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PHYTOCHEMISTRY OF THE *SALICACEAE*

III. A GAS–LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE IDENTIFICATION OF PLANT PHENOLS

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SUMMARY

A method is described for the gas chromatographic analysis of a number of phenols and related compounds that are commonly found in members of the *Salicaceae* and in other plants. The phenols listed can be resolved by using one or more columns. The procedure was successfully applied to various plant samples and is extremely useful for identification of aglycones from phenolic glycosides. Application of the procedure to *Populus* samples was not uniformly successful.

INTRODUCTION

Gas–liquid chromatography (GLC) has proved to be an extremely useful tool in the examination of samples of *Populus*[1,2], *Salix*[1,2], *Chosenia*[3] and *Toisusu*[4] species for phenolic glycosides. Examination of micro-samples by this technique has permitted investigation of chemotaxonomic problems, seasonal variation[5], changes with age, geographical variation and hybridisation problems. It was thought that the use of a similar technique for free phenols in *Salicaceae* should be possible and, if successful, would shed further light on the above phytochemical problems. A screening procedure for simple plant phenols would also be adaptable for investigation of other genera.

EXPERIMENTAL

The gas chromatograph used throughout this work was a Varian Aerograph 1840, equipped with dual flame ionization detectors and a matrix temperature programmer. The signal was recorded on a Varian Aerograph recorder, 1 mV full-scale deflection. Nitrogen was used as the carrier gas.

The column packings used were 3% OV-1 on Chromosorb G, AW/DMCS, 80–100 mesh; 2% OV-17 on Chromosorb G, AW/DMCS, 80–100 mesh; 2% OV-25 on Chromo-

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sorb G, AW/DMCS, 80–100 mesh; and 4% OV-25 on Chromosorb G, AW/DMCS, 100–120 mesh. All columns were prepared and conditioned as described earlier[1]. The temperature programme used for all columns was 10 min under isothermal conditions (100°) then an increase of 4°/min for 10 min, 10°/min for a further 10 min and finally 15°/min to the maximum temperature (350°). The maximum temperature was maintained until all components were eluted. Other condition parameters are listed in Table I. Owing to the high thermal stability of the column packings, dual compensatory columns were not necessary.

The compounds listed in Table I were prepared for injection as their trimethylsilyl derivatives by dissolving approximately 20 μg in 100 μl of Tri-Sil (Pierce Chemical Co.) and allowing the mixture to stand for 10 min. A Hamilton 25-μl syringe was used to inject 10 μl of the resulting reaction mixture. Retention times were recorded relative to the internal standards (naphthalene or dimethyl phthalate), which were injected simultaneously as solutions in diethyl ether.

Synthetic mixtures of phenols were also tested but the results in Table I and Fig. 1 were derived from injections of the individual compounds with either naphthalene or dimethyl phthalate.

The above GLC procedure was then applied to various natural products as follows.

Bark from Populus species

Consistent results were obtained by refluxing coarsely ground, dried bark (10 mg) for 1 h with acetone (2 ml), followed by removal of the extract via a cotton-tipped teat pipette. The extract and washings (1 ml) were combined in a test-tube with a capillary tube bottom (100-μl capacity in the capillary portion)[6] and evaporated to dryness on a rotary evaporator. Tri-Sil (100 μl) was added to the residue and allowed to react for 10 min before injecting.

Powdered cloves (Eugenia caryophyllus)

This sample (10 mg) was extracted and treated with Tri-Sil as described above for bark from *Populus* species. Pure eugenol was trimethylsilylated in the usual way and used as a reference compound.

Vanilla pods (Vanilla planifolia)

Vanilla pods were coarsely powdered and extracted in the cold by stirring with 95% ethanol. One drop of the resulting filtrate was evaporated to dryness at as low a temperature as possible. The residue was then treated with Tri-Sil (100 μl) and injected after 10 min.

Leaves of Berberis vulgaris

Red and green leaves were ground and separately extracted as described for bark from *Populus* species.

