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APPLICATION OF GAS CHROMATOGRAPHY TO THE NEUTRAL, WATER-SOLUBLE CONSTITUENTS OF TWIGS OF SOME GYMNOSPERMS*

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Abstract—Residues from the hot aqueous ethanol extracts of twigs were fractionated to obtain water-soluble fractions which were subsequently deionized, and then methylated by a modification of Kuhn's procedure. Gas–liquid partition chromatography (GLPC) was used for the qualitative analysis of the products of methylation, and to isolate individual constituents. The identities of the latter were established or confirmed by infrared and proton magnetic resonance (PMR) studies. This general procedure appears to be useful for chemo-taxonomical surveys, for the detection of new constituents, and for biosynthetic studies using tracer techniques. When the method was applied to twigs of *Picea mariana* (Mill.) BSP., *Picea glauca* (Moench) Voss and *Taxus* sp., monosaccharides, sucrose, and raffinose were detected readily. *P. glauca* contained in addition both picein and pungenin, whereas *P. mariana* did not contain these glucosides. PMR studies revealed that the acetyl groups in picein and pungenin were converted to isobutyryl groups, in quantitative yield, during the methylation procedure. Taxicatin was found in *Taxus* together with several unidentified constituents.

INTRODUCTION

INVESTIGATIONS on the separation of methylated carbohydrates by gas–liquid partition chromatography (GLPC) have been reported.[1–7] However, little use has been made of this method for investigation of the soluble sugars and glycosides of plants. The GLPC analysis of naturally occurring glucosides and their aglycones is practically an unexplored field,[8] although it offers considerable promise as a technique in biosynthetic investigations, as well as in analytical surveys. This paper describes a simple method which can be used for the determination and isolation of the methylated derivatives of some oligosaccharides and phenolic glucosides found in gymnosperms, and also describes the separation of their methanolysis products. Since the compounds are obtained in a form suitable for liquid-scintillation counting, the procedure should be useful for studies on the biosynthesis of these compounds by isotopic tracer methods. Its possible application to studies on the biosynthesis

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[1] A. G. MCINNES, D. H. BALL, F. P. COOPER and C. T. BISHOP, *J. Chromatog.* **1**, 556 (1958).
[2] C. T. BISHOP and F. P. COOPER, *Can. J. Chem.* **38**, 388 (1960).
[3] H. W. KIRCHER, *Anal. Chem.* **32**, 1103 (1960).
[4] M. GEE and H. G. WALKER, *Anal. Chem.* **34**, 650 (1962).
[5] H. G. JONES and M. B. PERRY, *Can. J. Chem.* **40**, 1339 (1962).
[6] G. O. ASPINALL, *J. Chem. Soc.* 1676 (1963).
[7] C. T. BISHOP, *Methods of Biochemical Analysis*, vol. 10, p. 1. Interscience, New York (1962).
[8] H. P. BURCHFIELD and ELEANOR E. STORRS, *Biochemical Applications of Gas Chromatography*. Academic Press (New York and London) (1962).

of raffinose, picein (β-D-glucopyranoside of 4-hydroxyacetophenone), pungenin (3-β-D-glucopyranoside of 3,4-dihydroxyacetophenone) and taxicatin (β-D-glucopyranoside of di-*O*-methylphloroglucinol) is indicated.

RESULTS AND DISCUSSION

The GLPC analysis of the methylated neutral constituents of *Picea mariana* (black spruce), *P. glauca* (white spruce) and a horticultural variety of *Taxus* sp. (yew) are given in Fig. 1. The retention volumes of some compounds, which are likely to occur in the neutral fraction of plant extracts, relative to octamethyl sucrose, are given in Table 1. It can be seen that the neutral fractions of all species contain monosaccharides, sucrose and raffinose. Previous work[9] and investigations in this laboratory have shown a seasonal variation of raffinose synthesis. The present work has been carried out on plant extracts prepared at the beginning

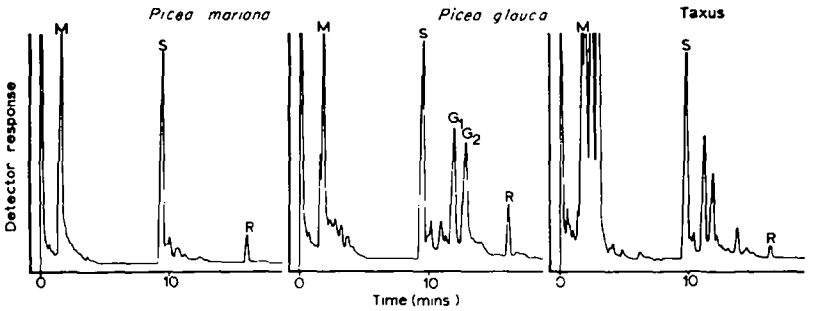


FIG. 1. CHROMATOGRAPHIC SEPARATION OF THE METHYLATED WATER-SOLUBLE FRACTIONS OF TWIGS OF THREE SPECIES OF GYMNOSPERMS.

