Triacylglycerols of Evening Primrose (*Oenothera biennis*) Seed Oil

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The triacylglycerol (TG) composition of evening primrose *(Oenothera biennis)* seed oil (EPO) was studied using a combination of silver nitrate thin layer chromatography ($AgNO_3$-TLC), reverse phase high performance liquid chromatography (HPLC) and capillary gas liquid chromatography (GLC). The important TGs in EPO are LLL (24.4%), LLO (23.9%), LLP (11.5%), LOO (7.2%), LOP (6.8%), LLS (4.8%), γLnLp (3.7%), LOS (3.3%), γLnLS (2.0%), γLnLL (2.0%), LPP (1.9%), OOO (1.7%), LSP (1.3%) and γLnLO (1.0%).

Evening primrose *(Oenothera biennis)* seed oil (EPO) has attracted interest as a very convenient natural source of γ-linolenic (18:3n6 or γ-Ln) acid (1-3). Clinical studies have indicated that γ-linolenic acid in EPO is capable of abating or reducing the risk of diseases linked to deficiencies in certain essential fatty acids (3-9). Included among these are atopic eczema, rheumatoid arthritis, high serum cholesterol, thrombosis, mastalgia, and premenstrual syndrome. Since EPO is believed to be clinically and pharmaceutically of considerable value, there is need for an overall characterization of this oil. The fatty acid composition and the components in the unsaponifiable matter have been well documented in the literature (1,2,10). A recent publication gives the triacylglycerol stereospecific structure (11). We chose to determine the triacylglycerol composition of EPO in detail.

EXPERIMENTAL PROCEDURES

Evening primrose oil was supplied by the Efamol Research Institute, Kentville, Nova Scota. The triacylglycerol (TG) fraction of EPO was obtained by passing the oil through a silica gel column with hexane as the eluting solvent. The isolated TG was fractionated on $AgNO_3$-TLC plates prepared as follows: Pre-coated silica gel thin-layer chromatographic (TLC) plates (Adsorbosil-PLUS-1, 20 cm × 20 cm, 250 microns particle size; Alltech Associates, Inc., Applied Science Labs, Deerfield, Illinois) were developed in ethyl acetate overnight. The plates were dried in an oven at 120°C for 30 minutes and then were allowed to cool to room temperature in a desiccator covered with aluminum foil. The cleaned, dried plates were placed horizontally in a tray containing a solution of 10% $AgNO_3$ in acetonitrile for 20 min. The $AgNO_3$-TLC plates were carefully removed in a horizontal position and activated by heating in an oven at 120°C for 60 min. A chloroform solution of the TGs of EPO (12 μg/100 μl $CHCl_3$) was streaked on each $AgNO_3$-TLC plate (12 plates in total). The plates were developed in the solvent system benzene:MeOH (99.5:0.5, v/v) and then sprayed with 2′,7′-dichlorofluorescein (0.1% in ethanol) and examined under UV light. Eight bands were visible with the following R_f values: Band 1 (R_f 0.0), band 2 (R_f 0.07), band 3 (R_f 0.28), band 4 (R_f 0.40), band 5 (R_f 0.55), band 6 (R_f 0.62), band 7 (R_f 0.70), and band 8 (R_f 0.81). The bands were recovered and the silica gel extracted five times with $CHCl_3$.

Each $CHCl_3$ extract was evaporated to dryness under N_2 and redissolved in exactly 8 ml of benzene. To convert TGs to fatty acid methyl esters, 2 ml of the benzene solution was pipetted into a 10-ml screw-capped centrifuge tube (Teflon-lined cap) and heated for one hour with 7% BF_3-MeOH (12). A known amount of an internal standard, methyl eicosanoate, was added to each of the fatty acid methyl ester (FAME) fractions of the $AgNO_3$-TLC TG bands. The FAME mixture was then analyzed with a SILAR-5CP open-tubular stainless steel capillary column (47 m × 0.25 mm i.d.) in a Perkin Elmer Model 900 gas liquid chromatograph (GLC) equipped with a flame ionization detector (FID). Helium (60 psi) was used as the carrier gas, and the column was operated at 175°C.

