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Structure Determination of Long-Chain Polyunsaturated Triacylglycerols by High-Resolution ^{13}C Nuclear Magnetic Resonance

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ABSTRACT: The application of ^{13}C nuclear magnetic resonance to determine the positional distribution of fatty acids on the glycerol backbone has been investigated. A systematic approach and synthetic triacylglycerols were used to measure the effect on the carbonyl chemical shifts of triacylglycerols by the positional distribution on the glycerol backbone and the number and position of the double bonds within the fatty acids. The correlation of ^{13}C carbonyl chemical shift to the molecular structure of triacylglycerol was delineated. The assignments for the chemical shifts of the carbonyl nuclei of monoacyltriacylglycerol standards were compiled. The resonance from the carbonyl carbons at the 1,3 positions is resolved from that at the 2 position. The ^{13}C carbonyl chemical shift was more dependent on the position of the double bonds than the degree of unsaturation of the fatty acids. In particular, little effect was observed in the chemical shifts for fatty acids containing more than two double bonds. However, the chemical shifts were influenced significantly by the position of the first double bond. The difference in the chemical shift of the unsaturated species from that of the saturated species was plotted against the position of the first double bond. A natural logarithmic relationship was found between carbon numbers 5 and 11. Inflection points were found outside of this region at carbon numbers 4 and 13. In addition, the resonances from the saturated species, independent of their chainlength, were degenerate in oil systems, even though small differences were observed in the standards. The applicability of this method was demonstrated in the determination of the composition and positional distribution of the fatty acids in borage and evening primrose oils.
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KEY WORDS: Borage oil, chemical shifts, evening primrose oil, fatty acid analysis, long-chain polyunsaturated triacylglycerol, ^{13}C NMR, positional distribution.

Long-chain polyunsaturated fatty acids (LCPUFA) have been suggested to cause specific biological outcomes in addition to their caloric contributions to energy metabolism. The lower incidence of coronary heart disease in Eskimos in Greenland, compared with the incidence of coronary heart disease of the general populations in the United States and Denmark, has been attributed to their high dietary content of n-3 LCPUFA (1). The importance of n-3 and n-6 PUFA in neural and retinal development in infants has also been widely acknowledged by infant nutritionists (2,3). γ-Linolenic acid, an n-6 LCPUFA, has been reported to circumvent the inadequate Δ6-desaturation step and to provide therapeutic benefits to atopic eczema and diabetic neuropathy (4). The absorption of fatty acids has been reported in the literature to be dependent on the molecular structure of the triacylglycerol. Investigation of the absorption of fatty acids in a canine model suggests that the positional distribution of the fatty acids within the triacylglycerol may affect the metabolic fate of the fatty acids (5). Similar observations in human infants also have been reported in the literature (6,7).

Analytical methodologies that have been reported in the literature for the determination of fatty acid composition and positional distribution of triacylglycerols generally require hydrolysis of the triacylglycerols by enzymes or chemical processes and subsequent analysis of the mono- and diacylglycerol components by chromatography techniques, i.e., high-performance liquid chromatography (8–12), supercritical fluid chromatography (13), and gas chromatography (14,15). Some of these report the capability to distinguish the complete stereospecific distribution, i.e., positions 1, 2, and 3 of the triacylglycerol (10–12). These methods are destructive and do not allow the recovery of the original triacylglycerols. The hydrolysis procedure gives rise to the possibility of acyl migration, resulting in erroneous measurements of the positional distribution. In addition, these methods are time-consuming and labor-intensive.

There are several unique properties of ^{13}C nuclear magnetic resonance (NMR) that make its application to this type of study useful. First, the chemical shift is sensitive to molecular structure, thereby producing a spectrum where each nucleus is represented by a peak at a specific frequency. The resolution of the nuclei in each environment is determined by the linewidth and the chemical shift differences between adjacent peaks (16). Second, the area under the peak, arising from each ^{13}C nucleus, is proportional to the number of nuclei in that environment because all ^{13}C nuclei exhibit the same absorption coefficient (16). Therefore, the chemical shift and the integrated area of each peak can be used for both

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qualitative and quantitative measurements of the specific nucleus. Third, the preparation of the sample for this application is simple, resulting in large savings in resources. Lastly, NMR is a nondestructive technique that enables the investigator to recover the sample for other analyses. Some papers have been presented that describe the application of ^{13}C NMR to triacylglycerols, where the carbonyl region was used to determine the positional distribution of fatty acids on the glycerol backbone (16–21). These studies showed that ^{13}C resonances of the carbonyls from the 1 and 3 positions of the glycerol were well- resolved from those esterified at the 2 position. In addition, the data from these studies showed that most unsaturated fatty acids in the same position on the glycerol backbone were nondegenerate, allowing differentiation of the unsaturated fatty acids in the sample. This application was used to study vegetable oils that contained fatty acids with two or fewer double bonds, such as palm (17), corn (21), peanut (21), and canola (21). More recently, this application has been extended to include some fish oils that contain LCPUFA (18).

