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Retention behavior of triglycerides in subcritical fluid chromatography with carbon dioxide mobile phase

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Abstract

The retention behavior of triglycerides (TGs) was studied in subcritical fluid chromatography (SubFC) at 150 atm and in the temperature range of 0 to 25°C using an octadecyl-silica (ODS) column and carbon dioxide as the mobile phase without modifier. Both the retention and selectivity drastically changed with temperature. The contributions of the carbon number and the number of double bonds to the retention were similar to those in reversed-phase liquid chromatography with ODS column, which means that the concept of equivalent carbon number (*ECN*) can be applied to SubFC. In addition, *ECN* was expressed as a function of temperature, so that the retention of TGs can be predicted at any temperature. Predicted retentions based on five standard TGs were in good agreement with experimental ones within 2.1% error. Hold-up time was also correlated to the column temperature, so that simulated chromatograms can be produced. This method can be used to optimize the separation of various plant oils. Moreover, separations at different temperatures allowed us to identify each TG in oils.

Keywords: Triglycerides

1. Introduction

The analysis of plant oils is important in the food industry and the dietetics. The analysis methods of plant oils have been reviewed [1–3]. The complete separation of plant oils is very difficult because they are complex mixtures of triglycerides (TGs) which have very similar chemical and physical properties. Gas chromatography (GC) is not suitable for analysis of highly unsaturated TGs because they are thermally labile and unvolatile compounds. Therefore, the analysis of TGs in natural fats and oils has usually been performed by high-performance liquid chromatography (HPLC) [4–22]. Supercritical fluid chromatography (SFC) has been recognized to be an alternative for the analysis of TGs. The analysis time is shorter than in HPLC due to the low viscosity and high diffusivity of the mobile phase. A flame ionization detector (FID) which is a universal detector can be used, when carbon dioxide is used as the mobile phase.

In HPLC, TGs are primarily separated in reversed-phase (RP) mode using octadecyl-silica (ODS) columns and sometimes in normal-phase (NP) mode using silver impregnated columns. In RP-HPLC with ODS columns, the retention depends on the hydrophobicity of TGs. Linear relationships have been observed between log k' and the carbon number (*CN*) for saturated TGs, and also between log k' and the number of double bonds for particular series of

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unsaturated TGs [4–11]. The presence of one double bond in TGs roughly corresponds to a reduction of two methylene units [4,19–22]. More exactly, the contribution of double bonds to the retention depends on the type of fatty acids composing TGs [5–7,12–15]. Thus, the retention of TGs has been related to the equivalent carbon number (*ECN*) [5,7,12–18], which is defined as $ECN=CN-f\cdot DB$ (f, double bond coefficient; *DB*, number of double bonds). *ECN* is a useful parameter for identification of TGs under a given condition. However, double bond coefficients vary depending on the experimental conditions as well as the type of fatty acids and the detailed study has not been reported.

In this study, effects of temperature on the retention of TGs were studied in subcritical fluid chromatography (SubFC) with ODS column. Based on the results, a method for predicting the retention of TGs as a function of temperature was established using *ECN*. The method was applied to the separation of various plant oils.

2. Experimental

2.1. Apparatus

The chromatographic system was based on a SUPER-200 SFE/SFC system (Jasco, Tokyo, Japan), which consisted of a 880-PU HPLC pump, a 880-81 back pressure regulator and a Uvidec 100-V spectrophotometer (Jasco). The UV detector was connected to a C-R4A integrator (Shimadzu, Kyoto, Japan). Samples were injected with a Rheodyne 7125 injector (Cotati, CA, USA) with a 20 μl loop. Injection volume was 5 μl for analytical work and 10 μl for preparative work. The oven was cooled by decompressing liquid carbon dioxide through a nozzle, and the temperature was controlled using an HPV-6A autoswitching valve (GL Science, Tokyo, Japan) and an E5L temperature controller (Omron, Tokyo, Japan). A Model 5890 series II gas chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a FID was used to analyze fatty acid methyl esters (FAMEs).

