Journal of Chromatography, 211 (1981) 61–78
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,745

ROLE OF WATER IN QUALITATIVE AND QUANTITATIVE DETERMINATION OF POLYMETHOXYLATED FLAVONES BY STRAIGHT-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: APPLICATION TO ORANGE PEEL OILS

J. P. BIANCHINI*

Ecole Supérieure de Chimie de Marseille, Centre Universitaire de Saint-Jérôme, Rue Henri Poincaré, 13 397 Marseille Cedex 4 (France)

and

E. M. GAYDOU

Etablissement d'Enseignement Supérieur des Sciences Agronomiques, Département des Industries Agricoles et Alimentaires, Université de Madagascar, B.P. 175, Antananarivo (Madagascar)

(Received February 16th, 1981)

SUMMARY

The separation of seventeen polymethoxylated flavones was studied by straight-phase high-performance liquid chromatography using a LiChrosorb Si 60 packed column and four solvent systems (*n*-heptane–ethanol, 90:10 and 75:25, and *n*-heptane–isopropanol, 70:30 and 60:40). The role of water was studied for each solvent system in the range 0.025–0.57%. The solvent mixtures giving the best separation were *n*-heptane–ethanol (75:25) and *n*-heptane–isopropanol (60:40) for water concentrations between 0.05 and 0.02%. The influence of the position and the number of methoxy groups of the flavonic skeleton on the retention is discussed.

The response factors of these polymethoxylated flavones were determined by UV measurements at 254, 280 and 365 nm and by chromatography. The detection limits averaged between 5 and 10 μg.

The results were used for the quantitation of polymethoxylated flavones in three samples of industrial orange oils. Heptamethoxyflavone was the major product in two oils (Guinea and Israel) and tangeretine was the major product in Brazil oil. In all the samples, sinensetin had the lowest concentration of the six flavones identified and quantitated.

INTRODUCTION

Flavones, which are widespread in the vegetable kingdom, generally occur in the form of hydroxylated or glycosylated derivatives[1]. Polymethoxylated flavones constitute a special group that is found in certain citrus species[2–4], and oranges, tangerines and related species contain them in characteristic distributions[5]. The peel oil of these fruits contain the highest concentrations, but it is possible to detect them in the leaves[5] and juice[6].

0021-9673/81/0000–0000/$02.50

The world production of orange oil, which is several tens of thousands of tons, is an important source of polymethoxylated flavones. If part of these good quality oils is used as food flavour component (beverage, biscuits, cakes), the excess, after vacuum or steam distillation to recover volatile fractions rich in terpenic compounds such as limonene, gives a residue containing concentrated polymethoxylated flavones.

Pharmacological applications of flavones are well known and have been reviewed[7,8]. The polymethoxylated flavones have some peculiar pharmacodynamic properties, *e.g.*, tetra-O-methylscutellarein is a cytotoxic agent towards different strains of carcinoma cells[9] and nobiletin and sinensetin are very active in regulating erythrocyte aggregation in human blood[10]. Dietary control has been suggested for the high blood viscosity syndrome[10].

Flavonoid determination is also important in chimiotaxinomic studies on citrus[5]. An easy to perform high-performance liquid chromatographic (HPLC) method for quantitative determination should enable more information to be obtained on the species and hybrids of the plants concerned.

Recent analytical studies using HPLC were carried out only on some of the polymethoxylated flavones claimed to be present in citrus[11,12]. Most of the polymethoxylated flavones present in citrus are not commercially available[13] and serious risks of interferences may exist between the main flavones and the minor ones not identified by HPLC[12,13].

In this work, we describe an HPLC method for the separation of seventeen flavones with O-7 methoxy groups at different positions on the flavonic skeleton. The flavones investigated were natural or synthetic compounds[14,15]. Their capacity factors were determined using four solvent systems with various concentrations of water as eluents. The determination of response factors of these flavones at different UV wavelengths will permit quantitative studies of industrial orange oils of various origins to be made.

EXPERIMENTAL

Reagents and standards

All chromatographic solvents were of Spectrograde quality from E. Merck (Darmstadt, G.F.R.) and were dried in the usual manner and distilled. Deionized water was used for varying the water content.

The formulae and some physical characteristics of the seventeen flavones are shown in Table I. Most of them are found in oils of some citrus species. Products 2–4, 6, 7 (tetra-O-methylscutellarein), 8 (sinensetin) 10, 15 (tangeretin), 16 (nobiletin) and 17 (heptamethoxyflavone) have been isolated from oranges, tangerines and pomelos[22–28]. Product 13 (auranetin) was isolated from a peculiar variety, *Citrus aurantium*[29].

Products 15, 16 and 17 (methoxylated in the 5-, 6-, 7- and 8-positions) were obtained using preparative chromatography of orange oil. The residue obtained, after steam distillation, was fractionated on a silica Si 60 column (E. Merck) using *n*-heptane–isopropanol as eluent. Fractions rich in flavones were recovered using preparative thin-layer chromatography (TLC) according to the method described by Tatum and Berry[23]. Products 3–12 were obtained using Baker–Venkataraman syntheses as recently improved[14,15] and by the Algar, Flynn and Oyamata method[19].

Products 13 and 14 were prepared from the corresponding acetophenones[1]. Flavone 2 was obtained by methylation of apigenine (5,7,4′-trihydroxyflavone), already available in our laboratory[15].

Melting points (Table I) were uncorrected. Proton NMR spectra were recorded on a Perkin-Elmer R-32 NMR spectrometer (Perkin-Elmer, Norwalk, CT, U.S.A.). The solvent was deuterochloroform with tetramethylsilane as internal standard. Chemical shifts are given in parts per million.

Products 5, 9, 11 and 12 have not been described previously to our knowledge, so we give (see footnote of Table I) the UV spectra of theses compounds, which were recorded in ethanolic (95%) solution on a Beckmann DB-G UV–visible spectrometer.

