The Structure of the Triacylglycerols of Meadowfoam Oil

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The triacylglycerols of meadowfoam oil have been resolved by HPLC in the silver ion and reversed-phase modes, and by the two techniques used in a complementary fashion. The fractions obtained were collected and quantified by gas chromatography of their methyl esters in the presence of an internal standard. Silver ion chromatography gave a distinctive resolution in which fractions differing solely in the position and chain-length of a single monoenoic fatty acyl group were resolved, the order of elution being 11-20:1, 5-20:1, 13-22:1, 5-18:1 and 9-18:1. Reversed-phase chromatography also gave fractions containing single positional isomers, (11-20:1 < 5-20:1 < 13-22:1 < 5-22:1), but the pattern was more difficult to discern since fractions containing 22:2 tended to overlap with those containing 20:1. The species (5-20:1)(5-20:1)(22:2), (5-20:1)(5-20:1)(5-20:1) and (5-20:1)(5-20:1)(13-20:1) were found to be the most abundant, and together comprised 67% of the total. A small but significant trilinolein fraction was detected and its presence may have biosynthetic implications.

KEY WORDS: High-performance liquid chromatography, *Limnanthes alba*, meadowfoam oil, reversed-phase chromatography, silver ion chromatography, triacylglycerols.

The seed oil of the Meadowfoam plant (*Limnanthes alba* Benth.) is unusual in that the triacylglycerols contain mainly C_{20} and C_{22} fatty acids with double bonds in positions 5 and 13, i.e. 66 mol% of 5*c*-20:1, 2 mol% of 5*c*-22:1, 8 mol% of 13*c*-22:1 and 16 mol% of 5*c*,13*c*-22:2 (1). This combination of fatty acids gives the oil distinctive chemical and physical properties, including resistance to oxidation, and has lead to some industrial interest (2). Similar fatty acids had earlier been found in the related seed oil *Limnanthes douglasii* (3,4). It appeared likely that the molecular arrangement of the triacylglycerols in meadowfoam oil would also be distinctive, since stereospecific analyses procedures have given valuable data with *L. douglasii* (5). In this study, the triacylglycerol structure of the seed oil of *L. alba* has been determined by high-performance liquid chromatography (HPLC) in both the silver ion and reversed-phase modes used on their own and to complement each other.

EXPERIMENTAL PROCEDURES

Materials. Triacylglycerols of meadowfoam oil were obtained from the Oregon Meadowfoam Growers' Association, Salem, Oregon. Purity was confirmed by HPLC on a column of silica gel. All solvents were Analar or HPLC grades and were supplied by FSA Scientific (Loughborough, U.K.).

Silver ion HPLC. A Spectra-Physics Model 8700 solvent delivery system (Spectra-Physics Ltd, St. Albans, U.K.) was used in all HPLC separations, together with an ACS Model 750/14 Mass detector (Applied Chromatography Systems (ACS), Macclesfield, U.K.). When required, a stream-splitter (approximately 10:1) was inserted between the column and the detector. A column (4.6 × 250 mm) of Nucleosil™ 5SA (kindly donated by ACS) was converted to the silver ion form as described previously (6).

The procedure for silver ion chromatography of triacylglycerols has been described in detail elsewhere, and was modified only slightly here to suit the Meadowfoam sample (7). In brief, the three solvent reservoirs of the ternary gradient system contained the following: A, 1,2-dichloroethane-dichloromethane (1:1, v/v); B, acetone; C, acetone-acetonitrile (9:1, v/v). A linear gradient of A to 50% A - 50% B was generated over 50 min, and this then was changed to 50% B - 50% C over a further 50 min at a flow-rate of 0.75 mL/min. Samples (0.25 to 0.8 mg) were applied to the column in dichloroethane solution (5 to 10 microliters). Fractions were collected manually via the stream splitter, and methyl nonadecanoate was added to each as an internal standard.

Reversed-phase HPLC. Two columns (250 × 4.6mm) of Spherisorb™ ODS2 (5 micron; ACS Ltd) were employed in series, and were eluted isocratically with dichloromethane-dichloroethane-acetonitrile (32:8:60 v/v/v) at a flow-rate of 0.75 mL/min. The same pump, detection and collection systems as before were utilized. Samples were injected in 5-10 microliters of the mobile phase.

