RESULTS AND DISCUSSION

Yield and characteristics of the sample oils are shown in Table I. Each achene sample of the family Urticaceae had a lower oil content (11-18%), as compared to the 2 seed samples of the family Liliaceae (22 and 29%). UV and IR examinations of the oils revealed no evidence for conjugated unsaturation or unusual functional groups.

Fatty acid profiles of the oils, based on GLC analyses of their methyl esters, are given in Table II. All the oils were characterized by the presence of high levels of linoleic acid (80.2-83.5%). Another remarkable feature of these oils was the predominance of unsaturated components (91.0-93.0%) consisting exclusively of C_{18} acids, except for the *H. longipes* oil with 0.1% of C_{16}-monoenoic acid.

ACKNOWLEDGMENTS

Experimental assistance was provided by K. Yamada.

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[Received April 9, 1981]

High Performance Reversed Phase Chromatography of Natural Triglyceride Mixtures: Critical Pair Separation

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ABSTRACT

The separation of the critical triglyceride pairs C48:0, C50:1, C54:3*ccc* and C54:3*ttt* as well as C54:2, C52:1 and C50:1 has been accomplished without the aid of any interacting ion such as silver. A theoretical carbon number (TCN) for the unsaturated triglycerides can be calculated from the carbon number (CN) and capacity factor (k') relationship of the saturated triglycerides, and used to predict the separation of critical pairs. A mathematical equation was derived for the identification of not only the triglycerides by their carbon number and number of double bonds but also the possible acyl groups present in these triglycerides. The pattern of triglyceride elution sequence within each triglyceride category with the same equivalent carbon number (ECN) starts with the triglyceride with the highest number of double bonds and terminates with those with the lowest number of double bonds, with the lower ECN triglycerides eluting ahead of those with higher ECN. A possible mechanism for the separation of these triglycerides on highly efficient columns packed with 5 μ silica bonded with the octadecyl stationary phase is postulated.

INTRODUCTION

The increasingly efficient separation of individual triglycerides present in fats and oils which can now be carried out is gradually increasing the understanding of their structural composition. The availability of such data would facilitate the understanding of triglyceride biosynthesis and deposition in plant and animal cells.

Increasingly efficient separation of triglycerides by high performance reversed-phase chromatography (HPRC) has been achieved on μ-Bondapac C18 (1-5), Spherosorb 5-ODS (6), Vydac (6), Zorbax (7,8), Supelcosil LC-8, Supelcosil LC-18 and Partisil ODS-2 (8) column packings using various mobile phases. Triglyceride separations have been attempted with silica columns, but satisfactory separations (9) were not achieved. Mobile phases containing silver nitrate (10) have aided the separation of saturated and unsaturated triglycerides where highly efficient packings were not available. However, argentation liquid chromatography showed a lack of reproducibility of k' value. Furthermore, possible silver mirror formation on the detector cell windows remains a strong deterrent toward general use of this type of approach. The effects of changes in mobile phase polarity and composition on the separation and resolution of triglycerides as well as the characteristics of the packing (e.g., particle size, bonded chain length, percent coverage) have also been reported (8). In the present study, the separation of critical pairs and triglyceride isomers has been accomplished with the aid of highly efficient octadecyl bonded column packings operated in the reversed phase mode. A possible mechanism for the separation of triglyceride critical pairs by reversed-phase chromatography is postulated.

