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Separation and quantification of the triacylglycerols in evening primrose and borage oils by reversed-phase high-performance liquid chromatography

Peter R. Redden[a,*], Yung-Sheng Huang[b], Xiaorong Lin[a], David F. Horrobin[a]

[a]*Efamol Research Institute, Kentville, Nova Scotia, B4N 4H8, Canada*

[b]*Medical Nutritional R and D, Ross Products Division, Abbott Laboratories, 625 Cleveland Avenue, Columbus, OH 43215, USA*

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Abstract

Evening primrose and borage oil are used frequently in nutritional and clinical studies where an impaired Δ^6-desaturase enzyme activity may be bypassed by supplementation with γ-linolenic acid (GLA, 18:3*n* − 6). The separation and quantification of the triglycerides of borage oil and evening primrose oil has been carried out using reversed-phase HPLC with UV detection. Borage oil was found to have 34 UV-detectable fractions and evening primrose 22. The TG fractions were collected manually, their fatty acid composition determined and quantified with an internal standard. The probable identity of the individual TG fractions was deduced using the fatty acid composition of the TG fractions, calculated theoretical carbon numbers (TCN) for the various TG species and the predicted probability of occurrence. Correction factors, U_i, for GLA (18:3*n* − 6), gadoleic acid (20:1*n* − 9), erucic acid (22:1*n* − 9) and nervonic acid (24:1*n* − 9) were estimated to be 0.3–0.4, 0.6, 0.4 and 0.3, respectively, and are used along with other known U_i correction factors for unsaturated fatty acids to calculate TCN values for all the TG species. These U_i values represent the loss in affinity of the unsaturated fatty acid for the reversed-phase C-18 stationary phase. The reversed-phase HPLC trace of borage oil is much more complex compared to evening primrose oil. Apart from differences in the total fatty acid composition there are substantial differences in the quantity of individual TG species present in the two oils. The clinically important fatty acid, γ-linolenic acid, is distributed much more widely throughout the TG species of borage oil compared to evening primrose oil. Over 90% of the GLA present in evening primrose occurs in the first 9 eluting TG species whereas only about 65% is found in these TG species of borage oil.

1. Introduction

Clinical evidence has shown that diseases possibly associated with an impaired Δ^6-desaturase activity as indicated by deficits of linoleic acid metabolites, may be alleviated by dietary supplementation of γ-linolenic acid (GLA, 18:3*n* − 6), an intermediate Δ^6-desaturation product of linoleic acid (LA, 18:2*n* − 6) [1–5]. In human and animal tissues, GLA levels are generally low because GLA is rapidly metabolized to 20:3*n* − 6 and subsequently to 20:4*n* − 6 [6]. However, significant levels of GLA, mainly in

* Corresponding author.

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the triacylglycerol (TG) form, have been found in evening primrose oil (EPO, *Oenothera biennis*), borage oil (BO, *Borago officinalis*), black currant seed oil (*Ribes nigrum*) and fungal oils (e.g. *Mucor javanicus*) [7,8]. Among these, EPO and BO are used most frequently in nutritional and clinical studies. However, the biological potency of these two oils appears to be quite different [9–15]. Thus BO and EPO do not have equivalent effects explicable on the basis of the GLA content of the oils.

There are several possible explanations for this. Apart from GLA there are substantial differences in the overall fatty acid compositions of the two oils (Tables 1 and 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 TG molecules may modulate GLA potency in exerting its beneficial effects. Additionally, fats with similar fatty acid composition but different TG structures exert different biological activities [16–18]. For example peanut oil in its native state is relatively atherogenic, however, when peanut oil is randomized it loses its atherogenicity [16,17]. In this article we were interested in comparing the identification and quantitation of the individual TG species between the two oils.

