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Note

Separation of peracetylated flavanoid and flavonoid polyphenols by normal-phase high-performance liquid chromatography on a cyano-silica column and their determination

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The characteristics of many plant products, *i.e.*, taste, palatability, nutritional value, pharmacological and toxic effects and microbial decomposition, depend substantially on their polyphenol content. For this reason, studies on such widespread natural compounds are not only of scientific interest, but also of considerable practical significance. In the last 10 years, in this journal alone, at least 40 papers relating to high-performance liquid chromatographic (HPLC) separations of flavanoid and flavonoid polyphenols have been published. Usually the separations were carried out by reversed-phase HPLC, and only in about 10 instances was the normal-phase mode used. Recently, however, Cooper and Smith[1,2] published two important papers describing the advantages of a cyanopropyl-bonded phase as a normal phase. This phase, in fact, seems to be a "universal phase"[2], which can exhibit a different character depending on the polarity of the liquid system, which normally consists of a binary mixture of *n*-hexane and another more polar solvent[1,2].

In this work we studied the effectiveness and versatility of this phase for the separation of complex mixtures of paracetylated polyphenols.

EXPERIMENTAL

Materials

In the course of previous investigations on polyphenols from grapes (*Vitis vinifera*), we were able to isolate in the pure state, as their corresponding peracetyl derivatives, (+)-catechin, (−)-epicatechin, kaempferol glucoside (glucose position not known), quercetin 3-glucoside (isoquercitrin) and the four procyanidins B_1–B_4[3–7]. (−)-Epigallocatechin, (−)-epicatechin 3-gallate and (−)-epigallocatechin 3-gallate

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were recently isolated from green tea and (+)-gallocatechin was obtained from *Trebbiano* grapes[5]. Apigenin and tricin were isolated from *Alfalfa*[8], persicoside was extracted from the bark of *Prunus persica*[9] and naringin was supplied by Fluka (Buchs, Switzerland).

Hesperetin and naringenin, which are the aglycones of persicoside and naringin, respectively, were obtained by acidic hydrolysis of the corresponding glycoside according to Harborne[10]. Quercetin and kaempferol were prepared in the same way.

Polyphenol acetylation

A 100-mg amount of each polyphenolic compound was dissolved in a mixture of 150 μl of anhydrous pyridine and 500 μl of anhydrous acetic anhydride. The solutions were allowed to stand at room temperature. After *ca.* 12 h the solutions were poured into ice–water and the precipitated acetates were recovered by filtration. Incomplete acetylations were never observed.

Thin-layer chromatography (TLC)

Silica gel plates (20 × 20 cm, thickness 250 nm; Stratocrom SI RS, Carlo Erba, Milan, Italy) were employed and were developed with benzene–acetone (8:2) at room temperature. After development, the plates were sprayed with sulphuric acid–40% formaldehyde (9:1, v/v) and heated at 150°C until coloured spots appeared.

HPLC conditions

All measurements were obtained using a Varian LC 5060 ternary liquid chromatograph equipped with a 10-μl loop, combined with a Varian UV-100 variable-wavelength detector. The retention times and the quantitative parameters were determined with a Spectra-Physics Model 4270 integrator. The data system output was fixed at 2.0 absorbance/V and the integrator attenuation was maintained at × 8.

A 5-μm Spherisorb S-5 Nitrile column (250 × 4.6 mm I.D.) (Phase Separations, Queensferry, U.K.) was used, coupled with a precolumn (100 × 4.6 mm I.D.) packed with Partisil-10 PAC (Whatman, Clifton, NJ, U.S.A.).

n-Hexane (A) and ethyl acetate (B) solvents of HPLC grade were supplied by Merck-Bracco (Milan, Italy). Two different elution profiles were chosen for the separations of the mixtures of the above-mentioned compounds: the first was isocratic with A–B (60:40) (Fig. 1), and the second was programmed as follows: 0–15 min, isocratic with A–B (60:40); 15–17 min, linear gradient to A–B (55:45); 17–30 min (end of chromatogram), isocratic with A–B (55:45) (Fig. 2). Separations were usually performed by injecting 10-μl aliquots of each sample in ethyl acetate solution, using a flow-rate of 1 ml/min. Peaks were monitored at 278 nm.

