

✧ A Time Normalization Study of the Separation of Olive Oil Triglycerides

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ABSTRACT

A brief study on the selection of the optimal mobile phase for the reversed-phase liquid chromatographic separation of olive oil triglycerides was carried out using a time normalization approach. Two parameters, the solvents comprising the binary mobile phase and the proportion of strong solvent in the binary mixture, were simultaneously varied in order to maintain the retention time of the last component of interest fixed. Seven binary mobile phases were examined in the study. Plots of $\log k'$ against percent strong solvent were found to be linear for all seven solvent pairs. This allowed prediction of the solvent composition needed to produce a given k' . A mixture of dichloromethane/acetonitrile gave optimal resolution for the triglycerides under study. This time normalization approach offers a simple method directly to study effects of the variation of experimental parameters upon resolution.

INTRODUCTION

High performance reversed-phase liquid chromatography has been widely employed for the separation of triglyceride mixtures (1-6). Nonaqueous solvents and octadecyl bonded silica are normally employed in these separations. El-Hamdy and Perkins (1) recently discussed the effect of solvent strength and column packing upon triglyceride retention and resolution. They found that longer carbon chains and higher percent carbon loadings on the packing yielded improved separations of triglycerides. They followed this study with an examination (2) of the separation of critical pairs of triglycerides and postulated a potential mechanism for the retention and separation of triglycerides on reversed-phase columns.

Jensen (4) recently studied the influence of column temperature upon the retention of triglycerides. Retention was found to increase as column temperature decreased and linear plots between $\log k'$ and $1/T$ were obtained. Plattner et al. (5) briefly examined the effects of solvent composition upon triglyceride separations. Mixtures of acetone/methanol and acetone/acetonitrile were examined. In this work, acetone/acetonitrile mobile phases were reported to give less complex chromatograms of triglyceride mixtures than acetone/methanol mixtures. Vonach and Schomberg (7) examined the effect of the addition of silver ion to the mobile phase upon the separation of a number of classes of compounds, including triglycerides. Silver complexation reduced retention times of unsaturated triglycerides and was reported to provide improved resolution of triglyceride mixtures.

In this work, the effect of the composition of binary solvent mixtures upon the selectivity and resolution of olive oil triglyceride components was examined. Separation factors (α values) and resolution were calculated for the linolyldiolein (LOO) - linolylpalmitylolein (LPO) and triolein (OOO) - palmityldiolein (POO) pairs in olive oil. Five strong solvents (isopropanol, dichloromethane, chloroform, tetrahydrofuran, and acetone), as well as two weak solvents (methanol and acetonitrile) were employed. In all, seven solvent pairs were examined.

The technique of time normalization (8-10) was employed in order to compare the effect of varying solvent composition upon triglyceride resolution under conditions of a fixed analysis time. In time normalization, two variables are simultaneously varied so that the retention time of the last eluting component remains fixed. Thus all comparisons are carried out within the same analysis time. Under these conditions, changes in resolution could be directly related to changes in solvent properties. This approach allowed selection of the optimal mobile phase yielding the greatest resolution.

In this study, the two variables modified were the components comprising the binary mobile phase and the percent of strong solvent in the binary mixture. Retention of the last component of interest, POO, was maintained at a k' of 7.5 by adjusting the amount of strong solvent. Plots of $\log k'$ against percent strong solvent for all seven solvent pairs were found to be linear and this allowed prediction of the solvent composition yielding a k' of 7.5.

EXPERIMENTAL

The liquid chromatograph employed in this study consisted of an Altex 100 A pump and pulse dampener (Beckman Instruments, Berkeley, CA), a Rheodyne Model 7125 syringe loaded injection valve (Cotati, CA) equipped with a 20 μ L sample loop, and a Waters Associates Model R401 refractive index detector (Milford, MA). The column employed was a Beckman Ultrasphere-ODS, 250 \times 4.6 mm containing 5 μ m packing. In all studies a mobile phase flow rate of 2.0 mL/min was employed. Chromatograms were recorded on a Linear Instruments (Irvine, CA) Model 255 chart recorder operated at a chart speed of 2.5 cm/min. All measurements of retention time and peak width were made directly from the strip chart recordings.

Acetonitrile (ACN), isopropanol (IPA), dichloromethane (DCM) and tetrahydrofuran (THF), were all distilled in glass grade (Burdick and Jackson, Muskegon, MI). Tetrahydrofuran was obtained without an inhibitor. Chloroform (CHL) (Omnisolv grade, Matheson, Coleman, Bell, Cincinnati, OH) as well as acetone (ACE) and methanol (MEOH) (Baker analyzed reagent grade, Phillipsburg, NJ) were used as received. Batches of mobile phase were prepared by adding appropriate known volumes of each solvent to a flask. The mixtures were then degassed by rapidly stirring. Preparation of the solvent mixture in this manner overcame problems associated with the volume changes which take place upon mixing. Reported compositions are volume percents.

