

## Effect of mobile phase composition on retention factor in supercritical fluid chromatography

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### Abstract

In supercritical fluid chromatography with a mixture of carbon dioxide and a modifier as the mobile phase, the relationship between retention factor  $k$  and modifier mole fraction  $x$  was derived as  $1/k_{\text{mix}} = x_{\text{CO}_2}/k_{\text{CO}_2}^0 + x_{\text{mod}}/k_{\text{mod}}^0$ , where  $k_{\text{mix}}$ ,  $k_{\text{CO}_2}^0$ , and  $k_{\text{mod}}^0$  are the retention factors for the mixture solvent, pure CO<sub>2</sub> and pure modifier, respectively, and  $x_{\text{CO}_2}$  and  $x_{\text{mod}}$  are the mole fractions of CO<sub>2</sub> and a modifier, respectively. Since  $k_{\text{CO}_2}^0$  and  $k_{\text{mod}}^0$  are often difficult to determine due to too large of a value for  $k_{\text{CO}_2}^0$  and an invalid value of  $k_{\text{mod}}^0$  for the liquid phase, both values were estimated experimentally from the two retention factors available at the lowest and highest modifier mole fractions at each temperature and pressure. The equation was effective for the retention factors of the R- and S-forms of racemic trans-stilbene oxide measured in the present study by supercritical fluid chromatography using a modifier such as methanol, ethanol or acetonitrile. Moreover, the equation was also valid for the retention data of various enantioselective separations as well as achiral separations reported in the literature.

Key words: supercritical fluid chromatography, correlation, retention factor, modifier, enantioselective separation

### Introduction

Since enantiomers often exhibit significant differences in biological activities, accurate and efficient separation/fractionation of racemic compounds is increasingly demanded in analytical chemistry as well as in various industries such as bio, pharmaceutical, nutraceutical, cosmetic, fragrance, and food. Compared to other separation/fractionation methods, supercritical fluid chromatography (SFC) is attractive for a number of reasons [1-3]. For example, supercritical carbon dioxide, mainly employed as a mobile phase, has unique physical properties: it has low viscosity and readily solubilizes various compounds, and its properties can be tuned by changing the pressure. The pressure drop across analytical packed columns can be suppressed even when the flow rate of the solvent is increased, especially when using fine particles packed in separation columns, due to the lower viscosity of supercritical carbon dioxide compared to other liquids. Moreover, by adding particular organic solvents, i.e., a so-called modifier, co-solvent or entrainer such as a polar solvent like methanol, ethanol, 2-propanol, or acetonitrile, the polarity of the mobile phase can be changed extensively.

Various commercial columns for analyzing/separating enantiomers are available that use stationary

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phases based on sugar derivatives. There has therefore been a large increase in both fundamental and application studies on the analysis and separation/fractionation of enantiomers by SFC using both packed [4-24] and open capillary [25-29] columns. In analysis/separation with SFC, a modifier is often added to the mobile phase of sub- and supercritical carbon dioxide. Consequently, exploring the optimum operation conditions of SFC is more difficult because the retention factors are sensitive to mobile phase density, as well as the temperature, flow rate, and modifier composition.

Since density affects peak retention time significantly, the effects of solvent properties on peak retention times at certain temperatures and pressures should be determined to optimize the analytical/separation conditions. Indeed, various approaches to estimate retention factors for chiral and achiral compounds have been investigated with either packed or open capillary columns: (1) by expressing  $k$  values by a polynomial function of temperature, pressure/density, or properties related to chemical structure [2,9,10,21,24,27,29-33], determining the constants involved statistically [6], and with an artificial neural network [34], (2) by a function of thermodynamic properties such as heat of vaporization and entropy [4,5,10,13,15,16,18,21,22,35], e.g., the van't Hoff equation, (3) with fugacity coefficients and an equation of state under equilibrium conditions between a mobile phase and a stationary phase [36], and (4) by a mathematical model describing solute concentration in the packed [19,23] and open columns [33,37]. However, the relationship between retention factor and mobile phase composition is not well understood, and thus it is typically estimated experimentally. The relationship between  $k$  values and solvent composition must be known to optimize the separation/analytical conditions. In particular, the retention factor is sensitive to the modifier composition in the lean concentration region.

