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Stereospecific analysis of the major triacylglycerol species containing γ-linolenic acid in Evening Primrose oil and borage oil

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Abstract

Evening Primrose oil (EPO) and borage oil (BO) are used frequently in nutritional and clinical studies involving a disease condition with an impaired or inadequate Δ^6-desaturase enzyme activity. This impairment may be bypassed by supplementation with γ-linolenic acid (GLA, 18:3n-6), an intermediate metabolite of linoleic acid (LA, 18:2n-6). The major individual triacylglycerol (TG) species comprising all potential positional isomers (molecular species) from both EPO and BO were separated, isolated by reversed-phase HPLC and subjected to HPLC stereospecific analysis as naphthylethyl urethane derivatives. The method of analysis is useful since only small quantities of the individual TG species are required and prior experimentally demanding fractionation steps are eliminated. Over 90% of the important clinical fatty acid, GLA, present in EPO and over 80% of the GLA in BO have been identified and quantified in the molecular species of their respective analyzed TG fractions. Generally, within the individual GLA-containing TG species from both oils, GLA is distributed asymmetrically among the three positions, preferentially at the sn-2 and sn-3 positions, although more so for the TG species in BO. The positional isomers of the diacid TG species were determined directly from the stereospecific analysis. For the triacid TG species computer aided linear regression was used to determine the positional isomers. The predicted positional isomeric distributions for the individual TG species calculated using the 1-random, 2-random, 3-random distribution theory from the stereospecific analysis of the native oils were in good agreement with the experimentally determined values. In contrast to other seed oils the pairs of individual TGs possessing chirality do not exist as racemic mixtures in either oil.

1. Introduction

EPO and BO are used most frequently in nutritional and clinical studies where it has been shown clinically that diseases are possibly associated with an impaired or inadequate Δ^6-desaturase activity. This impairment may be alleviated by dietary supplementation with γ-linolenic acid (GLA, 18:3n-6) an intermediate Δ^6-desaturation product of linoleic acid (LA, 18:2n-6) [1–5]. A number of animal and human studies have suggested, however, that the biological activities of these two oils are quite different [6–12].

Therefore, BO and EPO do not have equivalent effects, which however can not be explained on the basis of the GLA content alone. There are several possible explanations for this. Apart from GLA there are substantial differences in

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the overall fatty acid compositions of the two oils (see Table 2). There may be minor non-triglyceride components of the oils which exert pharmacological effects. Alternatively, the association of GLA with other fatty acids in the triacylglycerol (TG) species of the oils may modulate GLA potency in exerting its beneficial effects. Indeed, fats with similar or even identical fatty acid composition, but different TG structures, have already been shown to exert different biological activities. Peanut oil in its native state is relatively atherogenic; however, when randomized so that all the fatty acids are equally distributed among the three positions of the TG, peanut oil loses its atherogenicity [13,14]. Moreover, in rats given a structured TG, the absorption into the lymph was enhanced for fatty acids that were located in the sn-2 position of the TG [15]. In addition, there is evidence suggesting that the TG structure of human breast milk, as compared to that of formula, affects infant plasma TG and phospholipid fatty acid composition [16]. Thus it appears that the structure of the TGs in fats and oils may play an important part in the absorption, biological action and distribution of the fatty acids into tissue lipids.

Previously, the stereospecific distribution of the fatty acids in native EPO [17,18] and BO [17,19] have been determined. The GLA in both oils was distributed asymmetrically and was preferentially located at the sn-2 and sn-3 positions although more so for BO. The stereospecific distribution of individual TG fractions cannot be assumed on the basis of the analysis of the total oil. This applies to all fats and oils and the TG structures can only be determined by first fractionating the fat or oil into the individual TG species and carrying out a stereospecific analysis on each of them [20].

Previously we have described the HPLC separation and quantification of the TG species of both EPO and BO [21]. In this report we have isolated the major TG species of both EPO and BO by reversed-phase HPLC, subjected them to stereospecific analysis, and compared the positional isomer distribution ratio of the individual GLA containing TG species. There are several different methods available to determine the fatty acid stereospecific or positional distribution of TGs based on TLC, chiral-phase or normal-phase HPLC [22–27]. For our study the latter approach is the most convenient [27]. Smaller quantities of TGs can be analyzed since no intermediate TLC purification step is required and numerous samples can be handled at the same time. Moreover, this method is considered robust for the stereospecific analysis of the fatty acids in fats and oils. Recently, a comparison between the classical TLC procedure and this HPLC method demonstrated that there was no significant difference between the two methods using the TGs from olive oil [28].

2. Experimental

All solvents were HPLC grade and were obtained from BDH (Toronto, Ont., Canada). 1,3-Dioleoyl-2-palmitoyl-sn-glycerol (sn-OPO), 1,2-dioleoyl-2-linoleoyl-*rac*-glycerol (*rac*-OLL) and 1-palmitoyl-2-oleoyl-3-stearoyl-*rac*-glycerol (*rac*-POS) were purchased from Sigma (St. Louis, MO, USA). BF_3–methanol (14%) was supplied by Pierce Chemical Co. (St. Louis, MO, USA). *S*-(+)-1-(1-Naphthyl)ethyl isocyanate, 4-pyrrolidinopyridine, ethyl bromide and magnesium turnings were purchased from Aldrich (Milwaukee, WI, USA). Bakerbond Octadecyl SPE (500 mg) cartridges were obtained from J.T. Baker (Toronto, Ont., Canada) The TGs of Evening Primrose oil (Efamol, Guildford, UK) and borage oil (Callanish, Breasclete, UK) were purified on 20×20 cm silica TLC plates with hexane–diethyl ether–formic acid (80:20:2, v/v) as the developing solvent and extracted from the silica gel with chloroform–methanol (1:1, v/v).

