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Note

Separation of polymethoxylated flavones by straight-phase high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) is considered to be one of the most promising method for the analysis of flavonoids¹. Some papers dealing with separation of flavonoids² or flavonoid glycosides²⁻⁷ by HPLC have been published. Recently a reversed-phase procedure has been reported^{8,9} which separated the usual polymethoxylated flavones. These compounds, which are found in peel oils and juice from tangerines and oranges¹⁰⁻¹², have been separated in the past by column chromatography¹³. A thin-layer chromatographic (TLC) procedure has also been reported^{14,15}.

This paper describes a new method using a straight-phase HPLC procedure with LiChrosorb Si 60 for the resolution of some of these naturally occurring flavones.

EXPERIMENTAL

Materials

Nobiletin, tangeretin and heptamethoxyflavone were obtained from orange peel oil (kindly supplied by C.F.P. Orangina, Vitrolles, France) using preparative TLC according to the procedure reported by Tatum *et al.*^{14,15}. They were identified by comparison of their R_f , UV and m.p. data with those published earlier^{14,15} and by ¹H nuclear magnetic resonance spectrometry. Tetra-O-methylscutellarein and sinensetin were synthesized from 2-hydroxy-4,5,6-trimethoxyacetophenone¹⁶ and anisoyl or veratroyl chloride using a modified Baker-Venkataraman procedure^{17,18}.

Natural extracts were obtained from peels of commercial tangerines or oranges. Sundried materials (ca. 30 g) were extracted in a Soxhlet apparatus with benzene for 4 h. The benzene extracts were dried and evaporated *in vacuo* below 50°. The standards were dissolved in benzene to a concentration of ca. 0.2 µg/µl. The pure orange and diluted (1:4) tangerine extracts were injected without further purification.

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Chromatographic system

We used home-built equipment consisting of an Orlita (Giessen, G.F.R.) DMP AE 1044 dual-stroke pump, an ISCO (Lincoln, Nebr., U.S.A.) Model UA 5, dual-beam UV-visible detector with a filter kit (254, 280 and 360 nm), cell volume 10 μ l. Injections were carried out with a 1- μ l syringe (SGE, Melbourne, Australia). The columns (25 cm \times 4 mm I.D.) were slurry-packed with LiChrosorb Si 60 (6 μ m) purchased from Merck (Darmstadt, G.F.R.) A Spectra-Physics (Santa Clara, Calif., U.S.A.) integrator, Mingrator type, was used for all computations.

Analytical method

Each flavone was examined by HPLC in order to determine retention time, order of elution and capacity factor.

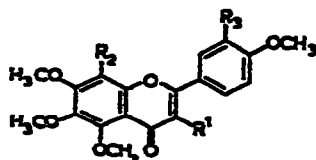
Separations were performed by isocratic elution with solvent A, heptane-ethanol (75:25), or solvent B heptane-isopropanol (60:40), containing 0.08% and 0.11% of water by Karl-Fisher determinations. The columns were equilibrated with the solvent by recycle during at least 10 h before a run at ambient temperature. Detection was at 280 nm and 0.04 a.u.f.s.

RESULTS AND DISCUSSION

Retention data for polymethoxylated flavones are listed in Table I. With solvent A, tetra-O-methylscutellarein and nobiletin elute very close together ($\alpha = 1.07$). The separation is rather difficult to achieve with an average LiChrosorb Si 60 column (Fig. 1).

TABLE I

STRUCTURES AND CAPACITY FACTORS OF POLYMETHOXYLATED FLAVONES



Compound	R^1, R^2, R^3			k'	
				Solvent A	Solvent B
1 Tangeretin	H	OCH ₃	H	0.69	0.69
2 Tetra-O-methylscutellarein	H	H	H	1.04	0.99
3 Heptamethoxyflavone	OCH ₃	OCH ₃	OCH ₃	0.94	1.14
4 Nobiletin	H	OCH ₃	OCH ₃	1.11	1.46
5 Sinensetin	H	H	OCH ₃	1.76	2.34

The separation between the five compounds is complete using solvent B. The most closely eluted compounds are tetra-O-methylscutellarein and heptamethoxyflavone ($\alpha = 1.15$). A column of *ca.* 3300 plates is sufficient to ensure a resolution of unity. A typical separation is shown in Fig. 2. The column exhibited 4800 plates as calculated from the nobiletin peak. Further investigation is now in progress on other polymethoxylated flavones.

