THIN-LAYER CHROMATOGRAPHY OF PHENOLIC GLYCOSIDES AND ITS USE AS A SCREENING PROCEDURE FOR THE GENUS *SALIX*

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(Received May 13th, 1966)

The phenolic glycoside salicin is of widespread occurrence in the genus *Salix* and has been used in medicine for the treatment of acute rheumatism and influenza[1]. Other phenolic glycosides have been isolated from this genus[2], including several during the past few years from European species[3,4]. There are many *Salix* species indigenous to North America, but of these very few have received any chemical investigation, in particular those of the Canadian prairie regions. As part of an investigation into the distribution of phenolic glycosides in the *Salix* species native to Manitoba, a screening procedure was sought for these compounds. The present work describes thin-layer chromatographic separations which are applicable to *Salix* extracts for this purpose.

EXPERIMENTAL

Air-dried silica gel G (Research Specialities Co.) layers, 250 μ thick, were prepared by the method of FIKE AND SUNSHINE[5] on glass plates 20 × 20 cm. Polyamide–cellulose plates were made by suspending 5 g polyamide TLC grade (Woelm) and 2.5 g cellulose powder TLC grade (Gerard Pleuger) in 50 ml ethanol in a 125 ml glass-stoppered flask and shaking vigorously for at least 5 min before spreading 5 plates with a layer 250 μ thick. The plates were left to air dry overnight before use.

An alcoholic solution of reference phenolic glycosides was applied near one corner of a plate, the spot dried and then developed using the super-saturated method of STAHL[6]. Two-dimensional chromatograms were prepared with a development distance from the origin to the solvent front of 15 cm in each direction. Air-dried silica gel G plates were used as well as plates that had been subjected to humidity control, but this treatment was unnecessary for the polyamide–cellulose layers. For humidity control, the plate was spotted and then suspended for 16 h in an enclosed, filter-paper lined chamber, containing 100 ml of either a saturated aqueous sodium bromide solution, which gave a relative humidity of 58 %, or a saturated solution of sodium thiosulphate, producing a relative humidity of 78 %[7]. After humidification, the plate was developed in the first direction, dried and re-humidified for 16 h before development in the second direction.

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Solvent systems

The solvent systems are summarized in Table I.

TABLE I

Thin layer		*Solvent system*
A. Silica gel G	a. No humidity control	(i) Ethyl acetate–methanol (9:1, v/v)
		(ii) Benzene–methanol (7:3, v/v)
	b. 58% relative humidity	(iii) Ether–methyl ethyl ketone–formic acid–water (7:1:1:1, v/v)
		(iv) Ethyl acetate–ether–formic acid–water (67:4:4:5, v/v)
		(v) Ethyl acetate–xylene–formic acid–water (35:1:2:2, v/v)
	c. 78% relative humidity	(v) As above
		(vi) Ethyl acetate–methyl ethyl ketone–formic acid–water (15:3:1:1, v/v)
B. Polyamide–cellulose (2:1, w/w)		(vii) Ethyl acetate–acetic acid–water (23:1:1, v/v)
		(viii) Water–ether (24:1, v/v)

Locating reagents

(i) Millon's reagent B.P.
(ii) Silver nitrate reagent[8].
(iii) Sulphuric acid (4%, v/v) in absolute ethanol.

Preparation and examination of Salix extract

S. gracilis Anderss. var. *textoris* Fern. (*S. petiolaris* Sm.) bark was collected in June, 1965 from trees growing near Stonewall, a small township about 20 miles north-west of Winnipeg. The bark was oven dried below 70° and powdered. The powdered bark, 10 g, was extracted in a Soxhlet apparatus with 95% ethanol for 10 h, the ethanol recovered, and the residue extracted with 4 × 20 ml water. The aqueous solution was shaken with benzene, the benzene layer discarded, and the aqueous solution treated with 10 ml lead subacetate solution B.P. The precipitate was filtered off and washed with a small volume of water. The clear filtrate was treated with hydrogen sulphide, and the resultant precipitate filtered off. The filtrate was concentrated to about 20 ml and extracted with ethyl acetate by continuous liquid–liquid extraction. The final ethyl acetate solution, concentrated to small volume, was used for the examination of the phenolic glycosides.

