Gas–liquid chromatography of silylated mixtures from lipolysis of triglycerides containing unusual fatty acyl groups

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ABSTRACT A convenient and rapid procedure involving methylation, silylation, and temperature-programmed gas–liquid chromatography (GLC) is described for analyzing unfractionated products from the hydrolysis of triglycerides with pancreatic lipase. The conditions employed for GLC were selected to provide maximum and rapid separation of silylated monoglycerides in which the acyl moieties differ in chain length or degree of substitution with oxygen-containing functional groups. Derivatives differing only in the number of double bonds present were not separated. In the GLC curves obtained, peaks representing methyl esters are generally readily distinguishable from those of other components. Therefore, the extent of lipolysis and the composition (with respect to chain length and substitution of acyl groups) of monoglycerides formed are determined simultaneously.

The accuracy of the new method was demonstrated with standard mixtures and by comparison of results for several lipolysis products with data obtained by conventional procedures.

KEY WORDS gas–liquid chromatography · pancreatic lipase · silylation · triglycerides · diglycerides · monoglycerides · vernolic acid · lesquerolic acid · ricinoleic acid · *Cephalaria joppica* · *Crepis aurea* · *Limnanthes douglasii* · *Cuphea carthagenensis* · unusual seed oils

IN THE COURSE of the plant-screening program that has been underway in this laboratory for several years (1), the structures of many new fatty acids obtained from seed oils have been determined (2 and references cited therein). With the recent rapid accumulation of evidence that individual fatty acids are not necessarily distributed randomly in triglyceride molecules (e.g. 3–6), we became interested in carrying one step further the chemical characterization of many of the discovered oils; namely, determining the distribution of the unusual acyl groups in them. Published procedures for determining intraglyceride distribution of acyl groups involve analysis of monoglyceride fractions isolated by column (7) or preparative thin-layer (8) chromatography, after preferential removal of the 1- and 3-acyl groups by treatment of the oil with pancreatic lipase. Such procedures proved inconveniently time-consuming. Moreover, these conventional methods were often complicated by unusual chemical and physical properties of acyl groups under study.

The method described in this report involves the direct silylation[1] and temperature-programmed GLC of methylated samples from unfractionated, partial hydrolysates obtained by treatment of vegetable oils with pancreatic lipase. The successful application of silylated derivatives in the GLC analysis of carbohydrates by Sweeley, Bentley, Makita, and Wells (10) and more recently the GLC separation of silylated monoglycerides by Wood, Raju, and Reiser (11) suggested to us that this approach should be both workable and convenient. In practice the new approach has proved to be particularly useful for determination of the intraglyceride distribution of those unusual acyl groups which cause complications in other procedures. Our procedure gives simultaneously the extent of lipolysis and the com-

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography or gas–liquid chromatographic.

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[1] Birkofer and Ritter (9) have introduced and defined the useful term "silylation." For present purposes it means simply the conversion of hydroxy to trimethylsilyloxy groups.

position (with respect to chain length and substitution of acyl groups) of the monoglycerides formed.

The examples presented here have been selected to illustrate the wide applicability of the new method. Intraglyceride distribution data are reported for two hydroxy acids [lesquerolic or 14-hydroxy-*cis*-11-eicosenoic acid (12) and ricinoleic acid] as well as vernolic [*cis*-12,13-epoxy-*cis*-9-octadecenoic (13)] acid. Results are also given for oils containing respectively unusually long- or short-chain fatty acids; namely, *Limnanthes douglasii* var. *nivea* (14) and *Cuphea carthagenensis* (15) oils.

MATERIALS

Pancreatic lipase (steapsin) was obtained from Nutritional Biochemicals Corp.,[2] Cleveland, Ohio, and used without further purification. All reference compounds except methyl vernolate were acquired from The Hormel Institute, Austin, Minn. Methyl vernolate was prepared from *Cephalaria joppica* oil (16). Karl Fischer reagent grade pyridine (No. 214 H, Eastman Organic Chemicals, Rochester, N.Y.), further purified by distillation from barium oxide, was used as the solvent for the silylation reaction.

Arrangements for collection and botanical identification of seeds were made by Dr. Quentin Jones, Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md. Pure trivernolin prepared from *Vernonia anthelmintica* oil (17) was graciously supplied by Dr. C. F. Krewson of the Eastern Regional Research Laboratory, Philadelphia, Pa.