2.2. Conditions

The column used was an L-column ODS (4.6 mm I.D.×250 mm, 5 μm particle size) purchased from Chemicals Inspection and Testing Institute (Tokyo, Japan). The mobile phase was pure carbon dioxide (99.99% purity) at a flow-rate of 3 ml/min, which was obtained from Showa-Tansan (Yokkaichi, Japan). The column outlet pressure was kept at 150 atm and the detection wavelength was set at 210 nm throughout this work. The column temperature was ranged from 0 to 45°C. Data analyses were performed by using Microsoft Excel.

Table 1
Observed and calculated retention factors

TGs	Temperature (°C)						Calc[b]
	0	5	10	15	20	25	
LnLnLn	12.17	9.84	8.35	7.50	7.10	7.07	7.00
LnLnL	14.13	11.29	9.43	8.36	7.82	7.72	7.63
LnLnO	17.22	13.47	11.01	9.62	8.86	8.63	8.52
LLL	19.11	14.82	11.99	10.39	9.48	9.16	9.06
LLP	23.24	17.05	13.29	11.21	9.98	9.43	9.39
LLO	23.24	17.66	14.01	11.94	10.74	10.23	10.12
LOP	28.15	20.37	15.58	12.90	11.30	10.54	10.49
OOO	34.75	25.10	19.13	15.78	13.75	12.78	12.62
LaLaLa	4.91	3.87	3.20	2.79	2.55	2.45	2.46
MMM	13.52	9.60	7.39	6.15	5.41	5.03	4.98
PPP	37.24	23.83	16.94	13.34	11.16	10.22	10.09
SSS	(102.6)[a]	(59.13)	(39.00)	28.56	23.19	20.29	20.41
t_0 (min)	1.49	1.46	1.43	1.40	1.37	1.34	–

[a] Extrapolated value.
[b] Calculated value at 25°C.

2.3. Samples

All plant oils were dissolved in *n*-hexane in concentrations of 1.5% for analytical separation and 10% for preparative separation. Plant oils used in this work were perilla oil, soybean oil, rapeseed oil, sesame oil, and safflower oil, which were obtained from Kishida Chemical (Osaka, Japan). These oils are composed of palmitate (P, 16:0), stearate (S, 18:0), oleate (O, 18:1), linoleate (L, 18:2), and linolenate (Ln, 18:3). In this case, 35 TGs can theoretically be formed. The TG composition of oils was estimated from their fatty acid compositions. For this purpose, each oil was converted into FAMEs by ester exchange reaction in methanol using sulfuric acid as catalyst and fatty acid composition was determined by GC analysis.

Saturated TGs, trilaurin (LaLaLa), trimyristin (MMM), tripalmitin (PPP), and tristearin (SSS), were obtained from Tokyo Kasei (Tokyo, Japan). Various unsaturated TGs listed in Table 1 were fractionated from perilla oil, soybean oil, and rapeseed oil based on the preliminary experimental results. Purities of these TGs were at least 95% from the UV chromatograms.

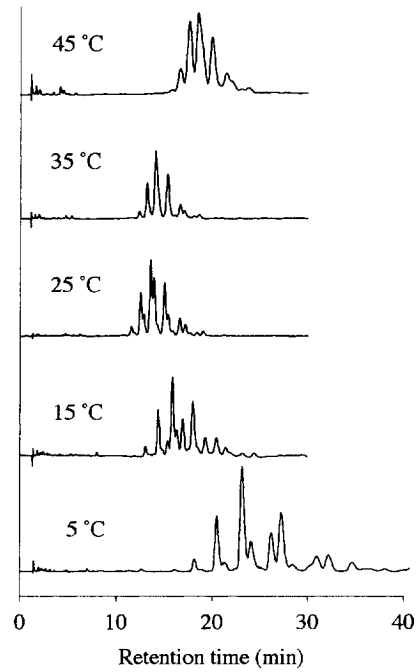

Fig. 1. Chromatograms of soybean oil at various temperatures. Column: L-column ODS, 5 μm particle, 250 mm×4.6 mm I.D. Mobile phase: carbon dioxide at a flow-rate of 3 ml/min. Outlet pressure: 150 atm. Detection wavelength: 210 nm.