The HPLC separation of the TG species of natural fats and oils has been carried out by numerous workers with a wide variety of solvents and methods of detection [19–24]. Although the separations in most cases was good, the identification of the individual TG fractions was made solely on the basis of their retention times; consequently the assignments were incomplete and in some cases incorrect. Here we describe the separation, identification and distribution of the TG species in EPO and in borage oil using highly efficient reversed-phase HPLC columns. The individual TG fractions as they elute from the HPLC column were collected, their fatty acid composition determined and quantitated by GC using an internal standard. In addition, a comparison of the TG species was made between quantification with an internal standard and integration of the areas of the TG species obtained with an evaporative light-scattering detector.

2. Experimental

HPLC grade isopropanol, chloroform, and acetonitrile were purchased from BDH (Toronto, Ont., Canada). BF_3-methanol was supplied by Pierce Chemical Co. (St. Louis, MO, USA). The TG standards tri-γ-linolenin (GGG) and trilinolein (LLL) were purchased from Nu-Chek Prep (Elysian, MN, USA) Pure di-γ-linoleneoyl-monolinolein (LGG), and dilinoleoyl-mono-γ-linolenin (LGL) were purified by semi-prep HPLC as outlined below from a sample of EPO enriched in unsaturated TG species (Callanish, Breasclete, Scotland). Note that the abbreviations used for TG's 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 most highly unsaturated fatty acid chain in the middle. The triacylglycerol components in cold pressed olive oil, evening primrose oil (Efamol, Guildford, U.K.) and borage oil (Callanish) were purified by silica gel column chromatography using 3% diethyl ether in hexane.

HPLC analysis was carried out using a Beckman System Gold solvent module 126, autosampler Model 507 and variable UV wavelength detector Model 166. The following column, conditions and solvent system were used for the purification of LGG and LGL from the enriched unsaturated TG EPO sample. The column used was a μ-Bondapak C-18 radial-pak 10 cm × 25 mm, 6 μm (Millipore Corp., Milford, MA, USA). The solvent system was acetonitrile–2-propanol (65:35 v/v) at a flow rate of 10 ml/min with UV detection at 218 nm. The following columns, conditions and solvent system were used for the separation of the TG species of the various oils. A reversed-phase Supelcosil LC-18 column (25 cm × 4.6 mm, 5 μm, Supelco, Bellefonte, PA, USA), was used for the separation and analysis. The purified oils were dissolved in chloroform (200 mg/ml) and eluted with

Table 1
Fatty acid composition (area % of the total fatty acids) of the TG fractions of borage oil separated by reversed-phase HPLC

Fraction no.	16:0	18:0	18:1 $n-9$	18:1 $n-7$	18:2 $n-6$	18:3 $n-6$	20:1 $n-9$	22:1 $n-9$	24:1 $n-9$	Other
Total oil	11.3	3.9	18.9	0.1	37.6	20.6	3.5	1.8	0.8	1.5
1						99.9				0.1
2	2.1	1.1	3.5		33.1	59.1				1.1
3	0.8	0.4	0.8		64.7	32.4				1.0
4	3.2	2.2	26.8	2.8	9.3	55.8				
5	29.0	1.7	2.5		3.1	62.9				
6	1.7	1.0	1.3		93.8	1.7				0.5
7	0.7	0.4	30.9	1.4	33.8	31.7	0.3			0.8
8	27.3	0.5	2.2		32.9	34.8	1.7			0.6
9	15.4	20.4	7.9		11.9	38.1				6.3
10	0.7	0.7	31.6	1.2	64.1	0.9	0.5			0.4
11	14.7	0.5	15.5	0.6	43.6	15.4	8.6	0.4		0.7
12	16.3	13.7	19.1	0.8	16.8	32.2	0.5	0.6		
13	51.8	3.1	3.8		4.9	36.3				
14	2.4	1.2	7.3	1.4	55.0	1.6	29.9			1.2
15	1.4	1.7	39.8	1.1	29.4	9.1	2.3	9.2	1.5	3.1
16	17.4	9.0	24.3	0.8	40.7	3.9	3.0	0.9		
17	31.6	9.7	10.5	0.5	17.9	18.8	8.8			1.8
18	27.4	30.7	5.4		8.9	27.7				
19	1.7	2.1	6.1		52.1		7.0	31.0		
20	3.0	3.0	28.7		32.8		32.4			
21	1.8	0.8	53.2	1.1	11.7	11.2	4.9	2.9	10.3	2.1
22	9.9	6.8	9.6		30.2	3.1	31.9	4.6		4.0
23	12.0	18.1	41.7	1.2	21.9		3.8			1.3
24	19.6	8.8	36.2		6.5	12.6	4.7	11.6		
25	38.1	22.5	16.7		20.8	1.8				
26	13.7	37.7				20.3			28.3	
27			8.8		41.5		5.5	12.8	25.7	5.7
28			35.7		19.6		14.7	25.5		4.4
29	11.1	6.2	22.1		16.7	3.5	19.7	13.8	3.4	3.2
30	8.5	21.2	37.8		2.9	10.2	4.5	4.0	9.4	1.5
31	19.7	43.0	25.6		11.7					
32	6.0	3.8	32.5		13.4	2.3	6.7	17.2	15.7	2.4
33	17.6	8.8	12.4		22.4		3.2	18.5	17.1	
34	9.7	17.3	26.1		9.0	5.9	10.5	7.0		14.5
Total recom.	10.9	5.2	18.7	1.0	34.8	19.3	4.4	2.9	1.4	