The TG species of EPO and BO (~ 500 μg injected) were separated and identified by reversed-phase HPLC as previously described [21]. Individual TG species were collected manually (up to ten injections were made to ensure enough material was collected) for the stereochemical analysis in duplicate. An aliquot of

each individual TG fraction was kept for total fatty acid composition.

The standard TGs, the isolated individual TG species as well as samples of native EPO and BO were each dissolved in diethyl ether (2 ml) previously dried over molecular sieves (4 Å). Freshly prepared 0.5 *M* ethyl magnesium bromide (Grignard reagent) in dry diethyl ether (0.5 ml) was added and the mixture shaken for 30–40 s before glacial acetic acid (50 μl) and water (2 ml) were added to stop the reaction. The products were extracted with diethyl ether (5 ml) and washed with 2% sodium bicarbonate and distilled water. To minimize isomerization of the fatty acids within the diacyglycerols (DGs), the solvent was evaporated at room temperature and the residue derivatized immediately. The Grignard hydrolysis mixture was dissolved in toluene (0.5 ml) previously dried over molecular sieves (4 Å). (*S*)-(+)-1-(1-Naphthyl)ethyl isocyanate (12.5 μl) and 4-pyrrolidinopyridine (4 mg) were added and the mixture heated overnight at 50°C. After evaporation of the solvent, the products were dissolved in warm methanol–water (95:5, v/v, 6 ml) and added to a Bakerbond ODS solid-phase extraction column previously solvated with methanol–water (95:5, v/v, 10 ml). An additional 15 ml of this solvent was washed through and the required products were then eluted with acetone (10 ml).

The HPLC separation of the diastereomeric sn-1,2(2,3)-DG urethane derivatives was carried out with the Beckman HPLC system described above. Two columns of silica gel (Supelcosil, Supelco, Bellefonte, PA, USA, 3 μ, 15 cm × 4.6 mm I.D. and Hypersil, HiChrom, Reading, UK, 3 μ, 25 cm × 4.6 mm I.D.) were used in series with 0.3% 1-propanol (containing 2% water) in iso-octane as the mobile phase at a flow-rate of 1 ml/min and UV detection at 280 nm. The solvent system was prepared fresh daily and the sample was injected in a minimum amount of solvent. The corresponding sn-1,2- and sn-2,3-DG naphthylethyl urethane derivatives were collected manually and subsequently transmethylated [27]. In practice the diastereomeric sn-1,2- and sn-2,3-DG urethane derivatives were each collected, usually as a group of peaks, and there was baseline separation of these two groups of derivatives with the exception of samples containing long chain fatty acids (see Results section). Although the sn-1,3-DG urethane derivatives can be collected, they are usually contaminated as a result of acyl migration of the sn-1,2(2,3)-DG urethanes, and hence do not give accurate results for the sn-2 position. The individual TG species and the collected diastereomeric sn-1,2- and sn-2,3-DG urethane derivatives were methylated with 14% BF_3–methanol (2 ml) for 30 min, extracted with hexane (5 ml), concentrated and the entire sample injected.

GLC analysis was carried out using a Hewlett-Packard Model 5890 chromatograph equipped with a flame ionization detector. Separation of the methyl esters was achieved using a fused capillary Omegawax 320 column (Supelco, 30 m × 0.32 mm, 0.25 μm film thickness) at a split ratio of 1:25, injection port temperature of 200°C, detector temperature of 220°C and an oven temperature programmed at 165°C for 2 min then 6°C/min to 180°C and then held at 180°C for 3 min then 1°C/min to 190°C and held for 5 min then 0.8°C/min to 200°C then held for 5 min.

3. Results

To ensure that our methodology was reliable, three standard TGs, sn-OPO, *rac*-POS and *rac*-OLL were subjected to stereospecific analysis. After collection and methylation of the sn-1,2-DG and sn-2,3-DG urethane derivatives, the fatty acids at the three positions (expressed as mol%) within all the TGs were determined from the following equations (the brackets are meant to represent mol% values):

$$\text{Position[sn-1]} = 3 \times [\text{TG}] - 2 \times [\text{sn-2,3-DG urethanes}]$$

$$\text{Position[sn-3]} = 3 \times [\text{TG}] - 2 \times [\text{sn-1,2-DG urethanes}]$$

$$\text{Position[sn-2]} = 3 \times [\text{TG}] - [\text{sn-1}] - [\text{sn-3}]$$

Assuming these standard TGs were positionally pure, we found about 4% of the fatty acids had migrated to the outside sn-1 and sn-3 positions (Table 1) indicating a 4% absolute error associated with our methodology. This error most probably arises from acyl migration within the diacylglycerols prior to or during the derivatization procedure but is comparable to the error associated with this method of stereospecific analysis [27].