For studies on the relationship between $\log k'$ and percent strong solvent, olive oil was dissolved in the appropriate strong solvent at a concentration of 50 mg/mL. For time normalization studies, olive oil was dissolved in the mobile phase mixture at this same concentration level

TABLE I

Solvent Pair Properties

Solvent pair	% Strong solvent to yield $k' 7.5^a$	Slope from Figure 1 ^b
CHL-ACN	35.9	-0.051
THF-ACN	36.8	-0.052
DCM-ACN	42.3	-0.049
IPA-ACN	56.2	-0.030
ACE-ACN	79.2	-0.027
IPA-MEOH	53.1	-0.021
ACE-MEOH	71.5	-0.021

^aFor POO peak.

^bRate of change in k' for POO as a function of change in percent strong solvent.

whenever possible. In cases where olive oil was not soluble in the mobile phase, it was dissolved in the strong solvent.

The column void time was determined by measuring the average retention time of tetrahydrofuran, isopropanol and acetonitrile. A mobile phase of tetrahydrofuran/acetonitrile (35/65) at a flow rate of 2.0 mL/min was employed for this measurement. An average void time of 75.6 ± 1.1 sec was obtained.

Capacity factors, k' , and separation factors, α , were calculated by standard procedures (11).

Resolution, R , was calculated by

$$R = 2(t_2 - t_1)/(w_1 + w_2) \quad [1]$$

where t_1 and t_2 are the retention times of the two components, and w_1 and w_2 are the peak widths at the base line. In cases where the peaks were too badly overlapped to allow measurement of the total peak width, the width of the back half of the peak was doubled. A more fundamental expression for resolution is given by Equation 2:

$$R = (N)^{1/2}/4 \{(\alpha-1)/\alpha\} [(k'/(k'+1))] \quad [2]$$

where N is the number of theoretical plates and k' is the capacity factor of the second peak.

RESULTS AND DISCUSSION

In order to carry out the time normalization, the composition of each solvent pair which would yield a given k' for POO had to be determined. Plots of $\log k'$ for POO against the percent strong solvent were made for the seven solvent pairs for this purpose. In five of the seven cases, acetonitrile was employed as the weak solvent, and methanol was employed in the other two. The seven solvent pairs examined were acetone/acetonitrile, acetone/methanol, isopropanol/acetonitrile, isopropanol/methanol, dichloromethane/acetonitrile, chloroform/acetonitrile and tetrahydrofuran/acetonitrile. Three or four k' values were determined for each solvent pair. These data are plotted in Figure 1. Over the range of k' values examined, all the plots appeared to be linear. Thus the solvent composition necessary to provide a given k' value could be easily determined.

A k' value of 7.5 ($\log k'$ of 0.875) for the last peak of interest, POO, was selected for this study. This fixed the retention time of this component at ca. 11 min under the given chromatographic conditions. Table I contains the calculated solvent composition necessary to yield a k' value of 7.5 for POO based upon the data in Figure 1. Chloro-

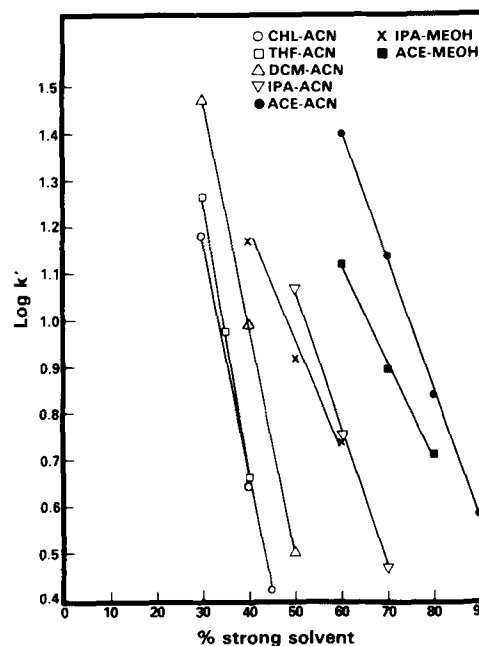


FIG. 1. Plot of $\log k'$ POO as a function of percent strong solvent.

form and tetrahydrofuran appeared to have the greatest solvent strength for the elution of these triglycerides, since a lower proportion of these solvents (ca. 36%) was required to give a k' of 7.5 for POO. With acetonitrile as weak solvent, these two were followed by dichloromethane (42%), isopropanol (56%) and acetone (79%). The substitution of methanol for acetonitrile as weak solvent tended slightly to decrease the percent of strong solvent required, indicating that methanol is a stronger solvent than acetonitrile for these species.

