Journal of Chromatography, 596 (1992) 185–195
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 973

Separation of monoacylglycerols by reversed-phase high-performance liquid chromatography

B. G. Semporé and J. A. Bézard*

Unité de Nutrition Cellulaire et Métabolique, Université de Bourgogne, B.P. 138, 21004 Dijon Cedex (France)

(First received April 18th, 1991; revised manuscript received December 22nd, 1991)

ABSTRACT

Reversed-phase high-performance liquid chromatography on a thermostated octadecylsilyl column was used to separate complex mixtures of monoacylglycerols formed by chemical deacylation of natural oil triacylglycerols. Acetonitrile–water mixtures were used for elution of the underivatized monoacylglycerols and a differential refractometer was used for their detection. The order of elution of monoacylglycerols in complex mixtures varies as a function of the chain length, unsaturation, positional isomerism, analysis temperature and eluent water concentration. The peak areas were representative of the amounts of monoacylglycerols detected. The method can be applied to compositional analysis and to the collection of non-contaminated fractions for further stereospecific analysis.

INTRODUCTION

Theoretically the simplest method for studying the stereospecific distribution of the fatty acids in triacylglycerol molecules is to determine the fatty acid profile of the three *sn*-1-, *sn*-2- and *sn*-3-monoacylglycerol isomers, formed by a deacylation procedure. The prerequisite is to use a deacylation technique which produces "representative" monoacylglycerols, *i.e.*, whose fatty acid profiles are exactly those expected from the fatty acid distribution in the triacylglycerol molecules. This means that the deacylating reagent should not have undesirable specificities for certain fatty acids or triacylglycerols, for certain positions in the triacylglycerol molecules, and should not promote acyl migration from one position to another, during or after hydrolysis.

Mammalian pancreatic lipase which specifically catalyses the hydrolysis of primary ester linkages (*sn*-1 and *sn*-3) in triacylglycerols [1] has been widely used in studying the fatty acid distribution at the *sn*-2-position of triacylglycerols. However, its specificity precludes the formation of *sn*-1- and *sn*-3-monoacylglycerols. In general, enzymatic deacylation procedures do not produce representative monoacylglycerols for the three positions of the glycerol moiety.

Chemical deacylation by a Grignard reagent has been widely used to produce representative diacylglycerols, especially *sn*-1,2(2,3)-diacylglycerols. Those were then utilized to study the fatty acid distribution at the *sn*-1- and *sn*-3-positions of triacylglycerols by Brockerhoff's enzymatic method [2–4] or by physical methods in which the diacylglycerol isomers were separated by high-performance liquid chromatography (HPLC) as chiral derivatives [5] or by HPLC on a chiral column [6–8].

However, the *sn*-1(3)- and *sn*-2-monoacylglycerols formed by Grignard deacylation could not be used as their fatty acid composition was not representative of the original triacylglycerol structure [9]. Extensive isomerization seemed to occur between *sn*-1(3)- and *sn*-2-isomers in both directions.

The possibility of optimizing the reaction conditions in order to minimize the extent of isomerization to an acceptable level cannot be discarded. In this regard we have examined the possibility of separating monoacylglycerols by HPLC both in the reversed-phase mode and in the presence of a chiral phase. Previously Maruyama and Yonese [10] devel-

0021-9673/92/$05.00 © 1992 Elsevier Science Publishers B.V. All rights reserved

oped reversed-phase HPLC methods for the separation and determination of saturated and unsaturated simple underivatized monoacylglycerols. However, these methods were not entirely convenient for the simultaneous determination of the homologous distribution and the ratio of positional isomers of monoacylglycerols. Kondoh and Takano developed an original detection method for acylglycerols which they applicated to the simultaneous determination of mono-, di- and triacylglycerols [11] and then to mixtures of monoacylglycerols [12]. Homologous series and positional isomers of monoacylglycerols were well separated but the detection method involves destruction of monoacylglycerols and does not allow their collection for further analysis.

To study further the stereospecific distribution of fatty acids in triacylglycerols, we first developed the HPLC separation of complex mixtures of monoacylglycerols according to the nature of the constituent fatty acid (chain length and unsaturation) and to its positioning [*sn*-1(3)- and *sn*-2-monoacylglycerols]. Analyses were principally carried out on underivatized monoacylglycerols.

Some experiments were performed on monoacylglycerols derivatized with 3,5-dinitrophenyl isocyanate. These urethane derivatives are used in chiral-phase HPLC analyses of monoacylglycerol stereoisomerism [13].

EXPERIMENTAL

Samples

Optically active *sn*-3-monopalmitoylglycerol (*sn*-3-16:0) was obtained from Fluka (Buchs, Switzerland). *rac*-1-monopalmitoylglycerol (*rac*-1-16:0), *rac*-1-monostearoylglycerol (*rac*-1-18:0), *rac*-1-monooleoylglycerol (*rac*-1-18:1) and *rac*-1-monolinoleoylglycerol (*rac*-1-18:2) were purchased from Serdary (London, Ontario, Canada), as were optically inactive *sn*-2-isomers, namely *sn*-2-monopalmitoylglycerol (*sn*-2-16:0) and *sn*-2-monooleoylglycerol (*sn*-2-18:1). These monoacylglycerols were used without prior purification.

Natural source monoacylglycerols were prepared by Grignard degradation with ethylmagnesium bromide [14,15] and by pancreatic lipase hydrolysis [16,17] of triacylglycerols. Those were isolated from complex mixtures of peanut oil and cottonseed oil triacylglycerols by combined argentation thin-layer chromatography (TLC)–reversed-phase HPLC [18]. The fractionated triacylglycerols submitted to partial deacylation were palmitoyldioleoylglycerol (16:0 18:1 18:1), trioleoylglycerol (18:1 18:1 18:1), palmitoyloleoyllinoleoylglycerol (16:0 18:1 18:2), dioleoyllinoleoylglycerol (18:1 18:1 18:2), oleoyldilinoleoylglycerol (18:1 18:2 18:2) from peanut oil and palmitoyloleoyllinoleoylglycerol (16:0 18:1 18:2) from cottonseed oil (no distinction is being made among the *sn*-1-, *sn*-2- and *sn*-3-positions of these triacylglycerols). Monoacylglycerols formed by chemical or enzymatic deacylation of triacylglycerols were isolated by TLC on silica gel G (Merck, Darmstadt, Germany) impregnated with 5% (w/w) boric acid to prevent izomerization [19].