The HPLC separation of the TG species of BO and EPO is shown in Fig. 1. The identities or probable identities of the peaks have been reported previously [21]. The individual TG fractions collected from BO for stereochemical analysis were the major peaks labelled 2, 3, 4, 5,

Table 1
Positional fatty acid analysis of standard TGs and the individual TG fractions isolated from EPO and BO (expressed as mole%)

Fatty acid[a]	TG species[b]	All	sn-1[c]	sn-2[d]	sn-3[e]	Fatty acid[a]	TG species[b]	All	sn-1[c]	sn-2[d]	sn-3[e]
	sn-OPO					*Borage oil*					
16:0		33.3	3.6	92.8	3.6		LGG				
18:1[f]		66.7	96.5	7.1	96.5	18:2		33.9	88.8	5.6	7.2
	rac-POS					18:3		66.1	12.0	93.5	92.8
16:0		32.4	48.2	−0.9	49.8		LGL				
18:0		33.9	48.7	7.0	46.1	18:2		65.4	92.1	60.8	43.3
18:1[f]		33.7	3.9	93.3	4.1	18:3		34.8	8.4	38.8	57.2
	rac-OLL						OGG				
18:1[f]		33.7	46.6	4.0	50.6	18:1[f]		27.5	65.4	2.8	14.2
18:2		66.3	53.4	96.1	49.4	18:2		12.2	14.5	47.1	79.7
EPO						18:3		60.4	20.2	10.1	6.1
	LGG						PGG				
18:2		35.2	59.8	25.5	20.5	16:0		30.4	57.4	38.4	−4.6
18:3		64.8	39.5	75.3	79.6	18:2		3.3	3.9	9.3	−3.3
	LGL					18:3		66.3	38.5	53.0	107
18:2		66.8	79.8	68.9	51.7		OGL				
18:3		33.2	20.2	32.4	47.0	18:1[f]		33.0	20.2	23.4	55.4
	OGL					18:2		33.9	68.1	19.5	14.1
18:1[f]		34.4	36.8	32.6	33.8	18:3		33.0	11.2	57.4	30.4
18:2		35.8	46.8	41.8	18.8		PGL				
18:3		29.9	16.7	25.3	47.7	16:0		31.3	43.7	27.3	22.9
	PGL					18:2		33.4	44.0	27.6	28.6
16:0		28.5	59.1	1.5	24.9	18:3		35.3	12.5	44.9	48.5
18:2		36.8	24.6	52.0	33.8		OLL				
18:3		34.6	16.2	49.6	38.0	18:1[f]		33.6	15.4	21.0	64.4
	OLL					18:2		66.4	84.6	30.0	35.5
18:1[f]		33.6	27.2	41.4	32.2		SGL and PGO				
18:2		66.4	72.8	58.6	67.8	16:0		18.6	36.5	11.3	8.0
	PLL					18:0		13.5	2.4	22.2	15.6
16:0		30.0	48.2	6.4	35.4	18:1[f]		19.1	8.5	24.3	24.5
18:1[f]		2.0	nd	nd	nd	18:2		15.5	32.9	1.7	11.9
18:2		67.0	42.8	103	55.6	18:3		33.4	19.8	40.6	39.8
18:3		1.0	nd	nd	nd						

Abbreviations: nd = not detected. P = palmitic, S = stearic; O = oleic, L = linoleic, G = γ-linolenic acid.
[a] Unless otherwise indicated the unsaturated fatty acids are from the n-6 family.
[b] Each TG species analyzed in duplicate.
[c] 3 × [TG] − 2 × [sn-2,3-DG urethanes].
[d] 3 × [TG] − [sn-1] − [sn-3].
[e] 3 × [TG] − 2 × [sn-1,2-DG urethanes].
[f] n-7 and n-9.

