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High Performance Size Exclusion Chromatography of Fatty Acids, Mono-, Di- and Triglyceride Mixtures

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A high performance size exclusion chromatographic (HPSEC) method is described for the separation and quantitation of fatty acids, mono-, di- and triglyceride mixtures. The various lipid components were separated on two columns packed with 5 μm styrene/divinylbenzene copolymer and connected in series. Toluene was employed as eluant, and components were monitored by refractometry. A formula derived for calculation of total weighted correction factors (WCF) for the various lipid classes based on known values of correction factors of simple lipid components and the fatty acid composition of the sample allowed quantitation of lipid mixtures containing a variety of different molecules. The precision of the experiments is such that the relative standard deviation for each lipid component was 1-5%, and a component could be detected at 0.05% level.

Analytical methods based on liquid, thin layer and gas liquid chromatography are used to analyze mixtures of fatty acids and acylglycerols, in emulsifiers, polymer additives and in many other areas of chemistry and biochemistry. These methods permit satisfactory separation of such lipids, but quantitation is often tedious and inaccurate. In recent years high performance liquid chromatographic methods have been developed using polar column packings and nonaqueous gradient elution systems. However, the requirement for special types of detectors (1,2) as well as poor quantitation of unsaturated components (3) has limited their use.

The development of high resolution size exclusion chromatographic columns has allowed simple and rapid separation of low molecular weight compounds with relatively small differences in molecular weight. In this study we report the development of a method for the separation of methyl esters, mono-, di- and triglycerides by high performance size exclusion chromatography (HPSEC).

EXPERIMENTAL

High performance size exclusion chromatography: Apparatus. The chromatographic system consisted of a Tracor 995 Isochromatographic Pump (Tracor, Inc., Austin, Texas); a Rheodyne 7120 syringe loading sample injector with a 20 μl loop (Rheodyne, Berkeley, California), and a Waters Model 401 Differential Refractometer (Waters Associates, Framingham, Massachusetts). Chromatograms were recorded and peak areas determined using an HP 3390 A Integrator (Hewlett-Packard, Avondale, Pennsylvania).

Analyses were performed on a pair of LiChrogel PS_4 and LiChrogel PS_1 columns connected in series with the LiChrogel PS_4 column placed first (EM Science, Gibbstown, New Jersey). The columns were 25 cm × 0.7 cm ID, packed with spherical, styrene/divinylbenzene copolymer beads with an average particle size of 5 μm. The upper molecular weight exclusion limit was 5.10^3 daltons and 2.10^3 daltons for LiChrogel PS_4 and LiChrogel PS_1, respectively; 100 daltons was the lower exclusion limit for both columns.

Materials and reagents. Standards used for chromatographic studies were methyl esters, mono-, di- and triglycerides containing C-12:0 to C-18:2 fatty acids (Nu-Chek Prep., Inc., Elysian, Minnesota); Myverol 18-00 (Eastman Chemical Products, Inc., Kingsport, Tennessee), and safflower oil (Hollywood Health Foods, Los Angeles, California). The purity of standards was >99% as determined by GLC. Chromatographic solvents, toluene (A.C.S. grade, Fisher Scientific Co., Fair Lawn, New Jersey), tetrahydrofuran and dichloromethane (A.C.S. grade, MBC Manufacturing Chemists, Inc.,Cincinnati, Ohio) had been distilled in glass.

The sample concentration was 25 mg/ml in either toluene or tetrahydrofuran; 0.5 mg were injected onto the column with a 20 μl sample loop. All samples and eluants were pre-cleaned by passing them through a filter (<2 microns). Toluene was used as the eluant at a flow rate of 0.5 ml/min.