Preparation of monoacylglycerol urethane derivatives

The procedure used to prepare the urethane derivatives of monoacylglycerols was derived from that described by Oi and Kitahara [20] for chiral alcohols and adapted to monoacylglycerols by Takagi and co-workers [13,21]. A 20-μmol amount of monoacylglycerol (6–8 mg) was dissolved in 450 μl of dry toluene in a 0.5-ml glass vial with a PTFE-lined screw-cap, then 45 μmol (*ca.* 10 mg) of 3,5-dinitrophenyl isocyanate powder (Sumitomo, Osaka, Japan) and 45 μl of dry pyridine were added. The mixture was heated at 70°C for 1 h in an oven (or left for 3 h at ambient temperature) with occasional shaking.

At the end of the reaction, the mixture was cooled, the solvent was removed under nitrogen and the resulting urethane derivatives were dissolved in 0.2 ml of chloroform and purified by TLC on a silica gel 60 F_{254} precoated plate (20 × 20 cm, 0.25 mm thick layer) from Merck. The plates, containing a fluorescence indicator, were previously activated at 110–120°C for 1 h. They were developed twice with hexane–ethylene dichloride (or dichloromethane)–ethanol (40:15:3, v/v/v).

Crude urethane derivatives were alternatively purified by reversed-phase HPLC instead of TLC. In this instance, at the end of the derivatization procedure the mixture was decanted. The limpid upper phase was filtered through hyperfine glass-wool into another vial. The solvent was evaporated under nitrogen and the urethane derivatives were dissolved in chloroform for storage, in acetonitrile

or in the mixture used as the mobile phase for reversed-phase HPLC fractionation, *i.e.*, acetonitrile–water (95:5 or 90:10, v/v). Purification was carried out isocratically at ambient temperature (*ca.* 20°C).

Liquid chromatography

HPLC analyses were performed using a Model 6000 A solvent-delivery system (Waters, Milford, MA, USA) connected either to an R 401 differential refractometer or to a Model 450 variable-wavelength UV detector (Waters). The column was a stainless-steel prepacked 250 mm × 4 mm I.D. Hibar LiChroCART HPLC cartridge, LiChrospher 100 CH-18/II Super (4-μm particles) column purchased from Merck. A Guard-Pak precolumn, LiChroCART 4-4 filled with LiChrosorb 100 RP-18 (Merck), was attached to the column inlet. The mobile phase was acetonitrile–water in various proportions (80:20, 85:15, 90:10 and 95:5, v/v), depending on the monoacylglycerol mixtures to be analysed. It was delivered at a constant flow-rate of 1.0 or 1.2 ml min^{-1}. The analysis temperature was ambient (*ca.* 20°C) or a constant thermostatically controlled temperature [22].

Acetonitrile (Hypersolv "far UV") was obtained from BDH (Poole, UK). Water was doubly distilled. Solvents were filtered through a Millipore membrane (pore size 0.5 μm) and the HPLC solvent mixture was vacuum degassed for 2 min before use.

Peak areas were measured by means of an Enica 21 integrator–calculator (Delsi, Suresnes, France).

Gas chromatography

The fatty acid composition of the underivatized monoacylglycerols recovered from the hydrolysis products and that of the HPLC-collected monoacylglycerol urethane derivatives was determined by gas chromatography (GC) of the methyl esters prepared with methanol–boron trifluoride [23]. The analyses were carried out on a Becker-Packard Model 417 gas chromatograph equipped with a laboratory-made 30 m × 0.4 mm I.D. glass capillary column coated with Carbowax 20M (Applied Science Labs., State College, PA, USA) at a constant temperature of 195°C and a nitrogen flow-rate of 3 ml min^{-1}. The apparatus was equipped with a Ros injector [24] (Spiral, Dijon, France) and a flame ionization detector. Peak areas were measured with an Enica 21 integrator–calculator (Delsi). Calibration factors were calculated using standard mixtures of fatty acids (Nu-Chek Prep, Elysian, MN, USA).

Definitions

Monoacylglycerols were characterized by their partition number (*PN*), calculated from the equation [1]

$$PN = CN - 2\,DB$$

where *CN* is the number of carbon atoms in the acyl chains and *DB* the number of double bonds.

Chromatographic separations were characterized by three parameters: the retention time, generally corrected from the column void volume (t_R); the separation factor (α) between two successive peaks 1 and 2 (in the elution order), expressed as the ratio t_{R_2}/t_{R_1} [25]; and the resolution factor (R_s) calculated from the equation [25]

$$R_s = 2\,(t_{R_2} - t_{R_1})/(w_2 + w_1)$$

where t_R is the retention time and *w* the peak width at the baseline. For $R_s \geqslant 1$ two peaks are reasonably well separated.

RESULTS AND DISCUSSION

Separation of monoacylglycerols as urethane derivatives

Reversed-phase HPLC can separate a mixture of monoacylglycerol isomers into two groups, the optically inactive *sn*-2-monoacylglycerols and the optically active group of the two *sn*-1- and *sn*-3-isomers eluted together [12]. Separation of the two enantiomers in the latter can be achieved by HPLC in the presence of a chiral phase as monoacylglycerol urethane derivatives [13,21].

Chiral-phase HPLC separation of enantiomers generally cannot be applied directly to *sn*-2- and *sn*-1(3)-isomers resulting from chemical deacylation of natural triacylglycerols because of their complexity. A previous fractionation by reversed-phase HPLC according to chain length and unsaturation should be carried out.