Apparatus

The HPLC equipment consisted of an Orlita (Giessen. G.F.R.) DMP AE 1044 dual-stroke pump and an ISCO (Lincoln, NE, U.S.A.) Mocel UA5 dual-beam UV–visible detector with a filter kit (254, 280 and 360 nm). Two cells of volume 10 μl and path length 5 mm were used. A Perkin-Elmer Model 56 recorder and a Spectra-Physics (Santa Clara, CA, U.S.A.) integrator were used for the measurement of retention times and peak areas. Injections were carried out with a Model 1B7 1-μl syringe (S.G.E., Melbourne, Australia) or with a Rheodyne 70-10 valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 10-μl loop.

Water determinations by Karl-Fisher titration were carried out using a Prolabo (Paris, France) apparatus.

Chromatographic conditions

Two Hibar (E. Merck) columns (25 cm × 4.6 mm I.D.) slurry-packed with LiChrosorb Si 60 (6 μm) were used. The compositions of the solvents used for separations by isocratic elution are shown in Table II. The experiments were carried out at ambient temperature (20–22°C).

The solvents were degassed under vacuum using a sonicator for 5 min. Appropriate water concentrations were obtained by addition of known volumes of deionised water to 1 l of anhydrous solvent. After mixing of the solvent system, the pump was started, the first 100 ml of eluent were discarded and the solvent system was recycled overnight in order to improve the stability of the column. An aliquot was taken for Karl-Fisher determination of water. During all the experiments a magnetic stirrer was used for solvent mixing. The column head pressure was maintained between 25 and 50 bar in order to obtain a flow-rate of about 1 ml/min.

Required analyses for the determination of the retention parameters for a solvent system of known composition and water concentration were made in one day.

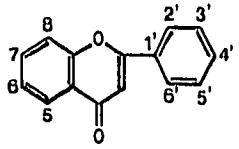
Samples preparation

Pure compounds. A 10-mg amount, exactly weighed, of each of the polymethoxylated flavones was dissolved in exactly 10 ml of pure benzene using a sonicator.

Natural samples. A 30-g amount of sun-dried orange peels were finely ground, then extracted in a Soxhlet apparatus with methylene chloride for 2 h. The solutions were dried with anhydrous sodium sulphate and the solvent was removed by vacuum distillation at 50°C on a rotary evaporator. The residue obtained was weighed, then injected either directly or after dilution with benzene or methylene chloride.

TABLE I

FORMULAE AND PHYSICAL DATA FOR POLYMETHOXYLATED FLAVONES



No.	*Compound*	*Trivial name*	*Occurrence in citrus fruits*	*M.p. (°C)***		*^1H NMR shifts (ppm)****							
				Exptl.	*Literature*	*3-*	*5-*	*6-*	*8-*	*2'-*	*3'-*	*5'-*	*6'-*
1	Flavone												
2	5,7,4'-Trimethoxyflavone		+	156	156–7[16]	6.52		6.33	6.50	7.76	6.95	6.95	7.76
3	5,7,8,4'-Tetramethoxy-flavone		+	209	209–10[17]	6.5		6.4		7.83	6.98	6.98	7.83
4	5,7,8,3',4'-Pentameth-oxyflavone		+	196	198–9[18]	6.55		6.39		7.36		6.93	7.53
5*	3,5,7,8,4'-Pentameth-oxyflavone			157–8	157–8[16]			6.38		8.11	6.98	6.98	8.11
6	3,5,7,8,3',4'-Hexame-thoxyflavone		+	168	171–2[16]			6.35		7.78		6.93	7.73
7	5,6,7,4'-Tetramethoxy-flavone	Tetra-O-methyl-scutellarein	+	140	142[17]	6.43			6.66	7.79	6.95	6.95	7.79
8	5,6,7,3',4'-Pentame-thoxyflavone		+	172–4	177–8[18]	6.51			6.74	7.24		6.90	7.43
9*	3,5,6,7,4'-Pentame-thoxyflavone			153–4	153–4[16]				6.70	8.02	6.97	6.97	8.02

10	3,5,6,7,3′,4′-Hexamethoxyflavone		+	136–7	142–3[16]			6.72	7.66		6.95	7.66
11*	5,7,8,4′-Tetramethoxyflavone			174–5	175–6[19]	6.69	7.36		7.86	6.99	6.99	7.86
12*	6,7,8,3′,4′-Pentamethoxyflavone			191–3	191–2[19]	6.66	7.35		7.35		6.94	7.51
13	3,6,7,8,4′-Pentamethoxyflavone	Auranetin	+	137–8	141[19]		7.38		8.08	6.98	6.98	8.08
14	3,6,7,8,3′,4′-Hexamethoxyflavone			164–5	168–9[19]		7.32		7.70		6.93	7.76
15	5,6,7,8,4′-Hexamethoxyflavone	Tangeretin	+	151–2	152–3.5[20]	6.53			7.80	6.99	6.99	7.80
16	5,6,7,8,3′,4′-Hexamethoxyflavone	Nobiletin	+	135–6	136.5–7.5[21]	6.56			7.38		6.95	7.53
17	3,5,6,7,8,3′,4′-Heptamethoxyflavone	Heptamethoxyflavone	+	130–1	130–1[22]				7.79		6.99	7.74

* UV spectrum (ethanol), λ_{max} (log ε):
5: 269 (4.34), 306 (4.17), 342 (4.21);
9: 260 (4.26), 320 (4.40);
11: 274 (4.54), 324 (4.77);
12: 250 (4.54), 274 (4.47), 334 (4.72).

** Melting points were uncorrected.

*** The NMR spectra were obtained with a Perkin-Elmer Model R-32 magnetic resonance spectrometer in deuterochloroform with tetramethylsilane as internal standard.

TABLE II

COMPOSITION OF ELUTING SOLVENTS USED IN THE SEPARATION OF POLYMETHOXYLATED FLAVONES

	Solvent system			
	A	*B*	*C*	*D*
Main components	*n*-Heptane–ethanol (90:10)	*n*-Heptane–ethanol (75:25)	*n*-Heptane–isopropanol (70:30)	*n*-Heptane–isopropanol (60:40)
Water content (%)*	0.025–0.2	0.025–0.15	0.035–0.54	0.025–0.57

* Determined by Karl Fisher titration.

Peak identification

Mixtures of four or seven flavones, depending on their ease of separation, were prepared, each flavone being present at least in two mixtures. For simple identification, compounds were mixed in different proportions and for each injection one or two flavones were increased in concentration. Results were calculated from the area counts on the integrator.