Quantitation. Actual correction factors were calculated for methyl esters, mono-, di- and triglycerides of C-16:0, C-18:0, C-18:1 and C-18:2 fatty acids. Monolaurin was used as the internal standard for mixtures containing components with C-16:0 to C-18:2 fatty acids. A mixture of methyl ester, mono-, di- and triglyceride of each acid was prepared gravimetrically, and a known amount of internal standard added. Each mixture was analyzed three times by the HPSEC method. Correction factors were

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calculated for the components of the mixture. The values of correction factors for each component were summed, and means calculated to yield the correction factors for that particular component. Correction factors were calculated using the following equation:

$$CF = (A_{is}/W_{is}) \times (A_i/W_i)^{-1} \quad [1]$$

where W_i, A_i = weight and area of component of interest
W_{is}, A_{is} = weight and area of internal standard

The weight percentage of each component in the mixture was calculated using the following equation:

$$C_i\% = (A_i \times w_{is} \times CF \times 100)/(A_{is} \times \Sigma w_i) \quad [2]$$

where $C_i\%$ = % of the component of interest and
w_i = weight of each component in the sample analyzed

Gas liquid chromatography. Fatty acids, mono-, di- and triglyceride mixtures were determined according to the method of Goh and Timms (4). Trimethylsilyloxy (TMS) derivatives of fatty acids, mono- and diglycerides were prepared, and the samples were analyzed on a Hewlett-Packard Model 5840A Gas Chromatograph using a glass column, 0.3 m × 0.3 cm ID packed with 3% OV-1 on 80/100 Chromosorb HP. The chromatographic conditions were: Detector (FID) and injector at 360 C, oven programmed from 120 C to 350 C at 8 C/min followed by 5 min at 350 C, and nitrogen carrier gas at 60 ml/min. Calculation of correction factors and quantitation were performed as described for HPSEC. Docotriacontane was used as the internal standard.

The fatty acid composition of samples was determined according to AOCS Official Method Ce 1-62 (5).

Preparation of lipolyzed samples. Safflower oil was lipolyzed according to the method of Luddy et al. (6). The extracted lipid mixtures were treated with diazomethane to convert the free fatty acids into methyl esters for HPSEC analysis (7). Trimethylsilyloxy and methyl esters derivatives of the samples also were prepared for GLC analysis.

RESULTS AND DISCUSSION

A standard mixture of methyl stearate, mono-, di- and tristearin was employed in order to determine the various experimental variables that affect separation. A typical chromatogram of the separation of the standard mixture is presented in Figure 1. Lichrogel PS_4 and LiChrogel PS_1 columns were used in series, and toluene was the eluting solvent at a flow rate of 0.5 ml/min.

The effect of gel pore dimensions on the separation was studied for the test mixture components. Although monostearin and methyl stearate were well separated using either column separately, the resolution of tristearin and distearin required both columns used in series.

The effect of the solvent on separation relates mainly to its polarity. Nonpolar solvents favor swelling, which subsequently enhances resolution by increasing the fractionation range of the gel. Based on sample solubility, physical properties and safety information, a variety of solvents were tested as swelling agents and eluting solvents. The separations obtained with both tetrahydrofuran and methylene chloride as eluants are shown in Figure 2. It was concluded that solvents with a solvent strength parameter (ε^0) greater than that of toluene (ε^0 = 0.29) would result in incomplete resolution of the components of interest (8).

An important variable which can be used to control separation is the eluant flow rate because column length and particle diameter are fixed. As flow rates increase, resolution decreased for the tristearin-distearin peaks. Thus, a flow rate of 0.5 ml/min was considered the optimum in terms of resolution and analysis time.

The relationship between sample concentration and detector response was also studied for the test mixture components. Calibration curves were prepared by plotting the sample concentration vs the peak area for each component. A linear response was obtained for up to 5% of component which corresponded to 1 mg injected. As little as 0.05% of each component can be determined, thus the lower detection limit for this method was 10 μg for each component.

The retention times obtained using the conditions defined previously for methyl esters, mono-, di- and triglycerides containing various fatty acids are given in Table 1. Separation of the various molecular species of individual lipid classes was not possible under the present experimental conditions. However, only the complete separation of individual lipid species was desired. The presence of unsaturation in the fatty acids increased the retention time of components by approximately 0.25 min for introduction of one double bond in each fatty acid moiety of the molecule compared to that of the corresponding saturated compound. Increased retention of unsaturated molecules can occur as a result of an increase in their "effective size" due to the steric hindrance caused by the presence of double bonds.

