Separation of Polyhydroxylflavonoids by Packed-Column Supercritical Fluid Chromatography

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Abstract

Polyhydroxylflavonoids, isorhamnetin, kampcetin, quercetin, and fisetin are efficiently separated using supercritical carbon dioxide modified by ethanol with phosphoric acid as an additive on packed-column supercritical fluid chromatography. The effect of the silica-based stationary phase, composition of mobile phase, temperature, and pressure are investigated. The addition of phosphoric acid as a polar additive improves peak shapes.

Introduction

Supercritical fluid chromatography (SFC) has been used as a powerful separation technique complementing or being superior to gas chromatography (GC) and high-performance liquid chromatography (HPLC), because supercritical fluid as a mobile phase exhibits high diffusivity, low viscosity, and high solvating power. Supercritical carbon dioxide is the most popular supercritical fluid because it is nontoxic, nonflammable, and easy to obtain and has a near-ambient critical temperature. However, CO_2 has weak solvating power for polar compounds, so it is often modified by polar organic solvents (such as methanol, ethanol, etc.), termed modifiers, to be used in SFC mobile phases (1,2). However, this binary mobile phase cannot elute very polar compounds efficiently. To widen the application of SFC, a small amount (< 1%) of very polar solvent was added to a modifier and then to a mobile phase. This very polar solvent, called an additive (3–5), is not directly miscible with CO_2 but can be added to the modifier to form ternary mixture with CO_2. Organic acids (such as trifluoroacetic acid and citric acid) were used as additives to cause polar solutes (such as hydroxybenzoic and polycarboxylic acids) to be eluted rapidly and efficiently from packed SFC (3–5). So far, packed-column SFC using binary or ternary mobile phases (1–13) has been applied to the separation of various kinds of polar substances.

Generally, flavonoids are separated and analyzed by HPLC using organic solvent (methanol, acetonitrile, etc.) aqueous solutions as mobile phases with an ultraviolet–visible detector. In recent years, SFC was used to separate this type of compound. Morin et al. (14) successfully separated polymethoxylated flavones by packed-column SFC using supercritical CO_2 modified by methanol. Hadj-Mahammed et al. (15) analyzed some polyhydroxy- and polymethoxy-flavones using capillary column SFC with flame ionization detection and Fourier transfer infrared detection. No reports about the separation of polyhydroxyflavonoids using SFC were found in the literature.

In this paper, flavonoids with 4 or 5 hydroxyl groups were chosen in order to investigate their retention behavior on several stationary phases using ternary mobile phases. The effects of temperature, pressure, and mobile phase composition on retention were investigated, and the role of additive in influencing retention and in improving peak shapes was also studied.

Experimental

Instrumentation

The SFC apparatus was designed and set up in our laboratory, which was previously described in detail (16). The mobile phase delivery system included two syringe pumps (100DM, Isco, Lincoln, NB), one of which pumped liquid CO_2 with a chilled pump head, and the other delivered modifier. The two flows were combined and mixed in a mixer in terms of preset volume proportions, which was carried out through a controller that controlled the operating modes of the two pumps at the same time. The preheating tube and column were mounted in a oven modified by a GC oven (model SQ-204, Beifen Gases, Beijing, China). The Rheodyne (Rohnert Park, CA) model 7520 injection valve with a 1-µL loop was mounted on top of the oven. A variable-wavelength detector (Spectra 100, Thermo Separation Products, San Jose, CA) equipped with a 250-nL high-pressure flowcell, was set at 254 nm. A manual back-pressure regulator was used to control the flow rate at about 1.0 mL/min, which kept the column pressure drop less than 1.0 MPa under the constant inlet pressure mode.