The remaining portions of the benzene solutions (6 ml) of the $AgNO_3$-TLC TG bands were evaporated to dryness, redissolved in acetonitrile-methyl ethyl ketone (2:1, v/v), and fractionated by HPLC on a reverse-phase column (Bondapak C_{18}, 10 μ, 30 cm × 3.9 mm i.d., Waters Associates Inc., Milford, Massachusetts). The samples were run on a Waters Associates Model 6000A solvent delivery system HPLC unit using a mixture of acetonitrile-methyl ethyl ketone (2:1, v/v) at a flow rate of 1.8 ml min^{-1}. The components were detected using a Model R401 (Waters Associates) refractive index detector. Each TG peak in acetonitrile-methyl ethyl ketone solution was collected, evaporated to dryness and redissolved in benzene. The TGs were transmethylated with BF_3-MeOH and analyzed by GLC.

The identifications of fatty acid components are abbreviated as follows: M, myristic; P, palmitic; Po, palmitoleic; S, stearic; O, oleic; L, linoleic, γLn, γ-linolenic. Thus TGs are abbreviated as OOM, SSP, LnLP, etc. It should be noted here that the TG structure written as above does not necessarily represent the positional distribution of the component fatty acids.

RESULTS AND DISCUSSION

The fatty acid composition of the TG fraction of EPO used in this study is given in Table 1 with results quite compatible with those reported in the literature for this oil (1,2). Hudson (2) surveyed 192 samples of EPO and reported that γ-linolenic content varied from 2-20% with an average value of 10.4%. The 4.9% found here is on the lower range of Hudson's scale.

EPO is characterized by eight important fatty acids, of which five are major (>1%) and three are minor (>0.1%, Table 1). Theoretically, without distinguishing the isomers, one could expect at least 120 TG species in EPO for the eight important fatty acids (13). The first step in the analysis was the TLC fractionation of the TG according to their total degree of unsaturation. The solvent system benzene:MeOH gave a very clean separation of authentic tristearin (R_f 0.89), glycerol 1,3-stearate 2-oleate (R_f 0.83), triolein (R_f 0.53), glycerol 1-palmitate

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TABLE 1

Experimental[a] and Calculated[b] Fatty Acid Compositions of Evening Primrose Oil

Fatty acid	Experimental (mol %)[c]	Calculated (mol %)[c]
14:0	0.1	0.2
16:0	9.1	10.3
18:0	3.1	4.1
20:0	0.6	ND
14:1	TR	TR
16:1	0.2	0.2
18:1n9	17.7	19.2
18:2n6	64.3	61.9
18:3n6	4.9	4.1
18:3n3	TR	ND

[a]From GLC analysis of FAME.
[b]From TG fraction composition.
[c]TR, trace, <0.05%; ND, not detected.

2-oleate 3-linoleate (R_f 0.49), triolein (R_f 0.07) and trilinolenin (R_f 0.0). The TGs of EPO could be separated into eight bands on $AgNO_3$-TLC. Most of the TGs were concentrated in bands 2, 3 and 4 (Table 2) and were sandwiched between authentic triolein and trilinolein. In the second step of the analytical procedure, the isolated TGs were divided quantitatively into two portions. One portion was transmethylated and analyzed by GLC along with the internal standard, methyl eicosanoate. Table 2 gives the weight percentage of each TG fraction calculated from the internal standard and the fatty acid compositions. The remaining portions of each of the $AgNO_3$-TLC TG bands were further fractionated by reversed phase HPLC. Each HPLC fraction was collected, transmethylated and the fatty acid composition studied. TGs on reversed phase HPLC, especially on 10 μ Bondapak, separate mainly according to their partition numbers (14,15). Thus, the HPLC partition numbers and the fatty acid compositions of HPLC fractions provided more information on the types of TGs present in each $AgNO_3$-TLC band. By combining this HPLC information with the position of TG bands on $AgNO_3$-TLC and also with the fatty acid composition of each TG fraction, the TG composition of each $AgNO_3$-TLC fraction was computed with the results given in Table 3. Finally, knowing the weight of $AgNO_3$-TLC bands, the weight percentage of each TG in EPO was calculated (Table 3). An indirect approach was used to check the accuracy of the proposed TG composition. The fatty acid composition calculated from the TGs (Table 3) was compared with that experimentally determined by GLC. The results (Table 1) show that the values are remarkably similar. This confirms the correctness of the TG composition proposed for EPO.

