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Reversed-phase liquid chromatography–mass spectrometry of complex mixtures of natural triacylglycerols with chloride-attachment negative chemical ionization

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ABSTRACT

Short chain triacylglycerols of butteroil and long chain triacylglycerols of menhaden oil were resolved by conventional reversed-phase high-performance liquid chromatography (HPLC) using a linear gradient of 10–90% propionitrile in acetonitrile as the mobile phase. The triacylglycerol species were identified by positive chemical ionization mass spectrometry, which provided $[MH–RCOOH]^+$ and $[MH]^+$ ions and by negative chemical ionization mass spectrometry with chloride attachment, which yielded exclusively the pseudomolecular ions $[M + Cl]^-$. The negative ions were produced by the inclusion of 1% methylene chloride in the HPLC mobile phase, which did not affect the triacylglycerol elution profile. The restriction of ionization to the chloride-attachment pseudomolecular ions increased about 100-fold the sensitivity of detection of all molecular species and facilitated their quantitation by mass spectrometry. By combining the results of positive and negative chemical ionization mass spectrometry of the eluted peaks it was observed that the complex short chain triacylglycerols showed extensive resolution within isologous saturated carbon number while the polyunsaturated fish oil triacylglycerols showed readily detectable resolution within isologous carbon and double bond number*. These observations permit the identification and quantitation of molecular species of triacylglycerols in such complex mixtures as butterfat and fish oil, which have thus far proved difficult or impossible.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) of triacylglycerols has been extensively studied and the sequence of elution of the common molecular species well established [1]. Identification of molecular species is obtained by means of theoretical carbon numbers (TCN) that are determined from a plot of capacity factors k' *vs* carbon number of the corresponding saturated triacylglycerols [2,3]. The TCN is calculated as the equivalent carbon number (ECN) minus the sum of the emperical factors determined separately for each fatty acid. This method is adequate for oils and fats made up of the common medium and long chain fatty acids [1–3]. By changing the ratio of polar to non-polar solvents, it is possible to obtain a wider variety of mobile phase polarities, which affect the migration of the triacylglycerol species resulting in different retention times for different members of a given ECN [4]. It is therefore necessary to determine the exact retention times for each combination of saturated and unsaturated fatty acids in each solvent system, which may be impossible in the absence of standards. Furthermore, the more complex mixtures of natural triacylglycerols require HPLC with gradient elution [3,4] or temper-

* These are subsets of triacylglycerols: isologous saturated triacylglycerols contain same number of total acyl carbons but differ in their distribution among the fatty chains; isologous polyunsaturated triacylglycerols contain same number of total acyl carbons and number of double bonds, but differ in their distribution among the fatty chains.

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ature programming [5,6] which complicates the calculation of the TCN and peak identification. Since peak collection from poorly resolved profiles is impractical, HPLC with mass spectrometry (MS) appears to provide the only effective method of identification of the molecular species of the triacylglycerols. However, conventional LC–MS is not well suited for peak quantitation in absence of extensive calibration of the system because electron impact and positive chemical ionization (PCI) provide disproportional yields of the diacylglycerol fragment ions from mixed acid triacylglycerols, as well as little or no molecular ion [7,8].

In the present report we wish to describe the application of chloride attachment negative chemical ionization (NCI) mass spectrometry, which yields exclusively the pseudomolecular ions of triacylglycerols [9], as an aid in the identification and quantitation of triacylglycerol species in two complex natural fats by reversed-phase LC–MS.

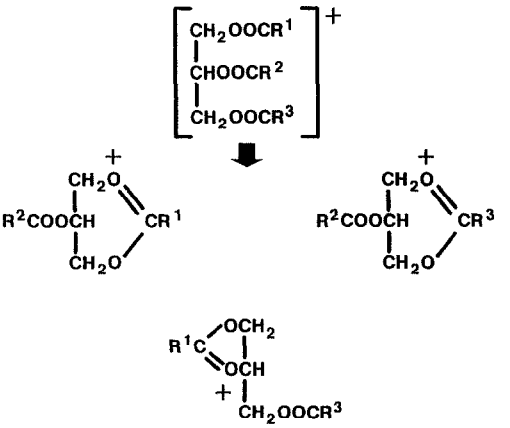
EXPERIMENTAL

Fats and oils

The third most volatile 2.5% distillate (R-3) of butteroil was available in the laboratory [10]. Menhaden oil triacylglycerols were obtained from Zapata Haynie (Reedville, VA, USA). Simple monoacid triacylglycerols were obtained from Serdary Research Laboratories (London, Ontario, Canada). Prior to LC–MS analysis the triacylglycerols were purified by normal-phase thin layer chromatography (TLC).

Reversed-phase HPLC and LC–MS

Reversed-phase C_{18} 5-μm columns (30 cm × 0.46 cm I.D.) were purchased from Supelco Canada (Mississauga, Ontario, Canada) and were installed in a Hewlett-Packard Model 1084B liquid chromatograph interfaced with a Hewlett-Packard Model 5895B quadrupole mass spectrometer via a direct liquid inlet interface as previously described [7]. The mobile phase consisted of a linear gradient of 10–90% propionitrile in acetonitrile for PCI and of a linear gradient of 10–90% propionitrile in acetonitrile containing 1% dichloromethane for NCI with chloride attachment. The columns were run at a flow-rate of 1.5 ml/min with 1% of the effluent being admitted to the mass spectrometer. The PCI and NCI spectra were recorded at 210 eV. The ion source temperature was 200 and 150°C for PCI and NCI, respectively. The mass spectrometer scans were restricted to masses above 200 and were taken at rates of one scan per 4–5 s (m/z 200–1000) over the entire elution profile. In some instances background subtractions were performed by the computer to compensate for impurities in the solvents. For detailed examination and quantitation, the data were recalled from the computer and displayed in a suitable manner. The limit of detection was defined as the lowest detectable concentration yielding a signal to noise ratio of at least two. The general pattern of fragmentation of triacylglycerols in LC–MS under PCI conditions may be represented as shown in Scheme 1 [7].



Scheme 1.

Polarizable capillary gas chromatography (GC)

This was performed with the R-3 distillate of butteroil as previously described for the R-4 distillate of butteroil [11].

RESULTS

R-3 distillate of butteroil

Fig. 1 shows the reversed-phase LC–MS profile of the third most volatile 2.5% molecular distillate in relation to that of whole butteroil triacylglycerols. The distillate represents the more complex part of the triacylglycerol mixture corresponding largely to that of the saturated short and medium chain species as shown elsewhere [12]. Peaks 27 to

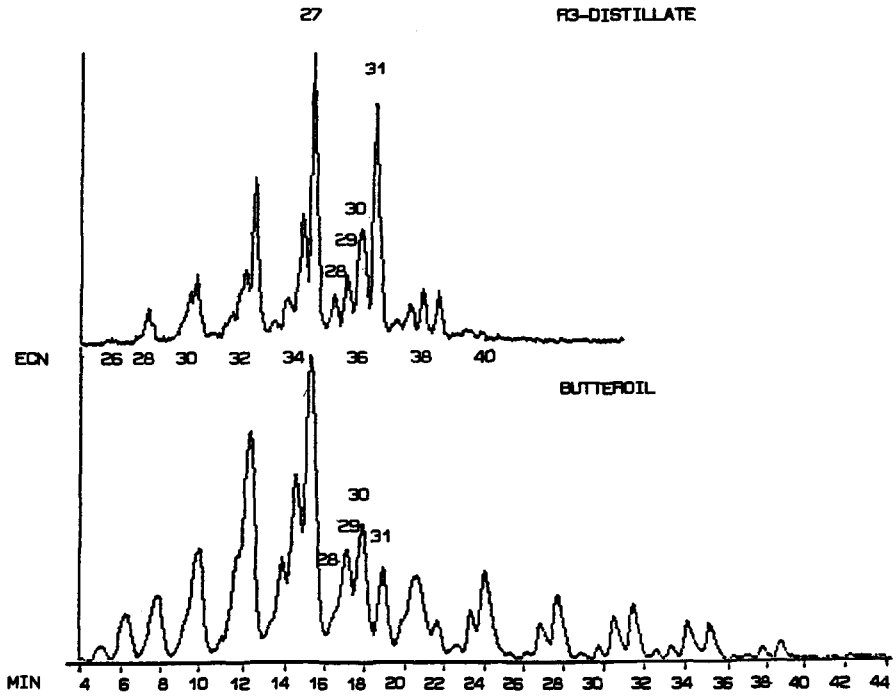


Fig. 1. Reversed-phase LC–MS profile of a butteroil distillate (R-3) and whole butteroil. HPLC conditions: C_{18} column (25 cm × 0.4 cm I.D. tube containing 5 μm packing); solvent system, 10–90% linear gradient of propionitrile in acetonitrile. Other LC–MS conditions as given in text. Peak detection by PCI. Mass range, 270–900 a.m.u.