The total recombined fatty acid composition was determined by computation using an internal standard.

acetonitrile–2-propanol (65:35 v/v) at a flow of 1 ml/min and UV detection at 210 nm. The eluting TG species emerging from the UV detector were collected manually for fatty acid analysis by GC. When the evaporative light-scattering detector (ACS Model 740/14, Applied Chromatography System, Macclesfield, England) was used the settings were as follows: attenuation range, 16; photomultiplier sensitivity, 5; time constant, 5 s; evaporator set, 40 and internal air pressure, 187 kPa. The output signal analysis and integration were performed with an IBM PS/2 computer using the System Gold software (version 3.1).

GC analysis was carried out using a Hewlett-

Table 2
Fatty acid composition (area % of the total fatty acids) of the TG fractions of EPO separated by reversed-phase HPLC

Fraction no.	16:0	16:1 $n-7$	18:0	18:1 $n-9$	18:1 $n-7$	18:2 $n-6$	18:3 $n-6$	Other
Total oil	5.3	0.1	1.0	6.4	0.6	75.1	11.3	0.4
1							tr	
2	7.3	1.1	1.9	6.3		37.6	43.9	1.8
3	1.0		0.2	1.1		65.7	31.4	0.5
4	10.7		7.5	20.7		32.6	28.5	
5	24.4		8.8	11.6		20.4	34.8	
6	0.2	0.1	0.1	0.2		99.4	0.1	
7	2.3		1.3	26.9	2.6	39.0	27.2	0.7
8	28.8	1.4	1.3	2.0		35.3	30.6	0.5
9	20.1		24.3			33.5	22.2	0.6
10	1.2	0.7	0.4	30.1	2.6	64.4		
11	28.8			2.0		68.2	1.0	
12	10.7		24.1	10.4		28.7	26.1	
13	33.2		10.4	8.5		29.7	18.1	
14	17.2	5.5	8.4	14.7		54.3		
15	2.7	0.8	1.6	57.9	3.4	33.6		
16	14.0		18.6	15.3	1.0	51.1		
17	44.7		6.7	5.9		34.6	8.1	
18	20.8		20.2			32.7	26.3	
19	11.7		7.7	55.2		25.4		
20	11.9	4.2	16.3	23.9		38.0		5.7
21	31.9		28.3	8.5		28.1	3.2	
22	13.7	2.7	7.8	6.0		66.6	1.6	1.5
Total recom.	7.0	0.3	2.4	8.6	0.5	72.3	8.9	

The total recombined fatty acid composition was determined by computation using an internal standard.

Packard Model 5890 chromatograph equipped with a flame ionization detector [25]. The column was a fused capillary Omegawax column (30 m × 0.32 mm, 0.25 μm film thickness, Supelco) 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 13 min. The individual TG species collected after evaporation of the solvent were methylated along with an internal standard (heptadecanoic acid) in 2 ml of toluene and 2 ml of 14% BF_3-methanol at 90°C for 30 min, cooled, 2 ml of 0.9% saline added and the required methyl esters extracted with hexane (5 ml).