METHODS

The instrument used consisted of a Tracor 995 isochromatographic pump (Tracor Instruments, Austin, TX), a Rheodyne loop (20 μL) injector (Model 7120), and a Waters R401 Differential Refractometer Detector (Waters Assoc., Milford, CT). Separations were recorded with the aid of a Hewlett Packard 3385A electronic integrator (Hewlett Packard, Palo Alto, CA) at a chart speed of 0.1-0.2 cm/min. Retention times were automatically printed by the recording integrator. Two 250 mm x 4.6 mm commercially packed columns from different manufacturers were used in this study: a Supelcosil LC-18 column with a 5-μ octadecyl bonded spherical silica (Supelco, Supelco Park, Bellefonte, PA) and a Zorbax ODS column with octadecyl bonded silica of 6-7-μ diameter (DuPont Co., Wilmington, DE). Mixtures of analytical grade acetone and glass-distilled acetonitrile (63.6:36.4 [v/v]) were used as the mobile phase. Triglycerides used as standards were obtained from Nu-Chek-Prep (Elysian, MN) and Supelco (Supelco, Bellefonte, PA). Triglycerides were solubilized in tetrahydrofuran (THF) or acetone at 100 mg/mL for each triglyceride. Vegetable oils were solubilized in THF at 200-250 mg/mL.

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RESULTS AND DISCUSSION

During the analysis and separation of triglycerides, difficulties in obtaining superior resolution arise as a result of the complex mixtures of varying triglyceride structure caused by varied acyl components. One of these difficulties is the formation of "critical pairs" which has made it more difficult to separate certain methyl esters from each other as well as their triglycerides on reversed-phase chromatography using 5 μ octyl or 10 μ octadecyl bonded phases. Critical pairs have been found to have close behavior on reversed-phase chromatography in spite of the difference in chain lengths, number of double bonds, and geometrical configuration. Critical pairs, therefore, have been defined as those structures with an equivalent carbon number (ECN):

$$ECN = CN-2n,$$

where CN = actual carbon number
n = number of double bonds per molecule.

Thus oleic and palmitic acids are critical pairs because they have the same ECN (18-2=16). Linoleic and myristic acids, as well as linoleic and lauric acids, are therefore also critical pairs because they have an equivalent ECN of 14:0 and 12:0, respectively.

Triglycerides are also critical pairs if they have the same ECN. This includes critical pairs which contain one or more critical acyl groups on the glycerol such as triolein (54:3), palmityldiolein (52:2), oleyldipalmitin (50:1) and tripalmitin all having the same ECN of 48:0 (CN:n). The triglycerides SOO (54:2) and SOP (52:1) are critical pairs with SPP (50:0). (The designation SOO, SOP, e.g., does not imply the triglyceride SOO but a mixture of all isomers: SOO, OSO and OOS.)

Positional isomers of triglycerides also are critical pairs because they have the same acyl groups but differ in the position of these acyl groups. Triglycerides containing geometrical isomers also form critical pairs; they differ only in the configuration of the double bond whether *cis* or *trans*. Oleic and elaidic acids can therefore be considered critical pairs because they have the same ECN although oleic acid has a *cis* double bond whereas elaidic acid has the *trans* configuration. This separation of critical pairs requires optimization of mobile phase composition and highly efficient stationary phases that have enough theoretical plates to resolve such critical pairs into their individual components.

The separation of triglyceride critical pairs was accomplished employing a mobile phase of acetone/acetonitrile at a ratio of 63.6:36.4 (v/v). A homologous series of saturated triglycerides were well resolved with both Supelcosil LC-18 (Fig. 1a) and Zorbax ODS (Fig. 1b) columns. Baseline resolution of the critical pairs of triglycerides with ECN of 48:0 and 50:0 was also achieved in the same chromatogram under isocratic conditions. A good resolution of the triglyceride critical pairs of OOO (54:3), POP (50:1) and PPP (48:0) is shown in Figure 1, as well as the separation of SOO (54:2), SOP (52:1) and SPP (50:0). The separation of triglycerides from trilaurin (36:0) to SOP (52:1) required 75 min on the Zorbax ODS column, and the separation of triglycerides up to SSO (54:1) required less than 60 min on a Supelcosil LC-18 column; lower k′ values and sharper bands were obtained with the latter column. The relationship between the capacity factor k′ vs the carbon number of the saturated triglycerides separated on both columns is shown in Figure 2. The data obtained indicated that the elution of SSS (54:0) from the Zorbax column would require more than 30 k′ values and about 20 k′ on the Supelcosil LC-18 column.