Gas chromatography. The methyl esters of the fatty acids from each fraction were prepared by sodium methoxide-catalyzed transesterification (8). A Carlo Erba Model 4130 capillary gas chromatograph (Carlo Erba, Crawley, U.K.), fitted with split/splitless injection, was equipped with a capillary column (25 m × 0.22 mm i.d.) of fused silica coated with Carbowax 20M (Chrompak Ltd, London, U.K.). It was temperature-programmed from 165°C (held at this for 3 min) at 4°C/min to 195°C, and held at this point for an additional 20 min. Hydrogen was the carrier gas. The component fatty acids were quantified by electronic integration.

Gas Chromatography-Mass Spectrometry. The fatty acids were identified as the picolinyl esters by means of gas chromatography-mass spectrometry (GC-MS) as described elsewhere (except that the upper temperature of the column was 10°C lower), i.e. a fused-silica capillary column (25 m × 0.2 mm i.d.), coated with a cross-linked (5% phenylmethyl) silicone (Hewlett-Packard Ltd, Wokingham, Berks, U.K.), with helium as carrier gas, was temperature-programmed from 60°C to 220°C at 50°C/min then to 250°C at 1°C/min (9). The column outlet was connected directly into the ion source of a Hewlett-Packard 5970 Mass Selective Detector, operated at an ionization energy of 70 eV.

RESULTS AND DISCUSSION

Meadowfoam oil contains an unusual range of fatty acids, and its composition is listed in Table 1. In particular, it contains fatty acids with double bonds in positions 5 and

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TABLE 1

Fatty Acid Compositions (mol % of the total) of the Triacylglycerols of Meadowfoam Oil and of Fractions of This Obtained by Silver Ion HPLC

	Total	Fraction																			Check
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
16:0	0.5	4.0	3.2																						0.3
18:0	0.2	3.5	2.4																						0.3
5–18:1	0.6					1.7		4.7																	0.1
9–18:1	1.8		4.5		1.2		6.8		5.4	15.1				15.6					13.9				34.9		1.0
18:2	2.9																		5.7		23.4		65.1	100.0	0.9
18:3	0.2																								0.0
20:0	0.6	26.7	1.2						4.6																0.8
5–20:1	66.6	62.3	60.4	96.4	78.1	76.6	57.3	40.3	71.8	37.4	65.8	48.1	45.6	20.3	59.3	59.3	31.2	28.0	11.2	70.5	7.8	63.3			69.6
11–20:1	0.4	1.2	26.8					13.6		22.7			21.9							6.4					1.2
5–22:1	1.8	2.3	1.4	3.6							2.0						1.5		9.3						1.8
13–22:1	8.4				20.7	15.6	35.9	41.5	18.2	4.2		21.5	5.3	29.9	13.9			3.5		4.6		36.7			7.0
5,13–22:2	16.0					6.2				20.6	32.3	30.4	27.2	34.1	26.8	40.7	67.3	68.5	59.9	18.5	68.7				17.1
Percentage (%) of the total		1.2	1.7	28.0	16.5	2.5	1.1	1.1	0.7	1.1	25.2	6.8	0.6	0.6	0.4	0.2	4.2	2.2	1.3	0.5	1.0	0.4	0.3	0.4	

13 and dienoic acids with several methylene groups between the double bonds. To ensure that these fatty acids were identified correctly, their structures were determined by GC-MS after conversion to the picolinyl esters, since these derivatives give spectra which are easy to interpret in terms of the positions of the double bonds (10). Examples of the spectra are illustrated in Figure 1. There is always a prominent molecular ion, and abundant ions at *m/e* = 92/93, 108, 151 and 164 are characteristic of the pyridine ring in the ester moiety. With monoenes such as eicos-5-enoate (Fig. 1A), simple radical-induced cleavage occurs from the terminal end of the molecule giving a regular series of ions 14 amu apart, until the double bond is reached (11). Usually, a prominent doublet of ions is seen, resulting from abstraction of hydrogen atoms from either side of the double bond and rearrangement; when the double bond is near the carboxyl group, the doublet is no longer evident, and a double bond in position 5 is recognized by a single ion, produced in a similar manner, at *m/z* = 232. Spectra of dienes with more than one methylene group between the double bonds are not often easy to interpret (9,12). On the other hand, the double bonds in picolinyl docos-5,13-dienoate are sufficiently far apart that the spectrum (Fig. 1B) can be interpreted in terms of isolated double bonds. It has the ion at *m/z* = 232, characteristic of a double bond in position 5.