Alternatively, fresh material in broken pieces was extracted by macerating with 95% ethanol for 3 h, the mixture ground in a high speed blender, the clear liquid decanted and the residue extracted in a Soxhlet apparatus for 10 h. The extracts were combined and processed as before.

Two-dimensional thin-layer chromatograms were prepared of *S. gracilis* var. *textoris* bark extract using both the silica gel G and polyamide–cellulose layers. Sulphuric acid (4%, v/v) in absolute ethanol was used for locating the compounds on silica gel G layers and the silver nitrate reagent for the polyamide–cellulose layers.

Millon's reagent was also used to confirm the location of the phenolic glycosides on both types of layers. The identity of individual compounds was determined by the characteristic colour produced with the sulphuric acid reagent and by co-chromatography when running the plant extract with individual reference phenolic glycosides.

RESULTS

Ten *Salix* phenolic glycosides were available as reference compounds, *viz.* salicin, populin, tremuloidin, picein, vimalin, triandrin, fragilin, salireposide, salicortin and grandidentatin. On air-dried silica gel G layers, used without humidity control, none of the six solvent systems listed achieved complete separation of all ten reference compounds. Two-dimensional chromatograms, using ethyl acetate–methanol in the first direction and benzene–methanol in the second direction, separated nine of the reference compounds, but picein and triandrin were not separated and the separation between the triandrin–picein spot and grandidentatin was incomplete. With the silica gel G layers under controlled humidity conditions, all ten reference compounds were clearly separated using the solvent systems listed (Fig. 1). Two-dimensional chromatograms on polyamide–cellulose layers also clearly separated all of the reference glycosides used (Fig. 2).

The phenolic glycosides were detected on silica gel G layers by spraying with 4% sulphuric acid in absolute ethanol. After spraying, the plates were heated at 110° for 10–15 min, by which time all ten reference compounds gave a specific colour. Tremuloidin, populin, fragilin and salicin gave red colours, salireposide yellow-orange, picein orange-brown, grandidentatin yellow, salicortin yellow changing to pink,

