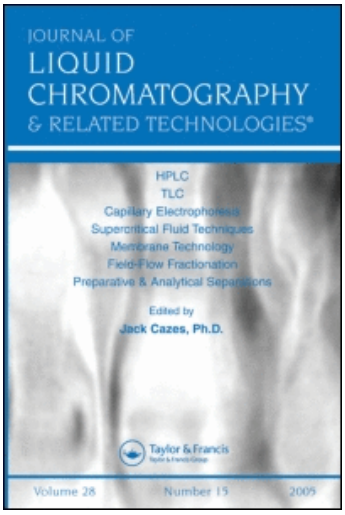

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SEPARATION OF STEROIDS BY COUNTERCURRENT CHROMATOGRAPHY USING SUPERCRITICAL FLUID CARBON DIOXIDE

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ABSTRACT

Since the introduction of the modern apparatus and technology of countercurrent chromatography (CCC), researchers have found numerous applications in the analyses and preparations of natural products. Selection of appropriate solvent systems is a basic and important task for separation in CCC. Recently, we provided an alternative choice of the solvent system by replacing the liquid mobile phase with supercritical-fluid carbon dioxide ($SFCO_2$). In this work, three steroids - progesterone, Δ^4-androstene-3,17-dione, and (+)-4-cholesten-3-one were separated as test compounds using $SFCO_2$ as the mobile phase and methanol-water (30/70 v/v) solution as the stationary phase.

The polarity of the cholestenone steroid was low enough so that it could be used as a dead volume tracer. The $SFCO_2$ -(methanol-water 70/30 v/v) partition coefficients were accurately determined at different temperatures and pressures. At a constant pressure of 10 MPa (100 kg/cm^2, 1400 psi), Van't Hoff plots allowed to estimate the transfer enthalpy and entropy of the steroid compounds.

It is shown that the partition coefficients of the compounds are related to the $SFCO_2$ density. The significant effects of temperature and/or pressure changes on the partition of the solutes are seen on the chromatograms. The versatility of this new mobile phase is demonstrated.

INTRODUCTION

Countercurrent chromatography (CCC) is a chromatography technique in which the stationary phase is a liquid. This liquid stationary phase is usually retained in a dedicated column by centrifugal forces. Since the stationary-phase volume can be very large, it has become an important preparative technique in many areas. In addition, sample loss due to permanent adsorption would not occur because no solid packing material exists like most other liquid chromatography techniques. This unique characteristic makes it a flexible separation method for many natural products.[1-3]

Conventionally, CCC solvent systems are composed of two immiscible liquids saturated with each other; one serves as the mobile phase while the other one the stationary phase. Solutes are separated due to the partition between these two phases. Analyte capacity factors can be changed by modifying the solvent compositions. Recently, we substituted the mobile phase with supercritical-fluid carbon dioxide ($SFCO_2$).[4-5] Due to its relatively high diffusivity, low viscosity, and liquid-like solvent strength properties, $SFCO_2$ has been widely applied in supercritical fluid chromatography (SFC).[6-8] In addition, its environmental-benign nature makes it almost always the first choice as the mobile phase while applicable in SFC.

The use of $SFCO_2$ as the mobile phase in CCC has another advantage, i.e., the mobile phase turns into gas and separates from the analytes while the sample components elute out of the column. Accordingly, concentration of analytes is not required after elution. A mixture of three steroid compounds was separated in this work to demonstrate the versatility of changing solvent strength by manipulating the system pressure or temperature. It is also shown that physico-chemical parameters can easily be obtained through solute retention measurements.