Regeneration of column activity

When the column performance deteriorates, *i.e.* the separations are no longer optimum, the original activity can be regenerated by washing the column with ethyl acetate (flow-rate 1 ml/min) for 1 h.

Calibration graphs

All the quantitative determinations were performed under isocratic conditions

with A–B (60:40). For each pure compound, available in a sufficient amount, ten solutions were prepared with concentrations of 500, 400, 250, 200, 100, 75, 50, 25, 10 and 5 mg/l. The calibration graphs were constructed by plotting the peak-area counts of each chromatographic peak *versus* the corresponding concentration. In our graphical representations, the following units were chosen: 1 mm on the abscissa = 4 mg/l and 1 mm on the ordinate = 2000 peak-area counts. The slopes (m) of the curves $y = mx + q$ are given in Table II, together with the intercepts (q) and with the corresponding correlation coefficients; these parameters were determined according to the least-squares calibration mode. For this purpose, only the experimental data were used, and the points 0,0 were never considered.

Extraction of polyphenolic constituents from the peel of Golden Delicious apples and green tea

From 2 kg of Golden Delicious apples 150 g of peel were obtained, which was cut into small pieces and immediately immersed in 1500 ml of methanol. The mixture was heated at 30°C for 10 min with stirring and left for 24 h at room temperature. After filtration and distillation of the methanol, the residue was dissolved in 500 ml of distilled water and extracted five times with 100-ml portions of ethyl acetate. The remaining aqueous solution was discarded and the organic phase was dried over anhydrous sodium sulphate.

The solvent was then removed under vacuum and the residue was dispersed in 50 ml of chloroform, in which polyphenols are insoluble. After filtration, 360 mg of polyphenols were recovered.

One third of the material extracted was acetylated and analysed by HPLC (Fig. 3a). The remaining part was hydrolysed with ethanol–2 *M* hydrochloric acid according to Harborne[10].

After evaporation of the ethanol, the polyphenol aglycones were extracted from the reaction mixture with ethyl acetate as described above. The corresponding peracetylated material weighed 160 mg; its HPLC profile is shown in Fig. 3b.

In the same way, from 10 g of green tea leaves 1.3 g of polyphenols were extracted. After acetylation of such material 1.5 g of the peracetyl derivative was obtained, the composition of which determined by HPLC is shown in Fig. 3c.

Quantitative determinations

A 100-mg amount of peracetylated material from green tea was dissolved in ethyl acetate and diluted to 50 ml with the same solvent in a volumetric flask. The average values of the peak-area counts, recorded for each component for three successive 10-μl injections, are reported in Table III, together with the corresponding concentrations determined using our calibration graphs. In Table III are also reported the calculated percentage concentration of each component in its free state.

A 25-mg amount of the peracetyl aglycone (quercetin) isolated from the apple-peel extract was diluted to 50 ml with ethyl acetate in a volumetric flask. The average value of the peak-area counts, obtained as described above for the only peak in the chromatogram (Fig. 3b), was 175 371. According to our calibration graph this value corresponds to 30 mg/l of peracetylquercetin in the prepared solution. Hence, one can calculate that the polyphenolic material extracted from apple peel contains 4.3 mg of free quercetin per kg of apples or 5.7 mg per 100 g of peel.

RESULTS

Twenty peracetylated polyphenolic compounds, representative of the various classes (3-flavanols, 4-flavanones, flavones and 3-flavonols) were isolated as their peracetyl derivatives in the course of previous investigations[3–9]. This provided the opportunity to study their separation by normal-phase HPLC using a column packed with a cyanopropyl-bonded phase.

Figs. 1 and 2 show the HPLC profiles obtained under two different separation conditions, with isocratic elution (Fig. 1) and programmed elution (Fig. 2). The separation of peracetylated polyphenolic mixtures extracted from some vegetable matters is reported in Fig. 3.

