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Analysis of plant oils by subcritical fluid chromatography using pattern fitting

Yasuhiro Funada, Yukio Hirata*

School of Materials Science, Toyohashi University of Technology, Toyohashi 441, Japan

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Abstract

A new method for analyzing plant oils was developed, which allows determination of fatty acid (FA) and triglyceride (TG) compositions of oils directly from their chromatograms without complete separation and peak identification. TGs of oils were separated by subcritical fluid chromatography using an octadecyl-silica column, carbon dioxide mobile phase and flame ionization detector. Observed chromatograms were compared with simulated ones, which were generated using FA compositions and predicted retentions of TGs on the assumption that FAs are combined with glycerol at random. FA compositions were determined by minimizing the differences between observed and simulated chromatograms through trial and error. Compared with GC analysis, relative errors of calculated FA compositions were less than 10% for main components (mol fraction$>$0.2). However, cocoa butter presented large errors even for main components because of the selective bonding of FAs to glycerol. Application of this method to the analysis of blended oils was also demonstrated, where FA compositions and mixing ratio were determined. © 1998 Elsevier Science B.V.

Keywords: Subcritical fluid chromatography; Pattern fitting; Fatty acids; Triglycerides

1. Introduction

The analysis of plant oils is important in the food industry and in nutrition since the properties of edible oils and fats depend on the composition of triglycerides (TGs). The analytical methods for oils and fats have been reviewed [1–3]. The analysis of oils and fats has usually been performed by transesterification of TGs into methyl esters followed by gas chromatographic (GC) analysis, but the method has some problems. The transesterification of highly unsaturated TGs is not always quantitative and information about actual compositions of TGs is lost. Few papers have reported on the correlation between FA and TG compositions [4,5]. Although the analyses of TGs have usually been performed by high-performance liquid chromatography (HPLC) with ultraviolet (UV) and refractive index (RI) detection, there exist some drawbacks in terms of sensitivity, and the complete separation of plant oils is very difficult because they are complex mixtures of TGs. Super- and sub-critical fluid chromatography (SFC and SubFC) have several advantages over GC and HPLC in the analysis of TGs including high efficiency and ability to separate highly unsaturated TGs which are nonvolatile and thermally labile. In addition, a flame ionization detector (FID), which is a universal detector, can be used when carbon dioxide is used as mobile phase.

In reversed-phase HPLC with octadecyl-silica

*Corresponding author.

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(ODS), the retention of TGs has been related to the equivalent carbon number (ECN) [6–9]. We previously reported that the concept of ECN can also be applied to SubFC when an ODS column is used with carbon dioxide mobile phase [10]. ECN was expressed as a function of temperature, so that the retention of TGs could be predicted at any temperature. Simulated chromatograms of oils were produced on the assumption that FAs combine with glycerol at random. The method was useful to optimize the separation and identify each TG.

In this study, it was confirmed that when FID is used, peak areas of simulated chromatograms are in good agreement with those of observed chromatograms for various oils. The results suggested that FAs are randomly combined to glycerol in many oils with some exceptions, and FA and TG compositions of plant oils can be determined directly from their chromatograms. For this purpose, a 'pattern fitting' method was examined, where FA compositions were determined by minimizing the differences between observed and simulated chromatograms. The method was successfully applied to the analysis of various plant oils. In addition, this method could be applied to the analysis of blended oils.

2. Experimental

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). An FID of a GC-6A gas chromatograph (Shimadzu, Kyoto, Japan) was placed after the UV detector and connected to a C-R4A integrator (Shimadzu). An integral restrictor for the FID was prepared from 30 μm I.D. fused-silica tubing and about 1/20 of the effluent was introduced into the FID. Samples were injected with a Rheodyne 7125 injector (Cotati, CA, USA) with a 20-μl loop. Injection volume was 5 μl throughout this work. In the experiments under subcritical conditions, 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). The column used was an L-column ODS (250 mm×4.6 mm I.D., 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 200 atm.