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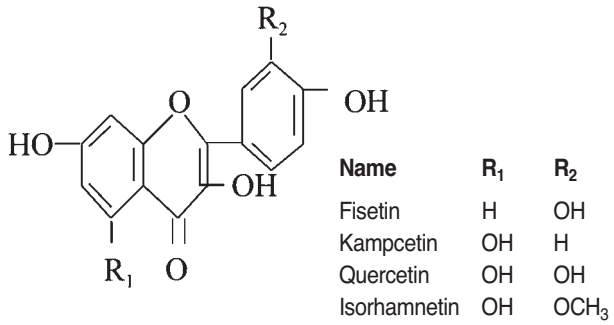
Consumables

Columns included a 5-µm-diameter spherisorb cyanopropyl column (4.6 × 200 mm), a 5-µm-diameter spherisorb phenyl column (4.6 × 200 mm), and a 5-µm Hypersil octodecanosiloxane (ODS) column (4.6 × 200 mm). Carbon dioxide from Beijing Oxygen Gas Company (Beijing, China) was high grade with purity of 99.995%. Ethanol and phosphoric acid from Beijing Chemical Reagent Factory (Beijing, China) were analytical grade. Solutes with a purity of 95.0%, whose molecular structures are shown in Figure 1, were purchased from Sigma (St. Louis, MO).



Name	R_1	R_2
Fisetin	H	OH
Kampcetin	OH	H
Quercetin	OH	OH
Isorhamnetin	OH	OCH_3

Figure 1. Molecular structure of flavonoids.

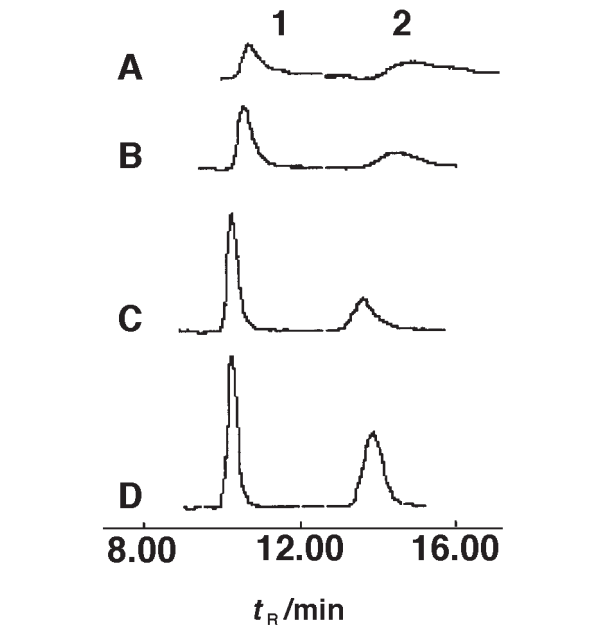


Figure 2. The effect of phosphoric acid on peak shapes.The phosphoric acid concentration (v/v) in ethanol was 0.025% (A), 0.05% (B), 0.1% (C), and 0.2% (D). Peaks: 1, quercetin; 2, risetin. Conditions: pressure, 25.0 MPa; temperature, 50.0°C; mobile phase, CO_2–ethanol–phosphoric acid; modifier concentration, 10% (v/v); flow rate, 1.05 mL/min; stationary phase, phenyl column.

Methods

Flavonoids were dissolved in ethanol at approximately 100 µg/mL before chromatographic operation. CF_4 was used as an unretained substance to measure the dead time (t_0) of the columns at the mobile phase flowrate of approximately 1.0 mL/min. All data were obtained three times.

Results and Discussion

The capacity factor (k') is an important thermodynamic parameter used to describe the retention of a solute in a chromatographic system; it can be expressed as $k' = (t_R - t_0)/t_0$, where t_R is the retention time of the solute and t_0 is the dead time. In the present study, t_0 was obtained by measuring the time of CF_4 to flow through the chromatographic column, because CF_4 was hardly retained on the silica-based column (17). The capacity factor and retention time were chosen to investigate the retention behavior of flavonoids.

Effect of composition of mobile phase

Both pure supercritical CO_2 and CO_2 modified by ethanol did not elute all the flavonoids tested in the present study because of the poor solvating power of the mobile phase or the strong interactions between the solutes and the stationary phases.

In this study, phosphoric acid, which is an inorganic acid and is often used in HPLC mobile phases, was chosen as an additive to the modifier in a very small amount, and then both were added to the mobile phase. It was found that the ternary mobile phase eluted all 4 solutes from these silica-based columns rapidly and efficiently. Changes in concentration of additive failed to significantly change solute retention, as shown in Figure 2. However, it can be seen that an increase in additive concentration improves the peak shapes, and that when the additive concentration is up to 0.01% (v/v) in 10% (v/v) modifier, the peaks exhibit symmetrical shapes. Retention time versus modifier concentration was measured using 4 concentrations of additive. The results are listed in Table I, which indicates that additive concentration has little effect on the retention time.

