CHROM. 3933

THE CHROMATOGRAPHIC SEPARATION OF HIGHER FATTY ACID MONOGLYCERIDES ACCORDING TO CHAIN LENGTH AND UNSATURATION*

GALINA V. NOVITSKAYA AND A. G. VERESHCHAGIN

Lipid Biochemistry Research Unit, Institute of Plant Physiology, Academy of Sciences, Moscow (U.S.S.R.)

(Received December 31st, 1968)

SUMMARY

A mixture of monostearin, monopalmitin, monoolein, monolinolein and monolinolenin (5 μg each or more) was obtained by high temperature glycerolysis of vegetable oils and isolated from an equilibrium mixture by liquid–liquid extraction in the system hexane–80% aqueous methanol saturated with boric acid. The individual monoglycerides were then separated by reversed-phase partition chromatography in the system octyl acetate–75% aqueous methanol saturated with $AgNO_3$ and octyl acetate and made visible on the chromatograms with a permanganate solution. The R_2' values of monoglycerides were 2.91, 4.94, 10.42, 12.26, and 13.47, respectively. Preparative separation of individual monoglycerides and gas-chromatographic determination of their fatty acid composition have shown that the main component content of the individual monoglycerides was equal to 96.3–99.5%. The purity of the monoglycerides at all stages of the procedure was checked by adsorption chromatography on alumina. There is no intermolecular isomerization of monoglycerides and no separation of their positional isomers in the reversed-phase system.

INTRODUCTION

The triglyceride molecular species composition of poppy, cotton and linseed oils, as well as oils of other plants has been established earlier in our laboratory[1–4]. As a further development of these studies we intend to investigate the structure (positional species composition) of individual di- and triacid triglycerides of natural oils, *i.e.* to determine the molar ratio of the positional isomers (AAB + BAA):(ABA) in diacid molecules having general formula A_2B, and the ratio of three isomers (ABC + CBA):(ACB + BCA):(CAB + BAC) in triacid molecules of the (ABC) kind.

Until recently it has been impossible to obtain any natural triglyceride in a

* Abbreviations and conventions: A, B, C = any fatty acids, and acyls of these acids in the triglyceride molecule; the sequence of the symbols indicates the position of the acyl in the triglyceride molecule. R_2' = ratio of R_F of monoglyceride to the R_F of butyl hexabromostearate[1]. V_R = retention volume in gas–liquid chromatography. 16:0, 18:0, 18:1, $18:2^{9,12}$, $18:3^{9,12,15}$ = palmitic, stearic, oleic, linoleic, and linolenic acids, respectively.

sufficiently pure individual state, and for this reason its structure was calculated indirectly from the fatty acid composition of total monoglycerides separated by adsorption chromatography of partial glycerides produced by positionally-specific lipase hydrolysis of a natural oil[5–7]. It is evident that in order to investigate directly the structure of an individual triglyceride, it is necessary to have available firstly a method of extraction and purification of the compound from its natural source, and secondly a method of chromatographic separation and identification of individual monoglycerides of definite fatty acid composition, which are produced by enzymic splitting of a triglyceride. The first of these requirements may be considered, at present, as being generally satisfied[1–3], but the chromatographic methods available have not as yet solved the other problem. Silicic acid thin-layer chromatography does not separate monoglycerides according to their fatty acid composition; furthermore, acyl migration caused by adsorption yields unidentified compounds which have the same R_F value as monoglycerides[8,9]. High-temperature gas–liquid chromatography fractionates the volatile trimethylsilyl derivatives of C_8–C_{16}-saturated monoglycerides according to chain length; however, synthesis of these derivatives is somewhat difficult, unsaturated and saturated monoglycerides of the same chain length do not resolve, and the peak of each monoglyceride is split into separate peaks of 1- and 2-isomers making evaluation of the chromatogram very complicated[10,11].

The aim of this investigation was to develop a simple analytical and preparative method for separating monoglycerides of the most common fatty acids occurring in plant tissues, according to chain length and unsaturation. The results obtained indicate that this separation can be achieved by partition chromatography of π-complexes of monoglycerides with silver ions on hydrophobic stationary phases of a new type.