31 represent triacylglycerols with ECNs 34 to 36, which are discussed in detail below Fig. 2 shows, on an expanded scale, the part of the chromatogram of interest along with the single ion plots for the major diacylglycerol ions detected in the different triacylglycerol peaks by PCI. Fig. 3 shows the corresponding total ion current profile along with the single pseudomolecular ion plots obtained by NCI with chloride attachment for the triacylglycerol species shown in Fig. 2. There were no diacylglycerol type ions seen in the NCI. PCI gave small and variable yields of molecular ions for the short chain triacylglycerols. By combining the data from diacylglycerol fragmentation and the formation of pseudomolecular ions in the NCI it was possible to obtain an unambiguous identification of all the major triacylglycerol species, which was consistent with the anticipated reversed-phase HPLC elution order of the molecules.

Thus, the major isologous triacylglycerols making up peak 31 are 16:0-16:0-4:0 (*m/z* 639, M + 1), as indicated by the presence in PCI (Fig. 2) of prominent diacylglycerol (DG) fragment ions ($[MH-RCOOH]^+$) corresponding to DG20:0 (*m/z* 383) and DG 32:0 (*m/z* 551), and much smaller amounts of 18:0-14:0-4:0 (*m/z* 639), as indicated by diacylglycerol fragment ions corresponding to DG18:0 (*m/z* 355), DG22:0 (*m/z* 411) and DG32:0 (*m/z* 551), as well as by the presence in NCI (Fig. 3) of the pseudomolecular ion $[M+35]^-$ at *m/z* 673. Peak 31 is preceded by peak 30, which is made up of two major triacylglycerols with pseudomolecular ions $[M+35]^-$, corresponding to TG38:1 (*m/z* 699), and $[M+35]^-$, corresponding to TG36:0 (*m/z* 673). The isologous triacylglycerols of the TG38:1 species were made up of 18:1-16:0-4:0, as indicated by the diacylglycerol fragment ions corresponding to DG20:0 (*m/z* 383), DG22:1 (*m/z* 409) and DG 34:1 (*m/z* 577), and very minor contribution of 18:0-16:1-4:0, as indicated by the diacylglycerol fragment ions corresponding to DG20:1 (*m/z* 381), DG22:1 (*m/z* 409) and DG34:1 (*m/z* 577). The 38:1 triacyl-

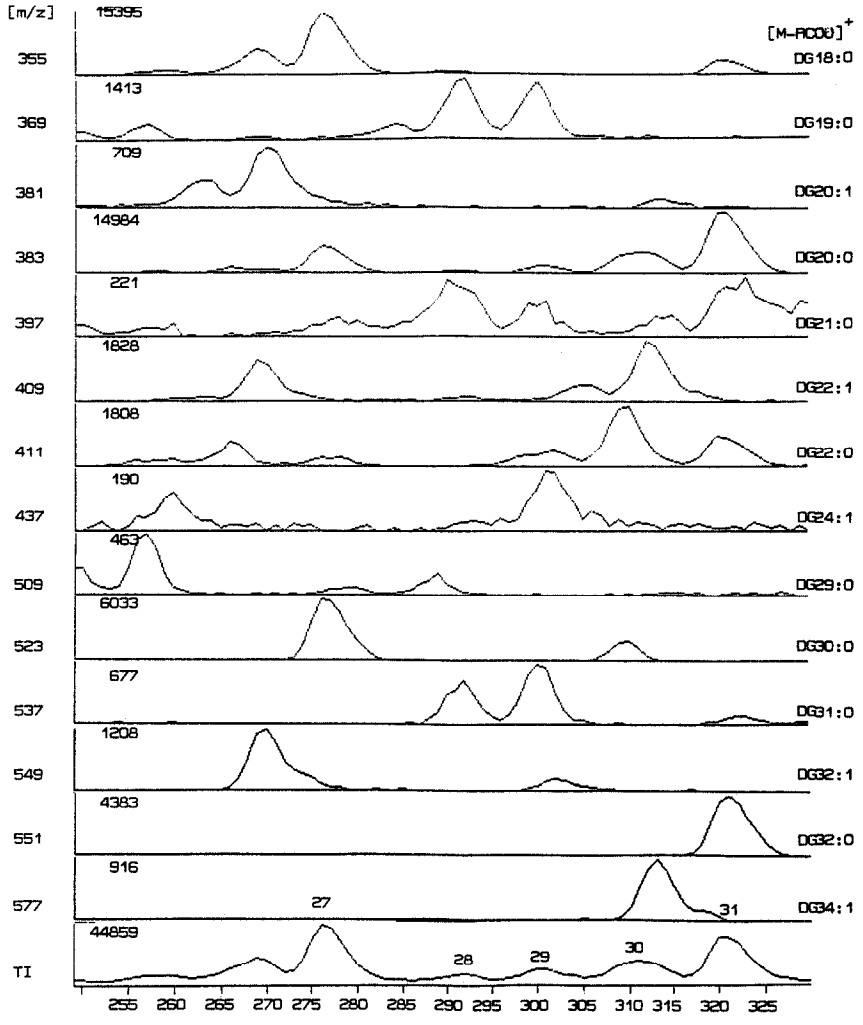


Fig. 2. Expanded scale representation of part of the LC–MS profile of R-3 in Fig. 1, along with single ion plots for major diacylglycerol fragments in PCI. TI, total PCI current; *m/z* values, $[M-RCOO]^+$ ions as identified by carbon number:double bond number; values inside panels, ion counts for each scan. Other LC–MS conditions as in Fig. 1.

glycerol species were preceded by another TG36:0 species, which was made up of 14:0-16:0-6:0, as indicated by the diacylglycerol fragment ions DG20:0 (*m/z* 383), DG22:0 (*m/z* 411) and DG30:0 (*m/z* 523), and smaller amounts of 18:0-12:0-6:0, as indicated by the appropriate diacylglycerol fragment ions. Peak 30 was preceded by peak 29 which was found to yield pseudomolecular ions $[M+35]^-$ at *m/z* 659, 673 and 699, corresponding to TG35:0, TG36:0 and TG38:1, with smaller amounts of TG37:1 and TG40:2, which were found to be eluted midway between peaks 29 and 30. The presence of the TG35:0 triacylglycerol was due to 15:0-16:0-4:0, as indicated by diacylglycerol fragment ions corresponding to DG19:0 (*m/z* 369), DG20:0 (*m/z* 383) and DG31:0 (*m/z* 537), while that of TG36:0 was due to 14:0-14:0-8:0, as indicated by diacylglycerol fragments DG22:0 (*m/z* 411) and DG28:0 (*m/z* 495), and that of TG38:1 duc to 14:0-18:1-6:0, as indicated by the diacylglycerol fragment ions DG20:0 (*m/z* 383), DG24:1 (*m/z* 437) and DG32:1 (*m/z* 549). There were small amounts of other triacylglycerols in this