Qualitative determinations

Retention times and capacity factors were determined for each flavone. At least two or three injections of each mixture were made, and 4–8 results were obtained for each flavone. Capacity factors were averaged.

Quantitative determinations

Linearity of response. The range of linearity of the detector response was determined by injecting mixed standard solutions of six principal flavones (2, 7, 8, 15, 16 and 17) using the injection loop valve and plotting peak areas against mass of flavone injected.

Sensitivity. The sensitivity of the method was determined for the principal flavones; sensitivity was defined as the smallest amount of flavone that would give a peak height of twice the peak-to-peak noise level.

Response factors. Response factors were determined chromatographically and using UV spectroscopy. Mixed standard solutions of flavones were chromatographed with eluent D (*n*-heptane–isopropanol, 60:40) containing 0.05% water. Peak areas were counted by the integrator. The values obtained were compared with those of nobiletin 16, which was chosen as a standard. The determinations were made at a wavelength of 280 nm.

Application to quantitation of industrial citrus oils

Samples of different origin (Guinea, Israel, Brazil) were diluted 10-fold with absolute ethanol and 10-μl volumes were injected using the loop valve. The amounts of the flavones present were calculated from the area counts of the integrator using the response factors for the standards.

RESULTS AND DISCUSSION

Qualitative analysis

Capacity factors of the polymethoxylated flavones were determined with eluents A–D. The role of water in adsorption chromatography on silica or alumina has often been studied[30–32]. The time necessary to obtain good water and mobile phase equilibrium is about 10 h[33] with apolar solvents and about 10 min with polar solvents mixed with water over a wide percentage range[34,35]. We observed in this work that after a few hours the column was readily stabilized. Capacity factors obtained for a particular flavone were stable for a whole day. The stabilization period may be shortened without adverse effects. Rapid equilibrium with any solvent mixture in Table II is due to the presence of water-miscible alcohols (ethanol or isopropanol). This observation is in agreement with those observed elsewhere[34]. The accuracy and reproducibility of capacity factor measurements was about 4–6%.

The results obtained are shown in Figs. 1–4. A logarithmic scale was used for k'

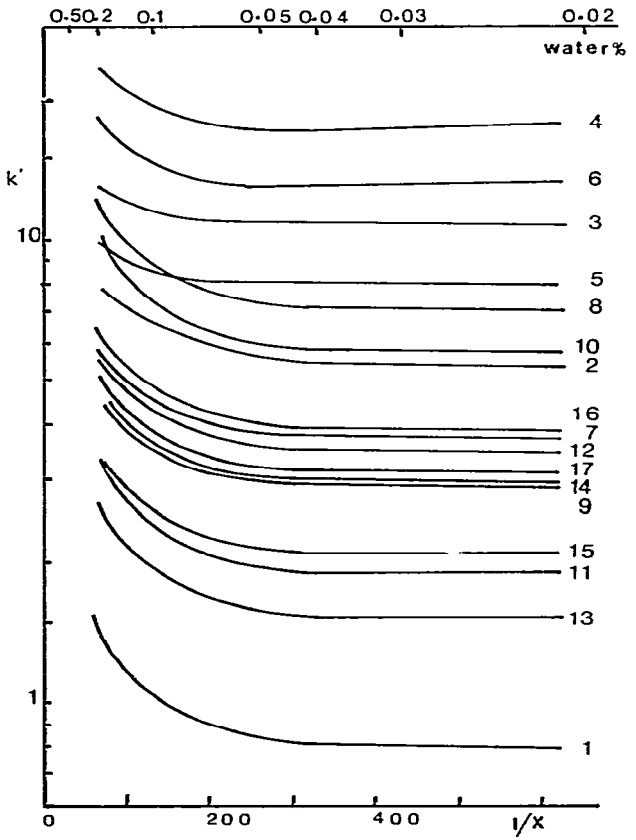


Fig. 1. Variation of the capacity factors of polymethoxylated flavones with concentration of water in the mobile phase. Solvent, *n*-heptane–ethanol (90:10). Compound identification as shown in Table I. k' = capacity factor; X = molar fraction of water in the solvent mixture. Column: 25 cm × 4 mm I.D. LiChrosorb Si 60 (6 μm).

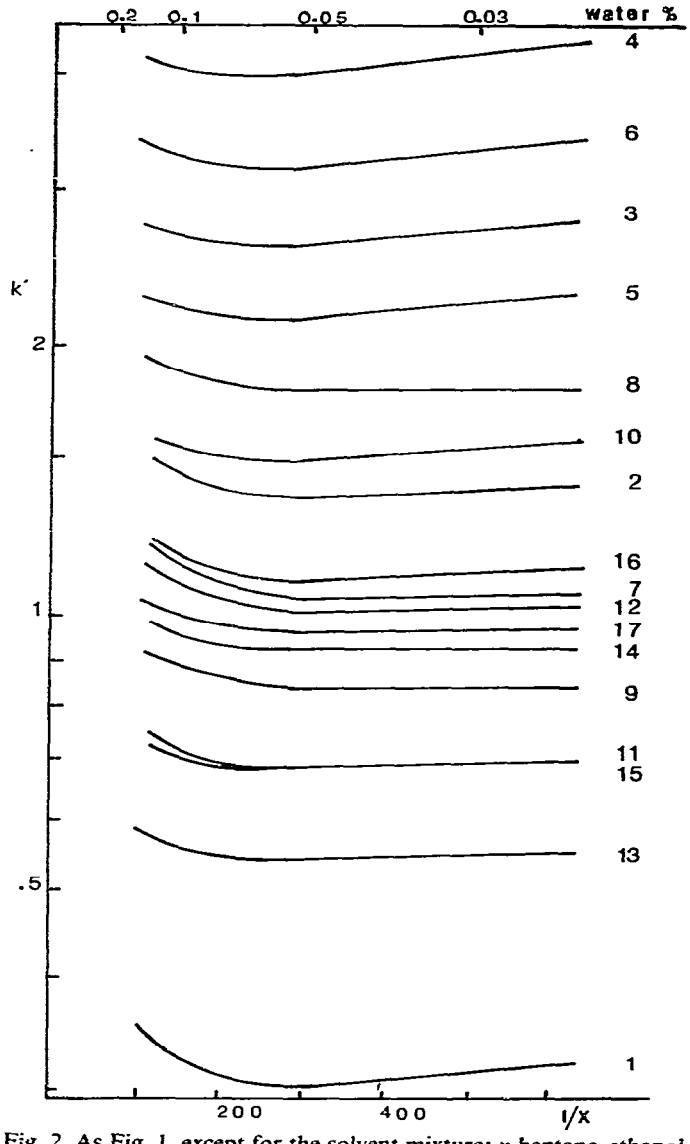


Fig. 2. As Fig. 1, except for the solvent mixture: *n*-heptane–ethanol (75:25).

