

Improvement in Resolution of Low Melting Point Triglyceride Molecular Species on Chilled Column Isocratic Liquid Chromatography

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Abstract

An isocratic reversed-phase high-performance liquid chromatographic system is used to achieve the separation and improved resolution of individual triglyceride molecular species that are rich in icosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The triglycerides are enzymatically synthesized and contain EPA and DHA. Various column temperatures, from -20°C to 20°C , are used to aid in calculation of the changes in chromatographic parameters. It is found that when column temperature decreases, the selectivity values and retention rate values in the equation used to describe resolution exhibit an increasing; however, there is a critical column temperature that gives optimum resolution. For this reason, we concluded that to obtain the best resolution of a triglyceride molecular species with a low melting point, it is important to lower the column temperature near to the critical point.

Introduction

A column oven is sometimes used in high-performance liquid chromatography (HPLC) to decrease retention time (t_R) and to increase the theoretical plate number (N) (1–3). Plate number decreases when the column is cooled. However, it is often observed that baseline resolution can be obtained for some peaks by cooling the column.

Fish oil contains many more kinds of fatty acids and highly unsaturated fatty acids than vegetable oils, thus it has a more complex triglyceride (TG) composition that may be difficult to resolve using the techniques applied to the separation of vegetable oil samples. However, fish oil has the advantage of a low melting point (approximately 0 – 5°C), and good resolution can be obtained for lipid species with the use of a column chilling system.

In this article, we discuss the theoretical aspects of improvement in resolution of TG molecular species via reversed-phase HPLC and lowering column temperature.

Experimental

Synthesis of triglyceride-containing icosapentaenoic acid and docosahexaenoic acid

Icosapentaenoic acid (0.6 mL, 90.9% pure) (EPA) and docosahexaenoic acid (0.6 mL, 88.0% pure) (DHA) were gifts of Nippon Chemical Feed (Hakodate, Japan). Both chemicals were added to 16 mL glycerin (Wako Pure Chemical; Osaka, Japan). A 0.5M phosphate buffer enzyme solution (0.22 mL) (pH 6.0) that contained 30.4 mg Lipase TOYO (*Chromobacterium viscosum*) (gift of Asahi Chemical; Fuji, Japan) was added to initiate triglyceride synthesis. The mixture was heated at 25°C and magnetically stirred under argon gas for 24 h. Incubation was terminated by adding ethanol. The triglycerides thus synthesized were extracted using ethylether and then rinsed several times with water. Finally, purified triglyceride was obtained after preparative thin-layer chromatography (Kieselgel 60 F₂₅₄, 20×20 cm, 0.5-mm thickness) (E. Merck; Darmstadt, Germany). The mobile phase was composed of *n*-hexane, ethylether, and acetic acid (70:30:1, v/v/v).

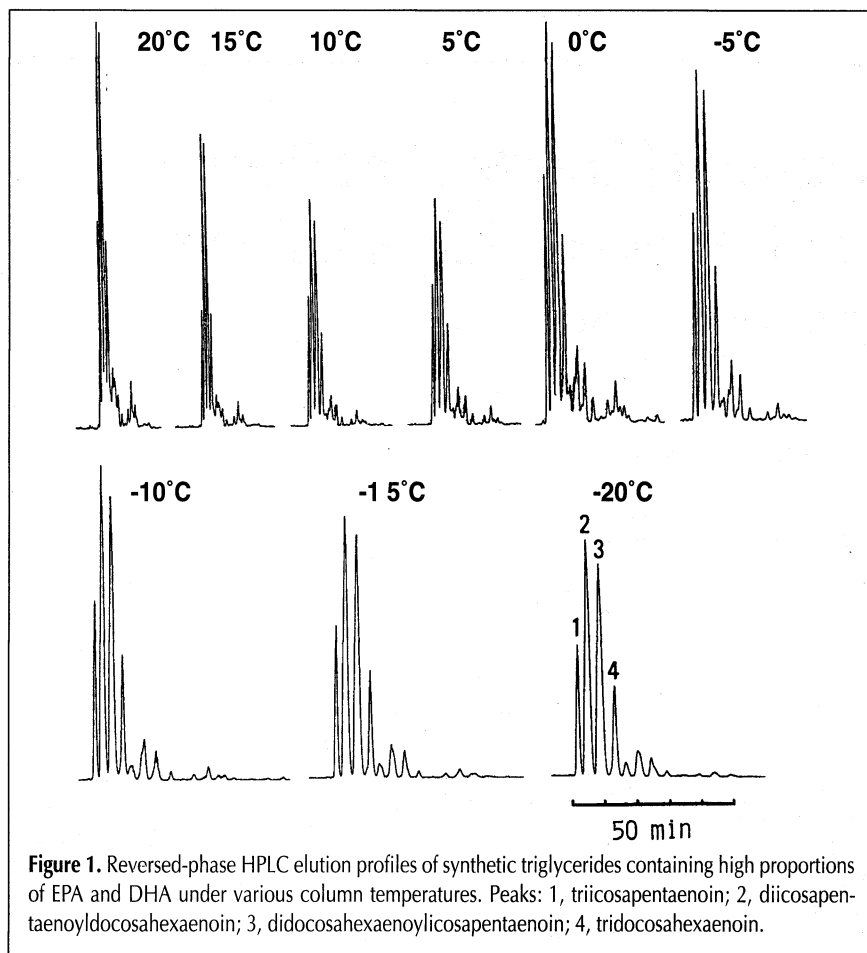
Reversed-phase HPLC

A Hitachi 638-50 liquid chromatograph (Tokyo, Japan) was used. It was equipped with a Wakosil 5C18N column (300×4 mm, $5 \mu\text{m}$) (Wako Pure Chemical) and a Supersphere RP-18 column (250×4 mm, $4 \mu\text{m}$) (E. Merck) in tandem. The solvent was acetone–acetonitrile (3:2 or 3:1 or 7:1, v/v). The flow rate was 0.7 mL/min. The following column temperatures were used: 20°C , 15°C , 10°C , 5°C , 0°C , -5°C , -10°C , -15°C , -20°C . A Model 750/14 mass detector was obtained from Applied Chromatographic Systems (Cheshire, U.K.). Other conditions were as follows: time constant, 5; attenuation, 2; photomultiplier gain, 1; air pressure of nebulizer, 0.8 kg/cm^2 ; sample volume, 1–2 μL . The outlet of the column was bifurcated to recover each peak fraction. Recovering timing was synchronized with a needle valve using oil red as a marker. Recovered peak fractions (Figure 1) were identified as previously reported (4).