METHODS

Seeds were cleaned and ground in the usual way (18) and extracted with petroleum ether (bp 30–60°C) in a Soxhlet apparatus to provide the oils studied. Lipolyses were performed as described by Mattson and Volpenhein (7). After removal of solvent from diethyl ether extracts of the resulting acidified reaction mixtures, 5–20 mg samples of the residues were treated with diazomethane (19, 20) to esterify free fatty acids, and silylated by the procedure of Wood et al. (11). Extraneous peaks appeared in the GLC curves of the final products if they were not stored in a cold (−10°C) *dry* atmosphere until analyzed, or if less pure pyridine than indicated under Materials was employed in their preparation. For the GLC analysis of these products, an F & M Model 810 gas chromatograph equipped with an on-column injection system and a hydrogen flame ionization detector was used. The column was 50 × 0.16 cm I.D. (0.64 cm O.D.) stainless steel packed with 100–120 mesh DMCS Chromosorb W coated with 2% SE-30 (methylpolysiloxy gum, General Electric). It was conditioned for 16 hr at 400°C. Operating conditions included a 75 ml/min helium flow rate, an injection port temperature of 360°C, and a detector bath temperature of 370°C. The column was held at 110°C for 1 min after the sample (0.2 μl) was injected via a Hamilton No. 7101 syringe. Then a linear temperature program of 4°/min up to 400°C was initiated.

In a few instances, monoglyceride fractions from lipolysis products were analyzed in order to confirm results of the new method. They were isolated by column chromatography (7) or preparative TLC (8). Methyl esters were then prepared from the monoglycerides by acid-catalyzed methanolysis or (in the case of those containing the acid-sensitive epoxide group) by saponification and treatment of the resulting acids with diazomethane. The methyl esters so obtained were analyzed by GLC (21). Preparative TLC was employed to separate the lipolysis product from *Cuphea carthagenensis* oil into fractions to provide more suitable samples for analysis by the new method.

RESULTS AND DISCUSSION

A typical result obtained for a mixture of reference compounds by silylation and temperature programmed GLC as described above is shown in Fig. 1, curve A. Data obtained from such curves for four different standard mixtures with compositions designed to equal those generally obtained by methylation of the lipolysis products (i.e., about 50% methyl esters and 30% monoglycerides) are given in Table 1. Before percentages of components were computed from the GLC results, areas of peaks in the mono- and diglyceride regions of the curves were corrected for the silyl methyl groups but not for the silicon (which apparently does not ionize in the hydrogen flame, see reference 22). Correction for the silicon also, or failure to make any correction, led to values that did not agree so well with the actual ones. Correction for silyl methyl groups only was therefore generally applied to similar curves subsequently obtained in the analysis of lipolysis products. The method of calculation is illustrated in Table 2.

If standard mixtures A, B, C, and D of Table 1 had been methylated samples from partially hydrolyzed seed oils, the extent of hydrolysis (i.e., the percentage of free fatty acids present in each before methylation) would have been calculable from the area percentages of the methyl ester peaks. Fig. 1 provides several additional examples in support of the validity of determining extent of hydrolysis from the GLC results. That so determined for the lipolysis product from *Limnanthes douglasii* oil

[2] Mention of trade products or firm names does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over similar products or other firms not mentioned.