All plant oils were dissolved in dichloromethane in concentration of 0.5%. Plant oils used in this work were perilla, soybean, rapeseed, sesame, safflower, palm, and cocoa butter, which were obtained from Kishida (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. Saturated TGs, trilaurin (LaLaLa), trimyristin (MMM), tripalmitin (PPP), and tristearin (SSS), were obtained from Tokyo Kasei (Tokyo, Japan).

FA compositions of oils were determined using GC-14A gas chromatograph (Shimadzu) equipped with an FID. Each oil was converted into methyl esters in the following way: a weighted amount (0.1 g) of oil was placed in a flask and 3 ml of methanol and several droplets of sulfuric acid were added. The solution was refluxed in a water bath at 70°C for 1 h. Subsequently, the solution was cooled to room temperature, and 10 ml of water and 5 ml of dichloromethane were added. The dichloromethane layer was diluted 30-fold and analyzed by GC using splitless injection. Injection volume was 1 μl. The column used was an UA-CW capillary column (10 m×0.25 mm I.D., PEG-20M, 0.25 μm film thickness) obtained from Frontier (Kohriyama, Japan). The carrier gas was nitrogen, and injector and detector temperatures were held at 250°C and 300°C, respectively. Column temperature was held at 60°C for 2 min, and then programmed at a rate of 15°C/min to 250°C. FA compositions of various oils are listed in Table 1, where mol fractions were calculated using effective carbon number for FID detection [11]. Data analyses and calculations including pattern fitting were performed with Microsoft EXCEL.

3. Results and discussion

3.1. Retention prediction of TGs as a function of temperature

The method for predicting the retention of TGs in

Table 1
Mol fractions of fatty acids determined by GC and pattern fitting

FA	Method	Perilla	Soybean	Sesame	Palm	Safflower	Rapeseed	Cocoa
P	GC	0.064	0.114	0.123	0.485	0.072	0.048	0.294
	Pattern fitting	0.093	0.122	0.105	0.530	0.096	0.072	0.207
S	GC	0.032	0.035	0.055	0.037	0.021	0.015	0.343
	Pattern fitting	0.042	0.037	0.062	0.027	0.025	0.024	0.449
O	GC	0.210	0.226	0.394	0.385	0.132	0.573	0.334
	Pattern fitting	0.201	0.215	0.389	0.362	0.112	0.569	0.316
L	GC	0.147	0.550	0.428	0.093	0.775	0.259	0.029
	Pattern fitting	0.156	0.540	0.435	0.072	0.765	0.233	0.012
Ln	GC	0.547	0.075	N.D.	N.D.	N.D.	0.105	N.D.
	Pattern fitting	0.508	0.086	0.009	0.009	0.002	0.102	0.016

N.D.: not detected.

SubFC with a carbon dioxide mobile phase has been previously reported [10]. Equations used are as follows:

$$\log k' = a\,\mathrm{ECN} + b \quad (1)$$

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

where ECN is equivalent carbon number, CN is carbon number, N is the number of FAs in TG, E is the double bond coefficient, and the subscripts denote the type of FAs. Coefficients in these equations (a, b and E) were correlated to the reciprocal of the column temperature, so that the retention can be predicted at any temperature. Errors were less than 2.1% in the temperature range of 0 to 25°C. In this study, temperature range was extended to supercritical region up to 80°C. Although errors were increased (less than 4.6%), a fairly good agreement was obtained between observed and predicted retentions, suggesting this method can also be used in SFC region. Fig. 1 shows plots of predicted retentions for all TGs which can be produced from five TGs. In the subcritical region, the retention decreased with increasing temperature, as usually observed in liquid chromatography. In the supercritical region, on the other hand, the retention increased with increasing temperature, which is a typical behavior of SFC at constant pressure. Although selectivities largely changed with temperature in both regions, a complete separation was still difficult at any temperature. Since overall resolution was better in the subcritical region than in the supercritical