The data given in Table I show the good agreement between the TLC separation of the above-mentioned polyphenolic compounds on silica gel plates and their corresponding HPLC separation by programmed elution. In Table II the analytical parameters are reported, by means of which it is possible to construct the calibration graphs, useful for performing quantitative determinations of some of the compounds under examination. Using such calibration graphs the quantitative composition of the polyphenols contained in a sample of green tea was calculated. The results obtained employing 10 g of vegetable matter (Table III) are in good agreement with those in the

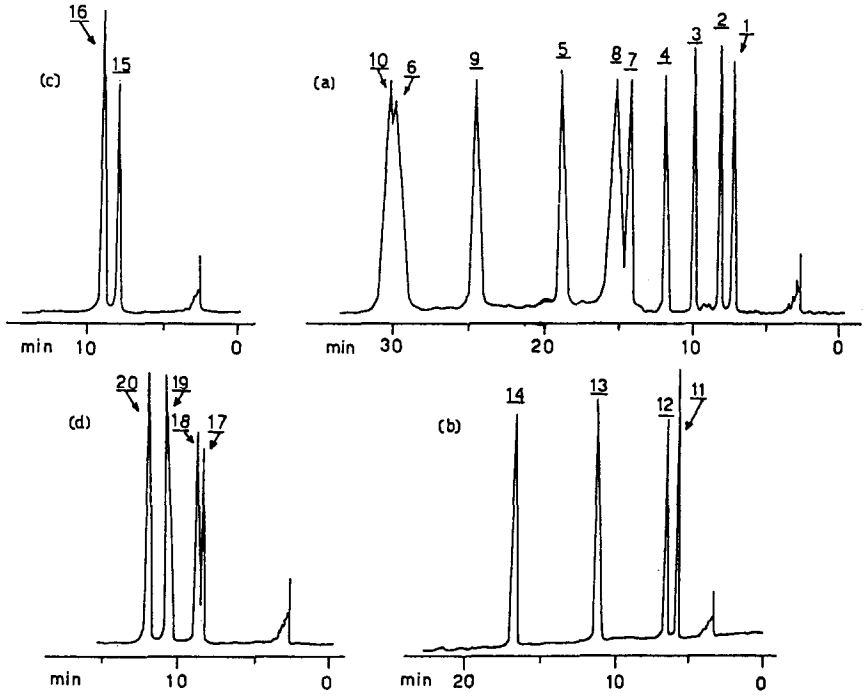

Fig. 1. Separation of selected mixtures of peracetylated polyphenolic compounds by isocratic elution (see Experimental). (a) 3-Flavanols; (b) 4-flavanones; (c) flavones; (d) 3-flavonols. The chromatograms were obtained by injection of 10 μl of a solution containing amounts of each peracetylated polyphenol sufficient to produce chromatographic peaks higher than 50% of full-scale. For peak identification, see Table I.

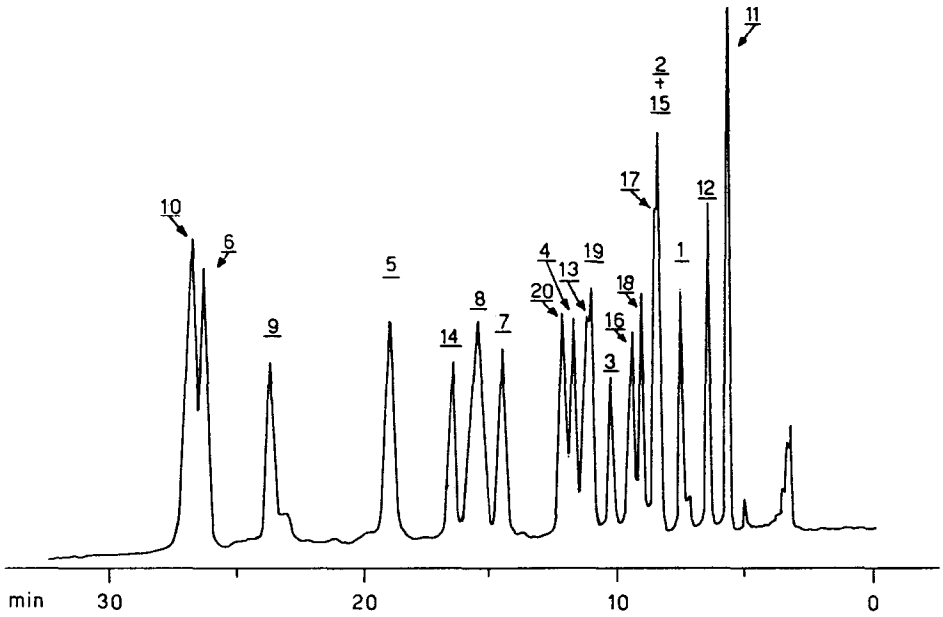

Fig. 2. Separation of a mixture of all the peracetylated polyphenolic compounds by programmed elution (see Experimental). The chromatogram was obtained by injection of 10 μl of a solution containing amounts of each peracetylated polyphenol sufficient to produce chromatographic peaks higher than 50% of full-scale. For peak identification, see Table I.