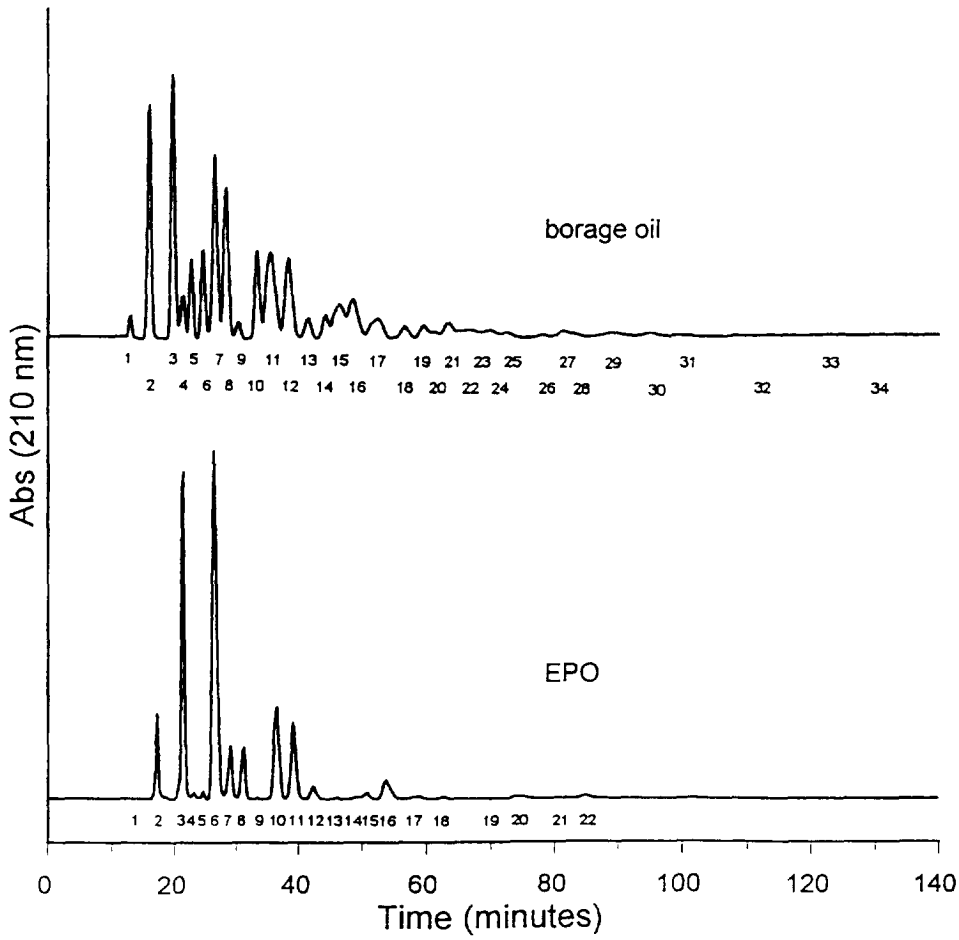

Fig. 1. Reversed-phase HPLC profile of the triacylglycerols of EPO and borage oil. Reproduced from Ref. [21].

7, 8, 10, 11, and 12 corresponding to LGG [1], LGL, OGG, PGG, OGL, PGL, OLL, a mixture of OGO, GaGL and PLL, and a mixture of SGL and PGO, respectively. For EPO the TG fractions collected were 2, 3, 7, 8, 10, and 11 corresponding to LGG, LGL, OGL, PGL, OLL, and a mixture of PLL and OGO, respectively.

The HPLC profiles of the sn-1,2(2,3)-DG urethane derivatives of native BO and EPO are shown in Fig. 2 with the results of the stereospecific analysis given in Table 2. Example HPLC profiles of the sn-1,2(2,3)-DG urethane derivatives of the individual diacid species, LGL, from BO and EPO are shown in Fig. 3. Similarly the profiles from the individual triacid species, OGL from both BO and EPO, are shown in Fig. 4. Since the mobile phase was prepared daily, differences in day-to-day retention times were observed, however, there was nearly baseline resolution of sn-1,2-DG urethanes from the sn-

[1] Note that the abbreviations (P = palmitic; S = stearic; O = oleic; L = LA; G = GLA and Ga = gadoleic or 20:1n-9) used for the TGs are meant to mean all positional isomers of a particular TG and according to a rule often used in reversed-phase HPLC are named with the shortest or most saturated fatty acid chain first and the most highly unsaturated fatty acid chain in the middle. When discussing positional isomers within an individual TG species the notation, sn (stereochemical numbering), will be used to indicate positional specificity. For example sn-OGL refers to a TG with oleic acid located at the sn-1 position, GLA at the sn-2 position and LA at the sn-3 position.

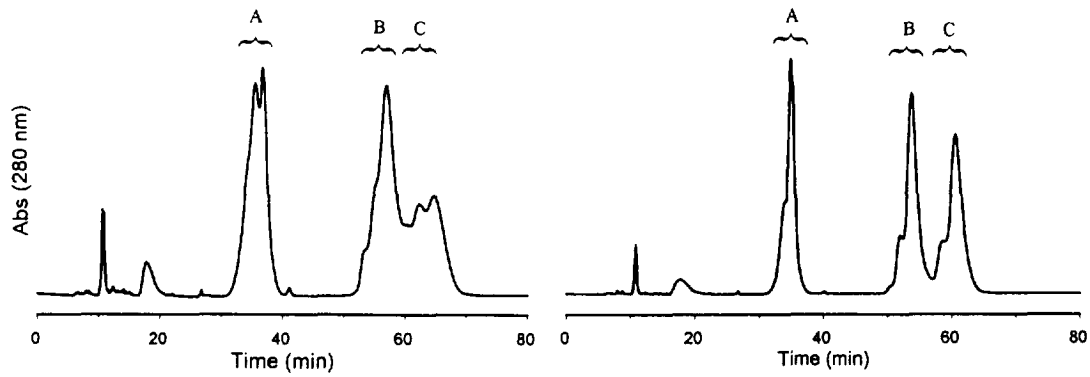

Fig. 2. HPLC profiles of the sn-1-3-, sn-1,2- and sn-2,3-DG naphthylethyl urethane derivatives obtained from BO (left) and EPO (right). The peaks under the letter A represent the sn-1,3-DG derivatives, under the letter B, the sn-1,2-DG derivatives and under the letter C the sn-2,3-DG derivatives. Conditions: two silica columns in series (150 × 4.6 mm I.D., 3-μm and 250 × 4.6 mm I.D., 3-μm); mobile phase, iso-octane–2-propanol (containing 2% water) (99.7:0.3, v/v) at 1 ml/min and UV detection at 280 nm.

2,3-DG urethane derivatives with the exception of samples containing long chain fatty acids. It was not possible to obtain baseline resolution for samples containing long chain fatty acids, most probably because the retention times of the sn-1,2-DG urethane derivatives containing long chain fatty acids overlap with the sn-2,3-DG urethane derivatives containing the normal range of fatty acids [27].