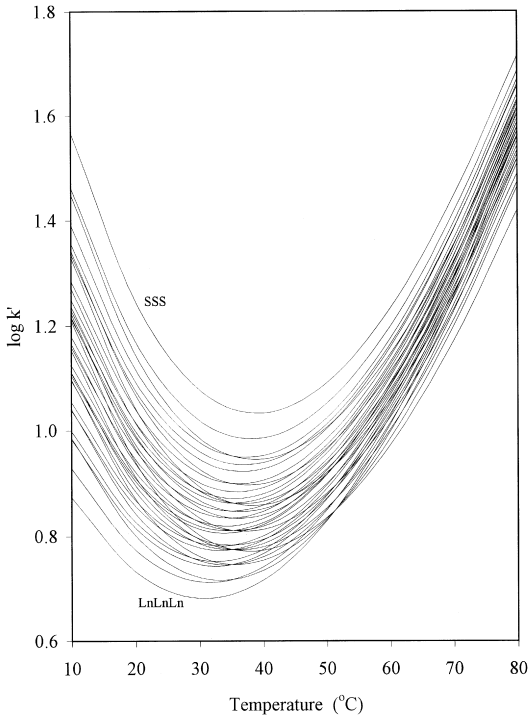

Fig. 1. Plots of predicted log k' of 35 TGs versus temperature. Retention prediction was performed under the following conditions. Column: L-column ODS, 5-μm particle, 250 mm×4.6 mm I.D. Mobile phase: carbon dioxide at a flow-rate of 3 ml/min. Outlet pressure: 200 atm. Detection: FID. Retentions were calculated for all TGs, which compose of palmitate (P, 16:0), stearate (S, 18:0), oleate (O, 18:1), linoleate (L, 18:2) and linolenate (Ln, 18:3).

region, further work was performed in the subcritical region.

3.2. Simulation of chromatogram using FA compositions determined by GC

Fig. 2 shows a comparison between observed and simulated chromatograms of soybean oil at 10°C. Observed chromatogram (Fig. 2A) was obtained using FID detection. Each TG can be identified by collecting fractions and separating them at different temperature, as demonstrated previously. Therefore, quantitative analysis of TGs and then FA compositions may be possible, but it would be a time consuming task. If the FA composition is known, identification of TGs is much easier. Simulated chromatogram (Fig. 2B) was produced on the assumption that FAs are randomly combined to glycerol. FA compositions were determined by GC after transesterification (see Table 1) and then TG compositions were calculated using following equation:

$$Y_{ABC} = nX_A X_B X_C \quad (3)$$

where X and Y are mol fractions for FA and TG, respectively, and subscripts denote the type of FAs. Values of n are 1 (for AAA), 3 (for AAB) or 6 (for ABC) because positional isomers cannot be resolved in the present work. Effective carbon number in FID detection, theoretical plate number ($N = 15\,000$), and hold-up time were taken into account and the peak shape was expressed as Gaussian distribution. Comparison between observed and simulated chromatograms shows good agreement. This suggests that FA and TG compositions of unknown oils can be determined by minimizing the differences between observed and simulated chromatograms. Such a method does not require complete separation and peak identification of TGs.