In addition, Table I contains the slopes obtained by a linear least-squares curve fit of the seven lines plotted in Figure 1. These slopes are measures of the sensitivity of POO retention to shifts in the amount of strong solvent present. For the five solvent pairs containing acetonitrile as weak solvent, the slope values roughly follow the same pattern as the solvent strengths discussed above. Tetrahydrofuran, chloroform and dichloromethane gave the steepest slopes followed by isopropanol and acetone. In the cases where methanol replaced acetonitrile as weak solvent, the slope decreased upon substitution.

The information contained in Table I has practical implications, since it indicates how closely solvent composition must be controlled in order to maintain adequate retention time reproducibility. Solutions containing tetrahydrofuran, chloroform or dichloromethane as strong solvents must be prepared more precisely than those with isopropanol in order to achieve similar retention time reproducibility. As an example, under conditions of k' equal 7.5 for POO, a 1% shift in tetrahydrofuran content from 37% to 36% would shift the retention time from 10.5 to 11.7 min, roughly an 11% shift. In contrast, a 1% shift in isopropanol content from 56% to 55%, with acetonitrile as weak solvent, would shift the retention time from 10.9 to 11.6 min, roughly a 6% shift. This problem of the high sensitivity of retention times to solvent composition is compounded by the high volatility of some of these solvents, especially dichloromethane.

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TABLE II

Results of Time Normalization Study on the Resolution of Olive Oil Triglycerides^a

Solvent pair	% Strong solvent	k' POO	LOO - LPO		OOO - POO	
			α	R	α	R
CHL-ACN	37	7.7	1.09	1.19	1.11	1.22
THF-ACN	37	7.4	1.08	0.88	1.08	0.89
DCM-ACN	42	7.8	1.11	1.35	1.12	1.22
IPA-ACN	56	7.5	1.07	0.82	1.08	0.97
ACE-ACN	79	7.7	1.07	0.63	1.07	0.68
IPA-MEOH ^b	53	7.4	—	—	—	—
ACE-MEOH	71	7.4	1.04	0.41	1.05	0.47

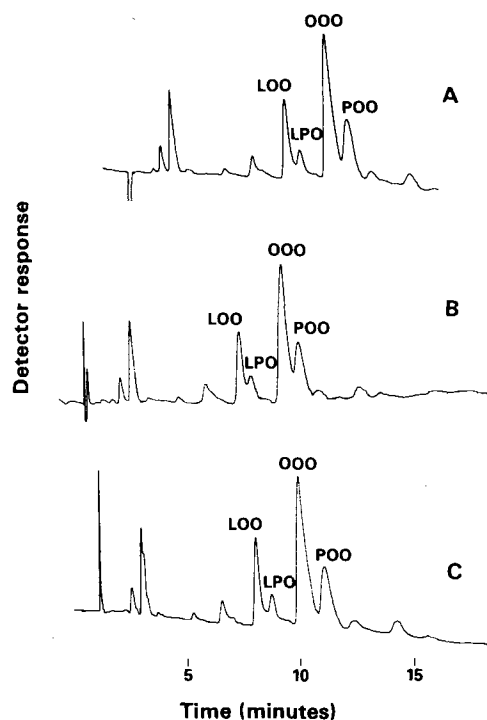
^aAverage of duplicate runs.^bResolution was not sufficient to allow measurement.

FIG. 2. Chromatogram of olive oil triglycerides with: (A) CHL-ACN (37:63, v/v); (B) THF-ACN (37:63, v/v); and (C) DCM-ACN (42:58, v/v) mobile phases.

The results of the time normalization study are provided in Table II. The composition of each of the seven solvent mixtures, k' values for POO, and the α value and resolution for the LOO-LPO and OOO-POO pairs are given. Values provided for α and resolution are averages of duplicate runs with the same batch of mobile phase. For resolution, these duplicate values normally agreed to within $\pm 5\%$ of each other. The variability in resolution values was much greater than that obtained for α values because of the greater uncertainty in measuring peak widths compared to retention times. In all cases, the difference in α values was less than 1%. Thus the differences between α values reported in Table II are greater than experimental uncertainties. All the k' values were within $\pm 5\%$ of the targeted value of 7.5. It was difficult to obtain values of exactly 7.5 because of the sensitivity of retention to exact mobile phase composition as discussed above. Equation 2 indicates that these slight shifts in k' values would not have any discernable effects upon resolution.