To simplify this double-step analysis and to minimize the isomerization of underivatized monoacylglycerols, we first checked the possibility of fractionating by reversed-phase HPLC the natural complex mixture of monoacylglycerols in the obliga-

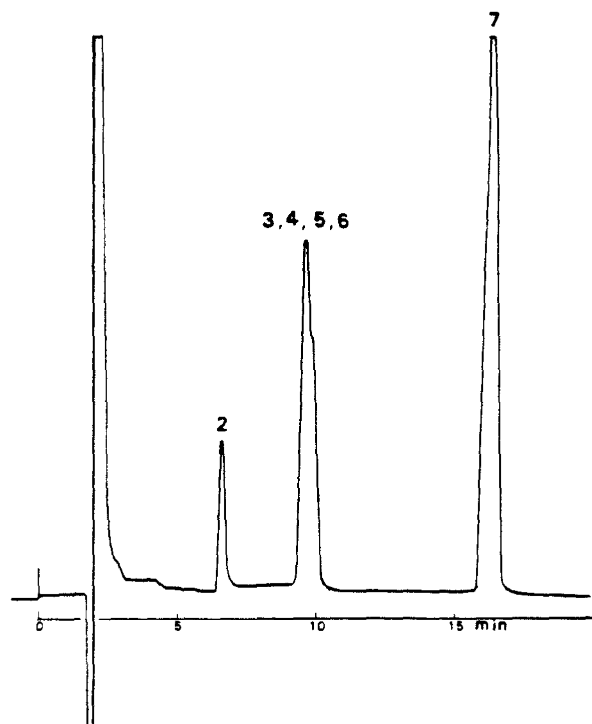

Fig. 1. HPLC separation of a mixture of standard isomeric monoacylglycerols (2 = *rac*-1-18:2; 3 = *sn*-2-18:1; 4 = *sn*-2-16:0; 5 = *rac*-1-18:1; 6 = *rac*-1-16:0; 7 = *rac*-1-18:0) as 3,5-dinitrophenyl isocyanate derivatives on an RP-18 column. The numbering of the different monoacylglycerols separated is done with respect of the elution order after reversed-phase HPLC separation of the total monoacylglycerol isomers analysed in this work as derivatives, *i.e.*, 1 = *sn*-2-monolinoleoylglycerol (*sn*-2-18:2); 2 = *sn*-1(3)- or *rac*-1-monolinoleoylglycerol [*sn*-1(3)- or *rac*-1-18:2]; 3 = *sn*-2-monooleoylglycerol (*sn*-2-18:1); 4 = *sn*-2-monopalmitoylglycerol (*sn*-2-16:0); 5 = *sn*-1(3)- or *rac*-1-monooleoylglycerol [*sn*-1(3)- or *rac*-1-18:1]; 6 = *sn*-1(3)- or *rac*-1-monopalmitoylglycerol [*sn*-1(3)- or *rac*-1-16:0]; 7 = *sn*-1(3)- or *rac*-1-monostearoylglycerol [*sn*-1(3)- or *rac*-1-18:0]. Analytical conditions: stainless-steel column (250 mm × 4 mm I.D.) packed with 4-μm octadecylsilyl (C_{18}) reversed-phase material; eluent, acetonitrile–water (95:5, v/v) at 1.2 ml min^{-1}; isocratic analysis at ambient temperature *ca.* 21°C; refractive index detection.

tory form of urethane derivatives prior to further separation of the enantiomers by chiral-phase HPLC. Six groups of urethane derivatives were studied. They were first injected separately on to the RP-18 column and then together.

Fig. 1 shows the results obtained with the synthetic mixture of the six groups of isomers. The resolution factors calculated from the corrected retention times of the monoacylglycerols injected individually indicated that for monopalmitoylglycerols the two groups of isomers [(*sn*-2 and *sn*-1(3)] were poorly separated, the calculated resolution factor R_s being only 0.56. For monooleoylglycerols, separation was just achieved (R_s = 1.00). Moreover, for the same group of isomers [*sn*-2 or *sn*-1(3)] the separation of monooleoyl- and monopalmitoylglycerol was very poor, because for the *sn*-2-isomers the retention time were 9.4 and 9.5 min, respectively, and hardly more different for the *sn*-1(3)-isomers, 9.7 and 10 min, respectively. On the other hand, in the chromatogram in Fig. 1 the four isomers [*sn*-2- and *sn*-1(3)-16:0, *sn*-2- and *sn*-1(3)-18:1] eluted almost together in the second peak (marked 3, 4, 5, 6).

Modifications of the analytical conditions (temperature, nature and flow-rate of the eluent) did not really improve the separations. This is the reason why previous fractionation of monoacylglycerol complex mitures was carried out by reversed-phase HPLC as underivatized monoacylglycerols.

Separation of underivatized monoacylglycerols

In preliminary experiments, various analytical conditions were checked: pure acetonitrile, pure propionitrile, mixtures of acetonitrile with different proportions of acetone, propionitrile and water were tested as mobile phases; and pure acetone, acetonitrile, chloroform and mixtures of these three solvents in different proportions were checked for dissolution of the monoacylglycerols samples.

The results showed that the best separations were obtained with a mixture of acetonitrile and water as the mobile phase and when the sample was dissolved in pure acetonitrile. These optimum conditions were similar to those observed previously [10,12].

Elution order. Fig. 2 shows the separation obtained in analysing a mixture of the three isomers *sn*-1-, *sn*-2- and *sn*-3- of monopalmitoylglycerol by reversed-phase HPLC at ambient temperature. Under all conditions only two peaks appeared on the chromatograms. Separate injections of commerc ial *rac*-1- and *sn*-2-monopalmitoylglycerols showed that the *sn*-2-isomer was first eluted and that the two *sn*-1- and *sn*-3-isomers were later eluted together. This elution pattern was also verified with monooleoylglycerols (Fig. 3) and monolinoleoylglycerols (Fig. 5).