In the present study, the objectives were to study the effects of modifier species and its composition on the enantioselective separation of racemic trans-stilbene oxide in supercritical fluid chromatography, and to demonstrate the effectiveness of the equation describing  $k$  with the derived solvent composition.

## Theoretical background

The relationship between the retention factor  $k$  and the retention time  $t_R$  for the solute in chromatography is described in Eq. (1) [37].

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

where  $t_0$  is the retention time of a marker having no adsorption onto the adsorbent particles. The  $k$  value is also defined in Eq. (2) [37] as the ratio of the amounts in number of moles for the solute in the stationary phase to those in the mobile phase, respectively.

$$k = \frac{\xi n_s}{\zeta n_m} \quad (2)$$

where  $\xi$  and  $\zeta$  are the mole-fractions of the solute in the stationary phase and in the mobile phase, and  $n_s$  and  $n_m$  are the total number of moles in the two phases, respectively. When the mobile phase is pure CO<sub>2</sub> (retention factor  $k_{CO_2}^0$ ), the amounts of the solute in the mobile phase ( $\zeta n_m$ )<sub>CO<sub>2</sub></sub> is:

$$(\zeta n_m)_{CO_2} = \frac{\xi n_s}{k_{CO_2}^0} \quad (3)$$

Similarly, the amounts of the solute in a pure modifier and in the mixture mobile phase are expressed by Eqs (4) and (5), respectively.

$$(\zeta n_m)_{mod} = \frac{\xi n_s}{k_{mod}^0} \quad (4)$$

$$(\zeta n_m)_{\text{mix}} = \frac{\zeta n_s}{k_{\text{mix}}} \quad (5)$$

where the subscripts of mod and mix are modifier and mixture, respectively. When the mole fractions of CO<sub>2</sub> and a modifier are  $x_{\text{CO}_2}$  and  $x_{\text{mod}}$ , respectively, the amount of the solute in the mixture  $(\zeta n_m)_{\text{mix}}$  can be expressed by

$$(\zeta n_m)_{\text{mix}} = x_{\text{CO}_2}(\zeta n_m)_{\text{CO}_2} + x_{\text{mod}}(\zeta n_m)_{\text{mod}} \quad (6)$$

From Eqs (4) to (6)

$$(\zeta n_m)_{\text{mix}} = \frac{(\zeta n_s)_{\text{mix}}}{k_{\text{mix}}} = x_{\text{CO}_2} \frac{(\zeta n_s)_{\text{CO}_2}}{k_{\text{CO}_2}^0} + x_{\text{mod}} \frac{(\zeta n_s)_{\text{mod}}}{k_{\text{mod}}^0} \quad (7)$$

In addition, the amounts of the solute in pure CO<sub>2</sub>, pure modifier, and the mixture can be assumed to be the same at the same temperature and pressure; namely those are dependent on the stationary phase species not the mobile phase species.

$$(\zeta n_s)_{\text{mix}} = (\zeta n_s)_{\text{CO}_2} = (\zeta n_s)_{\text{mod}} \quad (8)$$

Thus,

$$\frac{1}{k_{\text{mix}}} = \frac{x_{\text{CO}_2}}{k_{\text{CO}_2}^0} + \frac{x_{\text{mod}}}{k_{\text{mod}}^0} \quad (9)$$

$k_{\text{CO}_2}^0$  and  $k_{\text{mod}}^0$  are often difficult to determine due to too large of a value for  $k_{\text{CO}_2}^0$  and an invalid value of  $k_{\text{mod}}^0$  for the liquid phase. For instance, when a mixture of CO<sub>2</sub> and the modifier is not a single phase at higher modifier compositions, and/or the retention factor  $k_{\text{CO}_2}^0$  is too large, namely, not eluted without a modifier,  $k_{\text{CO}_2}^0$  and  $k_{\text{mod}}^0$  can be estimated using two  $k_{\text{mix}}$  values at different  $x_{\text{CO}_2}$ 's. In this study, those at the lowest and highest  $x_{\text{CO}_2}$  were used. Moreover, since a volume fraction  $\phi$  of a modifier is often used, Eq. (9) can be alternatively rewritten as Eq. (10).

