Journal of Chromatography, 586 (1991) 171–176
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 630

Short Communication

Fast separation of polymethoxylated flavones by carbon dioxide supercritical fluid chromatography

Ph. Morin*

Laboratoire de Chimie Bioorganique et Analytique, Université d'Orléans, 45067 Orléans Cédex 2 (France)

A. Gallois☆ and H. Richard

Laboratoire de Chimie, ENSIA, Massy (France)

E. Gaydou

Laboratoire de Phytochimie, Faculté des Sciences et Techniques de Saint-Jérôme, Marseille (France)

(First received March 27th, 1991; revised manuscript received July 16th, 1991)

ABSTRACT

The application of supercritical fluid chromatography (SFC) with modified carbon dioxide for the separation of polymethoxylated flavones (PMFs) is reported. The chromatographic system consists of a bare silica column (250 × 4.6 mm I.D.) with a carbon dioxide–methanol mobile phase and UV detection (313 nm). Selectivities are found to be different with this SFS system than with high-performance liquid chromatography (HPLC). All these naturally occurring PMFs (sinensetin, nobiletin, tangeretin, heptamethoxyflavone, tetramethylscutellarein) could be satisfactorily determined using an internal standard. The method was applied to the determination of PMFs in several *Citrus* oils. The SFC procedure is considerably faster than HPLC with good resolution and an adequate accuracy for the quantitative identification of PMFs.

INTRODUCTION

Flavones, which are widespread in the vegetable kingdom, generally occur as hydroxylated or glycosylated derivatives [1]. Polymethoxylated flavones (PMFs) constitute a special group which are present in certain *Citrus* species. The peel of these fruits contains higher concentrations of PMFs than their leaves or juices. Flavonoid determination is useful in chimiotaxonomic studies on *Citrus* and to authenticate *Citrus* oils. Many papers have been published on the determination of PMFs by high-performance liquid chromatography (HPLC) [2–11].

The results of supercritical fluid chromatographic (SFC) separations of PMFs with carbon dioxide on silica-packed columns are described in this paper.

EXPERIMENTAL

Separations were performed on a stainless-steel column (250 mm × 4.6 mm I.D.) packed with Du-

☆ Present address: Dionex S.A., 103 av. Grenier, 92100 Boulogne, France.

0021-9673/91/$03.50

Pont Zorbax silica (5 μm) equipped with a precolumn (50 mm × 4.6 mm I.D.) filled with the same silica. The mobile phase, carbon dioxide (N 45 grade, 99,995% purity) (Air Liquide, Bois d'Arcy, France) was pumped through the system by a Varian (Palo Alto, CA, USA) Model 2510 pump. The polar modifier (HPLC-grade methanol; Rathburn Chemicals, Walkerburn, UK) was added using a Gilson (Villiers-le-Bel, France) Model 302 pump. The two solvents were mixed in a Gilson mixing chamber. The pump heads were cooled at 4°C to promote filling of the pump with liquid mobile phase from the carbon dioxide tank. Samples were introduced onto the column via a Rheodyne Model 7010 injector fitted with a 20-μl sample loop. A Varian Model 2550 variable-wavelength UV detector, equipped with an 8-μl high-pressure flowcell, was set at 313 nm (range 0.08 a.u.f.s.). The pressure was controlled by a manual back-pressure regulator (Model 26-3220-24004, Tescom, MN, USA) connected in series after the detector and maintained at 40°C by a water-bath. All stainless-steel tubing between the injector and the column, and also between the outlet of the column and the pressure regulator, was immersed in a water-bath at 40°C to reduce detector noise.

The PMFs used as standards in this work were donated from several sources.

RESULTS AND DISCUSSION

The major aim of this study was to investigate the ability of packed columns for SFC separation of the PMFs. These compounds have the basic flavone structure shown in Table I. As they differ only in the position and the number of methoxy groups on the A, B and C rings of the flavone, differences in polarity and solubility are weak and difficult to interpret. Owing to the similar chemical structures of these compounds, a bare silica stationary phase with a high specific surface area was considered to give higher selectivity than a partitioning stationary phase, and used for this work. All these flavones have their UV absorbance maxima in the range 310–350 nm range [2].