3. Results and discussion

3.1. Separation of plant oils at various temperatures

Oils were separated under super- and sub-critical conditions at 150 atm. Fig. 1 shows the chromatograms of soybean oil at various temperatures. Although the retention increased with increasing temperature in the supercritical region due to the decrease of density, the separation profile was hardly improved. On the other hand, lowering temperature in the subcritical region (lower than 31.3°C) allowed more peaks to be resolved, suggesting that the selectivity largely changed. In this case, the retention increased with decreasing temperature as normally observed in HPLC. The results indicate that temperature is a more effective parameter to change selectivity than the mobile phase density. Therefore, the retention behavior of TGs was studied in the temperature range of 0 to 25°C in further work. Table 1 lists retention factors of selected TGs together with hold-up times measured by injecting *n*-hexane. Extrapolated values were estimated with a linear relationship discussed later.

3.2. Retention behavior of TGs

In RPLC with ODS columns, it is well known that a linear relationship exists between log k' and the *CN* for saturated TGs. Another linear relationship also exists between log k' and *DB* for the particular series such as LnLnLn, LnLnL, LnLL, and LLL [4–11]. These relationships are expressed as follows:

$$\log k' = aCN + b \quad (1)$$

$$\log k' = -a'DB + b' \quad (2)$$

In Eq. (2) each series has different values for the coefficients. These equations can be combined into Eq. (3) by using the *ECN* expressed by Eq. (4) [6,12,13,15].

$$\log k' = aECN + b \quad (3)$$

$$ECN = CN - (3N_{Ln}E_{Ln} + 2N_{L}E_{L} + N_{O}E_{O}) \quad (4)$$

where N is the number of fatty acids in TG, E is the double bond coefficient, and the subscripts denote the type of the fatty acids.

In this work these relationships were found to exist at various temperatures. Fig. 2 shows the plots of log k' versus CN for saturated TGs, where $R>0.9999$. The plots of log k' versus DB for various series (L–O, Ln–O, and L–P series) at 20°C are shown in Fig. 3. In both the Ln–L and L–O series, successive replacement of the fatty acid moiety varies DB by one, and in the Ln–O series by two. These series have the same CN while in the L–P series simultaneous changes of DB and CN occurred. Good linear relationships ($R>0.9999$) were also observed except for the L–P series ($R>0.999$).

In many SFC works using carbon dioxide, elution behaviors have been reported to be similar to NP mode on silica gel [23] and RP mode on ODS [24,25], respectively. Such behaviors may be caused by the properties of carbon dioxide: carbon dioxide is a non-polar solvent in terms of dipolarity, but it is more polar than saturated hydrocarbons in terms of polarizability [26]. The solvating properties of carbon dioxide has been described to be intermediate between n-pentane and dichloromethane [27]. Although linear relationships exist between log k' and CN in GC, a GC model based on volatility may be unsuitable to account for the results of the present work, because TGs are low volatility compounds and column temperatures are much lower than that of GC required to separate TGs. In addition, the mobile phase in the present work is liquid carbon dioxide and Table 1 indicates that an increase in DB (i.e., an increase in polarity) allows the faster elution for TGs with same CN. Therefore, the present separation mode is most likely to be RP.

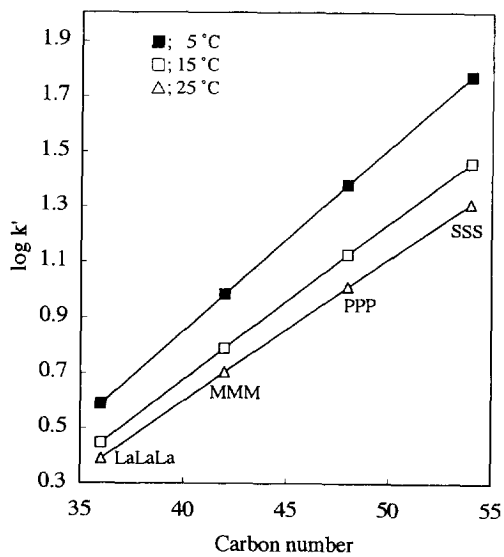

Fig. 2. Plots of log k' versus carbon number for saturated series. Symbols: La, laurate (12:0); M, myristate (14:0); P, palmitate (16:0); S, stearate (18:0).