$$\frac{1}{k_{\text{mix}}} = \frac{\phi_{\text{CO}_2}}{k_{\text{CO}_2}^0} + \frac{\phi_{\text{mod}}}{k_{\text{mod}}^0} \quad (10)$$

## Experimental

Figure 1 shows a schematic diagram of the experimental apparatus, which consists of two syringe pumps (Models 260D and 100DM, Teledyne ISCO, Lincoln, U.S.A.), which were used for supplying CO<sub>2</sub> and the modifier, respectively, a preheater tube (1/16 inch O.D. × 2 m long), a packed adsorption column

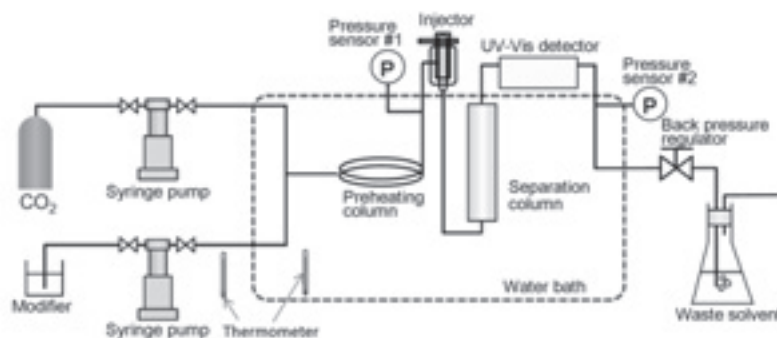


Figure 1. A schematic diagram of the experimental apparatus.

(Chiralpak AD-H,  $4.6 \times 250$  mm,  $5 \mu\text{m}$ , 3, 5-dimethylphenylcarbamate derivative of amylose, Daicel, Tokyo, Japan), a UV-VIS multi photodiode-array detector (MD-1510 or MD-2018, JASCO, Tokyo, Japan), and a back pressure regulator (Model 880-81, JASCO). The adsorption column was installed vertically in the water bath, which was maintained within a  $\pm 0.2$  K temperature range.

Liquid  $\text{CO}_2$  and a modifier (methanol, ethanol, or acetonitrile) were used to fill the cylinder of each syringe pump, and were held until the temperatures of the cylinders reached room temperature.  $\text{CO}_2$  and the modifier were separately fed at constant flow rates from each syringe pump, and the two fluids were mixed upstream at the inlet to the preheating tube. When the flow rates, composition of mobile phase, temperature and pressure of the column were stabilized,  $5 \mu\text{L}$  of a methanol solution of racemic trans-stilbene oxide was injected via an injector (Model: 7520, Rheodyne, California, U.S.A.). The time-concentration profile of the solute was monitored by the detector by scanning from 195 to 600 nm in increments of 1 nm at an interval of 0.2 to 1.6 s. The measurements were made at temperatures from 308.2 to 313.2 K, pressures of 10, 20, and 30 MPa, and mole fractions of modifier of 0 to 0.35. 1,3,5-tri-tert-Butylbenzene was used as an inert marker to determine the residence times of the mobile phase. The measurement pressures at the inlet and outlet column were recorded by pressure sensors #1 and #2, respectively. The pressure drop between the inlet and outlet of the separation column was less than 1 MPa.

The retention factors are known to be sensitive to modifier composition of the mobile phase in the lean modifier region. Thus, the modifier compositions should be carefully adjusted by setting the flow rates of the pumps. The modifier compositions were estimated from the flow rates of carbon dioxide and the modifier, and assumed that the inside temperatures of the syringe pump cylinders were equal to room temperature. The flow rates were confirmed by directly measuring the flow rate of carbon dioxide using a soap bubble film meter, and the flow rate of the modifier was determined by weighing the collected liquid mass obtained from the exit of the back pressure regulator. The flow rate settings in the two pumps were consistent within  $\pm 2\%$  with the values directly measured.