Triglycerides prepared from geometrical isomers have also been resolved. Triolein (54:3*ccc*) was separated from trielaidin (54:3*ttt*) on both the Supelcosil LC-18 (Fig. 3a) and Zorbax ODS (Fig. 3b) columns. A comparison of the critical pair behavior of the triglycerides with ECN of 48:0 in these separations shows that EEE (54:3*ttt*) has the same k′ value as POP (50:1) indicating that, using this system, 3 *trans* double bonds are equivalent to 1 *cis* double bond.

The theoretical carbon number (TCN) of any triglyceride can be determined from a plot of k′ vs carbon number of the corresponding saturated triglycerides by extrapolation from a known k′ value (Table I). Thus, for any saturated triglyceride, the TCN is equal to the actual carbon number.

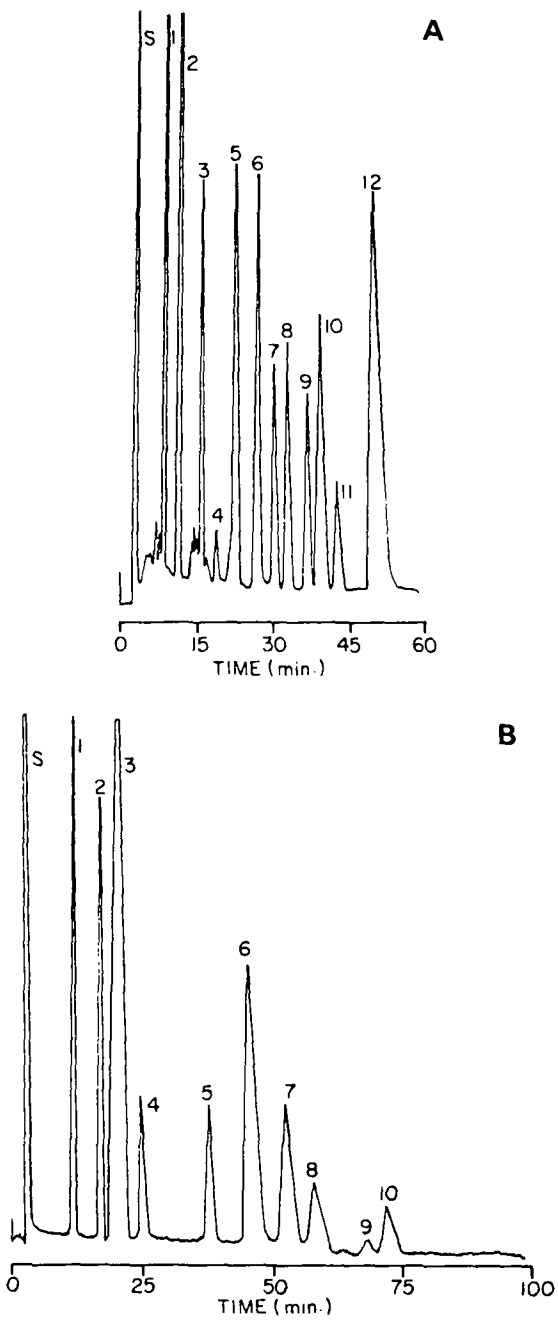

FIG. 1. Separation of triglyceride standards on 250 X 4.6 mm (a) Supelcosil LC-18; (b) Zorbax ODS, using acetone/acetonitrile, 63.6: 36.4 (v/v), as a mobile phase at a flow rate of 1 mL/min. Triglycerides: (1) LaLaLa (36:0); (2) tridecanoin (39:0); (3) MMM (42:0); (5) tripentadecanoin (45:0); (6) triolein (54:3); (7) POP (50:1); (8) PPP (48:0); (9) SOO (54:2); (10) SPO (52:1); (11) SPP (50:0); and (12) SOS (54:1).