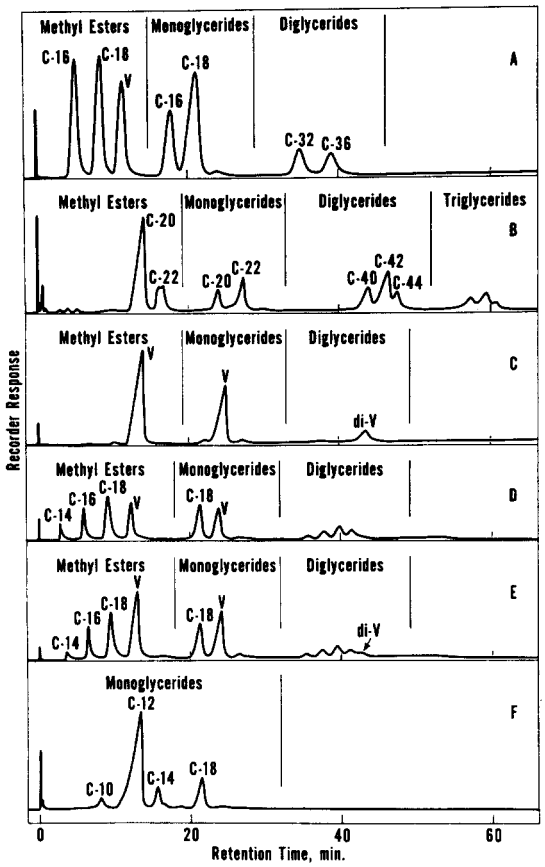

FIG. 1. Temperature-programmed GLC of silylated samples. Peaks representing methyl esters and monoglycerides are identified by the chain length of the acyl groups or *V* for methyl vernolate and monovernolin. Diglyceride peaks are identified by the sum of the chain lengths of the two acyl groups or *di-V* for divernolin. Curve A, standard mixture A of Table 1; curve B, methylated lipolysis product of *Limnanthes douglasii* oil (note slight separation of 22:1 and 22:2 methyl esters); curve C, methylated lipolysis product of trivernolin; curve D, methylated lipolysis product of *Cephalaria joppica* oil; curve E, mixture of the samples used for curves C and D; curve F, isolated monoglycerides from lipolysis product of *Cuphea carthagenensis* oil.

(curve B) was 46%, vs. 44% by titration. Similarly, trivernolin was 55% hydrolyzed by the enzymatic treatment according to curve C and 53% according to titration. The values for *Cephalaria joppica* oil are 54% from curve D and 51% by titration.

Likewise, if the standard mixtures had been samples prepared from lipolysis products of oils, the mole per cent of the C_{16} and C_{18} components in the monoglycerides would have been calculable from the areas of the peaks in the monoglyceride region. To confirm the accuracy of the new procedure further, we analyzed the lipolysis products from four oils by it and also by isolation of monoglycerides, preparation of methyl esters from the monoglycerides, and GLC of the esters. As can be seen in Table 3, the difference between the new and conventional procedures is very small for hydroxy and unusually long acyl groups. In the course of application of the conventional procedure to epoxy oils, however, we encountered several difficulties. As a result, monovernolin contents of the monoglycerides were somewhat higher (46 vs. 37% for *Cephalaria joppica* oil and 84 vs. 71% for *Crepis aurea* oil) when determined by the new method. Our experience with these epoxy oils emphasizes the advantages of analysis of lipolysis products from such oils by silylation and temperature-programmed GLC.

Three points need to be made in connection with identification of the GLC peaks:

First, the particular GLC column and conditions used in this investigation were selected to provide maximum separation (without unduly large retention times or decomposition) of silylated monoglycerides in which the acyl groups differ in chain length or degree of substitution with oxygen-containing functional groups. Derivatives differing only in the number of double bonds present were not resolved. This point is illustrated in Fig. 1, curve A, in which the C_{18} monoglyceride peak represents both monoolein and monostearin.

Secondly, we encountered no evidence for the separation of derivatives of 1- and 2-monoglyceride isomers or 1,2- and 1,3-diglycerides. These isomers were almost certainly present, for example, in the lipolysis product from trivernolin; yet curve C of Fig. 1 shows a single monoglyceride and a single diglyceride peak. This failure to separate mono- and diglyceride isomers was expected from the results of Wood et al. (11) with SE-30 columns. For present purposes such separation is not desirable because it would make the GLC curves more complicated and difficult to interpret.

Thirdly, since in curves obtained by temperature-programmed GLC two variables, time and temperature, are superimposed on the horizontal axis, some variation in retention times for individual components might be expected, thus making the identification of peaks uncertain. Actually, as inspection of Fig. 1 reveals, such variation in retention times was surprisingly small; and for oils of simple composition, the assignment of monoglyceride peaks posed no particular problem. For example, 98 moles per cent of the acyl groups in the sample of *Limnanthes douglasii* oil studied have chain lengths of 20 or 22 carbon atoms, so there can be little doubt concerning the chain lengths of the acyl groups in the derivatives represented by the only two significant peaks in the monoglyceride region of curve B. Often, however, it is desirable to add internal standards to facilitate identification of peaks. The trivernolin lipolysis product was used for this purpose in the case of the enzymatic hydrolysates from epoxy-containing materials, as illustrated in Fig. 1, curve E vs. curve D, for *Cephalaria joppica* oil. The monovernolin peak is smaller than the C_{18} monoglyceride peak in curve D but larger in curve E, i.e., with the

