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Note

High-performance liquid, gas–liquid and thin-layer chromatography of naturally occurring flavonoids, phenolic and related compounds

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Numerous studies on the isolation, separation and determination of naturally occurring phenolic compounds have been reported. They were recently discussed by Strack and Krause[1], by Soczewiński *et al.*[2] and by Felice and Kissinger[3]. Gas–liquid chromatography (GLC) on packed columns, high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) have been widely used for this purpose.

This paper describes the separation of these phenolic compounds by various chromatographic methods, including GLC in glass capillary columns, gradient elution from an alkylphenyl column in HPLC and reversed-phase high-performance TLC (HPTLC).

EXPERIMENTAL

HPTLC pre-coated plates of silica gel 60 F_{254}, RP-2, RP-8 and RP-18 $F_{254}S$ were obtained from Merck (Darmstadt, G.F.R.), and pre-coated plastic sheets of Wang-Polyamide for TLC from Macherey, Nagel and Co. (Düren, G.F.R.). Samples of flavonoids, aromatic acids and phenolic compounds were purchased from K. Roth (Karlsruhe, G.F.R.), Fluka (Buchs, Switzerland) or Aldrich (Milwaukee, Wisc., U.S.A.). The investigated compounds (1-μg amounts) were applied 12 mm from the lower edges of the plates with 0.75-μl capillaries. The mobile phases are listed in Table I; detection was in UV radiation (254 and 360 nm).

The liquid chromatograph was an apparatus from Waters Assoc. (Milford, Mass., U.S.A.) equipped with two pumps (Model 6000 A), a solvent programmer (Model 660), a sample loop (Model U6K), a UV detector (Model 440) operating at 254 nm and a 300 × 3.9 mm I.D. stainless-steel column pre-packed with μBondapak alkylphenyl (mean particle size 10 μm). The Packard-Becker model 421 gas chromatograph (Delft, The Netherlands) was equipped with either a packed column (1 m × 1/8 in. I.D.) of 2% OV-17 on Chromosorb W HP (100–120 mesh) or a capillary column (50 m × 0.5 mm I.D.) coated with SE-52.

RESULTS AND DISCUSSION

High-performance thin-layer chromatography

Silica gel, bonded phases and polyamide layers were compared on the basis of the chromatographic behaviour of 25 phenolic compounds including flavonoids (aglycones), phenolic acids and related compounds of general interest in plant analysis and found in *Propolis*.

The mobile phases were selected after studying numerous results reported in the literature or obtained in our laboratory; they were optimized as far as possible. For HPTLC on silica gel and Wang-polyamide, the mobile phases described by Hiermann and Kartnig[4] and by Wollenweber and Egger[5] were used. Partition chromatography was first investigated with RP-2, RP-8 and RP-18 chromatoplates. In the present application, the RP-8 bonded phase afforded the best resolution with a simple mobile phase of ethanol and water. The results of HPTLC on silica gel, polyamide and RP-8 silica gel are listed in Table I; 62% of the compounds studied were separated on poly-

TABLE II

THIN-LAYER CHROMATOGRAPHIC BEHAVIOUR OF AROMATIC ACIDS, PHENOLS AND FLAVONOIDS ON THREE ADSORBENTS

Compound	R_F *value on*		
	*Polyamide**	*Silica gel***	*Silica gel RP-8 S****
Tectochrysin	0.91	0.81	0.11
Chrysin	0.78	0.70	0.23
Acacetin	0.74	0.65	0.23
Galangin	0.70	0.78	0.28
Kaempferid	0.67	0.74	0.50
Rhamnetin	0.43	0.54	0.38
Apigenin	0.34	0.49	0.46
Kaempferol	0.26	0.59	0.28
Quercetin	0.09	0.41	0.61
Benzoic acid	0.80	0.79	0.50
Caffeic acid	0.24	0.45	0.75
Cinnamic acid	0.83	0.78	0.41
m-Coumaric acid	0.54	0.62	0.64
o-Coumaric acid	0.47	0.66	0.59
p-Coumaric acid	0.50	0.63	0.67
Ferulic acid	0.66	0.63	0.69
Gallic acid	0.07	0.25	0.82
Gentisic acid	0.07	0.55	0.75
Hydrocaffeic acid	0.30	0.37	0.75
4-Hydroxybenzoic acid	0.48	0.62	0.70
Isoferulic acid	0.61	0.58	0.67
Isovanillin	0.82	0.56	0.67
Protocatechic acid	0.19	0.45	0.76
Salicylic acid	0.20	0.76	0.76
Vanillin	0.84	0.67	0.62

* Mobile phase, benzene–ethyl methyl ketone–methanol (60:26:14, v/v).
** Mobile phase, benzene–ethyl acetate–formic acid (40:10:5, v/v).
*** Mobile phase, ethanol–water (55:45, v/v).

