Journal of Chromatography, 189 (1980) 217–224
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,435

ANALYSIS OF FLAVONOIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RUDOLF GALENSA and KARL HERRMANN

Lehrstuhl für Lebensmittelchemie der Universität Hannover, Wunstorfer Str. 14, D-3000 Hannover 91 (G.F.R.)

(Received October 8th, 1979)

SUMMARY

Numerous acetates of flavonoids (flavones, flavonols and flavanone aglycones and glycosides) have been separated and determined on silica gel, using four different liquid systems (benzene–acetone; benzene–acetonitrile; benzene–ethanol; isooctane–ethanol–acetonitrile) at temperatures between 45° and 0°. This method has been applied to the determination of analytically pure substances and fruit and vegetable extracts.

INTRODUCTION

Flavonoids exist in almost all plants and can be present in considerable concentrations, *e.g.*, in leaves. In the past, thin-layer chromatography was mainly used for their analysis. Recently some workers have applied reversed-phase high-performance liquid chromatography (HPLC) to particular flavonoids[1–3] and to the flavonoids in citrus fruit[4], tobacco[5], soya[6] and species of *Larix*[7] and *Cedrus*[8]. The great advantage of the commonly used gradient elution process is the saving of time and solvents by the direct injection of only a slightly purified complex extract, but expensive columns are required. The lack of a preliminary clean-up, however, may lead to errors in identification if only one analysis system is used.

Another possibility is to acetylate the compounds, the products being separated isocratically on silica gel. The selective clean-up and acetylation method that is used guarantees durability of the columns, definite identification and quantitative determination of flavonoids in plant material. Such a method is described in this paper.

EXPERIMENTAL

Apparatus

A Siemens S 200 high-performance liquid chromatograph (isocratic system with an injection dosage of 20 μl) was used with a Pye Unicam LC 3 UV detector. The column system consisted of a series of three stainless steel columns (each 200 × 3 mm I.D.) (directly connected), packed with 5-μm LiChrosorb Si 60 (Merck, Darmstadt,

G.F.R.). Benzene–acetone, benzene–acetonitrile, benzene–ethanol and isooctane–ethanol–acetonitrile were used as mobile phases. The flow-rates were between 0.8 and 1.2 ml/min.

A Pye Unicam SP 800 instrument was used for the determination of the electronic absorption spectra of the three groups of compounds analysed (see Fig. 1). Other equipment used consisted of an Ultraturrax (IKA, Staufen, G.F.R.), a Labofuge 3 centrifuge (4300 *g*) (Heraeus-Christ, Osterrode, G.F.R.), an L2 freeze drier (WKF, Brandau, G.F.R.) and a rotary evaporator (Heidolph Kelheim, G.F.R.). All evaporations were performed at a temperature not higher than 40°.

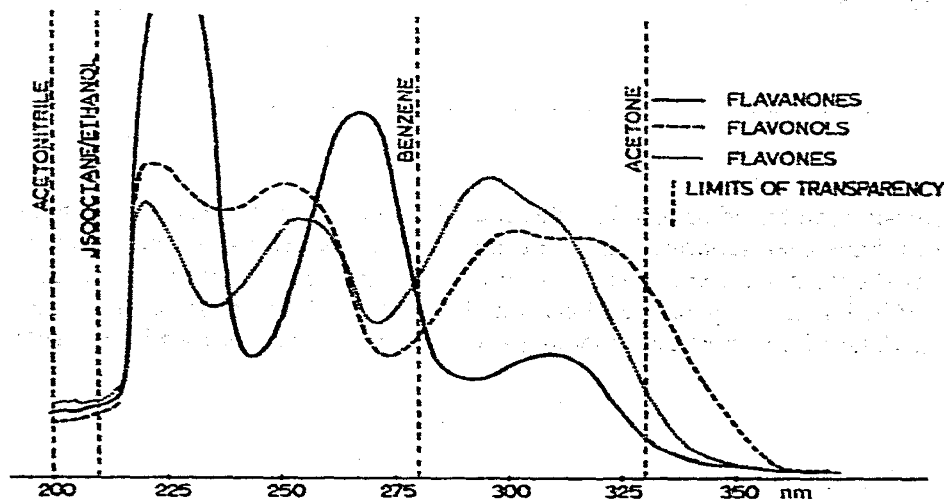

Fig. 1. UV spectrum of acetylated flavonoids (solvent: acetonitrile).

