosorb G AW/DMCS 60/80 mesh.

Temperature programmes were determined empirically for each column. The following programmes were used:

(I) 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.

(II) For OV-17, start at 190°, hold for 10 min, then 6° per min rise to 250° and hold until the last component is eluted.

(III) For DEGS, start at 130° and hold for 10 min, then 4.55° per min rise to 175.5° and hold for 10 min. Finally, 4.9° per min rise to 195° and hold until the last component is eluted.

(IV) For CHDMS, start at 180° and hold for 10 min, then 2° per min rise to 198°. Hold for 5 min then 5.25° per min rise to 240° and hold until the last component is eluted.

Dual compensatory columns were only necessary for the CHDMS system.

Each glycoside was injected into the chromatograph as its trimethylsilyl ether derivative, prepared by dissolving 0.01 to 0.05 mg of the pure glycoside in 50 μl of Tri-Sil (Pierce Chemical Co.) and, after shaking, allowing the mixture to stand at room

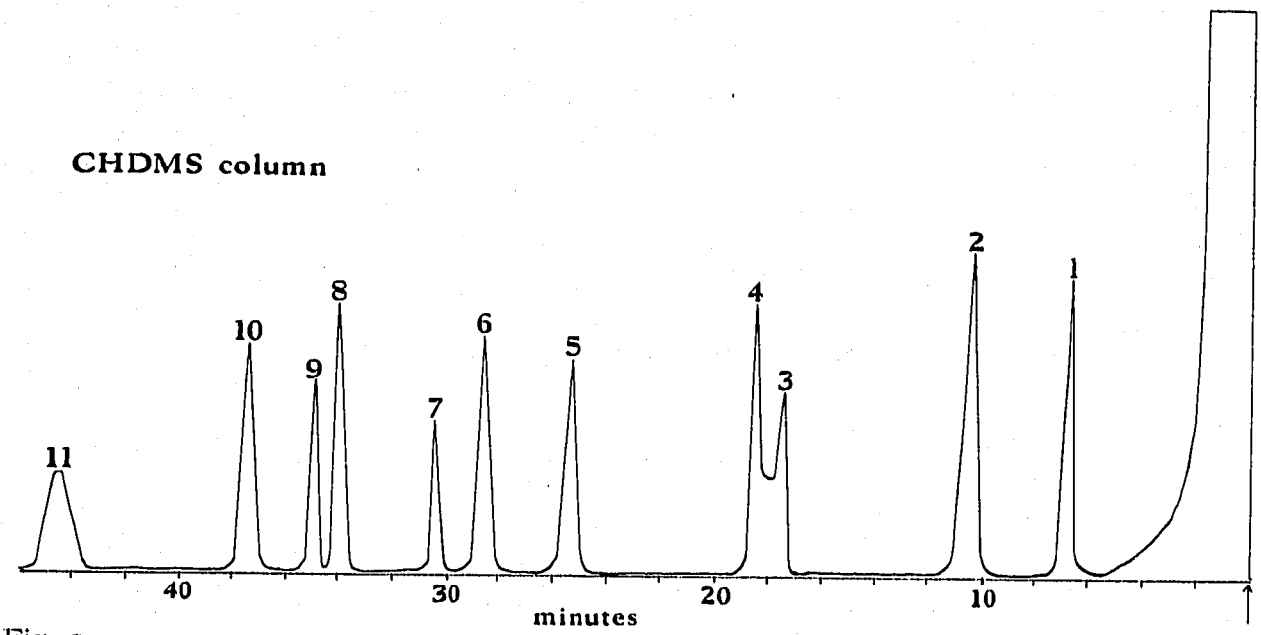


Fig. 1

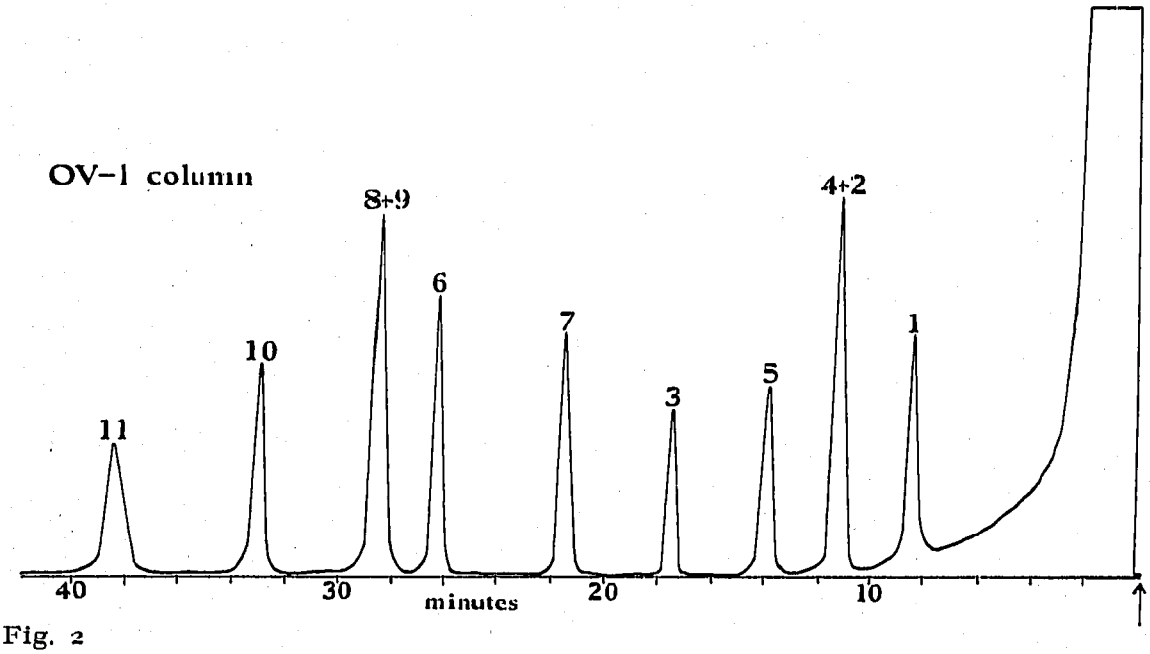


Fig. 2

temperature for 15 min. This method gave a clear solution and reproducible results. A Hamilton 0–10 μl syringe was used to inject 2 to 5 μl of this solution into the gas chromatograph. Solutions of both the natural and artificial mixtures described below were analysed by dissolving 0.1 to 0.3 mg of the solid in 50 μl of Tri-Sil and injecting 5 to 10 μl of this solution into the chromatograph.

Retention times were recorded relative to trimethylsilyl arbutin to allow for minor variations in carrier gas flow rate.

The trimethylsilyl derivatives of the reference compounds were injected into the chromatograph both individually and admixed with trimethylsilyl arbutin.