The column was a 4 ft × ¼ in. stainless steel coil packed with silicone gum rubber on acid-washed Chromosorb W (5% w/w). The temperature was programmed from 150° to 295° at 7·9°/min. Helium (60 ml/min) was used as carrier gas. M: methylated monosaccharides and pinitol, S: octamethyl sucrose, G_1: methylation product of picein, G_2: methylation product of pungenin, R: hendecamethyl raffinose.

of December. A major difference in the composition of *P. mariana* and *P. glauca* is the presence of two compounds in the latter, corresponding to peaks G_1 and G_2. Infrared and GLPC analysis established that peak G_1 was identical to the product of methylation of an authentic sample of picein, and peak G_2 to the corresponding derivative prepared from an authentic sample of pungenin. The occurrence of picein in white spruce was confirmed by isolating it from another batch of *P. glauca* using charcoal chromatography.

The proton magnetic resonance (PMR) spectra of compounds G_1 and G_2 contained no signal for an acetyl methyl group. Consequently, these compounds were not tetramethyl picein and pentamethyl pungenin. The spectrum of both compounds, however, contained a doublet, with a spacing of 6·5 counts/sec, at an average τ value of 8·85, and had an intensity corresponding to six protons. Such a doublet is characteristic of two methyl groups substituted on a carbon atom carrying one hydrogen.[10] The acetyl group of both picein and pungenin had been converted, therefore, to an *iso*butyryl group in quantitative yield during the methylation procedure. This conclusion was substantiated when the PMR spectrum of

[9] A. C. NEISH, *Can. J. Botany*, **36**, 649 (1958).
[10] F. A. L. ANET, *Can. J. Chem.* **39**, 2262 (1961).

p-hydroxy acetophenone, which had been methylated under our standard conditions, showed a doublet for two methyl groups, at an average τ value of 8·85, and a septet for a single proton, at an average τ value of 6·58, with a common spin–spin coupling constant of 6·5 counts/sec. These data can only be explained if the acetyl group of *p*-hydroxy acetophenone was converted to an *iso*butyryl group during methylation. A similar septet for a single proton and a doublet for two methyl groups were obtained in the PMR spectra of the aglycones of methylated picein and pungenin which were isolated by GLPC. The aglycone of G_1 is therefore *p-iso*butyryl phenol and o fG_2 5-*iso*butyryl guaiacol. The change in the structure of the aglycone accounts for the difference in the relative retention volumes of G_2 and authentic pentamethyl pungenin (see Table 1).

TABLE 1. THE RETENTION VOLUMES OF SOME FULLY-METHYLATED COMPOUNDS RELATIVE TO OCTA-METHYL SUCROSE ON SILICONE GUM*

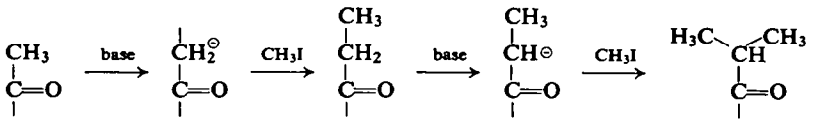
Compound methylated	
Monosaccharides (including pinitol)	0·19
Sucrose	1·00
Melibiose (β-anomer)†	1·14
Melibiose (α-anomer)†	1·18
Maltose (α- and β-anomer)	1·17
Picein‡	1·24
Pungenin‡	1·33
Raffinose	1·68

* Chromatographed as described in Fig. 1.

† These compounds have been provisionally assigned these relative RV-values, since the axial anomer might be expected to have the higher retention volume.

‡ The aglycone of methylated picein is present as *p-iso*butyryl phenol, and of pungenin as 5-*iso*butyrylguaiacol (see text). Authentic pentamethyl pungenin has a relative retention volume of 1·26.

The conversion of an acetyl to an *iso*butyryl group was probably due to the successive reaction of carbanions with methyl iodide by an S_N2 mechanism, as shown below.



Although considerable care was taken to remove traces of base from the silver oxide used in the methylation procedure, it would appear that the reaction medium was sufficiently basic to catalyse this reaction. In this respect, it is perhaps pertinent to mention that the N,N-dimethylformamide, the solvent in the methylation procedure, was used as supplied by the manufacturer without further purification.