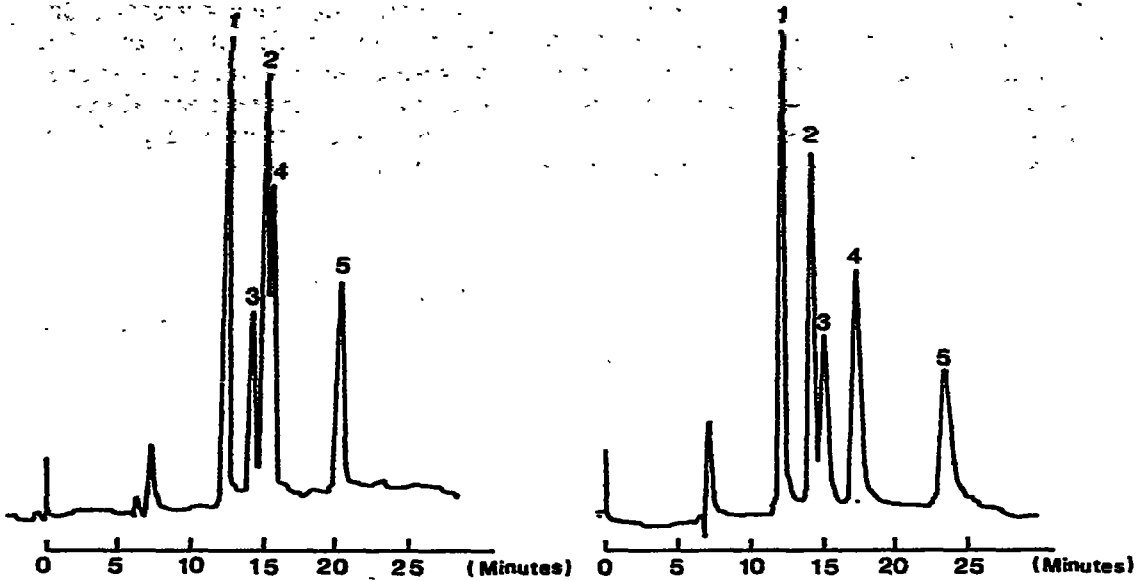


Fig. 1. Separation of polymethoxylated flavones. Column 25 cm \times 4 mm I.D., LiChrosorb Si 60 (6 μ m); solvent heptane-ethanol (75:25); flow-rate 0.45 ml/min; sample volume 1 μ l (ca. 0.2 μ g of each compound); detection, UV absorbance at 280 nm. Peak identifications as in Table I.

Fig. 2. Conditions and compounds are the same as in Fig. 1 except the solvent, which is heptane-isopropanol (60:40).

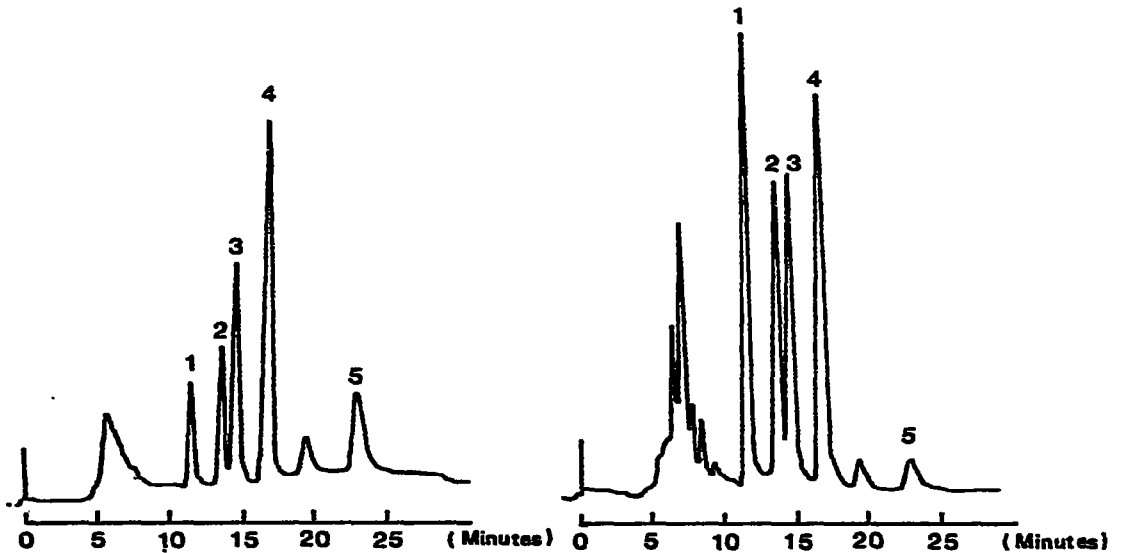


Fig. 3. Crude extract of tangerine peel. Conditions as Fig. 2.

Fig. 4. Crude extract of orange peel. Conditions as Fig. 2.

Figs. 3 and 4 represent results from the injection of crude extracts from tangerine and orange peels. These results corroborate previous determinations^{8,9,15}, which list nobiletin as the major component of tangerine peel oil, whereas flavones 1-4 (Table I) are the most important ones in orange peel. Assuming approximately the same detector response for all the flavones except heptamethoxyflavone, as shown in Figs. 1 and 2, a quick determination of the nature of the oil is possible.

CONCLUSION

HPLC using LiChrosorb Si 60 as adsorbent and a mixture of heptane-isopropanol (60:40) as eluent is a very efficient method for the separation of the most important polymethoxylated flavones from citrus fruits.

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