3. Results and discussion

The reversed-phase HPLC traces of borage oil and EPO with UV detection at 210 nm are given in Fig. 1. Broadly speaking the TG profile of borage oil is more complex than EPO: EPO was found to have 22 UV detectable TG fractions and borage oil 34. The fatty acid profiles of the individual TG fractions for borage oil and EPO are given in Tables 1 and 2, respectively. The analysis and separation of TG's by reversed-phase HPLC is sometimes difficult as both the degree of unsaturation and chain length of the fatty acyl groups are important. One of these difficulties is the formation of "critical pairs"

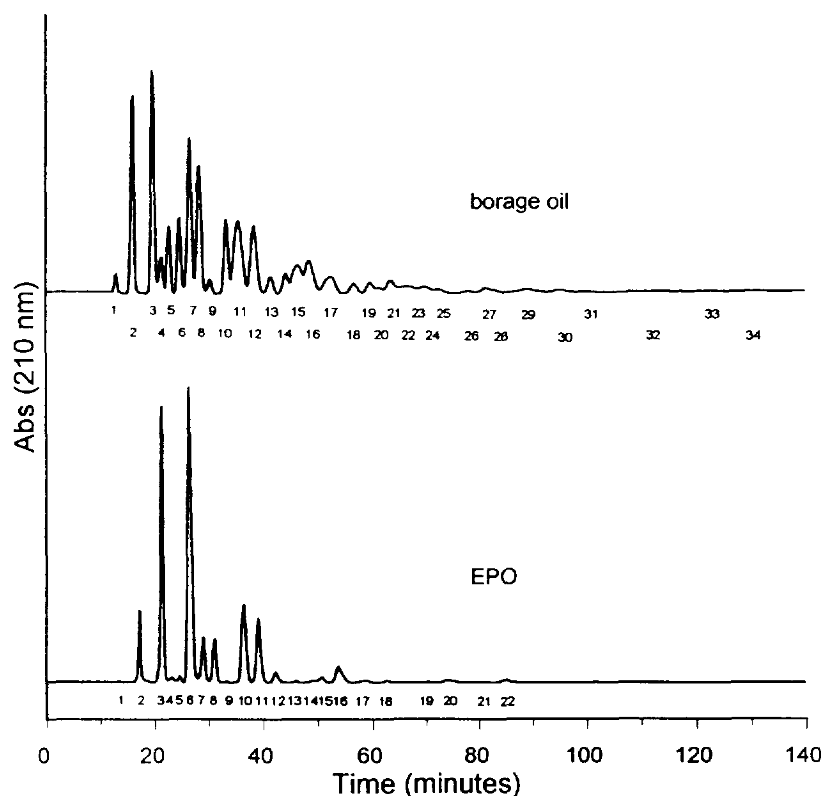

Fig. 1. Reversed-phase HPLC profile of the triglycerides of EPO and borage oil using a Supelcosil LC-18 column (250 × 4.6 mm I.D., 5 μm) with isopropanol–acetonitrile (35:65, v/v) at a flow rate of 1 ml/min and detection by UV absorption at 210 nm. The fractions collected for GC analysis are shown above the baseline.

which elute close to one another during reversed-phase chromatography in spite of differences in chain lengths and number of double bonds. Critical pairs are defined as structures with an equivalent carbon number (ECN).

$$ECN = CN - 2n$$

where CN equals the number of carbon atoms in the fatty acids and n equals the number of double bonds per molecule. It has, however, been demonstrated [22] that the reversed-phase HPLC retention times of TG's are in a positive logarithmic relationship to their theoretical carbon number, TCN, defined as

$$TCN = ECN - \sum U_i$$

where ECN is the equivalent chain length defined as above and U_i represents a correction factor summed for all three fatty acids of the TG. These U_i values are determined experimentally and were determined to be zero for saturated fatty acids, 0.6–0.65 for oleic acid and 0.7–0.8 for linoleic acid [21].