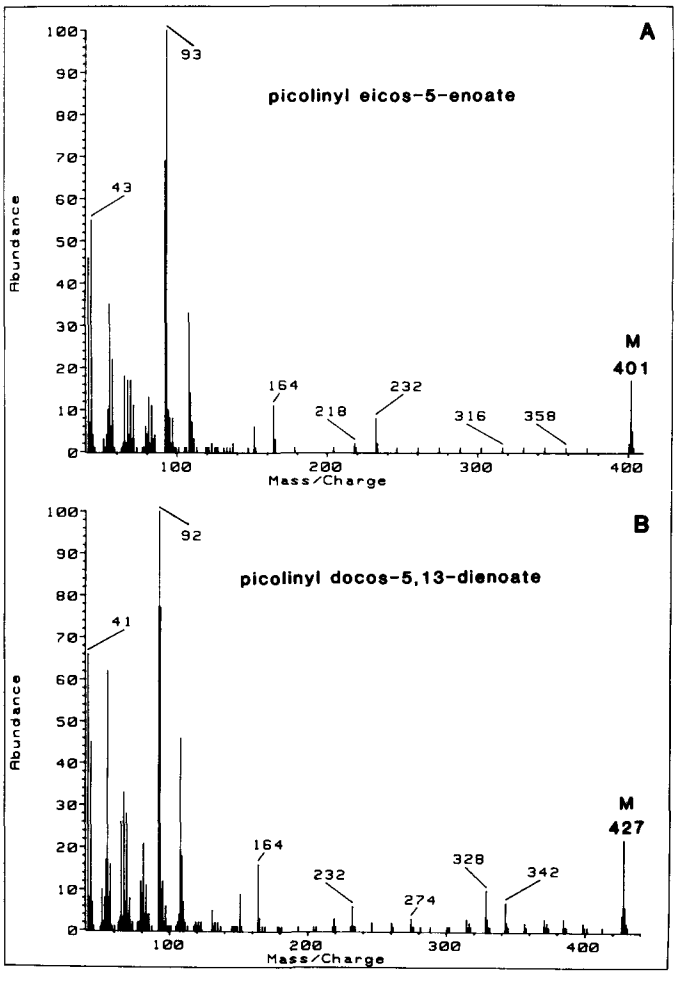

FIG. 1. Mass spectra of (A) picolinyl eicos-5-enoate and (B) picolinyl docos-5,13-dienoate.

An isolated double bond in position 13 would normally have a distinctive doublet at m/z = 330 and 344 (11), but because of the presence of the double bond in position 5 they are in fact found at m/z = 328 and 342.

In recent years, HPLC in the reversed-phase mode has become the favored technique for the structural analysis of triacylglycerols (13). However, because components are separated both by chain-length and degree of unsaturation, one double bond reducing the effective chain-length by about two methylene groups, the order of elution of fractions can sometimes be confusing, especially with novel samples. Silver ion chromatography is a powerful tool for the separation of molecular species of triacylglycerols by degree of unsaturation only, but to date has been used mainly in conjunction with thin-layer chromatography (14). Because separation is based on a single molecular property, it is easier to identify fractions. Recently, a stable silver ion column has been developed for the analysis of triacylglycerols, that gives fractions uncontaminated by silver ions or other intrusive materials (6,7). When this was used with meadowfoam oil with some minor modifications to the elution scheme to optimize resolution, the separation illustrated in Figure 2 was achieved. The abbreviations S, M and D are used to denote saturated, monoenoic and dienoic fatty acyl residues respectively. The peaks were identified and quantified by GC of the methyl ester derivatives of the component fatty acids with an added internal standard following collection via a stream splitter. (The mass or light-scattering detector can only be used for direct determination of lipids following careful calibration.) The results are listed in Table 1. To check the recoveries, the fatty acid composition of the whole was reconstituted from the relative proportions in each of the fractions. There is a tendency to overemphasize the relative proportions of some of the minor peaks, which were not fully resolved, because of the practical difficulty of starting the collection of components at the precise point intended.