The above procedure revealed the presence of 35 TGs which are present above the 0.1 wt% level in EPO. The LLL and LLO were the major TGs and nearly equal. The dominant role of LLL is obvious, as 64% of the total fatty acids in EPO is linoleic acid. Other TGs of importance were LLP, LOO, LOP, LLS, γLnLP, γLnLS, γLnLL, LPP, OOO, LSP and γLnLO. Further support for the proposed TG composition of EPO was obtained by examining the total oil by reversed phase HPLC. Analysis of whole EPO by reversed phase HPLC on the same 10 μ Bondapak C_{18} column showed six TG peaks of partition numbers corresponding to 40, 42, 44, 46, 48 and 50 (Fig. 1). The partition numbers of some of these peaks were assigned using authentic TG standards and the others using elution patterns published in the literature (14–18). The major components in the two peaks of partition numbers 42 and 44 were obviously LLL and LLO, respectively. The respective major components in peaks of PN 40, 46, 48 and 50 were γLnLL, LOO, LOS and OOS. The other important TGs present in all of these peaks are illustrated in Figure 1. It should be noted that EPO is a special case for the application of HPLC, as α-linolenic acid is virtually absent. The two isomers of linolenic acid (γ or 18:3n6 and α or 18:3n3) do not separate on reversed phase HPLC (10). The area percentages of the HPLC TG peaks (Fig. 1) are given in Table 4 and are compared to the weight percentages calculated from Table 3 for each partition number.

The main finding of this study of the natural occurrence of one of the rarer essential fatty acids is that a high proportion of γ-linolenic acid occurs in combination with linoleic acid (9.5% of TG), and relatively little in other

TABLE 2

Weight Percentages of the $AgNO_3$-TLC Triacylglycerol Bands of Evening Primrose Oil and Their Fatty Acid Compositions in Mol %

$AgNO_3$-TLC TG band no.	1	2	3	4	5	6	7	8
$AgNO_3$-TLC R_f	0.0	0.07	0.28	0.40	0.55	0.62	0.70	0.81
Wt %	5.8	29.4	23.8	23.4	10.6	1.9	4.2	0.8
Fatty acid				Mol %				
14:0	1.2	0.2	0.1	0.1	0.4	4.3	0.4	1.8
16:0	2.9	2.6	2.2	16.9	23.1	9.0	44.6	46.5
18:0	0.6	1.5	0.9	6.3	7.9	17.4	15.5	13.8
14:1	ND	0.1	ND	ND	ND	ND	ND	ND
16:1	ND	0.6	0.1	ND	ND	ND	ND	ND
18:1n9	8.1	5.1	27.2	18.1	37.7	51.3	12.2	32.6
18:2n6	57.4	85.7	66.2	58.3	29.7	20.0	27.2	4.3
18:3n6	29.7	4.2	3.3	0.3	1.1	ND	ND	ND
Calculated IV	183.7	164.0	146.1	118.2	88.0	79.4	59.5	37.8

TABLE 3

Triacylglycerol Components of Whole Evening Primrose Oil, HPLC Partition Numbers, and Weight Percent Distributions in Oil and $AgNO_3$-TLC Bands[a]