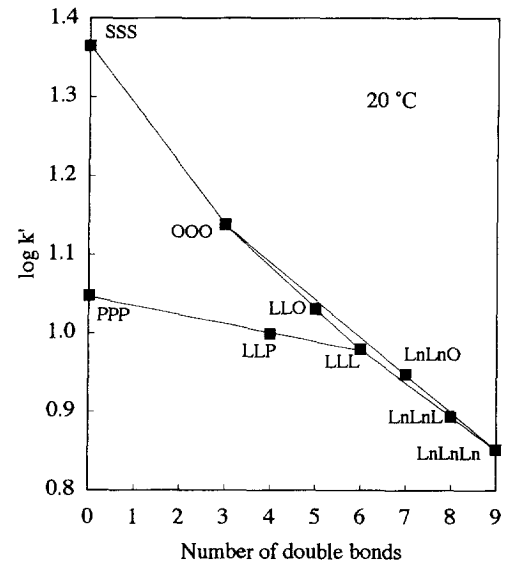

Fig. 3. Plots of log k' versus number of double bonds for all TGs listed in Table 1. Symbols: P, palmitate (16:0); S, stearate (18:0); O, oleate (18:1); L, linoleate (18:2); Ln, linolenate (18:3).

Each double bond coefficient can be determined as a value which give a maximum correlation coefficient in the linear regression analysis of retention data in Table 1. To estimate the value of E_{Ln}, for example, retention data for all saturates and Ln–L series can be used. Similarly, values of E_{L} and E_{O} can be estimated. However, it is not always practical to use many standards, because TGs are difficult to obtain in high purity specially for unsaturates. Moreover, measurement of retention of SSS takes a long time and is difficult to perform with the UV detector at lower temperatures. As the simplest set, therefore, we selected PPP and MMM as saturates, and LnLnLn, LLL, OOO as unsaturates. Firstly coeffi-

cients in Eq. (3), a and b, were determined from retention data of PPP and MMM. Then, the double bond coefficients of E_{Ln}, E_L and E_O were determined by using Eq. (4). For example, ECN of LnLnLn was found to be 44.892 at 25°C. Since CN and N_{Ln} for LnLnLn are 54 and 3, respectively, E_{Ln} was determined to be 1.012 from Eq. (4). Similarly, E_L and E_O were also determined. Using all saturates and unsaturates in Table 1 did not provide appreciable improvement. Thus, the retention of all of 35 TGs can be predicted based on five standard TGs at each temperature.

3.3. Correlation of retention to temperature

In HPLC the retention generally increases with decreasing temperature. When log k' is plotted against a reciprocal temperature ($1/T$), a linear relation can be obtained (i.e. van't Hoff plot). The retention under the subcritical conditions in this work also increased with decreasing temperature. However, such a linear relation was not observed. This may be due to the simultaneous density change because of the high compressibility of carbon dioxide even under the subcritical conditions. In this work, quadratic equations as a function of $1/T$ were examined to correlate the coefficients in Eqs. (3) and (4) to temperature. Plots of the coefficients versus temperature are shown in Fig. 4. The best correlation was achieved by using a function of $300/T$ rather than $1/T$. Although cubic equations were also examined, little difference was observed. Thus, Eqs. (3) and (4) were expressed as function of temperature as follows:

$$\log k' = a(T)ECN(T) + b(T) \quad (5)$$

$$ECN(T) = CN - \{3N_{Ln}E_{Ln}(T) + 2N_LE_L(T) + N_OE_O(T)\} \quad (6)$$

These equations allows to calculate the retention of TGs at any temperature. Table 1 shows a comparison of calculated and observed retention factors for typical TGs at 25°C, indicating a good agreement. The errors were less than 2.1% in the range of 0 to 25°C.

In RPLC, double bond coefficients are around 2, so that such solutes as OOO and OOP, LLL and LnLO tend to co-elute. In SubFC, however, double bond coefficients are dependent on the type of fatty acids and largely change with temperature as shown in Fig. 4 (E_O = 1.37–2.14, E_L = 1.15–1.66, E_{Ln} = 1.01–1.40).