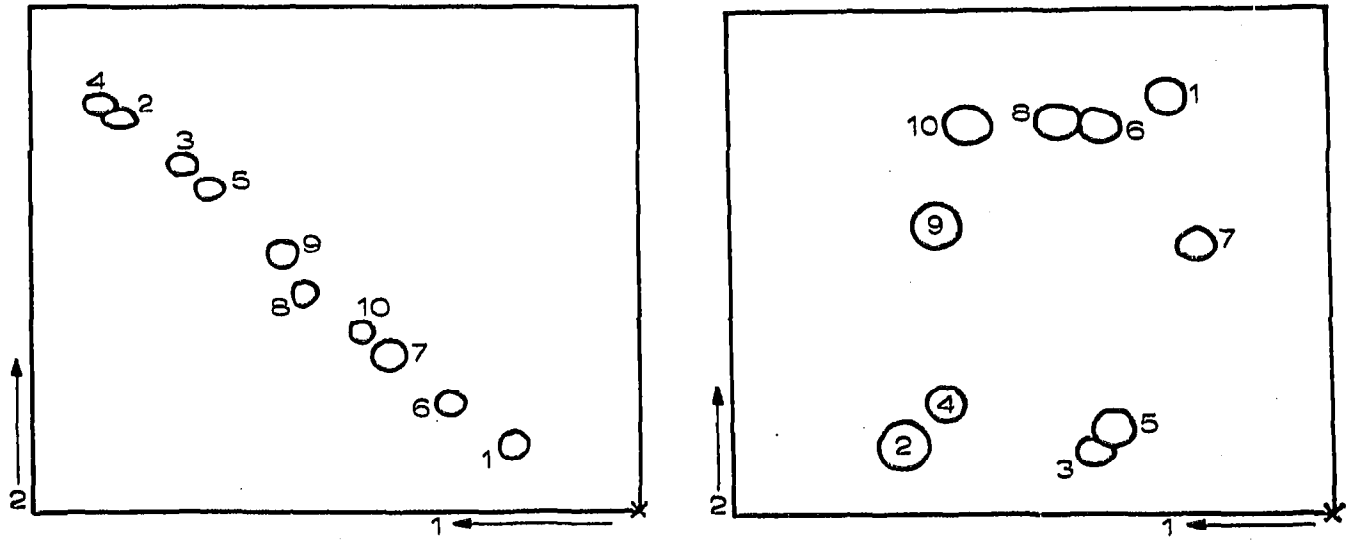

Fig. 1. Two-dimensional thin-layer chromatogram of phenolic glycosides on silica gel G layers at 78% relative humidity. Solvent system 1 = ethyl acetate–xylene–formic acid–water (35:1:2:2, v/v); solvent system 2 = ethyl acetate–methyl ethyl ketone–formic acid–water (15:3:1:1, v/v). Reference compounds: 1 = salicin; 2 = populin; 3 = salireposide; 4 = tremuloidin; 5 = grandidentatin; 6 = picein; 7 = triandrin; 8 = fragilin; 9 = vimalin; 10 = salicortin.

Fig. 2. Two-dimensional chromatogram of phenolic glycosides on polyamide–cellulose (2:1, w/w) layers. Solvent system 1 = water–ether (24:1); solvent system 2 = ethyl acetate–acetic acid–water (23:1:1). Reference compounds: 1 = salicin; 2 = populin; 3 = salireposide; 4 = tremuloidin; 5 = grandidentatin; 6 = picein; 7 = triandrin; 8 = fragilin; 9 = vimalin; 10 = salicortin.

vimalin blue-green and triandrin grey-blue. Salireposide, triandrin and vimalin developed their colours within 5–10 min, but the other compounds required a longer period of heating.

The silver nitrate reagent was used to detect the phenolic glycosides on polyamide–cellulose layers. The plates were sprayed with the silver nitrate solution and then, after 5–10 min, sprayed again with $N/2$ ethanolic sodium hydroxide solution and left at room temperature. The glycosides produced dark-brown spots against a lighter brown background, but the spots of salireposide and triandrin were darker than those of the others. If necessary, the plates were sprayed with 10% aqueous sodium thiosulphate solution, which reduced the background colour of the plate on standing.

Millon's reagent was used to confirm the location of the phenolic glycosides on both the layers of silica gel G and polyamide–cellulose. After spraying the plates, some of the compounds produced coloured spots on standing at room temperature, but all of the glycosides developed red or yellow colours on heating at 90–95° for 10–15 min. Millon's reagent was particularly useful in the detection of picein on silica gel G layers. By spraying the chromatogram first with sulphuric acid in absolute ethanol and then again with Millon's reagent, picein was detected as a very distinct rose red coloured spot.

On the basis of the colours produced on silica gel G layers with the sulphuric acid spray reagent and chromatographic behaviour in relationship to the reference phenolic glycosides, *S. gracilis* var. *textoris* bark was shown to contain tremuloidin, salicortin, salireposide, salicin, fragilin and picein. Trace amounts of populin, grandidentatin and triandrin also appeared to be present. A number of other coloured spots were detected, but these could not be identified with any of the available reference compounds.

DISCUSSION

Good separation of all ten reference phenolic glycosides was achieved on silica gel G layers under the specified conditions of humidity, particularly at 78% relative humidity. The disadvantage of using humidity control was the length of time involved, but the effect of humidity on the separation of the phenolic glycosides was most pronounced and changing the degree of humidity of the plate brought about marked changes in the R_F values. A two-dimensional chromatogram, run in both directions with the same solvent system, ethyl acetate–xylene–formic acid–water (35:1:2:2, v/v), but run under different humidity conditions, gave results comparable with those obtained with different solvent systems at the same humidity level.