We utilize here a systematic approach and synthetic triacylglycerol standards of LCPUFA to measure carbonyl chemical shifts of fatty acids with specific degrees and positions of unsaturation. It is hoped that this work will allow more assignments of the carbonyl carbons of various fatty acids and establish the trends to correlate chemical shifts with molecular structure. Therefore, these data can be used in studying the relationship between structure and function in edible oils with specific biological benefits, such as fish, evening primrose (EPO), and borage oils (BO).

EXPERIMENTAL PROCEDURES

Materials and sample preparation. Monoacyltriacylglycerol standard materials that contained fatty acids of different degrees of unsaturation and their positional isomers were purchased from Sigma Chemicals (St. Louis, MO) and Nu-Chek-Prep Inc. (Elysian, MN): tridecanoin (Tri 10:0), tristearin (Tri 18:0), tridocosanoin (Tri 22:0), triolein (Tri18:1 Δ9), trilinolein (Tri 18:2 Δ9,12), trilinolenin (Tri 18:3, Δ9,12,15), trigammalinolenin (Tri 18:3, Δ6,9,12), triarachidonin (Tri 20:4, Δ5,8,11,14), trieicosapentaenoin (Tri 20:5, Δ 5,8,11,14,17), and tridocosahexaenoin (Tri 22:6, Δ4,7,10,13,16,19). EPO is a product of Traco Laboratory (Seymour, IL), and BO is a product of Bioriginal Inc. (Alberta, Canada). Approximately 100 mg of total lipids in 700 μL deuterochloroform was used throughout the study.

^{13}C NMR data collection. The proton-decoupled ^{13}C NMR data were collected at 30°C in a 5-mm broadband probe on a Varian Unity 500 MHz spectrometer (Palo Alto, CA) operating at 125.728 MHz. Waltz decoupling was the chosen mode of decoupling and was gated on only during acquisition. The spectral window used was *ca.* 23,000 Hertz with approximately a 3-s acquisition time. The spectra were internally referenced to deuterochloroform at 77.0 ppm. A 7-s pre-pulse delay and 4.5 μs pulse length, corresponding to a 45° pulse angle, were applied for quantitative purposes. Typically, the approximate number of scans collected for adequate signal-to-noise ranged from 300 to 1200 scans, depending on the complexity of the mixture. The total acquisition time for the experiments, therefore, ranged between approximately 1–4 h (BO = 1272 scans/3.5 h).

RESULTS AND DISCUSSION

The ^{13}C carbonyl chemical shifts of the triacylglycerol standards investigated in this study are listed in Table 1. Some results from earlier work, investigating isomers of palmitic and stearic acids by ^{13}C NMR (125.728 MHz), showed that the resonances from these saturated fatty acids at the same position in the triacylglycerol were not resolved in the carbonyl region (data not shown). Similar results have been reported by other investigators as well (17,20). The chainlengths of these fatty acids were similar and not expected to yield different chemical shifts. Therefore, we investigated saturated fatty acids of triacylglycerols with medium and long chains, i.e., Tri 10:0, Tri 18:0, and Tri 22:0, as pure standards and as a mixture. As pure standards, the chemical shifts at the 1,3 positions of Tri 10:0, Tri 18:0, and Tri 22:0 were 173.162, 173.186, and 173.208 ppm, respectively. These three triacylglycerols were unresolved at both the 1,3 and 2 positions when analyzed as a mixture (Fig. 1).