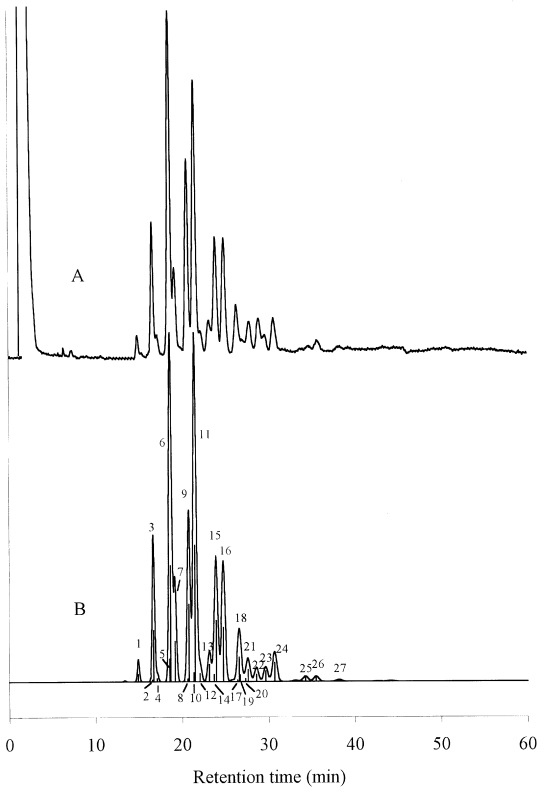

Fig. 2. Comparison between observed and simulated chromatograms for soybean oil at 10°C. (A) observed chromatogram; (B) simulated chromatogram from FA composition determined by GC (bar graph represents relative abundances of TGs). Column: L-column ODS, 5-μm particle, 250 mm×4.6 mm I.D. Mobile phase: carbon dioxide at a flow-rate of 3 ml/min. Outlet pressure: 200 atm. Detection: FID. Key: (1) LnLnL (2) LnLnP (3) LnLL (4) LnLnO (5) LnLP (6) LLL (7) LnLO (8) LnPP (9) LLP (10) LnOP (11) LLO (12) LnOO (13) LPP (14) LnLS (15) LOP (16) LOO (17) LnSP (18) LLS (19) OPP (20) LnOS (21) OOP (22) OOO (23) LSP (24) LOS (25) OSP (26) OOS (27) LSS.

3.3. Pattern fitting between observed and simulated chromatograms

Comparison between observed and simulated chromatograms were performed with pattern fitting, and various important parameters were examined. For this purpose, the extent of overlapping (EO) of two chromatograms was defined as follows:

$$EO(\%) = 100[1 - (\sum|H_{obs} - H_{sim}|/2\sum H_{obs})] \quad (4)$$

where H is peak height at each data point and an EO value of 100 means perfect fitting. FA compositions were determined as a set of values which give a maximum EO value.

Initial mol fraction of each FA was set at 0.2 as a matter of convenience, since varying the initial compositions (keeping their sum to be one) did not affect the results. In this case, H_{sim} in Eq. (4) is a function of FA compositions and other parameters

described in Section 3.2. These relations were constructed using EXCEL. Then FA compositions were selected as variables and determined as a set of values which give a maximum EO value using Solver Add-in of EXCEL. The data interval of chromatograms was 0.02 min (1500 points for analysis time of 30 min). Unnecessary peaks such as solvent peak were eliminated from observed chromatogram and the level of baseline was corrected to zero. Total peak area of observed chromatogram was normalized ($\Sigma H_{obs} = 50$ because the data interval is 0.02 min).

Retention reproducibility is a very important factor when using pattern fitting. Relative standard deviations (R.S.D.s) for retention times of main components of soybean oil at 25°C were less than 0.2% ($n = 4$) within a day, but 2.3% ($n = 4$) over a week. The value is relatively good, but large errors in FA compositions were generated. Increased R.S.D. values may be due to the slight fluctuations of column temperature and flow-rate. Therefore, temperature and hold-up time used for prediction of retention times were corrected so as to minimize the differences between predicted and observed retention times. Known TGs in oils can be used, but it is not practical because of difficulty of peak assignment in the analysis of unknown oils. Therefore, saturated TGs (LaLaLa, MMM, and SSS) were selected as reference markers for correction. They were spiked into oil samples which did not contain them or separated just after the separation of oil sample. With this method errors in retention time were significantly decreased, for example less than 0.7% for main components of soybean oil.

Both selectivity and retention drastically changed with temperature as shown in Fig. 1. In the subcritical region, a better resolution was obtained at a lower temperature, but analysis time was longer. Therefore, the effect of temperature on the pattern fitting method was examined for the analysis of soybean oil at temperatures of 10, 15, 20, and 25°C. R.S.D. values ($n = 4$) of calculated mol fractions of FAs were less than 6.7%. The results indicate that temperature is not an important factor in this method.