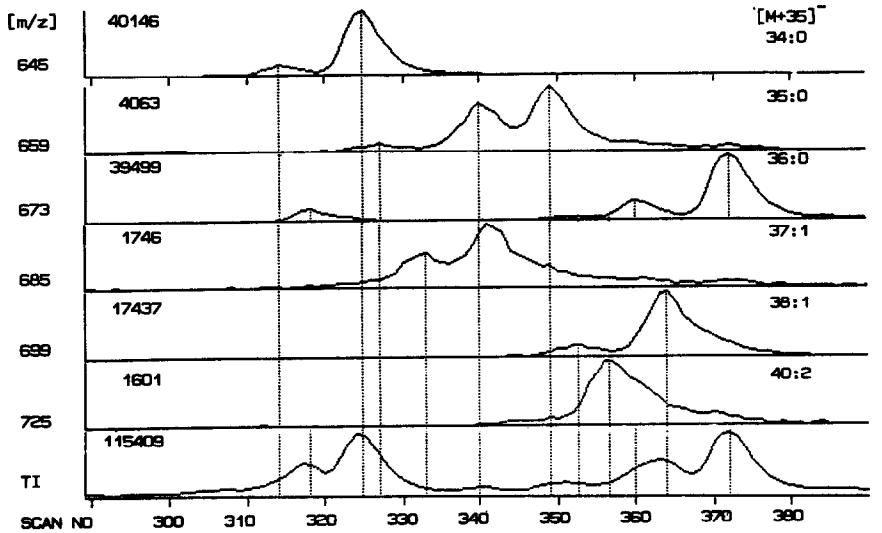


Fig. 3. Expanded scale representation of part of the LC–MS profile of R-3 in Fig. 3 as obtained by chloride attachment NCI. TI, total NCI current; *m/z* values, $[M+35]^-$ pseudomolecular ions as identified by carbon number:double bond number; values inside panels, ion counts for each scan. Solvent system: 10–90% linear gradient of propionitrile in acetonitrile containing 1% dichloromethane. Other LC–MS conditions as in Fig. 1. Peak detection by NCI.

minor peak either because of overlapping with other isologous triacylglycerols or because of the tailing of major triacylglycerols preceding the minor peaks. Peak 29 was preceded by peak 28, which also was made up of several triacylglycerols giving mainly pseudomolecular ions $[M+35]^-$ at *m/z* 645, 659 and 685 corresponding to TG34:0, TG35:0 and TG37:1, with smaller amounts of pseudomolecular ions of *m/z* 671 and 699 corresponding to TG36:1 and TG38:1. The 35:0 triacylglycerol was due to 15:0-14:0-6:0, as indicated by the presence of diacylglycerol fragment ions of DG20:0 (*m/z* 383), DG21:0 (*m/z* 397) and DG29:0 (*m/z* 509), while the presence of the TG37:1 was due to 15:0-16:1-6:0, as indicated by the diacylglycerol fragment ions DG21:0 (*m/z* 397), DG22:1 (*m/z* 409), and DG31:1 (*m/z* 535). Peak 28 also contained significant amounts of the 14:0-16:0-4:0 species due to tailing of the major peak 27, which contained this triacylglycerol species nearly exclusively. Fig. 4 compares the PCI and NCI spectra recorded for the center sections of peak 29, while Fig. 5 compares the PCI and NCI spectra for peak 28. As already explained, the short chain saturated and monounsaturated triacylglycerols give low yields of molecular ions and variable yields of diacylglycerol type of fragments in PCI. NCI with chloride attachment yields only the pseudomolecular ions from which the molecular weight of the triacylglycerol species can be reliably obtained.

Table I compares the quantitative estimates for the major triacylglycerols in the R-3 distillate as obtained from the negative pseudomolecular ions in LC–MS and from polarizable capillary GLC with flame ionization detection. The triacylglycerol species are matched on basis of total carbon and double bond number and include separate estimates for the major isologous species within each carbon and double bond number. The LC–MS data show good agreement with the data obtained by polar capillary GLC of the intact triacylglycerols of R-3 distillate. From the close agreement, it would appear that chloride attachment NCI yields quantitatively correct proportions for the component short chain length triacylglycerol when analyzed in a complex mixture.

Menhaden oil

Fig. 6 shows a three-dimensional reversed-phase LC–MS profile of menhaden oil triacylglycerols as obtained using PCI as a means of peak detection. This profile is similar to that reported for fish oil

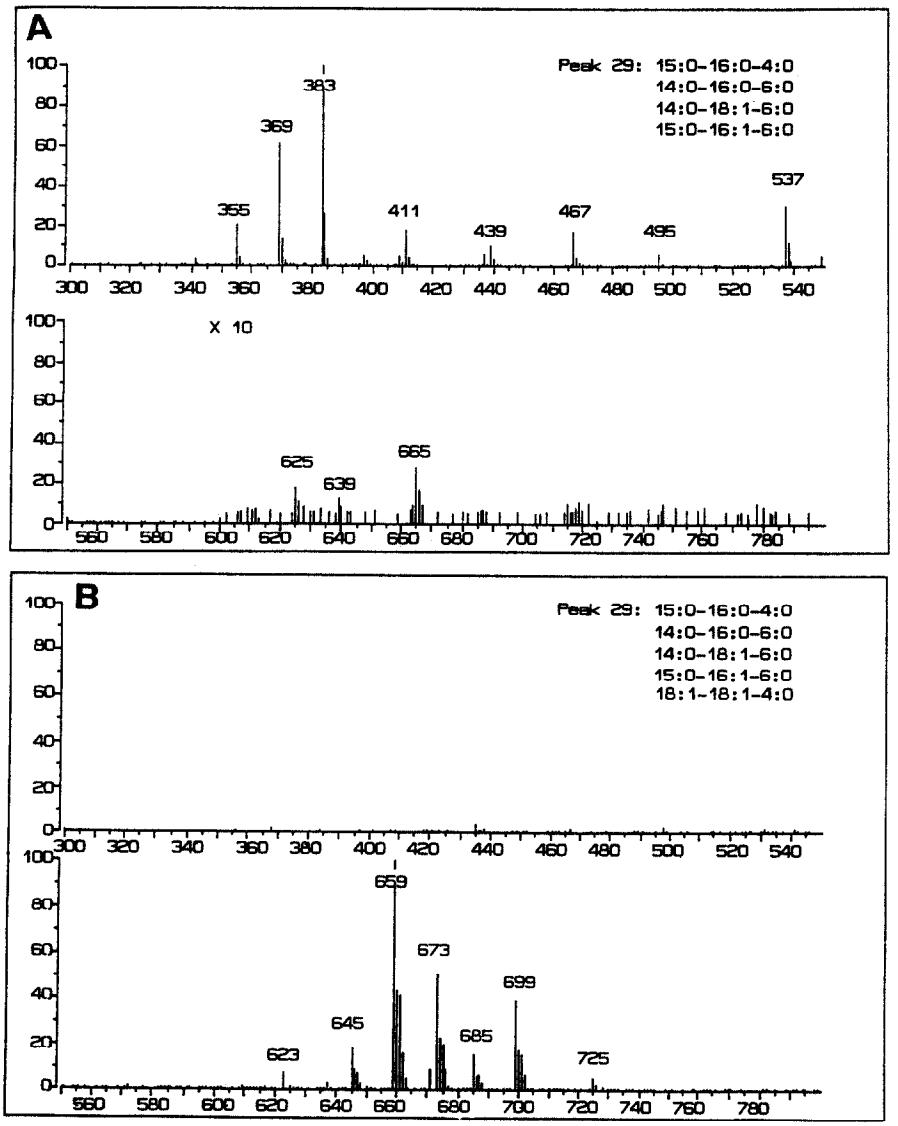


Fig. 4. Comparison of (A) PCI and (B) NCI mass spectra for the center cut of peak 29 (Fig. 2). LC–MS conditions as in Figs. 1 and 3 for the PCI and NCI, respectively. Major triacylglycerols identified by carbon number:double bond number. Ion identification as in Table I.

triacylglycerols by Wojtusik *et al.* [13]. The HPLC profile shows extensive peak overlapping and possible peak tailing. The ion plot which represents largely the distribution of the diacylglycerol type of fragments confirms the extensive peak overlapping and interdigitation but gives no evidence of peak tailing. The application of LC–MS to the identification and quantitation of the fish oil triacylglycerol peaks, all of which contain extremely complex mixtures of numerous molecular species [14], is illustrated by examining selected sections of the chromatographic profile.

