Analysis of fatty acid methyl esters by a gas–liquid chromatography–chemical ionization mass spectrometry computer system[1]

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Abstract The technique of gas–liquid chromatography–chemical ionization mass spectrometry can easily identify trace peaks and unresolved peaks on gas–liquid chromatography, utilizing MH^+ ions of chemical ionization mass spectra. In polyunsaturated fatty acid methyl esters such as C22:5 and C22:6, the determination of molecular weights that are difficult to determine by electron impact mass spectrometry could be easily identified by chemical ionization mass spectrometry. The identification could be performed even more easily from a mass chromatogram obtained by means of a gas–liquid chromatography–chemical ionization mass spectrometry–computer system. This technique was applied to analysis of fatty acid methyl esters of triglycerides, cholesteryl esters, nonesterified fatty acids, and phospholipids obtained from normal human serum. It was observed that odd-carbon-number fatty acids contained more isomers of different types than even-carbon-number fatty acids, whether the fatty acids were saturated or unsaturated. As for the types of isomers, we presume the existence of iso, anteiso, branched, cyclic fatty acids, and, in the case of unsaturated fatty acids, positional isomers. The qualitative determination of the group of fatty acid isomers containing 20 carbon atoms was also investigated as well as the application of procedures for quantitative determination. Sensitivities for saturated fatty acids differ from those for unsaturated fatty acids of the same carbon number, and sensitivities for unsaturated fatty acids of the same carbon number differ with the number of double bonds because the amounts of $MH^+ - 32$ and $MH^+ - 32 - 18$ fragment ions formed are different.

Supplementary key words triglycerides · cholesteryl esters · nonesterified fatty acids · phospholipid · protonated molecular ion · fragment ion

Though there are a wide variety of reports on the gas–liquid chromatography–electron impact mass spectrometry (GLC–EI–MS) of long chain fatty acid methyl esters, it is rather difficult to determine the molecular ions of small gas chromatographic peaks, especially an unresolved peak, or of polyunsaturated fatty acids (1, 2). Capillary gas–liquid chromatography has also been used for analysis of fatty acid methyl esters, but the results are not satisfactory (3–5). In contrast, chemical ionization mass spectrometry (CI–MS) (6–10) is useful for the determination of molecular ions of saturated and unsaturated fatty acid methyl esters, because a large part of the total ion current occurs in the pseudo-molecular ion.

Previous papers referred to structural analysis of long chain fatty acids (11), sterol esters (12), and triglycerides (13) by chemical ionization mass spectrometry using a direct probe inlet system. Preliminary reports about analysis of fatty acid methyl esters by gas–liquid chromatography–chemical ionization mass spectrometry (GLC–CI–MS) (14) and mass fragmentography using a multi-ion detector have also been presented (15). In the present study, an on-line computer was interfaced to the GLC–CI–MS, and fatty acid methyl esters of triglycerides, cholesteryl esters, phospholipids, and nonesterified fatty acids from normal human serum were analyzed.

MATERIALS AND METHODS

Materials

Methyl esters of palmitic acid, stearic acid, oleic acid, α-linoleic acid, γ-linolenic acid, arachidic acid, and docosahexaenoic acid were purchased from Applied Science Lab. Inc. (State College, PA). Normal serum was obtained from a 30-year-old healthy male.

Preparation of fatty acid methyl esters of triglycerides, cholesteryl esters, phospholipids, and nonesterified fatty acids from normal serum

Extraction of lipid was essentially done according to the procedure of Folch, Lees, and Sloane Stanley

Abbreviations: GLC–CI–MS, gas–liquid chromatography–chemical ionization mass spectrometry; GLC–EI–MS, gas–liquid chromatography–electron impact mass spectrometry; MH^+, protonated molecular ion.