3.4. Application of pattern fitting to various oils

FA compositions of various oils were determined at 25°C using the pattern fitting method, where the most retained TG (SSS) was eluted at 24 min. The results and relative errors from the compositions determined by GC are summarized in Table 1. Relative errors are plotted in Fig. 3. They were less than 10% for main components (more than 0.2 mol fraction), but absolute errors were less than 4.5%. However, cocoa butter presented large errors even for main components because of the selective bonding of FAs to glycerol [12]. From the results, bonding of FAs to glycerol seems to be relatively random in many oils.

Fig. 4 shows plots of EO values for various oils. The broken line is a comparison between observed and simulated chromatograms from FA compositions determined by GC. This can account for the degree of selective bonding of FAs to glycerol. Cocoa butter with lower EO value is clearly different from the others because of the highly selective bonding of FAs to glycerol. The solid line is a comparison between observed and simulated chromatograms obtained by pattern fitting. The shape is similar to the broken line, indicating the pattern fitting method has been successfully applied. Except for cocoa butter, these oils have large EO values, but the

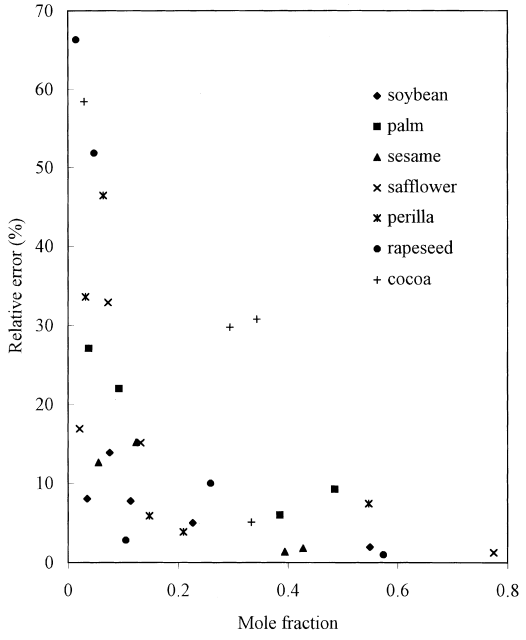

Fig. 3. Relative error of FA composition determined by pattern fitting to FA composition determined by GC.

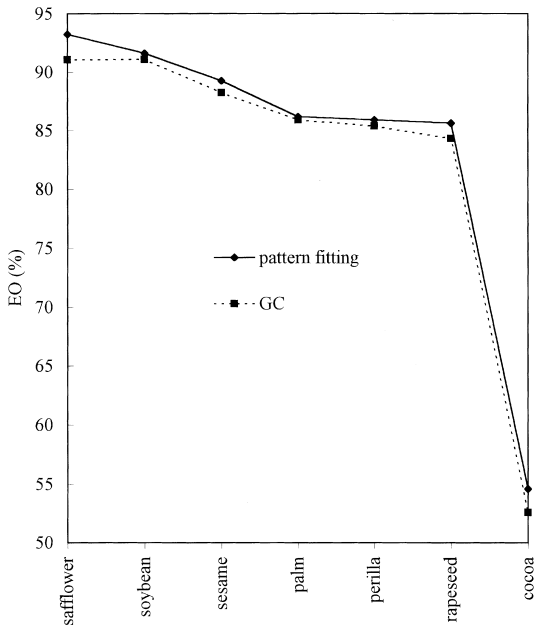

Fig. 4. Plots of EO values for various oils. Broken line: comparison between observed and simulated chromatograms from FA compositions determined by GC. Solid line: comparison between observed and simulated chromatograms obtained by pattern fitting.