The U_i values for GLA found in borage oil and EPO and for gadoleic acid, 20:1$n-9$; erucic acid, 22:1$n-9$ and nervonic acid, 24:1$n-9$ found in borage oil have not been determined. However, a good estimation of the U_i value for GLA was determined by co-eluting isocratically a mixture of the pure TG's, GGG, LGG, LGL and LLL along with olive oil rich in OLL, OLO, PLO, PLP, OOO and SOO. By plotting the log

RRT for the olive oil TG species against their corresponding known TCN values and extrapolating the line through the observed log RRT values for the GLA-containing TG species it was possible to assess the TCN values for GGG, LGG and LGL to be 35.0, 36.5 and 38.0, respectively (Fig. 2). Relative Retention Time, RRT, values (relative to LLL) are used instead of retention times or capacity factors since the latter are subject to drifting during the analysis. Using the value of 0.8 for the U_i for L sets the U_i value for GLA at 0.3–0.4. Although it has been stated that the values of U_i vary with elution conditions we have assumed the values for linoleic acid and oleic acid to be the same or at least similar under our elution conditions [19].

Similarly an estimation of the U_i values for $20{:}1n-9$, abbreviated Ga; $22{:}1n-9$, abbreviated E; and $24{:}1n-9$, abbreviated N, can also be determined using Fig. 2. As given in Table 1 the 14th fraction of borage oil was found to be predominantly GaLL. This fraction was observed to have a TCN value of 44.0, corresponding to a U_i value for $20{:}1n-9$ of 0.6. Similarly fraction 19 of borage oil was determined to be mainly ELL and fraction 27 was determined to be predominantly NLL allowing calculation of a U_i value for $22{:}1n-9$ of 0.4 and a U_i value for $24{:}1n-9$ of 0.3. Using these estimated U_i values allows calculation of the

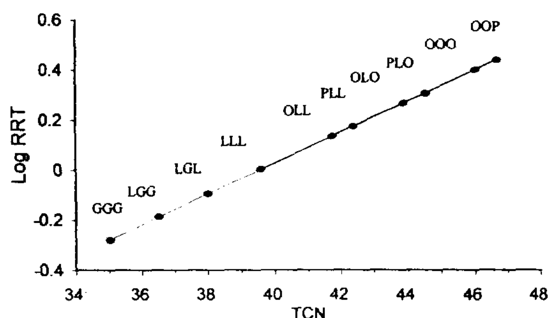

Fig. 2. Log RRT of triglyceride standards of olive oil versus their theoretical carbon number, (TCN), $r^2 = 0.9993$. The separation of the TG's was performed with the same conditions as described for Fig. 1. The TCN values for the GLA-containing TG's were estimated by extrapolating the plot through the observed log RRT values for the GLA-containing TG's.

TCN values for the various other TG species found in the two oils.

To a first approximation there is general agreement that the distribution of fatty acids among the TG's of unsaturated vegetable oils follows a random distribution. Previously an algorithm has been devised which follows the random theory of distribution [26]. The proportion of each TG is then calculated from probability. The algorithm used is

$$\eta = n \cdot p$$

where η is the probability of occurrence of a given TG, n is a weighting factor for the number of times the same fatty acid occurs in the TG (n is either 1, 3 or 6 depending on whether a fatty acid occurs 3 times, 2 times or only once, respectively in the TG) and p is the percentage abundance of the fatty acid present in the fat. For the borage oil and EPO samples examined here the probability of the various TG species occurring in the oils are given in Tables 3 and 4, respectively, with TG species less than 0.05% being excluded.

Hence, using these probability values, along with the fatty acid composition of the TG fractions collected, and comparing the observed TCN values with calculated TCN values, the identity or probable identity of the TG species present in the fractions was deduced. These are shown in Tables 3 and 4 as well. Since the fatty acid composition of the individual TG fractions were determined and their retention times are related to their TCN values, reasonably reliable identification was possible. Certainly, if available, mass spectroscopy would provide verification of the assignments.