The above side reaction in no way detracts from the value of the method since the products are formed in quantitative yield. If this method were used to isolate compounds for structural

analysis, care would have to be taken in the interpretation of the results if the presence of C—CH_3 groups were established, since the latter could be formed during the methylation procedure from any carbon atom carrying an acidic hydrogen.

A quantitative difference was found between *P. glauca* samples obtained from Saskatoon and Halifax. The material collected in Halifax contained approximately equimolar amounts of pungenin and picein (see Fig. 1), whereas the concentration of pungenin was twice that of picein in the Saskatoon sample.

Taxus gave three major peaks between sucrose and raffinose (see Fig. 1). The largest of these was probably tetramethyl taxicatin since GLPC analysis of the products of methanolysis gave the expected glucose derivatives (see Table 2) and an aglycone which had the same retention volume as an authentic sample of phloroglucinol dimethyl ether. The latter had a

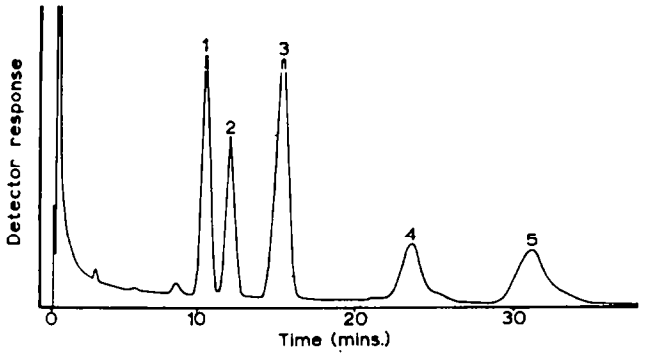


FIG. 2. CHROMATOGRAPHIC SEPARATION OF THE METHANOLYSIS PRODUCTS OF FULLY METHYLATED RAFFINOSE.

A 5 ft × ¼ in. stainless steel coil packed with Carbowax 20M on Chromosorb W (20% w/w) was operated isothermally at 170°. Other conditions were the same as described for Fig. 1.
(1),(2): methyl 1,3,4,6-tetra-O-methyl-α-&-β-D-fructofuranoside
(3): methyl 2,3,4,6-tetra-O-methyl-α-&-β-D-galactopyranoside
(4),(5): methyl 2,3,4-tri-O-methyl-β-&-α-D-glucopyranoside

much higher retention volume than the pentamethyl glucosides and therefore was analysed at 215°.

In view of past researches, the monosaccharide fractions could be further separated into their individual components if desired by the method, for example, of Gee and Walker.[4] A more interesting application of this method described above would be studies on the composition and labelling patterns of oligomers. The usefulness of this procedure is illustrated for the case of raffinose in Fig. 2. Methanolysis of hendecamethyl raffinose yields a mixture of partially and fully methylated glycosides. The relative retention volumes of these compounds together with the two anomers of fully methylated glucose are given in Table 2. The derivatives of the individual monosaccharides are well separated and can be collected for radioactivity measurements. It is therefore a relatively simple matter to determine the specific activities of the glucose, fructose and galactose moieties in a sample of ^{14}C-labelled raffinose. In this laboratory the activities are determined with a liquid scintillation counter. The methylated sugars can be eluted quantitatively from the GLPC capillary collecting tubes with commonly used scintillation fluids (e.g. ethanol–toluene). This method is at present being used to investigate the biosynthesis of raffinose.

Another application of the technique would be the determination of labelling patterns in aromatic glucosides. For example, pungenin methylated under our standard conditions yields the two anomers of pentamethyl glucose (see Table 2), and the aglycone 5-*iso*butyrylguaiacol (see above). These compounds are readily separated on our standard GLPC columns. The retention volume of the aglycone relative to methyl 2,3,4,6-tetra-O-methyl-α-D-glucopyranoside on silicone gum is 2·69 at 130° (isothermal) and 3·58 on Carbowax 20M at 215° (isothermal). On both of these columns the total time for analysis is less than 20 min. This procedure has obvious advantages over the more cumbersome method used previously in studying the biosynthesis of pungenin which was based on charcoal chromatography.[11]

TABLE 2. THE RETENTION VOLUMES OF METHYLATED HEXOSES RELATIVE TO METHYL 2,3,4,6-TETRA-O-METHYL-α-D-GLUCOPYRANOSIDE ON CARBOWAX 20M*

Compound	
Methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside	0·72
Methyl 1,3,4,6-tetra-O-methyl-α-D-fructofuranoside	0·76
Methyl 1,3,4,6-tetra-O-methyl-β-D-fructofuranoside	0·89
Methyl 2,3,4,6-tetra-O-methyl-α-D-glucopyranoside	1·00
Methyl 2,3,4,6-tetra-O-methyl-α-&-β-D-galactopyranoside	1·17
Methyl 2,3,4-tri-O-methyl-β-D-glucopyranoside	1·87
Methyl 2,3,4-tri-O-methyl-α-D-glucopyranoside	2·56

* Chromatographed as described in Fig. 2.