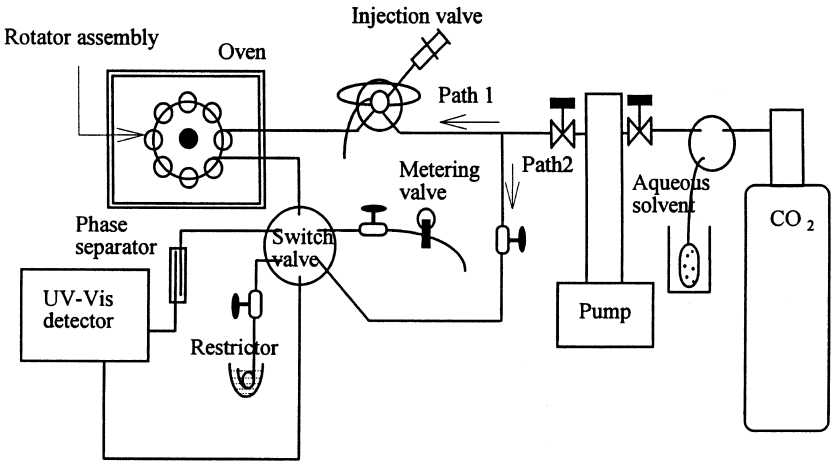

Figure 1. Schematic diagram of the experimental set-up.

EXPERIMENTAL

The instrumental layout is shown in Fig. 1. Refer to our previous works[4, 5] for more details. All valves used were high pressure valves and connected by 1.59 mm O.D. × 0.25 mm I.D. PEEK tubing which endures pressures up to 41 MPa (400 kg/cm^2, 6000 psi). An ISCO (Lincoln, NE) Model 260 syringe pump was used to deliver fluids while an ISCO V^4 detector was used to monitor analytes. To maintain a back pressure in the column a 30 cm × 50 μm I.D. fused-silica tubing was attached to the outlet of the system. The laboratory-made slow-speed rotor assembly[9] was wound solar-coaxially from a 10.9 m long 6.35 mm O.D. × 5.33 mm I.D. stainless steel tube which gave a total volume of 240 mL. The temperature of the rotor was controlled by an oven within 1.0°C. A 0.5 mL phase separator was introduced before the detector in order to remove solvent droplets to reduce monitoring noise.

The oven was set at the experimental temperature. The stationary phase was pumped into the column via Path 1 until liquid flow was observed at the metering valve outlet. Note that the stationary phase flowed directly from the column to the metering valve without going into the detector at this stage. Liquid CO_2 was then drawn to the pump, compressed to the desired pressure and delivered to the column through the same path while the rotor was revolving at 70 rpm. Phase-retention ratios could be adjusted by the $SFCO_2$ flow rate during the displacement of the stationary phase. The $SFCO_2$ flow rate was controlled through the metering valve. Once the desirable phase-retention volume was brought about, the metering valve was then closed. Subsequently, a $SFCO_2$ flow was delivered to the detector via

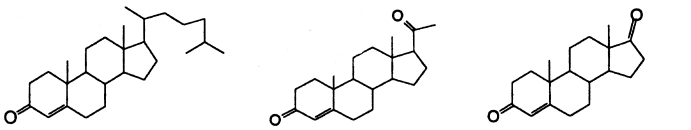

Figure 2. The steroid solutes. **Ch** is the least polar, **An** is the most polar.

Path 2 and finally to the fused-silica restrictor that made the whole system under same pressure. The valve on Path 2 was closed and the switch valve was operated in a way to let the flow move via Path 1 to the column, the phase separator, the detector, and finally to the restrictor. The purpose of the above operation simply avoided pressure disturbance during the switching of the flow. Once the solvent system reached hydrodynamic equilibrium, samples (all of 100 μL) were ready to be injected.

Methanol used in this work was HPLC grade. SFC-grade CO_2 was purchased from Scott Specialty Products (Plumsteadville, PA). Progesterone (Pr) (>98%), (+)-4-cholesten-3-one (Ch) (>95%) and Δ^4-Androstene-3,17-dione (An) (>99%) were obtained from Tokyo Chemical Industry (Chuo-Ku, Tokyo, Japan). Their chemical structures are shown by Figure 2. The sample solutions were prepared in methanol.

RESULTS AND DISCUSSION

As generally known, the $SFCO_2$ solvent strength can be manipulated by changing pressure and temperature. There is very little work done on biphasic supercritical-liquid systems. The CO_2 solubility in water-methanol phase is dependent on pressure and temperature. Similarly, the methanol and water solubilities in $SFCO_2$ are also altered by temperature and pressure modifications. The partition of a solute between these two phases of different nature may have a great fundamental interest.