Fig. 7 shows the PCI plots for the molecular ions of selected triacylglycerol species of ECN 42, 40 and 38, which are eluted over a wide range of the chromatographic profile due to differences in the con-

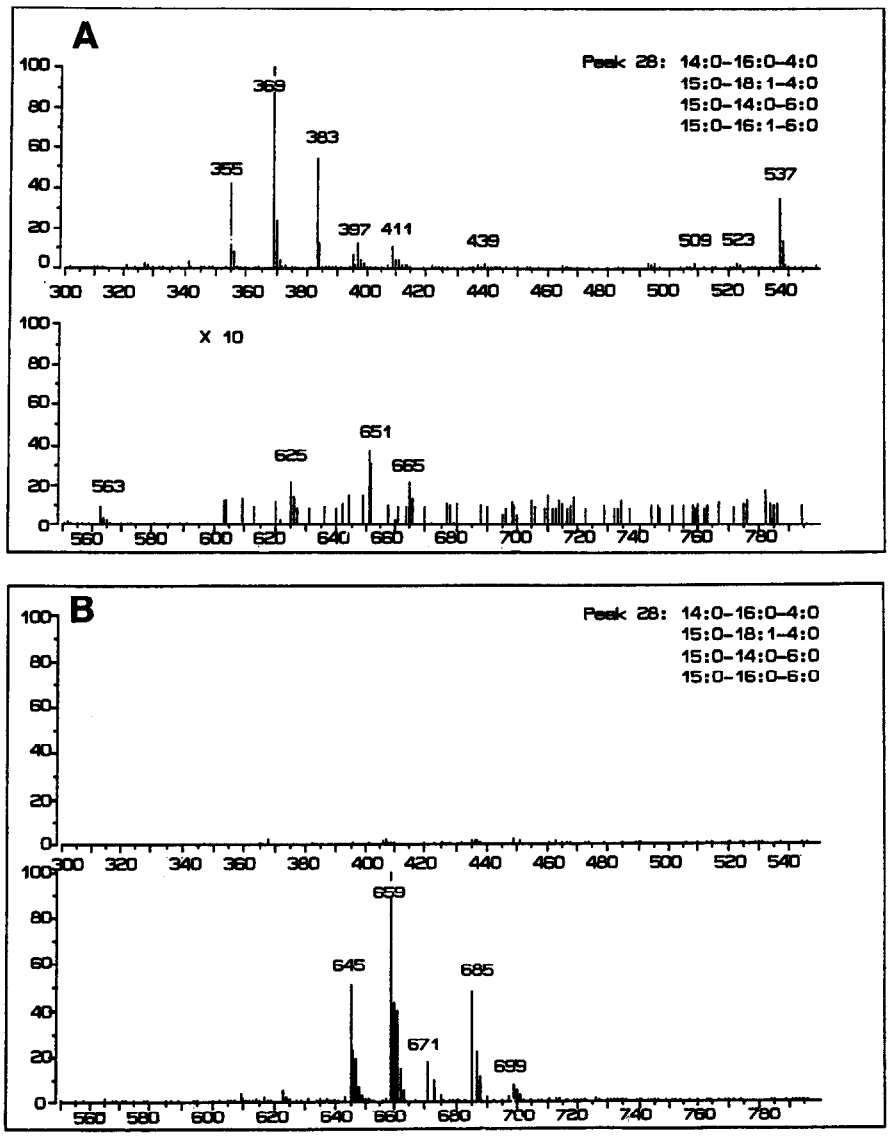


Fig. 5. Comparison of (A) PCI and (B) NCI mass spectra for a center cut of peak 28 (Fig. 2). LC–MS conditions as in Fig. 4. Major triacylglycerols identified by carbon number:double bond number. Ion identification as in Table I.

tent of acyl carbons and double bonds among the fatty chains. The molecular species have been selected to illustrate isologous triacylglycerol separation during reversed-phase HPLC of fish oil triacylglycerols, which makes peak identification based on theoretical carbon numbers difficult or impossible.

The positive ions corresponding to the diacylglycerol fragments in Fig. 8 show that the major isologous 52:5 triacylglycerols (*m/z* 853) in peak 4 are made up 16:0-16:0-20:5, as indicated by the presence of a prominent diacylglycerol fragment ion DG32:0 (*m/z* 551), although the corresponding DG36:5 ion (*m/z* 597) is seen in lesser abundance, along with a smaller amount of 18:0-14:0-20:5, as indicated by the presence of DG32:0 (*m/z* 551), DG34:5 (*m/z* 569) and still smaller amounts of

TABLE I

COMPOSITION OF TRIACYLGLYCEROLS IN R-3 DISTILLATE OF BOVINE MILK FAT AS ESTIMATED BY REVERSED-PHASE LC-MS WITH CHLORIDE-ATTACHMENT NEGATIVE CHEMICAL IONIZATION (AREA %)

Molecular species	NCI $[M+35]^-$	GC–FID[c]	Molecular species	NCI $[M+35]^-$	GC—FID
28:1	0.04 (11.65)[a]		36:0	4.53 (23.38)	6.46
30:1	0.46 (13.33)	0.63	38:1	7.66 (23.65)	6.24
32:1	0.21 (13.40)		39:1	0.14 (23.78)	0.48
28:0	0.31 (13.47)	0.75	41:2	0.09 (24.00)	n.d.
31:1	0.02 (14.45)	n.d.[b]	40:2	0.59 (24.03)	0.88
32:1	0.21 (14.97)	n.d.	36:0	15.91 (24.17)	15.22
30:0	0.27 (15.22)	0.37	37:0	1.36 (24.30)	1.12
32:1	1.53 (15.42)	1.58	39:1	0.27 (24.42)	n.d.
34:2	0.26 (15.48)	n.d.	37:0	2.39 (25:13)	1.18
30:0	1.26 (15.73)	2.16	39:1	0.27 (25.20)	0.48
33:1	0.07 (16.20)	n.d.	40:1	0.65 (25.33)	n.d.
31:0	0.20 (16.45)	0.40	42:2	0.27 (25.40)	n.d.
33:1	0.08 (16.72)	n.d.	37:0	2.39 (25.78)	1.22
31:1	0.02 (16.97)	n.d.	40:1	0.66 (25.92)	1.32
34:1	0.52 (17.37)	2.37	38:0	1.19 (25.98)	1.78
32:0	1.05 (17.62)	1.31	38:0	3.18 (26.57)	3.72
36:2	0.26 (17.75)	n.d.	40:1	0.66 (26.70)	0.75
34:1	3.03 (17.95)	2.37	41:1	0.14 (26.88)	n.d.
36:2	0.26 (18.13)	n.d.	38:0	3.32 (27.33)	4.45
32:0	4.43 (18.33)	6.11	39:0	0.33 (27.40)	n.d.
35:1	0.28 (18.72)	n.d.	44:2	– (27.98)	n.d.
33:0	0.55 (19.18)	0.99	39:0	0.22 (28.25)	n.d.
35:1	0.28 (19.30)	n.d.	42:1	0.15 (28.50)	n.d.
33:0	0.69 (19.70)	0.88	39:0	0.11 (28.90)	n.d.
40:3	0.12 (20.22)	n.d.	42:1	0.15 (28.97)	n.d.
34:0	2.57 (20.40)	3.13	40:0	0.68 (29.03)	1.38
38:2	0.06 (20.53)	n.d.	40:0	0.68 (29.67)	0.97
36:1	7.63 (20.67)	6.55	43:1	– (29.93)	n.d.
38:2	0.86 (21.05)	n.d.	41:0	0.14 (30.45)	n.d.
34:0	14.13 (21.12)	15.1	44:1	– (31.33)	n.d.
35:0	0.21 (21.25)	n.d.	42:0	– (31.88)	n.d.
37:1	0.82 (21.57)	n.d.	Other		
39:2	0.11 (21.70)	n.d.			
35:0	1.51 (22.10)	2.34			
37:1	1.40 (22.15)	0.97			
35:0	1.61 (22.68)	2.00			
38:1	1.24 (22.87)	n.d.			
40:2	0.11 (23.13)	n.d.			