The results of the stereospecific analysis for the above TG samples and all the other isolated individual TG species are given in Table 1. Additionally for all the diacid TG species the results from the stereospecific analysis give the TG positional isomer ratios (normalized to 100%) and these are shown in Tables 3 and 4. With regards to the diacid TG species containing GLA there are some empirical observations worthy of comment. There is a significantly greater proportion of the positional isomers

Table 2
Borage oil and EPO positional fatty acid composition (expressed as mol%)

	16:0	18:0	18:1	18:2n-6	18:3n-6	20:1n-9	22:1n-9	24:1n-9
Borage Oil								
Total	11.5	4.2	18.0	42.0	17.7	3.3	2.0	1.2
sn-1[a]	20.2	4.9	17.5	38.5	3.5	5.9	6.1	3.5
sn-2[b]	−3.1	1.4	18.9	53.4	32.2	0.0	−1.7	−0.9
sn-3[c]	17.5	6.4	17.5	34.2	17.4	4.0	1.7	0.9
EPO								
Total	6.0	2.2	9.0	74.4	8.5			
sn-1[a]	11.3	3.9	9.8	70.0	4.9			
sn-2[b]	−0.4	0.7	7.8	81.5	10.3			
sn-3[c]	7.1	2.0	9.4	71.6	10.2			

[a] 3 × [TG] − 2 × [sn-2,3-DG urethanes].
[b] 3 × [TG] − [sn-1] − [sn-3].
[c] 3 × [TG] − 2 × [sn-1,2-DG urethanes].

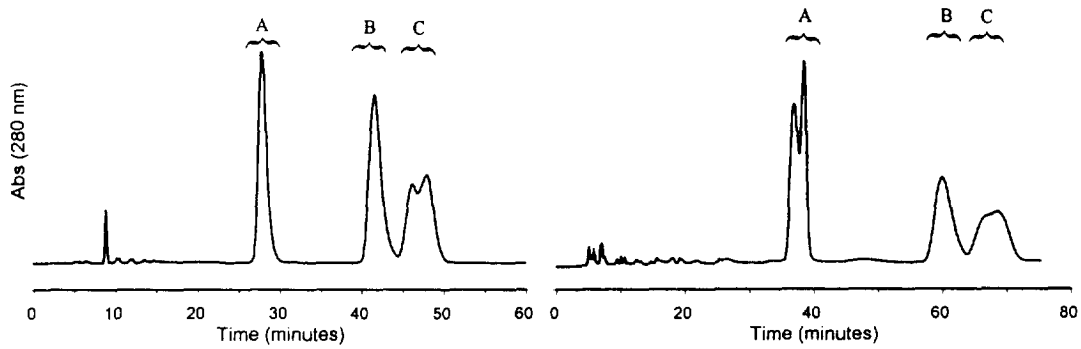

Fig. 3. HPLC profiles of the sn-1,3-, sn-1,2- and sn-2,3-DG naphthylethyl urethane derivatives obtained from the diacid TG species, LGL, isolated from BO (left) and EPO (right). The conditions and letters as described in Fig. 2.

containing GLA at the sn-1 and sn-2 positions in EPO than in BO. For example for the LGG species in EPO, sn-GGL and sn-GLG correspond to 19.4% and 24.1%, respectively, of the three possible isomers compared to only 7.1% and 5.5%, respectively, for BO. Similarly for the LGL species, sn-GLL in EPO accounts for 20.7% of the three isomers compared to only 8.0% in BO.

Within the triacid OGL and PGL species, GLA was distributed throughout the three positions slightly more in EPO than in BO. For the OGL species from EPO the percentages of the sn-GXX, sn-XGX and sn-XXG isomers are 20.1, 28.9 and 51.1%, respectively, compared to 11.6, 57.6 and 30.7%, respectively, for BO. For the PGL species from EPO the percentages of the sn-GXX, sn-XGX and sn-XXG isomers are 15.3, 49.4 and 35.6%, respectively, compared to 10.4, 43.9 and 45.7%, respectively, for BO. The different molecular species or positional isomers present in the individual triacid TG species can be mathematically determined by solving a set of equations. The equations are derived from the data in Table 1. Examples for the *rac*-POS TG and the OGL TG species isolated from EPO are given in Table 5. There are nine independent equations with a total of six unknowns. Although there are more equations than unknowns none of the equations are exactly mathematically redundant because of the small experimental inaccuracies. Consequently an exact solution using

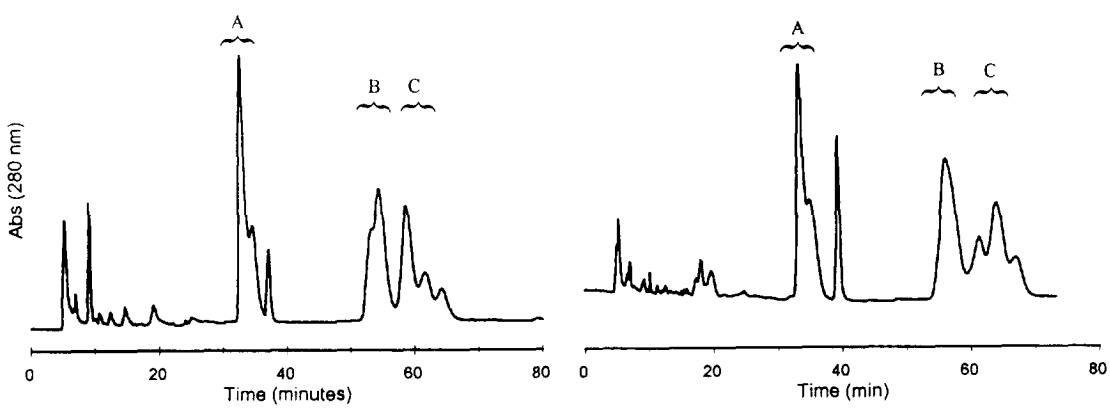

Fig. 4. HPLC profiles of the sn-1,3-, sn-1,2- and sn-2,3-DG naphthylethyl urethane derivatives obtained from the triacid TG species, OGL, isolated from BO (left) and EPO (right). The conditions and letters as described in Fig 2.