EXPERIMENTAL

Purification of reagents

Methanol, ether, *n*-hexane and *n*-decane were purified according to YURYEV[12]; *n*-octanol-1 and *n*-decanol-1 were distilled before use. Reagent grade glacial acetic acid, acetic anhydride, 96% ethanol, glycerol, acetone, benzene for cryoscopy, $AgNO_3$, H_3BO_3, $KMnO_4$, anhydrous Na_2SO_4, NaOH, NaCl and argon were used without further purification.

Technical grade boric acid was recrystallized, 40 g of H_3BO_3 were dispersed in 100 ml of water and a small amount of charcoal was added; the mixture was heated with frequent stirring until all the H_3BO_3 dissolved, then filtered on a heated funnel and kept overnight at 0°. The H_3BO_3 crystals were washed with iced water on a Buchner funnel and dried to constant weight over concentrated sulphuric acid.

Glycerolysis of linseed and cottonseed oils

A mixture of 10 g of linseed oil obtained by cold pressing, 2.5 g of glycerol and 0.01 g of NaOH as a catalyst was placed in a 100 ml double-necked pear-shaped flask and heated at 180° ± 2° (20 mm Hg) for 4 h with a constant flow of argon. The contents of the flask were cooled to room temperature in the inert atmosphere and transferred with a minimal volume of ether into a 100 ml separatory funnel. The excess of glycerol was removed by 4- or 5-fold extraction with 20% (w/v) aqueous

NaCl; the ether layer was dried with Na_2SO_4, the solvent was distilled off and a 10% (w/v) solution of the glyceride equilibrium mixture in hexane or benzene was prepared. If it was necessary to obtain an equilibrium mixture with a higher content of saturated monoglycerides, a mixture of 9 g of solvent extracted cottonseed oil and 1 g of stearic acid of 96% purity was used instead of linseed oil.

Isolation of monoglycerides

The monoglycerides were extracted five or six times from a hexane solution of the equilibrium mixture with equal volumes of 80% (v/v) aqueous methanol saturated with H_3BO_3. The methanol was distilled off *in vacuo* and the monoglycerides were extracted by 5 × 4 ml of ether, dissolved in benzene to obtain 1% (w/v) solutions, and stored in dry ice to exclude any acyl migration and autooxidation. The purity of the monoglyceride preparation and the completeness of extraction was checked by chromatography on paper impregnated with Al_2O_3 (ref. 2) and also treated with a saturated solution of H_3BO_3 in methanol. The glycerides (100–500 μg) were applied from a micropipette on the start line of a 2.3 × 13 cm paper strip. The separation was carried out as described earlier[2] using 5 ml of ether–decane (4:1 v/v) as the mobile phase.

After the solvent front had reached the upper edge of the chromatogram the paper strip was removed from the chamber, the solvent was removed in a current of warm air, and the glycerides were visualized as brown spots on a light background by immersing the strip for 30–60 sec in a freshly prepared 0.1% (w/v) aqueous solution of $KMnO_4$ (ref. 13). An equilibrium mixture of mono-, di-, and triglycerides has been separated by the same method.

Preparation of stationary phases for reversed-phase chromatography

To synthesize octyl acetate, a mixture of octanol and acetic anhydride (2:3, v/v) was heated to 100° for 48 h and the b.p. 231–232° fraction was separated. In a similar way decyl acetate (b.p. 247–248°) was derived from decanol[14]. The refraction index was measured on an IRF-22 refractometer. The composition of the ester preparations was determined by gas–liquid chromatography.

Chromatographic separation of monoglycerides

Monoglycerides (5 μg or more) from a 1% (w/v) solution were applied as described above on a standard strip[15] of the "Goznak" or "Filtrac N 1" (VEB Spezialpapierfabrik, Niederschlag, G.D.R.) chromatographic paper. The strip was then impregnated with a 5% (v/v) benzene solution of octyl acetate. The monoglycerides were separated for 15–18 h at 20° (ref. 15), using 5 ml of 75% (v/v) aqueous methanol saturated with $AgNO_3$ and octyl acetate as a mobile phase. After evaporation of the methanol the stationary phase was removed in a current of warm air. The strips were then washed of the excess of silver ions and stained with a $KMnO_4$ solution (see above). The R_2' values were determined in relation to trilinolein[16].