This work was done to show the capabilities of the CCC technique for the separation of solutes from medium to low polarity and for fundamental physico-chemical studies in such supercritical-liquid biphasic systems. The liquid phase composition (amount of methanol in the aqueous phase) plays one of the major roles in separation. However, only the temperature and pressure effects on the steroid partition and separation were studied here. Accordingly, the same composition: methanol-water (30/70% v/v) solution was used as the stationary phase.

Table 1

The Experimental Data

Supercritical CO_2			Solute Retention Volumes				Partition Coefficients				Figure 4 and 5	
Temp. °C	Pressure kg/cm^2	Density g/cm^3	1/T K^{-1}x1000	V_R Ch mL	V_R Pr mL	V_R An mL	P Pr	P An	V_S mL	Sf %	Log P Pr	Log P An
35	100	0.70	3.25	131	146	201	0.14	0.64	109	45.4	-0.861	-0.192
40	100	0.62	3.19	125	162	296	0.32	1.49	115	47.9	-0.492	0.172
50	100	0.37	3.10	98	464	(1100)[a]	2.58	(7.06)[a]	142	59.2	0.411	0.849
40	100	0.62	3.19	116	154	285	0.31	1.36	124	51.7	-0.514	0.134
50	118	0.48	3.10	115	196	408	0.65	2.34	125	52.1	-0.188	0.370

[a] Extrapolated value. $SFCO_2$ density values obtained from the Hewlett Packard P, T, density abacus.

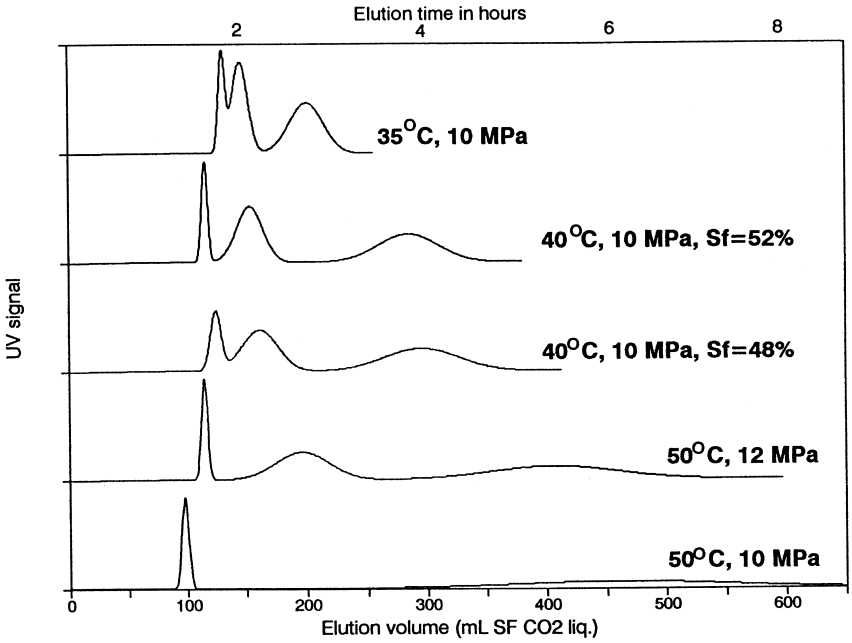

Figure 3. Separation of **Pr**, **An** and **Ch** at different temperatures, pressure and phase retention ratio. Experimental conditions: stationary phase, methanol-water (30/70 v/v); mobile phase, supercritical CO_2 1.2 mL/min measured in the liquid CO_2 state and moving in the tail to head direction, 70 rpm, analyte concentration : 4000, 4000, 2000 ppm for **Pr**, **An** and **Ch**, respectively. In all chromatograms, **Pr** is the first eluting compound, **An** is intermediate and **Ch** is the final eluting compound.

With 10 MPa (100 kg/cm^2, 1400 psi) and ~50% stationary phase retention ratio, chromatograms were obtained at three different temperatures, i.e., 35, 40, and 50°C. The results are listed in Table 1 and the chromatograms shown in Fig. 3. A constant flow-rate of 1.2 mL/min was measured at the pump head.