Chemicals

Commercial products (see Table I) or compounds synthesized by Koeppen[9] or isolated by our group were used. The commercial products usually needed chemical purification.

Solvents of analytical-reagent grade were purified with Extrelut (Merck) (always 1 l per column).

Polyamide SC G was obtained from Macherey, Nagel & Co. (Düren, G.F.R.).

Procedure

Clean up of plant material[10,11]. The material was homogenized with methanol in an Ultraturrax, followed by centrifugation and a triple methanol extraction of the remainder of tissue. The methanol was evaporated, leaving an aqueous solution, then lipoid substances were extracted by repeated shaking with light petroleum (b.p. 40–60°). The aqueous extract was placed on a polyamide column, which was washed with water (500–1000 ml). The flavonoids were eluted with methanol (500–1000 ml), and glucuronides by addition of ammonia[11]; the latter were not analysed in this study. The eluate was evaporated to dryness; freeze drying is possible after evaporation of methanol. Finally acetylation was carried out.

Acetylation[12]. Equal portions of pyridine and acetic anhydride (2 ml per 500 mg) were added to the dry plant extract or to the pure substances, and dissolved with

stirring at boiling temperature. The solution was then cooled and allowed to stand. Esterification was complete after several hours at ambient temperature (overnight) or after 1 h in a drier at 70°. A 30-fold surplus of cold water was added, the acetates being completely precipitated and are allowed to stand, preferably overnight in a refrigerator (normally 1–2 h will be adequate). The precipitate was filtered through a G-4 frit and washed with distilled water. Incomplete acetylation of the hydroxyl groups was not observed in any experiment. The dry residue was eluted with 150 ml of benzene–acetone (85:15). The eluate was evaporated to dryness and the residue, dissolved in a solvent and suitably concentrated, was then subjected to HPLC.

RESULTS AND DISCUSSION

Table I gives the retention times of the examined substances in different systems but with the same column. This comparison shows occasional reversal of retention in

TABLE I

RETENTION TIMES OF ACETATES

B/N = benzene–acetonitrile; B/A = benzene–acetone; B/E = benzene–ethanol; I/E/N = isooctane–ethanol–acetonitrile.

Peak No.	*Compound*	*B/N (85:22), 170 bar, 45°*	*B/A (90:15), 100 bar*		*B/E (80:0.8), 220 bar 16°*	*I/E/N (70:16:5.5), 155 bar, 45°*
			20°	*0°*		
1	Naringenin	284			405	450
2	Hesperetin	300			459	483
3	Chrysin	309				
4	Kämpferol	343				528
5	Tectochrysin	362				
6	Acacetin	392				
7	Quercetin	392	639	774	618	627
8	Quercitrin	446	639	785		699
9	Apigenin	447				674
10	Myricetin	448				
11	Kämpferol-3-glucoside	485			824	680
12	Robinetin	489				
13	Kämpferol-3-galactoside	520			824	685
14	Myricitrin	520				
15	Luteolin	541				
16	Quercetin-3-glucoside	583	774	945	1040	808
17	Quercetin-3-arabinoside	600			1124	760
18	Quercetin-3-galactoside	623	774	900	1025	808
19	Narirutin 1	700			950	870
20	Narirutin 2	707			1002	870
21	Naringin 1	708			942	860
22	Naringin 2	708			950	860
23	Kämpferol-3-rutinoside	745	837	1026	1359	855
24	Hesperidin	774			1211	980
25	Spiraeoside	820	1052			860
26	Rutin	832	954	1170	1680	990
27	Apigenin-7-glucoside	915				
28	Robinin	1000			1682	1030

the four systems, which assists in better identification. Some examples of the separation of standards are shown in Figs. 2–4.