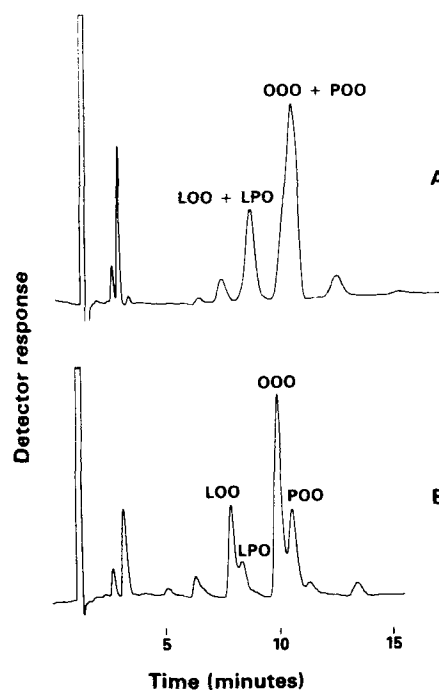


FIG. 3. Chromatogram of olive oil triglycerides with: (A) IPA-MEOH (53:47, v/v); and (b) IPA-ACN (56:44, v/v) mobile phases.

For the LOO-LPO pair the greatest resolution, with acetonitrile as the weak solvent, was obtained with dichloromethane as strong solvent followed by chloroform, tetrahydrofuran, isopropanol and acetone. No resolution was obtained for this pair with isopropanol/methanol as mobile phase, while the resolution obtained with acetone/methanol was significantly less than that obtained for acetone/acetonitrile.

For the OOO-POO pair, a similar trend was observed with acetonitrile as weak solvent. Optimal resolution was obtained with either dichloromethane or chloroform as strong solvent followed by isopropanol, tetrahydrofuran and acetone. Results with methanol as weak solvent were similar to those obtained for the LOO-LPO pair. In general, dichloromethane appeared to provide the greatest resolution, followed by chloroform. This advantage is somewhat offset by potential problems with retention time reproducibility noted earlier with these two solvents, especially with the more volatile dichloromethane. Isopropanol and tetrahydrofuran gave the next best resolution, followed by acetone. With the exception of tetrahydrofuran, the best reso-

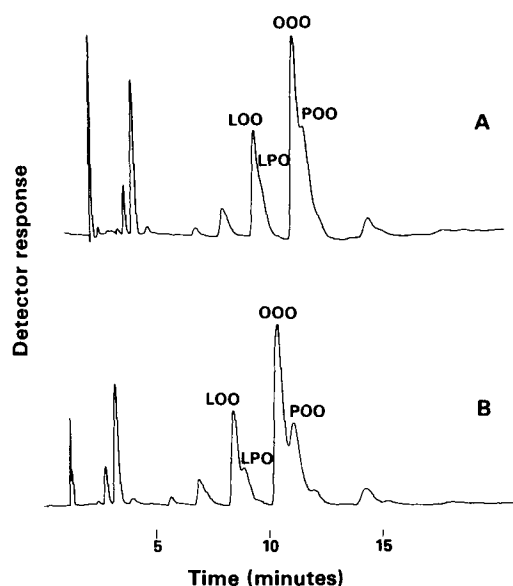


FIG. 4. Chromatogram of olive oil triglycerides with: (A) ACE-MEOH (71:29, v/v); and (b) ACE-ACN (79:21, v/v) mobile phases.

lution was obtained with the strongest solvents. Acetonitrile as weak solvent provided greater resolution than methanol.

Chromatograms obtained with the seven solvent pairs are provided in Figures 2-4. Note in Figure 3 the dramatic loss in resolution with the isopropanol/methanol pair.

Resolution, as given by Equation 2, is determined by three factors: column efficiency (N), selectivity (α) and retention (k'). In this study, retention was held constant and changes in resolution would result primarily from changes in column efficiency and α . For mixtures with acetonitrile as weak solvent, with the exception of acetone/acetonitrile, the number of theoretical plates obtained for POO did not shift significantly as the solvent composition was varied. Any slight shift in plate count would have a minimal effect

upon resolution because of the square root dependence of resolution upon N . The reason for the lower plate count with acetone is unknown. This value was roughly half that obtained with other solvents. However, this factor alone does not explain the much lower resolution obtained with this mobile phase compared to dichloromethane/acetonitrile.

Thus changes in resolution noted in this study result primarily from changes in α values. This is confirmed by the data in Table III since resolution followed the same trend as a α values. Except for acetone, the ratio of resolution to the $(\alpha-1)/\alpha$ term was roughly constant for all strong solvent/acetonitrile pairs. Therefore optimal resolution was obtained with those solvent mixtures which produced the highest α values.

In each of the triglyceride pairs under study (LOO-LPO and OOO-POO), the first triglyceride contains two more carbon atoms and one more double bond than the second triglyceride. The dependence of resolution upon solvent composition may be a function of the extent to which a solvent can shift retention per double bond compared to the extent to which it shifts retention per carbon unit. The influence of double bonds upon retention with different solvent mixtures will be investigated in the future.

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