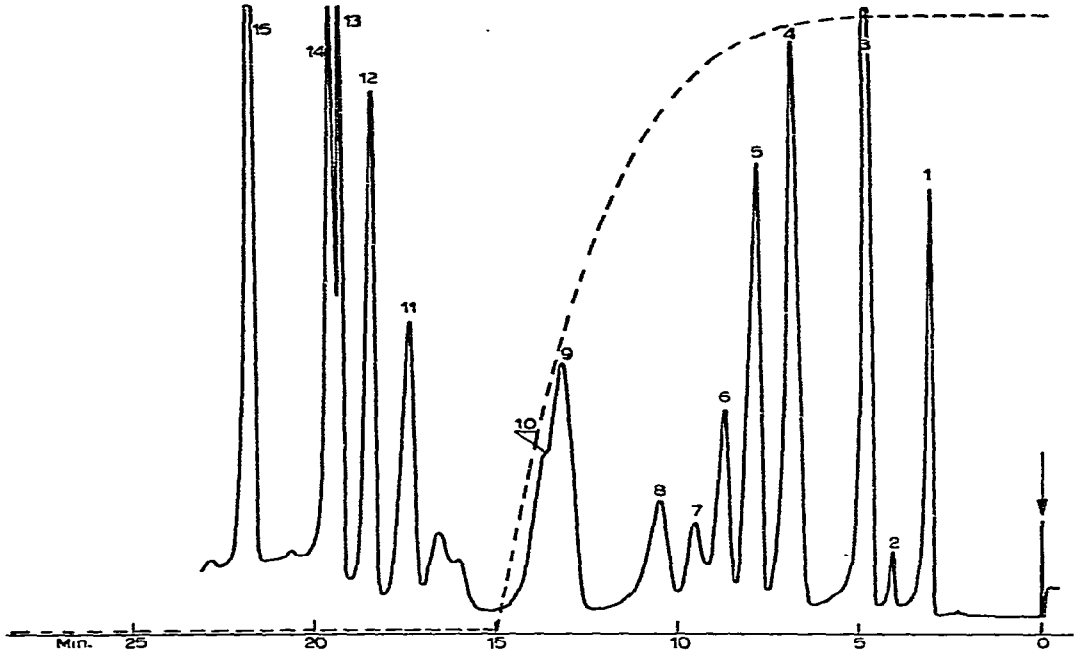

Fig. 1. Chromatogram of the HPLC of a synthetic mixture of aromatic acids and phenols on a column of μBondapak alkylphenyl (10 μm). Mobile phase: gradient following programme curve 10; programme time, 15 min; initial conditions, ethanol–water containing 0.1% of acetic acid (12.5:87.5, v/v); final conditions (maintained for 10 min after the programme end), ethanol–water containing 0.1% of acetic acid (40:60, v/v); flow-rate, 1.5 ml/min. Peaks: 1 = gallic acid; 2 = gentisic acid; 3 = protocatechic acid; 4 = 2,4-dihydroxybenzoic + hydrocaffeic acids; 5 = 4-hydroxybenzoic acid; 6 = 3-hydroxybenzoic acid; 7 = salicylic acid; 8 = caffeic acid; 9 = *p*-coumaric acid + isovanillin; 10 = vanillin; 11 = benzoic acid; 12 = *m*-coumaric + ferulic acids + scopoletol; 13 = isoferulic acid; 14 = *o*-coumaric + piperonylic acids + coumarin; 15 = cinnamic acid.

amide, 48% on silica gel and 44% on RP-8 silica gel. Polyamide afforded the best selectivity (larger dispersion of R_F values in all groups for the compounds investigated), but was least efficient (larger spot areas after chromatography).

High-performance liquid chromatography

Because of the widely differing polarities of the compounds investigated and the constant presence in crude plant extracts of extraneous components (which shorten column life), adsorption chromatography is not advised.

After several attempts, the alkylphenyl bonded phase was selected because of its high selectivity. As in GLC, the presence of phenyl groups on the phase improved the resolution of aromatic compounds.

Although the separation of some aromatic acids was better when isocratic elution was used, a programmed mobile phase gradient was necessary to obtain the best separation of all the compounds listed in Fig. 1.