in order to avoid crowding of small values. The water content was plotted as a percentage and also inverse molar fraction[34]. The percentage of water varied between 0.025 and 0.5%. For lower water contents serious peak distortion of the more retained compounds occurred so that the chromatograms were difficult to interpret. For higher water contents partition phenomena are important. For each solvent system, capacity factors were determined with four different water contents.

n-Heptane–ethanol mixtures

With eluent A (Fig. 1) the capacity factors are fairly constant for water con-

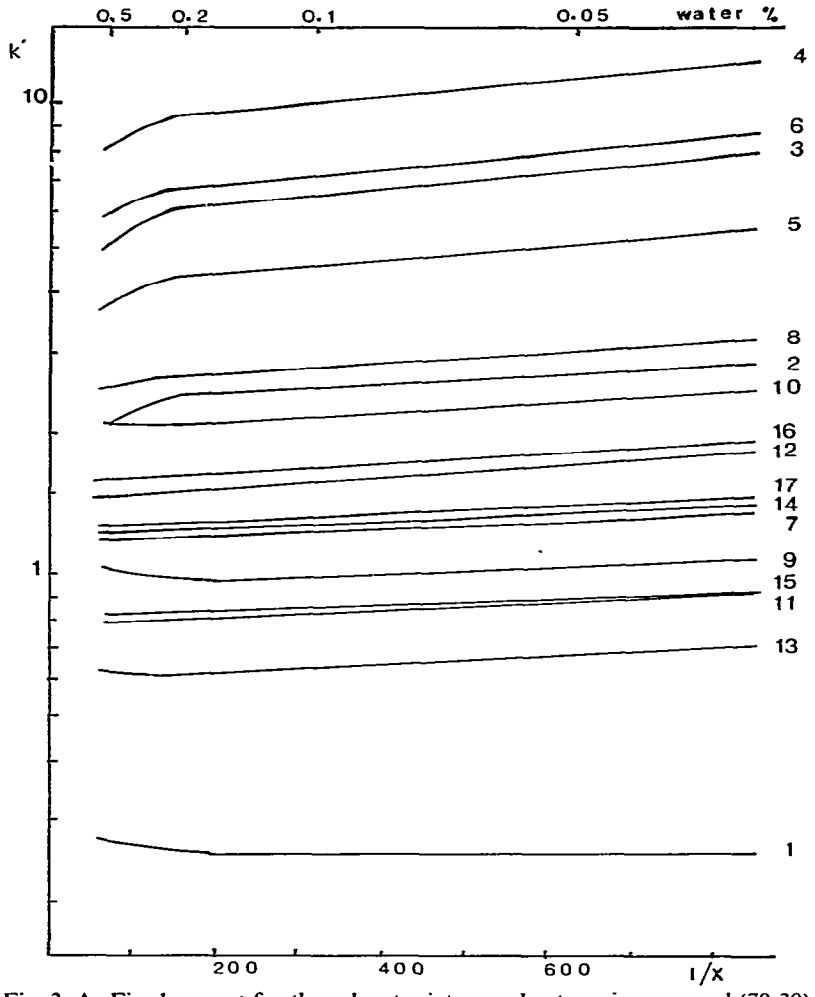


Fig. 3. As Fig. 1, except for the solvent mixture: *n*-heptane–isopropanol (70:30).

tents up to 0.06%, with an increase in retention at higher water contents. Saturation with water for this solvent mixture is about 0.4% at ambient temperature (20–22°C). For such a high water content there is competition between polar solvent molecules, which are generally fixed at the stationary phase surface, and molecules of water[36]. Partition phenomena then play an important role in the separation. The groups 10–2, 16–7 and 17–14–9 are inseparable under such conditions with columns of the usual efficiency. When the proportion of polar solvent is increased to 25% (solvent mixture B, Fig. 2) the capacity factors depend slightly on the water content, especially for the most retained compounds (3–6). Separation of compounds 11 and 15 (tangeretin) is possible only at higher water contents. With heptamethoxyflavone (17), tetra-O-methylscutellarein (7) and nobiletin (16), which are major components of orange oil, separation may be effected with low or medium water contents (Fig. 5), but at high water contents the peaks of tetra-O-methylscutellarein and nobiletin (compounds 7

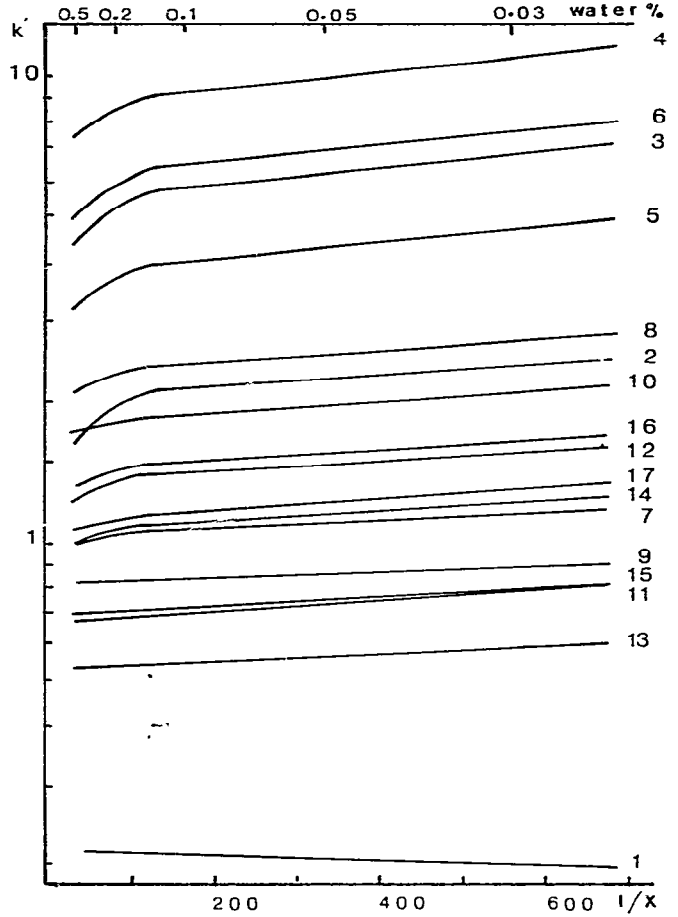


Fig. 4. As Fig. 1, except for the solvent mixture: *n*-heptane–isopropanol (60:40).