Effect of solvent. The effect of increasing the

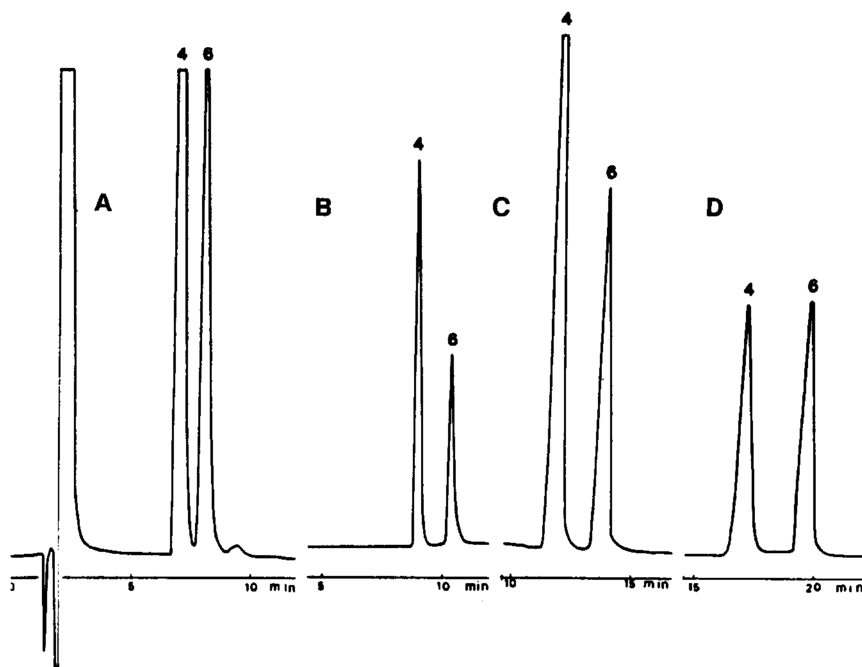

Fig. 2. HPLC separations of two standard mixtures (A–C, mixture 1; D, mixture 2) of *sn*-2- and *rac*-1-monopalmitoylglycerols (peaks 4 and 6, respectively) on an RP-18 column with different mixtures of acetonitrile–water [(A) 95:5, (B) 90:10, (C) 85:15, (D) 80:20, v/v] as the mobile phases. Analysis at ambient temperature (*ca.* 19–20°C); flow-rate, 1.2 ml min^{-1}; other conditions as in Fig. 1.

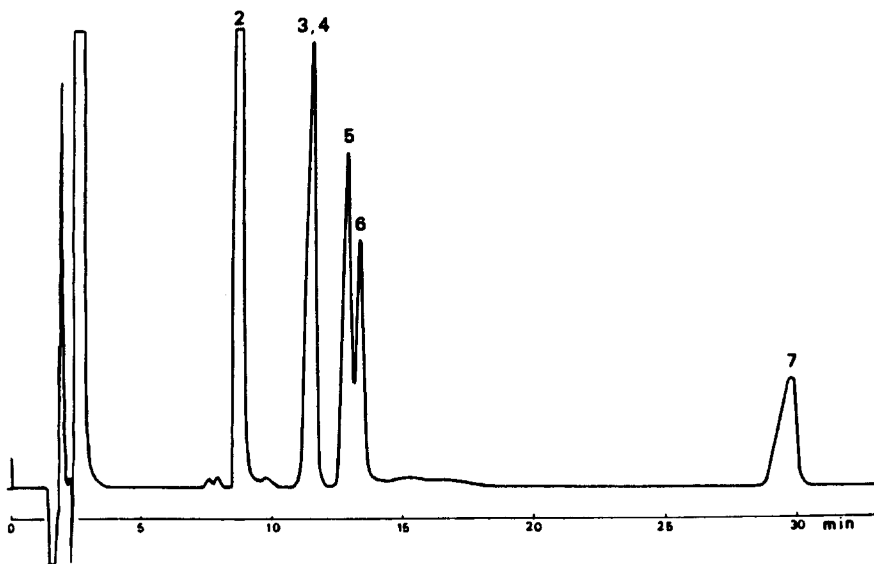

Fig. 3. RP-18 HPLC separation of a mixture of standard underivatized monoacylglycerols at ambient temperature (*ca.* 19°C). (2) = *rac*-1-18:2; (3, 4) = *sn*-2-18:1 and *sn*-2-16:0; (5) = *rac*-1-18:1; (6) = *rac*-1-16:0; (7) = *rac*-1-18:0. Mobile phase, acetonitrile–water (85:15, v/v); flow-rate, 1.2 ml min^{-1}; refractive index detection.