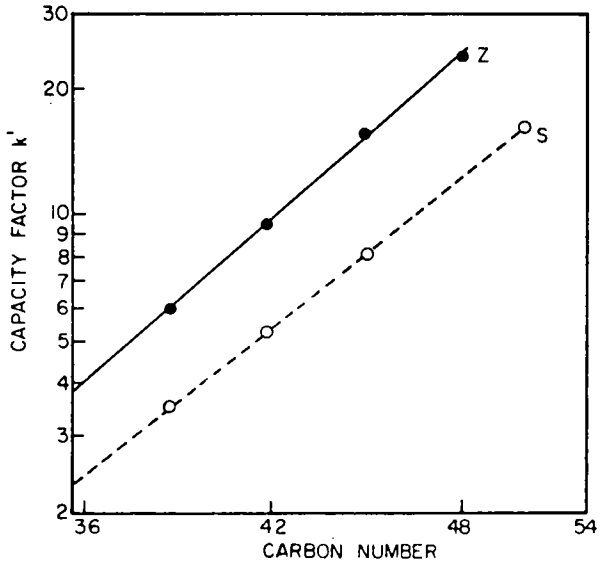

FIG. 2. Carbon number of saturated triglycerides vs their capacity factor k' on Supelcosil LC-18 (S) and Zorbax ODS (Z).

The theoretical carbon number can also be calculated from the following formula:

$$TCN=ECN-(\Sigma_1^3 U_i), \quad [1]$$

where TCN = theoretical carbon number; ECN = equivalent carbon number; U_i = a factor determined experimentally for several fatty acids and was found to be: 0.6-0.65 for oleyl, 0.7-0.8 for linoleyl, 0.2 for elaidyl acyl groups and 0.0 for saturated acyl groups; $\Sigma_1^3 U_i$ = the total U_i of individual fatty acids present in the triglyceride, e.g., the TCN of POL = 46.0-[0 (for palmityl) + 0.6 (for oleyl) + 0.7 (for linoleyl)] = 46.0-1.3 = 44.7.

During the analysis of triglyceride mixtures, it is possible to identify triglycerides by their ECN, or their carbon number and double bonds. In addition, it is feasible to identify their acyl components as well from their k' and TCN. For example, if a triglyceride peak has a TCN of 44.8 and ECN of 46.0, respectively, then: $\Sigma_1^3 U_i$ = 46.0-44.8 = 1.2. The combination of fatty acids in triglyceride with $\Sigma_1^3 U_i$ of 1.2 would be 2 oleic, and 1 myristic acid. Thus, the most probably triglyceride with a TCN of 44.8 and ECN of 46.0 would be MOO (0 + 0.6 ± 0.6).

This calculation also allows one to predict the feasibility of separating 2 components such as OOO and OSL which have the same carbon number and number of double bonds (TCN of SOL = ECN-[U_S+U_O+U_L] = 48.0-[0+0.6+0.7] = 46.7 and therefore, OOO = 48.0-[1.8] = 46.2). The differences between the TCN of OOO and SOL indicate that there is a possibility of separating these 2 critical pairs. However, this difference can decrease or increase, depending on the solvent system used as the mobile phase and the triglyceride mixture analyzed, since the TCN of the same triglycerides would differ slightly if they were present in a complex mixture due to mutual solubility and affinity factors.

The results of this study suggest a possible pattern of triglyceride separation by high efficiency reversed-phase chromatography. The separation of a triglyceride mixture by a high efficiency octyl bonded reversed phase column is by equivalent carbon number. The same mixture is separated by molecular weight or carbon number when gas liquid chromatography is used and according to the number of double bonds in each triglyceride by argentation thin layer chromatography. The equivalent carbon number or