Table 3
Positional isomer distribution for the individual TG species isolated from BO

TG mol%[a]		LGG 4.7 mol%			LGL 8.3 mol%			OGG 1.0 mol%	
		TG[b]	Oil[c]		TG[b]	Oil[c]		TG[b]	Oil[c]
	sn-LGG	87.4	4.1	sn-GLL	8.0	0.7	sn-OGG	79.4	0.8
	sn-GLG	5.5	0.3	sn-LGL	37.2	3.1	sn-GOG	3.4	0.03
	sn-GGL	7.1	0.3	sn-LLG	54.8	4.5	sn-GGO	17.2	0.2
TG mol%[a]		PGG 1.9 mol%			OGL 7.4 mol%			PGL 6.1 mol%	
		TG[b]	Oil[c]		TG[b]	Oil[c]		TG[b]	Oil[c]
	sn-PGG	59.9	1.1	sn-OGL	13.5	1	sn-PGL	23.3	1.4
	sn-GPG	40.1	0.8	sn-LGO	44.1	3.3	sn-LGP	20.6	1.3
	sn-GGP	nd	nd	sn-GLO	11.6	0.9	sn-GLP	4.3	0.3
				sn-OLG	7.1	0.5	sn-PLG	22.4	1.4
				sn-LOG	23.6	1.7	sn-LPG	23.3	1.4
				sn-GOL	0	0	sn-GPL	6.1	0.4
TG mol%[a]		OLL 5.1 mol%			SGL and PGO 5.6 mol%				
		TG[b]	Oil[c]		TG[b]	Oil[c]			
	sn-OLL	15.3	0.8	sn-SGL	3.3	0.2			
	sn-LOL	20.8	1.1	sn-LGS	16.2	0.9			
	sn-LLO	63.9	3.2	sn-GLS	0.5	0.03			
				sn-SLG	0.2	0.01			
				sn-LSG	15.6	0.9			
				sn-GSL	7.7	0.4			
				sn-PGO	17.9	1.0			
				sn-OGP	3.1	0.2			
				sn-GOP	5.2	0.3			
				sn-POG	18.8	1.1			
				sn-OPG	5.2	0.3			
				sn-GPO	6.4	0.4			

[a] mol% of total oil TGs; from reference [21], [b] mol% of the individual TGs, [c] mol% of the total oil TGs.

elementary linear algebra is not possible. These slight inconsistencies resulting from the experimental inaccuracies can be resolved either by ignoring some of the equations, by making simplifying assumptions (See Ref. [26]), or by calculating an approximate solution to this overdetermined system using linear regression. To eliminate any further error that may arise from simplifying assumptions the latter approach was used. Linear regression was carried out with the numerical values in column 4 of Table 5 as the dependent variables and six independent indicator variables, A–F, corresponding to the six positional isomers of *rac*-POS or OGL. The

Table 4
Positional isomer distribution for the individual TG species isolated from EPO

TG mol%[a]		LGG 2.7 mol%			LGL 14.7 mol%			OGL 3.3 mol%	
		TG[b]	Oil[c]		TG[b]	Oil[c]		TG[b]	Oil[c]
	sn-LGG	56.5	1.5	sn-GLL	20.3	3.0	sn-OGL	10.3	0.3
	sn-GLG	24.1	0.7	sn-LGL	32.5	4.8	sn-LGO	18.6	0.6
	sn-GGL	19.4	0.5	sn-LLG	47.2	6.9	sn-GLO	14.1	0.5
				sn-OLG	25.4	0.8			
				sn-LOG	25.7	0.8			
				sn-GOL	6.0	0.2			
TG mol%[a]		PGL 3.2 mol%			OLL 12.3 mol%			PLL 10.9 mol%	
		TG[b]	Oil[c]		TG[b]	Oil[c]		TG[b]	Oil[c]
	sn-PGL	33.0	1.1	sn-OLL	27.0	3.3	sn-PLL	53.6	5.8
	sn-LGP	16.4	0.5	sn-LOL	41.1	5.1	sn-LPL	7.1	0.8
	sn-GLP	14.8	0.5	sn-LLO	31.9	3.9	sn-LLP	39.3	4.3
	sn-PLG	30.9	1.0						
	sn-LPG	4.7	0.2						
	sn-GPL	0.5	0.02						

[a] mol% of total oil TGs; from Ref. [21].
[b] mol% of the individual TGs.
[c] mol% of the total oil TGs.