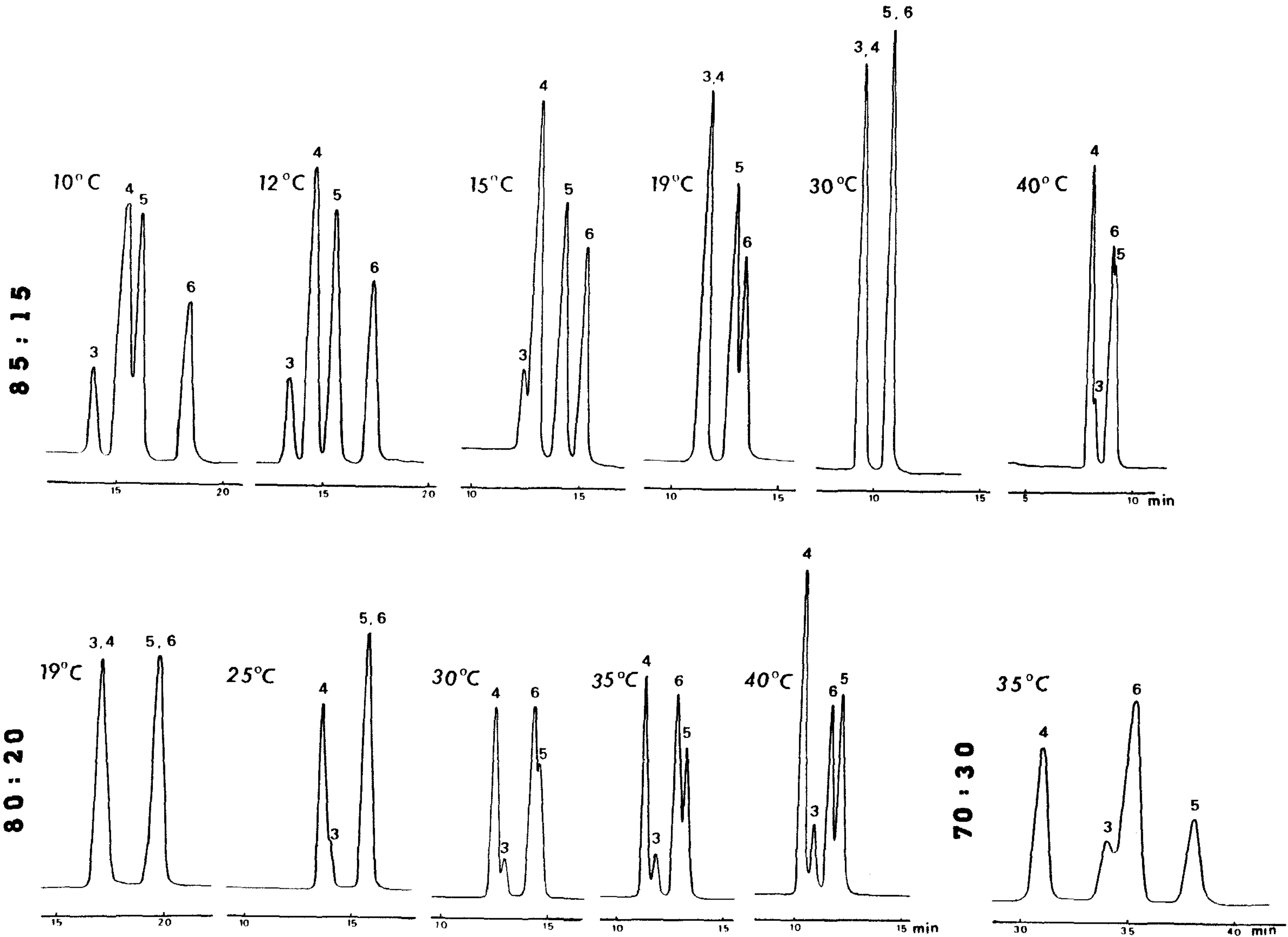

Fig. 4. RP-18 HPLC isocratic separations of a mixture of underivatized monoacylglycerols (3 = *sn*-2-18:1; 4 = *sn*-2-16:0; 5 = *rac*-1-18:1; 6 = *rac*-1-16:0) at ambient (19°C) and different thermostatically controlled temperatures (10–40°C) and with different mobile phases [acetonitrile–water (85:15, 80:20 and 70:30, v/v], as indicated. Flow-rate, 1 ml min^{-1} for the other analyses. Other conditions as in Fig. 1.

proportion of water (5, 10, 15 and 20%) in the mobile phase on the separation of the two groups of isomers [*sn*-2-16:0 and *sn*-1(3)-16:0] is illustrated in Fig. 2.

An increase in the proportion of water greatly increased the retention times of both groups of isomers and in the same proportion. The effect was more pronounced at higher proportions of water (almost a 50% increase in retention time when the water content was increased from 15 to 20%, whereas the retention time increase was only *ca.* 30% when the water content was increased from 5 to 10%).

The separation factor between the two peaks was hardly affected (1.16 to 1.18) by varying the solvent polarity, indicating that the retention times of the two peaks were similarly affected. In contrast, the resolution factor between the two peaks increased considerably (from 1.65 to 3.00) with an increase from 5 to 10% of water in the mobile phase. The resolution factor then slightly decreased (2.39 at 15% of water and 2.77 at 20%), showing that the peak widths increased more rapidly than retention times above 10% of water. At this water concentration the resolution was sufficiently improved for pure fractions of monoacylglycerol isomers to be collected for further stereospecific analysis.

Fig. 3 illustrates the separation obtained in the HPLC analysis of a more complex mixture of monoacylglycerol isomers. The synthetic mixture contained *rac*-1-monolinoleoylglycerol (*rac*-1-18:2), *rac*-1-monooleoylglycerol (*rac*-1-18:1), *rac*-1-monopalmitoylglycerol (*rac*-1-16:0), *rac*-1-monostearoylglycerol (*rac*-1-18:0), *sn*-2-monooleoylglycerol (*sn*-2-18:1) and *sn*-2-monopalmitoylglycerol (*sn*-2-16:0). The mixture was analysed at ambient temperature and with acetonitrile–water (85:15, v/v) as the mobile phase.

Separate injection of the different isomers present in the synthetic mixture showed that the first-eluted peak corresponded to a mixture of *sn*-1- and *sn*-3-18:2, which were not separated. The second peak contained the two *sn*-2-isomers of monopalmitoyl- and monooleoylglycerols, which were not separated. They form a critical pair (similar partition number). The two components of *rac*-1-16:0 and -18:1, also constituting a critical pair, were partially separated (resolution factor 0.86). The *rac*-1-18:0 was eluted very late in 29 min, but the two *sn*-1- and *sn*-3-isomers were not separated. This very long retention time implies that with monoacylglycerols of longer chain length (20:0, 22:0, 24:0) such as those encountered in peanut oil triacylglycerols after partial deacylation, the solvent polarity should be decreased and/or the analysis temperature should be increased to reduce the retention times. Even under these conditions, the resolution factors between homologous monoacylglycerol isomers would be acceptable, when considering the resolution observed between the last two peaks in the chromatogram.

In addition to the impossibility of separating the *sn*-1- and *sn*-3-isomers of monoacylglycerols, the only problem encountered in the mixture analysis was the separation of the two critical pairs of monopalmitoyl- and monooleoylglycerols in both series of *sn*-2- and *sn*-1(3)-isomers. The resolution was studied by modifying the analysis temperature.

Effect of temperature. A mixture of the two series of monopalmitoyl- and monooleoylglycerols was analysed at increasing temperature from 7.5 to 40°C with acetonitrile–water (85:15, v/v) as the mobile phase, by using the thermostating device described previously [22]. Fig. 4 shows that at 12°C the two critical pairs in both isomer series were completely separated within the reasonable time of 18 min. The observed elution order was *sn*-2-18:1, *sn*-2-16:0, *sn*-1(3)-18:1, *sn*-1(3)-16:0. This elution order was different from that obtained by Takano and Kondoh [12] on an octylsilyl column (C_8) with an acetonitrile–water mobile phase, *viz.*, *sn*-2-16:0, *sn*-1(3)-16:0, *sn*-2-18:1, *sn*-1(3)-18:1. The resolution between peaks was also better under our analytical conditions.