Chromatography of monoglycerides on a preparative scale

The sample was applied and separated as described above, but its amount was increased up to 5000–6000 μg, and the time of separation up to 24 h. The monoglyceride spots were cut out from one or several stained chromatograms, cut finely

with scissors and extracted with 1% (v/v) CH_3COOH in ether. Five minutes later the liquid was decanted and the residue was washed several times with ether. Individual monoglycerides were dissolved in benzene and stored in dry ice. The fatty acid composition of the equilibrium mixtures, of the total monoglycerides, and of the individual monoglyceride preparations was determined by gas–liquid chromatography[2].

RESULTS AND DISCUSSION

For a preparative separation of total monoglycerides use was made of an equilibrium mixture of mono-, di-, and triglycerides obtained by glycerolysis of vegetable oils. The concentrations of all components of this system are random[17,18]. Therefore, it was first necessary to determine to what extent the finally adopted parameters of the reaction provide an optimal yield of monoglycerides. As shown in Fig. 1a, our mixture contains approximately equal amounts of mono- and diglycerides

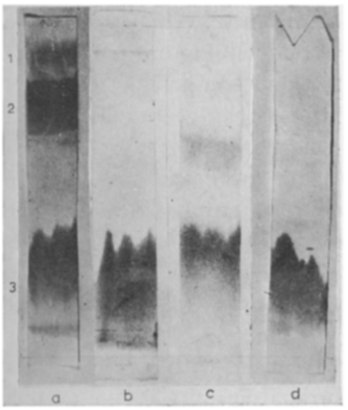

Fig. 1. Adsorption chromatograms of mono-, di-, and triglycerides. For the conditions of separation and development see "EXPERIMENTAL: Isolation of monoglycerides". 1 = Triglycerides; 2 = diglycerides; 3 = monoglycerides. (a) Equilibrium glyceride mixture; (b) monoglycerides isolated by liquid–liquid extraction; (c) the same as (b) +1% of diglycerides; (d) monoolein eluted from a partition chromatogram.

and a small amount of triglyceride. These results agree with the data of RYBICKA[17] and CHOUDHURY[18] according to whom the quantities of the respective products of glycerolysis of linseed oil amounted to 44–45, 44–51, and 5–10%. It is clear that our equilibrium system contains enough of the monoglycerides for their preparative isolation.

The total monoglycerides were isolated by liquid–liquid extraction according to the modified technique of GALANOS AND KAPOULAS[19] originally used by these authors to separate polar lipids in the two-phase system petroleum ether–87% ethanol. Boric acid saturated aqueous methanol served as the lower phase for the separation of the monoglycerides, the borate anions being capable of forming hydrophilic complexes with free hydroxyl groups of monoglyceride[20]. The method suggested can effect almost complete extraction of the monoglycerides from a non-polar phase.

TABLE I

FATTY ACID COMPOSITION OF EQUILIBRIUM MIXTURES AND ISOLATED MONOGLYCERIDES (moles %)

Original oil	*Products of glycerolysis*	*16:0*	*18:0*	*18:1*[9]	*18:2*[9,12]	*18:3*[9,12,15]	*Average lipophily of fatty acids*[21]
Linseed oil	Equilibrium mixture	6.8	4.6	17.6	10.7	60.2	13.4
	Monoglycerides	4.8	2.5	13.8	10.4	68.5	13.1
Cottonseed oil + stearic acid (9:1)	Equilibrium mixture	27.4	9.2	15.5	47.8	—	15.2
	Monoglycerides	13.0	8.6	16.9	61.5	—	14.9

The diglycerides do not pass into the aqueous phase: their visually estimated content in the final monoglyceride preparation amounted to less than 1% (Fig. 1b, c). The extractability of various monoglycerides may be different depending on chain length and unsaturation. A parallel determination of the fatty acid composition (Table I) has shown that, compared with the original mixture, the monoglycerides exhibit a somewhat lower lipophily and, consequently, a greater polarity of their acyl radicals[21]. Nevertheless, the concentrations of fatty acids found are quite sufficient for the separation of each individual monoglyceride. It should be emphasized that the extraction method has certain advantages over vacuum distillation[22,23] which yields no monoglycerides with a purity better than 50–60% and leads to a considerable acyl migration; it also compares favourably with the dialysis through a rubber membrane[24], which is very time-consuming.