[a] HPLC retention times (minutes) in brackets.
[b] n.d. = Not determined.
[c] FID = Flame ionization detection.

DG38:5 (*m/z* 625), and of 16:0-14:0-22:5, as indicated by the diacylglycerol fragment ions DG30:0 (*m/z* 523), DG36:5 (*m/z* 597) and DG38:5 (*m/z* 625). Peak 4 is preceded by peak 3, which is seen to be made up of 16:0-18:1-18:4 as indicated by the diacylglycerol fragment ions DG34:1 (*m/z* 577), DG34:4 (*m/z* 571) and DG36:5 (*m/z* 597), and of 14:0-18:1-20:4, as indicated by the diacylglycerol fragment ions corresponding to DG32:1 (*m/z* 549), DG34:4 (*m/z* 571) and DG38:5 (*m/z* 625). Peak 1 is due to the M + 2 ions from the 52:6 triacylglycerols (*m/z* 851), while peak 2 represents a complex mix-

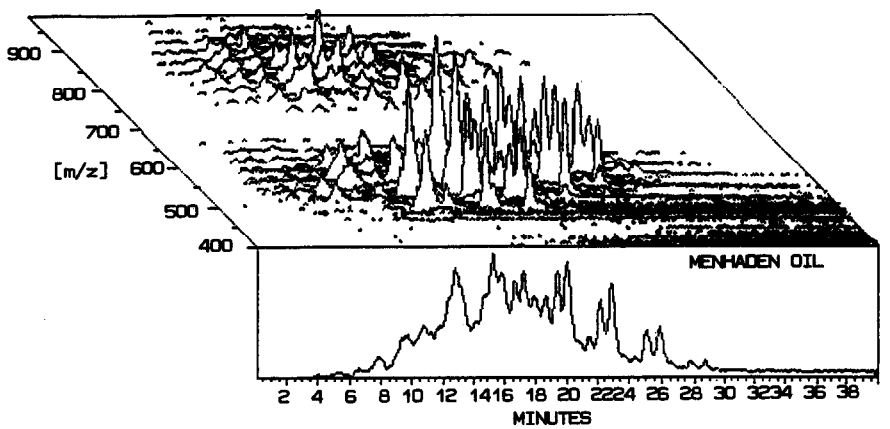


Fig. 6. Three-dimensional LC–PCI-MS profile of menhaden oil triacylglycerols. TI, total ion current; *m/z* 450–700, diacylglycerol fragment ions $[M-RCOO]^+$; *m/z* 700–1000, molecular ions of triacylglycerols $[M]^+$. HPLC solvent: 20–90% linear gradient of propionitrile in acetonitrile. Other LC–MS conditions as in Fig. 1.

ture of triacylglycerols of ECN 42 which contains very little of the isologous triacylglycerols (*m/z* 853) identified in peaks 3 and 4.

The diacylglycerol fragment ions in Fig. 9 show that the major isologous 54:6 triacylglycerols (*m/z* 879) in peak 3 are made up of 16:0-16:0-22:6, as indicated by the fragment ions DG32:0 (*m/z* 551) and DG38:6 (*m/z* 623), and 18:0-14:0-22:6, as indicated by the fragment ions DG32:0 (*m/z* 551), DG36:6 (*m/z* 595) and DG40:6 (*m/z* 651). Peak 3 is preceded by peak 2, which is made up of 16:0-18:1-20:5, as indicated by major fragment ions corresponding to DG34:1 (*m/z* 577), DG36:5 (*m/z* 597) and DG38:6 (*m/z* 623). Peak 1 contains 18:1-18:1-18:4, as indicated by major fragment ions corresponding to DG36:2 (*m/z* 603) and DG36:5 (*m/z* 597).

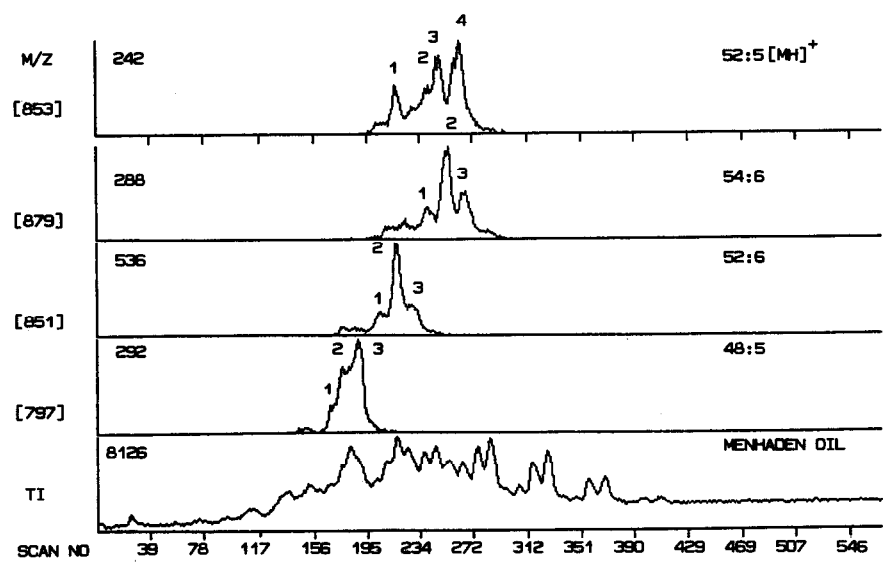


Fig. 7. Single ion plots in PCI for selected triacylglycerol (TG) species of ECN 38 (48:5), 40 (52:6) and 42 (52:5 and 54:6). TI, total PCI current; *m/z* values represent molecular ions $[M]^+$ of isologous triacylglycerol. Values inside panels, ion counts for each scan. LC–MS conditions as in Fig. 6.