## Results and discussion

Figure 2 shows retention factors  $k_1$  and  $k_2$  ( $k_1 < k_2$ ), corresponding to  $k_1$  and  $k_2$  for the R- and S-forms, respectively, vs. various compositions of modifiers such as methanol, ethanol and acetonitrile at 313.2 K and 10 MPa. Solid, broken and dotted-solid lines were obtained from Eq. (9). All the data were averaged from four or more measurements under each condition of  $k_1$  and  $k_2$  measured in the present study. Note that trans-stilbene oxide was not eluted for 250 min without methanol at a  $\text{CO}_2$  flow rate of 0.8 mL/min. All the values of the retention factors ( $k_1$  and  $k_2$ ) decreased with increasing modifier mole fractions  $x_{\text{mod}}$  for the three modifiers. The significant changes in  $k_1$  and  $k_2$  values were seen up to  $x_{\text{mod}} = 0.1$ , and the changes gradually decreased above  $x_{\text{mod}} = 0.1$ . The tendency is commonly observed for many solutes in the literature [4,5,10,12,13,15-17,19,21]. Although the differences in  $k_1$  values are not evident, the  $k_2$  values at  $x_{\text{mod}} = 0.05$  decreased in the order: ethanol > methanol > acetonitrile, corresponding to the inverse order of the dielectric constant values, which show the solvent polarities. Note that the dielectric constants at room temperature are 24.5, 32.7 and 37.5 for ethanol, methanol and acetonitrile, respectively [38].

Table 1 presents the accuracy of Eq. (9), in terms of average absolute relative deviation (AARD) defined as Eq. (11), for  $k_1$  and  $k_2$  measured in the present study. The measured  $k_1$  and  $k_2$  decreased with increasing  $x_{\text{mod}}$  for the three modifiers, consistent with the literature, as mentioned above, and Eq. (9) represented the  $k_1$  and  $k_2$  values well.

$$\text{AARD}(\%) = 100 \times \frac{1}{N} \sum \left| 1 - \frac{k_{\text{mix}}^{\text{prd}}}{k_{\text{mix}}^{\text{exp}}} \right| \quad (11)$$

Herein,  $N$  is the number of data points for examining accuracy, equal to  $N_{\text{total}} - 2$ ;  $N_{\text{total}}$  is the number of data points reported, and the superscripts, prd and exp, are prediction and experiment, respectively. The accuracies in Eq. (9) were quite good for most of the conditions, except for acetonitrile. The reason for this

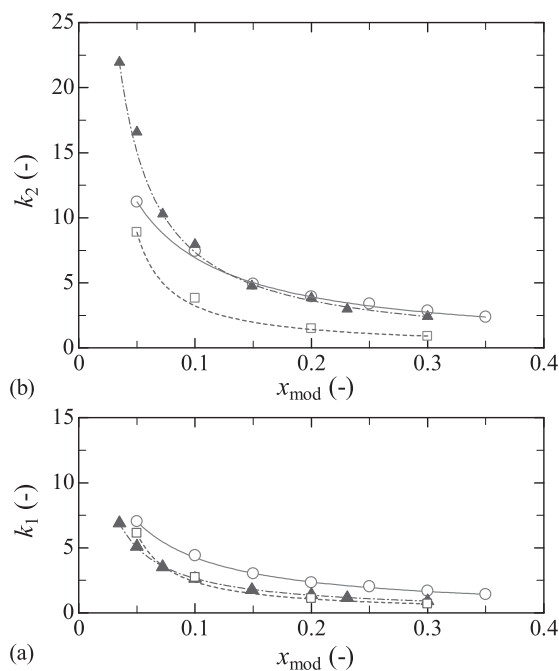


Figure 2. Retention factors (a)  $k_2$  and (b)  $k_1$  for trans-stilbene oxide at 313.2 K and 10 MPa in a mixture of  $\text{CO}_2$  and a modifier, ( $\circ$ ) methanol, ( $\blacktriangle$ ) ethanol and ( $\square$ ) acetonitrile. The solid, broken and solid-dotted lines were estimated by Eq. (9).