Measurement of the viscosity of the eluent

Viscosity was measured on an Ostwald viscometer (Nippon

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Rikagaku Kikai Co. Ltd., Tokyo). The Q-value examination (proposed by Dean and Dixon) was used to minimize the error after 10 replicate analyses for each temperature.

Results and Discussion

The resolution value (R_s) is often used as an index of resolution in HPLC. R_s is expressed in the following way:

$$R_s = (k_1 / 4)(\alpha - 1)\sqrt{N_1} / (k' + 1) \quad \text{Eq 1}$$

where k_1 and k_2 are capacity factors and N_1 and N_2 ($N_1 \approx N_2$) are theoretical plate numbers of the adjacent two peaks (5). Here, selectivity (α) is defined as k_2/k_1 and k' is defined as $(k_2 + k_1)/2$.

In this study, we considered the changes in resolution values when column temperature varied by examining each term in Equation 1.

Selectivity

Selectivity (α) corresponds to the ratio of retention values (t_{R1} , t_{R2}) of two adjacent peaks (TG_1 , TG_2). For example, $\alpha = (k_2/k_1) = (t_{R2}/t_{R1})$.

According to the law of Martin (6), the following two relationships can be induced:

$$\therefore \ln t_{R1} \propto (\Delta\mu_{TG1}/RT) \quad \text{Eq 2}$$

$$\ln t_{R2} \propto (\Delta\mu_{TG2}/RT) \quad \text{Eq 3}$$

where $\Delta\mu_{TG1}$ and $\Delta\mu_{TG2}$ are chemical potentials of molecular species adjacent to each other in a chromatographic system (TG_1 and TG_2). By introducing constants (P_1 , P_2) into Equations 2 and 3, we obtained the following equations:

$$\ln t_{R1} = (\Delta\mu_{TG1}/RT) - P_1 \quad \text{Eq 4}$$

$$\ln t_{R2} = (\Delta\mu_{TG2}/RT) - P_2 \quad \text{Eq 5}$$

If Equation 4 is subtracted from Equation 5, the following is obtained:

$$\ln (t_{R2}/t_{R1}) = \left(\frac{\Delta\mu_{TG2} - \Delta\mu_{TG1}}{RT} \right) + P_1 - P_2 \quad \text{Eq 6}$$

If $\Delta\mu_X$ is substituted for $\Delta\mu_{TG2} - \Delta\mu_{TG1}$ and if α is substituted for t_{R2}/t_{R1} , the following should hold true:

$$\ln \alpha = (\Delta\mu_X/RT) + P_1 - P_2 \quad \text{Eq 7}$$

In terms of column temperature, there might be a certain change in state; that is, there may be a bend or an association of molecules in the mobile phase, the stationary phase, or the solutes. If this is taken into consideration, $\Delta\mu_X$ is a function of column temperature (T). Therefore, from Equation 7, the following equation can be derived:

$$\ln \alpha = (\Delta\mu_X/[T]RT) + P_1 - P_2 \quad \text{Eq 7A}$$

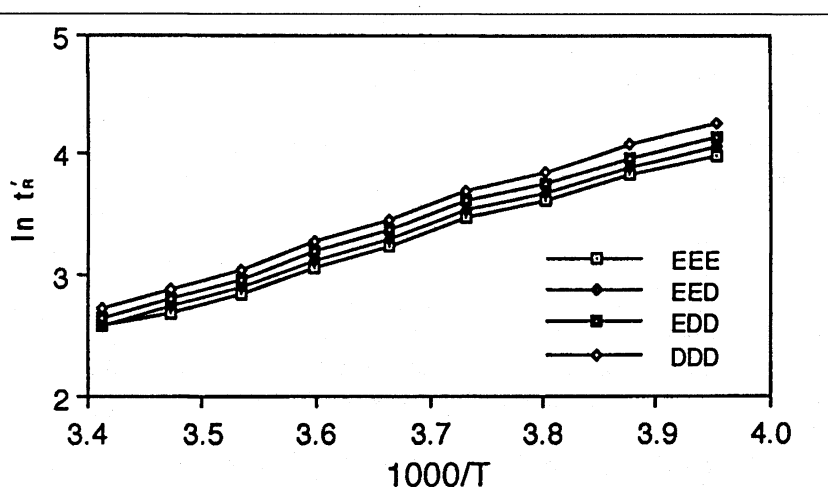


Figure 2. Relationship between retention time of the individual triglyceride molecular species versus absolute temperature (T) of the column. E, icosapentaenoil; D, docosahexaenoil. Chromatograms shown in Figure 1 were used for this analysis.