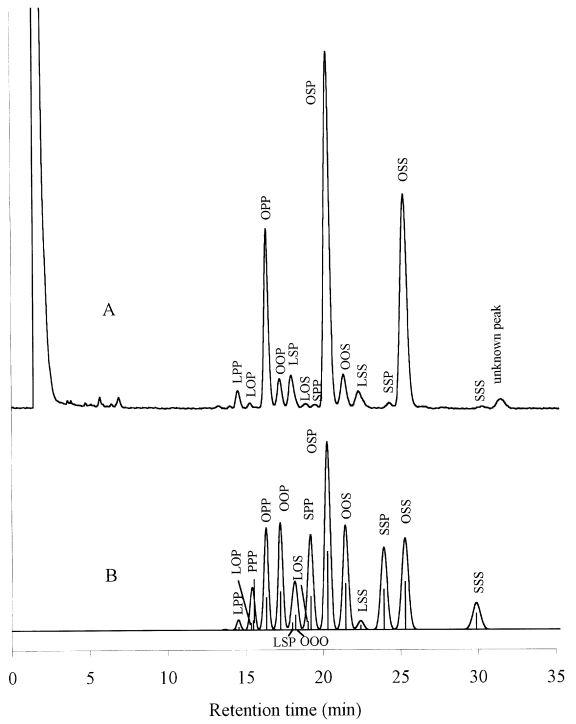

Fig. 5. Simulated and observed chromatograms for cocoa butter at 18°C. Conditions as in Fig. 2. (A) observed chromatogram; (B) simulated chromatogram from FA composition determined by GC.

values are slightly different from each other. This may indicate the selectivity differences between these oils. Palm oil has smaller EO value and larger errors in FA composition than soybean oil, suggesting TGs in palm oil are formed more selectively than in soybean oil. With this pattern fitting method, FA compositions could be determined within absolute errors of ~2% provided that FAs are bonded to glycerol at random. Since errors may arise from various sources including plate number, retention time and peak shape, further study is required.

Fig. 5 shows observed and simulated chromatograms for cocoa butter which is the most selectively formed. Separation was carried out at 18°C, where best separation can be expected from Fig. 1. Almost all the TGs are resolved. The simulated chromatogram was produced from FA compositions determined by GC. The tendency to form TGs containing one unsaturated FA (LPP, LSS, LSP, OPP, OSP, OSS) can be clearly seen. In other words, TGs containing two unsaturated FAs (LOP, OOS. etc.) and saturated TGs (SSS, PPP, etc.) are much less than would be expected in random bonding. Such an observation has been confirmed with high temperature capillary GC [13]. This behavior can be attributed to the selective bonding of unsaturated FAs to the *sn*-2 position of glycerol [1,12]. This information may be useful for characterization of oils. Further study is under way without using the assumption of random bonding, with which more detailed information about selectivity will be obtained.

3.5. Analysis of blended oils

Plant oils are often blended to obtain compositions to suit its usage. FA composition of blended oil can be easily determined, however information about processing and TG compositions are lost. Here, rapeseed oil and soybean oil were blended in various ratios (Table 2) to prepare test sample for the pattern fitting method. Chromatograms of blended oil III (soybean–rapeseed, 43:57, v/v) are shown in Fig. 6. Fig. 6A shows chromatogram at 20°C. Fig. 6B is simulated chromatogram by pattern fitting, where

Table 2
Mol fractions of fatty acids determined by GC and pattern fitting in blended oils composed of soybean and rapeseed oils