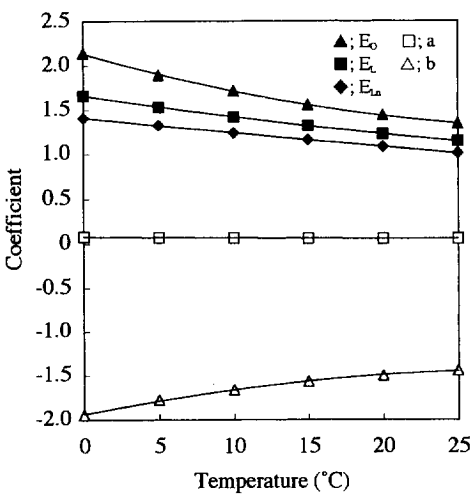

Fig. 4. Plots of coefficients in Eqs. (3) and (4) versus temperature.

3.4. Simulated chromatogram and application

It is more practical to obtain retention times rather than retention factors. Furthermore, if simulated chromatograms can be produced, visual comparison is possible. This requires a correlation of hold-up time to temperature and number of theoretical plates. Hold-up time listed in Table 1 linearly increased with decreasing temperature. Theoretical plates of the column used in this work were around 15 000. Fig. 5 shows a comparison of observed and simulated chromatograms of soybean oil at 25°C. The peak areas in simulated chromatograms correspond to the TG composition, which was estimated on the assumption that FAMEs are combined to glycerol at random. There exist a very good agreement between observed and simulated chromatograms. There are some differences in the peak height, because the UV absorption coefficient depends on the DB in TGs. These differences may decrease by using a FID instead of a UV detector.

Fig. 6 shows the plots of calculated log k' versus temperature for main TGs in perilla oil. It can be seen that selectivity largely changes with tempera-

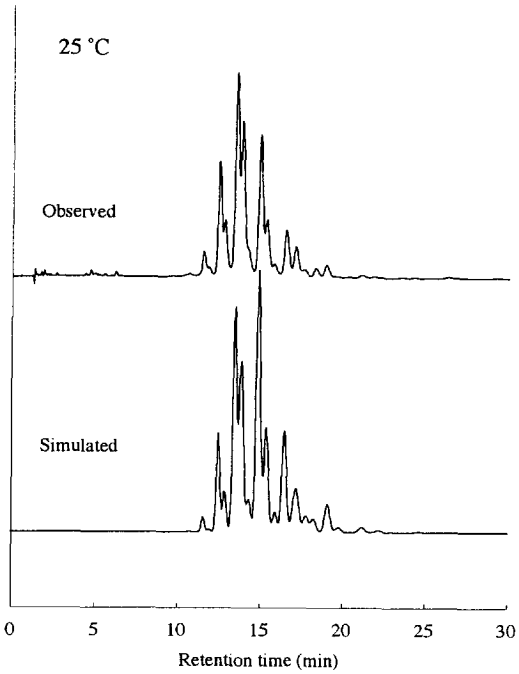

Fig. 5. Comparison between simulated and observed chromatograms for soybean oil at 25°C. Conditions as in Fig. 1. The peak areas in the simulated chromatogram correspond to the TG compositions in soybean oil.

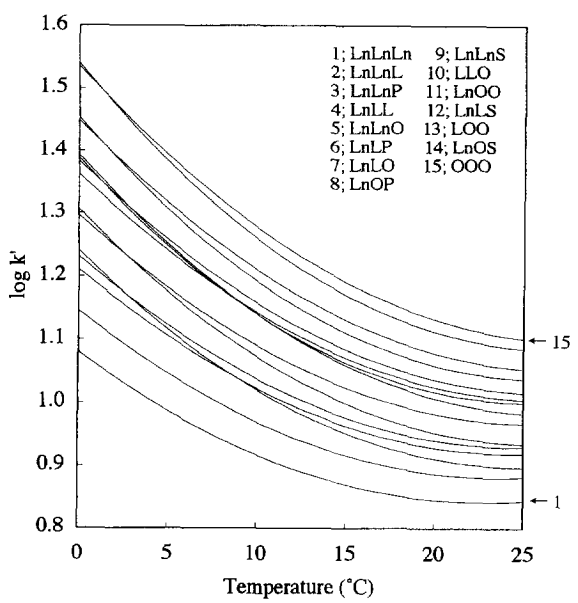

Fig. 6. Plots of calculated log k' of main components in perilla oil versus temperature. Retentions at 25°C increase with increasing number.