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(16). To 3 ml of serum, 40 ml of chloroform–methanol 2:1 was added, and the total lipids were extracted by heating at 50–60°C in a water bath for 10 min. After cooling, the solution was filtered, 50 ml was added to 10 ml of 0.85% NaCl, and the solution was centrifuged at 2,000 rpm for 5 min. The chloroform-containing lower phase, which contained most of the total lipids, was fractionated on a silicic acid column (Mallinckrodt Inco., 100 mesh) using 15 vol. each of the following solvents: (I) *n*-hexane–diethyl ether 99:1, (II) *n*-hexane–diethyl ether 94:6, (III) *n*-hexane–diethyl ether 50:50, and (IV) methanol at a flow rate of 0.5 ml/min, under nitrogen pressure. The fractions were evaporated under reduced pressure in the presence of nitrogen, and the lipids were hydrolyzed with alcoholic potassium hydroxide (6 ml of 33% KOH + 94 ml of ethyl alcohol). Diazomethane was added to form methyl esters of fatty acids of triglycerides (I), cholesteryl esters (II), nonesterified fatty acids (III), and phospholipids (IV).

Gas–liquid chromatography–chemical ionization mass spectrometry computer

The equipment used was a Shimadzu LKB 9000 gas chromatograph–mass spectrometer system equipped with a chemical ionization source (Shimadzu Seisakusho Ltd.). The data processing system, the GCMS-PAC 300 DG (Shimadzu Seisakusho Ltd.) consisted of an OKITAC-4300C minicomputer with 12K core, a typewriter, an incremental plotter, a magnetic disc, and an interface for the gas chromatograph–mass spectrometer.

The gas–liquid chromatographic conditions were as follows. The 2m × 3mm (ID) glass column was packed with 15% diethylene glycol succinate polyester on Chromosorb W 80–100 mesh. The column temperature was programmed from 170 to 210°C at 5°/min. The flow rate of helium carrier gas was 30 ml/min. The chemical ionization–mass spectrometric conditions were as follows. The ion source temperature was held at 200°C. The mass spectra were obtained at 500 eV of electron energy, 3.5 kV of accelerating voltage, and 500 μA of emission current. Isobutane gas was used as the reagent gas at 0.9 torr in the ionization chamber. For the electron impact–mass spectrometry, conditions were 70 eV electron energy, 3.5 kV of accelerating voltage, and 60 μA of filament current.

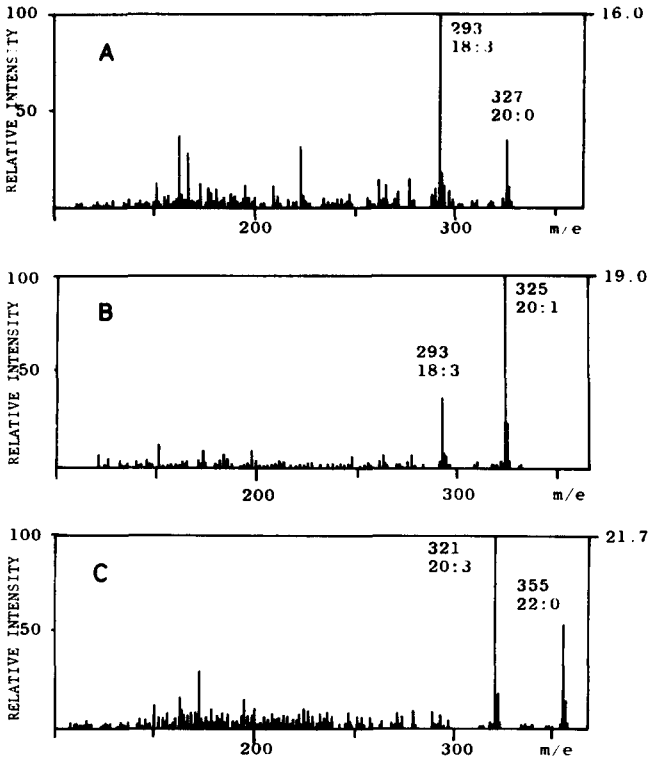

Fig. 1. Chemical ionization mass spectra of the fatty acid methyl esters of cholesteryl esters from normal serum; the methyl esters were not separated by gas–liquid chromatography.