Identification of aglycones from phenolic glycosides

Picein was chosen as a representative phenolic glycoside found in many members of the *Salicaceae*. Picein (1–2 mg) was added to 10% hydrochloric acid (0.5 ml) and the mixture was refluxed for 15 min. After cooling, the reaction mixture was

TABLE I

RELATIVE RETENTION VALUES OF TRIMETHYLSILYLATED PHENOLS AND RELATED COMPOUNDS WITH RESPECT TO AN INTERNAL STANDARD

No.	*Compound*	*Column*			
		3% OV-1	*2% OV-17*	*2% OV-25*	*4% OV-25*
1	Furfural[a]	0.21	0.37	—	—
2	Cyclohexanediol	0.44	0.59	0.43	0.39
3	Piperonal[a]	0.58	2.79	—	—
4	4-Hydroxybenzaldehyde	0.73	2.37	2.04	1.47
5	Coumarin[a]	0.91	4.05	—	—
6	Salicyl alcohol	1.06	2.26	1.93	1.34
7	4-Hydroxyacetophenone	1.10	3.29	3.18	1.91
8	Eugenol	1.15	2.80	—	1.82
9	Salicylic acid	1.33	2.90	—	—
10	4-Hydroxy-3-methoxybenzaldehyde (vanillin)	1.36	3.75	—	2.16
11	Cinnamic acid	1.37	3.81	3.69	2.06
12	4-Hydroxybenzoic acid	1.82	3.88	—	1.90
13	2,5-Dihydroxybenzaldehyde	1.94	4.33	3.92	2.23
14	Syringaldehyde	1.96	5.26	5.11	2.75
15	4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	2.25	5.10	4.88	2.57
16	2,3-Dihydroxybenzoic acid	2.32	4.74	4.21	2.37
17	2,5-Dihydroxybenzoic acid	2.33	4.90	4.70	2.47
18	2-Hydroxycinnamic acid (*o*-coumaric acid)	2.35	5.26	5.03	2.66
19	4-Hydroxy-3,5-dimethoxybenzoic acid	2.42	5.74	5.59	2.94
20	3,4-Dihydroxybenzoic acid	2.46	5.14	4.50	2.53
21	4-Hydroxycinnamic acid (*p*-coumaric acid)	2.61	5.74	5.50	2.94
22	4-Hydroxy-3-methoxycinnamic acid	2.86	6.36	6.14	3.09
23	3,4-Dihydroxycinnamic acid	—	—	—	2.77
	Internal standards				
24	Retention time of naphthalene[a] (min)		3.90	4.04	7.99
25	Retention time of dimethyl phthalate[a] (min)	8.30			
	Carrier gas flow-rate at outlet (ml/min)	30	30	30	30
	Column temperature (°C)	100	100	100	100
	Inlet temperature (°C)	250	225	225	225
	Detector temperature (°C)	350	350	350	350
	Column length (ft.)	4	6	6	6

[a] These compounds do not react with Tri-Sil under the conditions given.

evaporated to dryness in a rotary evaporator and extracted twice with 2-ml volumes of diethyl ether. The ethereal extract was evaporated to dryness in a capillary-bottom test-tube and Tri-Sil (100 μl) was added. The sample was injected after 10 min.

RESULTS

The four columns used gave symmetrical peaks with little or no tailing effect. Table I shows the relative retention values for a number of simple phenols and related compounds that are commonly found in plants. Fig. 1 gives an indication of the re-