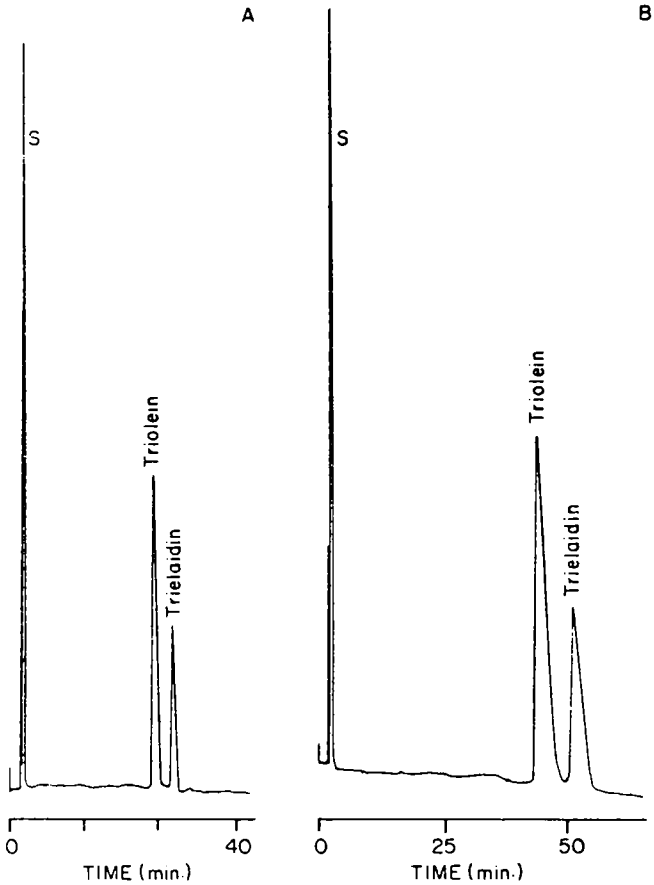

FIG. 3. Separation of *cis-trans* triglyceride isomers: (A) on Supelcosil LC-18; (B) on Zorbax ODS. Conditions as in Fig. 1.

carbon number and degree of unsaturation for triglycerides obtained in this way can be correlated with ECN by means of a "triglyceride separation triangle" (Fig. 4). The same mixture of triglycerides, when separated on an octadecyl bonded column with nonaqueous mobile phase appears to follow a combination of the reversed-phase mode of separation and that obtained by gas liquid and argentation chromatography. The order of elution from the octadecyl bonded phase column is reversed during liquid chromatography on the bonded phase with nonaqueous solvents compared to the other modes of separation mentioned

TABLE I

Theoretical Carbon Number of Unsaturated Triglycerides Deduced from k' vs Carbon Number of Saturated Triglycerides (Standards Curve)

Triglyceride	C:m[a]	ECN[b]	TCN[c]
LLL	54:6	42:0	39.6
MOM	46:1	44:0	43.4
PPL	50:2	46:0	45.2
OOO	54:3	48:0	46.2
POO	52:2	48:0	46.8
POP	50:1	48:0	47.4
EEE	54:3	48:0	47.4
SOO	54:2	50:0	48.8
SPO	52:1	50:0	49.4
SSO	54:1	52:0	51.4

[a]Actual carbon number:number of double bonds in the molecule.
[b]Equivalent carbon number.
[c]Theoretical carbon number.