Detection limits are listed for five typical compounds in Table II. Recoveries were determined ($n = 10$) for rutin (93 ± 5%), hesperidin (92.5 ± 4%) and naringenin (83 ± 6%). These values are less than 100% because of the presence of impurities.

The acetate of the commercial triglycoside robinin (Roth, Karlsruhe, G.F.R.) is often suitable for use as an internal standard.

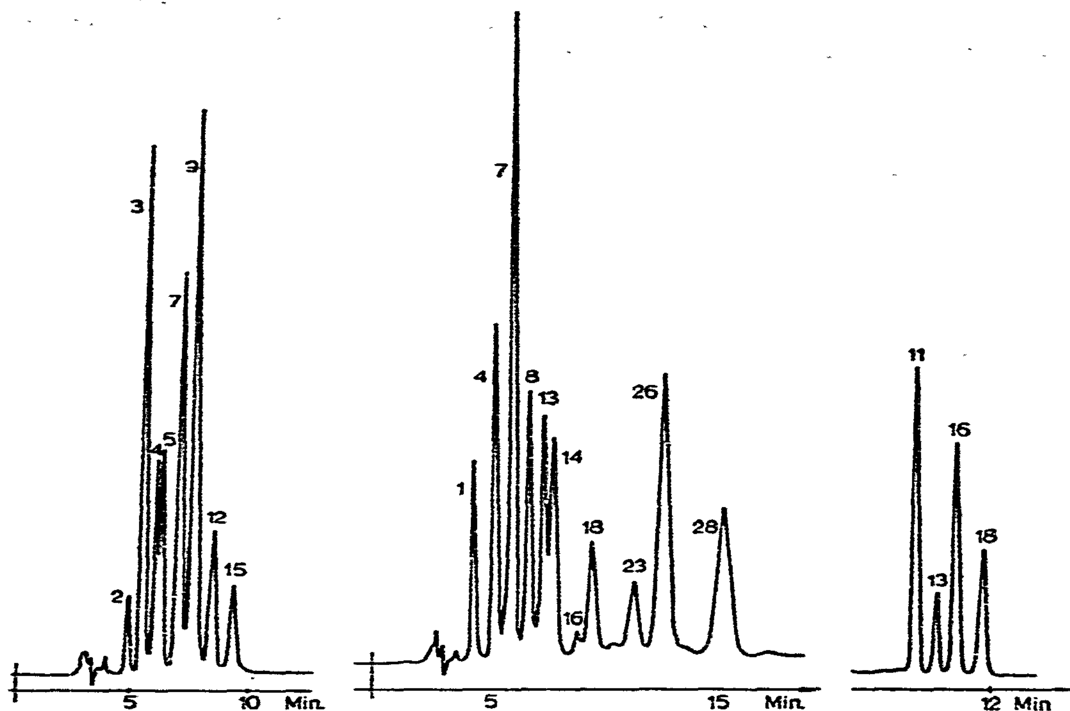

Fig. 2. Separation of aglycones (for peaks see Table I; for conditions see Table III).
Fig. 3. Separation of some standards (for peaks see Table I; for conditions see Table III).
Fig. 4. Separation of glycosides (for peaks see Table I; for conditions see Table III).

TABLE II

DETECTION LIMITS OF ACETATES

Injection: 20 μl. Eluents: see Table I.

Compound	*B/N (80:25), 175 bar, 45°*				*B/E (80:1), 180 bar, 17°, 300 nm*		*B/A (90:15), 100 bar, 20°, 335 nm*		*I/E/N (70:16:6), 160 bar, 37°*			
	300 nm		*312 nm*						*300 nm*		*270 nm*	
	ppm	*ng*	*ppm*	*ng*	*ppm*	*ng*	*ppm*	*ng*	*ppm*	*ng*	*ppm*	*ng*
Naringenin	1.2	24	0.7	14	2	40	20	400	1.2	24	0.3	6
Quercetin	0.5	10	0.5	10	1	20	4	80	0.5	10	0.5	10
Apigenin	0.75	15	0.75	15	1.5	30	4	80	0.5	10	—	
Rutin	5	100	5	100	10	200	25	500	5	100	5	100
Hesperidin	12	240	10	200	25	500	—		12	240	3	60