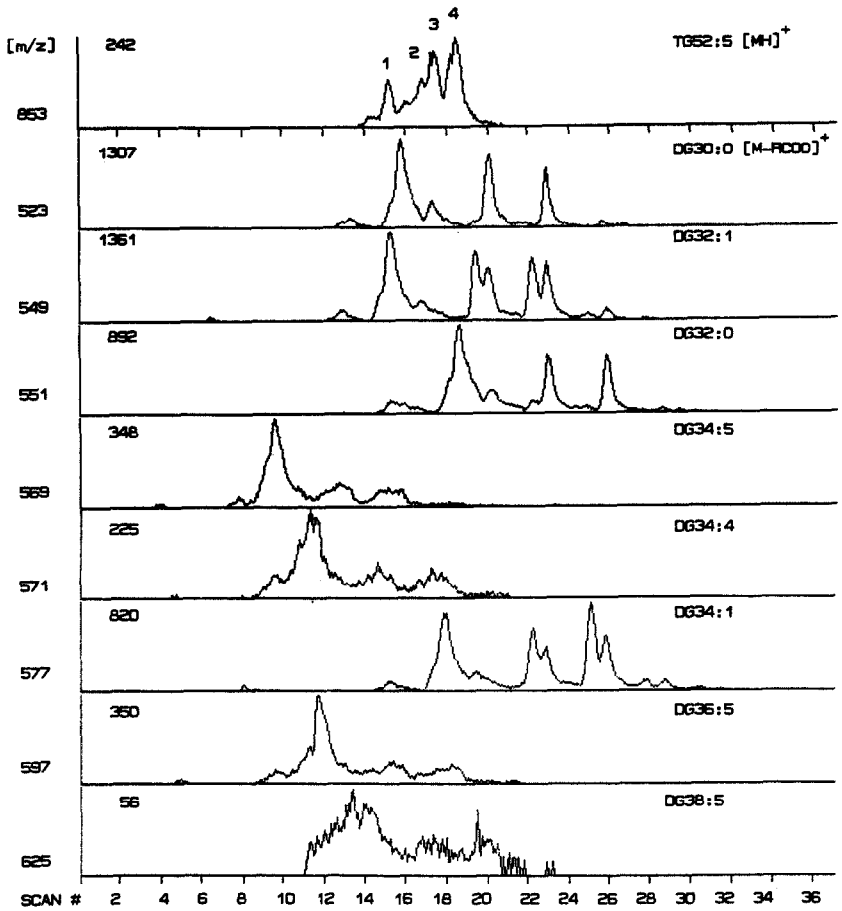


Fig. 8. Single ion plots in PCI for major diacylglycerol (DG) fragment ions $[M-RCOO]^+$ of isologous 52:5 triacylglycerols. TI, total PCI current; *m/z* values as identified by carbon number:double bond number. Values inside panels, ion counts for each scan. LC–MS conditions as in Fig. 6.

The major isologous 52:6 triacylglycerols (*m/z* 851) in Fig. 7 were made up of 16:1-18:1-18:4 (peak 1), as indicated by major fragment ions corresponding to DG34:2 (*m/z* 575), DG34:5 (*m/z* 569) and DG36:5 (*m/z* 597), along with 14:0-18:1-20:5 (peak 2), as indicated by major fragment ions corresponding to DG32:1 (*m/z* 549), DG34:5 (*m/z* 569), and DG38:6 (*m/z* 623) and 14:0-16:0-22:6 (peak 3), as indicated by the diacylglycerol fragment ions DG30:0 (*m/z* 523), DG36:6 (*m/z* 595) and DG38:6 (*m/z* 623), while the major isologous 48:5 triacylglycerols (*m/z* 797) could be identified as 16:1-16:1-16:3 (peak 1), as indicated by the major fragment ions corresponding to DG32:2 (*m/z* 547) and DG32:4 (*m/z* 543); 14:0-16:4-18:1 (peak 2), as indicated by fragment ions DG30:4 (*m/z* 515), DG32:1 (*m/z* 549) and DG34:5 (*m/z* 569), and 14:0-14:0-20:5 (peak 3), as indicated by the fragment ions corresponding to DG28:0 (*m/z* 495) and DG34:5 (*m/z* 569) (data not shown). It should be noted that in these instances the various isologous triacylglycerols were eluted in order of increasing content of saturated fatty chains. Those with two such chains were retained longer than those with one saturated fatty chain, while those containing three unsaturated fatty chains were eluted first within each isologous series. This elution order was confirmed for several other mixtures of isologous triacylglycerols, including 54:8 and 56:7 (data not shown).

In all instances the molecular weight distribution

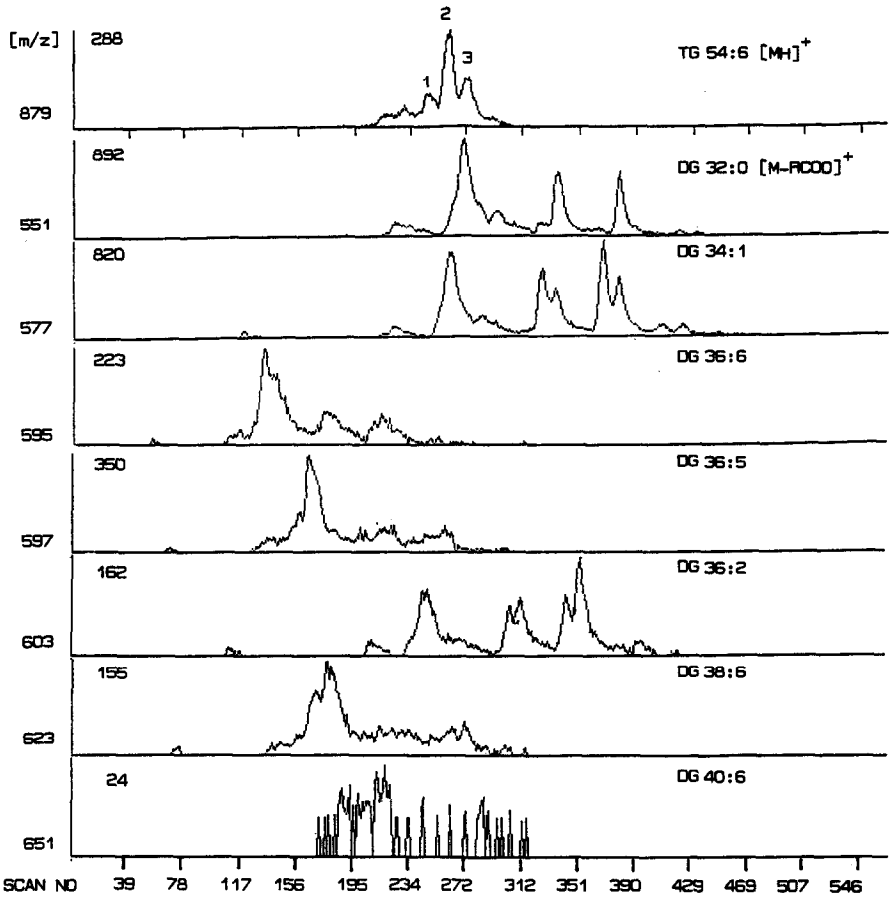


Fig. 9. Single ion plots in PCI for major diacylglycerol (DG) fragment ions $[M-RCOO]^+$ of isologous 54:6 triacylglycerols. TI, total PCI current; *m/z* values as identified by carbon number:double bond number. Values inside panels, ion counts for each scan. LC–MS conditions as in Fig. 6.

of the triacylglycerols was confirmed by NCI mass spectrometry with chloride attachment. Fig. 10 compares the full mass spectra obtained by PCI (panel A) and NCI (panel B) for the center section of the HPLC peak 4 (Fig. 7) corresponding to TG52:5. In addition to the molecular ions corresponding to triacylglycerols 50:4 (*m/z* 827), 52:5 (*m/z* 853), 54:6 (*m/z* 879), 56:7 (*m/z* 905), and 58:8 (*m/z* 931) seen in PCI, NCI reveals the presence of pseudomolecular ions for triacylglycerols 44:1 (*m/z* 783), 46:2 (*m/z* 809), 48:3 (*m/z* 835), 50:4 (*m/z* 861), 52:5 (*m/z* 887), 54:6 (*m/z* 913), 56:7 (*m/z* 939), 58:8 (*m/z* 965) and 60:9 (*m/z* 991). Fig. 11 compares the full mass spectra obtained by PCI (panel A) and NCI (panel B) for the center section of the HPLC peak 2 (Fig. 7) corresponding to the isologous TG54:6. The PCI spectrum yielded molecular ions only for the major polyunsaturated triacylglycerol species: TG48:3 (*m/z* 801); TG50:4 (*m/z* 827); TG52:5 (*m/z* 853); TG54:6 (*m/z* 879); TG56:7 (*m/z* 905); and TG58:8 (*m/z* 931), along with the corresponding diacylglycerol type of ions. From the NCI spectrum it is obvious that the triacylglycerol mixture is more complex and contains other species belonging to ECN 42: TG42:0 (*m/z* 757); TG44:1 (*m/z* 783); TG46:2 (*m/z* 809); TG48:3 (*m/z* 835); TG50:4 (*m/z* 861); TG52:5 (*m/z* 887); TG54:6 (*m/z* 913); TG56:7 (*m/z* 939); TG58:8 (*m/z* 965); and TG60:9