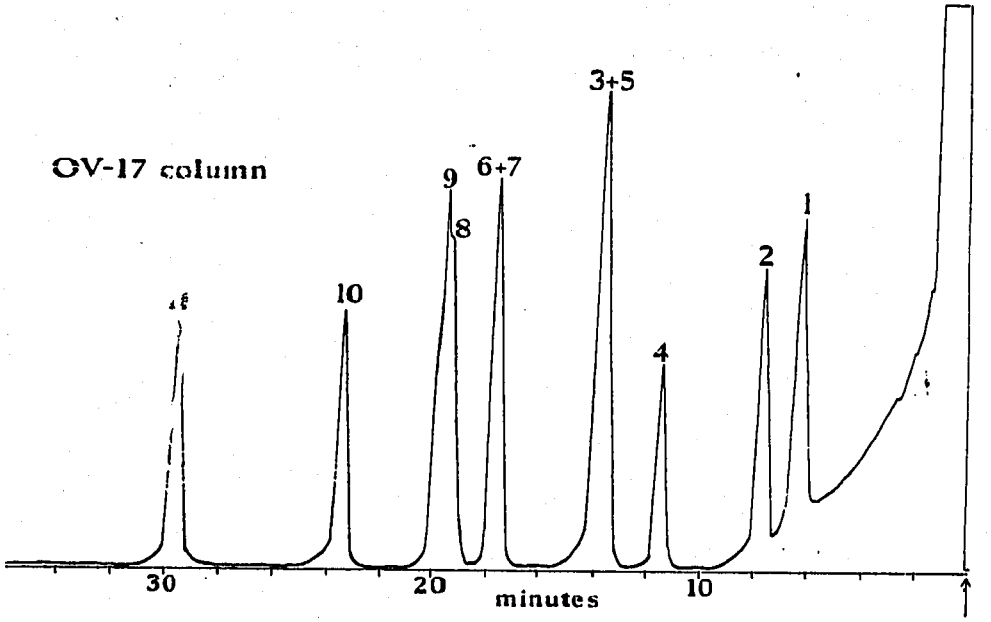


Fig. 3

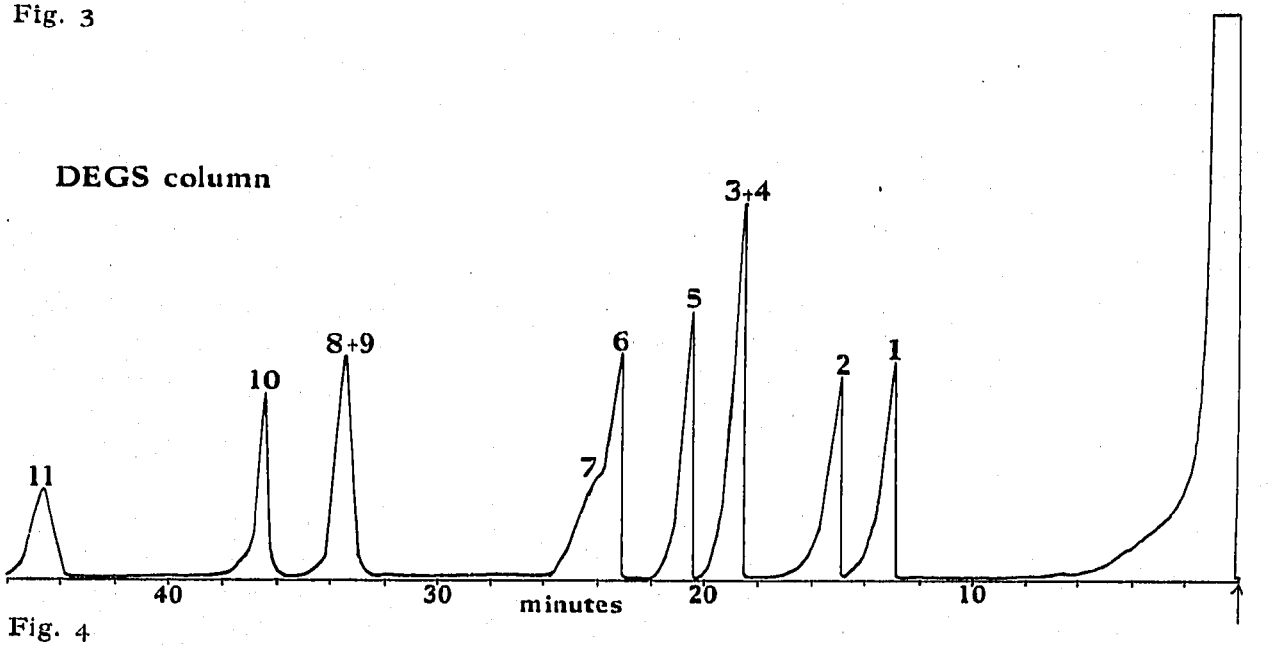


Fig. 4

Figs. 1–4. Gas chromatograms of a mixture of the trimethylsilyl derivatives of eleven phenolic glycosides. 1 = Salicin; 2 = arbutin; 3 = salidroside; 4 = fragilin; 5 = picein; 6 = triandrin; 7 = vimalin; 8 = tremuloidin; 9 = populin; 10 = salireposide; 11 = grandidentatin.

Various mixtures of the glycosides were also tested, including a mixture containing all of the reference compounds with arbutin (Figs. 1–4) and the relative retention times of the individual glycosides were calculated, where possible, from this mixture (Table I). In the case of compounds which were eluted at the same time, the relative retention times were calculated individually. A crystalline fraction obtained from a glycoside extract of a species of *Salix* was used to test the efficiency of the procedure (Fig. 5). Relative retention times for the components of this mixture were calculated after mixing some arbutin with the fraction.