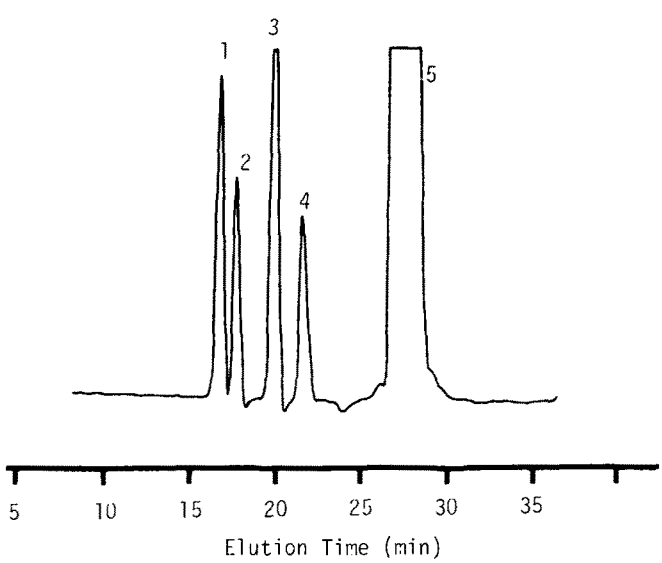

FIG. 1. HPSEC separation of standard mixture. Columns: LiChrogel PS_4 + LiChrogel PS, (each 25 cm × 0.7 cm ID). Eluant: Toluene at a flow rate of 0.5 ml/min. Detection: RI. Injection volume: 20 μl. Sample conc.: 2.5%. Temp.: Ambient. Peaks: 1, tristearin; 2, distearin; 3, monostearin; 4, methyl stearate, and 5, THF (employed as solvent for the sample).

Chromatography of a free fatty acid as a component of a lipid mixture resulted in incomplete separation from the monoglyceride peak (Fig. 3). The increased retention times of fatty acids compared to those of methyl esters may be due to adsorption effects between the stationary phase and the fatty acid. It is common practice to combine true GPC separation mechanism and adsorption effects to achieve optimum resolution for components with varying polarity. However, the chromatographic behavior of monoglycerides did not allow complete separation from the acid peak. Thus, esterification of any free fatty acids present in the sample before analysis is necessary for complete separation from the monoglycerides. Diazomethane was used for esterification of the free fatty acids present in mixtures, because this reaction does not involve the hydroxyl groups present in the mono- and diglycerides molecules.

Quantitation of mixtures of methyl esters, mono-, di- and triglycerides with C-16:0 to C-18:2 fatty acids, commonly found in vegetable oils, was of primary interest. In order to obtain absolute concentration values for the components in the above mixtures and maintain satisfactory quantitative reliability, an internal standard, monolaurin, was employed. Monolaurin is well resolved from the monoglyceride peak in mixtures such as those with the above composition (Fig. 4). The correction factors were calculated for methyl esters, mono-, di- and triglycerides containing C-16:0, C-18:0, C-18:1 and C-18:2 fatty acids from HPSEC data and are shown in Table 2. These correction factors were used for quantitation of individual components of standard or actual mixtures of methyl esters, mono-, di- and triglycerides containing C-16:0 to C-18:2 fatty acids. In general, correction factors within the same lipid group (i.e. triglyceride) increased with unsaturation and exhibited a small standard deviation. Since the correction factors calculated for the mixture containing C-16:0 fatty acids were very close to those containing C-18:0 fatty acids, the correction factor values for components derived from C-18:0 fatty acids were used for quantitation of saturated components. An example of the quantitation of a standard mixture using these correction factors shown in Table 2 is illustrated in Table 3.