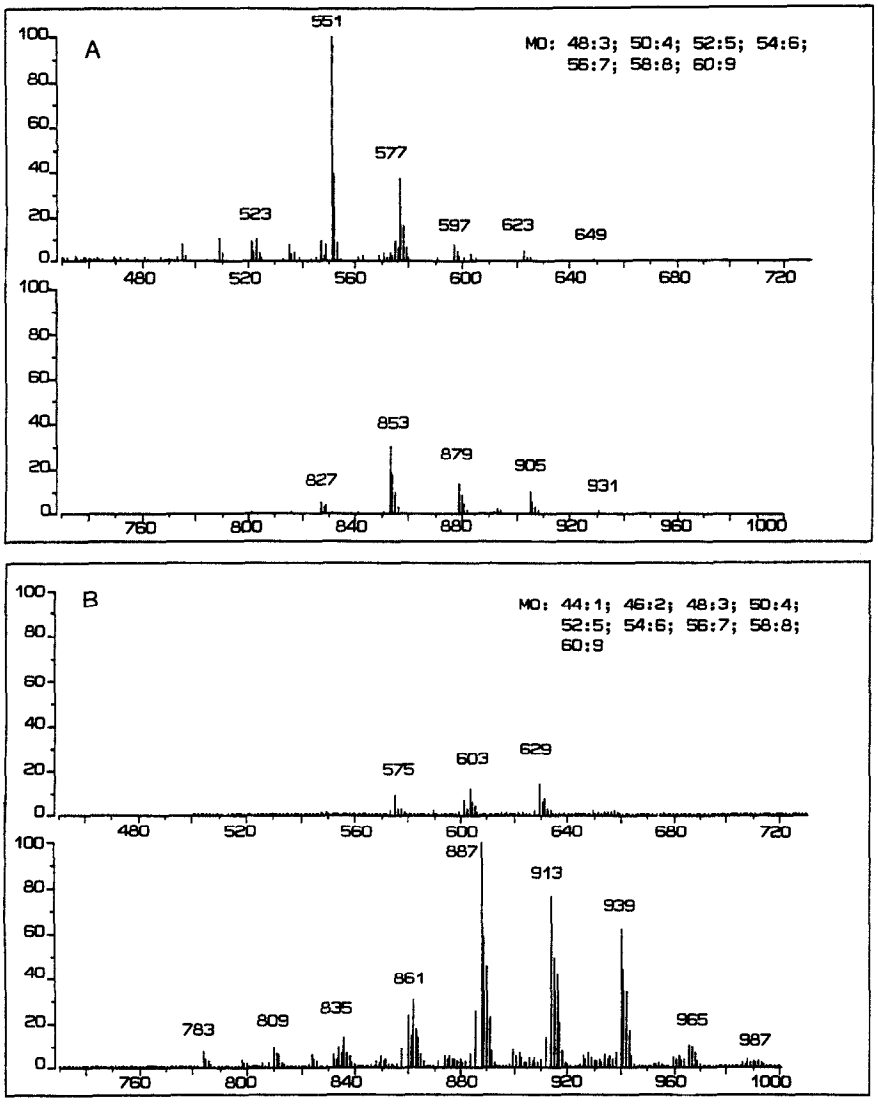


Fig. 10. Comparison of full mass spectra in (A) PCI and (B) NCI for the center sections of HPLC peak 4 (Fig. 7) corresponding to isologous 52:5 triacylglycerols. Solvent system: 20–90% linear gradient of propionitrile in acetonitrile containing 1% dichloromethane. Other LC–MS conditions as in Fig. 6.

(*m/z* 991). Only traces of diacylglycerol fragment ions were observed in the NCI spectrum obtained with chloride attachment.

Table II compares the recoveries of the different menhaden oil triacylglycerol species within ECN 36, 38 and 42 series as estimated by reversed-phase LC–MS with PCI and NCI and by calculation of the 1-random 2-random 3-random association based on the knowledge of the positional distribution of the fatty acids in menhaden oil triacylglycerols [14]. There is a reasonable agreement between the estimates from the PCI and NCI mass spectrometry, even though molecular species having more than 60 acyl carbons could not be estimated in the chloride attachment mode because the combined molecular weight exceeded the 1000 mass limit of the instrument. Furthermore, the measured values are reasonably close to the values calculated from the 1-random 2-random 3-random association of the fatty acids in the triacylglycerol species. This

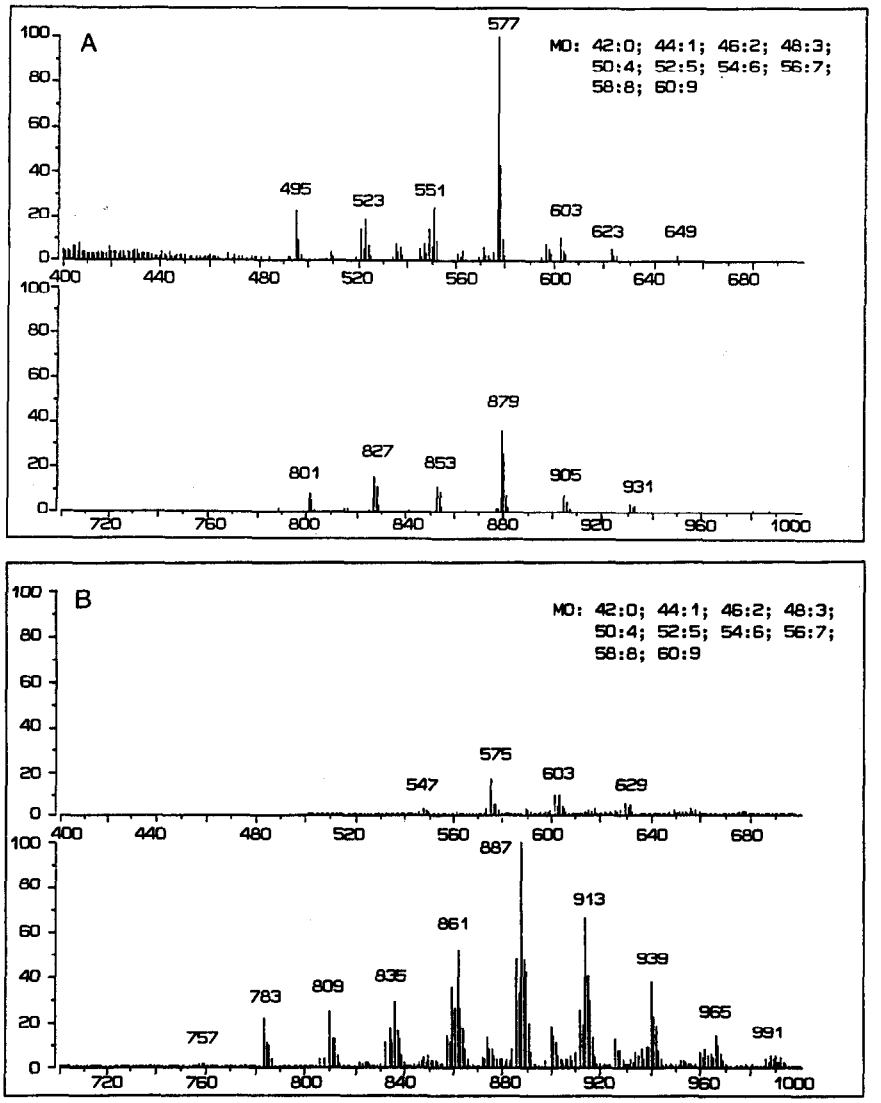


Fig. 11. Comparison of full mass spectra in (A) PCI and (B) NCI for the center section of HPLC peak 2 (Fig. 7) corresponding to a series of isologous 54:6 triacylglycerols. LC–MS conditions as in Fig. 10.

indicates that PCI of the polyunsaturated triacylglycerols yields nearly correct proportions of the molecular species, unlike the short and medium chain saturated fatty triacylglycerols, which gave low and variable yields of molecular ions in PCI. NCI with chloride attachment gave apparently correct proportions of molecular ions with both saturated and unsaturated triacylglycerols of short and long chain lengths.