Table 1. Accuracy of Eq. (9) for the  $k_1$  and  $k_2$  data ( $t_{R,1}$  for  $k_1 < t_{R,2}$  for  $k_2$ ) measured in the present study.

$T(\text{K})$	$P(\text{MPa})$	Co-solvent	$N^{\text{a}}$	$k_1$		$k_2$	
				Max ARD (%)	AARD (%)	Max ARD (%)	AARD (%)
313.2	10	Acetonitrile	2	12.9	8.27	15.8	10.7
	10	Ethanol	6	2.03	1.38	9.20	5.22
	10	Methanol	5	4.22	2.54	6.87	3.81
	20	Methanol	2	5.24	4.8	3.63	2.84
	30	Methanol	2	4.05	2.44	4.36	2.62
308.2	10	Methanol	5	3.95	2.06	4.82	2.45
	20	Methanol	2	8.32	6.12	7.00	4.49
	30	Methanol	2	5.54	4.46	3.55	3.17
303.2	10	Methanol	5	3.99	2.20	4.75	3.29
	20	Methanol	2	3.22	2.85	9.18	6.55
	30	Methanol	2	6.81	6.13	3.67	2.08

\*a: Number of data points for examining accuracy in Eq(9); namely,  $N - 2$   
 Max ARD: maximum absolute relative deviation  
 AARD: average absolute relative deviation

is not clear, but the assumption in Eq. (8) for the derivation of Eq. (9) could partially fail. Further studies on the phase diagram of the mixture of CO<sub>2</sub> and acetonitrile and excess molar volume of the mixture may be required.

Figure 3 shows the composition dependence of separation factors  $\alpha = k_2/k_1$  determined from  $k_1$  and  $k_2$  values measured experimentally, where the solid lines were obtained from  $k_1$  and  $k_2$  values estimated by Eq. (9). The separation factors were also well predicted by Eq. (9). The composition dependences of methanol and acetonitrile were not evident, while that of ethanol slightly decreased with ethanol mole fraction at low ethanol mole fractions, corresponding to the region showing the significant decreases in  $k_1$  and  $k_2$  values. Whereas the  $\alpha$  values with ethanol ranged from 2.6 to 3.3 and the peak separation is good, it could be time consuming. The  $\alpha$  values with methanol and acetonitrile were nearly equal to about 1.7 and 1.4, respectively, over all  $x_{\text{mod}}$  range studied. Thus, the separations with methanol and acetonitrile are

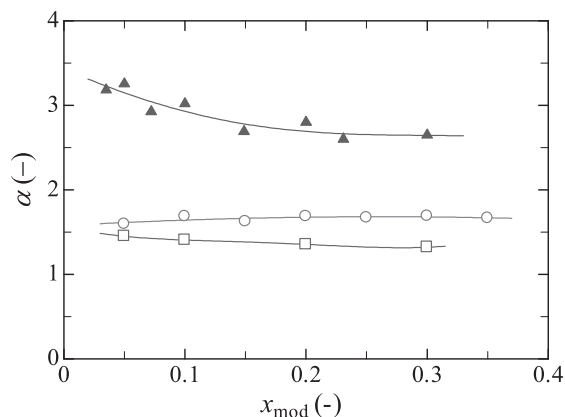


Figure 3. Separation factor  $\alpha$  vs. modifier mole fraction for trans-stilbene oxide at 313.2 K and 10 MPa in a mixture of CO<sub>2</sub> and a modifier, (○) methanol, (▲) ethanol, and (□) acetonitrile. The solid lines were obtained from  $k_1$  and  $k_2$  values estimated by Eq. (9).