RESULTS AND DISCUSSION

Chemical ionization mass spectrometry of fatty acid methyl esters

The protonated molecular ions, MH^+, of fatty acid methyl esters have strong intensities and, hence, their molecular weights are easy to determine from MH^+ ions, even when sample sizes are small and when they have a high degree of unsaturation. Methods using electron impact (EI) and chemical ionization (CI) mass spectra of fatty acid methyl esters of C22:5 and C22:6 were compared. It was clear that, because it provides only a little information on molecular ions, EI mass spectrometry is not suitable for structural studies of polyunsaturated fatty acid methyl esters. In CI–MS, in contrast, an MH^+ of mass 345 (amounting to 22.3% total ionization) was observed for C22:5 and an MH^+ of mass 343 (12.1% total ionization) was observed for C22:6. The $MH^+ - 32$ and $MH^+ - 32 - 18$ were recorded as major fragment ions. These fragment ions from C22:5 were recorded at *m/e* 313 and 295 and those from C22:6 at *m/e* 311 and 293. The other fragment ions were few and had low intensities. The applicability of mass spectrometry to analysis of fatty acids that were not completely resolved on packed columns was investigated; the methyl esters of fatty acids from fraction (II) were used. **Fig. 1** shows the mass spectra of three different GLC fractions. In the CI

mass spectrum in Fig. 1*A*, C18:3 and C20:0 can be identified from the MH^+ ions at *m/e* 293 and 327. In the spectrum in Fig. 1*B*, C18:3 and C20:1 can be identified. C20:1 gave a fragment ion of $MH^+ - 32$ at *m/e* 293 with an intensity of about 5% relative to the MH^+. In this mass spectrum, *m/e* 293 had an intensity of 41% compared with *m/e* 325, which suggests that the mass spectrum corresponds to a mixture of C20:1 and C18:3. In the spectrum in Fig. 1*C*, C20:3 and C22:0 can be identified from the ions at *m/e* 321 and 355.

Mass chromatography of fatty acid methyl esters of normal serum

Since the MH^+ ions of fatty acid methyl esters have strong intensities in IC–MS, the mass chromatogram of the MH^+ ions was used to determine fatty acid methyl esters from normal serum. The mass spectra were stored in a magnetic disc at 6-sec intervals and the mass chromatograms of desired *m/e* values were recorded. **Fig. 2** shows the mass chromatograms of methyl esters prepared from the nonesterified fatty acid fraction (III) presented in Fig. 1. Fig. 2*A* shows the mass chromatogram of the C18 and C19 groups; the mass chromatogram shows the relationship of retention times for C19:0, C18:1, and C19:1 and for C18:2 and C19:1, which were not separated under the gas–liquid chromatography conditions used. Fig. 2*B* shows the retention times of C22:2 and C21:0 and also of C18:3 which was included as a reference. Fig. 2*C* shows the relative retention times of C20:3, C22:0, and C20:4. Fig. 2*D* shows that γ-C18:3 and C18:3 overlap with C20:0 and C20:1, respectively. Fig. 2*D* is the mass chromatogram that corresponds to the mass spectra of Figs. 1*A* and *B*.

The mass chromatograms were used to evaluate mass spectral data in the analysis of fatty acid methyl esters of the 20-carbon molecules (**Fig. 3**). The methyl esters of fatty acids of C20:0, C20:1, C20:2, C20:3, C20:4, and C20:5 were all well identified in a mass range from *m/e* 327 to 317. Some methylated members of the 20-carbon molecule fatty acids did not produce abundant peaks on the total ion current, but they did produce readily detectable peaks in mass chromatograms. $MH^+ + 1$ and $MH^+ + 2$ ions may be produced from isotopic peaks and, in CI–MS, an $(M - H^+)$ ion may also be produced; however these ions can be easily identified from the retention times and by measuring the ratio of isotope ions. For example, a large quantity of C20:1 ($MH^+ = 325$) may also produce a peak in the ion current profile of *m/e* 327 ($MH^+ = 327$ for C20:0) due to the second isotope peak of C20:1; however, the fact that this small peak in the ion current profile at *m/e* 327 occurred at the retention time for C20:1 and not that for C20:0 assures proper identification. **Table 1** shows the type of fatty acids of triglycerides (I), cholesteryl esters (II), nonesterified fatty acids (III), and phospholipids (IV) as deduced from the mass chromatograms.