Sample no. of blended oils[a]	FA	GC	Pattern fitting			
			One set[b]	Two sets[c]		
				Oil A	Oil B	Average[d]
I	P	0.11	0.13	0.15	0.06	0.13
	S	0.03	0.05	0.04	0.04	0.04
	O	0.26	0.23	0.16	0.56	0.26
	L	0.52	0.52	0.58	0.24	0.49
	Ln	0.08	0.07	0.07	0.10	0.08
	Determined mixing ratio			75	25	
II	P	0.09	0.11	0.14	0.04	0.10
	S	0.03	0.05	0.06	0.01	0.04
	O	0.37	0.30	0.18	0.67	0.37
	L	0.42	0.46	0.54	0.19	0.41
	Ln	0.09	0.08	0.08	0.09	0.08
	Determined mixing ratio			62	38	
III	P	0.08	0.07	0.16	0.04	0.10
	S	0.02	0.03	0.05	0.02	0.03
	O	0.43	0.49	0.19	0.66	0.43
	L	0.39	0.30	0.53	0.17	0.35
	Ln	0.08	0.11	0.07	0.11	0.09
	Determined mixing ratio			50	50	
IV	P	0.06	0.07	0.16	0.03	0.08
	S	0.02	0.02	0.07	0.01	0.04
	O	0.50	0.54	0.20	0.70	0.49
	L	0.32	0.28	0.49	0.18	0.31
	Ln	0.10	0.09	0.08	0.08	0.08
	Determined mixing ratio			42	58	

[a] Prepared mixing ratio of soybean oil and rapeseed oil, I (89:11), II (57:43), III (43:57), IV (21:79).
[b,c] Details of pattern fitting methods are described in the text.
[d] Calculated from FA compositions of oil A and oil B and determined mixing ratio.

one set of FA compositions was determined in the same way as in the pattern fitting of pure oils discussed above. Large differences can be seen between two chromatograms. FA compositions determined with this method for blended oils with various mixing ratios are listed in the column denoted by 'one set' in Table 2. Fig. 6C is also a simulated chromatogram, but with the assumption that the oil is composed of two pure oils, two sets of FA compositions and their mixing ratios were simultaneously determined. In the pattern fitting two distinct TG compositions were calculated from each FA composition and then added using the mixing ratio. A very good agreement between two chromatograms (A and C) can be seen. Determined values for two sets of FA compositions (oil A and oil B), the average compositions and the mixing ratios for various blended oils are listed in the columns denoted by 'two sets' in Table 2. It should be noted that FA compositions of oil A and oil B are similar to those of soybean and rapeseed oils, respectively, shown in Table 1.

EO values are plotted against mixing ratio in Fig. 7. The broken line is a comparison between observed and simulated chromatograms, where one set of FA compositions was determined (i.e., comparison between A and B in Fig. 6). EO values approach to those of pure oils as one of components is increased (I and IV). However, EO values of intermediate mixtures (II and III) are smaller. This is natural because FAs cannot be evenly distributed in blended oils, even if FAs in each oil are bonded to glycerol at

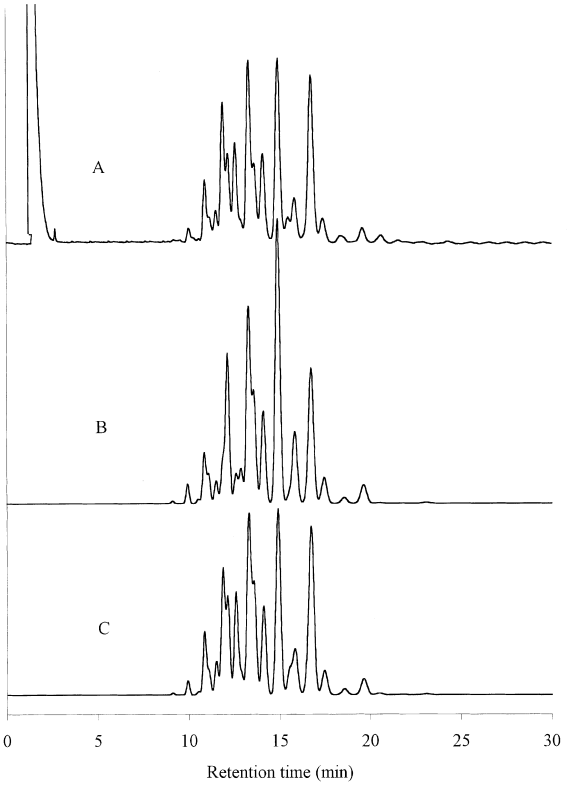

Fig. 6. Simulated and observed chromatograms for blended oil III (soybean–rapeseed, 43:57, v/v) at 20°C. Conditions as in Fig. 2. (A) observed chromatogram; (B) simulated chromatogram from one set of FA composition determined by pattern fitting; (C) simulated chromatogram from two sets of FA compositions and mixing ratio determined by pattern fitting.

random. Accordingly, errors of FA compositions are smaller for I and IV (absolute errors less than 4%) than II and III (less than 9%) as shown in Table 2.