Benzene–acetonitrile was the most suitable mobile phase, especially at 45°. Measurements up to 285 nm can be made without difficulty, and flavanones can therefore also be determined. The extremely different properties of flavonol and flavanone glycosides on the one hand and of flavone glycosides on the other are useful from the analytical point of view. Benzene–acetonitrile (85:40) separated the flavone glycosides very well, whereas flavonol and flavanone glycosides have very short retention times.

Interesting results were obtained by cooling the column (Fig. 5). These results could not be achieved by changing the flow-rate or the proportions of the ratio components in the mobile phase. They are based on differences in the solubilities of the saccharides (which are also acetates), which depend on temperature. Cooling was employed equally succesfully with the other systems and plant extracts. Cooling could also be useful in reversed-phase HPLC.

Benzene–ethanol often gave the best separation, but the sensitivity was lower than with B/N. Small differences in the proportions of the components gave totally different retention times. With benzene–ethanol it was possible to separate *R*- and *S*-isomers of flavanone glycosides if cooling was applied (Fig. 6), but long retention times resulted.

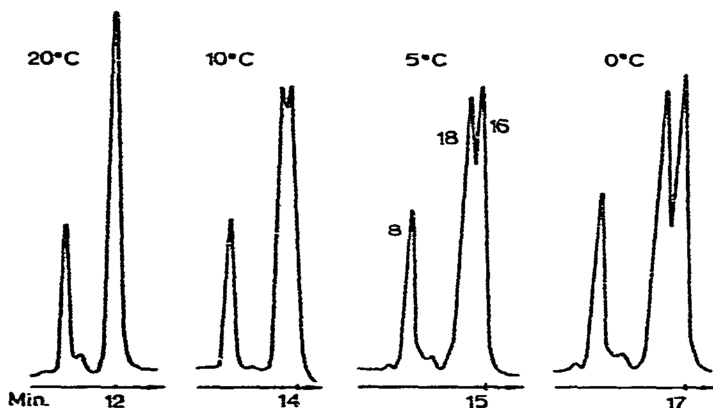

Fig. 5. Influence of cooling on separation (for peaks see Table I; for conditions see Table III).

TABLE III

OPERATING CONDITIONS

Fig. No.	*Pressure (bar)*	*UV wavelength (nm)*	*Eluent (see Table I)*	*Temperature (°C)*
2, 4, 8	180	300	B/N (85:20)	45
3	180	300	B/N (85:22)	45
5	100	335	B/A (90:15)	See figure
6	220	312	B/E (80:0.5)	15
7	155	300/270	I/E/N (70:16:6)	45
9	180	300	B/N (80:35)	45
10	155	270	I/E/N (70:16:5.5)	37

None of these eluents permits useful detection below 285 nm because of the presence of benzene. Therefore, isooctane–ethanol–acetonitrile was used for the