All fatty acids ranging from C10 to C23, including minor components and polyunsaturated fatty acids, were identified. Cholesteryl esters and triglycerides had the fewest types of fatty acids; C21-, C22-, C23-molecule groups were barely detected. Even-carbon-number fatty acids contained two types of saturated fatty acids, while odd-carbon-number fatty acids contained two to three types of saturated fatty acids. These data seem to indicate the existence of branched and cyclic fatty acids. there may be two to four types of isomers for unsaturated fatty acids with the same degree of unsaturation; they may be attributable to iso, anteiso, branched, and cyclic acids, or double bonds at different locations. For

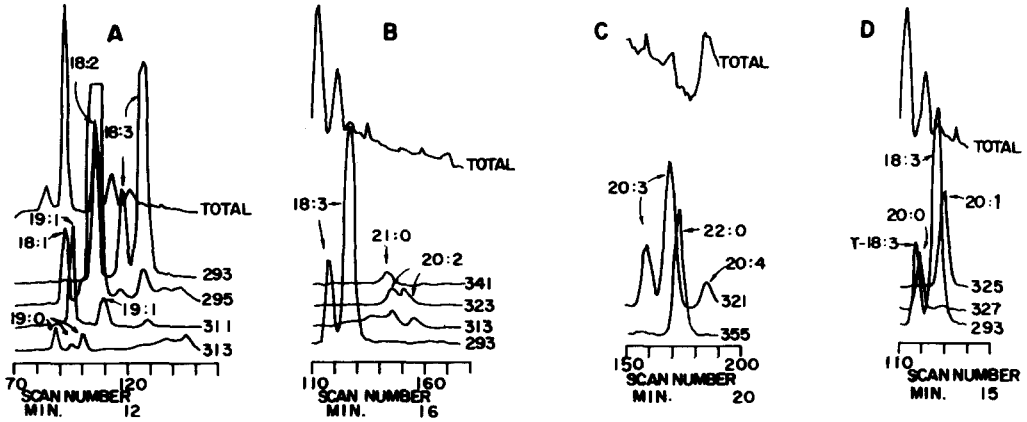

Fig. 2. Chemical ionization mass chromatograms of unresolved fatty acid methyl esters of the nonesterified fatty acid fraction of normal human serum.