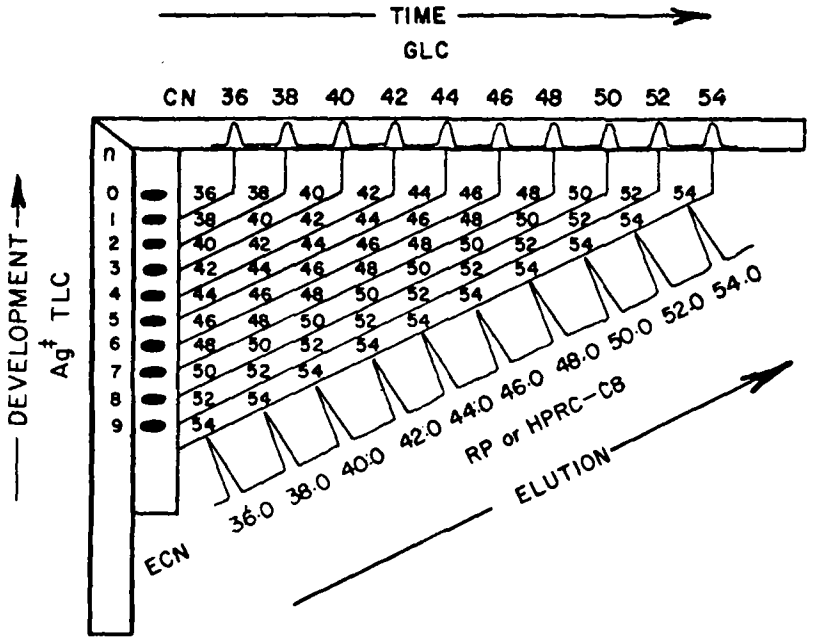

FIG. 4. Triglyceride Separation Triangle "TST": the relationship of the different chromatographic methods to each other in triglyceride analysis.

above and the triglycerides present in the mixture are separated according to their carbon number and total unsaturation. Results of this study indicate that, at lower ECN, the k′ differences between critical pairs are smaller and therefore their resolution is more difficult.

A simple hypothesis for the mechanism of critical pairs separation on very high efficiency octadecyl bonded columns can be suggested and illustrate the potential of this technique in lipid analysis and separation.

Critical pairs of triglycerides travel together through the bonded column because of their similar characteristics on reversed-phase packings (assuming no treatment of the mobile phase to enchance the elution of one triglyceride over another). Upon contact and interaction with the long chain hydrocarbon stationary phase, and among the triglycerides with the same ECN, the triglycerides containing the more saturated acyl groups will tend to travel more slowly due to their higher affinity with the stationary phase. Those triglycerides with more unsaturated acyl groups will have a shorter relative residence time due to decreased affinity with the stationary phase. This can be more clearly illustrated in the case of methyl oleate and methyl palmitate. These esters will not separate easily on a 5-μ octyl bonded phase. With an octadecyl bonded phase with small particle size packing, oleate will have a shorter residence time than palmitate which in turn results in a shorter retention time and thus will elute faster. This preferential retention of palmitate by the packing is because the saturated hydrocarbon chain of methyl palmitate (Fig. 5a) will be more compatible with the saturated bonded hydrocarbon chain than the oleate chain due to the presence of the double bond which tends to decrease the attraction between the 2 chains (Fig. 5b, c). Thus, carbons 9 and 10 on both chains (Fig. 5b) or carbon number 10 on the oleate chain and carbon 18 on the octadecyl chain (Fig. 5c) will not have the same attraction as do the other carbon atoms in both chains; this results in decreasing the relative residence time by the equivalent of 2 units from 18 to 16 units with the end result that, at this point, palmitate and oleate have the same relative residence time of 16. This is equal to the carbon number of palmitate and thus both methyl esters will not separate at this point. However, the repulsion between the hydrocarbon stationary phase and the oleate chain increases as the result of the presence of the double bond with the result that the distance between carbons 8 and 11 on both chains (Fig. 5b) and between carbons 8, 9, 11 and 12 on the oleate chain and 8, 9, 10 and 11 on the hydrocarbon chain of the stationary phase (Fig. 5c) will increase, resulting in a looser attraction of these carbons, and thus, shorter relative residence time than 16. If we assume that the relative retention time of palmitate is 16 and if we assume that the repulsion caused by the double bond is 2 and the further decrease in attraction as a result of all other carbons is U_O (for oleate), then the oleate relative residence time will be:

$$[(\#\text{ of carbon atoms}) - (\text{carbon equivalent of c=c}) - U_O] = 18\text{-}2\text{-}0.6 = 15.4, \quad [11]$$