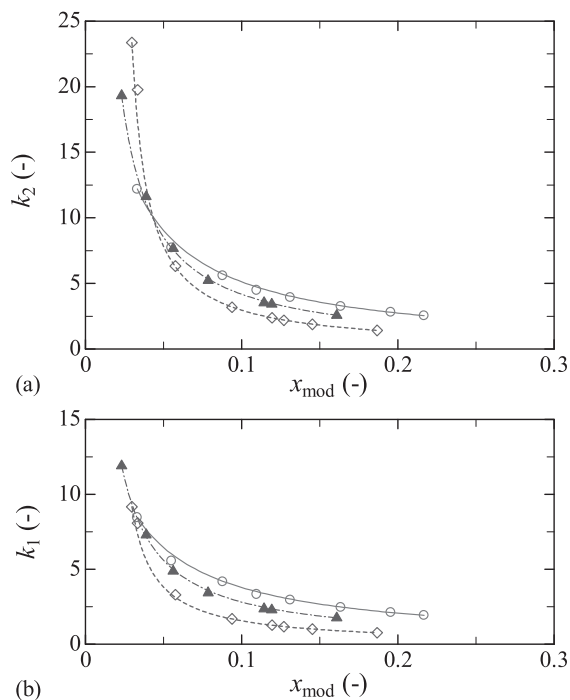


Figure 4. Retention factors (a)  $k_2$  and (b)  $k_1$  for paroxetine at 35 °C and 15 MPa in a mixture of CO<sub>2</sub> and a modifier, (○) methanol, (▲) ethanol and (◇) 2-propanol, obtained by Su et al. [16]. The solid, broken and dotted-solid lines were estimated by Eq. (9).

good and fast separation can be achieved. However, it is notable that the peak separation between two enantiomers cannot be drastically changed by changing modifier concentration for the three modifiers.

Figure 4 demonstrates the validity of Eq. (9) for paroxetine at 35 °C and 15 MPa in a mixture of CO<sub>2</sub> and a modifier such as methanol, ethanol, and 2-propanol reported by Su et al. [16], whose data with the three modifiers are available over a wide range of the modifier mole fraction. Although both  $k_1$  and  $k_2$  values sharply decreased with increasing  $x_{\text{mod}}$  in the lean modifier region, Eq. (9) represented the measured  $k_1$  and  $k_2$  data well. In the region showing significant decreases in  $k_1$  and  $k_2$  values, up to  $x_{\text{mod}} = 0.025$ , the values of  $k_1$  and  $k_2$  decreased in the order: 2-propanol > ethanol > methanol. As seen in Fig.2, the orders for  $k_1$  and  $k_2$  are consistent with the inverse order of the modifier polarity, represented by the dielectric constant values, where that of 2-propanol at room temperature is 17.9 [38].

Table 2 shows the percentages of the maximum absolute relative deviation (ARD) ranges for the retention factors reported in the literature [5,10,12,13,15-17,19,21]. A total of 122 systems/conditions were examined for accuracy using Eq. (9), and there were 80 systems with maximum ARD lower than 20% and 49 lower than 10%. The large deviations result partly from low reproducibilities for each dataset, and in particular the  $k$  values at low  $x_{\text{mod}}$  values, where the  $k$  values drastically decrease with increasing  $x_{\text{mod}}$ . Note that Eq. (9) was also valid for the retention data [10] for separation of tocopherols, which are achiral compounds.

Table 2. Accuracy of Eq.(9) for the retention data reported in the literature.

Range for Maximum	Percentage of the system/condition
< 5%	16.4
5 to 10 %	23.8
10 to 15 %	14.8
15 to 20 %	10.7
20 % <	34.3

Total: 122 systems/conditions  
 Data sources: Bernal, J. L. et al. [5],  
 Jiang, C. et al. [10], Toribio, L. et al. [12],  
 Yang, Y. et al. [13], Toribio, L. et al. [15],  
 Su, B. et al. [16], Bao, Z. et al. [17],  
 Kamarei, F. et al. [19], Li, M. et al. [21]

## Conclusions

The retention factors  $k_1$  and  $k_2$  for enantiomers were derived as a function of mobile phase compositions. The equation was valid for retention factors of the R- and S-forms in the enantioselective separation of racemic trans-stilbene oxide with methanol, ethanol and acetonitrile as a modifier measured. In addition, the equation was valid for data on various enantiomers as well as achiral compounds reported in the literature.

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