CHROM. 3957

PHYTOCHEMISTRY OF THE *SALICACEAE*

II. THE EFFECT OF EXTRACTION PROCEDURES ON THE APPARENT FREE PHENOLIC GLYCOSIDE CONTENT OF *SALIX* SPECIES

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(Received January 20th, 1969)

SUMMARY

A gas–liquid chromatography procedure was used to examine the effect of different extraction methods on the apparent free phenolic glycoside content of the bark of *Salix petiolaris* Sm. Previously reported decomposition of phenolic glycosides in plant extracts treated with lead subacetate was confirmed. Decomposition and interconversion of certain pure glycosides in commonly used extraction solvents were demonstrated and the effect of solvents on extracts of the above bark is described.

INTRODUCTION

If any examination of the distribution of phenolic glycosides in plants is to be significant for chemotaxonomic purposes, it is essential that the extraction procedures used should give reproducible results.

Several different extraction procedures have been used for the examination of the phenolic glycosides of both *Salix* and *Populus* species[1–5]. These methods vary greatly in minor details but usually involve extraction of the fresh or dried plant material with hot solvent; some include a purification step in which lead subacetate is used to precipitate unwanted components. In some cases, lead subacetate treatment may be followed or replaced by column chromatography, solvent extraction of the original extract, or both.

PEARL AND DARLING[6] reported that lead subacetate treatment changed the apparent glycoside content in extracts of *Populus* species to a varying degree, depending on the temperature and amount of lead subacetate used. They concluded that some of the glycosides found after this purification procedure are probably artifacts. PEARL AND DARLING currently employ a hot water extraction process in which lead subacetate is not used[7]. Despite this, apparent variations in the glycoside pattern of *Populus* species have been found.

THIEME[1] has suggested that lead subacetate treatment, or treatment with other bases, is undesirable in the examination of extracts of *Salix* species, but he only

mentions occlusion of the glycosides by the resulting precipitate rather than decomposition. He currently uses a column chromatographic method to purify extracts[8,9].

A recently developed gas–liquid chromatography procedure for the separation of phenolic glycosides[10] enabled a critical study to be made of some of the factors which influence the apparent free phenolic glycoside content of plant extracts.

EXPERIMENTAL

To separate and identify the phenolic glycosides, the gas–liquid chromatography procedure of BOLAN AND STEELE[10] was used. Two columns were employed in this work; 0.3% OV-1 on Chromosorb G acid washed/dimethyldichlorosilane (a.w./DMCS) 60–80 mesh and 0.5% cyclohexanedimethanol succinate (CHDMS) on Chromosorb G a.w./DMCS 60–80 mesh.

The glycoside extracts and pure glycosides were dissolved in Tri-Sil (Pierce Chem. Co.) as reported previously[10]. The plant extracts were prepared from the dried, powdered bark of *Salix petiolaris* Sm. Thin-layer chromatographic analysis was by the method of AUDETTE *et al.*[11].

Extraction procedures

Method 1. Stage A. 1 g of the bark was extracted with solvent in a Soxhlet apparatus for 8 h. Extracts were prepared with ethanol, methanol, water, acetone and ethyl acetate. The solvent was removed *in vacuo*, the residue taken up in several portions of warm water and the combined aqueous extracts were filtered and cooled. One quarter of the filtrate was evaporated to dryness in a vacuum rotary evaporator, dried overnight in a vacuum desiccator, treated with Tri-Sil and analysed by gas chromatography.

Stage B. The remaining aqueous filtrate was treated with cold lead subacetate solution until no further precipitate was formed. The mixture was filtered and the precipitate was washed with water. Hydrogen sulphide was bubbled through the combined filtrate and washings until all of the excess lead had precipitated. After filtering, the precipitate was washed with water and the combined filtrate and washings were made up to a convenient volume with water. One-third of this solution was treated and analysed in the same way as the aliquot from Stage A.

Stage C. The remaining clear, aqueous solution from Stage B, equivalent to 0.5 g of bark, was continuously extracted with ethyl acetate in a liquid–liquid extractor for 8 h. Both the ethyl acetate and the aqueous portions were evaporated to dryness *in vacuo*, dried overnight in a vacuum desiccator, treated with Tri-Sil and analysed by gas chromatography.