A pure carbon dioxide mobile phase was first tested, but none of the PMFs in Table I could be eluted from Zorbax silica-packed columns at 40°C and 300 atm. As expected on such a column, the flavonoids were strongly adsorbed on the activated silanol sites present in the column and the elution strength of pure carbon dioxide was not sufficient for these medium-polarity compounds.

TABLE I

STRUCTURES OF POLYMETHOXYLATED FLAVONES [1]

PMF	Systematic name
Tangeretin	5,6,7,8,4′-Pentamethoxyflavone
Heptamethoxyflavone	3,5,6,7,8,3′,4′-Heptamethoxyflavone
Nobiletin	5,6,7,8,3′,4′-Hexamethoxyflavone
Sinensetin	5,6,7,3′,4′-Pentamethoxyflavone
Tetramethylisoscutellarein	5,8,7,4′-Tetramethoxyflavone
Isosinensetin	5,7,8,3′,4′-Pentamethoxyflavone
Tetramethylscutellarein	5,6,7,4′-Tetramethoxyflavone
Hexamethoxyflavone	3,5,6,7,3′,4′-Hexamethoxyflavone

Methanol–carbon dioxide mixtures as mobile phase

The addition of methanol to supercritical carbon dioxide alters the retention in packed-column SFC by masking the active silanol sites. A preliminary of the separation of a test mixture containing five PMFs was carried out to determine their retention behaviour on a bare silica column using supercritical carbon dioxide modified with methanol in the range 5–30%. The capacity factor of each flavone (tangeretin, nobiletin, sinensetin, tetramethylisoscutellarein and isosinensetin) on a Zorbax silica column was determined at the constant temperature (40°C) for several different methanol contents in carbon dioxide (Table II). Each solute was injected four times and the relative standard deviation (R.S.D.) of the capacity factor was less than 0.5%. The elution order obtained on a silica column with a carbon dioxide–methanol mixture as mobile phase is the same that observed in adsorption HPLC with heptane–ethanol or heptane–isopropanol mixtures [1], and the opposite of the elution order in reversed-phase HPLC [9,10]. In our work, the retention behaviour observed on the silica column confirms that the retention mechanism is based on adsorption of solutes on silanol groups. Tangeretin and heptamethoxyflavone, which contain five and seven methoxy groups, respectively, elute before nobiletin and tetramethylisoscutellarein, which contain six and four methoxy groups, respectively.

TABLE II

INFLUENCE OF METHANOL ADDED TO CARBON DIOXIDE ON THE CAPACITY FACTORS OF POLYMETHOXYLATED FLAVONES

Stationary phase, Zorbax silica (250 mm × 4.6 mm I.D.); outlet pressure, 200 atm; temperature, 40°C.

Methanol (%, v/v)	Capacity factor (k')				
	Tangeretin	Nobiletin	Sinensetin	Isoscutellarein	Isosinensetin
5	3.94	5.64	15.35	21.00	–
7	2.40	3.34	5.75	8.35	11.00
10	1.55	2.25	3.60	4.75	5.00
20	0.56	0.73	1.17	1.52	–
30	0.40	0.50	0.76	0.99	–

This indicates that the flavonoid retention is determined not only by the number of methoxy groups present, but also by steric effects to an extent that becomes greater as the number of methoxy groups increases. Similar effects were observed in HPLC and reported by Bianchini and Gaydou [1], who studied the retention of seventeen polymethoxylated flavones on a silica packing (LiChrosorb Si 60) using mainly *n*-heptane–ethanol (75:25, v/v) or *n*-heptane–isopropanol (60:40, v/v) as mobile phase. They discussed the influence of both position and number of methoxy groups in the flavonoid skeleton on the retention. We observed in SFC the same sequence for the capacity factors as obtained by Bianchini and Gaydou using adsorption HPLC.