Partition Coefficient Determination

A great advantage of CCC is its very simple retention equation:

$$V_R = V_M + PV_S \quad (1)$$

in which the subscripts R, M and S refer to the retention volume, the mobile phase volume (the $SFCO_2$ volume or dead volume), and the stationary phase volume (the water-methanol phase volume), respectively. Since the CCC column contains only

the stationary phase and the mobile phase, its volume, V_T, is equal to the sum $V_M + V_S$. If the V_M volume is known, partition coefficients can be measured using the solute retention volume:

$$P = (V_R - V_M)/V_S \quad (2)$$

The problem is to know accurately the dead volume, V_M. In our case, we selected a very apolar solute, (+)-4-cholesten-3-one (**Ch** in Fig. 1), with an octyl side chain. **Ch** is likely sparingly soluble in the polar stationary phase consequently it should not be retained and could act as a dead volume marker.

The exact knowledge of the V_M value is critical to obtain relevant values for the partition coefficient. Also, the V_M value can be used to obtain the experimental stationary phase retention parameter, Sf:

$$Sf = V_S/V_T = 1 - V_M/V_T \quad (3)$$

V_T is the CCC apparatus volume (240 mL).

An experimental phase-retention volume was obtained directly by flushing the stationary phase out of the column at the end of each chromatographic run and collecting it in a graduated cylinder. This experimental value is only indicative since the strong wetting of the water-methanol liquid phase and possible evaporation reduced the collected liquid phase volume. It was always very inferior, by more than 20%, than the Sf value obtained using the **Ch** dead volume tracer. For the partition coefficient calculations, the later Sf values were always used (Eq. 3).

Steroid Separation

Figure 3 shows the CCC chromatograms whose chromatographic parameters are listed in Table 1. At 35°C compounds **Ch** and **Pr** were not well resolved. The $SFCO_2$ solvent strength at this temperature apparently was too large for **Ch** and **Pr** to separate. At 50°C compounds **Ch** and **Pr** were well separated. However, the $SFCO_2$ solvent strength at this temperature was not large enough to elute compound **An** at all. To take the advantage of the unique property of supercritical fluid, the $SFCO_2$ solvent strength could be fine-tuned by varying the temperature. At 40°C, the three compounds were nearly separated to baseline-resolution.

In general, both the vapor pressure of the solute and the density of the mobile phase are temperature dependent. When the temperature rises, the solute vapor pressure increases and solute distribution favors the mobile phase. However, higher temperatures can also increase the solute solubility in the polar stationary

phase. Furthermore, a temperature increase at constant pressure lowers the mobile phase density which results in a lower solvent strength[10]. Most often the density effect dominates the retention of the compounds.

Two separations were done with the same supercritical fluid conditions (40°C and 10 MPa). The phase retention ratio was adjusted by the $SFCO_2$ flow rate during the initial equilibrium of the CCC machine as described in the experimental section. Evidently the resolution was superior with the higher stationary phase ratio, just like the conventional liquid/liquid CCC system. The Sf values listed in Table 1 shows the trend first described by Berthod:[11] the stationary phase retention ratio increases when the density difference between the mobile and the stationary phase increases, i.e., the mobile phase density decreases. The highest temperature used, 50°C, produced the lowest $SFCO_2$ density, 0.37 g/cm^3, and also the highest stationary phase retention, Sf=59.2%, when the CCC machine was filled in the same way as it was with the other temperatures.

In addition to temperature effect, the effect of pressure changes was also briefly examined. At 50°C the sample mixture was eluted at 12 MPa (118 kg/cm^2, 1700 psi). The chromatogram clearly demonstrates that the higher pressure granted greater solvent strength for compounds **Pr** and **An**. Therefore, compound **Pr** was eluted out much earlier than when the pressure was only 10 MPa (100 kg/cm^2, 1430 psi). Compound **An** was able to be eluted out in approximately six hours when, after eight hours **Pr** was not completely eluted at 10 MPa (Figure 3).