Method 2. 10 mg of bark were refluxed with 2 ml of solvent for 2 h. The mixture was filtered, the residue was washed with 1 ml of the same solvent and the washings and filtrate were combined. The extract was then treated and analysed in the same way as the aliquots from *Method 1*. Extracts were prepared with ethanol, methanol, water, acetone, ethyl acetate, chloroform, dichloromethane and pyridine.

Method 3. 10 mg of bark were refluxed for 1 h with 2 ml of Tri-Sil. The mixture was filtered and the marc refluxed with a further 2 ml of Tri-Sil for 1 h. The mixture was again filtered and the combined filtrates were evaporated to small volume (approx.

100 μl) in a vacuum rotary evaporator and then analysed by gas chromatography.

Effect of solvents on pure glycosides

Samples of about 0.5 mg of pure tremuloidin, populin, fragilin and grandidentatin were refluxed individually for 2 h with one or more of the solvents described in *Method 2*. The solutions were taken to dryness and dried overnight in a vacuum desiccator. The residues were then treated with Tri-Sil and analysed by gas chromatography.

RESULTS

Due to the previously reported results of PEARL AND DARLING[6] and the suggestion of THIEME[1] on the effects of lead subacetate treatment on the apparent glycoside content of *Populus* and *Salix* species, trials were run to establish whether or not this procedure was necessary and what effect, if any, it had on extracts of the dried bark of *Salix petiolaris* Sm.

Chromatograms of the dried, initial Soxhlet extract (*Method 1*, Stage A) showed picein to be the major component. Salicin was only present in the extracts of this species in relatively small proportions. Numerous other small peaks which corresponded with other previously reported glycosides[11] were also noted. However, after treatment of the extracts with lead subacetate solution and subsequent removal of excess lead (*Method 1*, Stage B) the proportion of salicin in the filtrate was found to have greatly increased. In some cases, salicin had become the major component of the extract while other components, with the exception of picein, had almost disappeared. Although disappearance of many of the components could be attributed to occlusion of the molecules by the precipitated lead salts[1], the amount of picein present in the extracts remained almost constant and rendered this explanation unlikely.

Further purification of the extracts by continuous liquid–liquid extraction with ethyl acetate was found to be undesirable in a screening procedure since, even after 12 h, glycosidic material could still be detected in the aqueous phase.

The second extraction procedure was developed in order to minimise glycoside decomposition by eliminating the lead subacetate treatment and reducing the time in which the extracts were in contact with hot solvent. Samples of bark were extracted for different lengths of time and it was found that after 2 h, extraction was virtually complete. Despite this, extracts prepared with the various solvents described *(Method 2)* showed that the number and proportions of glycosides in each extract were different (Figs. 1–4). Salicin was present in these extracts only in very small amounts and picein was always the major component. Thin-layer chromatography was used to confirm the above results and in some cases the salicin content of the extracts was so small that it could not be detected by this means. Extracts prepared with chloroform or dichloromethane showed no detectable quantities of glycosidic material.

Extracts prepared with pyridine *(Method 2)* or Tri-Sil *(Method 3)* gave similar results. The extracts contained a much larger proportion of the higher molecular weight components but later it was found that pyridine caused a substantial amount of decomposition and neither of these solvents was suitable for extraction purposes.