On the other hand, as shown in Figure 2, there is a linear relationship between $\ln t_R'$ and $1/T$ such that the following empirical relationships can be obtained:

For DDD, $\ln t_R'(\text{DDD}) = 2.943 \times 10^3/T - 7.33$ Eq 8

For EDD, $\ln t_R'(\text{EDD}) = 2.869 \times 10^3/T - 7.15$ Eq 9

For EED, $\ln t_R'(\text{EED}) = 2818 \times 10^3/T - 7.04$ Eq 10

For EEE, $\ln t_R'(\text{EEE}) = 2.786 \times 10^3/T - 6.98$ Eq 11

From Equations 8–11, the following equations can be derived:

$\ln[t_R'(\text{DDD})/t_R'(\text{EEE})] = 0.157 \times 10^3/T - 0.35$ Eq 12

$\ln[t_R'(\text{DDD})/t_R'(\text{EED})] = 0.125 \times 10^3/T - 0.29$ Eq 13

$\ln[t_R'(\text{DDD})/t_R'(\text{EDD})] = 0.074 \times 10^3/T - 0.18$ Eq 14

$\ln[t_R'(\text{EDD})/t_R'(\text{EEE})] = 0.083 \times 10^3/T - 0.17$ Eq 15

$\ln[t_R'(\text{EDD})/t_R'(\text{EED})] = 0.051 \times 10^3/T - 0.11$ Eq 16

$\ln[t_R'(\text{EED})/t_R'(\text{EEE})] = 0.032 \times 10^3/T - 0.06$ Eq 17

Since the left members of Equations 12–17 correspond to $\ln \alpha$, the empirically derived linear relationships shown in Equations 12–17 corroborate that $\Delta\mu_X(T)$ from Equation 7A is almost constant under the column temperatures used (-20°C to $+20^\circ\text{C}$). In addition, $\ln \alpha$ and $1/T$ are proportional, and it is evident from Equations 12–17 that when α increases, $\Delta\mu_X$ also increases. Therefore, Equation 7A should be true.

To examine the temperature dependence of $\Delta\mu_X(T)$ more precisely, we calculated the values obtained from the following equation (Table I):

$(P_2 - P_1 + \ln \alpha_{T1}) / (P_2 - P_1 + \ln \alpha_{T2}) = (\Delta\mu_X T_1 / \Delta\mu_X T_2) T_2 / T_1$ Eq 18

If $\Delta\mu_X(T)$ is not dependent on T , $[\Delta\mu_X(T_1) / \Delta\mu_X(T_2)]$ in Equation 18 should be equal to 1. In fact, as shown in Table I, $[\Delta\mu_X(T_1) / \Delta\mu_X(T_2)]$ is close to 1. Therefore, the hypothesis is corroborated.

Table I. Relationship Between $(P_2 - P_1 + \ln \alpha)$ and T_2/T_1 of the Column						
T_2/T_1	$(P_2 - P_1 + \ln \alpha_{T1}) / (P_2 - P_1 + \ln \alpha_{T2})^*$					
	EED/EEE	EDD/EED	DDD/EDD	EDD/EEE	DDD/EED	DDD/EEE
258/253(1.020)	0.9919	1.0460	1.0370	1.0254	1.0410	1.0299
263/253(1.040)	1.0099	1.0630	1.0550	1.0427	1.0590	1.0471
268/253(1.059)	1.0480	1.0940	1.0880	1.0799	1.0910	1.0792
273/253(1.079)	1.0645	1.0990	1.0910	1.0859	1.0940	1.0854
278/253(1.099)	1.0882	1.1190	1.1120	1.1075	1.1150	1.1063
283/253(1.119)	1.0930	1.1360	1.1280	1.1197	1.1310	1.1194
288/253(1.138)	1.1252	1.1570	1.1510	1.1455	1.1540	1.1428
293/253(1.158)	1.1378	1.1740	1.1670	1.1601	1.1700	1.1578

*E=icosapentaenyl; D=docosahexaenyl.

Column efficiency

The column efficiency is given as the square root of the plate number. The theoretical plate number is as follows:

$N = L/H$ Eq 19

where L is column length, and H is height equivalent to a theoretical plate. H is a function of particle diameter of column packing (d_p), the overall velocity of the mobile phase (u), the molecular diffusion coefficient in the mobile phase (D_m), and the molecular diffusion coefficient in the stationary phase (D_s). In other words,

$$H = 1 / \left(\frac{1}{(1/C_e d_p) + (1/C_m d_p^2 u / D_m)} \right) + \frac{C_d D_m}{u} + \frac{C_{sm} d_p^2 u}{D_m} + \frac{C_s d_f^2 u}{D_s}$$
 Eq 20

where C_e , C_m , C_d , C_{sm} , and C_s are constants and d_f is the thickness of the stationary phase. Each term in Equation 20 is defined as follows:

- eddy diffusion $C_e d_p$
- mobile phase mass transfer $C_m d_p^2 u / D_m$
- longitudinal diffusion $C_d D_m / u$
- stagnant mobile phase mass transfer $C_{sm} d_p^2 u / D_m$
- stationary phase mass transfer $C_s d_f^2 u / D_s$

In liquid–liquid chromatography, the stationary phase mass transfer is negligibly small. In other words, $C_s d_f^2 u / D_s = 0$. These terms are functions of velocity and the molecular diffusion coefficient in the mobile phase. If we take notice of a certain analytical temperature, all the signs except u and D_m can be considered invariable. Then, we can focus attention on u and D_m . From the law of Darcy, the following is obtained:

$u = \kappa P / L$ Eq 21

where L is column length, κ is transmittance, and P is the depression between both ends of the column. From the law of Kozeny–Carmen (7), κ can be defined in the following way:

$\kappa = d_p^2 / 1000 \eta f$ Eq 22

where η is the viscosity of the solvent (mobile phase), and f is the totally porous proportion of column packing. By eliminating κ and using the definitions, we obtain the following equation:

$u = (d_p)^2 p / 1000 f L \eta$ Eq 23

From the law of Wilke–Chang (7), D_m is approximated by the following equation:

$D_m = (7.4 \times 10^{-8}) (\psi M)^{0.5} T / \eta V_1^{0.6}$ Eq 24

where M is the molecular weight of the solvent, V_1 is the molecular volume, and ψ is the association modulus. In general, when the temperature changes, the viscosity (η) of the solvent (mobile phase) also changes. There is a linear relationship between the logarithm of viscosity and the reciprocal value of temperature which is expressed as follows (the law of Andrade):

$$\eta = Ae^{\Delta E/RT} \quad \text{Eq 25}$$

where A is a constant, ΔE is the activation energy for flow; and R is the gas constant. As shown in Figure 3, Equation 25 was empirically borne out by the present data. In HPLC systems in general, changes in overall flow rate (u) suppressed by changes in viscosity (η) of solvent owing to temperature (T) change are compensated for by changing the solvent pressure simultaneously. Therefore, u is considered to be constant. In Equation 20, H can be considered to be a function of D_m . By eliminating η through substitution of Equation 25 in the definition of stag-

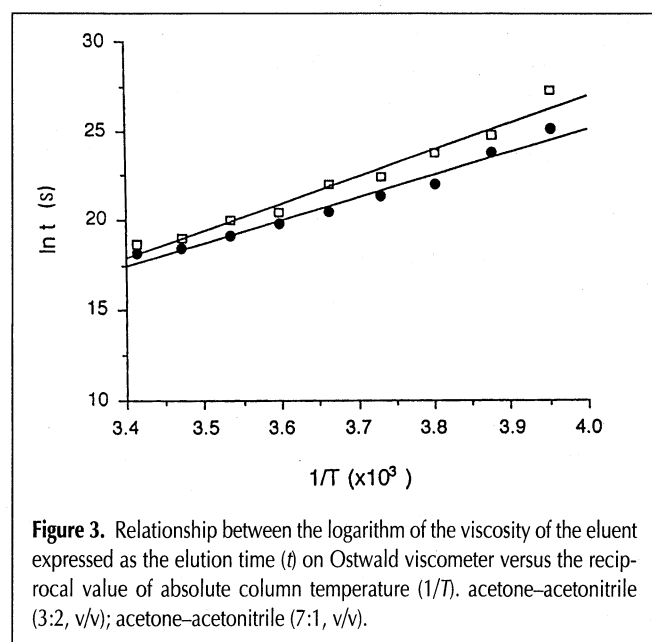


Figure 3. Relationship between the logarithm of the viscosity of the eluent expressed as the elution time (t) on Ostwald viscometer versus the reciprocal value of absolute column temperature ($1/T$). acetone-acetonitrile (3:2, v/v); acetone-acetonitrile (7:1, v/v).

nant mobile phase mass transfer and using that of stationary phase mass transfer in Equation 24, we can differentiate the equation and the following will be obtained:

$$\frac{\partial D_m}{\partial T} = \frac{(7.4 \times 10^{-8})(\psi M)^{0.5}(1 + \Delta E/RT)}{V_1^{0.6} A e^{\Delta E/RT}} \quad \text{Eq 26}$$

$$= (D_m/T)(1 + \Delta E/RT) > 0$$

From this inequality, we can derive that when T becomes decreases, D_m also decreases. On the other hand, by substituting $C_s d_p^2 u / D_s = 0$ into Equation 20, we can differentiate H with respect to D_m , and the following equation can be obtained:

$$\frac{\partial H}{\partial D_m} = \left(\frac{C_d}{u} \right) - \left(\frac{1/C_m d_p^2 u}{(1/C_e d_p + D_m / C_m d_p^2 u)^2} + \frac{C_{sm} d_p^2 u}{D_m^2} \right) \quad \text{Eq 27}$$

When T is small and when the following relationship is true,

$$\frac{1/C_m d_p^2 u}{(1/C_e d_p + D_m / C_m d_p^2 u)^2} + \frac{C_{sm} d_p^2 u}{D_m^2} > \frac{C_d}{u} (> 0) \quad \text{Eq 28}$$

H becomes large when D_m becomes small.

$$\frac{\partial H}{\partial D_m} < 0 \quad \text{Eq 29}$$

On the other hand, when T is large and when the following relationship is true,

$$(0 <) \frac{1/C_m d_p^2 u}{(1/C_e d_p + D_m / C_m d_p^2 u)^2} + \frac{C_{sm} d_p^2 u}{D_m^2} < \frac{C_d}{u} \quad \text{Eq 30}$$

H becomes small when D_m becomes small.

$$\frac{\partial H}{\partial D_m} > 0 \quad \text{Eq 31}$$

By the differentiation theory of composite function,

$$\frac{\partial H}{\partial T} = (\frac{\partial H}{\partial D_m})(\frac{\partial D_m}{\partial T}) \quad \text{Eq 32}$$

should hold true. By differentiating Equation 19 with respect to T , we obtained the following equation:

$$\frac{\partial N}{\partial T} = -(L/H^2)(\frac{\partial H}{\partial T}) \quad \text{Eq 33}$$

By substituting Equations 32, 27, and 26 into Equation 33, we obtained Equation 34:

$$\frac{\partial N}{\partial T} = \left(\frac{L(1 + \Delta E/RT)}{H^2 T} \right) \times \left(\frac{1/C_m d_p^2 u}{(1/C_e d_p + D_m / C_m d_p^2 u)^2} + \frac{C_{sm} d_p^2 u}{D_m^2} - \frac{C_d}{u} \right) \quad \text{Eq 34}$$

Therefore, when T is low and when the inequality in Equation 28 is true, N becomes small in accordance with the decrease in T . In other words,

$$\frac{\partial N}{\partial T} > 0 \quad \text{Eq 35}$$

On the other hand, when T is large and when the inequality in Equation 30 is true, N becomes large with a decrease in T :

$$\frac{\partial N}{\partial T} < 0 \quad \text{Eq 36}$$

From the results demonstrated so far, we can induce the following points:

- $\partial D_m / \partial T$ an increasing function. When T becomes infinitely large, $\partial D_m / \partial T$ will be $7.4 \times 10^{-8} (\psi M)^{0.5} / V_1^{0.6} A$; that is, if T decreases, D_m will also decrease.
- When T is large (when D_m is large) and when the in-

equality in Equation 30 is true, $\partial N/\partial T$ becomes negative. In this temperature range, N increases in accordance with the decrease in T .