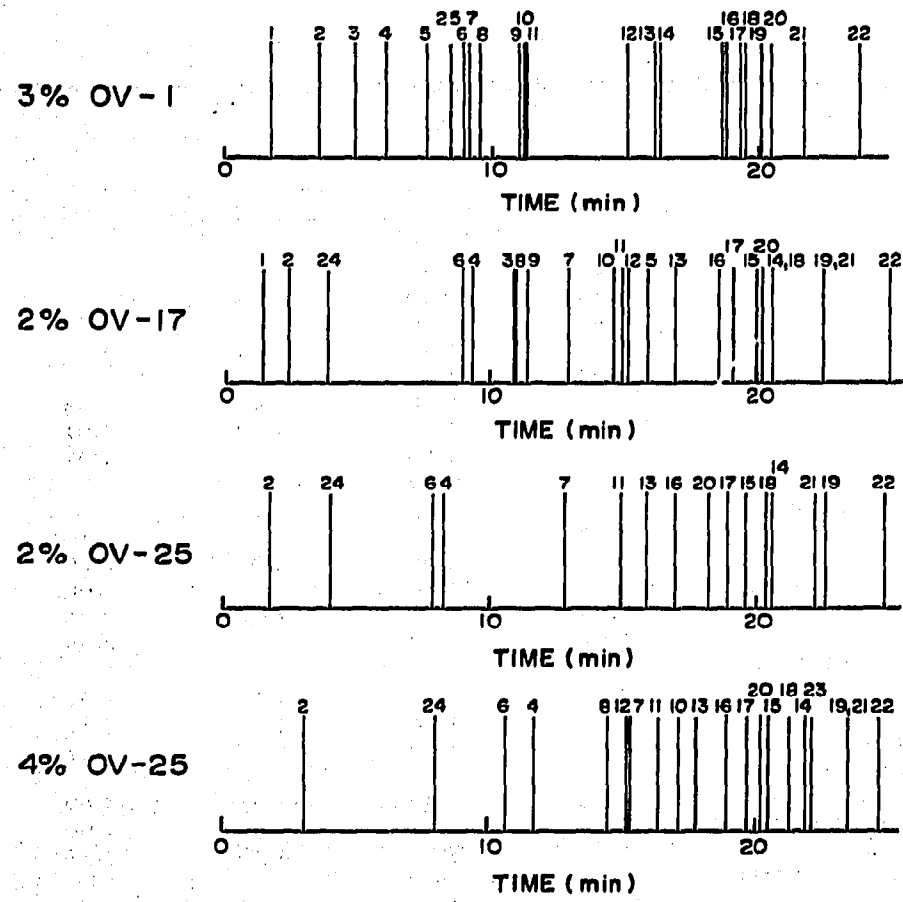

Fig. 1. Retention times for trimethylsilylated phenols and related compounds.

tention times on each column and can be used to select the most appropriate column(s) for resolution of particular compounds.

The extracts of *Populus* bark were run on the OV-1 and 4% OV-25 columns. Representative chromatograms are shown in Figs. 2a and 2b (*Populus canadensis* cv. "Erecta", 1 year old and 2 years old bark, respectively), Figs. 3a *(P. balsamifera)*, 3b *(P. deltoides)* and 3c *(P. petrowskyana)*.

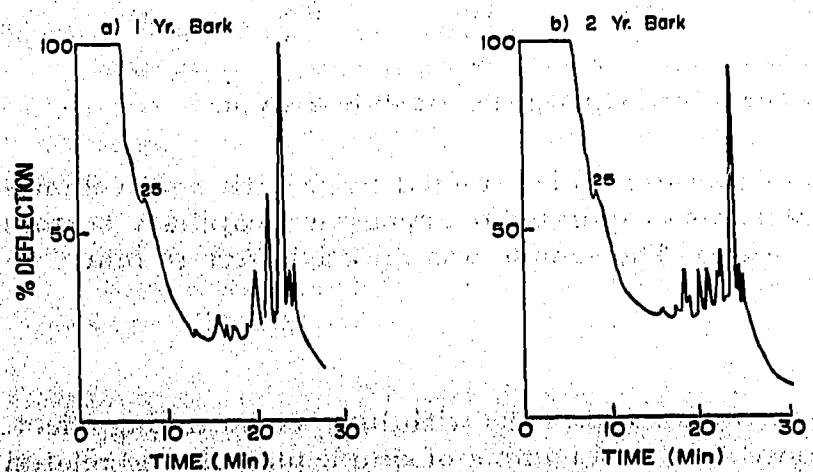

Fig. 2. Chromatograms of *P. canadensis* cv. 'Erecta' on OV-1.

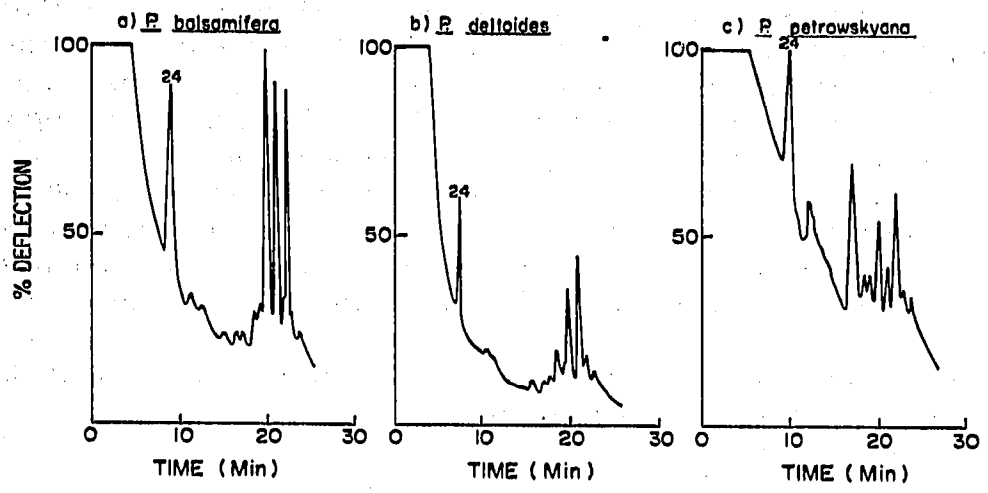

Fig. 3. Chromatograms of *Populus* species on 4% OV-25.