Extracts prepared using *Method 2* and subsequently treated with lead sub-

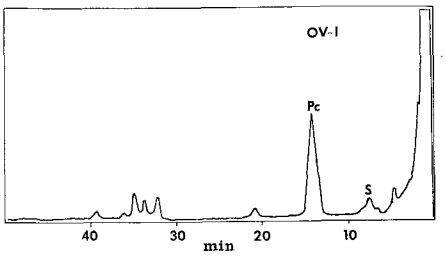

Fig. 1

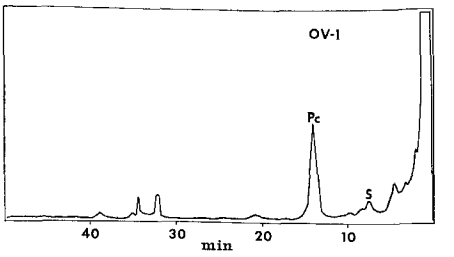

Fig. 2

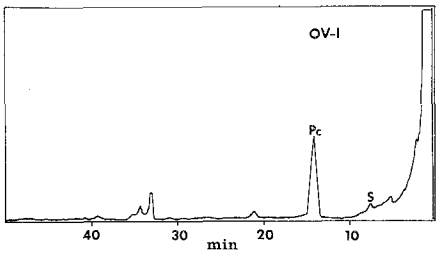

Fig. 3

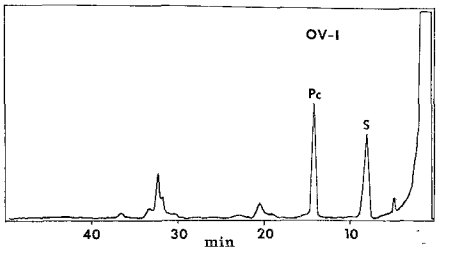

Fig. 4

Fig. 1. Gas chromatogram of ethanol extract of bark. S = salicin; Pc = picein.

Fig. 2. Gas chromatogram of methanol extract of bark. S = salicin; Pc = picein.

Fig. 3. Gas chromatogram of acetone extract of bark. S = salicin; Pc = picein.

Fig. 4. Gas chromatogram of aqueous extract of bark. S = salicin; Pc = picein.

acetate solution gave a completely different pattern of the apparent glycoside content of the extracts. Fig. 5 serves as a typical example of this effect and shows equal amounts of salicin and picein. These results confirmed that this treatment was responsible for large qualitative and quantitative changes in the glycoside portion of the extract.

Due to the variations in apparent glycoside content of extracts prepared with different solvents, a series of experiments was carried out to determine the effect of boiling solvents on pure glycosides. These tests were restricted to small quantities of a few glycosides due to the limited supply of reference compounds available.

When tremuloidin was refluxed in methanol or ethanol, gas chromatographic analysis of the residue showed that it had partially decomposed to give an unknown compound and a small amount of salicin (Fig. 6). When refluxed with water, tremuloidin was almost completely converted to populin and salicin although two other small, unidentified peaks were also noted (Fig. 7). In dimethylformamide or pyridine, tremuloidin decomposed to give large numbers of peaks, one of which corresponded to salicin, the remainder being unidentified (Fig. 8). Neither acetone nor ethyl acetate had any detectable effect on tremuloidin and refluxing tremuloidin with dichloromethane produced only traces of salicin.

After refluxing populin with water a small amount of salicin was detected in the residue and two other small peaks were also noted (Fig. 9). These two peaks

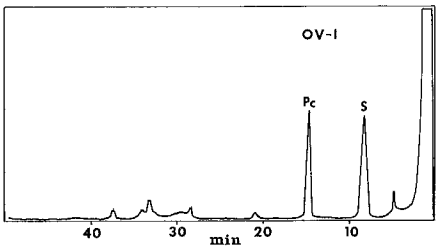

Fig. 5

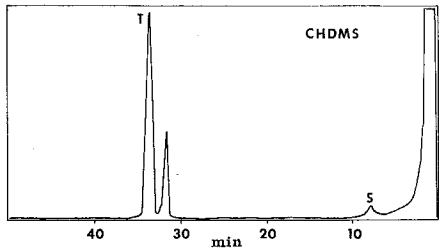

Fig. 6

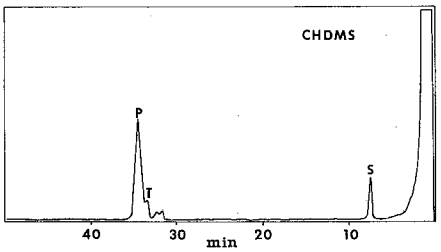

Fig. 7

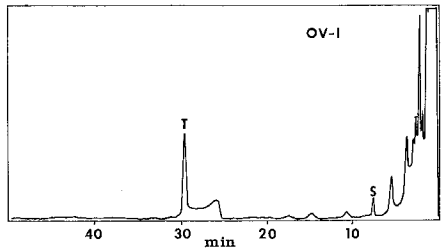

Fig. 8

Fig. 5. Gas chromatogram of ethanol extract of bark after treatment with lead subacetate solution. S = salicin; Pc = picein.