(c) On the other hand, when T is low (when D_m is small) and when the inequality in Equation 28 is true, $\partial N/\partial T$ becomes positive. In this temperature range, N decreases in accordance with the decrease in T .

(d) By combining points b and c, we can say that in a high T range (in the range where Equation 30 is true), N increases in accordance with the decrease in T . However, when T becomes significantly low (in the range where Equation 28 is true), N decreases with a decrease in T .

(e) If $\partial N/\partial T = 0$ as a critical temperature (T_c), T_c is the optimum temperature for obtaining the best N value. In the present study, -15°C corresponds to this T_c , as can be seen in Figure 4.

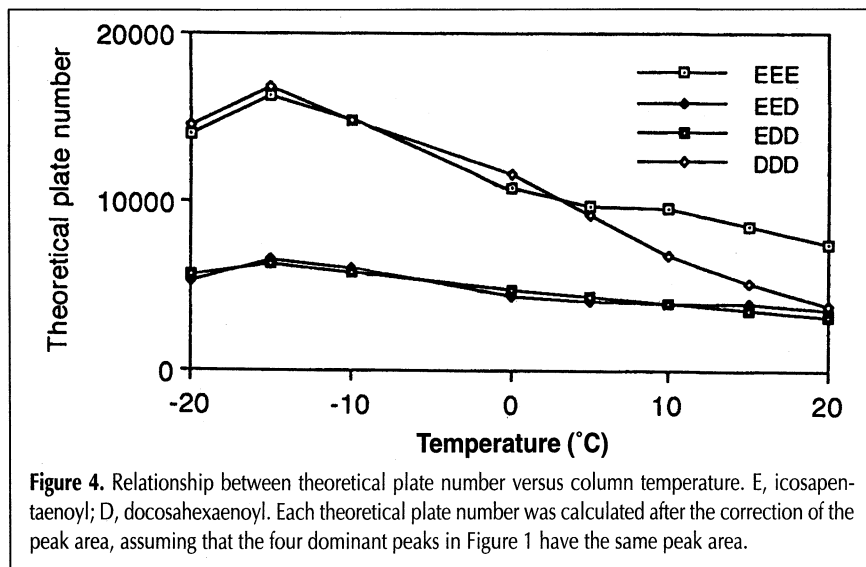


Figure 4. Relationship between theoretical plate number versus column temperature. E, icosapentaenoyl; D, docosahexaenoyl. Each theoretical plate number was calculated after the correction of the peak area, assuming that the four dominant peaks in Figure 1 have the same peak area.

(f) If T reaches the range where orientation of molecules occur (i.e., when a change in states takes place), the solubility of TG decreases. The efficiency in partition between both phases (the mobile and stationary phases) also decreases. It is thought that much time is required to reach a partition equilibrium. The theoretical plate number is thought to consequently decrease at this state.

(g) The phenomenon in point f can be explained in the following way: the modulus of D_m in Equation 24 decreases when there is a change in the state of the solvent. In other words, the coefficient in Equation 24 is thought to decrease at this state.

(h) As mentioned previously, when D_m decreases because of a change of state of the solvent, T_c increases (since T_c is the temperature that gives $\partial N/\partial T = 0$ in Equation 24), and if D_m decreases, T_c increases from the proportional relationship between T and D_m .

(i) As seen in Figure 3, a discrepancy was observed below -15°C (at -20°C , which corresponds to 3.95 on the x -axis). This suggests that a change in state at this temperature range should occur.

(j) Above the temperature at which a change of state occurs (e.g., above -15°C in this study), Equation 30 should be true.

In this temperature range, a lower T results in a better N value, and when the change of state starts to take place, T_c increases and suppresses the optimum value of N .

Based on our observations to this point, in order to obtain the best N value, we deduce the following points:

(a) Solvents of the mobile phase should be low in molecular weight and viscosity but high in solubility of TG. These three factors should be well balanced at a high level (solvents that have a high D_m value should be selected).

(b) Solvents in the mobile phase should be resistant to changes in state at low temperatures. It is desirable to select solvents that have a lower temperature at which they change state than to select solvents with a high or low T_c value.

(c) The temperature that gives $\partial N/\partial T = 0$ (i.e., T_c) is the best analytical temperature to obtain N_{\max} . However, T_c depends on u , which is the flow rate of the mobile phase. Therefore, if we take this into consideration, we can consider the following condition should be the best; in other words, when $\partial N/\partial \beta = 0$ ($\beta = D_m/u$), β gives the optimum condition:

$$\frac{\partial N}{\partial \beta} = -(L/H^2)(\partial H/\partial \beta) = \left(\frac{L}{H_2}\right) \left(\frac{1/C_m d_p^2}{(1/C_e d_p + \beta/C_m d_p)^2} + \frac{C_{sm} d_p^2}{\beta^2 - C_d} \right) \quad \text{Eq 37}$$