The individual TG fractions of both borage oil and EPO were quantitated using heptadecanoic acid as an internal standard. Of all the detection methods available for quantification, GC analysis of the fatty acid constituents of the TG species in the presence of an internal standard would be expected to give a high degree of precision [19]. These results are presented in Tables 3 and 4, respectively. With regards to quantification of the TG fractions, a check on the recovery of the fatty acids by computation (Total Recom. Tables

Table 3
Probable TG species, observed theoretical carbon number (TCN), calculated TCN, probability of occurrence and weight% distribution for the TG fractions of borage oil separated by reversed-phase HPLC

Fraction #	Prob. TG	Obs. TCN	Cal. TCN	Prob. (%)	Weight% (IS)	Weight% (ELS)
1	GGG	35.0	34.8	0.9	0.6	0.3
2	LGG	36.5	36.4	4.8	4.7	7.4
3	LGL	38.0	38.0	8.7	8.3	12.9
4	OGG	38.5	38.6	2.4	1.0	0.6
5	PGG	39.1	39.2	1.4	1.9	1.5
6	LLL	39.6	39.6	5.3	4.0	5.3
7	OGL	40.2	40.2	8.8	7.4	11.6
8	PGL, GaGG	40.6	40.6, 40.8	5.3, 0.4	6.1	9.2
9	SGG	41.3	41.2	0.5	1.0	0.1
10	OLL	41.8	41.8	8.0	5.1	9.6
11	GaGL, OGO	42.3	42.2, 42.3	1.6, 2.2	9.0	12.4
	PLL		42.4	4.8		
12	SGL, EGG	42.8	42.8, 42.8	1.8, 0.2	5.6	5.9
	PGO		43.0	2.7		
13	PGP	43.4	43.6	0.8	1.3	0.6
14	GaLL	43.8	43.8	1.5	2.3	1.5
15	OLO	44.2	43.9	4.9	5.8	5.4
16	SLL, OGGa	44.5	44.4, 44.4	1.7, 0.8	7.2	7.8
	EGL, PLO		44.4, 44.6	0.9, 4.8		
17	SGO, PGGa	45.1	45.0, 45.0	0.9, 0.5	3.4	1.8
	PLP		45.2	1.4		
18	PGS	45.6	45.6	0.6	1.1	0.2
19	ELL	46.0	46.0	0.8	1.8	0.6
20	OLGa	46.1	46.0	3.2	1.1	0.4
21	OOO, GaGGa	46.4	46.1, 46.4	0.7, 0.1	3.1	1.6
	NGL		46.5	0.4		
22	PLGa, SLO	46.7	46.6, 46.6	0.9, 1.7	1.2	nd
	OGE		46.6	0.4		
23	POO	46.8	46.7	1.2	2.6	2.6
24	SGGa, PGE	47.0	47.0, 47.2	0.2, 0.3	2.2	nd
25	PLS, POP	47.4	47.2, 47.4	1.0, 0.7	1.6	0.4
26	SGS, PPP	47.9	47.6, 48.0	0.1, 0.2	0.4	nd
27	GaLGa, NLL	48.1	48.0, 48.1	0.1, 0.3	1.6	0.1
28	OOGa, OLE	48.3	48.1, 48.2	0.4, 0.8	0.7	nd
29	SLGa, GaGE	48.8	48.6, 48.6	0.3, 0.1	2.4	nd
	OGN, SOO		48.7, 48.7	0.2, 0.4		
	POGa, PLE		48.8, 48.8	0.4, 0.5		
30	SLS, SGE	49.3	49.2, 49.2	0.2, 0.1	1.7	0.3
	PGN, PGaP		49.3, 49.4	0.1, 0.1		
31	POS	49.6	49.4	0.5	0.8	nd
32	PSP, OGaGa	50.5	50.0, 50.2	0.2, 0.1	1.5	nd
	GaLE, OOE		50.2, 50.3	0.1, 0.2		
	OLN, OGaE		50.3, 50.4	0.4, 0.1		
33	SOGa, PLN	51.0	50.8, 50.9	0.2, 0.2	0.9	0.04
	SLE, POE		50.9, 51.0	0.2, 0.2		
34	PGaS, PEP	51.5	51.4, 51.6	0.1, 0.1	0.6	nd

Abbreviations: nd, not detected; P, Palmitic; Po, Palmitoleic; S, Stearic; O, oleic; L, linoleic; G, γ-linolenic; Ga, Gadoleic; E, erucic; N, nervonic acids; IS, internal standard quantitation with heptadecanoic acid and ELS, quantitation based on integration of the TG peaks using an evaporative light-scattering detector.