Our preliminary attempts were to separate monoglycerides with regard to their fatty acid composition by reversed-phase partition chromatography in the system *n*-dodecane–silver nitrate saturated 80–95% methanol[16], because this method had been used successfully in the original form or in a somewhat modified form[25,26] for the fractionation of triglycerides and methyl esters of saturated and unsaturated fatty acids according to chain length and number of double bonds. However, because of the poor solubility of the coordination complexes of monoglycerides with silver ions in *n*-dodecane, their separation in this system proved unsatisfactory. The use of the aromatic hydrocarbon tetralin as a lipophilic phase did not improve the results. We assumed that the affinity of aliphatic stationary phase to more polar substances will increase if some hydrophilic functional group is introduced into the hydrocarbon chain. It finally turned out that if the *n*-dodecane used in the previous system was replaced by *n*-octyl or *n*-decyl aliphatic alcohols the differences in the chromatographic mobility of individual unsaturated monoglycerides considerably increased. However, the time of their separation also increased, and the separation of the monoglycerides of palmitic and stearic acids in the system *n*-octanol–silver nitrate saturated 50–60% methanol was extremely slow. Their affinity to the mobile phase could not be increased by reducing the water content in the phase because of the illimitable solubility of *n*-octanol in such solutions. Therefore, it became necessary to find a stationary phase which

TABLE II

CHARACTERISTICS OF STATIONARY PHASES FOR CHROMATOGRAPHIC SEPARATION OF MONOGLYCERIDES*

Stationary phases	*Boiling point (°C, 760 mm Hg)*		*Refraction index* n_D^{20}		V_R, *ml of carrier gas***	*Ratio between* V_R *values*	
	Found	*Literature*	*Found*	*Literature*		*Found*	*Literature*
n-Octanol-1	195	195[27]	1.4303	1.4304[28]	492	0.55	0.59[29]
n-Decanol-1	231	231[27]	1.4380	1.4368[27]	902		
n-Octyl acetate	207	210[30]	1.4207	1.4204[31]	456	0.59	0.56[33]
n-Decyl acetate	242	244[32]	1.4279	1.4273[32]	770		

* The superior figures are literature references.

** Operating conditions are similar to those described earlier[2], but column temperature is 125°, and the carrier gas flow rate 15 ml/min.

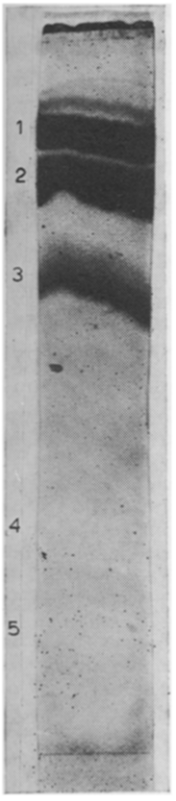

Fig. 2. Partition chromatogram of monoglycerides. For the conditions of separation and development see "EXPERIMENTAL: Chromatographic separation of monoglycerides". Sample weight 1000 μg. 1 = Monolinolenin; 2 = monolinolein; 3 = monoolein; 4 = monopalmitin; 5 = monostearin.

would be intermediate, by the degree of polarity, between a hydrocarbon and an aliphatic alcohol. It was suggested that the required value of polarity could be obtained by introducing an ester group into the aliphatic chain. To check this hypothesis, the synthesis of acetates of the two aliphatic alcohols was carried out. Some properties of the resultant esters as well as of the original alcohols are shown in Table II. It can be seen that physico-chemical constants found for the compounds investigated are close to those published elsewhere.