Physico-chemical Studies

It is interesting to consider the supercritical fluid as a classical fluid provided the pressure is maintained constant. Figure 4 shows the Van't Hoff plots (log partition coefficient *versus* 1/T in Kelvin) obtained with the Table 1 partition coefficients for the four experiments done at 10 MPa. The classical equation:

$$RT \log P = \Delta H_o + T \Delta S_o \qquad (4)$$

shows that the slope of the straight line gives the enthalpy of the supercritical fluid to liquid solute transfer and the intercept is related to the entropy change for the same transfer. Table 2 lists the regression parameters obtained for the Van't Hoff plots shown in Figure 4. The ΔH and ΔS values obtained have an order of magnitude corresponding to the one of liquid-gas similar values obtained by gas chromatography with a variety of solutes.[12]

The molecular differences between **Pr** and **An** are a methyl group and a methylene group (Figure 2). These disparities produce a 1.5 Kcal/mol energy difference between the enthalpy phase transfer of the **Pr** and **An** molecules. **Ch** has

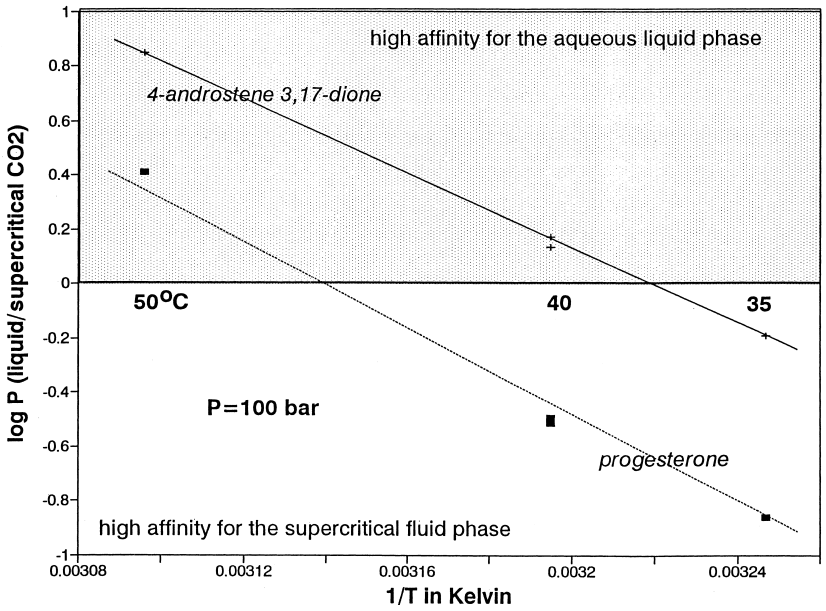

Figure 4. Van't Hoff plots for androstene-dione and progesterone. The regression parameters of the straight lines are listed in Table 2.

Table 2

Thermodynamic Parameters Obtained from the Figure 4 Data Plots

Compound	Slope	Intercept	r^2 Param.	ΔH_o Kcal/mol	ΔS_o cal*K/mol
Van't Hoff Plots					
Progesterone	-8600	27	0.993	-7.5	120
Androstenedione	-6930	22	0.999	-6.0	100
Density Plots					
Progesterone	-3.57	1.66	0.968		
Androstenedione	-2.84	1.85	0.950		

ΔH_o is the enthalpy value for the transfer of the compound from the liquid polar phase to the supercritical apolar phase, ΔS_o is the entropy value of the same transfer.