TABLE I

RELATIVE RETENTION VALUES OF TRIMETHYLSILYLATED GLYCOSIDES WITH RESPECT TO TRIMETHYLSILYL ARBUTIN

Compound	*Column*				*Structure reference*
	OV-1	*OV-17*	*DEGS*	*CHDMS*	
TMS* salicin	0.76	0.82	0.89	0.64	3
TMS salidroside	1.61	1.78	1.25	1.65	4
TMS fragilin	1.03	1.48	1.23	1.75	3
TMS picein	1.27	1.75	1.38	2.41	3
TMS triandrin	2.45	2.29	1.56	2.70	3
TMS vimalin	1.97	2.23	1.61	2.86	4
TMS populin	2.58	2.62	2.26	3.30	3
TMS tremuloidin	2.58	2.57	2.22	3.23	3
TMS salireposide	3.00	3.03	2.46	3.54	11
TMS grandidentatin	3.50	3.78	3.05	4.21	3
Retention time of TMS arbutin (min)	10.93	7.90	14.83	10.58	2
Carrier gas flow rate at outlet (ml/min)	100	100	100	100	
Column temperature (°C)	190–250	190–250	130–195	180–240	
Programme No.	I	II	III	IV	
Inlet temperature (°C)	250	250	250	250	
Detector temperature (°C)	300	300	300	300	
Column length (ft.)	6	6	4	6	

* TMS = Trimethylsilyl.

Trimethylsilyl salicin, trimethylsilyl picein and the two major components of the natural mixture were collected from the OV-1 analytical column by installing a 10:1 stream splitter between the column outlet and the detector. A metal line, heated to 250°, carried the major part of the column effluent to the collection vessels.

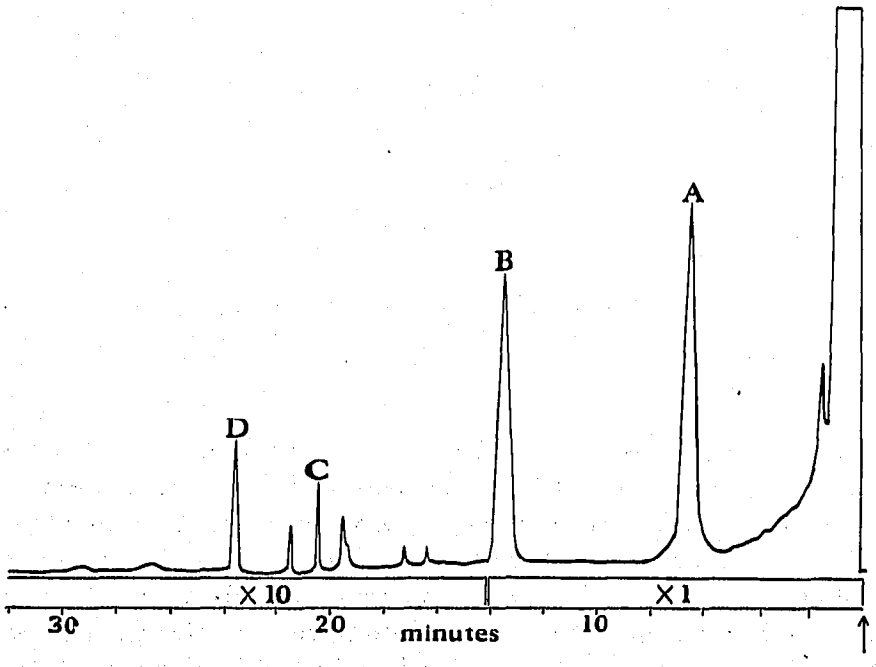


Fig. 5. Gas chromatogram of a crystalline fraction of a crude phenolic glycoside extract from a species of *Salix*, on an OV-17 column.

The trimethylsilylated salicin was a liquid and was collected in a 0.2 μl capacity silver chloride infrared cell of 0.010 mm pathlength (Beckman Extrocell). After the infrared spectrum had been recorded, the liquid was centrifuged out and the cell was washed several times with isopropyl alcohol. The combined trimethylsilyl salicin and washings, after suitable dilution with the same solvent, were transferred into a silica cell of 1 cm pathlength and the qualitative ultraviolet spectrum of the compound was determined. Trimethylsilylated picein was a solid and was collected directly from the gas chromatograph on dry potassium bromide powder[5]. After collection, the potassium bromide was formed into a disc and the infrared spectrum of the adsorbed compound was recorded. The qualitative ultraviolet spectrum of this compound was determined after extracting the potassium bromide disc with isopropyl alcohol, filtering and diluting the filtrate to an appropriate concentration.