Next, we investigated the effect on the ^{13}C carbonyl chemical shift by the degree of unsaturation, with the first double bond beginning at the same Δ9 position in triacylglycerols Tri 18:1 Δ9, Tri 18:2 Δ9,12, and Tri 18:3 Δ9,12,15. The carbonyl chemical shifts of these triacylglycerols are listed in Table 1. The resonances shifted to lower frequencies as the degree of unsaturation increased. The largest shift was found with the addition of the first double bond (Tri 18:1 Δ9, δ173.133 ppm) relative to the corresponding saturated triacylglycerol (Tri 18:0, δ173.186 ppm). A smaller shift was observed with the addition of the second double bond (Tri 18:2 Δ9,12, δ173.111) relative to the corresponding monounsaturated species. No significant differences in chemical shifts were found between the Tri 18:2 Δ9,12 and Tri 18:3 Δ9,12,15 at both positions (Fig. 2). Similarly, small differences in carbonyl chemical shifts between tri-

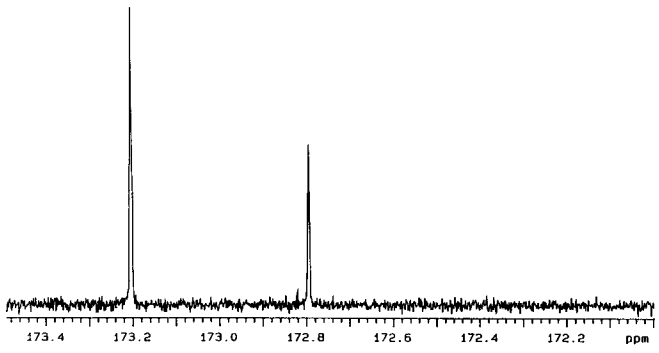

FIG. 1. The ^{13}C nuclear magnetic resonance spectrum of a mixture of tridecanoin, tristearin, and tridocosanoin.

TABLE 1
^{13}C Chemical Shifts (ppm) of the Carbonyl Nuclei of Monoacyltriacylglycerol Standards[a]

	1,3 Position				2 Position			
Fatty acid	Sat.	Unsat. mononene ≥Δ9	Unsat. polyene ≥Δ9	Unsat. polyene <Δ9	Sat.	Unsat. monoene ≥Δ9	Unsat. polyene ≥Δ9	Unsat. polyene <Δ9
10:0	173.162				172.754			
18:0	173.186				172.778			
22:0[b]	173.208				172.798			
18:1 Δ9		173.133				172.728		
18:2 Δ9,12			173.111				172.706	
18:3 Δ9,12,15			173.107				172.702	
18:3 Δ6,9,12				172.959				172.563
20:2 Δ11,14			173.145				172.738	
22:1 Δ13		173.208				172.797		
20:4 Δ5,8,11,14				172.893				172.510
20:5 Δ5,11,14,17				172.896				172.513
22:6 Δ4,7,10,13,16,19				172.403				172.025
Mixture 1[c]	173.207				172.797			
Mixture 2[c]	173.169	173.134	173.123		172.764	172.729	172.716	
Mixture 3[c]		173.130	173.116	172.949		172.723	172.710	172.533
				172.882				172.500
				172.409				172.031

[a]Sat., saturated; Unsat., unsaturated.
[b]Collected at 35°C.
[c]Mixture 1 = 10:0/18:0/22:0; Mixture 2 = 18:0/18:1 Δ9/18:2 Δ9,12/18:3 Δ9,12,15; and Mixture 3 = 18:1 Δ9/18:3 Δ9,12,15/18:3 Δ6,9,12/20:5 Δ5,8,11,14,17/22:6 Δ4,7,10,13,16,19.

acylglycerols Tri 20:4 Δ5, 8, 11,14 (1,3 positions: δ172.893, 2 position: δ172.510 ppm) and Tri 20:5 Δ5, 8, 11,14,17 (1,3 positions: δ172.896, 2 position: δ172.513 ppm) suggest that the increase of the unsaturation beyond two bonds has little effect, even when the unsaturation occurs much closer to the carbonyl (Table 1). Based on these examples, we conclude that the chemical shifts were not significantly affected by the presence of unsaturation beyond two double bonds.

Conversely, we studied the effect of the position of the double bond. First, we compared the data of two triacylglycerols that possess the same number of double bonds but at different positions Tri 18:3 Δ6,9,12 and Tri 18:3 Δ9,12,15 (Fig. 3). We found that the position of the double bond has a significant influence on the chemical shift and, in general, as the unsaturation occurs closer to the carbonyl, the resonances were shifted to lower frequencies for both the 1,3 and 2 positions. This was further demonstrated with triacylglycerols Tri 20:4 Δ5,8,11,14; Tri 20:5 Δ5,8,11,14,17; and Tri 22:6 Δ4,7,10,13,16,19 (Table 1). It was observed with these triacylglycerols that, as the first double bond occurs closer to the carbonyl, the difference in the chemical shift from the corresponding saturated species increases. Dramatic shifts were observed for triacylglycerol Tri 22:6 Δ4,7,10,13,16,19. The chemical shifts for the 1,3 positions were observed at a lower frequency than the 2 position for all fatty acids in this study. It appears that the first double bond at the fourth carbon, γ position, provides a unique chemical shift. The characteristic chemical shift of Δ4 fatty acids will allow this technique to be applied more readily to complex systems, such as marine oil. An example of a mixture