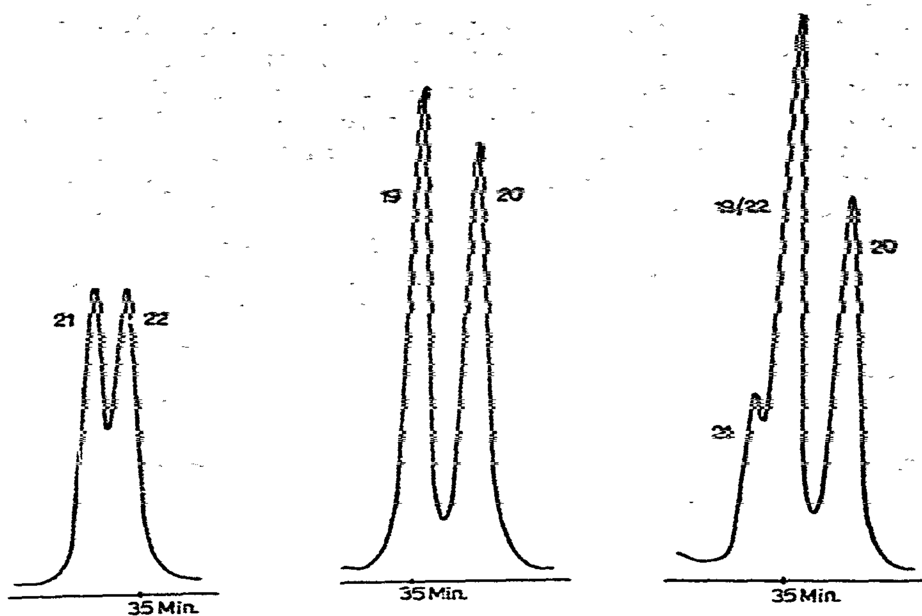

Fig. 6. Separation of *R*- and *S*-isomers (for peaks see Table I; for conditions see Table III).

Fig. 7. Detection at different wavelengths; (a) 300 nm; (b) 270 nm. For peaks see Table I; for conditions see Table III.