The use of ethanol instead of the methanol previously described led to an im-

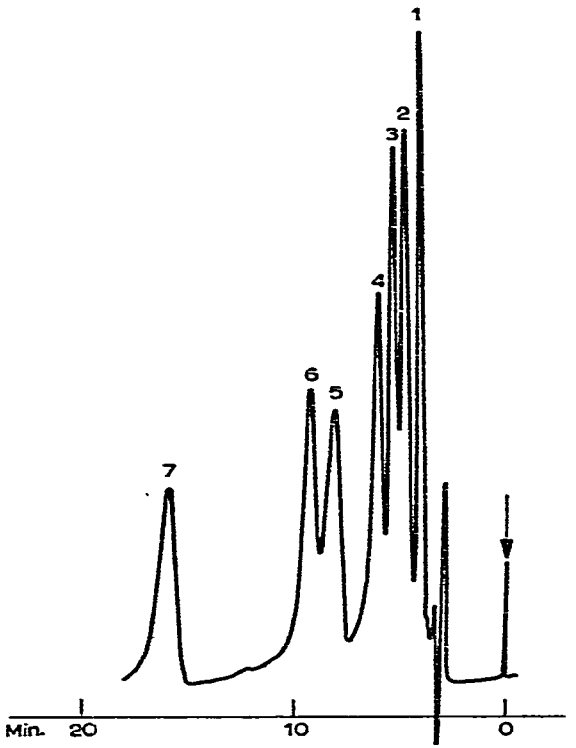

Fig. 2. Chromatogram of the HPLC of a synthetic mixture of flavonoids on a column of μBondapak alkylphenyl (10 μm). Mobile phase: ethanol–water-acetic acid (47.5:47.5:5.0, v/v); flow-rate 1 ml/min. Peaks: 1 = quercetin; 2 = kaempferol; 3 = apigenin; 4 = rhamnetin; 5 = galangin + kaempferid; 6 = chrysin + acacetin; 7 = tectochrysin.

portant improvement in the general resolution of the compounds under investigation. Using the same bonded phase, flavonoids were separated under isocratic conditions, as shown in Fig. 2. Acetic acid was added to the mobile phases to decrease the ionization of the acids.

The identification of the aromatic acids and phenols was unaffected by the presence of flavonoids in crude extracts; however, some of the aromatic acids and phenols interfered with separation of the flavonoids. Thus, it is advisable to carry out prior separation of these two groups of compounds before HPLC.

Gas–liquid chromatography

Silylation with N,O-bis(trimethylsilyl)trifluoroacetamide is a convenient method for quantitatively preparing volatile derivatives of the compounds investigated[6]. The GLC of such derivatives on capillary columns of SE-52 gave, as shown in Fig. 3, better results than those obtained with the packed columns hitherto used for similar separations.

The temperature and the gas flow-rate required for eluting the flavonoids are inconsistent with the use of capillary columns. A stationary phase such as OV-17 in packed columns led to improved resolution in comparison with the SE-30 or SE-52