solution values A–F that were obtained are given in Table 5, column 6. This method gives reasonable answers in most cases but can yield solution values that are negative when one of the fatty acid positional mol% values from Table 1 is small or close to zero. To prevent this, instead of minimizing the sum of squared differences between columns 4 and 5 of Table 5, as is done in routine linear regression, we minimized this sum with a large penalty added for any elements of column 6 that were negative. This minimization can be done easily using the iterative capabilities of non-linear regression software. The macros for implementing such a scheme in SPlus for Windows (Version 3.1, Statistical Sciences, Seattle, WA, USA) are available from the third author. The results obtained for *rac*-POS demonstrate that the linear regression analysis is in agreement with that expected for a 50:50 mixture of sn-POS and sn-SOP. The error of about 4% is a result of the error associated with the positional fatty acid stereospecific analysis. A check of the solution values by substituting them into the nine equations demonstrates that good matches were obtained. Analogous sets of equations and solutions were obtained for the OGL species isolated from BO and the PGL species isolated from both oils with the positional isomer results given in Tables 3 and 4. Fraction 11 isolated from BO contains P, O, L, G and Ga and corresponds to a mixture of PLL, OGO and GaGL based on its fatty acid composition and relative retention time [21]. No positional analysis is reported for this fraction because of errors associated with the stereospecific analysis that occur as a result of the presence of the long chain fatty acid 20:1n-9. Fraction 12 from BO contains P, S, O, L and G and corresponds to a mixture of SGL and PGO based on its fatty acid composition and relative retention time [21]. As

Table 5
Linear regression determination of the positional isomer distribution for *rac*-POS and the OGL TG species isolated from EPO

TG	sn-1,2,3-TG	Mol%	Equations[a]	Check[b]	Solution[c]	Theoretical[d]
rac-POS	PSO	A	A + B = 48.2	48.8	A = 3.2	A = 0
	POS	B	C + D = 0	0.17	B = 45.6	B = 50.0
	SPO	C	E + F = 49.8	50.7	C = 0.07	C = 0
	OPS	D	C + E = 48.7	47.47	D = 0.1	D = 0
	SOP	E	A + F = 7.0	6.5	E = 47.4	E = 50.0
	OSP	F	B + D = 46.1	45.7	F = 3.3	F = 0
			D + F = 3.9	3.4		
			B + E = 93.3	93.0		
			A + C = 4.1	3.21		
OGL	OLG	A	A + B = 36.8	35.7	A = 25.4	A = 23.8
	OGL	B	C + D = 32.6	31.7	B = 10.3	B = 21.1
	LOG	C	E + F = 33.9	32.7	C = 25.7	C = 16.3
	GOL	D	C + E = 46.8	44.3	D = 6.0	D = 8.0
	LGO	E	A + F = 41.8	39.5	E = 18.6	E = 19.8
	GLO	F	B + D = 18.8	16.3	F = 14.1	F = 11.0
			D + F = 16.7	20.1		
			B + E = 25.3	28.9		
			A + C = 47.7	51.1		

[a] Values from Table 1.
[b] Substitute solution into equations.
[c] Computer-aided non-linear least squares regression, see Results.
[d] Based on 50:50 mixture for *rac*-POS or on the data in Table 2 for EPO and using the 1-random, 2-random, 3-random distribution theory.

with the triacid TG species the positional isomer distribution for this mixture can be determined by linear regression of the set of equations derived from the data in Table 1. In this case there were 15 equations and 12 unknowns with the positional isomer distribution results given in Table 3.

The six TG species analyzed from EPO represent 47.1% of the total TGs. If trilinolein (38.0% of the total TGs) is included then over 85% of the molecular species (or positional isomers) in EPO and over 50% of the molecular species in BO have been identified and quantified. More importantly, over 90% of the GLA present in EPO and over 80% of the GLA in BO can be accounted for in their respective analyzed TG fractions.

4. Discussion

In this study the stereospecific analysis of native EPO agrees with previous analyses [17,18]. The results for BO are also in broad agreement with those obtained previously [17,19], although differences regarding the long chain fatty acids, 22:1n-9 and 24:1n-9, are evident. We found that these long chain fatty acids were almost exclusively located at the sn-1 position whereas previously 22:1n-9 was found almost exclusively at the sn-3 position [17]. However, it has been noted that fats or oils containing fatty acids greater than C_{18} in length are not suitable for stereospecific analysis by the present HPLC method, hence the results presented here for BO should be viewed with caution [27]. Indeed, the HPLC profile of the sn-1,2(2,3)-DG urethane derivatives from BO shown in Fig. 2 shows that there was no baseline separation between these two groups of diastereomeric DG urethane derivatives meaning there was overlap during their collection. A similar problem was encountered for fraction 11 isolated from BO that contains the long chain fatty acid, 20:1n-9. As mentioned earlier the most probable reason is the overlap of the sn-

1,2-DG urethanes containing long chain fatty acids with the sn-2,3-DG urethanes containing the normal range of fatty acids.

The stereospecific analysis of the individual TG species isolated by reversed-phase HPLC was relatively easy accomplished, although more than 10 injections of ~500 μg of BO or EPO were required to provide enough of the individual TG species for the stereospecific analysis. As can be seen from Figs. 3 and 4 the diastereomeric sn-1,2-DG urethane derivatives were virtually baseline resolved from the sn-2,3-DG urethane derivatives. Moreover, these profiles and the profiles of the sn-1,2- and sn-2,3-DG urethane derivatives for the other individual TGs isolated from the two oils are qualitatively different suggesting that the positional isomeric distributions are different between the same individual TG species from the two oils. Indeed, Tables 3 and 4 clearly show that the positional isomeric distributions are different for the same individual TG species in the two oils.