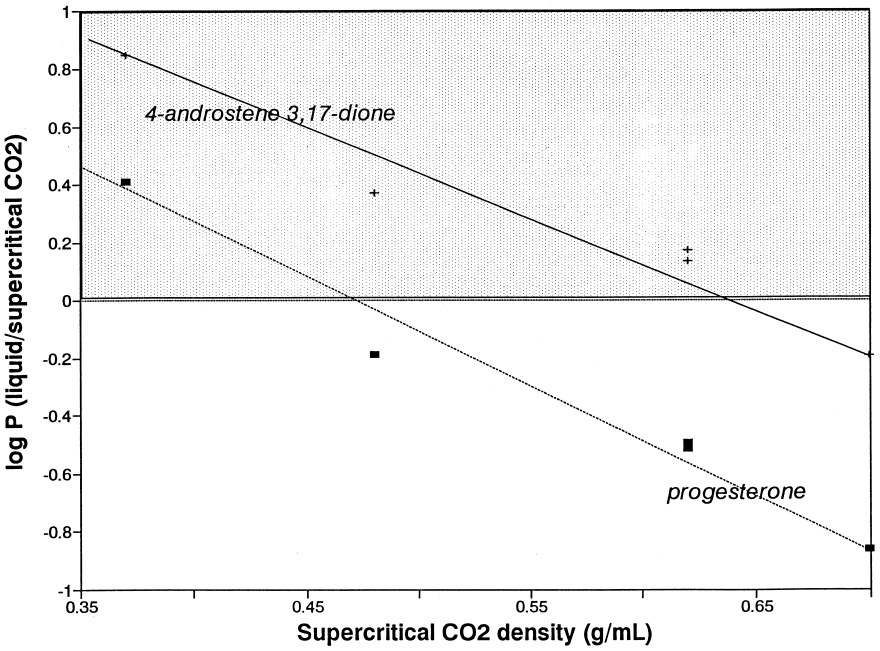

Figure 5. An increase of the supercritical fluid density decreases the affinity of the steroids for the polar aqueous liquid stationary phase. The regression parameters of the straight lines are listed in Table 2.

a dipolar carbonyl group missing and three methyl groups and four methylene groups more than **An** (Figure 2). This disparity may produce a five to six Kcal/mol energy difference. The **Ch** partition coefficients in the systems studied should be very low. This confirms that **Ch** is a valid dead volume tracer up to 50°C.

The plot obtained with the three retention values of the androstene-dione solute allowed to estimate its partition coefficient at 50°C (value in parenthesis in Table 1). The estimated retention volume (1100 mL) would correspond to more than 15 hours retention time. The peak would be so broad that it could note be detected.

It should be pointed out that it is not necessary to elute this last compound. Once the two steroids, **Ch** and **Pr**, eluted out of the CCC column, the $SFCO_2$ flow is stopped. The CCC column rotation is also stopped and the column content is collected. **An** is recovered in the polar liquid phase separated from the two other steroid.

Steroid Separation, Supercritical Fluid Density, and Peak Efficiency

Figure 5 shows the plot of the log P values versus the supercritical fluid density regardless of temperature and/or pressure. The regression parameters of these plots are listed in Table 2. The regression coefficients, 0.95 and 0.968, show that the fluid density is the main parameter responsible for the steroid solute solubility. When the fluid density increases, *i.e.*, the temperature decreases and/or the pressure increases, the P values decrease indicating a higher affinity for the supercritical phase and a lower affinity for the polar methanol-water phase.

Considering the quasi-linear relationship shown by Figure 5 and the importance of a high stationary phase retention to enhance the resolution parameter, it should be concluded that the best experimental conditions to separate the three studied steroids are the 40°C intermediate temperature with an as high as possible phase retention, Sf. The moderate temperature will produce moderate partition coefficients then acceptable retention times (equation 1 and Figure 3). A high Sf parameter maximizes the resolution. Sf could be enhanced by changing the CCC column design.

The resolution changes are mainly due to the solute partition coefficient changes described above. It is also partly due to peak efficiency changes. It is observed on the chromatograms that the chromatographic efficiency, *i.e.*, the peak sharpness, depends on the solute residence time in the CCC column. The peak efficiency decreases as the solute retention time increases (Figure 3). Peak efficiency is linked to the kinetics of the solute exchange between phases. Solute retention is linked to partition coefficient (equation 1). For the steroid solutes, it seems that a higher affinity for the polar liquid phase is linked to a slower $SFCO_2$-liquid phase exchange. This point needs further investigation to draw definite conclusion. In any cases CCC is a powerful tool to obtain valuable physico-chemical information on the solute exchange between a polar methanol-water liquid phase and an apolar supercritical phase. Its separation capability is also demonstrated in the case of this simple steroid mixture.

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