The two major components from the natural mixture, after preliminary identification by their relative retention times, were collected similarly and their spectra recorded.

In all four cases, ten consecutive injections, each of 30 μl of the trimethylsilyl reaction solution, gave sufficient material for good spectra.

RESULTS

Ten phenolic glycosides from *Salix* species were available, *viz.* salicin, salidroside, fragilin, picein, triandrin, vimalin, populin, tremuloidin, salireposide and grandidentatin. The phenolic glycoside arbutin was used as a reference standard as this glycoside has not been reported in *Salix* species.

Although the CHDMS column separated all ten glycosides (Fig. 1), including populin and tremuloidin, which were unresolved by the other systems, the peaks for salidroside and fragilin had overlapping bases. The OV-1 column resolved salidroside completely (Fig. 2) and was also particularly good for the separation of vimalin and triandrin. Fragilin was separated from all other components on the OV-17 column (Fig. 3). The DEGS column (Fig. 4) tended to cause some tailing of the peaks but was useful for obtaining additional evidence for the identification of the mixture components and as a check that no other separable components were present. Salicin, salireposide and grandidentatin gave distinct, separate peaks in every system. The relative retention times of the glycosides (Table I) were independent of the quantity injected

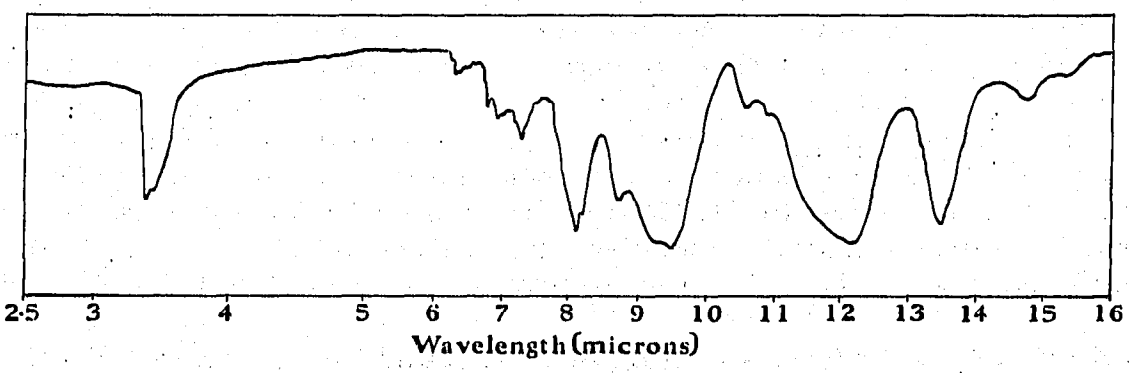


Fig. 6. Infrared spectrum of trimethylsilyl salicin (liquid film).

provided that this did not exceed 10 μg per component. Experiments showed that quantities as low as 0.02 μg of salicin could easily be detected.

The collected fraction of pure trimethylsilyl salicin gave a satisfactory infrared spectrum (Fig. 6). Absorption in the 5 to 7.5 μ range corresponded to the published spectra for pure salicin[6]. Strong bands at 8.1, 12.1 and 13.45 μ indicated the presence of $Si(CH_3)_3$ groups and the broad band at 9.3 to 9.55 μ was attributed to Si–O–C vibrations[7]. The expected peaks for CH, CH_2 and CH_3 absorptions were seen at 3.39 and 3.47 μ but only a faint shoulder at 3.28 μ showed the presence of =C–H (aromatic) stretching vibrations. No band was seen in this spectrum for OH absorption and this indicated that the trimethylsilylation reaction had proceeded to completion. The λ_{max} for trimethylsilyl salicin were at 268 and 274 mμ, corresponding with the recorded data for pure salicin[8]. The collected fraction of pure trimethylsilyl picein also gave a satisfactory infrared spectrum (Fig. 7) with the 5 to 7.5 μ region corresponding to the