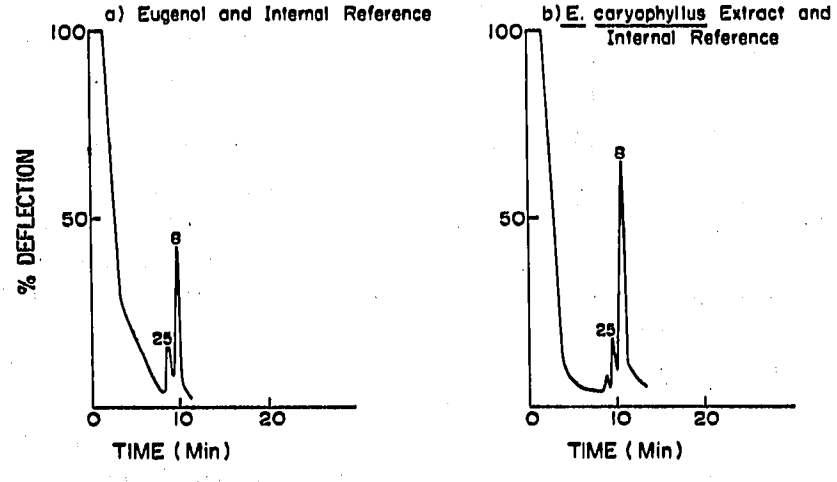

Fig. 4. Chromatograms of *Eugenia caryophyllus* on OV-1.

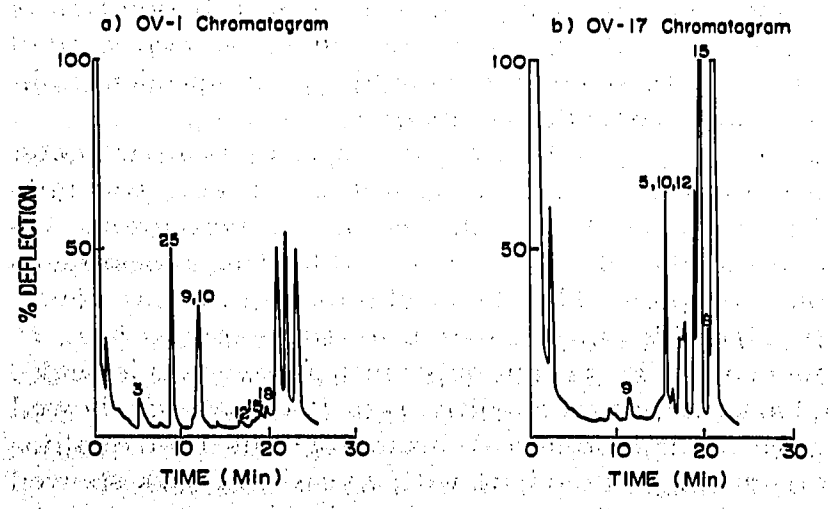

Fig. 5. Chromatograms of *Vanilla planifolia*.

Application of the simple extraction procedure to powdered cloves showed that eugenol was the principal constituent of the extract and that it was identified by the GLC procedure. The chromatograms of the extract showed only two significant peaks, the larger of which was shown to be eugenol using three columns. Figs. 4a and 4b show typical chromatograms with OV-1. The smaller peak was not identified.

Vanilla is known to contain many phenolic compounds. Figs. 5a and 5b show the chromatograms obtained on the OV-1 and OV-17 columns. The labelled peaks were confirmed by GLC analysis and co-chromatography with reference compounds on at least three columns. The other peaks in the chromatograms could not be identified.

The chromatograms from *Berberis vulgaris* on OV-1 are shown in Figs. 6a and 6b but no attempt was made to identify the components.