where U_O is the sum of the effect of polarity of the double

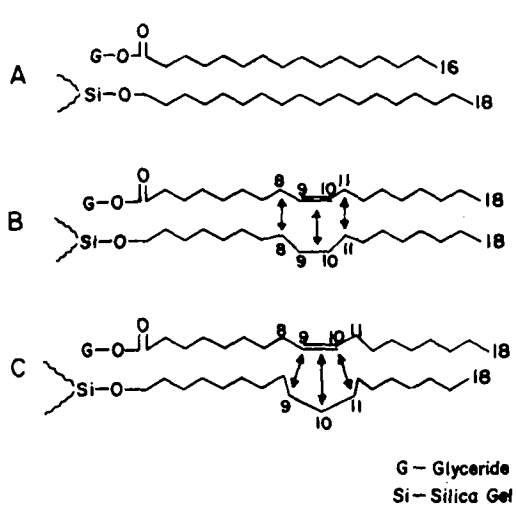

FIG. 5. The possible mechanism of triglyceride retention on HPRC. (A) Palmitate; (B&C) oleate; (G) glyceride; (Si) silica gel base.

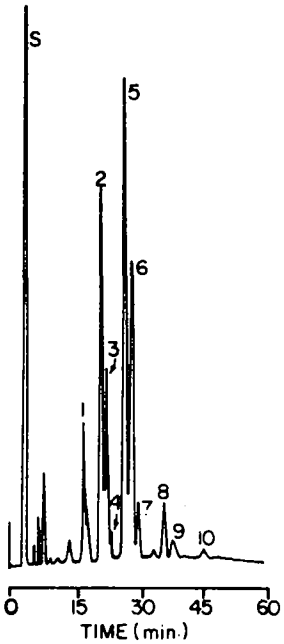

FIG. 6. Separation of olive oil triglycerides. Conditions as in Fig. 1a. Triglycerides present are (1) LOL, (2) LOO, (3) PLO, (4) PLP, (5) OOO, (6) POO, (7) POP, (8) SOO, (9) SOP and (10) SOS.

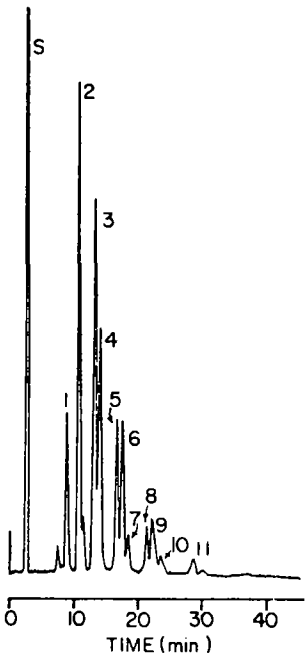

FIG. 7. Separations of soybean oil triglycerides. Conditions as in Fig. 1a. Triglycerides are (1) LLLe, (2) LLL, (3) LOL, (4) LLP, (5) LOO, (6) PLO, (7) LPP, (8) SOL, (9) SPL, (10) SOM and (11) SSL.

bond and the decrease in the affinity of the adjacent carbons to their counterparts in the bonded stationary phase. If the relative residence time of the triglycerides on the column packing is defined as TCN, we find that this equation is the same as Equation I which was deduced experimentally. Other decreases in the relative residence time for other molecules can also be explained. The value of U will decrease with the number of double bonds per molecule or decrease its chain length.

Incorporation of oleate and palmitate as different combinations in triglycerides would result in the formation of different critical pairs that can be separated from each other according to this mechanism. Thus if we assume that a triglyceride mixture formed from only oleic and palmitic acids is composed of OOO, POO, POP and PPP, then by calculation of the TCN we can determine if it may be possible to separate them:

$$\text{TCN of OOO} = (18\text{-}2\text{-}0.6)\times 3 = 46.2$$
$$\text{TCN of POO} = (18\text{-}2\text{-}0.6)\times 2+16 = 46.8$$
$$\text{TCN of POP} = (18\text{-}2\text{-}0.6)+16+16 = 47.4$$
$$\text{TCN of PPP} = 16+16+16 = 48.0$$

It is evident that the precise identification of the acyl groups in a triglyceride molecule with a lower ECN is more difficult than those with higher ECN where the difference of TCN is larger because of the close relative residence time of the group. This problem can be solved by using more efficient packings, longer columns an optimal mobile phase composition.