ture. The retention of TGs with saturated fatty acids (P and S) more largely changes with temperature than the others. This suggests that changes in solubility are larger for the former than the latter. Producing these plots is helpful to optimize the separation. Although from Fig. 6 the optimum temperature to resolve all the components in perilla oil appears to exist between 15 and 25°C, complete separation is still difficult as shown in Fig. 7, because of an insufficient number of theoretical plates. However, Fig. 6 suggests that co-eluting pairs at a certain temperature can be resolved at another temperature. Examples are shown in Fig. 8, where fractions (F1–F3) shown in Fig. 7 were collected and separated at different temperatures. F1 (containing LnLnL and LnLnP) and F2 (containing LnLL, LnLnO, and LnLP) collected at 25°C were

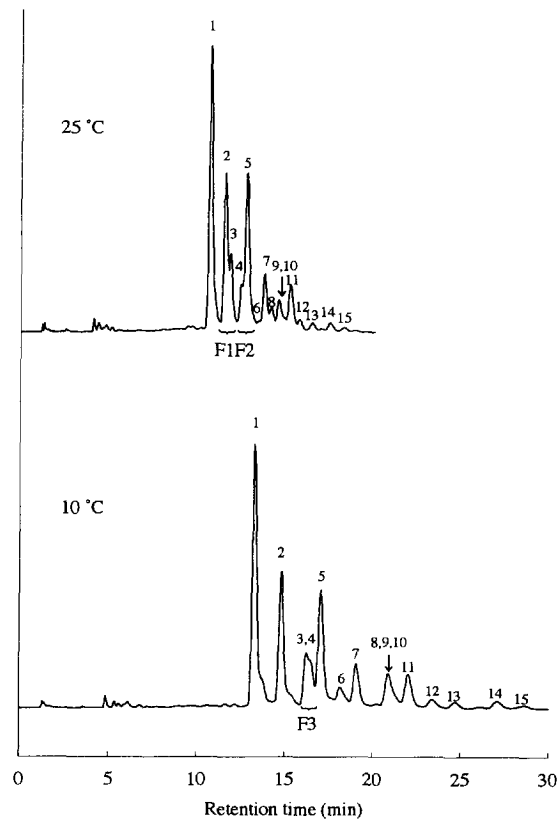

Fig. 7. Separation of perilla oil at 10°C and 25°C. Conditions as in Fig. 1. Key: 1) LnLnLn; 2) LnLnL; 3) LnLnP; 4) LnLL; 5) LnLnO; 6) LnLP; 7) LnLO; 8) LnOP; 9) LnLnS; 10) LLO; 11) LnOO; 12) LnLS; 13) LOO; 14) LnOS; 15) OOO.

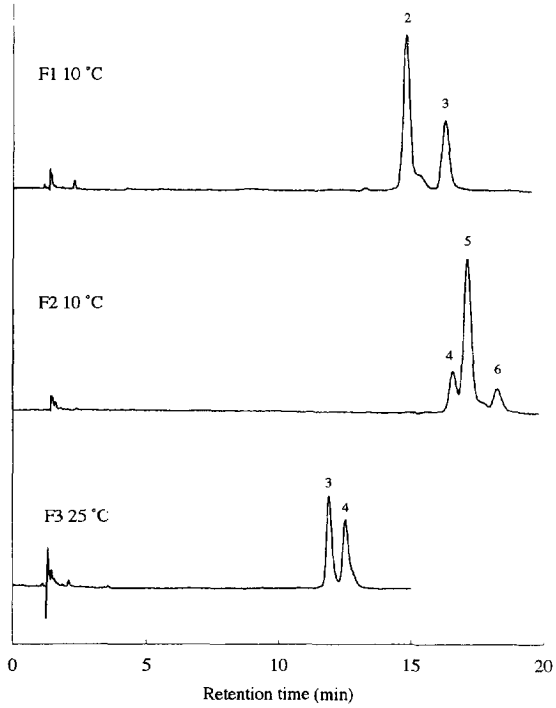

Fig. 8. Re-separations of collected fractions (F1–F3 in Fig. 7) from perilla oil. Conditions as in Fig. 1. Peak identification as in Fig. 7. Collection temperature: F1 and F2, 25°C; F3, 10°C. Re-separation temperature: F1 and F2, 10°C; F3, 25°C.

completely resolved at 10°C, and F3 (containing LnLnP and LnLL) collected at 10°C was also resolved at 25°C. This method will allow us to identify each component even for unknown samples.