The solid line is a comparison between observed and simulated chromatograms, where two sets of FA compositions and mixing ratios were determined (i.e., comparison between A and C in Fig. 6). EO values are greatly improved resulting in a decrease in errors in FA compositions (see the column 'average' in Table 2). Errors in FA compositions are comparable to those for pure oils and mixing ratios can be determined with relatively small errors for approximately equivalent mixture, allowing the analysis of each oil in blended oils. EO values with this method are higher than those of pure oils (see Fig. 4). FA compositions of pure oils could be more accurately determined with this method, because TGs in oils are

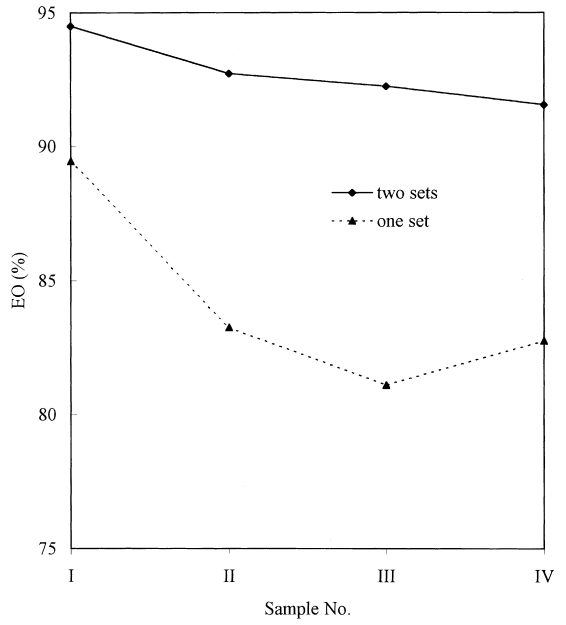

Fig. 7. Plots of EO values for blended oils listed in Table 2. Broken line: comparison between observed and simulated chromatograms from one set of FA compositions determined by pattern fitting. Solid line: comparison between observed and simulated chromatograms from two sets of FA compositions and mixing ratios determined by pattern fitting.

selectively formed. This method was applied to commercially available edible oil (salad oil). The results are summarized in Table 3. In this case, using one set of FA composition also yielded similar FA compositions, but agreements between chromatograms was poor. A better agreements was obtained by postulating that salad oil is composed of two oils. The calculated FA compositions suggested that salad

Table 3
Mol fractions of fatty acids determined by GC and pattern fitting in salad oil

FA	GC	Pattern fitting			
		One set	Two sets		
			Oil A	Oil B	Average
P	0.07	0.08	0.16	0.04	0.10
S	0.02	0.02	0.05	0.02	0.04
O	0.46	0.51	0.22	0.70	0.45
L	0.36	0.30	0.50	0.16	0.34
Ln	0.09	0.09	0.07	0.08	0.07
Determined mixing ratio			52	48	

oil is a mixture of soybean oil (oil A) and rapeseed oil (oil B). Mixing ratio was determined to be (48:52) for rapeseed and soybean oil.

4. Conclusions

A pattern fitting method for analyzing plant oils was developed. FA and TG compositions of plant oils could be determined directly from their SubFC chromatograms; complete separation and peak identification of TGs are not required. Compared with GC analysis, relative errors of FA compositions were less than 10% for main components (mol fraction > 0.2) for various oils. Furthermore, the mixing ratio as well as the FA compositions of blended oils can be determined.

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