TABLE 1

HPSEC Retention Times of Methyl Esters, Mono-, Di- and Triglycerides Containing Various Fatty Acids[a]

	Retention time (min)[b]			
Fatty acid	TG	DG	ME	MG
Lauric	17.98	19.26	21.88	24.25
Myristic	17.54	18.95	21.27	23.41
Palmitic	17.14	18.24	20.71	22.70
Stearic	16.86	17.88	20.20	22.15
Oleic	17.09	18.13	20.47	22.34
Linoleic	17.25	18.34	20.85	22.55

[a]Conditions as in Fig. 1.

[b]Average of 3 observations.

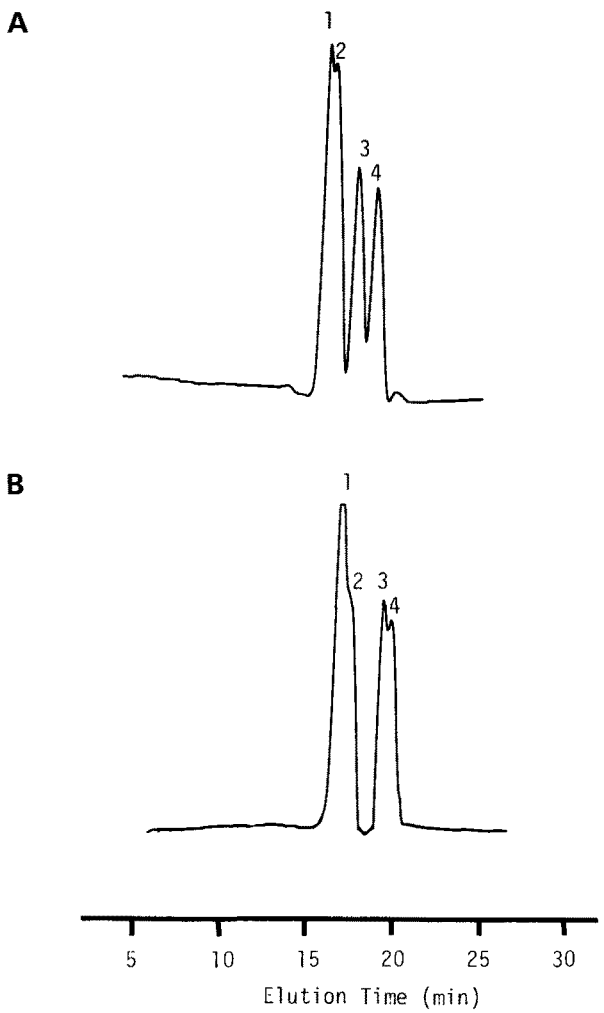

FIG. 2. Effect of solvent on the separation of standard mixture. A, THF, and B, CH_2Cl_2. (Chromatographic conditions and peaks identification as in Fig. 1.)

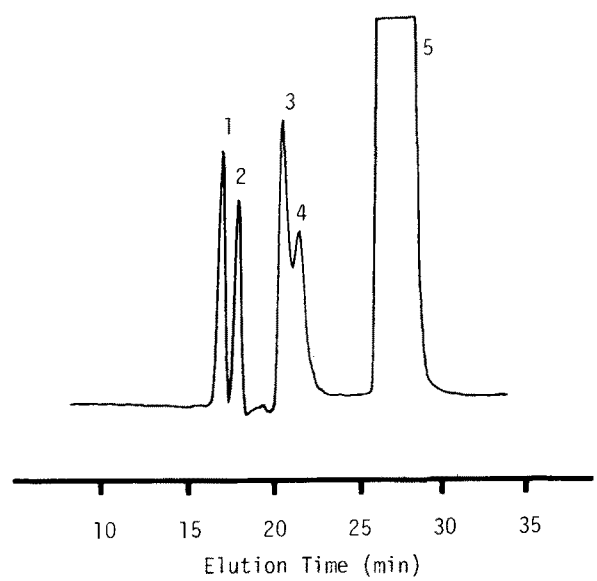

FIG. 3. HPSEC separation of a test mixture containing fatty acid. Peaks: 1, Tristearin; 2, distearin; 3, stearic acid; 4, monostearin, and 5, THF. (Chromatographic conditions as in Fig. 1.)