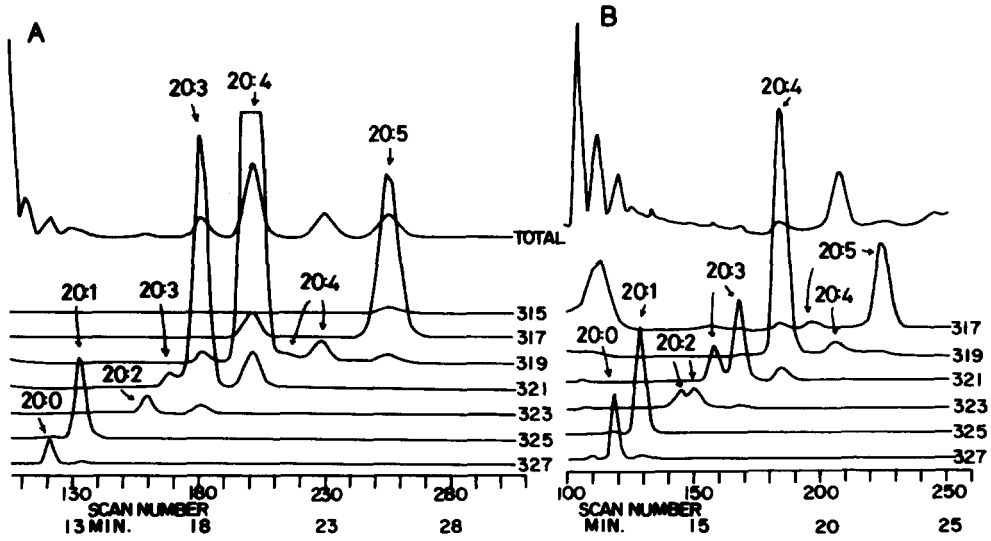

Fig. 3. Chemical ionization mass chromatograms of fatty acid methyl esters of 20-carbon fatty acids. *A*, phospholipid; *B*, nonesterified fatty acid. *m/e* 327, C20:0; *m/e* 325, C20:1; *m/e* 323, C20:2; *m/e* 321, C20:3; *m/e* 319, C20:4; *m/e* 317, C20:5.

the fatty acid methyl esters of the 18-carbon molecule group, the two C18:3 acids were identified, using standard samples, as γ-linolenic [18:3 (6, 9, 12)] and α-linolenic acid [18:3 (9, 12, 15)]. In the case of methyl esters of the phospholipid fraction (Fig. 3*A*), C20:4 gave three peaks at *m/e* 319 and C20:5 gave one peak at *m/e* 317; in the case of methyl esters of the nonesterified fatty acid (Fig. 3*B*), 20:4 and C20:5 each gave two peaks at *m/e* 319 and 317 respectively. In addition, the two peaks for 20:3 differed greatly in relative amounts. It must be noted that there was another peak after the main peak of C20:4. There was also a peak with nearly the same retention time on the total ion chromatogram. However the peak area of the total ion chromatogram was relatively larger and wider as compared with the

TABLE 1. Fatty acid methyl esters of phospholipids, nonesterified fatty acids, cholesterol esters, and triglycerides

Carbon Number of Fatty Acid	Phospholipid	Nonesterified Fatty Acid	Cholesteryl Ester	Triglyceride
10	0^1, 1^1, 2^1	0^1, 1^1, 2^1		
11	0^1, 0^2, 0^3, 1^1, 1^2, 1^3, 2^1, 2^2,	0^1, 0^2, 0^3, 1^1, 1^2, 2^1, 2^2		
12	0^1, 0^2, 1^1, 1^2, 2^1, 2^2,	0^1, 0^2, 1^1, 1^2, 2^1, 2^2,	0^1, 0^2, 1^1,	0^1, 0^2, 1^1,
13	0^1, 0^2, 0^3, 0^4, 0^5, 1^1, 1^2, 1^3,	0^1, 0^2, 0^3, 0^4, 1^1, 1^2, 1^3, 1^4	0^1, 0^2, 0^3, 1^1, 1^2, 2^1	0^1, 0^2, 1^1,
14	0^1, 0^2, 1^1	0^1, 0^2, 1^1, 1^2, 2^1, 2^2	0^1, 0^2, 1^1, 1^2	0^1, 0^2, 1^1
15	0^1, 0^2, 1^1, 1^2	0^1, 0^2, 0^3, 1^1, 2^2	0^1, 0^2, 0^3, 1^1, 1^2	0^1, 0^2, 0^3, 1^1, 1^2,1^3
16	0^1, 0^2, 1^1	0^1, 0^2, 1^1, 2^1, 2^2	0^1, 0^2, 1^1	0^1, 0^2, 1^1
17	0^1, 0^2, 0^3, 1^1, 1^2, 1^3, 1^4	0^1, 0^2, 0^3, 0^4, 1^1, 1^2, 1^3, 1^4	0^1, 0^2, 0^3, 1^1, 1^2	0^1, 0^2, 1^1, 1^2
18	0^1, 0^2, 1^1, 2^1, 3^1	0^1, 0^2, 1^1, 2^1, 3^1, 3^2	0^1, 0^2, 1^1, 2^1, 3^1, 3^2	0^1, 0^2, 1^1, 2^1, 3^1, 3^2
19	0^1, 0^2, 0^3, 1^1, 1^2, 1^3	0^1, 0^2, 0^3, 1^1, 1^2, 1^3, 2^1	0^1, 0^2, 0^3, 1^1, 1^2	0^1, 0^2, 1^3, 1^4, 3^1
20	0^1, 0^2, 1^1, 1^2, 2^1, 2^2, 3^1, 3^2, 4^1, 4^2, 5^1	0^1, 0^2, 1^1, 1^2, 2^1, 2^2, 3^1, 3^2 4^1, 4^2, 5^1, 5^2	0^1, 0^2, 1^1, 1^2, 2^1, 2^2 3^1, 3^2, 4^1, 4^2, 5^1	0^1, 0^2, 1^1, 2^1
21	0^1	0^1, 1^1		
22	0^1, 0^2, 1^1, 5^1, 5^2, 6^1	0^1, 1^1		
23	0^1			