The retention rate

The retention rate was defined as $k_1/(k'+1)$. For convenience sake, we will de-

fine δ as follows:

$$\delta \equiv k_1/(k'+1) = 2k_1/(k_1+k_2+2) \quad \text{Eq 38}$$

Since $k_1 = (t_{R1}' - t_0)/t_0$ and $k_2 = (t_{R2}' - t_0)/t_0$, the following equation should be true:

$$\delta = 2(t_{R1}' - t_0)/(t_{R1}' + t_{R2}') \quad \text{Eq 39}$$

t_0 can be defined in the following manner:

$$t_0 = L/u \quad \text{Eq 40}$$

If we substitute Equation 23 into Equation 40, the following equation will be obtained:

$$t_0 = 1000\eta/L^2/Pd_p^2 \quad \text{Eq 41}$$

In most cases, HPLC hardware compensates for solvent pressure to get a constant flow even though η changes because of a temperature change. Therefore, t_0 does not change even though T changes. By adjusting Equation 41, we obtain the following equations:

$$P=1000fL^2\eta/t_0d_p^2 \quad \text{Eq 42}$$

or

$$P/\eta = 1000fL^2/t_0d_p^2 \quad \text{Eq 43}$$

As shown in Figure 5, it is obvious that there is a linear relationship between P and η when t_0 is constant. Also, it can be seen from Table II that the values of P/t , which correspond to P/η ($\therefore \eta \propto t$) are almost constant despite the temperature employed. Therefore, it is clear that t_0 is not affected by T . However, if we take a precise look at the data of t_0 , as illustrated in Figure 6, in an expanded form, t_0 increases with the decrease in T . This contradiction can be explained by the following points:

(a) Strictly speaking, when T changes, the volume of the stationary phase changes so as to affect t_0 . For example, when T decreases, d_p in Equation 41 decreases and t_0 increases.

(b) When T is lowered, the kinetic energy of the stationary and mobile phase molecules decreases and affects the van der Waals force among these molecules. This will make the molecules attract much easier. If the attraction of molecules starts to take place, the available volume of the column will

increase. This phenomenon can be explained as follows: when T decreases, the column volume increases in Equation 41, resulting in an increase in t_0 .

(c) Partition of the solvent (the solvent used for sample dilution) itself may occur. If we denote the time required for a solvent to elute as t_0' , Equation 44 can be obtained by the law of Martin (6):

$$\ln t_0' \propto \Delta\mu_0/RT \quad \text{Eq 44}$$

However, Equation 44 can only be used when the solvent has a lower solubility than the mobile phase and when its affinity to the stationary phase is strong (i.e., when $\Delta\mu_0$ is large). This point can be checked by noting whether there is a separation between the loading shock and the solvent peak on the chromatogram. If there is no separation even when T varies, the effect of Equation 44 is negligible. The phenomenon of t_0 increasing in accordance with a decrease in T is attributed to the interactions of the above three factors. In fact, point c was consistent with the actual observations seen with *n*-hexane

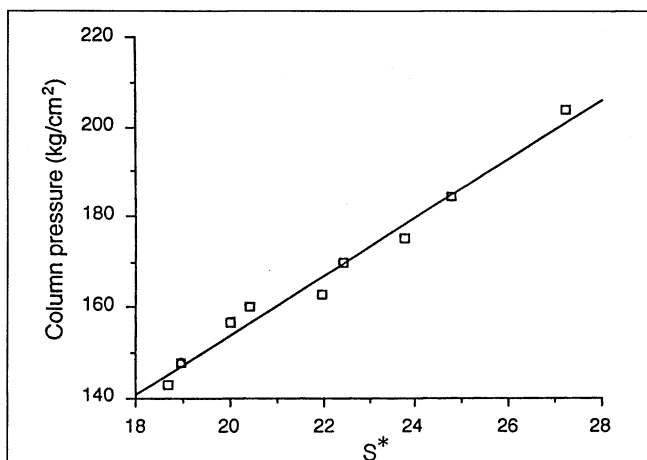


Figure 5. Relationship between column pressure and viscosity of the mobile phase. Measured by the flow time (s) of the mobile phase (acetone-acetonitrile, 3:2) as measured with an Ostwald viscometer.

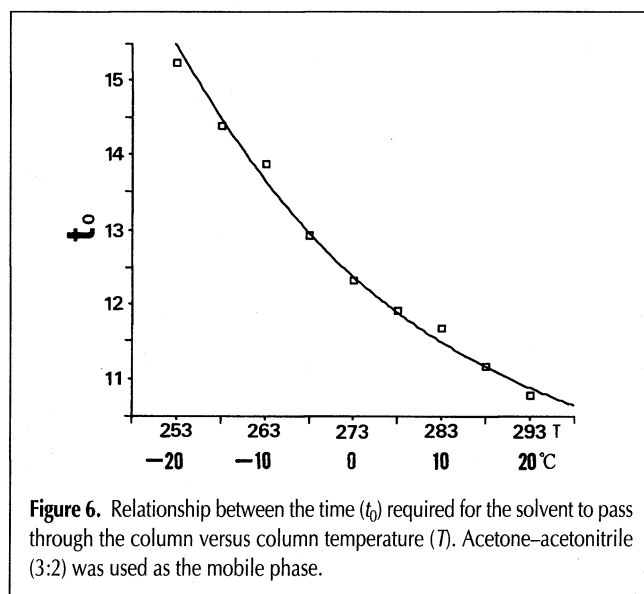


Figure 6. Relationship between the time (t_0) required for the solvent to pass through the column versus column temperature (T). Acetone-acetonitrile (3:2) was used as the mobile phase.

Table II. Values of Column Pressure and Flow Time of the Mobil Phase on Ostwald Viscometer at Various Absolute Column Temperatures

Column temp.	Column pressure (kg/cm ²)	Flow time (s)	P/t (kg/cm ² s)
293 (20°C)	142.9	18.8	7.6
288 (15)	148.0	19.1	7.7
283 (10)	156.9	20.0	7.8
278 (5)	160.0	20.5	7.8
273 (0)	162.7	22.0	7.4
268 (-5)	169.9	22.4	7.6
263 (-10)	175.0	23.9	7.3
258 (-15)	184.3	24.8	7.4
253 (-20)	203.6	27.2	7.5

*Same mobile phase as in Figure 5 was used.