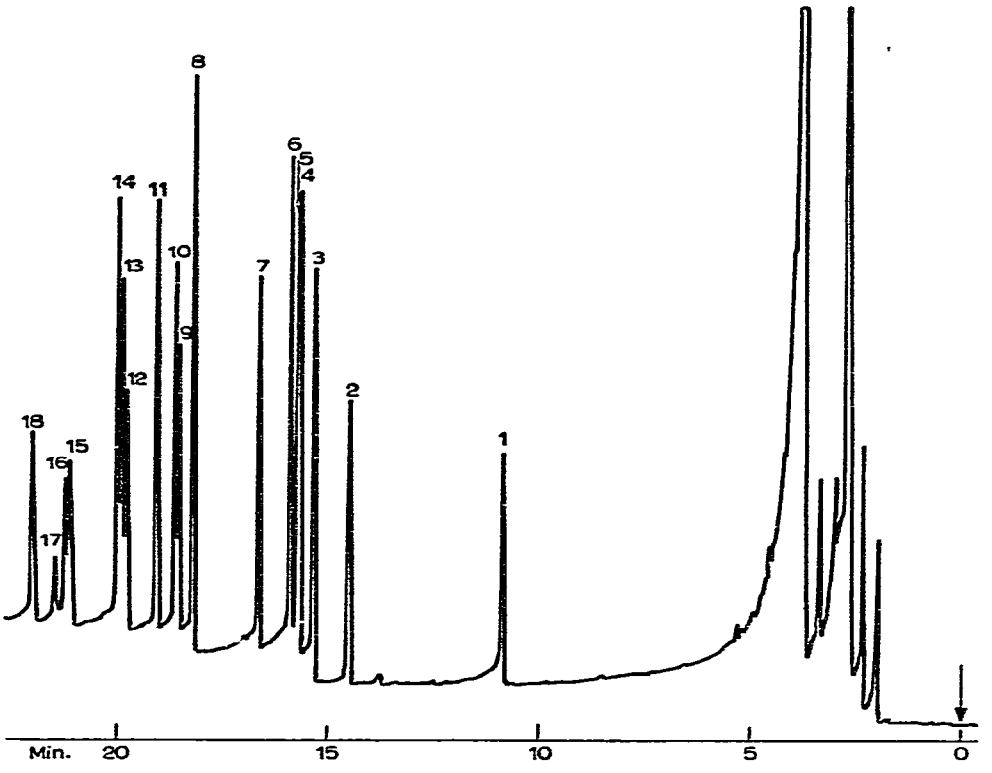

Fig. 3. Chromatogram of the GLC of a synthetic mixture of silylated derivatives of 18 aromatic acids and phenols. Conditions: glass capillary column (50 m × 0.5 mm I.D.) coated with SE-52; helium flow-rate, 3 ml/min; make-up: gas, 20 ml/min; detector and injector temperature, 310°; oven temperature, programmed from 175° to 255° at 5°/min, then to 275° at 7.5°/min and kept at 275°. Peaks: 1 = benzoic acid; 2 = coumarin; 3 = salicylic acid; 4 = cinnamic acid; 5 = vanillin; 6 = isovanillin; 7 = 4-hydroxybenzoic acid; 8 = gentisic acid; 9 = *o*-coumaric acid; 10 = protocatechic acid; 11 = *m*-coumaric acid; 12 = *p*-coumaric acid; 13 = hydrocaffeic acid; 14 = gallic acid; 15 = scopoletol; 16 = ferulic acid; 17 = isoferulic acid; 18 = caffeic acid.

generally recommended for these separations. Fig. 4 shows the separation obtained, within 8 min, of nine flavonoids (as silylated derivatives).

Critical pairs of flavonoids having, after derivatization, x silyloxy groups and a methoxy group are not always separated from the corresponding compounds having $(x + 1)$ silyloxy groups (acacetin and apigenin, for example); another limitation of GLC is that the elution of heterosidic flavonoids requires high temperatures. In spite of these restrictions, GLC remains the best method for separating the compounds investigated in crude extracts of plants.

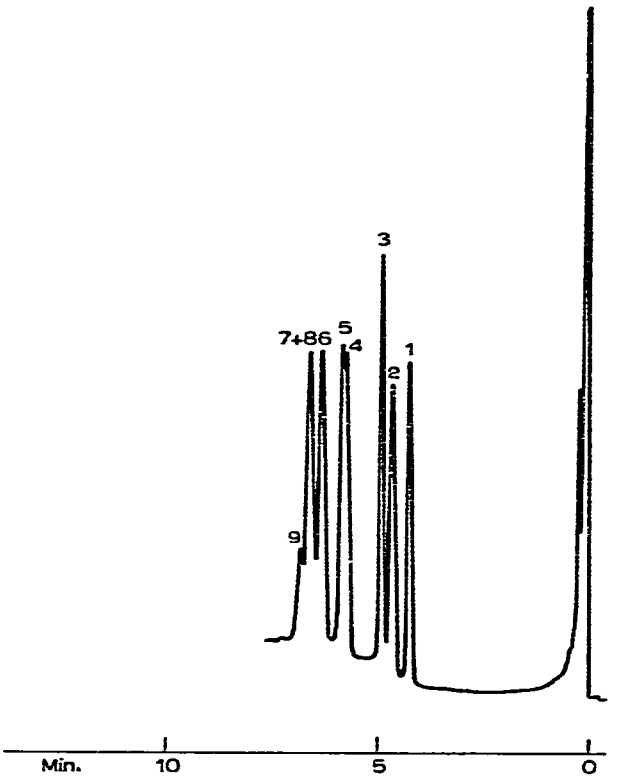

Fig. 4. Chromatogram of the GLC of a synthetic mixture of the silylated derivatives of nine flavonoids on a glass column packed with 2% of OV-17 on Chromosorb W HP (100–120 mesh); nitrogen flow-rate, 55 ml/min; detectors and injectors temperature, 310°; oven temperature, programmed from 200° to 275° at 15°min and kept at 275°. Peaks: 1 = galangin; 2 = chrysin; 3 = tectochrysin; 4 = kaempferol; 5 = kaempferid; 6 = quercetin; 7, 8 = acacetin + apigenin; 9 = rhamnetin.

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