Table II represents the effect of modifier concentration on the retention time of each solute, showing that the addition of modifier to the mobile phase dramatically decreases the retention. Two main reasons may exist: first, that the addition of modifier strengthens the solvating power of mobile phase, and second, that the stationary phase adsorbs modifier molecules on its active sites to reduce the opportunity for a solute to be adsorbed on stationary phase, thus decreasing the retention time of the solute.

Table I. Effect of Additive Concentration on the Retention Time (t_R/min)*

Phosphoric acid concentration in ethanol (%, v/v)	Isorhamnetin	Kampcetin	Quercetin	Fisetin
0.025	6.98	10.20	11.09	—
0.05	6.85	9.59	10.04	14.55
0.10	6.80	9.21	9.72	14.63
0.20	6.68	8.90	9.98	13.30
0.50	6.65	8.85	9.67	12.72

* Conditions: temperature, 50°C; pressure, 25.0 MPa; mobile phase, CO_2–ethanol–phosphoric acid (90:9.98:0.02, v/v/v); stationary phase, phenyl column; flow rate, 1.05 mL/min.

Effect of stationary phase

Three silica-based columns (a C_{18} column, a phenyl column, and a cyanopropyl column) were used to investigate the retention behavior of flavonoids. It was proven through experimentation that the solutes tested in this study could not be eluted from these columns without using phosphoric acid as an additive in the mobile phase, which indicated that the silica-based columns adsorbed polar solutes with polyhydroxyl groups so strongly that the supercritical CO_2 modified only by ethanol could not elute them. When a small amount of phosphoric acid was added to the mobile phase, the phosphoric acid molecules could be adsorbed onto the active sites of the stationary phase, which could prevent solute molecules from being strongly adsorbed; the interaction between solutes and stationary phase was released, thus the solutes could be easily eluted from the chromatographic system. These 3 columns have different polarities because of the difference in the bonded group on the silica. The increasing order of polarity of the columns is cyanopropyl column > phenyl column > C_{18} column. The solutes have the same elution order on these columns, but the retention time increased with polarity of the stationary phase, as shown in Table III. From Table III, it can be seen that the 4 solutes have the same retention time on the ODS column, which suggests that the ODS column is not suitable for their separation. Both the cyanoprophyl and phenyl columns could separate these solutes efficiently, but the latter separated them more rapidly.

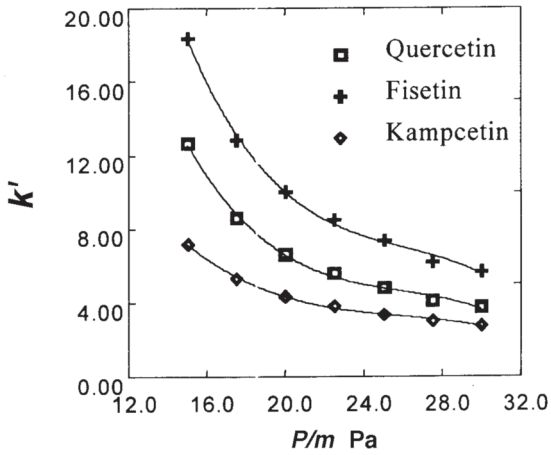


Figure 3. The effect of pressure on the capacity factor. Conditions: temperature, 50°C; mobile phase, CO_2–ethanol–phosphoric acid (90:9.98:0.02, v/v/v); stationary phase, phenyl column; flow rate, 1.05 mL/min.

Effect of pressure

In this study, modifier concentration was expressed as volume fraction, which resulted in a decrease in molar fraction of the modifier with pressure. It was previously discussed that increasing modifier concentration reduces the retention time of the solute. Therefore, a decrease in modifier concentration increases the retention time of the solute. From Figure 3, it can be seen that capacity factors decrease with pressure despite the effect of the decrease in modifier concentration, which suggests that the pressure effect is important. At low pressure, the capacity factor strongly depends on pressure, probably because the mobile phase is compressible; increasing pressure leads to a largely increased density of mobile phase, thus enhancing the solubility of the solute in the mobile phase.