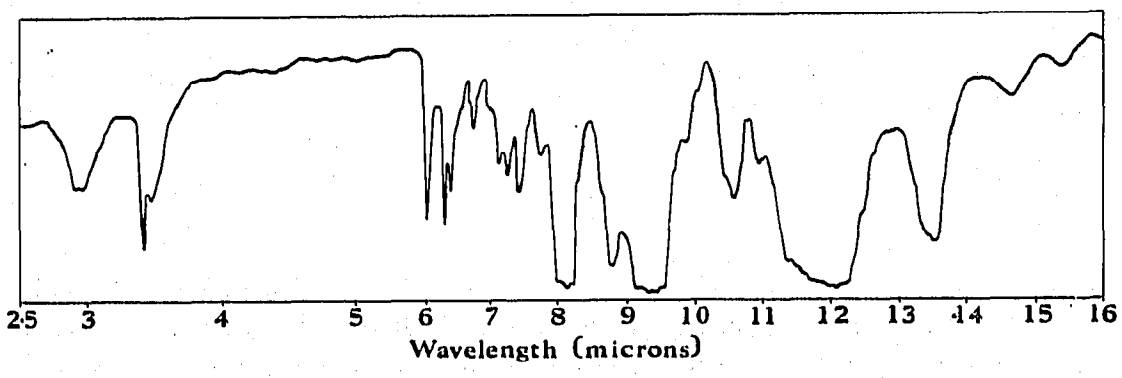
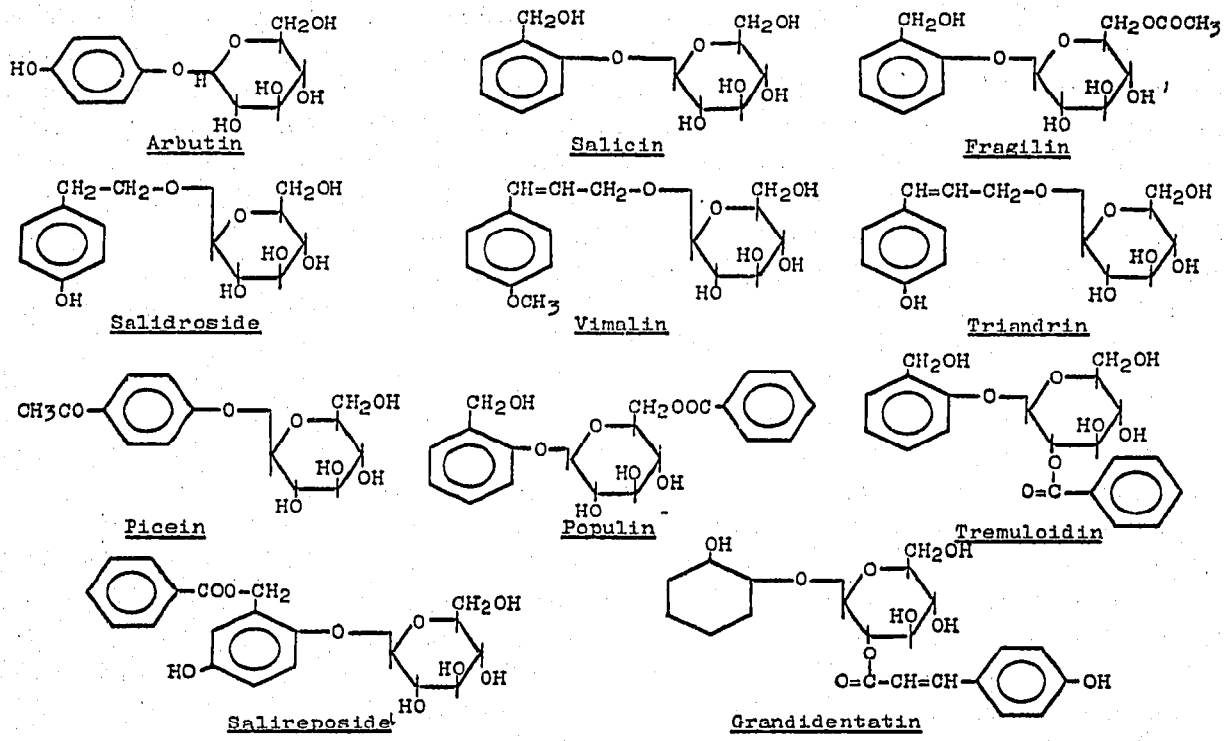


Fig. 7. Infrared spectrum of trimethylsilyl picein (potassium bromide disc).