and 16) become closer together, thus further decreasing the resolution (Fig. 6). A similar effect is observed with compounds 2 and 10 (Figs. 5 and 6).

The optimal solvent system has a water content ranging from 0.02 to 0.05%.

n-Heptane–isopropanol mixtures

In all instances, with these solvent systems (C and D, Table II) the retention decreases when the water content increases, as shown in Figs. 3 and 4. For lower water contents the peaks are very asymmetric, the last compounds being strongly retained. Such conditions are not useful for quantitative determinations.

In general the separations are slightly affected by variation in the water content, excepted for compounds 2 and 10, which became inseparable at water contents of 0.3–0.5% (Figs. 7 and 8). With solvent system D the separation of tetra-O-methylscutellarein and heptamethoxyflavone (compounds 7 and 17) is also more difficult.

With both *n*-heptane–ethanol and *n*-heptane–isopropanol solvent mixtures the selectivities for the most important polymethoxylated flavones in tangerine and

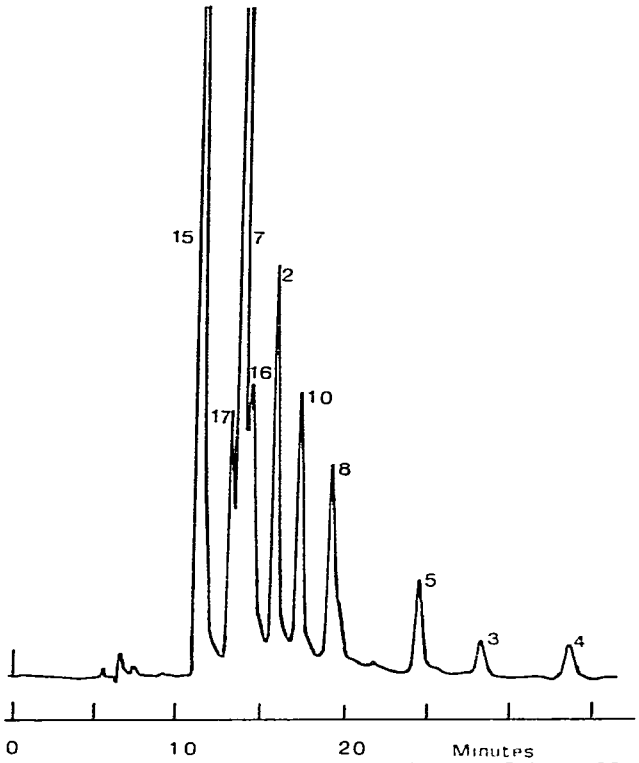


Fig. 5. Separation of polymethoxylated flavones. Column: 25 cm × 4 mm I.D., LiChrosorb Si 60 (6 μm). Solvent: *n*-heptane–ethanol (75:25)–0.02% water, flow-rate 0.45 ml/min. Sample volume: 10 μl (various amounts of compounds). Detection: UV absorbance at 280 nm. Peak identification as shown in Table I.

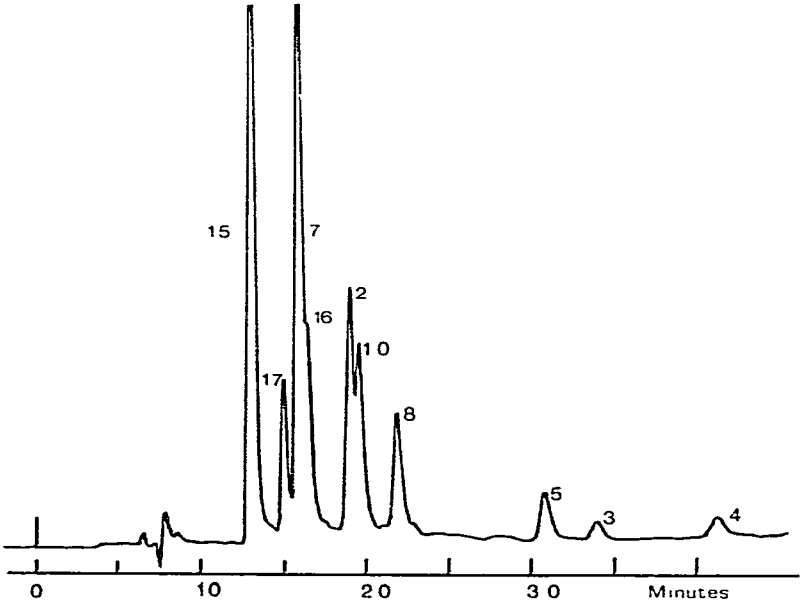


Fig. 6. As Fig. 5, except water content, 0.1%.