As shown in Fig. 2, the system *n*-octyl acetate–75% aqueous methanol saturated with $AgNO_3$ and octyl acetate allows a clear separation of individual monoglycerides. It should be emphasized that, disregarding earlier investigations[34,35] in which the paper was impregnated with triglycerides of olive oil to separate free fatty acids, the aliphatic stationary phases containing an ester group in the chain are now being used for the first time in the liquid partition chromatography of lipids. Up to now, hydrocarbon phases have been used almost exclusively in reversed-phase chromatography while stationary phases containing ester groups were only used in gas–liquid chromatography of lipids. It could be that acetates of higher fatty alcohols will find application in the partition chromatography of many classes of lipids.

Individual monoglycerides were identified by their relative chromatographic mobility, by the reaction of permanganate with their double bonds and by the fatty acid composition of their preparations. It is evident from Fig. 2 and Table III that the mixture of monoglycerides is separated into five zones having R_2' 2.91, 4.94, 10.42, 12.26, and 13.47. The three upper zones which are stained brown by a permanganate solution are apparently monoglycerides of unsaturated acids. The two lower spots do not give a colour reaction, but they are easily discernible in transmitted light on a moist chromatogram as dark areas against a more transparent background; in reflected light these zones are visible as white areas on the light-pink surface of the paper. It is suggested that the lower zones are produced by saturated monoglycerides. Determination of the fatty acid composition of the monoglycerides after their elution (Table III) allowed the final identification of the above zones as monostearin, monopalmitin, monoolein, monolinolein and monolinolenin, respectively. When separated in the reversed-phase system containing silver ions, individual monoglycerides are arranged on the chromatogram in the same order as the methyl esters

TABLE III

RELATIVE CHROMATOGRAPHIC MOBILITY, FATTY ACID COMPOSITION AND IDENTIFICATION OF INDIVIDUAL MONOGLYCERIDES

Number of chromatographic zone (Fig. 2)	R_2'	*Fatty acid composition of individual zones (moles %)*					*Identification*
		16:0	*18:0*	*18:1*[9]	*18:2*[9,12]	*18:3*[9,12,15]	
5	2.91	3.7	96.3	—	—	—	Monostearin
4	4.94	99.1	—	0.9	—	—	Monopalmitin
3	10.42	2.8	0.4	96.8	—	—	Monoolein
2	12.26	1.7	—	—	98.3	—	Monolinolein
1	13.47	0.5	—	—	—	99.5	Monolinolenin

of their respective fatty acids[16,26], *i.e.* in the order of the increasing polarity of the monoglycerides and their coordination complexes.

It is well known that under drastic conditions of separation and preparative isolation monoglycerides may undergo intermolecular isomerization with the appearance of di- and triglycerides, and intramolecular isomerization which causes the conversion of 2-monoglycerides into 1(3)-isomers[9,23]. As shown arbitrarily for monoolein (Fig. 1d), the eluted monoglycerides do not contain di- and triglyceride impurities, which points to the absence of intermolecular acyl migration. No definite answer can as yet be given to the question as to whether the partition chromatography and elution of the monoglycerides are or are not accompanied by their intramolecular isomerization, since monoglyceride positional isomers are not separated in our adsorption and reversed-phase chromatographic system.

Thus, it should be concluded, that the reversed-phase partition chromatographic system containing silver ions in the mobile phase and higher fatty alcohol acetates as a stationary phase permits an efficient analytical separation and identification of individual monoglycerides produced by lipase hydrolysis of natural triglycerides. The mild conditions of the liquid–liquid extraction and of the partition chromatographic procedure, which exclude the use of adsorption, higher temperatures and extreme pH's, make it possible to isolate individual fatty acid monoglycerides free from isomerization on a preparative scale.