After a minor peak of the SMM type is eluted, there follow groups of peaks corresponding in turn to MMM, MMD, MDD and DDD. Within the MMM group, a number of species are resolved differing from each other only in the position of a double bond in one of the fatty acid residues. Thus, fraction 2 can be represented as (5-20:1)(5-20:1)(11-20:1) predominantly, while the major fraction 3 is (5-20:1)(5-20:1(5-20:1) and fraction 4 is (5-20:1)(5-20:1)(13-22:1). The data for fraction 5 must be evaluated carefully, as it was not possible to isolate in it pure form without appreciable contamination with fraction 4, but it is considered to be (5-18:1)(5-20:1)(5-20:1). Fraction 6 is a mixture of (13-22:1)(13-22:1)(5:20:1) and (9-18:1)(5-20:1)(5:20:1), while fraction 7 is probably predominantly (5-20:1)(11:20:1)(13:22:1) and fraction 9 contains (9-18:1)(5-20:1)(11-20:1).

Analogous features are seen with the MMD group of peaks. Thus, if allowance is again made for some overlap in collecting components, fraction 10 to 13 represent the species (5-20:1)(5-20:1)(22:2), (5-20:1)(13-22:1)(22:2), (5-20:1)(11-20:1)(22:2) and (9-18:1)(13-22:1)(22:2) respectively. Similarly, the MDD group of peaks (16 to 19, 22) can be defined in this way.

Two tridienoic (DDD) peaks are seen. The first (fraction 20) appears to consist of tri-(22:2) and (22:2)(22:2)(18:2) while the second (fraction 23) contains only tri-(18:2). It seems remarkable that a significant proportion of a fatty acid, such as 18:2, that comprises only 2.9 mol% of the total should be found in this form.

With this silver ion HPLC system, the order of elution of the acyl groups or the strength of the interaction between the *pi*-electrons of the double bonds and the silver ions is 11-20:1, 5-20:1, 13-22:1, 5-18:1 and 9-18:1. It appears that both the chain-length and the position of the double bond in the fatty acid chain are important. Although it has yet to be studied rigorously, an effect of the position of the double bond on retention time was noted previously with this column (15), and is known from

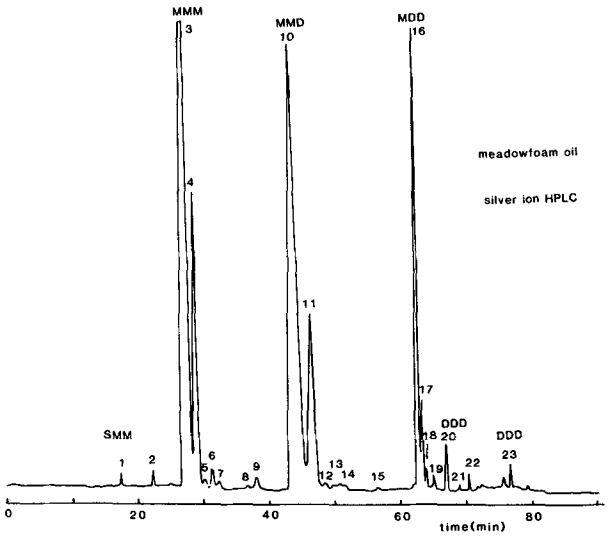

FIG. 2. Separation of the triacylglycerols from meadowfoam oil by HPLC with a silver ion column and mass detection. Fraction numbers are the same as in Table 1.

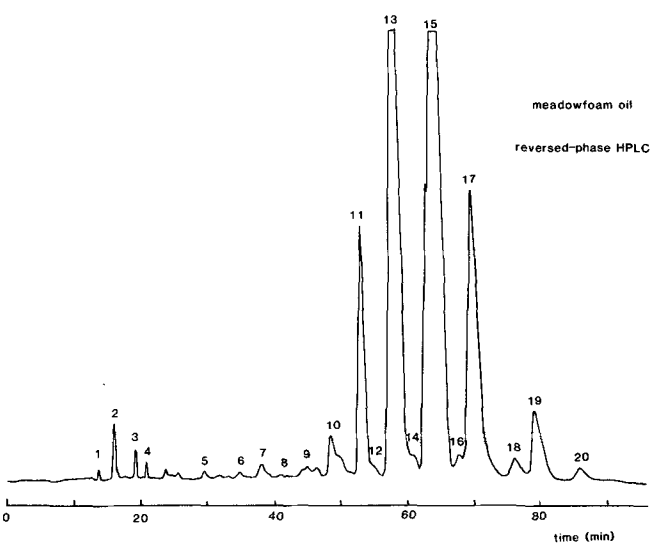

FIG. 3. Separation of the triacylglycerols from meadowfoam oil by HPLC in the reversed-phase mode with mass detection. Fraction numbers are the same as in Table 2.