TG	HPLC-PN	Whole oil	$AgNO_3$ bands							
			1	2	3	4	5	6	7	8
OSM	48	TR	—	—	—	—	—	—	—	5.7
PoSS	50	TR	—	—	—	—	—	—	—	3.3
OSP	50	0.3	—	—	—	—	—	—	—	32.9
OPP	48	0.5	—	—	—	—	—	—	—	58.1
OOM	46	TR	—	—	—	—	—	—	1.0	—
LPP	46	1.9	—	—	—	—	—	13.0	39.9	—
LSP	48	1.3	—	—	—	—	—	—	30.3	—
OOP	48	0.6	—	—	—	—	—	—	14.8	—
LSS	50	0.3	—	—	—	—	—	—	6.3	—
OOS	50	0.4	—	—	—	—	—	5.9	7.7	—
LSM	46	0.1	—	—	—	—	—	6.8	—	—
LOS	48	3.3	—	—	—	—	24.4	39.2	—	—
OOO	48	1.7	—	—	—	—	8.8	35.1	—	—
LOP	46	6.8	—	—	—	—	63.7	—	—	—
LOM	44	0.1	—	—	—	0.3	—	—	—	—
LLP	44	11.5	—	—	—	49.0	—	—	—	—
LLS	46	4.5	—	—	—	19.2	—	—	—	—
LOO	46	7.2	—	—	—	30.6	—	—	—	—
LLO	44	23.9	—	15.0	82.0	—	—	—	—	—
LLL	42	24.4	27.9	70.4	8.6	—	—	—	—	—
γLnMP	42	0.1	—	—	—	—	1.1	—	—	—
γLnPP	44	0.2	—	—	—	—	2.0	—	—	—
γLnOP	44	0.2	—	—	—	0.9	—	—	—	—
γLnLP	42	3.7	—	7.5	6.4	—	—	—	—	—
γLnLS	44	2.0	—	4.5	2.7	—	—	—	—	—
γLnPoM	40	0.1	—	—	0.3	—	—	—	—	—
γLnLM	40	0.4	3.4	0.6	—	—	—	—	—	—
γLnOMo	40	0.1	—	0.3	—	—	—	—	—	—
γLnLPo	40	0.5	—	1.7	—	—	—	—	—	—
γLnγLnP	40	0.5	6.6	—	—	—	—	—	—	—
γLnγLnO	40	0.4	8.5	—	—	—	—	—	—	—
γLnγLnS	42	0.1	1.8	—	—	—	—	—	—	—
γLnLO	42	1.0	17.8	—	—	—	—	—	—	—
γLnLL	40	2.0	34.0	—	—	—	—	—	—	—

[a]TR, Trace, <0.05%.

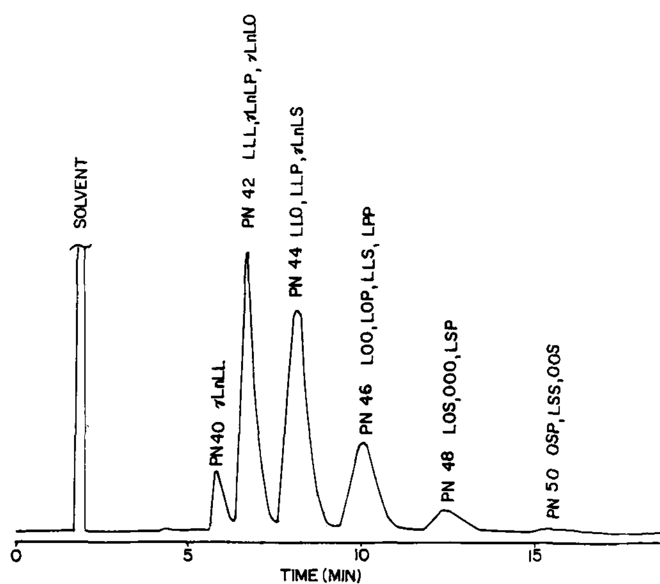

FIG. 1. HPLC separation of triacylglycerols of evening primrose oil on a C_{18} μ-Bondapak column (10 μ, 30 cm × 3.9 mm i.d.). Solvent system: acetonitrile:methyl ethyl ketone (2:1) at a forw rate of 1.8 ml min^{-1}.

TABLE 4

HPLC Peak Area Percentages of EPO TGs (Figure 1) and the Weight Percentages of the Corresponding Partition Numbers[a]

Partition number of the HPLC peak	Area %[b]	Theoretical wt%[a]
40	6.1	4.0
42	32.0	29.3
44	35.8	37.9
46	18.2	20.5
48	5.4	7.4
50	1.3	1.0

[a]Calculated from Table 3 results.
[b]Calculated from HPLC (Fig. 1).

combinations (1.7%). According to Lawson and Hughes (11) and Muderhwa et al. (19), γ-linolenic acid is mainly in the 3-position of the glycerol molecule. This co-occurrence may be of considerable interest in nutrition and biochemical studies as specific natural combinations of fatty acids in triglycerides may well modify animal biochemistry compared to the individual fatty acids or

their esters (20), or random combinations in triacylglycerols (21,22).

The co-occurrence in TG of the fatty acid with three ethylenic bonds and that with two ethylenic bonds makes it very difficult to find properties such as solubility (unpublished results of authors) which permit large-scale fractionation of this oil.

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