DISCUSSION

The present study shows that a combination of HPLC with mass spectrometry is necessary for identification and quantitation of the molecular species of triacylglycerols in the more complex natural fats and oils. This is largely so because of the presence in the HPLC peaks of triacylglycerol species with closely similar or identical theoretical car-

TABLE II

COMPOSITION OF TRIACYLGLYCEROLS IN ECN 36, 38 AND ECN 42 OF MENHADEN OIL TRIACYLGLYCEROLS AS ESTIMATED BY REVERSED-PHASE LC–MS WITH POSITIVE AND NEGATIVE CHEMICAL IONIZATION (AREA %)

Molecular species	1-R-2-R-3-R[a] (Calculated)	PCI $[M]^+$	NCI $[M+35]^-$
ECN 36			
44:4	0.6	2.3	1.7
46:5	2.1	7.0	4.2
48:6	3.4	9.2	5.2
50:7	5.9	12.5	6.2
52:8	11.6	18.7	11.4
54:9	17.4	18.2	17.2
56:10	22.8	18.2	25.1
58:11	22.0	10.5	20.4
60:12	11.4	3.2	8.5
62:13	2.4	0.1	?[b]
ECN 38			
44:3	0.5	1.4	1.0
46:4	4.8	8.9	6.3
48:5	12.6	21.1	16.3
50:6	17.9	22.1	19.8
52:7	18.6	18.7	16.4
54:8	14.7	11.2	11.9
56:9	10.9	8.4	11.6
58:10	10.1	5.6	10.7
60:11	7.1	2.8	6.0
62:12	2.2	trace	?[b]
64:13	0.2	?[b]	?[b]
ECN 42			
44:1	3.7	2.1	3.0
46:2	5.6	3.8	5.5
48:3	8.6	12.6	8.4
50:4	12.1	20.0	12.8
52:5	21.6	26.8	24.0
54:6	26.2	22.8	27.7
56:7	16.4	9.9	13.6
58:8	4.9	1.8	3.9
60:9	0.6	trace	1.1

[a] 1-Random 2-random 3-random calculation.
[b] Combined molecular weight of ions $[M+Cl]^-$ exceeds the calibrated mass range of the instrument.

bon numbers. Furthermore, the elution of critical pairs of triacylglycerols over a wide range of theoretical ECN leads to increased overlapping and interdigitation of peaks. In the short chain triacylglycerols this additional resolution takes place within the saturated triacylglycerols. Since the butyrates, caproates and caprylates make up a high proportion of total triacylglycerols in the R-3 distillate of butteroil, they appear as prominent peaks in the HPLC profile. The order of elution is recognized in a modified form in the monoenoic and dienoic triacylglycerols, which occur in much smaller amounts in the distillate. This order of reversed-phase HPLC elution of the short chain saturated triacylglycerols is similar to that reported by Takamura *et al.* [15] for the acetates, butyrates and hexanoates when combined with palmitic and stearic acids in diacylglycerol acetates. It led to an effective separation of the butyrates, caproates and caprylates among the saturated triacylglycerols and the resolution of triacylglycerols with 0, 1, 2 or 3 saturated fatty acids per molecule of triacylglycerol within an equivalent carbon and double bond number. Surprisingly, the order of elution of the short-chain saturated triacylglycerols from the reversed-phase HPLC column is similar to that noted during polar capillary GC [11,16]. The butyrates would have been expected to be more polar than the corresponding caproates and caprylates and thus lead to an earlier elution from the reversed-phase column, which was not observed.

The LC–MS combination was equally effective in establishing the order of elution of the complex polyunsaturated triacylglycerols present in menhaden oil. In addition to resolution based on ECN and the separation of the critical pairs within each ECN as established for seed oils [2,3] we were able to recognize resolution within isologous series of triacylglycerol species. This resolution was based on the relative distribution of the carbon and double bond numbers among the fatty chains in the triacylglycerol molecules. The order of elution is similar to that observed for diacylglycerophospholipids [17,18] and the diacylglycerol dinitrobenzoates [19] where the saturated-polyunsaturated fatty acid combination is retained longer than the combination of two oligounsaturated fatty acids within the same equivalent carbon number (*e.g.* 18:2-18:2 eluted earlier than 16:0-20:4). This order of elution differs from that seen on polar capillary GC where the combination of the polyunsaturated and saturated fatty acid is eluted earlier than the combination of two oligounsaturated fatty acids (*e.g.* 16:0-20:4 eluted ahead of 18:2-18:2). Comparable pairs and triplets were found to result from the combination of other polyunsaturated fatty acids with saturated and oli-

gounsaturated fatty acids within an equivalent carbon and double bond number, which were eluted in a similar order of increasing retention time with increasing number of saturated fatty chains in the molecule.

The present study also shows that chloride attachment NCI mass spectrometry can greatly improve the identification and particularly the quantitation of the molecular species of complex triacylglycerols and yields nearly correct proportional response for the pseudomolecular ion. The increased sensitivity of detection of the ions eliminates the need for overloading the HPLC column in order to obtain sufficient number of ions for molecular weight determination. This extra sensitivity is especially important when using the direct liquid inlet interface which can admit only 1% of the total column effluent to the mass spectrometer. We have established that the increased sensitivity obtained by chloride attachment NCI of triacylglycerols is about 100 fold greater than that seen in PCI for total ion current and many more-fold higher when compared to the molecular ion yield obtained from the short-chain saturated triacylglycerols [9]. The high sensitivity and the proportionally correct ionization response allows the detection of both major and minor triacylglycerol species in a reversed-phase LC–MS run. Knowledge of the diacylglycerol fragment ions obtained by PCI, which is less sensitive and yields variable amounts of the ions, however, is necessary for the identification, although this requirement greatly reduces the overall sensitivity of determination of the molecular species of the triacylglycerols. The detection of diacylglycerols containing polyunsaturated fatty acids remains a problem in those instances where the specific polyunsaturated ions are absent from the PCI mass spectrum. On the basis of the relative retention time and the presence of the complementary saturated diacylglycerol ions, however, it is usually possible to establish the identity of the main molecular species of triacylglycerols in the major HPLC peaks also from PCI spectra. The improved triacylglycerol profiles resulting from the chloride attachment NCI permit the determination of the molecular weight and the order of elution of even minor molecular species in a complex triacylglycerol mixture.

Finally, this study confirms the universal applicability and high resolving power of the propionitrile–acetonitrile gradient as the mobile phase in HPLC of neutral lipids. Earlier Schulte [20] had demonstrated excellent resolution of seed oil triacylglycerols with propionitrile alone, while acetonitrile has been generally recognized as an excellent solvent for neutral lipids [1]. The propionitrile–acetonitrile gradient is also capable of accommodating up to 10% dichloromethane without loss of peak resolution. It is concluded, however, that even this reversed-phase HPLC system in combination with both PCI and NCI mass spectrometry cannot completely resolve and identify all fish oil triacylglycerols and some prefractionation [21] is clearly necessary.

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