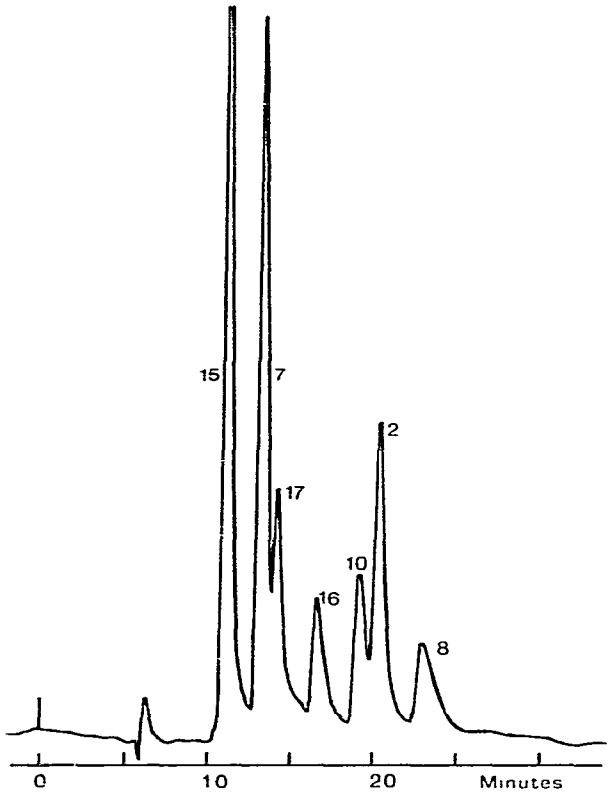


Fig. 7. As Fig. 5, except solvent mixture, *n*-heptane–isopropanol (60:40), and water content, 0.03%.

orange oils analyses are improved when larger proportions of polar solvent are used as can be seen if one compares solvent mixtures A and B (Figs. 1 and 2) with C and D (Figs. 3 and 4). However, the use of higher proportions of polar solvent is not convenient. The capacity factors decrease and if the relative retention of adjacent compounds increases, the effect on the separation according to the resolution equation[31] is not necessarily optimal. On the other hand, an increase in the viscosity of the mobile phase requires the use of lower flow-rates in order that the mass transfer resistance does not increase the HETP too much[32].

Of the four solvent mixtures studied, systems B and D are the most interesting for water contents of 0.05–0.02%.

Influence of substitution of the polymethoxylated flavones on retention

The retention of polymethoxylated flavones changes with variation in the number and position of the methoxy groups introduced into rings A, B and C of the flavone[37].

If we consider substitution on the B ring, the methoxy groups on rings A and C remaining unchanged, we observe the following sequence for the capacity factors: 6,7,8- $\leqslant$ 5,6,7,8- $<$ 5,6,7- $<$ 5,7- $<$ 5,7,8-. On the other hand, the introduction of a methoxy group in position 3 decreases the retention whereas introduction in position 3′ increases the retention. The simultaneous introduction of a methoxy group in

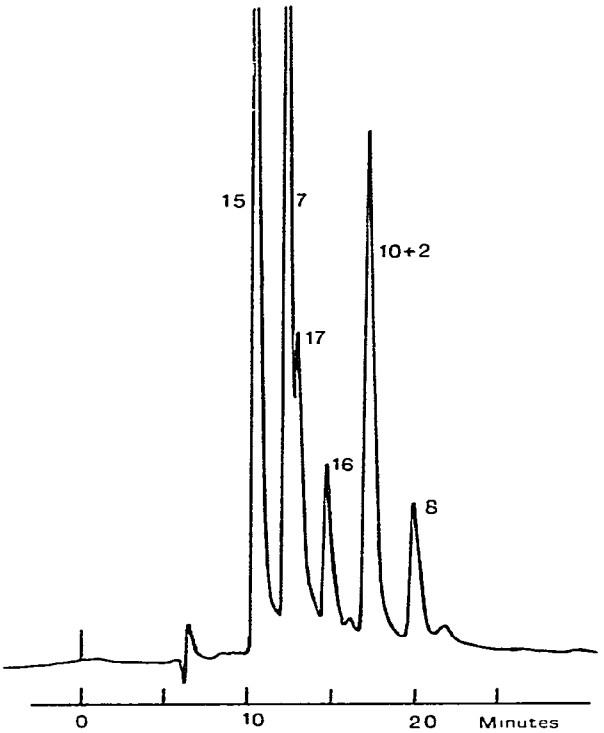


Fig. 8. As Fig. 7, except water content, 0.2%.

positions 3 and 3′ results in an increase in k'. For instance, with flavones 3, 4, 5 and 6 the following sequences are observed: 5 < 3, 4 > 3 and 6 > 3. With flavones 7, 8, 9 and 10 the following sequences are observed: 9 < 7, 8 > 7 and 10 > 7.

Quantitative determination

Linearity of response. This study was carried out using the polymethoxylated flavones 2, 7, 8, 10, 15, 16 and 17. The response of the detector was linear with amounts injected in the range 40–1000 ng, as shown in Fig. 9. It can be assumed that the other polymethoxylated flavones will give similar results under the same experimental conditions.

Sensitivity. In chromatographic analyses the sensitivity depends upon numerous factors, including the detector performance (response, stability), the path length of the cell, and the stability of the baseline, which is affected by the pump pulsations and recycling of solvent. The peak height depends upon column efficiency and capacity factors, which vary according to solvent polarity. Finally, the sensitivity is better when the absorbance for a particular wavelength is higher.

Under the experimental conditions used in this study, at 280 nm the sensitivity of the method averaged between 5 to 10 ng for the seven flavones studied. The sensitivity can be increased by improving some parameters, as pointed out above.

Response factors. Using UV spectroscopy, absorbance (for 1 mg/l) in the solvent mixture *n*-heptane–ethanol (90:10) containing 0.05% of water and for the three

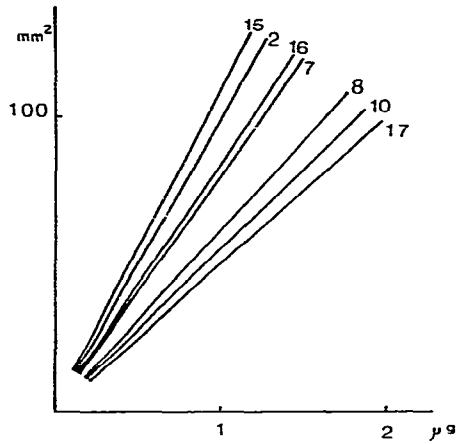


Fig. 9. Plot of peak area *versus* amount injected. Compounds identification as shown in Table I. Column and chromatographic conditions as in Fig. 7.

TABLE III

RESPONSE FACTORS OF POLYMETHOXYLATED FLAVONES*

Response factors determined using the solvent mixture *n*-heptane–isopropanol (60:40) containing 0.05% of water, flow-rate 0.45 ml/min; column, 25 cm × 4 mm I.D., LiChrosorb Si 60 (6 µm).