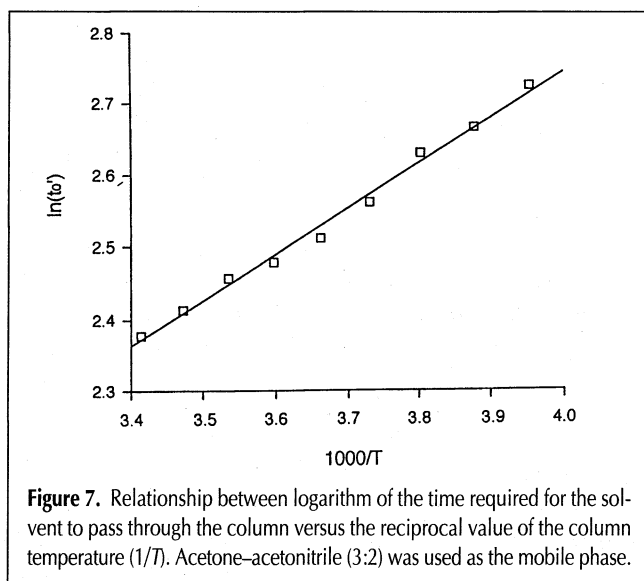


Figure 7. Relationship between logarithm of the time required for the solvent to pass through the column versus the reciprocal value of the column temperature ($1/T$). Acetone-acetonitrile (3:2) was used as the mobile phase.

Table III. Time Required to Detect the Solvent Peak and the Loading Shock

	-20°C		20°C	
	Solvent peak (min)	Loading shock (min)	Solvent peak (min)	Loading shock (min)
Acetone	7.24	7.26	6.67	6.67
	7.20	7.26	6.66	6.66
	7.27	7.29	6.64	6.68
	7.27	7.28	6.66	6.68
	7.24	7.24	6.66	6.68
	Mean	7.24	7.27	6.66
n-Hexane	13.15	7.12	10.07	6.68
	13.12	7.08	10.06	6.66
	13.11	7.14	10.03	6.67
	13.20	7.08	10.03	6.70
	13.26	7.14	10.03	6.70
	Mean	13.17	7.11	10.04

(Table III), which is less soluble in the mobile phase than acetone. However, in acetone, $t_0 = t_0'$ despite the temperature used (Table III). Acetone is generally used for the molecular species analysis of lipids. When it is used, we can neglect point c.

Meanwhile, as seen in Figure 7, $\ln t_0' \propto 1/T$ can be empirically derived. If we make this relationship into an equation by using $\Delta\mu_0/R$ as a coefficient and q_0 as a constant, the following equation can be obtained:

$$\ln t_0' = \Delta\mu_0/RT - q_0 \quad \text{Eq 45}$$

Based on the law of Martin (6), the following equations can be derived:

$$\ln t_{R1}' \propto \Delta\mu_{TG1}/RT \quad \text{Eq 46}$$

$$\ln t_{R2}' \propto \Delta\mu_{TG2}/RT \quad \text{Eq 47}$$

By using these equations and defining q_1 and q_2 as constants, Equations 48 and 49 can be obtained:

$$\ln t_{R1}' \propto \Delta\mu_{TG1}/RT - q_1 \quad \text{Eq 48}$$

$$\ln t_{R2}' \propto \Delta\mu_{TG2}/RT - q_2 \quad \text{Eq 49}$$

By substitution and rearrangement of Equations 45, 48, and 49, the following equations are obtained:

$$t_0' = e^{(\Delta\mu_0/RT - q_0)} \quad \text{Eq 50}$$

$$t_{R1}' = e^{(\Delta\mu_{TG1}/RT - q_1)} \quad \text{Eq 51}$$

$$t_{R2}' = e^{(\Delta\mu_{TG2}/RT - q_2)} \quad \text{Eq 52}$$

By substituting Equations 50–52 into Equation 39, we find that

$$\delta = 2 \frac{e^{(\Delta\mu_{TG1}/RT - q_1)} - e^{(\Delta\mu_0/RT - q_0)}}{e^{(\Delta\mu_{TG1}/RT - q_1)} + e^{(\Delta\mu_{TG2}/RT - q_2)}} \quad \text{Eq 53}$$

If we differentiate Equation 53 with respect to T ,

$$\begin{aligned} \frac{\partial \delta}{\partial T} = & [(\Delta\mu_0 - \Delta\mu_1) \{e^{(\Delta\mu_0 - \Delta\mu_1)/RT + q_1 - q_0}\} \\ & \{1 + e^{(\Delta\mu_2 - \Delta\mu_1)/RT + q_1 - q_2}\} + (\Delta\mu_2 - \Delta\mu_1) \\ & \{1 - e^{(\Delta\mu_0 - \Delta\mu_1)/RT + q_1 - q_0}\} \{e^{(\Delta\mu_2 - \Delta\mu_1)/RT + q_1 - q_2}\} \\ & / \{1 + e^{(\Delta\mu_2 - \Delta\mu_1)/RT + q_1 - q_2}\}^2 RT^2 / 2 \end{aligned} \quad \text{Eq 54}$$

The focus of our study is the resolution of adjacent peaks with close retention times. Therefore, $\Delta\mu_{TG2} - \Delta\mu_{TG1} = 0$. If this equation is substituted into Equation 54,

$$\begin{aligned} \frac{\partial \delta}{\partial T} = & [(\Delta\mu_0 - \Delta\mu_1) \{e^{(\Delta\mu_0 - \Delta\mu_1)/RT + q_1 - q_0}\} \\ & \{1 + e^{(\Delta\mu_2 - \Delta\mu_1)/RT + q_1 - q_2}\} / \{1 + \\ & e^{(\Delta\mu_2 - \Delta\mu_1)/RT + q_1 - q_0}\}^2 RT^2 / 2 < 0 \end{aligned} \quad \text{Eq 55}$$

should be true since $\Delta\mu_0 < \Delta\mu_{TG1}$. From Equations 54 and 55, the following two points pertaining to δ can be derived:

- Equation 55 demonstrates that when T decreases, δ increases between the adjacent two peaks (i.e., TG_2 and TG_1).
- However, this effect becomes moderate when the difference in retention times between the molecular species becomes significantly large (i.e., when $\Delta\mu_{TG2} - \Delta\mu_{TG1}$ is significantly large). Mathematically, when $\Delta\mu_{TG2} - \Delta\mu_{TG1}$ increases, the second term of the numerator in Equation 54 is offset so $\partial\delta/\partial T$ decreases. In fact, as illustrated in Figure 8, this was found to be true experimentally. For the term $\delta (= k_1/(k_1 + 1) =$

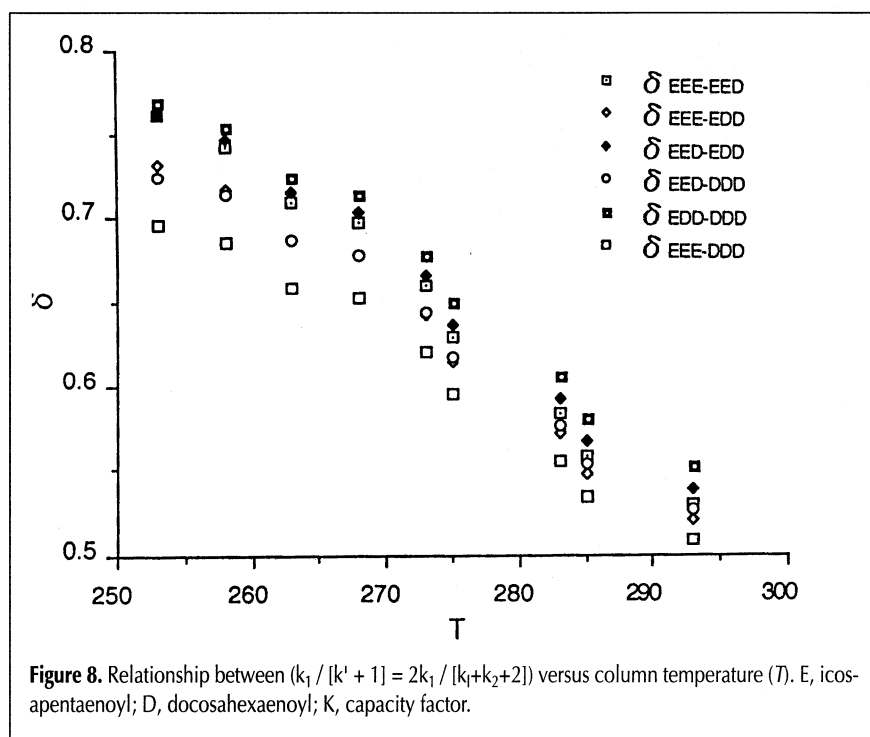


Figure 8. Relationship between $(k_1 / [k_1 + 1] = 2k_1 / [k_1 + k_2 + 2])$ versus column temperature (T). E, icosa-pentaenoyl; D, docosahexaenoyl; K, capacity factor.

$2k_1/(k_1+k_2+2)$), that is, the retention rate, we can conclude that the lower the T , the larger the value of δ becomes.

If we consider Equation 1 again,

$$R_s = (k_1/4)(\alpha - 1)\sqrt{N_1}/(k' + 1) \quad \text{Eq 56}$$

$$= (1/4)(\alpha - 1)\sqrt{N_1}\delta$$

we can deduce the following by summing up the explanation:

- Since $\partial\alpha/\partial T < 0$ in term $(\alpha - 1)$, it is obvious that when T decreases, α increases. Therefore, it is better to lower T pertaining to this term.

- In a higher T range, $\partial N/\partial T < 0$ should be true for N_1 . Therefore, N increases when T decreases. However, when T is below the critical temperature, $\partial N/\partial T > 0$ should be true. Therefore, in this case, when T decreases, N also decreases. T_c is the temperature that gives $\partial N/\partial T = 0$ and satisfies the following relationship:

$$\psi(T) = (C_d/u) - \frac{1/C_m d_p^2 u}{(1/C_e d_p + D_m / C_m d_p^2 u)^2} + (C_{sm} d_p^2 u / D_m^2) \quad \text{Eq 57}$$

where D_m is a function of T , and all others are constants under a certain analytical condition. In this study, -15°C is considered to be the value of T_c that gives the best value of N (Figure 4).

- In term $\delta (=k_1/[k' + 1] = 2k_1/[k_1 + k_2 + 2])$, $\partial\delta/\partial T < 0$ should be true. Therefore, when T decreases, δ increases. For this reason, it is better to lower T .

By summing up these points, we can conclude that terms $(\alpha - 1)$ and δ provide good resolution when T is low. However, when T reaches the critical temperature, $\sqrt{N_1}$ decreases and suppresses R_s . (Strictly speaking, $\partial R_s/\partial T = 0$ should be T_c). In fact, the critical temperature for R_s was between -15°C and -20°C in this study (data not shown).

We can conclude that the lowest temperature of the column without exceeding the critical temperature is best for the analysis of molecular species of TG rich in EPA and DHA by isocratic reversed-phase HPLC.

Unlike the analytical system introduced by Scott and Lawrence (8), we recognized the significant advantage of improving R_s through lowering column temperature. The only disadvantage that we found in lowering T is elevated retention times. Gradient elution may be useful in this system. Theoretical aspects of improving resolution of TG species with low melting points will be reported elsewhere.

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