An increase in methanol content results in enhanced solute solubility, a decreased solute adsorption and, consequently, a decrease in retention (Table II); nevertheless, no improvement in selectivity occurred in the 5–20% methanol range for these solutes.

Retention effects could be simply discussed in terms of additivity rules. For example, consider the logarithms of retention values with 7% modifier. A hypothetical 5,7,4′-trimethoxyflavone would have a log k' of 1.16. Introducing the increments $I_6 = -0.53$, $I_8 = -0.24$ and $I_{3'} = +0.13$ for substituents in positions 6, 8 and 3′, respectively, experimental and calculated log k' values compared as shown in Table III. A methoxy group in position 3 decreases the retention (see the elution order of heptamethoxyflavone and nobiletin), whereas a methoxy group in position 3′ increases the retention (see the capacity factors of tangeretin and nobiletin). Finally, methoxy groups in positions 3′ and 4′ contribute to an increase in the capacity factors (tangeretin and heptamethoxyflavone).

The SFC analysis of a standard mixture of six polymethoxylated flavones was carried out in less than 12 min using silica as the stationary phase, as shown in Fig. 1. The resolution between nobiletin and heptamethoxyflavone is greater than 1.5, whereas reversed-phase HPLC needed the use of water–tetrahydrofuran solvent to resolve these two compounds satisfactorily [8,9].

Fig. 2a and b show chromatograms of the same

TABLE III

EXPERIMENTAL AND CALCULATED LOG k' VALUES FOR POLYMETHOXYLATED FLAVONES

Stationary phase, Zorbax silica (250 mm × 4.6 mm I.D.); outlet pressure, 200 atm; temperature, 40°C; mobile phase, carbon dioxide modified with 7% of methanol.

Parameter	5,7,4′-Trimethoxyflavone	Tangeretin	Nobiletin	Sinensetin	Tetramethylisoscutellarein	Isosinensetin
$(\text{Log } k')_{\text{exp.}}$	–	0.380	0.524	0.760	0.922	1.04
$(\text{Log } k')_{\text{calc.}}$	1.160	0.390	0.520	0.760	0.920	1.05

flavonoid mixtures with different carbon dioxide and methanol flow-rates; at high flow-rate these solutes elute in 2 min without any significant loss of efficiency and resolution.