TABLE 2

HPSEC Correction Factors for Methyl Esters, Mono-, Di- and Triglycerides Containing Stearic, Oleic and Linoleic acids[a]

Fatty acid	Correction factor[b]			
	TG	DG	ME	MG
Oleic	0.83 ± 0.02	0.85 ± 0.03	0.60 ± 0.03	0.88 ± 0.01
Linoleic	1.42 ± 0.02	1.43 ± 0.01	0.70 ± 0.02	1.30 ± 0.05
Linolenic	2.85 ± 0.01	2.82 ± 0.04	1.10 ± 0.04	1.83 ± 0.06

[a]Conditions as in Fig. 1.

[b]Correction factors calculated using formula (1). Average of 3 observations.

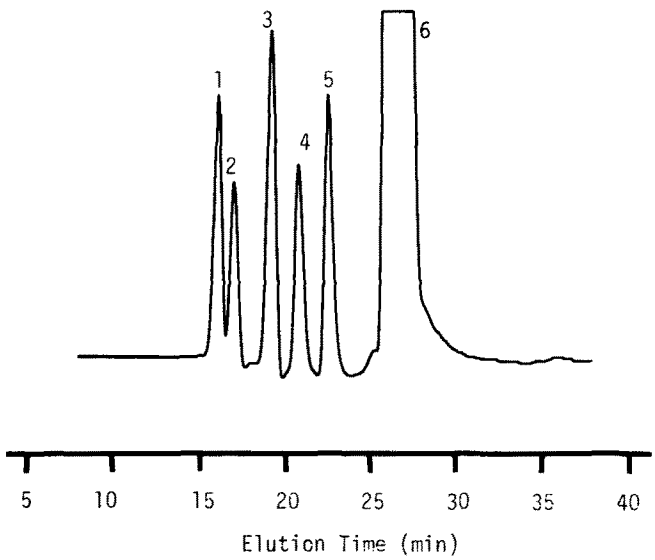

FIG. 4. HPSEC separation of a test mixture. Peaks: 1, simple triglycerides; 2, simple diglycerides; 3, methyl esters, and 4, monoglycerides containing C-16:0, C-18:0, C-18:1 and C-18:2 fatty acids; 5, monolaurin (internal standard), and 6, THF. (Chromatographic conditions as in Fig. 1.)

TABLE 3

HPSEC Quantitative Analysis of a Standard Mixture of Methyl Ester, Mono-, Di- and Triglyceride of Stearic Acid[a]

Compound	Composition (%)[b]		
	Actual	Found	
		HPSEC	GLC[c]
Tristearin	31.88	31.58 ± 0.05	31.23 ± 0.50
Distearin	21.31	21.22 ± 0.01	21.15 ± 0.09
Methyl stearate	22.63	22.79 ± 0.01	22.70 ± 0.05
Monostearin	24.18	24.25 ± 0.02	24.46 ± 0.03

[a]Conditions as in Fig. 1.

[b]Average of 4 observations.

[c]Conditions as described in experimental section.

However, mixtures to be analyzed may be composed of methyl esters, mono-, di- and triglycerides, containing different fatty acids. This results in chromatographic separation of the mixtures into "mixture peaks," each consisting of an entire group of lipids. Thus, a number of compounds each differing in molecular weight and in functional groups are eluted to give a single chromatographic peak. Quantitation of such eluted compouns by refractometry or ultraviolet detection may give incorrect results unless individual standards are available, because the refractive as well as the ultraviolet properties of the individual species comprising the eluted components peak can differ. The relatively large differences in the carbon contents of the various eluted components also limit the use of the transport flame ionization detector (9,10).