Without humidity control, separation of all ten reference compounds was not achieved. Two-dimensional chromatograms on silica gel G layers using solvent systems (i) and (ii) gave good resolution of most of the reference compounds, but the separation between picein, triandrin and grandidentatin was not complete. Although not fully separated, when spraying with the sulphuric acid reagent the orange-brown colour of picein was usually distinctly visible through the grey-blue of triandrin. When screening plant extracts that do not contain triandrin and grandidentatin, humidity control would not be necessary to effect good separation of all the compounds, although a few plates should be run under controlled humidity conditions for confirmation of the results.

The ten reference phenolic glycosides were separated on layers of polyamide alone, but the dried, developed layers tended to flake off on spraying with the locating reagents and so a mixture of two parts of polyamide and one part of cellulose was substituted. The resolution of the glycosides on this mixed layer was the same as on polyamide alone, but the mixture formed a better adhesive layer which did not crack and flake.

The best spray reagent for the detection of phenolic glycosides on silica gel G was found to be 4 % sulphuric acid in absolute ethanol. All ten compounds were easily detected with this reagent, which produced characteristic colours with each compound. Millon's reagent was used by THIEME[9] as a locating reagent for paper chromatography and was used in this study, but was found to be significantly less sensitive than the sulphuric acid reagent on silica gel G layers. Other spray reagents, such as Kedde's reagent, 2,3,5-triphenyltetrazolium chloride solution and antimony trichloride in chloroform[10], detected certain phenolic glycosides, but did not locate all ten reference compounds. All of the reference glycosides could be visualized with short wave ultra-violet light (2537 Å).

The reagents used for the detection of phenolic glycosides on polyamide–cellulose layers were restricted by the acid sensitivity of the polyamide. This excluded the use of 4 % sulphuric acid and other strongly acidic sprays. The compounds were best detected with the silver nitrate reagent, but this had the disadvantage that the colours produced were not specific and spots were produced by other compounds in the plant extract which were not phenolic glycosides. Coloured spots were produced by phenolic glycosides with other locating reagents used, such as Kedde's reagent and 2,3,5-triphenyltetrazolium chloride solution, but the spots were not easily detected.

Nine phenolic glycosides were identified in the bark of *S. gracilis* var. *textoris* including tremuloidin, which had not been detected previously in a willow bark[2]. The trace quantity of populin detected may have been formed from tremuloidin during the purification of the phenolic glycoside extract by migration of the benzoyl group from the 2 position to the 6 position[11].

On plates run in a single direction, with all the solvent systems listed, certain spots were detected which could not be identified. A few of these spots were in the same R_F range as the reference compounds and may prove to be unknown glycosides.

ACKNOWLEDGEMENTS

We thank Miss JEAN GREENHOUSE for her technical assistance and Dr. B. BOIVIN, Plant Research Institute, Ottawa for the authentication of the *S. gracilis* var. *textoris*. We are very grateful to Doz. Dr. H. THIEME, Karl Marx University, Leipzig, and Drs. I. A. PEARL and S. F. DARLING, Institute of Paper Chemistry, Appleton, Wisconsin, for the gift of the reference phenolic glycosides.

SUMMARY

The separation of phenolic glycosides on thin layers of silica gel G and polyamide–cellulose is given, and the effects of humidity control discussed. This technique is advocated for the detection of phenolic glycosides in the genus *Salix* and the results obtained from the bark of *S. gracilis* Anderss. var. *textoris* Fern. (*S. petiolaris* Sm.) are

recorded. Tremuloidin, which previously has been found in both the bark and leaves of *Populus* species, but only in the leaves of *Salix* species[2], was detected in the bark of *S. gracilis* var. *textoris*.

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