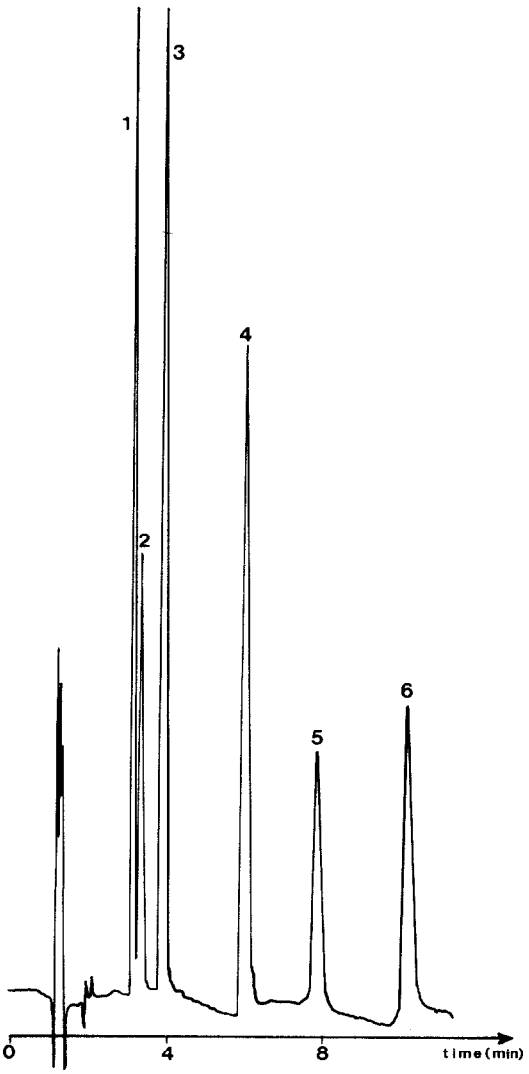

Fig. 1. SFC separation of synthetic mixture of polymethoxylated flavones. Column, 250 mm × 4.6 mm I.D.; stationary phase, Zorbax (5 μm) silica; mobile phase, carbon dioxide modified with 10% of methanol; inlet pressure, 220 atm; outlet pressure, 200 atm; column temperature, 40°C; carbon dioxide flow-rate, 3 ml/min; methanol flow-rate, 0.3 ml/min; UV detection at 313 nm. Solutes: 1 = tangeretin; 2 = heptamethoxyflavone; 3 = nobiletin; 4 = sinensetin; 5 = tetramethylisoscutellarein; 6 = isosinensetin.

Linearity of response and detection limits

The determination of the flavones was achieved using the described SFC system. Linearity ranges of peak area *versus* PMF concentration were established for the UV detector set at 313 nm by injecting 20 μl of the first sinensetin standards of various concentrations (238, 166, 142, 119, 100, 71, 47 and 25 p.p.m.) and second the tangeretin standards (196, 153, 130, 96, 65, 48 and 24 p.p.m.) (Fig. 3). The UV detector responses of these two flavones were linear in this concentration range up to 5 μg. Detection

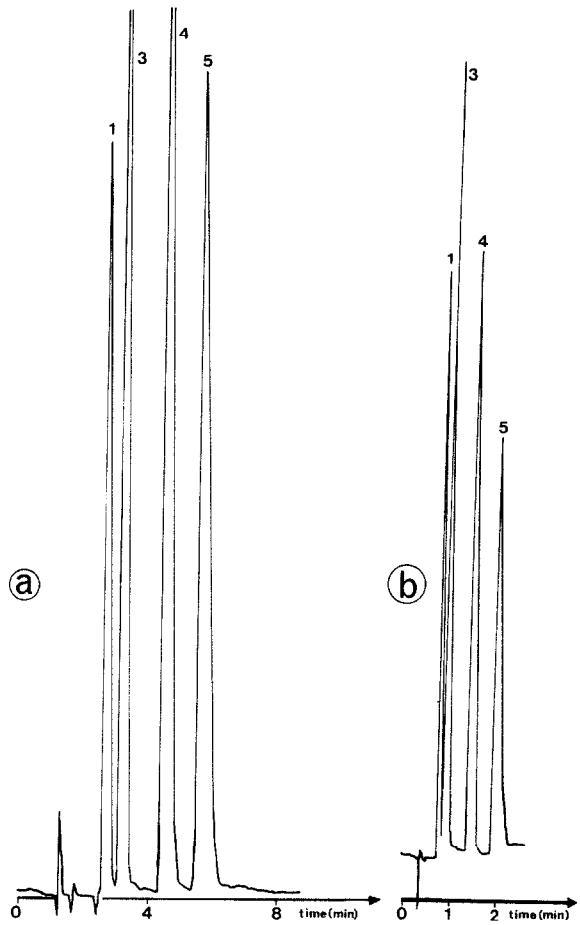

Fig. 2. Rapid separation of polymethoxylated flavones by packed-column SFC. Conditions as in Fig. 1, except as follows: (a) carbon dioxide flow-rate 3 ml/min, methanol flow rate 0.3 ml/min, inlet pressure 220 atm; (b) carbon dioxide flow-rate 9 ml/min, methanol flow-rate 0.9 ml/min, inlet pressure 258 atm. Solutes: 1 = tangeretin; 3 = nobiletin; 4 = sinensetin; 5 = tetramethylisoscutellarein.