Fig. 4 (top) also shows that when the analysis temperature was increased the retention of monopalmitoylglycerols decreased more rapidly than that of monooleoylglycerols in both series of isomers. At 30°C the same isomers of the two monoacylglycerols were eluted together. At higher temperature (40°C) the elution order of the two monoacylglycerols was the reverse of that observed at 12°C, and resolution was poorer. The separation can be greatly improved by combining an increase in solvent polarity and an increase in analysis temperature, as illustrated in Fig. 4 (bottom). With acetonitrile–water (80:20, v/v) the separation between the same isomers of the two different monoacylglycerols increased with increas-

ing temperature and at 40°C the four isomers were better separated with a more polar mobile phase with a similar sequence of elution (peaks 4, 3, 6, 5).

As shown qualitatively in Fig. 4, the calculated resolution factors indicated that the resolution between the two *sn*-2- (peaks 3 and 4) and the two *sn*-1(3)-18:1 and -16:0 isomers (peaks 5 and 6) decreased when the analysis temperature was increased whereas the reverse was true for *sn*-2-16:0 (peak 4) and *sn*-1(3)-18:1 (peak 5).

With the second more polar acetonitrile–water mixture (80:20, v/v), the resolution between isomer pairs (peaks 3 and 5 and peaks 4 and 6) greatly increased with increase in temperature.

Considering the overall results, it is clear that two optimum temperatures exist for the separation of critical pairs of monoacylglycerols (at least for 16:0 and 18:1) depending on the proportion of water in the mobile phase: a low temperature (12°C) for the lower proportion of water, with the elution order *sn*-2-18:1, *sn*-2-16:0, *sn*-1(3)-18:1, *sn*-1(3)-16:0 and a higher temperature (at least 40°C) for the higher proportion of water with a different elution order, *sn*-2-16:0, *sn*-2-18:1, *sn*-1(3)-16:0, *sn*-1(3)-18:1.

In practice, a low analysis temperature can be recommended for short-chain or unsaturated monoacylglycerols, which are fairly soluble in the usual solvents. Conversely, temperatures higher than ambient are more convenient for long-chain monoacylglycerols, especially when they are saturated. However, high analysis temperatures raise the problem of isomerization. Takano and Kondoh [12] observed an increased isomerization of *sn*-2- to *sn*-1(3)-monoacylglycerols at temperatures higher than 30°C, precluding quantitative analyses. The solution could be to increase the proportion of water in acetonitrile in order to obtain the same resolution at 30°C as at 40°C with a less polar mobile phase.

Application to natural monoacylglycerol mixtures

The purpose of this work was to fractionate by HPLC pure monoacylglycerol isomers for further stereospecific analysis as urethane derivatives by chiral-phase HPLC. The mixtures of isomers to be fractionated would be isolated by TLC from the chemical deacylation products of natural oil triacylglycerols.

For this reason, the analytical conditions studied above were applied to the following natural mixtures of monoacylglycerols. One mixture (A) was isolated after deacylation of peanut oil palmitoyldioleoylglycerol (16:0 18:1 18:1). It contained the four groups of isomers: *sn*-2- and *sn*-1(3)-monopalmitoyl- and -monooleoylglycerols. One mixture (B) resulted from deacylation of peanut oil palmitoyloleoyllinoleoylglycerol (16:0 18:1 18:2). It contained the six groups of isomers: *sn*-2- and *sn*-1(3)-monopalmitoyl-, -monooleoyl- and -monolinoleoylglycerols. Two mixtures (C and D) were obtained from deacylation of peanut oil dioleoyllinoleoylglycerol (18:1 18:1 18:2) and oleoyldilinoleoylglycerol (18:1 18:2 18:2), respectively. They contained the same four groups of isomers: *sn*-2- and *sn*-1(3)-monooleoyl- and monolinoleoylglycerols, but in different proportions.

The analyses were carried out at (A) 12.5°C, (B) 12°C and (C and D) 19°C with acetonitrile–water (85:15, v/v) as the mobile phase. Fig. 5 illustrates the separations obtained. Table I reports data which quantify the separations observed between two successively eluting peaks for the three analysis temperatures. The three chromatograms show a good resolution of the four or the six isomers present in the mixtures within relatively short retention times, less than 20 min in all instances.

The separation between two successive peaks was complete in all instances. The poorest separation was observed for the critical pair *sn*-2-18:1 and *sn*-2-16:0. However, the resolution factor (Table I) was 1.43 at 12.5°C (mixture A) and 1.45 at 12°C (mixture B). These good separations allow the collection of the separated fractions with minor cross-contamination for subsequent analysis of the *sn*-1(3)-isomers by chiral-phase HPLC to separate the two *sn*-1- and *sn*-3-monoacylglycerol enantiomers.

In the three mixtures, the proportions of the different isomers could be calculated from peak areas, assuming the latter is proportional to the mass of a compound. Such a determination was not made because the extent of isomerization cannot yet be determined. However, we can make interesting qualitative observations.

In mixture A (Fig. 5A), resulting from deacylation of 16:0 18:1 18:1, the 16:0 was rarely esterified at the *sn*-2 position (small amount of *sn*-2-16:0) to the benefit of 18:1. The peak areas of the monooleoylglycerols were roughly twice those of the mono-