literature[11].The amount of quercetin aglycone contained in apple peel was determined in the same way.

DISCUSSION

The results obtained clearly show that the cyano-silica column used to separate the peracetyl derivatives of flavanoid and flavonoid polyphenols by normal-phase HPLC under the conditions described here offers a good performance. Because of the necessity to evaluate simultaneously compounds with UV maxima at different wavelengths[12], 278 nm was chosen as a compromise. This wavelength is intermediate between the absorbance maxima of the acetylated flavones (*ca.* 270 nm) and the acetylated flavanones and flavonols (*ca.* 300 nm) and is sufficiently distant from that of ethyl acetate (256 nm), which is a component of the mobile phase used. The small amounts of substance (25–100 ng) necessary to produce a peak intensity corresponding to 5% of full-scale (Table II) suggest that the wavelength chosen allows satisfactory qualitative and quantitative evaluations.

We shall now discuss the reasons for choosing *n*-hexane–ethyl acetate in order to separate the acetylated polyphenols on a cyano-silica phase. According to the solvent classification proposed by Snyder[13], nitriles and esters belong to the same group VIa, which within the triangular diagram[13] occupies an almost equidistant position from the two oblique sides, shifted towards the base. In accord with their

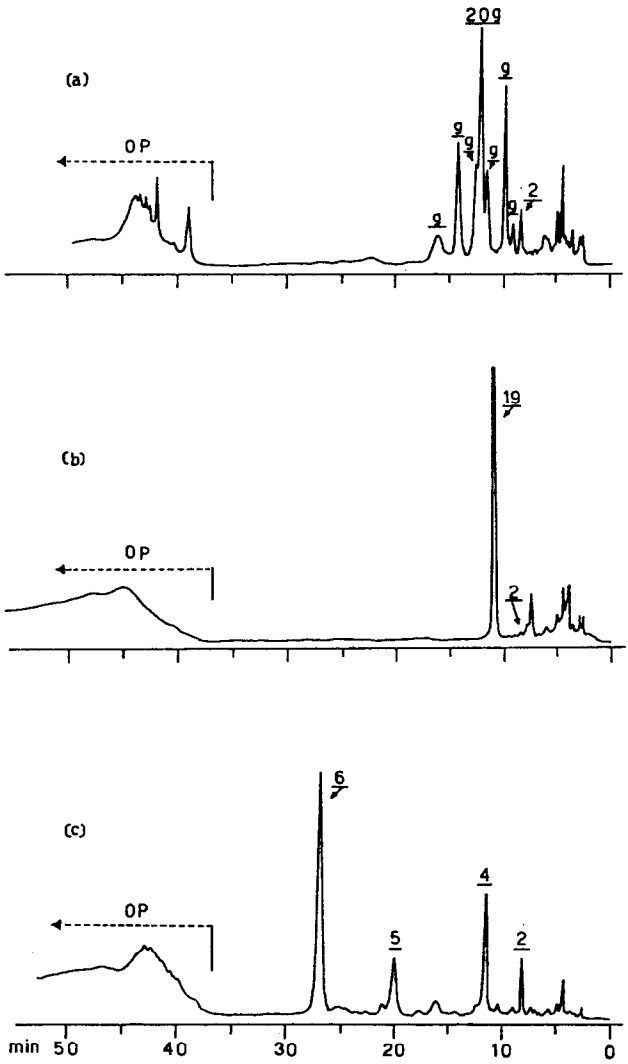

Fig. 3. Separation of peracetylated polyphenolic mixtures extracted from vegetable matter by programmed elution (see Experimental). (a) Quercetin glycosides from Golden Delicious apple peel. Peak 20 g corresponds to quercetin 3-glucoside (isoquercitrin); the others, indicated by g, are unknown glycosides. (b) Aglycones (quercetin only) of the above mixture. (c) Polyphenolic mixture extracted from green tea. The chromatograms were obtained by injection of 10 μl of a solution containing amounts of each peracetylated polyphenol sufficient to produce chromatographic peaks higher than 50% of full-scale. For peak identification in (b) and (c), see Table I.