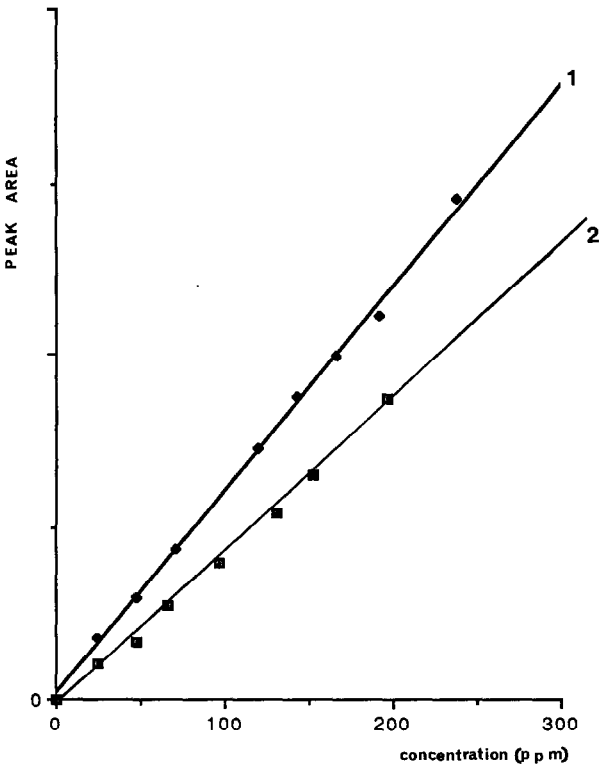

Fig. 3. Quantitative relationship between flavone peak area and concentration. Inlet pressure, 220 atm; outlet pressure, 200 atm; column temperature, 40°C; UV detection at 313 nm; Volume injected, 20 μl; sample dissolved in ethanol. Solutes: 1 = sinensetin; 2 = tangeretin.

limits (signal-to-noise ratio = 2) were 2.5 p.p.m. for tangeretin (50 ng) and 1.6 p.p.m. for sinensetin (32 ng).

Analysis of Citrus oils

In some instances, an internal standard (coumarin) was added to the *Citrus* oils in order to facilitate the identification of the peaks by comparison of their capacity factors. Finally, PMFs were determined in an orange oil (Fig. 4) and a tangerine oil (Fig. 5).

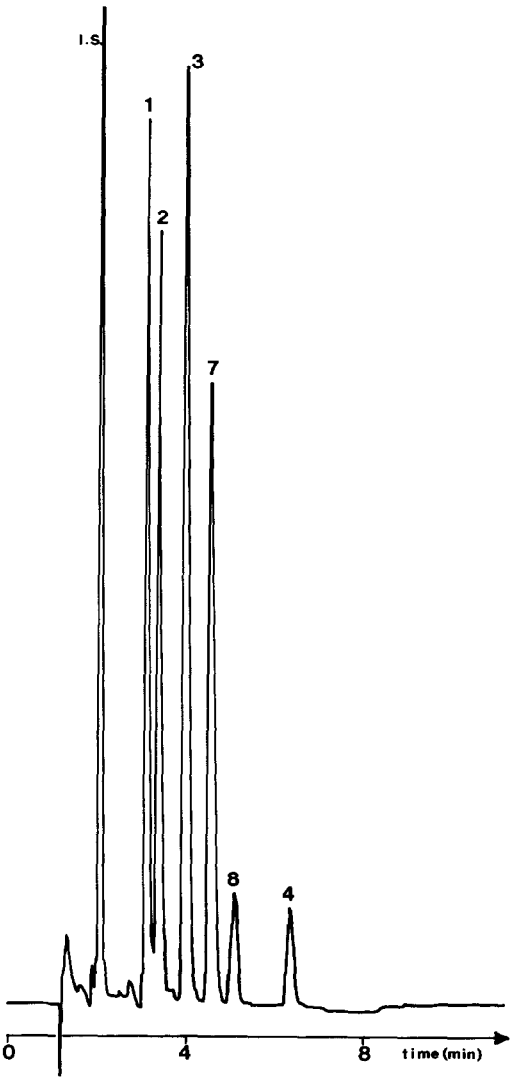

Fig. 4. SFC of the polymethoxylated flavones in orange oil. Conditions as in Fig. 1; orange oil diluted 1:15 in ethanol. Solutes: I.S. = internal standard (coumarin); 1 = tangeretin; 2 = heptamethoxyflavone; 3 = nobiletin; 4 = sinensetin; 7 = tetramethylscutellarein; 8 = hexamethoxyflavone.