TABLE 2

Fatty Acid Compositions (mol % of the total) of the Triacylglycerols of Meadowfoam Oil and of Fractions of This Obtained by Reversed-Phase HPLC

		Fraction																			
	Total	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Check
16:0	0.5				29.3					7.1										1.7		0.3
18:0	0.2									0.6										1.9	3.7	0.3
5–18:1	0.6									1.4	3.9	1.1										0.3
9–18:1	1.8			24.3	12.2	76.2	8.6			3.7	5.0	2.0										0.9
18:2	2.9	69.9	100.0	75.7	58.5					1.4												1.2
18:3	0.2	30.1								0.8												0.1
20:0	0.6									0.8								0.8		7.0	9.1	0.6
5–20:1	66.6					23.8	53.4	61.2	56.5	63.4	45.1	41.3	51.6	65.4	73.8	89.0	86.4	65.6	49.2	64.9	51.7	70.0
11–20:1	0.4														5.2		9.9	0.9				0.5
5–22:1	1.8									1.0	7.1									22.1	15.9	1.6
13–22:1	8.4							28.0	15.2	3.9				1.6	6.5	5.9	1.6	27.6	50.8	1.2	19.7	8.1
5,13–22:2	16.0						38.1	10.8	28.3	16.0	38.9	55.6	48.4	33.0	14.5	5.1	2.2	5.0		1.3		16.1
Percentage (%) of the total		0.1	0.4	0.2	0.2	0.2	0.3	0.5	0.4	4.2	2.1	6.4	1.5	21.1	2.1	33.4	2.7	15.7	1.4	5.2	1.2	

TABLE 3

The Principal Molecular Species of Meadowfoam Oil as Resolved by HPLC in the Reversed-Phase Mode (mol%)

Peak number	Species	Amount
1	(18:3)(18:2)(18:2)	0.1
2	(18:2)(18:2)(18:2)	0.4
3	(9–18:1)(18:2)(18:2)	0.2
4	(16:0)(18:2)(18:2)	0.1
	(16:0)(9–18:1)(18:2)	
5	(9–18:1)(9–18:1)(5–20:1)	0.2
6	(9–18:1)(22:2)(22:2)	0.3
	(5–20:1)(5–20:1(22:2)	
7	(5–20:1)(22:2)(13–22:1)	0.5
	(5–20:1)(5–20:1)(22:1)	
8	?	0.4
9	?	4.2
10	(22:2)(22:2)(22:2)	0.5
	(18:1)(5–20:1)(22:2)	1.6
11	(5–20:1)(22:2)(22:2)	7.1
12	(5–20:1)(11–20:1)(22:2)	0.8
13	(5–20:1)(5–20:1)(22:2)	22.8
14	(13–22:1)(22:2)(22:2)	0.4
15	(5–20:1)(5–20:1)(5–20:1)	28.6
	(5–20:1)(13–22:1)(22:2)	4.8
16	(5–20:1)(5–20:1)(11–20:1)	1.0
17	(5–20:1)(5–20:1)(13–22:1)	15.0
	(5–20:1)(5–22:1)(22:2)	2.4
18	(5–20:1)(13–22:1)(13–22:1)	1.1
19	(5–20:1)(5–20:1)(5–22:1)	3.4
	+ others	2.1
20	(5–20:1)(5–22:1)(13–22:1)	0.6
	+ others	0.6

other systems (16,17). No effect of the position of an unsaturated fatty acid within a triacylglycerol molecule can be seen here, in line with previous studies with this system (6,7), although some partial separation cannot be excluded entirely.