In the present study, differences in the refractive properties and the quantity of the individual components of a "mixture peak" have been taken into consideration in devising a method to quantitatively determine the composition of a mixture of species each consisting of a variety of components. A general formula was derived to calculate the total weighted correction factors (WCF) for a mixture of methyl esters, mono-, di- and triglycerides. This calculation is based on the correction factor values for saturated, mono- and di-unsaturated methyl esters, mono-, di- and triglycerides containing C-18 fatty acids obtained by the HPSEC method (Table 2) and on the fatty acid composition of the sample determined by GLC. It is expressed as follows:

$$\text{Total WCF} = (CF_s \times \%\ FA_s + CF_{u_1} \times \%\ FA_{u_1} + CF_{u_2} \times \%\ FA_{u_2})/100 \quad [3]$$

where CF_s, CF_{u_1}, CF_{u_2} are correction factors for saturated, mono- and di-unsaturated components of the species of interest, and
$\%\ FA_s$, $\%\ FA_{u_1}$, $\%\ FA_{u_2}$ are the percentages of saturated, mono- and di-unsaturated fatty acids in the sample to be analyzed.

Thus, the total WCF of a lipid species will be the sum of the correction factors of the saturated, mono- and di-unsaturated fraction of that species in the sample. Two assumptions are necessary for calculations using equation [3]: (a) the fatty acid composition (%) of each

TABLE 4

HPSEC Quantitative Analysis of a Standard Mixture of Methyl Esters, Mono-, Di- and Triglycerides of Stearic and Oleic Acids[a]

	Composition (%)[b]				
	Actual			Found	
Lipid species[c]	Total	Sat. (C-18:0)	Unsat. (C-18:1)	HPSEC[d]	GLC[e]
TG	30.22	27.51	72.49	29.20 ± 0.72	28.49 ± 0.22
DG	21.13	30.72	69.29	20.40 ± 0.52	20.31 ± 0.58
ME	24.03	35.29	64.71	23.61 ± 0.30	23.66 ± 0.26
MG	24.61	32.50	67.49	24.34 ± 0.20	24.23 ± 0.30

[a]Conditions as in Fig. 1.
[b]Average of 4 observations.
[c]TG, triglycerides; DG, diglycerides; ME, methyl esters; MG, monoglycerides.
[d]Calculations of total WCF using formula (3) and composition (%) using formula (2).
[e]Conditions as described in experimental section.

molecular lipid class is the same as that of the whole sample, and (b) only monoacid diglycerides and triglycerides are present in the sample. Results calculated using the correction factors based on the above assumptions are accurate and showed small standard deviation.

The values from the quantitative analysis of a standard mixture of methyl esters, mono-, di- and triglycerides containing C-18:0 and C-18:1 fatty acids are shown in Table 4. Total WCF for the lipid species were calculated based on the known values of correction factors for methyl esters, mono-, di- and triglycerides containing stearic and oleic acids, as well as on the % fatty acid composition of the sample determined by GLC as methyl esters (C-18:0, 34.22%; C-18:1, 65.78%). For example, total WCF for the triglyceride "mixture peak" was calculated as follows:

$$\text{Total WCF}_{Tg} = (CF_{Tg_s} \times \%\ FA_s + CF_{Tg_{u_1}} \times FA_{u_1})/100$$

$$= (0.83 \times 34.22 + 1.42 \times 65.78)/100$$

$$= 1.22$$

The weight (%) for each species was calculated using equation [2]. In the case of triglycerides, A_i = area of triglyceride peak; W_{is} = weight of monolaurin added in the sample; CF = total WCF for triglyceride species; A_{is} = area of monolaurin peak, and W_i = weight of sample.

The quantitative results obtained using the expressions described are in agreement with the gravimetric composition of the sample. The precision obtained for each lipid species was in the range of 1-5% relative standard deviation.