CONCLUSIONS

Packed-column carbon dioxide SFC appears to be useful for rapid analyses of the main polymethoxylated flavones in *Citrus* oils. Carbon dioxide modified with 10% of methanol was found to elute all the PMFs from a bare silica stationary phase. The SFC analysis is faster than reversed-phase HPLC, with a fair resolution and accurate and reproducible retention times and peak-area determinations.

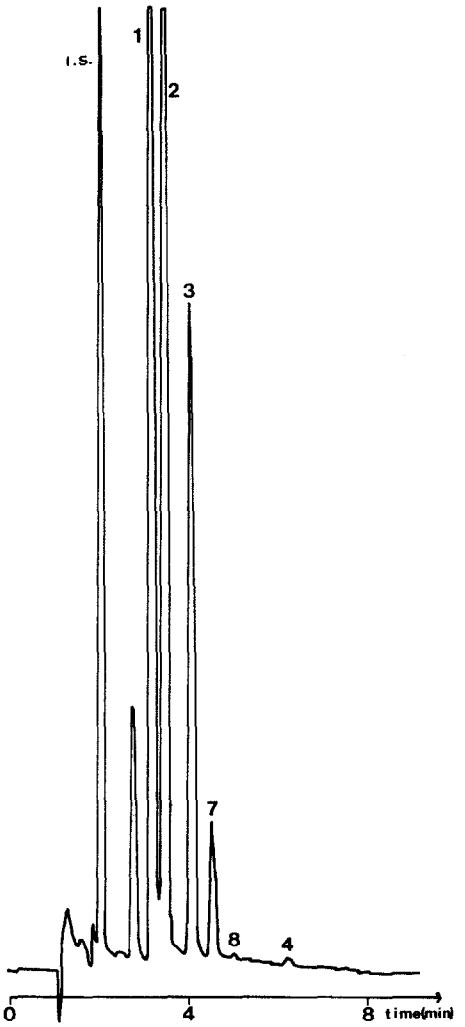

Fig. 5. SFC of the polymethoxylated flavones in tangerine oil. Conditions as in Fig. 1; tangeretin oil diluted 1:15 in ethanol. Solutes as in Fig. 4.

REFERENCES

1 J. P. Bianchini and E. M. Gaydou, *J. Chromatogr.*, 211 (1981) 61.
2 J. M. Sendra, J. L. Navarro and L. Izquierdo, *J. Chromatogr. Sci.*, 26 (1988) 443.
3 M. K. Veldhuis, L. J. Swift and W. C. Scott, *J. Agric. Food Chem.*, 18 (1970) 590.
4 R. Galensa and K. Herrmann, *J. Chromatogr.*, 189 (1980) 217.
5 B. Heimburger, R. Galensa and K. Herrmann, *J. Chromatogr.*, 439 (1988) 481.
6 E. M. Gaydou, J. P. Bianchini and R. Randriamiharisoa, *J. Agric. Food Chem.*, 35 (1987) 525.
7 M. Hadj-Mahammed and B. Y. Meklati, *Lebensm.-Wiss. Technol.*, 20 (1987) 111.
8 R. Rouseff and S. Ting, *J. Chromatogr.*, 176 (1979) 75.
9 S. V. Ting, R. L. Rouseff, M. H. Dougherty and J. A. Attaway, *J. Food Sci.*, 44 (1979) 69.
10 J. P. Bianchini, E. M. Gaydou, A. M. Siouffi, G. Mazerolles, D. Mathieu and R. Phan Tan Luu, *Chromatographia*, 23 (1987) 15.
11 J. P. Bianchini and E. M. Gaydou, *J. Chromatogr.*, 190 (1980) 233.