TABLE 1 GLC OF SILYLATED STANDARD MIXTURES

Component	A		B		C		D	
	Actual	Found	Actual	Found	Actual	Found	Actual	Found
				% of total				
Methyl palmitate	19	20	21	19	23	22	25	24
Methyl oleate	20	22	21	21	22	23	26	29
Methyl vernolate	18	16	—	—	—	—	—	—
Monopalmitin	11	10	12	13	13	14	18	16
Monostearin	10	21*	—	—	—	—	—	—
Monoolein	10		15	14	16	18	15	16
Dipalmitin	6	6	6	6	6	7	8	7
Distearin	6	5	6	7	7	7	8	7
Triolein	—	—	19	19	13	10	—	—

* Monostearin and monoolein were eluted as a single peak, labeled C_{18} in curve A, Fig. 1.

TABLE 2 SAMPLE CALCULATION: COMPOSITION OF STANDARD MIXTURE A FROM CURVE A OF FIG. 1

Component	Peak Area	Correction Factor (CF)*	Corrected Area	%†
Methyl palmitate	487	1.000	487	20
Methyl oleate	531	1.000	531	22
Methyl vernolate	398	1.000	398	16
Monopalmitin	312	0.789	246	10
Monostearin + Monoolein	646	0.802	518	21
Dipalmitin	150	0.928	139	6
Distearin	138	0.934	128	5
		Total	2447	

Peak areas were determined with a Wheelco electronic pipping integrator, Type B-2.

$$* \text{CF} = \frac{\text{Mol wt of component}}{\text{Mol wt of component} + \left(\begin{matrix}15.03 \times \text{No. of silyl methyl} \\ \text{groups in derivative}\end{matrix}\right) - \left(\begin{matrix}\text{No. of hydrogen} \\ \text{atoms replaced}\end{matrix}\right)}$$

e.g., for monopalmitin,

$$\text{CF} = \frac{330.49}{330.49 + (15.03 \times 6) - 2} = \frac{330.49}{418.67} = 0.789.$$

† Throughout this paper it is assumed that the area per cent of GLC peaks calculated as illustrated is equal to the weight per cent of components they represent. See reference 23 for a discussion of the response of the hydrogen flame ionization detector in GLC of glycerides.

TABLE 3 COMPARISON OF METHODS FOR DETERMINATION OF UNUSUAL ACYL GROUPS IN MONOGLYCERIDES

Source of Oil	Acyl Group	Mole % in Sample	Mole % in Monoglycerides	
			New Method	Conventional Method*
Lesquerella angustifolia (Nutt.) Wats.	Lesqueroloyl	63	18	19
Lesquerella angustifolia (Nutt.) Wats.	Ricinoleoyl	5	15	16
Cephalaria joppica (Spreng.) Beg.	Vernoloyl	27	46	37
Crepis aurea (L.) Cass. var. *aurea*	Vernoloyl	52	84	71
Limnanthes douglasii var. *nivea* Mason	20:0 + 20:1	69	38	39
Limnanthes douglasii var. *nivea* Mason	22:1 + 22:2	29	60	58

* Isolation of monoglycerides from lipolysis products, preparation of methyl esters from the monoglycerides, and GLC of the methyl esters.

trivernolin lipolysis product added. In other instances, such as in the analysis of the lipolysis product from the *Cuphea* oil, monostearin was used as the internal standard. The C_{18} monoglyceride peak was relatively much larger after the internal reference substance was added.

The GLC of the silylated methylated lipolysis product from *Cuphea carthagenensis* oil produced so many peaks that it was impossible to distinguish the monoglyceride region. Moreover, when the enzymatic hydrolysate was separated into mono-, di-, and triglyceride fractions, and curves such as that shown for the monoglyceride fraction (Fig. 1, curve F) were obtained for each, it became obvious that there was a marked overlap of peaks from adjacent regions in the curve for the unfractionated lipolysis product. In a case such as this, then, it is necessary to isolate the monoglyceride fraction even with our new procedure, but there is still an advantage in not having to prepare and analyze methyl esters when, as in the case of *Cuphea* oil (15), special precautions have to be taken to avoid loss of the more volatile ones.

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