limited tendency to act as proton donors or proton acceptors, these molecules exhibit low x_e and x_d values (0.33–0.34 and 0.26–0.25, respectively), while their x_n values (0.41 and 0.42, respectively) are markedly higher, as would be expected from their strong tendency to give rise to dipole–dipole interactions owing to their relatively high dipolar moment.

TABLE I

COMPARISON OF THE TLC AND HPLC BEHAVIOUR OF THE PERACETYL DERIVATIVES OF THE POLYPHENOLIC COMPOUNDS UNDER INVESTIGATION

No.	*Peracetyl derivative*	*TLC*[a]: R_F	*HPLC*[b]	
			Retention time (min)	k'[c]
1	(+)-Catechin	0.44	7.4	1.31
2	(−)-Epicatechin	0.40	8.4	1.62
3	(+)-Gallocatechin	0.37	10.2	2.19
4	(−)-Epigallocatechin	0.33	11.7	2.66
5	(−)-Epicatechin 3-gallate	0.26	19.9	5.22
6	(−)-Epigallocatechin 3-gallate	0.18	26.4	7.25
7	Procyanidin B_1	0.31	14.7	3.59
8	Procyanidin B_2	0.28	15.7	3.91
9	Procyanidin B_3	0.24	23.7	6.40
10	Procyanidin B_4	0.20	26.9	7.40
11	Naringenin	0.52	5.5	0.72
12	Hesperetin (persicoside aglycone)	0.50	6.3	0.97
13	Naringin	0.35	11.1	2.47
14	Persicoside (hesperetin glucoside)[d]	0.33	16.5	4.16
15	Apigenin	0.40	8.4	1.62
16	Tricin	0.39	9.4	1.94
17	Kaempferol	0.47	8.5	1.66
18	Kaempferol glucoside[d]	0.37	9.0	1.81
19	Quercetin	0.39	10.9	2.40
20	Isoquercitrin	0.32	12.2	2.81

[a] The conditions used for the TLC separation are described under Experimental.
[b] The data reported refer to programmed elution (see Experimental).
[c] Capacity factor.
[d] Glucose position not known.

In spite of their different polarities ($P' = 6.2$ for nitriles and 4.3 for esters)[13], these two classes of compounds exhibit almost identical selectivities. If the interaction between the solute and uncoated silanol groups can be disregarded, the interaction between the cyano-silica stationary phase and the solute molecules (acetylated polyphenols, *i.e.*, polyesters) must be attributed mainly to dipole–dipole interactions among molecules with different polarities but almost identical selectivities. This is why we considered that the most suitable eluent for separating the test compounds was a binary mixture of *n*-hexane (solvent of the group 0 according to Snyder) and ethyl acetate which, as an ester, according to Snyder belongs to the same group VIa as the solute and the stationary phase. The above mixture indeed possesses the same selectivity as the stationary phase and the solute with a more or less marked polarity depending on the concentration of ethyl acetate.

As shown by the results, our choice fully met the expectations. In order to eliminate possible overlappings which could originate during the separation of complex mixtures, it is necessary only to modify slightly the elution mixture considered to be the optimum, *i.e.*, *n*-hexane–ethyl acetate (60:40) (Fig. 2); in this way, only the polarity of the elution mixture and not its selectivity is changed.

TABLE II

HPLC DETERMINATION OF SOME PERACETYLATED POLYPHENOLS BY ISOCRATIC ELUTION

Slope, intercept and correlation coefficient (r) of the calibration graphs $y = mx + q$.