Flavone No.	*UV detection*						
	254 nm		*280 nm*			*365 nm*	
	*A**	*RA***	*A**	*RA***	*RFC****	*A**	*RA***
1	7.9	1.56	7.6	2.05		0.10	0.09
2	5.5	1.1	4.6	1.24	1.20	0.30	0.27
3	3.9	0.78	5.4	1.46		1.10	1.00
4	4.2	0.84	3.1	0.84		2.5	2.26
5	3.7	0.74	3.2	0.86		2.6	2.36
6	4.2	0.84	2.3	0.62		3.2	2.90
7	3.7	0.74	3.7	1.00	0.95	0.10	0.09
8	3.8	0.76	2.7	0.73	0.73	0.34	0.31
9	4.7	0.94	2.3	0.62		0.87	0.80
10	4.6	0.92	2.0	0.54	0.65	1.5	1.36
11	2.7	0.54	4.6	1.24		0.90	0.82
12	4.4	0.88	3.6	0.97		2.6	2.4
13	2.0	0.40	1.1	0.30		0.78	0.71
14	3.0	0.72	1.3	0.35		2.6	2.4
15	3.1	0.62	4.5	1.22	1.33	0.26	0.24
16	5.0	1.00	3.7	1.00	1.00	1.1	1.00
17	4.4	0.88	2.0	0.54	0.60	2.3	2.15

* Polymethoxylated flavone absorbance for the fixed-wavelength detector.

** RA = relative absorbance expressed as the ratio of the absorbance of the flavone to that of nobiletin (16).

*** RFC = response factor determined chromatographically.

wavelengths corresponding to the UV detector filters were determined (Table III). We have used nobiletin (compound 16) as a standard for relative absorbance (RA) calculations.

We determined response factors at 280 nm, comparing the slopes of the straight lines in Fig. 9 with nobiletin (16) as a standard. The values obtained are given in Table III, and show good agreement for the two methods.

At 280 nm, the response factors of polymethoxylated flavone can vary in the ratio 2.05:0.30 (flavones 1 and 13). For the most important flavones in citrus, the deviations are not so high (0.54:1.46 for flavones 10 and 3, respectively). At 254 nm slight differences are observed (0.62:1.1 for flavones 15 and 2). On the other hand, absorbances at 365 nm are very different because for some products the maximal absorption is very high[14,15]. The UV spectra show that there is no wavelength where the absorbances of polymethoxylated flavone are identical, so that a quantitative determination requires an accurate calibration under the experimental conditions used. Corrections can be made using the values given in Table III.

Response factors of flavones at various wavelengths should be an aid in the identification of compounds or for composite peak determinations.

For injections of identical amounts of compounds, under the same chromatographic conditions the peak heights are proportional to the absorbances of the compounds at the wavelength considered. We injected 10 μl of the same solution and recorded absorbances ast 254, 280 and 365 nm, as illustrated in Fig. 10. Peak-height ratios are given in Table IV, and also responses ratios calculated from Table III. The results for the two methods are in good agreement for 254 and 280 nm. On the other hand, for 280 and 365 nm the differences in the peak-height ratios are clearly higher, especially when the absorbance measured by the UV spectrophotometer at 365 nm is very low. The higher response of the ISCO detector may be explained by its wide-band frequency range.

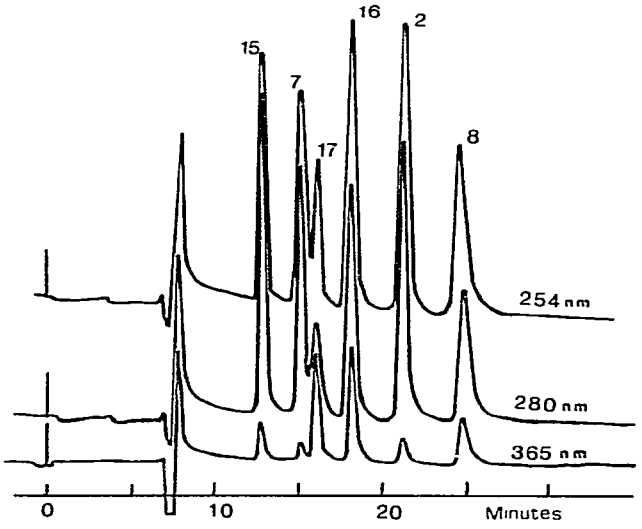


Fig. 10. Chromatograms of some polymethoxylated flavones at 254, 280 and 365 nm. Peak identification as shown in Table I. Column and chromatographic conditions as in Fig. 7.

TABLE IV

COMPARISON OF RESPONSE RATIOS OF POLYMETHOXYLATED FLAVONES AT DIFFERENT UV WAVELENGTHS

Response ratios determined using the solvent mixture *n*-heptane–isopropanol (60:40) containing 0.05% water, flow-rate 0.45 ml/min; column, 25 cm × 4 mm I.D., LiChrosorb Si 60 (6 μm).

Flavone No.	*Name*	*UV wavelength ratio*			
		254/280 nm		*280/365 nm*	
		*H**	*A***	*H**	*A***
2	5,7,4′-Trimethoxyflavone	1.03	1.20	11	15
7	Tetra-O-methylscutellarein	0.86	1.00	13	37
8	5,6,7,3′,4′-Pentamethoxyflavone	1.27	1.41	2.8	8
15	Tangeretin	0.78	0.69	8.0	17
16	Nobiletin	1.23	1.35	2.0	3.3
17	Heptamethoxyflavone	1.03	1.20	10.8	15.3

* H = peak-height ratio at 254 and 280 nm or 280 and 365 nm.
** A = absorbance ratio at 254 and 280 nm or 280 and 365 nm.

Quantitative determination of polymethoxylated flavones in industrial orange oils

Polymethoxylated flavone contents were determined for three samples of industrial orange oils of different origins (Guinea, Israel and Brazil). In previous determinations[5,11–13], nobiletin was listed as the major component of tangerine peel oil, whereas flavones 7, 8, 15 and 17 were the most important in orange peel.