4. Conclusion

The retention of TGs on ODS columns in SubFC with carbon dioxide mobile phase can be predicted as a function of temperature based on the retentions of five standard TGs. The predicted results were in good agreement with experimental ones. Selectivity changes drastically with temperature. With the method, the optimal separation temperature can be obtained. Co-eluting solutes at a certain temperature can easily be resolved at a different temperature. It is possible to identify each TG in various plant oils.

References

[1] M.J. Wojtusik, P.R. Brown and J.G. Turcotte, Chem. Rev., 89 (1989) 397–406.
[2] V. Ruiz-Gutierrez and L.J.R. Barron, J. Chromatogr. B, 671 (1995) 133–168.
[3] G. Dobson, W.W. Christie and B. Nikolova-Damyanova, J. Chromatogr. B, 671 (1995) 197–222.
[4] R.D. Plattner, G.F. Spencer and R. Kleiman, J. Am. Oil Chem. Soc., 54 (1977) 511–515.
[5] B. Herslof, O. Podlaha and B. Toregard, J. Am. Oil Chem. Soc., 56 (1979) 864–866.
[6] A.H. El-Hamdy and E.G. Perkins, J. Am. Oil Chem. Soc., 58 (1981) 867–872.
[7] G. Sempore and J. Bezard, J. Chromatogr., 366 (1986) 261–282.
[8] A. Stlyhwo, H. Colin and G. Guiochon, Anal. Chem., 57 (1985) 1342–1354.
[9] K. Takahashi, T. Hirano and K. Zama, J. Am. Oil Chem. Soc., 61 (1984) 1226–1229.
[10] K. Takahashi, T. Hirano, M. Egi, M. Hatano and K. Zama, J. Am. Oil Chem. Soc., 63 (1986) 1543–1546.
[11] K. Takahashi, T. Hirano, M. Egi and K. Zama, J. Am. Oil Chem. Soc., 62 (1985) 1489–11492.
[12] O. Podlaha and B. Toregard, J. High Resolut. Chromatogr. Chromatogr. Commun., 5 (1982) 553–558.
[13] O. Podlaha and B. Toregard, J. Chromatogr., 482 (1989) 215–226.
[14] T. Rezanka and P. Mares, J. Chromatogr., 542 (1991) 145–159.
[15] J.P. Wolff, F.X. Moredret and A. Dieffenbacher, Pure Appl. Chem., 63 (1991) 1173–1182.
[16] E. Frede, Chromatographia, 21 (1986) 29–36.
[17] L.J.R. Barron, M.V. Celaa, G. Santa-Maria and N. Corzo, Chromatographia, 25 (1988) 609–612.
[18] L.J.R. Barron and G. Santa-Maria, Chromatographia, 28 (1989) 183–188.
[19] A.G. Vereshchagin, J. Chromatogr., 14 (1964) 184–188.
[20] C. Litchfield, Lipids, 3 (1967) 170–177.
[21] S. Wada, C. Koizumi and J. Nonaka, Yukagaku, 26 (1977) 95–99.
[22] E.G. Perkins, D.J. Henderson, N. Pelick and J.E. Bauer, Lipids, 17 (1982) 460– 463.
[23] Y. Hirata, Y. Kawaguchi and K. Kitano, Chromatographia, 40 (1995) 42–46.
[24] A. Nomura, J. Yamada, K. Tsunoda, K. Sakaki and T. Yokochi, Anal. Chem., 61 (1989) 2076–2078.
[25] A. Nomura, J. Yamada, A. Takatsu, Y. Horimoto and T. Yarita, Anal. Chem., 65 (1993) 1994–1997.
[26] C.R. Yonker and R.D. Smith in T.G. Squires and M.E. Paulaitis (Editors), Supercritical Fluid Extraction and Chromatography: Techniques and Applications, ACS Symposium Series 329, American Chemical Society, Washington, 1987, p. 29.
[27] M.L. Lee and K.E. Markides (Editors), Analytical Supercritical Fluid Chromatography and Extraction, Chromatography Conferences Inc., Provo, UT, 1990, p.18.