Compound[a]	*Isocratic retention time (min)*[b]	m	q	r	*5% amount*[c] *($g \times 10^{-9}$)*
1	7.4	1.73	− 2.80	0.9993	100
2	8.4	1.28	− 0.17	0.9988	100
4	11.7	1.19	+ 0.13	0.9990	100
5	19.9	1.25	− 1.84	0.9993	100
6	29.8	1.17	+ 0.35	0.9998	100
7[d]	14.8	1.46	− 0.03	0.9988	100
8[d]	15.8	1.17	− 2.06	0.9965	100
9[d]	24.7	1.60	− 5.31	0.9993	100
10[d]	30.4	1.14	+ 4.98	0.9983	100
11	5.5	1.57	− 0.86	0.9998	50
13	11.1	2.97	+ 1.60	0.9986	50
14	16.8	3.55	+ 3.49	0.9995	50
15	8.4	18.96	+20.67	0.9996	25
16	9.4	11.32	− 7.08	0.9978	25
18	9.0	4.21	− 0.08	0.9981	50
19	10.9	11.60	+ 1.04	0.9996	25
20	12.2	5.63	+ 0.63	0.9980	50

[a] Compound numbers as in Table I.

[b] The retention times of compounds 6, 8, 9, 10 and 14 differ from those given by programmed elution (*cf.*, Table I). With isocratic elution these compounds show capacity factors of 8.31, 3.94, 6.72, 8.50 and 4.25, respectively.

[c] Amount sufficient to produce a peak intensity corresponding to 5% of full-scale.

[d] For the calibration graphs for these compounds the three most dilute solutions were disregarded (see Experimental).

TABLE III

QUANTITATIVE COMPOSITION OF THE POLYPHENOLIC CONSTITUENTS OF GREEN TEA

Compound[a]	*Peak-area counts*[b]	*Concentration as peracetyl derivative (mg/l)*	*Concentration as free compound (mg/l)*	*Content (%)*
1	5260	12	7	1
2	60 082	94	55	6
4	178 174	299	164	18
5	153 302	251	150	17
6	535 737	915	528	58

[a] Compound numbers as in Table I.

[b] Determined using a Spectra-Physics 4270 data system as integrator.

With complex mixtures (Fig. 1) where separation under isocratic conditions is not feasible, we believe that modification of the elution mixture at very short intervals (1–2 min) is more suitable than the use of a linear gradient for long periods, thus obtaining a step-like elution profile through a succession of isocratic states.

The observation that the performance of the column remains almost unchanged during use lends support to our choice of the eluent system for the separation of these mixtures. When a reduction in the retention times or a poor resolution of a pair of substances which were previously completely resolved was observed, the original activity could be regenerated simply by washing the column with ethyl acetate.

The column used for the separations described in this paper has been used every day in our laboratory for more than 4 years, and under the above conditions has retained its full starting efficiency.

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REFERENCES

1 W. T. Cooper and P. L. Smith, *J. Chromatogr.*, 355 (1986) 57.
2 P. L. Smith and W. T. Cooper, *J. Chromatogr.*, 410 (1987) 249.
3 K. Weinges and M. V. Piretti, *Justus Liebigs Ann. Chem.*, 748 (1971) 218.
4 K. Weinges and M. V. Piretti, *Ann. Chim. (Rome)*, 62 (1972) 29.
5 M. V. Piretti, M. Ghedini and G. Serrazanetti, *Ann. Chim. (Rome)*, 66 (1976) 429.
6 M. V. Piretti, M. Ghedini and G. Spezialetti, *Ann. Chim. (Rome)*, 66 (1976) 761.
7 M. V. Piretti, G. Serrazanetti and R. Pistore, *Ann. Chim. (Rome)*, 70 (1980) 615.
8 M. V. Piretti, F. Zeli and R. Pistore, *Gazz. Chim. Ital.*, 112 (1982) 47.
9 M. V. Piretti, M. Arias and S. Sansavini, *Frutticoltura*, 51 (1989) 53.
10 J. B. Harborne, *Phytochemistry*, 4 (1965) 107.
11 K. Weinges, W. Bähr, W. Ebert, K. Göritz and H. D. Marx, L. Zechmeister (Editor), *Progress of the Chemistry of Organic Natural Products*, Springer, Vienna and New York, 1969, pp. 158–260.
12 R. Galensa and K. Herrmann, *J. Chromatogr.*, 189 (1980) 217.
13 L. R. Snyder, *J. Chromatogr.*, 92 (1974) 223.