Each numeral indicates the number of double bonds and its superscript indicates the number of isomers having the given number of double bonds.

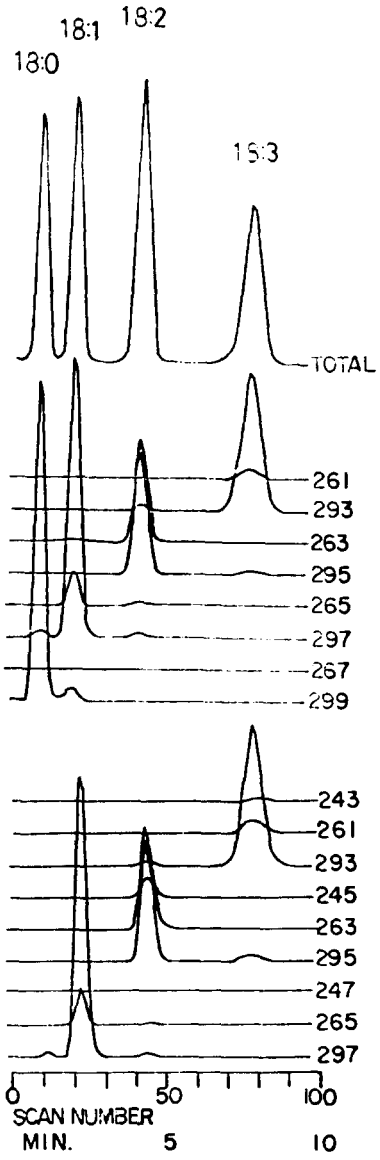

Fig. 4. Chemical ionization mass chromatograms of C18:0, C18:1, C18:2, and C18:3 mixture (0.5 μg each). Relationships between 18-carbon molecules and the *m/e* of the fragment ions are as follows.

18-carbon molecule	MH^+	$MH^+ - 32$	$MH^+ - 32 - 18$
C18:0	299	267	249
C18:1	297	265	247
C18:2	295	263	245
C18:3	293	261	243

peak of the mass chromatogram at *m/e* 319. This is because the retention times are not exactly the same; the peak of the total ion chromatogram includes a minor peak of C20:4.

Quantitative determination

I have also investigated the possibility of quantitative determination using mass chromatograms. The total ion chromatogram of samples of a mixture containing 0.5 μg each of C18:0, C18:1, C18:2, and C18:3 was examined. The sensitivities of total ion current were almost equal. The ratio of the peak areas was 1.00:1.03:1.01:1.05. In the mass chromatogram (**Fig. 4**) the peak area ratios for C18:0 at *m/e* 299, C18:1 at *m/e* 297, C18:2 at *m/e* 295, and C18:3 at *m/e* 293 were 1.00:0.93:0.50:0.73; they were remarkably different from the ratio of the peak areas of the total ion chromatogram. The difference is easily understood from the peak areas of the mass chromatogram for $MH^+ - 32 - 18$ of each fatty acid (see legend to Fig. 4).