As a typical application of the HPSEC method, safflower oil lipolysis mixtures were prepared and anayzed. The HPSEC chromatograms of the lipolyzed safflower oil after treatment with diazomethane are shown in Figure 5. The quantitative results obtained are presented in Table 5. Calculation of total WCF of the various lipid classes was based on the known values of correction factors for methyl esters, mono-, di- and triglycerides components containing C-18:0, C-18:1, C-18:2 fatty acids and the fatty acid composition of the safflower oil determined by GLC (sat., 13.25%; C-18:1, 12.59%, and C-18:2,

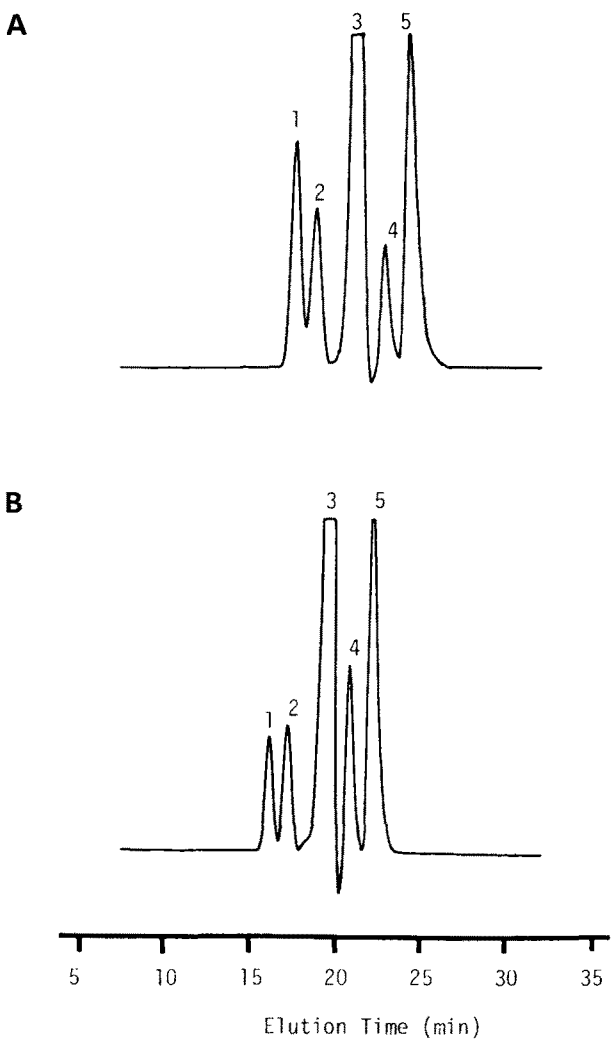

FIG. 5. HPSEC separation of safflower oil lipolysis mixtures after diazomethane methylation of the free fatty acids. A, 15 min lipolysis, B, 30 min lipolysis. Peaks: 1, triglycerides; 2, diglycerides; 3, methyl esters; 4, monoglycerides, and 5, monolaurin (internal standard). (Chromatographic conditions as in Fig. 1.)

TABLE 5

HPSEC Quantitative Analysis of Safflower Oil Lipolysis Mixtures[a]

	Composition (%)[b]			
	Sample A[c]		Sample B[d]	
Lipid species	HPSEC	GLC[e]	HPSEC	GLC
TG	28.27 ± 0.57	28.02 ± 0.13	7.54 ± 1.02	6.82 ± 0.55
DG	19.11 ± 0.21	20.33 ± 0.22	17.84 ± 0.85	17.84 ± 0.62
ME	42.09 ± 0.35	40.69 ± 0.45	56.38 ± 0.69	54.48 ± 0.49
MG	10.43 ± 0.54	10.98 ± 0.31	21.55 ± 0.96	20.87 ± 0.44

[a]Conditions as in Fig. 1.
[b]Average of 4 observations.
[c]Lipolysis time 15 min.
[d]Lipolysis time 30 min.
[e]Conditions as described in experimental section.

73.43%). The results obtained compare favorably with the GC analysis of the lipolyzed samples.

The applicability of the HPSEC method described here was determined on a wide variety of natural and synthetic samples containing methyl esters, mono-, di- and triglycerides species. This method is especially useful for the quantitative determination of the monoglyceride and diglyceride content of food emulsifiers. It is also applicable to the assay of mixtures obtained from studies on lipolysis and is useful in obtaining data regarding such enzyme action.

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