REFERENCES

1 A. G. VERESHCHAGIN, *Biokhimiya*, 27 (1962) 866.
2 A. G. VERESHCHAGIN, S. V. SKVORTSOVA AND N. I. ISKHAKOV, *Biokhimiya*, 28 (1963) 868.
3 A. G. VERESHCHAGIN AND G. V. NOVITSKAYA, *J. Am. Oil Chemists' Soc.*, 42 (1965) 970.
4 G. V. NOVITSKAYA AND V. I. MAL'TSEVA, *Biokhimiya*, 30 (1965) 543.
5 P. SAVARY, J. FLANZY AND P. DESNUELLE, *Biochim. Biophys. Acta*, 24 (1957) 414.
6 B. BORGSTRÖM, *Acta Physiol. Scand.*, 30 (1964) 231.
7 F. H. MATTSON AND R. A. VOLPENHEIN, *J. Biol. Chem.*, 236 (1961) 1891.
8 D. C. MALINS, in R. T. HOLMAN (Editor), *Progress in the Chemistry of Fats and other Lipids*, Pergamon Press, London, 1966, p. 303.
9 G. V. MARINETTI, *J. Lipid Res.*, 7 (1966) 786.
10 M. R. SAHASRABUDHE AND J. J. LEGARI, *J. Am. Oil Chemists' Soc.*, 44 (1967) 379.
11 R. D. WOOD, P. K. RAJU AND R. REISER, *J. Am. Oil Chemists' Soc.*, 42 (1965) 161.
12 YU. K. YURYEV, *Prakticheskiye raboty po organicheskoi khimii*, vyp. I i II, Izd. MGU, Moscow, 1961.
13 A. G. VERESHCHAGIN, *Usp. Khim.*, 34 (1965) 1448.
14 L. BOUVEAULT AND J. BLANK, *Compt. Rend.*, 136 (1903) 1676.
15 A. G. VERESHCHAGIN, *Biokhimiya*, 23 (1958) 721.
16 A. G. VERESHCHAGIN, *J. Chromatog.*, 17 (1965) 382.
17 S. M. RYBICKA, *Chem. Ind. (London)*, (1962) 1947.
18 R. B. CHOUDHURY, *J. Am. Oil Chemists' Soc.*, 37 (1960) 483.
19 D. S. GALANOS AND W. M. KAPOULAS, *J. Lipid Res.*, 3 (1962) 134.
20 J. B. MARTIN, *J. Am. Chem. Soc.*, 75 (1953) 5482.
21 C. LITCHFIELD, *Lipids*, 3 (1968) 170.
22 G. F. LONGMAN, in H. A. BOEKENOOGEN (Editor), *Analysis and Characterization of Oil, Fats and Fat Products*, Wiley, London, 1964, p. 234.
23 O. S. PRIVETT, M. L. BLANK AND W. O. LUNDBERG, *J. Am. Oil Chemists' Soc.*, 38 (1961) 312.
24 L. HARTMAN, *J. Lipid Res.*, 8 (1967) 285.
25 M. M. PAULOSE, *J. Chromatog.*, 21 (1966) 141.
26 W. O. ORD AND P. C. BAMFORD, *Chem. Ind. (London)*, (1967) 277.
27 S. M. LOKTEV, *Vysshiye zhirnyye spirty*, Izd. "Nauka", Moscow, 1964, p. 162.
28 *Chemical Reagents and Preparations*, Goskhimizdat Publishing House, Moscow-Leningrad, 1953, p. 438.
29 F. FALK, *J. Chromatog.*, 17 (1965) 450.

30 L. FIESER AND M. FIESER, *Organic Chemistry*, Vol. I, "Khimiya" Publishing House, Moscow, 1966, p. 433.
31 *Chemist's Manual*, Vol. 4, "Khimiya" Publishing House, Moscow, 1965, p. 830.
32 G. KOMPPA AND Y. J. TALVITIE, *J. Prakt. Chem.*, 135 (1932) 193.
33 M. SATO, H. MIYAKE, M. MITOOKA AND K. ASANO, *Bull. Chem. Soc. Japan*, 38 (1965) 884.
34 G. NUNEZ AND J. SPITERI, *Bull. Soc. Chim. Biol.*, 35 (1953) 851.
35 J. HORACEK AND V. KOBRLE, *Dermatol. Wochschr.*, 132 (1953) 1053.