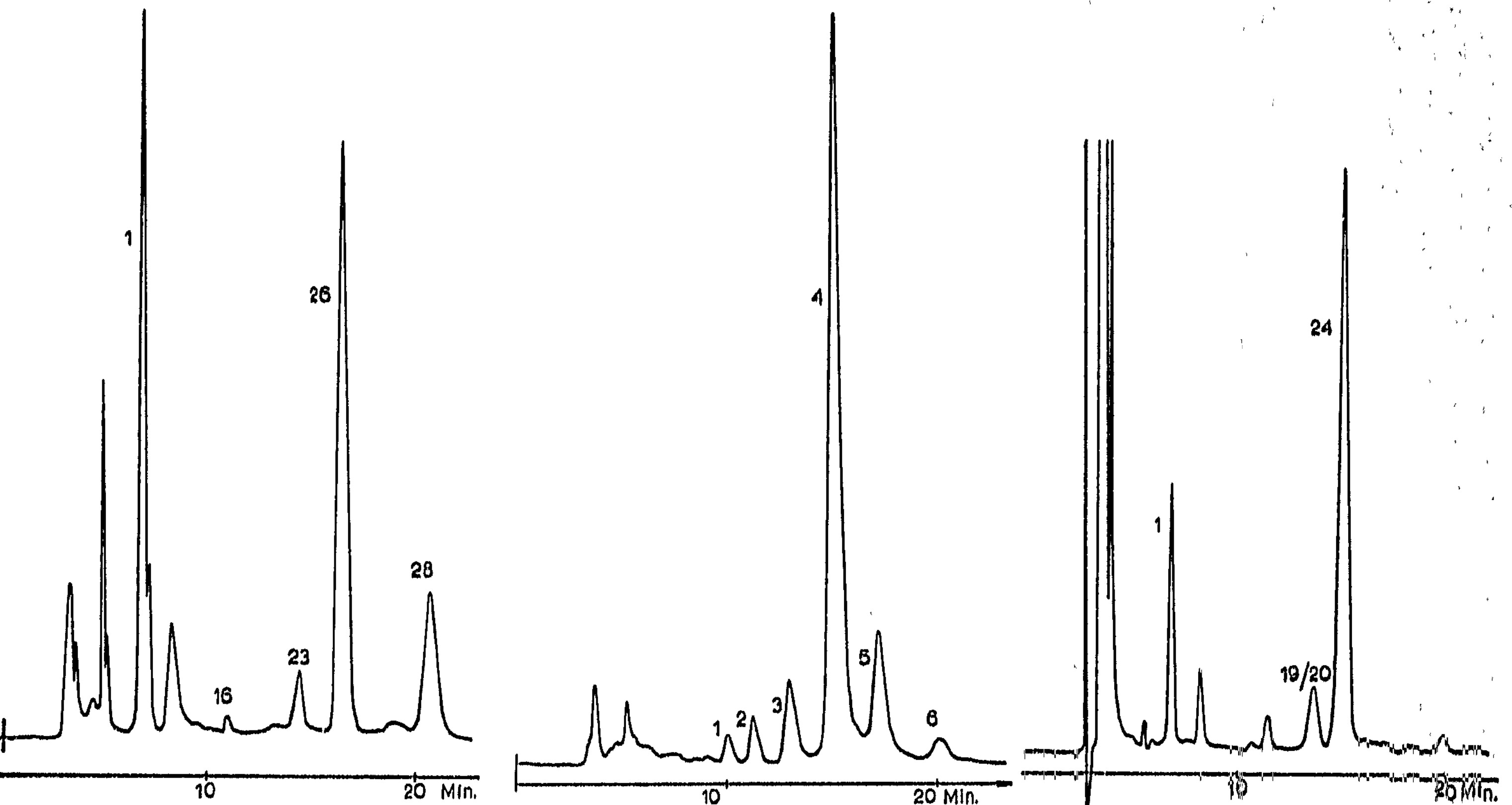

Fig. 8. Chromatogram of extract of skins of ripe tomatoes ("Frühzauber"). For peaks see Table I (28 = internal standard); for conditions see Table III.

Fig. 9. Chromatogram of extract of celery leaves ("Alba"). Peaks: 1 = apigenin-7-glucoside; 2 = luteolin-7-glucoside; 3 = probably chrysoeriol-7-glucoside; 4 = apiin; 5 = luteolin-7-apiosyl-glucoside; 6 = probably chrysoeriol-7-apiosylglucoside. For conditions see Table III.

Fig. 10. Chromatogram of extract of orange juice. For peaks see Table I (1 = internal standard); for conditions see Table III.

selective detection of flavanones at 270 nm because there are specific possibilities of determination at this wavelength, where flavones and flavonols show a minimum (Fig. 7a and b). The increase in the sensitivity of measuring flavanones at 270 nm, compared with measurements at 300 or 312 nm, permitted the presence of flavanones, flavones or flavonols in the examined extracts to be established.

Applications

Chromatograms of an extract of tomato, an extract of celery[13] and an extract of orange juice are shown in Figs. 8, 9 and 10, respectively. Detailed studies of the applications to tomato and orange juice will be published separately.

CONCLUSION

The expensive clean-up (in comparison with reversed-phase methods) is offset by the long durability of the columns. The use of the four mobile phase systems at different temperatures and wavelengths made possible the identification of flavonoids even in unknown mixtures.

REFERENCES

1 G. J. Niemann and J. van Brederode, *J. Chromatogr.*, 152 (1978) 523.
2 L. W. Wulf and C. W. Nagel, *J. Chromatogr.*, 116 (1976) 271.
3 H. Becker, G. Wilking and K. Hostettmann, *J. Chromatogr.*, 136 (1977) 174.
4 J. F. Fisher, *J. Agr. Food Chem.*, 26 (1978) 1459.
5 W. A. Court, *J. Chromatogr.*, 130 (1977) 287.
6 L. G. West, P. M. Birac and D. E. Pratt, *J. Chromatogr.*, 150 (1978) 266.
7 G. J. Niemann and J. W. Koerselman-Kooy, *Planta Med.*, 31 (1977) 297.
8 G. J. Niemann, *Z. Naturforsch. C.*, 32 (1977) 1015.
9 B. H. Koeppen, University of Stellenbosch, South Africa, personal communication.
10 W. Wildanger and K. Herrmann, *J. Chromatogr.*, 76 (1973) 433.
11 H. Teuber and K. Herrmann, *Z. Lebensm.-Unters.-Forsch.*, 165 (1977) 147.
12 R. Galensa and K. Herrmann, *Z. Lebensm.-Unters.-Forsch.*, 166 (1978) 355.
13 R. Galensa and K. Herrmann, *Z. Lebensm.-Unters.-Forsch.*, 169 (1979) 170.