The intensity of the ion for $MH^+ - 32$ at *m/e* 267 was weak, and the *m/e* 249 ion for $MH^+ - 32 - 18$ was barely detected in the case of C18:0. The intensities of $MH^+ - 32$ and $MH^+ - 32 - 18$ of C18:1 ions were a little higher compared with C18:0. C18:2, recorded at *m/e* 295, was about half as intense as C18:0 and C18:1. This is due to the fact that the *m/e* 263 ion had an intensity of about 80% and *m/e* 245 ion of about 5%, relative to the MH^+ ion. C18:3 was more intense than C18:2; 18:3 gave smaller peaks in the mass chromatogram at *m/e* 261 and 243 compared with the fragment ions of C18:2.

In the case of the 16-carbon molecules, C16:0 gave minor fragment ions of $MH^+ - 32$ and $MH^+ - 32 - 18$ at *m/e* 239 and 221. C16:1 gave MH^+ ion and $MH^+ - 32$ ion at a ratio of 1.00:0.13 which was exactly the same as that given by C18:1.

We used the mass chromatograms for quantitative analysis of methyl esters of the 20-carbon fatty acids of the nonesterified fatty acid fraction (**Fig. 5**). It was observed that C20:0 and C20:1 barely gave $MH^+ - 32$ ions at *m/e* 295 and 293, and also $MH^+ - 32 - 18$ ions at *m/e* 279 and 277, respectively. C20:2 as well as C18:2, gave considerable $MH^+ - 32$ ion at *m/e* 293 and intensities of MH^+ and $MH^+ - 32$ ions were nearly equal. C20:3, as well as C18:3, gave smaller amounts of $MH^+ - 32$ and $MH^+ - 32 - 18$ ions than did C20:2; C20:4 and C20:5 gave MH^+ and $MH^+ - 32$ ions at ratios of 1.00:0.12 and 1.00:0.15, respectively. It may be concluded that in mass chromatograms C20:4 and C20:5 are detected more easily than C20:2. It was observed also that fatty acids with two double bonds, such as C18:2 and C20:2, give more $MH^+ - 32$ and $MH^+ - 32 - 18$ ions.

In quantitation based on peak height or peak area of a mass chromatogram for MH^+ ion, it must be remembered that components of a same carbon number are detected at different sensitivities depending on the number of double bonds in the molecule.

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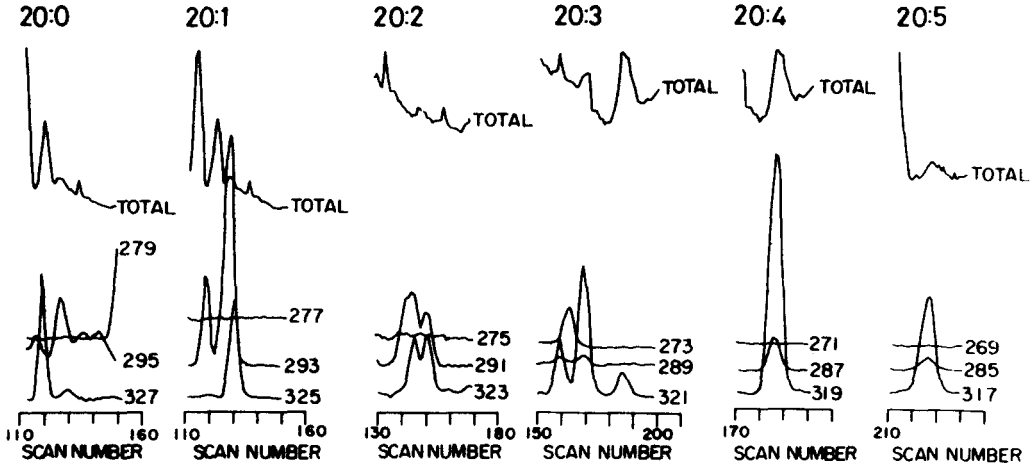

Fig. 5. Chemical ionization mass chromatograms of 20-carbon fatty acid methyl esters of the non-esterified fatty acid fraction.

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