Fig. 6. Gas chromatogram of tremuloidin after refluxing with ethanol. S = salicin; T = tremuloidin.

Fig. 7. Gas chromatogram of tremuloidin after refluxing with water. S = salicin; T = tremuloidin; P = populin.

Fig. 8. Gas chromatogram of tremuloidin after refluxing with pyridine. S = salicin; T = tremuloidin.

could not be identified but they corresponded with the two small unidentified peaks formed when tremuloidin was refluxed with water (Fig. 7).

Fragilin almost completely decomposed when refluxed with water. Gas chromatograms of the residue showed that two major components were formed in almost equal proportions. One of these was salicin and the other could not be identified.

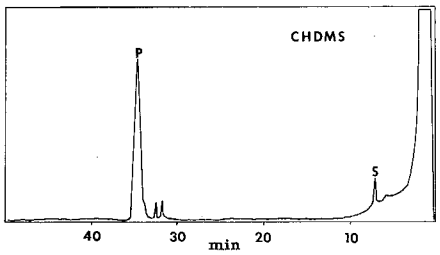

Fig. 9

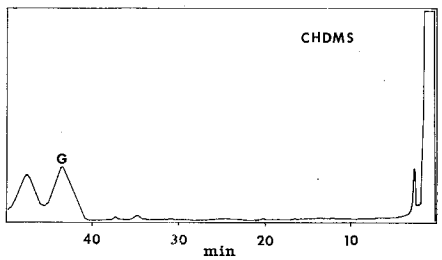

Fig. 10

Fig. 9. Gas chromatogram of populin after refluxing with water. S = salicin; P = populin.

Fig. 10. Gas chromatogram of grandidentatin after refluxing with water. G = grandidentatin.

The unidentified peak had a retention time between those of salicin and fragilin.

Some grandidentatin decomposed when refluxed with water to give several unidentified products, although a large proportion of the grandidentatin remained unchanged (Fig. 10). The major separable decomposition product was eluted shortly after grandidentatin which indicated that it probably had a molecular weight similar to the parent compound. This product may have been formed by a shift of the *p*-coumaroyl group from position 2 to position 6 on the glucose residue, similar to the shift of the benzoyl radical which occurs when tremuloidin is converted to populin[6].

DISCUSSION

The results of treating plant extracts with lead subacetate solution show that this procedure must be avoided if an accurate representation of the phenolic glycoside content of the plant is to be obtained.

It has been shown that treatment with basic substances can decompose acylated glycosides, such as tremuloidin, to form other products[2,6]. As the original extracts contained little free tremuloidin or other acylated salicin derivatives, this effect alone could not account for the large increase in salicin after lead subacetate treatment. The most likely alternative explanation for this increase is the decomposition of some unidentified glycoside complex in the extract, corresponding to the "polymeric material" suggested by PEARL AND DARLING[6], although the term "polymeric" may not be an accurate description of the nature of this unidentified component.

Since simple solvents decomposed all of the acylated glycosides tested, forming different mixtures of products, dependent upon the solvent used, it is likely that the glycoside complex in the plant material was similarly affected. This may help to explain the different glycoside patterns obtained with different extraction solvents. In addition, selective decomposition of any glycoside extracted by the hot solvent would probably enhance these differences.

These results suggest that most previous reports concerning the qualitative and quantitative phenolic glycoside content of members of the *Salicaceae* may require reinterpretation. Also, as some of the simple phenolic constituents isolated from the *Salicaceae* are known aglycones[4,12,13], some of these may not exist *per se* in the plant in the quantities reported.

ACKNOWLEDGEMENTS

We thank Doz. Dr. H. THIEME, Karl Marx University, Leipzig and Drs. I. A. PEARL and S. F. DARLING, Institute of Paper Chemistry, Appleton, Wisc. for the gift of the reference phenolic glycosides.

Financial assistance for J. W. STEELE from the National Research Council of Canada, the Canadian Foundation for the Advancement of Pharmacy and the Faculty of Graduate Studies and Research of the University of Manitoba and for R. C. S. AUDETTE from the Northern Studies Committee, University of Manitoba is gratefully acknowledged.

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