Table II. Effect of Modifier Concentration on the Retention Time (t_R/min)*

Ethanol concentration with 0.2% (v/v) additive	Isorhamnetin	Kampcetin	Quercetin	Visetin
8.0%	7.85	10.12	12.82	20.64
10.0%	6.68	8.90	9.68	13.70

* Conditions: temperature, 50°C; pressure, 25.0 MPa; mobile phase, CO_2–ethanol–phosphoric acid (90:9.98:0.02, v/v/v); stationary phase, phenyl column; flow rate, 1.05 mL/min.

Table III. Retention Time (t_R/min) of Solutes on Different Columns*

Column	Isorhamnetin	Kampcetin	Quercetin	Fisetin
C_{18}	6.32	5.31	5.33	5.35
Phenyl	6.68	8.90	9.98	13.30
Cyanopropyl	10.12	14.30	18.29	27.35

* Conditions: temperature, 50.0°C; pressure, 25.0 MPa; mobile phase, CO_2–ethanol–phosphoric acid (90:9.98:0.02, v/v/v); flow rate, 1.05 mL/min.

Table IV. Retention Time (t_R/min) of Solutes at Different Temperatures*

Temperature (°C)	Isorhamnetin	Kampcetin	Quercetin	Fisetin
40.0	6.48	8.40	9.48	12.80
45.0	6.55	8.72	9.76	13.05
50.0	6.68	8.90	9.98	13.30
55.0	6.76	8.98	10.08	13.41
60.0	6.82	9.06	10.12	13.46
65.0	6.86	9.12	10.19	13.52

* Conditions: pressure, 25.0 MPa; mobile phase, CO_2–ethanol–phosphoric acid (90:9.98:0.02, v/v); stationary phase, phenyl column; flow rate, 1.05 mL/min.

Effect of temperature

The retention time of solutes slightly increased with increasing temperature in the experimental temperature range 40.0–65.0°C. There are two effects of temperature on the retention, one of which is that the volatility of the solute increases with temperature, which is favorable to shortening the retention time of the solute. The other effect is that the density of the mobile phase decreases with temperature, which is not favorable to eluting the solute. The overall effects determine the retention behavior of solute. In the range of experimental temperatures in this paper, the retention time increased with temperature as shown in Table IV, showing that the second factor is dominant.

Figure 4 gives the linear plots of log k' versus

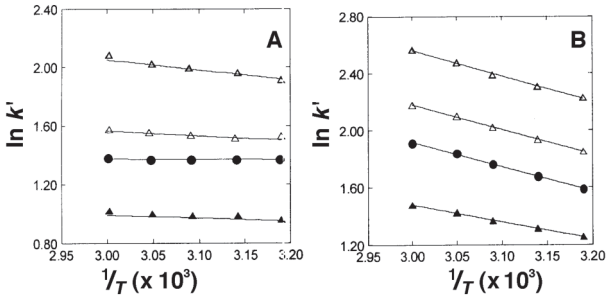


Figure 4. The plot of ln *k'* versus 1/*T* for the phenyl column (A) and cyanopropyl column (B). Conditions: temperature, 50°C; pressure, 25.0 MPa; mobile phase, CO_2–ethanol–phosphoric acid (90:9.98:0.02, v/v/v); flow rate, 1.05 mL/min. Flavonoids: **s**, fisetin; **s**, quercetin; **1**, kampcetin; **s**, isorhamnetin.

the reciprocal of absolute temperature, which is consistent with the literature (18). Comparing these two plots, one can also find that the slope of the plot on the cyanopropyl column is greater than that on the phenyl column, indicating that the temperature effect differs on different columns.

Conclusion

The solutes were rapidly and efficiently separated using a packed column and a ternary mobile phase. The additive, phosphoric acid, tends to improve peak shape and has little effect on retention time. Increases in modifier concentration and pressure are favorable to decreasing the retention time of solutes. The effect of temperature was minimal in the experimental temperature range.

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