The TGs of plant seed oils which are rich in oleic, linoleic and α-linolenic acids have a relatively low degree of asymmetry based on the stereospecific analysis of the total oil. In maize, soybean, linseed, and olive oils position sn-2 is almost exclusively occupied by unsaturated fatty acids, while saturated as well as unsaturated fatty acids occur to the same extent at the sn-1 and sn-3 positions [23]. The isolated TG classes from the monounsaturated TGs of cocoa butter were also found to be racemic [29]. However, in this report, examination of the individual TG species possessing chirality shows that they do not exist, either in BO or EPO, as racemic mixtures. In general, there is a substantial enantiomeric excess (ee) defined as

$$\text{Enantiomeric excess (ee)} = \frac{\%\text{difference between enantiomers}}{\%\text{total of enantiomers}} \cdot 100\%$$

which is larger in BO than in EPO. For the LGG species in EPO there was 49% ee for the sn-LGG optical isomer compared to 85% in BO. For the LGL species in EPO there was 40% ee for the sn-LLG optical isomer compared to 74% in BO. With regards to the three individual pairs of optical isomers in the OGL and PGL species, while there was an excess of one enantiomer, no striking differences were evident comparing the two oils. In contrast, the stereospecific analysis of the PLO TG species isolated from peanut and cottonseed oils showed that there was an enantiomeric excess within the pairs of optical isomers of PLO from peanut oil whereas the individual pairs of optical isomers occurred nearly to the same extent in cottonseed oil [26]. Although it has been shown that GLA is resistant to pancreatic lipase in vitro [17] it is uncertain whether the enzymes involved in the hydrolysis of TGs in vivo have a preference for one enantiomer.

The positional isomeric distribution for the individual TG species were also predicted using the 1-random, 2-random, 3-random distribution theory by multiplying the stereospecific results obtained for the native oils. For example, within EPO (Table 2) one would predict for sn-GLL, $0.049 \times 0.815 \times 0.716$, or 2.86 mol%; similarly 5.16 mol% for sn-LGL, and 5.82 mol% for sn-LLG. These values when converted to a percentage of the three possible isomers are 20.7%, 37.3% and 42.1%, respectively, which are in very good agreement with the values determined experimentally (Table 4). Similarly the predicted percentages of the three isomers for the LGG species from EPO are 24.0%, 27.1% and 48.9%, corresponding to sn-GGL, sn-GLG and sn-LGG, respectively. Again these predicted values are in good agreement with the values determined experimentally. The predicted positional distribution for the OGL species from EPO is given in Table 5 and broadly compares to the values determined experimentally by linear regression. A similar comparison was observed for the PGL species from EPO.

The values predicted for the isomeric distribution of the LGG and LGL species from BO do not agree as well with the experimental values. The differences in these cases are most probably a result of the errors associated with our stereospecific analysis of native BO as discussed earlier. However, using the data from Ref. [17], even though in that report there was significantly more GLA present, the predicted values for the isomeric distribution of the diacid LGG and

LGL species and triacid OGL and PGL species of BO are in much closer agreement to the values determined experimentally.

The agreement between the predicted (calculated) and experimentally determined positional isomeric distribution for individual TG species suggests that as long as the stereospecific analysis of an intact fat or oil is carried out with reasonable precision the results can be used as a guide to estimate the isomeric composition of individual TG species within a fat or oil. The exact composition, however, can only be determined by fractionating the fat or oil and analyzing the isolated individual TG species. In animal tissues where the TG species can be markedly asymmetric the 1-random, 2-random, 3-random distribution theory has been used to calculate the amounts of various positional isomers with precision [22,30]. Recently the major individual TG species of olive oil have been isolated by silver-ion HPLC and subjected to stereospecific analysis. Again, using the stereospecific analysis results for the intact olive oil and the 1-random, 2-random, 3-random distribution, there was very good agreement between the predicted and experimental positional isomer distributions for the isolated OLO and POO TG species [31].

The advantage of the present method for the stereospecific analysis of individual TG species is that they were isolated by HPLC directly from the oils. While it may be time-consuming to collect enough material, prior experimentally-demanding fractionation steps are eliminated. The positional isomer analysis of isolated diacid TG species was very straightforward, but for the triacid TG species computer-aided linear regression was utilized to solve the positional isomer equation set. Others utilizing a slightly different approach have, with regard to the isolated PLO species from peanut and cottonseed oils, assumed that the percentage of P at the sn-2 position was negligible as an approximation in solving their equation set [26]. This assumption was valid in their case, however, it would not be prudent to make any assumption in our case especially because of the asymmetric distribution of GLA. Moreover, while in this study only the sn-1 and sn-3 positions are determined directly with the sn-2 position calculated and, thus, subject to cumulative error, it has been demonstrated that no significant difference exists between the classical TLC and HPLC stereospecific methods of analyses for oils containing a normal range of fatty acids [28]. Further refinements are, however, required for fats and oils containing the full range of fatty acids.

In summary this study has established that there are substantial differences in positional isomerism of the major GLA-containing individual TG species between BO and EPO. Whether these differences can account for the demonstrated differences in biological activity is not known. A final explanation of the biological differences between the whole oils will require the specific biological testing of either the isolated individual TG positional isomers or the non-triglyceride fractions.

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