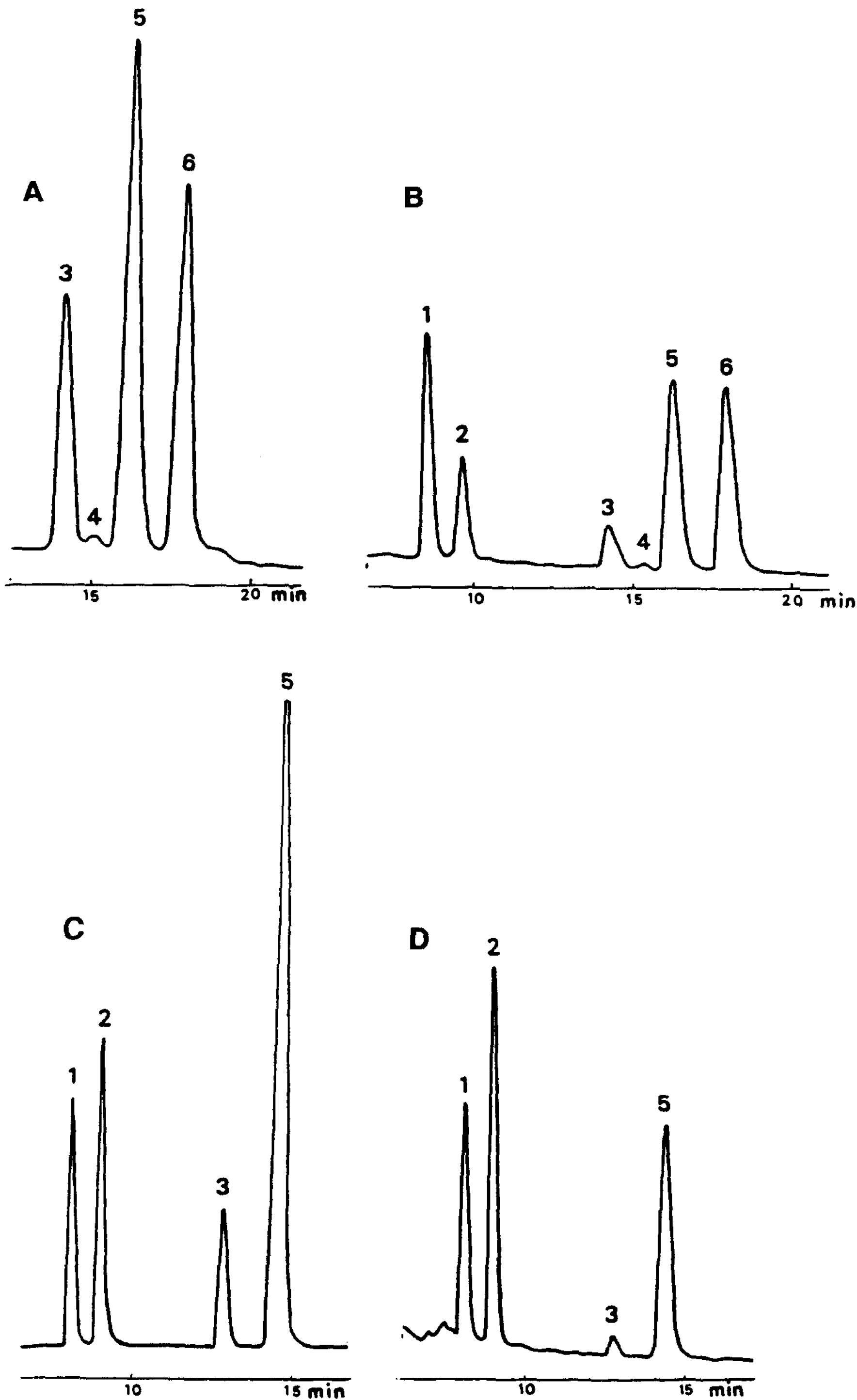

Fig. 5. RP-18 HPLC separations of mixtures of underivatized monoacylglycerols obtained after Grignard degradation of natural peanut oil triacylglycerols: (A) 16:0 18:1 18:1, (B) 16:0 18:1 18:2, (C) 18:1 18:1 18:2 and (D) 18:1 18:2 18:2. (A) Mixture of 3 = *sn*-2-18:1; 4 = *sn*-2-16:0; 5 = *sn*-1(3)-18:1; 6 = *sn*-1(3)-16:0. (B) Mixtures of 1 = *sn*-2-18:2; 2 = *sn*-1(3)-18:2; 3 = *sn*-2-18:1; 4 = *sn*-2-16:0; 5 = *sn*-1(3)-18:1; 6 = *sn*-1(3)-16:0. (C and D) Mixtures of 1 = *sn*-2-18:2; 2 = *sn*-1(3)-18:2; 3 = *sn*-2-18:1; 5 = *sn*-1(3)-18:1. Mobile phase, acetonitrile–water (85:15, v/v); flow-rate, 1.2 ml min^{-1}; analysis temperatures, (A) 12.5°C, (B) 12°C and (C and D) 19°C (ambient).

TABLE I

SEPARATION OF NATURAL MIXTURES OF MONOACYLGLYCEROL ISOMERS BY REVERSED-PHASE HPLC

Triacylglycerol[a]	16:0, 18:1, 18:1				16:0, 18:1, 18:2						18:1, 18:1, 18:2			
Monoacylglycerol[b]	*sn*-2-18:1	*sn*-2-16:0	*sn*-1(3)-18:1	*sn*-1(3)-16:0	*sn*-2-18:2	*sn*-1(3)-18:2	*sn*-2-18:1	*sn*-2-16:0	*sn*-1(3)-18:1	*sn*-1(3)-16:0	*sn*-2-18:2	*sn*-1(3)-18:2	*sn*-2-18:1	*sn*-1(3)-18:1
Temperature[c]	12.5°C				12°C						19°C			
Retention time (min)[d]	13.4	14.4	15.5	17.1	7.6	8.6	13.3	14.3	15.3	17.0	7.3	8.2	12.0	13.7
Separation factor[e]		1.07	1.08	1.10		1.14	1.54	1.08	1.07	1.11		1.12	1.47	1.14
Resolution factor[f]		1.43	1.54	1.79		1.87	6.76	1.45	1.36	2.00		2.43	9.11	3.17

[a] Triacylglycerols isolated from peanut oil by combined argentation TLC–reversed-phase HPLC.
[b] Monoacylglycerol isomers formed by chemical deacylation of the isolated triacylglycerols.
[c] Analysis at thermostated (12 and 12.5°C) and ambient (19°C) temperatures.
[d] Retention times corrected for the column void volume.
[e] Ratio of the corrected retention times of two successives peaks 1 and 2 (t_{R_2}/t_{R_1}).
[f] Calculated from the equation $R_s = 2(t_{R_2} - t_{R_1})/(w_2 + w_1)$, where t_R is the retention time and w the peak width at the baseline of two successive peaks 1 and 2.

palmitoylglycerol, *i.e.*, in proportion to the two acids in the original triacylglycerol.

The same remarks can be made for the localization of 16:0 in the triacylglycerol 16:0 18:1 18:2 (mixture B) and the roughly similar proportions of the three monoacylglycerols and the three fatty acids in the triacylglycerol (1:1:1).