The meadowfoam triacylglycerols were also resolved by HPLC in the reversed-phase mode with an octadecylsilyl stationary phase and isocratic elution, and the separation is illustrated in Figure 3. Fractions were identified and quantified as before; the results are listed in Table 2. The first group of minor peaks consists only of C_{16} and C_{18} fatty acids in combination, including species which can be represented as (18:3)(18:2)(18:2) (fraction 1) and tri-(18:2) (fraction 2), confirming the presence of the latter and its relative abundance. This is followed by (18:1)(18:2)(18:2) (fraction 3) and (16:0)(18:2)(18:2) (fraction 4) and other minor components. It is evident from the shape of the peaks that the major fractions contain more than one component, and it was not possible to accurately obtain the composition of partially resolved peaks directly, as before. However, it is feasible to make some deductions from the compositions of partially resolved peaks, when the identities of the major adjacent peaks are known. Most of the major fractions were identified in this way and the results are listed in Table 3 (data from the two techniques used in tandem were also helpful [see below]). When the data from the silver ion fractionation were used to calculate relative proportions of different components, very similar results were obtained (not shown). With the reversed-phase HPLC system, the main problem is that

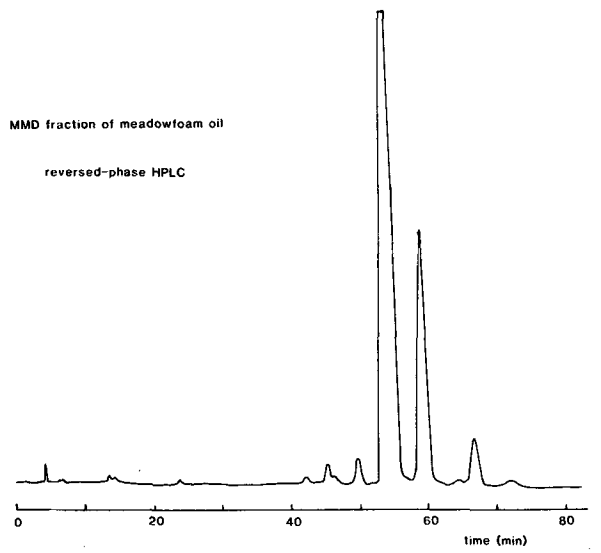

FIG. 4. Separation of the triacylglycerols from the MMD fraction of meadowfoam oil by HPLC in the reversed-phase mode with mass detection.

the 5-20:1 and 22:2 fatty acyl moieties, which comprise more than 80% of the total, have almost the same effect on the retention times (elution volumes) of molecular species. In fact, the order of elution is 18:3 < 18:2 < 18:1 < 16:0 < 11-20:1 < 5-20:1 < 22:2 < 13-22:1 < 5-22:1. The data in Table 2 reveal that molecular species containing 5-18:1, 11-20:1 and 5-22:1 emerge in distinct groups. As might be expected from the relative proportions of the fatty acyl constituents, the species (5-20:1)(5-20:1)(22:2), (5-20:1)(5-20:1)(5-20:1) and (5-20:1)(5-20:1)(13-20:1) are most abundant and together comprise 67% of the total.

In order to confirm the identities of some molecular species present in lower relative proportions, fractions corresponding to the MMM, MMD and MDD types of triacylglycerol were collected from the silver ion column on a micro-preparative scale, and these were each subjected to HPLC in the reversed-phase mode for analysis and quantification as before. As an example, the separation of the MMD fraction is illustrated in Figure 4. The most abundant peak is (5-20:1)(5-20:1)(22:2) and this is preceded by (5-20:1)(11-20:1)(22:2). The last two significant peaks (elution times—60 and 68 min) are (5-20:1)(13-22:1)(22:2) and (5-20:1)(5-22:1)(22:2) respectively. Thus, it is clearly demonstrated that the position of a single double bond in one of the fatty acyl-chains of a triacylglycerol can exert a sufficient effect for base-line separation to be possible. It had earlier been demonstrated that triolein could be separated from tripetroselinin (*cis*-double bonds in positions 9 and 6 respectively) by reversed-phase HPLC (18), but to our knowledge this is the first report of species in which positional isomers of only one fatty acid are resolved.

It is not possible to compare the relative merits of the HPLC in the silver ion and reversed-phase modes in a general manner, when the sole sample studied is so unusual. However, there is little doubt that silver ion chromatography is superior in this instance. In particular, the relative order of elution of fractions gives immediate information on their compositions, since the basis of separation is the single property of degree of unsaturation. Silver ion chromatography followed by reversed-phase chromatography is an especially useful combination.

The presence of appreciable amounts of molecular species of triacylglycerols composed mainly of C_{18} fatty acids, and especially of trilinolein, poses an interesting biosynthetic question. This would seem to provide confirmation of the suggestion that the C_{18} and C_{20}/C_{22} fatty acids are synthesized in distinct "compartments" of the seed, possibly in different regions or organelles where selective acylation can occur (19).

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