published spectrum for pure picein[6]. A strong band at 2.9 μ was seen on this spectrum, indicating the presence of a hydroxyl group or groups. A blank sample of potassium bromide exposed to the same conditions as those used to collect the fraction, showed the same absorption in this region. The absorption in the blank potassium bromide sample and the lack of any hydroxyl band in the spectrum of trimethylsilyl salicin strongly indicated that the band at 2.9 μ, seen in the spectrum of trimethylsilyl picein, was due to atmospheric moisture adsorbed onto the potassium bromide during the collection procedure. The spectrum of trimethylsilyl picein was very similar to the spectrum of trimethylsilyl salicin, with the exception of the OH band and the strong carbonyl absorption at 5.99 μ in the former spectrum. The trimethylsilyl picein had a λ_{max} of 266 mμ, which corresponded to that recorded for pure picein[8].

The gas chromatographic analysis of the trimethylsilylated crystalline fraction of the natural glycoside extract indicated that peak A was salicin, peak B was picein and that these were the principal constituents (Fig. 5). Several minor components were observed, two of which corresponded with tremuloidin and salireposide, peaks C and D respectively. The other constituents did not correspond with any of the reference compounds used. The infrared and ultraviolet spectra of the collected fractions of the two major components corresponded with those of trimethylsilylated salicin and picein.

The separated compounds (corresponding to peaks A and B, Fig. 5) were collected on Molecular Sieve 5A by the method of CARTWRIGHT AND HEYWOOD[9], but an attempt



to compare the mass spectra of these compounds with those of pure glycosides was unsuccessful. Both the collected fractions and the pure compounds exhibited rapid fragmentation and showed no peak for the parent compound, although peaks for some of the expected fragments could be identified.

DISCUSSION

Due to the chemical and physical nature of the phenolic glycoside molecule, the gas chromatographic analysis of these compounds must, at the present time, be carried out on a more volatile and less polar derivative. As a result of the work of SWEELEY *et al.*[10] and FURUYA[2], the trimethylsilyl derivative was selected for the gas chromatographic analysis of the phenolic glycosides from *Salix* species. A preliminary investigation showed that Tri-Sil (Pierce Chemical Co.), a premixed trimethylsilylation reagent containing hexamethyldisilazane and trimethylchlorosilane in pyridine, gave consistently reproducible results. Initial experiments showed that the reagent components and reaction by-products were eluted from the columns in a reasonable time. Mixtures of Tri-Sil concentrate with other silylation solvents, dimethylformamide, dimethylsulphoxide and tetrahydrofuran, were also tested, but it was found that the commercial mixture was as good or better than any of the test mixtures, and due to its consistency of formulation, this reagent was used for the remainder of the work.

The trimethylsilylation procedure used gave complete solution of the glycosides in 15 min. Samples injected into the chromatograph after this time gave single, sharp, symmetrical peaks and this was taken as an indication that the silylation reaction was complete. When excess glycoside was used in preparing the derivative, large

numbers of poorly defined peaks were seen on the chromatogram. To prevent this, the proportion of reagent used for the reaction was slightly greater than that recommended by the manufacturer. Although this method did not give very concentrated solutions, the amount of glycoside derivative present in the injected volume was sufficient to give good recorder deflections without undue interference from the solvent peak. Once prepared, the solutions of the trimethylsilylated glycosides were stored in stoppered vials at 0° for up to seven days without any detectable change taking place.

In all cases, the two isomers populin and tremuloidin, when individually injected onto the CHDMS column, showed only one peak, and this was taken as an indication that no interconversion had occurred during the silylation procedure.

Initial experiments showed that the higher molecular weight glycosides could only be eluted in a reasonable time from columns having low liquid phase loading. It was found that while a complete analysis could be carried out on all of the compounds tested using isothermal conditions, temperature programming gave much shorter analysis times and superior resolution of the higher molecular weight glycosides.

Using the columns described, complete separation of all of the glycosides tested was achieved. The columns proved to be particularly useful for the identification of individual glycosides of low molecular weight, as their change of elution order in the different systems was quite distinctive. Fractions of any component in a mixture, present in reasonable proportions, could be collected and their infrared and ultraviolet spectra recorded in as little as 6 h. Using any of the columns, a rapid screening procedure could be carried out for individual glycosides in 50 min.

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