As for mixture C, despite the affinity of 18:2 for the *sn*-2 position in triacylglycerols of vegetable origin, the proportion of *sn*-2-18:2 is lower than that of *sn*-2-18:1. The reason is that two molecules of 18:1 were in competition with one molecule of 18:2 for this position. The observed specificities of 16:0, 18:1 and 18:2 for the *sn*-2 position of peanut oil triacylglycerols confirm those generally observed for vegetable triacylglycerols [1].

Quantitative analysis

In stereospecific analyses of triacylglycerols by means of the monoacylglycerols formed by chemical

TABLE II

COMPOSITION OF NATURAL *sn*-2-MONOACYLGLYCEROL MIXTURES BY GC ANALYSIS OF FATTY ACIDS AND HPLC ANALYSIS OF MONOACYLGLYCEROLS

Fatty acid[a]	Triacylglycerol[b]									
	16:0 18:1 18:1		16:0 18:1 18:2		18:1 18:1 18:2		18:1 18:2 18:2		16:0 18:1 18:2	
	GC[c]	HPLC[c]	GC[c]	HPLC[c]	GC[c]	HPLC[c]	GC[c]	HPLC[c]	GC[c]	HPLC[c]
16:0	4.1	4.0	1.7	1.8	0.6	–	0.8	–	3.1	3.2
18:1	95.6	96.0	19.8	20.2	43.4	43.7	11.8	11.7	38.9	37.3
18:2	–	–	78.1	78.0	55.7	56.3	87.1	88.3	57.7	59.5

[a] Triacylglycerols isolated by combined argentation TLC–reversed-phase HPLC from peanut oil (first four triacylglycerols) and from cottonseed oil (last triacylglycerol).
[b] Traces of 16:1 (<0.1%) and 18:0 (<0.4%) were also found by GC analysis of fatty acids. Results are expressed as fatty acid mol%.
[c] *sn*-2-Monoacylglycerols formed by chemical deacylation of the triacylglycerols were isolated by TLC, analysed by HPLC and their fatty acids analysed by GC.

deacylation, one should know the exact relative proportions of the *sn*-2- and *sn*-1(3)-isomers separated by reversed-phase HPLC before analysing the *sn*-1(3)-isomers by chiral-phase HPLC for enantiomer composition. The easiest way to determine the relative proportions of the *sn*-2- and *sn*-1(3)-isomers is from peak areas, provided that the latter are proportional to mass.

To examine the possibility of using peak areas to calculate isomer composition, the following experiment was performed. Four triacylglycerols isolated from peanut oil (16:0 18:1 18:1, 16:0 18:1 18:2, 18:1 18:1 18:2 and 18:1 18:2 18:2) and one isolated from cottonseed oil (16:0 18:1 18:2) were submitted to chemical deacylation. The *sn*-2-monoacylglycerol fraction was isolated by borate-impregnated silica TLC [19]. An aliquot was analysed for fatty acid composition by GC as methyl esters. Another aliquot was analysed by HPLC and the composition was determined from peak areas.

The results reported in Table II show that the two series of figures are very similar, the variations not exceeding 4%.

As the GC analysis of fatty acids can be considered to be very accurate, the results reported here demonstrate that the same is true when the monoacylglycerol composition is calculated from peak areas.

CONCLUSIONS

This work has shown that monoacylglycerols can be completely separated by reversed-phase HPLC according to chain length and unsaturation and partially according to positional isomerism, as the *sn*-2-isomers were separated from the *sn*-1(3)-isomers. Peak areas are representative of the amount of compounds detected by their refractive indices (within the range tested). The *sn*-1(3)-isomers can be collected with minor contamination to be analysed further for *sn*-1- and *sn*-3-isomer composition by chiral-phase HPLC.

REFERENCES

1 C. Litchfield, *Analysis of Triglycerides*, Academic Press, New York, London, 1972.
2 H. Brockerhoff, *J. Lipid Res.*, 6 (1965) 10.
3 H. Brockerhoff, *J. Lipid Res.*, 8 (1967) 167.
4 H. Brockerhoff, *Lipids*, 6 (1971) 942.
5 P. Laakso and W. W. Christie, *Lipids*, 25 (1990) 349.
6 Y. Itabashi, A. Kuksis, L. Marai and T. Takagi, *J. Lipid Res.*, 31 (1990) 1711.
7 Y. Itabashi, A. Kuksis and J. J. Myher, *J. Lipid Res.*, 31 (1990) 2119.
8 B. G. Semporé and J. A. Bézard, *J. Chromatogr.*, 557 (1991) 227.
9 M. Yurkowski and H. Brockerhoff, *Biochim. Biophys. Acta*, 125 (1966) 55.
10 K. Maruyama and C. Yonese, *J. Am. Oil Chem. Soc.*, 63 (1986) 902.
11 Y. Kondoh and S. Takano, *J. Chromatogr.*, 393 (1987) 427.
12 S. Takano and Y. Kondoh, *J. Am. Oil Chem. Soc.*, 64 (1987) 1001.
13 Y. Itabashi and T. Takagi, *Lipids*, 21 (1986) 413.
14 W. W. Christie and J. H. Moore, *Biochim. Biophys. Acta*, 176 (1969) 445.
15 W. W. Christie and J. H. Moore, *Biochim. Biophys. Acta*, 210 (1970) 46.
16 F. E. Luddy, R. A. Barford, S. F. Herb, P. Magidman and R. W. Riemenschneider, *J. Am. Oil Chem. Soc.*, 41 (1964) 693.
17 F. H. Mattson and R. A. Volpenheim, *J. Lipid Res.*, 2 (1961) 58.
18 J. A. Bézard and M. A. Ouédraogo, *J. Chromatogr.*, 196 (1980) 279.
19 A. E. Thomas, III, J. E. Scharoun and H. Ralston, *J. Am. Oil Chem. Soc.*, 42 (1965) 789.
20 N. Oi and H. Kitahara, *J. Chromatogr.*, 265 (1983) 117.
21 T. Takagi and Y. Ando, *Lipids*, 25 (1990) 398.
22 M. Narce, J. Gresti and J. Bézard, *J. Chromatogr.*, 448 (1988) 249.
23 H. T. Slover and E. Lanza, *J. Am. Oil Chem. Soc.*, 58 (1979) 933.
24 A. Ros, *J. Gas Chromatogr.*, 3 (1965) 252.
25 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979.