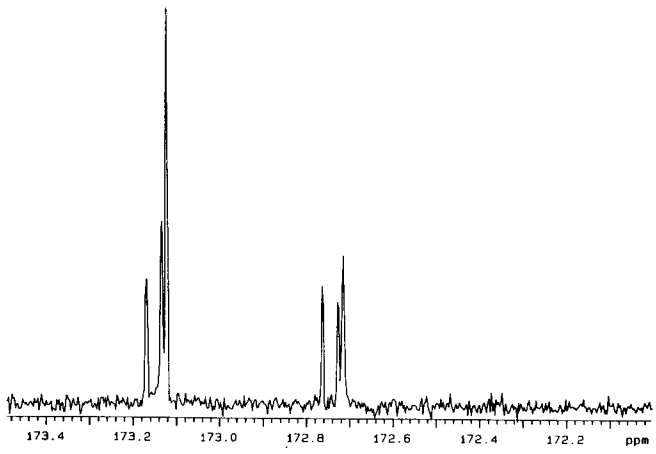

FIG. 2. The ^{13}C nuclear magnetic resonance spectrum of the carbonyl region of a mixture of tristearin, triolein, trilinolean, and trilinolenin.

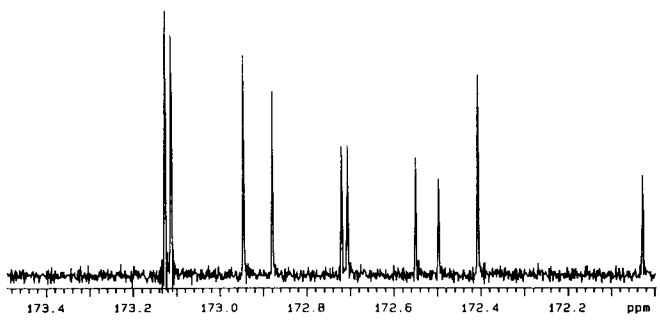

FIG. 3. The ^{13}C nuclear magnetic resonance spectrum of the carbonyl region of a mixture of triolein, trilinolenin, trigammalinolenin, trieicosapentaenoin, and tridocosahexaenoin.

TABLE 2
Correlation of Chemical Shift (ppm) and Position of the First Double (≥Δ9)

	1,3 Position	Diff.[a]	2 Position	Diff.
Saturated[b]	173.207		172.797	
18:1 Δ9	173.133	0.074	172.728	0.069
20:2 Δ11, 14	173.145	0.062	172.738	0.059
22:1 Δ13	173.208	0.001	172.797	0.000

[a]The difference (Diff.) is calculated by subtracting the frequency (in ppm) of the saturated species from that of the unsaturated species.
[b]The frequencies are taken from the data collected on a mixture of three saturated triacylglycerols: Tri 10:0/Tri 18:0/Tri 22:0 (Table 1).

with these types of triacylglycerols and their carbonyl chemical shifts is shown in Figure 3. Second, we compared the data of three triacylglycerols where the first double bond occurs farther from the carbonyl (>9) in triacylglycerols Tri 18:1 Δ9, Tri 20:2 Δ11,14, and Tri 22:1 Δ13. The differences in the chemical shifts between the saturated and the unsaturated species of these triacylglycerols are listed in Table 2. When the first double bond occurs farther from the carbonyl, the difference in the shift from the saturated species decreases. The difference in chemical shift between the saturated species and Tri 22:1 Δ13 was negligible at both the 1,3 and 2 positions.

Figure 4 summarizes the relationship between the position of the first double bond and the difference in the chemical shift of the saturated and the unsaturated species. For those species with the first double bond between carbon numbers 5 and 11, a linear correlation between the natural log of the difference in chemical shift and the carbon number of the first double bond was observed. Two inflection points were found outside of this region. A much larger difference in the chemical shifts was found with carbon number 4, where a large inflection occurs. No difference was found with carbon number 13; however, it is uncertain where the exponential relationship and the inflection meets in this region due to the lack of a data point at carbon number 12.