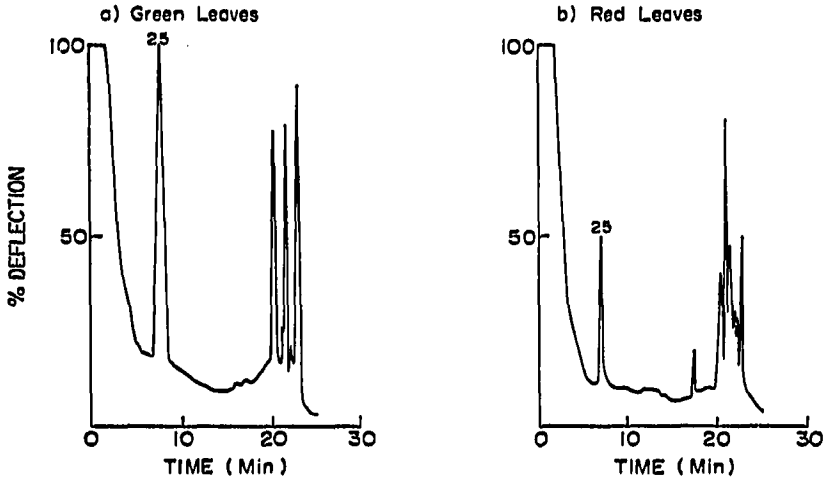

Fig. 6. Chromatograms of *Berberis vulgaris* on OV-1.

DISCUSSION

The columns described provided a method for identifying the simpler phenols, phenolic acids and related compounds that frequently occur in plants. Resolution of individual compounds was very good and, although no single column separated all of the component phenols, the use of two or three columns should give an excellent indication of the presence or absence of any particular compound.

This attempt to use GLC analysis of phenols in *Populus* species as a parameter in studies of chemotaxonomy, geographical variation, hybridisation, etc., was only partially successful. The content of free phenols that showed on chromatograms was more uniform than is the case with the phenolic glycosides, and for this reason tends to be less useful for chemotaxonomic purposes. The phenol chromatogram may nevertheless be more complex than the glycoside chromatogram for some species (*e.g.*, *P balsamifera* has three major phenol peaks (Fig. 3a) but only one major glycoside peak).

Study of a large number of bark samples from cultivars of *P. canadensis* showed that all samples of the same age gave virtually identical chromatograms, both qualitatively and quantitatively. Comparison of 1 year old bark with 2 years old bark showed some changes, but these lacked consistency. The shape of the latter portions of the

chromatograms (Figs. 2a and 2b) was consistent for most samples on OV-1. The results for the *P. canadensis* samples indicate that it would be impossible to identify such cultivars on the basis of the free phenol constituents. This is in agreement with the close morphological similarity of the various cultivars which have resulted from a single hybrid combination.

Samples of *P. deltoides*, *P. petrowskyana* and *P. balsamifera* could be consistently clearly differentiated on OV-1 and 4% OV-25 columns, (Figs. 3a–c) but hybrids of *P. balsamifera* × *P. petrowskyana* and *P. deltoides* × *P. petrowskyana* gave less clear results. With the latter hybrid, the only definite conclusion was that it tended to be more similar to the *P. deltoides* than to the *P. petrowskyana*. *P. petrowskyana* is itself reported to be a hybrid of *P. laurifolia* and *P. nigra* and may well be the underlying cause of some of the trouble in correlating gas–liquid chromatograms of its hybrids. This species is therefore contributing characteristics (both chemical and morphological) from two diverse parents. As the hybrids are composed of a 50% contribution from *P. deltoides* and 25% contributions each from *P. laurifolia* and *P. nigra*, it is not surprising that their chromatograms appear closer to those from *P. deltoides*.

It was encouraging to find that, apart from the hybrids, the same species gave virtually identical chromatograms for specimens from widely separated geographical locations (*e.g.*, samples of *P. deltoides* and *P. balsamifera*).

The application of the GLC technique to phenols in other phytochemical problems was uniformly successful. It offers an excellent means of identification of aglycones from phenolic glycosides. The experiments with picein showed that as little as 1–2 mg of glycoside gave sufficient phenol for identification on all four columns, including co-chromatography with 4-hydroxyacetophenone in each instance. As an extension of this aspect, two hydrolysates from phenolic glycosides sent to us by Dr. K. S. CHOPRA[7] were analysed by the GLC method and clearly shown (two columns) to be 4-hydroxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid. In these two experiments, the results were confirmed by mass spectral analysis of the purified aglycone hydrolysates.

Application of the technique to clove and vanilla extracts showed that the methods described could be used to isolate and identify the phenolic components. The extracts of *Berberis vulgaris* were chromatographed to see what sort of chromatogram would result and if there was a difference between extracts from green and red leaves.

It will be obvious that the extraction process used in this study is non-specific and caution should therefore be observed when examining samples whose constituents are totally unknown. In these circumstances, the techniques and results in this paper should be used only in conjunction with other more specific means of identification, such as derivatisation or mass spectral analysis.

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