Table 4
Probable TG Species, observed theoretical carbon number (TCN), calculated TCN, probability of occurrence and weight% distribution for the TG fractions of EPO separated by reversed-phase HPLC

Fraction #	Prob. TG	Obs. TCN	Cal. TCN	Prob. (%)	Weight% (IS)	Weight% (ELS)
1	GGG	35.3	34.8	0.1	nd	nd
2	LGG	36.9	36.4	2.9	2.7	1.2
3	LGL	38.3	38.0	19.0	14.7	20.8
4	OGG	38.9	38.6	0.3	0.5	nd
5	PGG	39.3	39.2	0.2	0.4	nd
6	LLL	39.6	39.6	42.3	38.0	48.3
7	OGL	40.4	40.2	3.5	3.3	1.7
8	PGL	40.9	40.8	2.7	3.2	1.6
9	SGG	41.4	41.2	0.04	0.3	nd
10	OLL	41.9	41.8	11.8	12.3	14.6
11	OGO, PLL	42.4	42.3, 42.4	0.2, 8.9	10.9	9.2
12	SGL, PGO	43.0	42.8, 43.0	0.5, 0.3	1.5	nd
13	PGP	43.6	43.6	0.09	0.3	nd
14	PoLL	44.0	–	–	0.5	nd
15	OLO	44.3	43.9	1.1	2.1	0.7
16	SLL, PLO	44.6	44.4, 44.6	1.7, 1.7	5.1	1.9
17	SGO, PLP	45.3	45.0, 45.2	0.05, 0.6	1.1	nd
18	PGS	45.7	45.6	0.04	0.4	nd
19	OOO, SLO	46.5	46.1, 46.6	0.03, 0.3	0.4	nd
20	POO	46.9	46.7	0.08	1.5	nd
21	PLS, POP	47.5	47.2, 47.4	0.24, 0.06	0.5	nd
22	PPP	47.5	48.0	0.01	0.5	nd

Abbreviations as described for Table 3.

1 and 2) confirms that recoveries were nearly quantitative using an internal standard. Overall, there is partial agreement between the ELS detector and internal standard quantification; however, the ELS results tend to be higher for the major fractions and lower for minor fractions. In fact, in most cases the minor TG's were not detected with the ELS detector. It is known that the response of the ELS detector with respect to mass is sigmoidal for TG standards [19]. This means that it is necessary to calibrate the ELS detector with a wide range of TG standards before quantification can be determined with an ELS detector.

The triglyceride structure of borage oil is clearly much more complex than that of EPO and some empirical observations can be noted. From a comparison of Tables 3 and 4, three fractions, LGG, LLL and LGL account for only 17.0% by weight of borage oil whereas they account for 55.4% by weight of EPO. Over 75% of EPO is accounted for by four fractions, whereas the most abundant four fractions of borage oil account for only 32.9% of the oil. Moreover, over 90% of the GLA present in EPO can be accounted for in the first 9 eluting TG species whereas only about 65% can be accounted for in these species in borage oil.

The predicted probability of a TG species occurring in the two oils agrees well with the values quantitated using an internal standard. This experimental observation agrees with that previously seen for other polyunsaturated oils [26]. However, this does not mean that, within a particular TG species, the fatty acids are distributed evenly: the positional isomeric composition of a particular TG species must be determined experimentally. Further studies determining which positional isomers of a particular TG species, in particular the GLA containing TG species, and to what extent each occurs in the two oils are in progress.

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