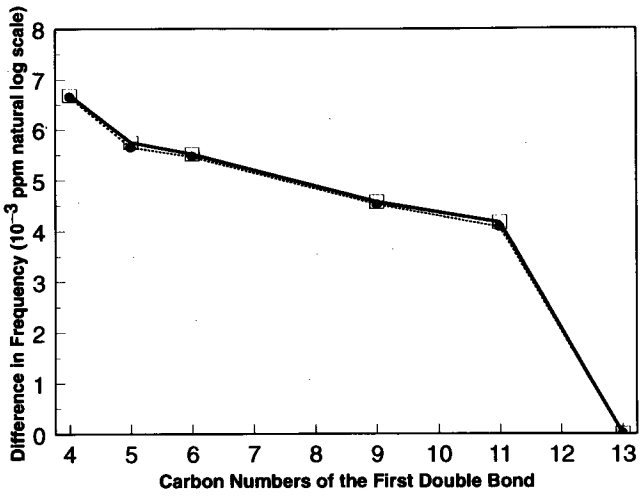

FIG. 4. The relationship between the position of the first double bond and the difference in the carbonyl chemical shifts of the saturated and unsaturated species; ⊟, 1,3 position, ···●···, 2 position.

^{13}C NMR data were collected on two natural oils, BO and EPO, and are shown in Figures 5 and 6, respectively. These oils were chosen for their high levels of γ-linolenic acid. The assignments of the fatty acids of these oils are listed in Tables 3 and 4. These assignments were based on the chemical shifts of the standards listed in Table 1. The chemical shift data of the unsaturated species agree well with that of the standards, i.e., average differences of .017 ± .005 and .005 ± .002 ppm for BO and EPO, respectively.

The saturated species in both oils resonated at a lower frequency as one peak than the standard mixture of saturated triacylglycerols with differences of .043 ppm for BO and 0.029 ppm for EPO. We attribute the differences observed between the natural oils and the standards partly to the differences in fatty acid composition within the triacylglycerol molecule.

The fatty acid profile and the positional distribution of the fatty acids were determined by integration of the peak areas in the NMR spectra for both BO and EPO. The total fatty acid compositions of these two lots of oils determined by NMR were compared to those obtained by gas chromatography and are summarized in Table 5. The results were in excellent agreement. The positional distribution determined by the NMR method also was compared to literature values for EPO and BO obtained by a gas chromatography-enzymatic method (22). The

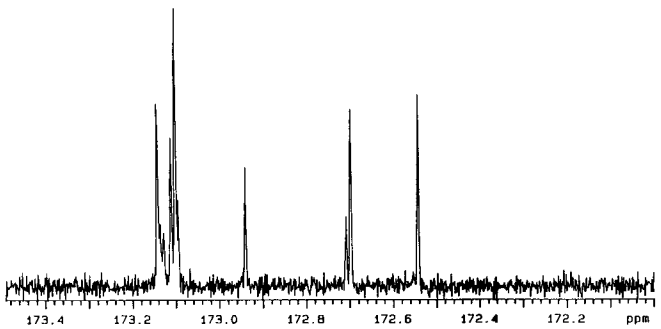

FIG. 5. The carbonyl region in the ^{13}C nuclear magnetic resonance spectrum of borage oil.

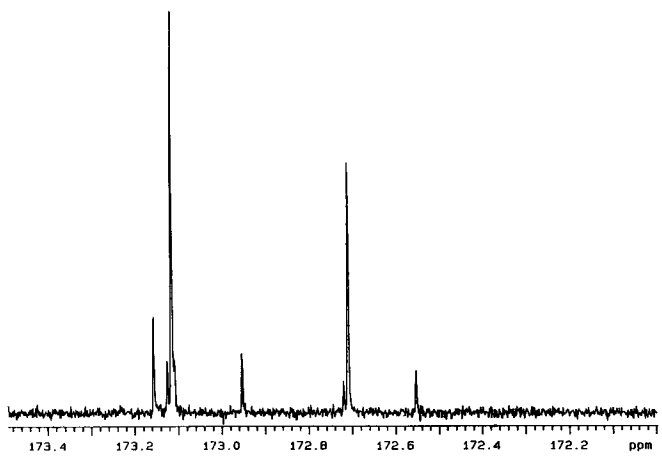

FIG. 6. The carbonyl region in the ^{13}C nuclear magnetic resonance spectrum of evening primrose oil.