APPLICATIONS

Different vegetable oils were separated by HPRC with a Supelcosil LC-18 column (Figs. 6-9). The major triglyceride components were identified from their TCN extrapolated for their k′ values and their identity was confirmed by GLC of the corresponding methyl esters. In olive oil (Fig. 6), the major triglycerides present are LOL (1) with ECN of 44:0 and LOO (2), PLO (3) (4), OOO (5), POO (6), POP (7), SOO (8) and SOP (9). Triolein was the major triglyceride with ECN of 48:0 and TCN of 46.2. However, the presence of SOL could not be detected because of the ratio of OOO to SOL (if present). Soybean oil analysis (Fig. 7) revealed the presence of LLLe (1), LLL (2), LOL (3), PLL (4), LOO (5) and PLO (6). Triolinolein was the major triglyceride with 2 minor triglycerides 52:5 which may be LLPo or LLeP and 50:4 which could be LLM as both show as shoulder peaks on LLL.

Analysis of corn oil by HPRC (Fig. 8) indicated that

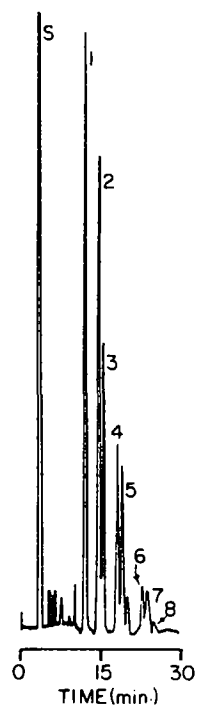

FIG. 8. Separation of corn oil. Conditions as in Fig. 1a. Triglycerides are (1) LLL, (2) LOL, (3) LLP, (4) LOO, (5) PLO, (6) SOL, (7) SPL and (8) unidentified.

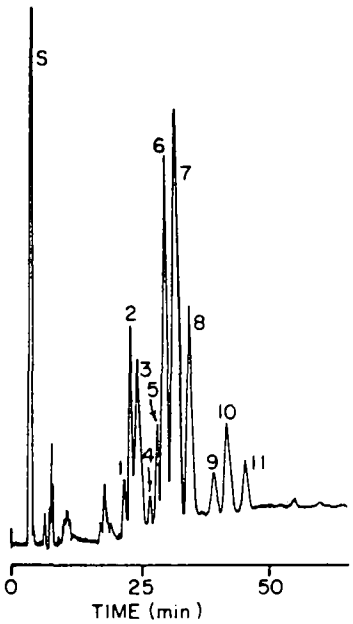

FIG. 9. Separation of palm oil. Conditions as in Fig. 1a. Triglycerides present in palm oil are (1) LOO, (2) PLO, (3) PPL, (4) MOP, (5) OOO+SOL, (6) POO, (7) POP, (8) PPP, (9) SOO, (10) SPO and (11) SPP.

trilinolein is the major triglyceride present (peak 1). The triglycerides of corn oil showed the same pattern as those of soybean oil, but a close look at both oils reveal some minor but significant differences. Soybean oil contains traces of 54:8 (LeLLe) which is not present in corn oil. Other minor components that eluted before LLL could not be identified due to the presence of other interfering components such as monoglycerides, diglycerides, phospholipids, which elute ahead of triglycerides due to their polarity and low molecular weight.

The analysis of palm oil is shown in Figure 9 and indicates that POP is the major triglyceride. Palm oil triglycerides are (1) LOO showed as a minor peak ahead of (2) PLO which is the major triglyceride in the ECN 46:0 group, (3) PLP, (4) MOP, (5) OOO, (6) POO, (7) POP, (8) PPP, (9) SOO, (10) SOP and (11) SPP. Triglyceride mixtures can be separated into their individual components using the system described. The mechanism proposed indicates the possibility of separation of lipid classes into their individual components according to their TCN, using the optimal phase composition and efficient packing.

ACKNOWLEDGMENT

Partial support was provided by the Illinois Agriculture Experiment Station, University of Illinois, Urbana, IL 61801; and the College of Agriculture, University of Al-Fateh, Tripoli, Libya.

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[Received March 9, 1981]