It is anticipated that this method will be used for surveying the composition of a large number of plant species, isolating new compounds, studying seasonal changes in composition and biosynthetic investigations.

EXPERIMENTAL

Preparation of Extract

Twigs collected from the tip of branches (about 20 g) were cut into pieces about 1 cm long and blended with 170 ml hot 90% ethanol containing a little sodium bicarbonate, using a VirTis homogenizer. The homogenate was filtered, the residue washed with boiling 80% ethanol and the filtrate evaporated at 40° on a rotary evaporator. The residue was taken up in 30% aqueous methanol (about 140 ml), and partitioned with two volumes of petroleum ether. The aqueous layer was separated, filtered through Celite Analytical Filter Aid and evaporated to dryness at 40°. This residue was dissolved in water (50 ml), filtered, and deionized by passage through a column composed of 45 ml Amberlite IR-120 (acid form) on top of 60 ml of Duolite A-4 (free base). The eluate, which contains the neutral, water-soluble, constituents of the plant, was evaporated to dryness under reduced pressure and stored *in vacuo* over phosphorus pentoxide until the methylation was carried out.

Methylation

The dry neutral fraction was methylated by a modification of Kuhn's procedure.[12] The sample (about 250 mg) was dissolved in 40 ml dimethylformamide and 16 g silver oxide and

[11] A. C. NEISH, *Can. J. Botany* **37**, 1086 (1959).
[12] R. KUHN, H. TRISCHMANN and I. LÖW, *Angew. Chem.* **67**, 32 (1955).

8 ml methyl iodide added. The reaction mixture (in a glass-stoppered flask) was shaken mechanically. One milliliter of methyl iodide was added after 24 and 48 hr. After 72 hr, the solid material was removed by filtration and washed with chloroform. The filtrate was evaporated to dryness at 50° under reduced pressure and the last traces of N,N-dimethylformamide removed by azeotropic distillation with ethanol. The residue was taken up in chloroform (30 ml), filtered through Celite Analytical Filter Aid, and the filtrate concentrated to make about a 50 per cent solution. At all times, care was taken to exclude water from the reaction solution. The silver oxide was freshly prepared, thoroughly washed with water (until free of base), methanol and finally with acetone and then dried *in vacuo*.

Gas-liquid Partition Chromatography

The methylated neutral fraction (2–10 microliters) was analysed on a silicone gum rubber column using an F & M model 500 gas chromatograph equipped with a katharometer detector. Details are given in the caption of Fig. 1. Identification of compounds was made by observation of peak enhancement following injection of a mixture of sample and known standard. In the case of glycosides (including oligosaccharides) the compounds were collected, subjected to methanolysis and the reaction products further analysed on a carbowax or silicone gum rubber column. The infrared spectra of material collected in this manner were taken as KBr pellets on a Baird Model 4-55 spectrophotometer, and proton magnetic resonance spectra on a Varian A60 spectrophotometer using deuterated chloroform as solvent, and tetramethyl silane as internal standard.

Isolation of Picein and Pungenin from Picea glauca *by Charcoal Column Chromatography*

A previous analysis of *P. glauca* collected in Saskatoon had yielded pungenin but not picein.[9] This was repeated using twigs collected from *P. glauca* growing in Point Pleasant Park, Halifax, N.S. The neutral, water-soluble, constituents from 109 g (fresh weight) of twigs were chromatographed on charcoal as described previously.[9] The fraction eluted by ethanol containing 20% benzene gave 2·04 g of crystalline material. This was recrystallized twice from absolute ethanol to give 0·38 g picein, m.p. 192–3°, identified by comparison of the infrared spectrum and a mixed melting point with an authentic sample of picein obtained from *P. excelsa*. The next fraction (about 2 g) was eluted from charcoal by 30% benzene in ethanol. This gave crystalline pungenin, which was also identified by its infrared spectrum and a mixed melting point. The yield of pure material was low compared with the previous experiment.[9]

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