TABLE 3
^{13}C Nuclear Magnetic Resonance of Borage Oil

Position	Fatty acid	Chemical shifts (ppm)			Rel. molar percent	
		Oil	Std.[a]	Diff.[b]	Int. Pk. A.[c]	Ref. 22[d]
1,3	Sat.	173.143	173.186	.043	13.1	13.2
1,3	22:1 Δ13	173.136	173.208	—	4.0	2.0
1,3	20:1 Δ11	173.128	173.145	.017	4.9	4.0
1,3	18:1 Δ9	173.112	173.133	.021	10.3	11.1
1,3	18:2 Δ9,12	173.095	173.111	.016	26.4	24.8
	18:3 Δ9,12,15	173.101	173.111	.010	[e]	[f]
1,3	18:3 Δ6,9,12	172.940	172.959	.019	7.9	11.7
2	Sat.	n.d.	—	—	n.d.	.9
2	22:1 Δ13	n.d.	—	—	n.d.	.2
2	20:1 Δ11	n.d.	—	—	n.d.	.2
2	18:1 Δ9	172.708	172.728	.020	4.7	4.6
2	18:2 Δ9,12	172.697	172.706	.009	14.4	14.1
	18:3 Δ9,12,15					
2	18:3 Δ6	172.542	172.563	.021	14.2	13.5

[a]Std: monoacyltriacylglycerol standard material. Other abbreviations as in Table 1. [b]Absolute value of the difference in chemical shifts (ppm) of oil and standard. [c]Integrated peak area. [d]Normalized 1,2 and 3 positions to 100%. [e]Integrated together with peak at 173.095 ppm. [f]Amounts of 18:2 Δ9,12 and 18:3 Δ9,12,15 are combined.

TABLE 4
^{13}C Nuclear Magnetic Resonance of Evening Primrose Oil[a]

Position	Fatty acid	Chemical shifts (ppm)			Rel. Molar Percent	
		Oil	Std.	Diff.[b]	Int. Pk. A.[c]	Ref. 22[d]
1,3	Sat.	173.157	173.186	.029	9.0	5.82
1,3	18:1 Δ9	173.126	173.133	.007	5.8	4.8
1,3	18:2 Δ9,12	173.115	173.111	.004	46.4	47.7
	18:3 Δ9,12,15	173.108	173.111	.003	[e]	[f]
1,3	18:3 Δ6,9,12	172.955	172.959	.004	5.5	8.3
2	Sat.	n.d.	172.778	—	—	1.1
2	18:1 Δ9	172.721	172.728	.007	3.6	2.5
2	18:2 Δ9,12	172.710	172.706	.004	25.4	26.3
	18:3 Δ9,12,15					
2	18:2 Δ9,12	172.554	172.563	.009	4.4	3.6

[a]Abbreviations as in Tables 1 and 3. [b]Absolute value of the difference in chemical shifts (ppm) of oil and standard. [c]Integrated peak area. [d]Normalized 1,2 and 3 positions to 100%. [e]Integrated together with peak at 173.095 ppm. [f]Amounts of 18:2 Δ9,12 and 18:3 Δ9,12,15 are combined.

TABLE 5
Comparison of Total Fatty Acid Composition of Evening Primrose Oil and Borage Oil Determined by Nuclear Magnetic Resonance (NMR) and Gas Chromatography (GC) Methods[a]

	NMR	GC
Evening primrose oil		
Sat.	9.0	8.1
18:2 Δ9	9.4	8.3
18:2 Δ9,12	71.8	73.4
18:2 Δ6,9,12	9.8	9.8
Borage oil		
Sat./22:1 Δ13	17.1	17.3
20.1 Δ11	4.9	4.9
24:1 Δ15		
18:1 Δ9	15.0	15.9
18:2 Δ9,12	40.8	39.8
18:3 Δ9,12,15		
18:2 Δ9,12	22.2	22.1

[a]Data presented as normalized molar percent. Abbreviation as in Table 1.

results agree well, as shown in Tables 3 and 4. The good agreement found in the quantitative measurements with other methods further supports the qualitative assignment of natural oils from the chemical shift table generated from triacylglycerol standards.

In this study, we have established a data base for the determination of positional distribution of triacylglycerols that contain fatty acids ranging from medium-chain saturated to long-chain polyunsaturated species. These data will enable us to further expand our capability to more complicated systems, such as fish oils and restructured lipids of various fatty acid compositions.

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