Six polymethoxylated flavones were identified and quantified. The results obtained are given in Table V. It can be seen that the total flavone content and the proportions of the six polymethoxylated flavones are different for the three samples, heptamethoxyflavone (17) being the major one in Guinea and Israel oils and tangeretine (15) in Brazil oil. In all three samples sinensetin (8) is the least abundant polymethoxylated flavone.

TABLE V

DETERMINATION OF QUANTITATIVE POLYMETHOXYLATED FLAVONES IN INDUSTRIAL ORANGE OILS

Determined using the solvent mixture *n*-heptane–isopropanol (60:40) with 0.05% of water, flow-rate 0.45 ml/min: column, 25 cm × 4 mm I.D., LiChrosorb Si 60 (6 μm). Injection of 10 μl of industrial oil diluted 10-fold with absolute ethanol.

Flavone No.	*Name*	*Amount found (g/l)*		
		Guinea	*Israel*	*Brazil*
15	Tangeretin	0.84	0.36	0.68
7	Tetra-O-methylscutellarein	0.50	0.16	0.18
17	Heptamethoxyflavone	1.24	0.42	0.36
16	Nobiletin	0.98	0.25	0.34
10	3,5,6,7,3′,4′-Hexamethoxyflavone	0.20	0.06	0.04
8	Sinensetin	0.16	0.05	0.04
Total		3.92	1.30	1.64

CONCLUSION

Studies of the capacity factors of various polymethoxylated flavones by straight-phase HPLC using four solvent systems with various water contents showed that systems B (*n*-heptane–ethanol, 75:25) and D (*n*-heptane–isopropanol, 60:40) are the most useful with water contents ranging from 0.05 to 0.02%.

The determination of response factors using chromatography or UV spectroscopy at different wavelengths allows a quantitative study of polymethoxylated flavones in citrus fruits. The results can be used to establish the amounts of polymethoxylated flavones ingested from orange juice for clinical experiments.

ACKNOWLEDGEMENT

The authors are grateful to C. F. P. Orangina Société, Vitrolles, France, for providing authentic industrial orange oils samples from Guinea, Israel and Brazil.

REFERENCES

1 J. B. Harborne, T. J. Mabry and H. Mabry, *The Flavonoids*, Chapman and Hall, London, 1975.
2 L. J. Swift, *J. Agr. Food Chem.*, 15 (1967) 99.
3 P. S. Sarin and T. R. Seshadri, *Tetrahedron*, 8 (1960) 64.
4 J. F. Kefford and B. V. Chandler, *The Chemical Constituents of Citrus Fruits*, Academic Press, New York, 1970.
5 J. H. Tatum, C. J. Hearn and R. E. Berry, *J. Amer. Soc. Hort. Sci.*, 103 (1978) 492.
6 M. K. Veldhuis, L. J. Swift and W. C. Scott, *J. Agr. Food Chem.*, 18 (1970) 590.
7 H. Wagner, in T. Swain, J. B. Harborne and C. F. van Sumere (Editors), *Recent Advances in Phytochemistry, Vol. 12, Biochemistry of Plant Phenolics*, Plenum Press, New York, 1974.
8 T. J. Mabry and A. Ulubelen, *J. Agr. Food Chem.*, 28 (1980) 188.
9 S. M. Kupchan, J. R. Knox and M. S. Udayamurthy, *J. Pharm. Sci.*, 54 (1965) 929.
10 R. C. Robbins, *Int. J. Vitam. Nutr. Res.*, 47 (1977) 373.
11 J. P. Bianchini and E. M. Gaydou, *J. Chromatogr.*, 190 (1980) 233.
12 S. V. Ting, R. L. Rousseff, M. H. Dougherty and J. A. Attaway, *J. Food Sci.*, 44 (1979) 69.
13 R. L. Rousseff and S. V. Ting, *J. Chromatogr.*, 176 (1979) 75.
14 E. M. Gaydou and J. P. Bianchini, *Ann. Chim. (Paris)*, 2 (1977) 303.
15 E. M. Gaydou and J. P. Bianchini, *Bull. Soc. Chim. Fr.*, II (1978) 43.
16 T. A. Geissman, *The Chemistry of Flavonoid Compounds*, Pergamon Press, Oxford, 1962.
17 F. Wessely, *Monatsh. Chem.*, 56 (1930) 97.
18 A. Olivero, G. B. Marini-Bettolo and G. Bargellini, *Gazz. Chim. Ital.*, 78 (1948) 363.
19 G. Bargellini and A. Olivero, *Chem. Ber.*, 75B (1942) 2083.
20 V. V. S. Murti, K. V. Rao and T. R. Seshadri, *Proc. Indian Acad. Aci.*, 26A (1947) 182.
21 V. V. S. Murti and T. R. Seshadri, *Proc. Indian Acad. Sci.*, 27A (1948) 217.
22 H. Böhme and P. E. Volcker, *Arch. Pharm.*, 292 (1959) 529.
23 J. H. Tatum and R. E. Berry, *Phytochemistry*, 11 (1972) 2283.
24 K. F. Tseng, *J. Chem. Soc.*, (1938) 1003.
25 L. J. Swift, *J. Org. Chem.*, 30 (1965) 2079.
26 R. Born, *Chem. Ind. (London)*, (1960) 264.
27 K. Walther, H. Rimpler and C. Leukert, *Planta Med.*, 14 (1966) 45.
28 E. K. Nelson, *J. Amer. Chem. Soc.*, 56 (1934) 1392.
29 V. V. S. Murti, S. Rangaswami and T. R. Seshadri, *Proc. Indian Acad. Sci.*, 28A (1948) 19.
30 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968.
31 J. J. Kirkland, *Chromatographie en Phase Liquide*, Gauthier-Villars, Paris,1973, p. 11.
32 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1974.

33 W. Boehme and H. Engelhardt, *J. Chromatogr.*, 133 (1977) 67.
34 J.-P. Thomas, A. Brun and J. P. Bounine, *J. Chromatogr.*, 139 (1977) 21.
35 J. P. Thomas, A. Brun and J.-P. Bounine, *Analysis*, 7 (1979) 221.
36 R. P. W. Scott, *J. Chromatogr. Sci.*, 18 (1980) 297.
37 J. P. Bianchini and E. M. Gaydou, *J. Chem. Soc. Chem. Commun.*, submitted for publication.