ISOLATION AND ANALYSIS OF TRIENOIC AND TETRAENOIC FATTY ACIDS WITH COMPLEMENTARY THIN LAYER AND GAS–LIQUID CHROMATOGRAPHY

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In an earlier report a complementary thin layer and gas–liquid chromatographic procedure was described for the analysis of fatty acid methyl esters[1]. Unsaturates were separated as their mercury adducts by thin-layer chromatography (TLC) into groups according to number of double bonds. Members within each unsaturation class were subsequently analyzed by gas–liquid chromatography (GLC). Synthetic fatty acid mixtures containing mostly saturates and monoenes were evaluated using this approach.

In the present report the procedure has been improved and used to separate the polyunsaturated components of a much more complicated mixture; in particular, the trienoic and tetraenoic fatty acids of menhaden fish oil. The experiments point out the value and capability of the complementary approach in analyzing complex mixtures at the submilligram level.

EXPERIMENTAL

Sample preparation

Fatty acid methyl esters from menhaden fish oil were obtained through a base-catalyzed interesterification procedure at room temperature[2]. These were separated from contaminating matter by TLC[3] using the developing solvent *n*-hexane–diethyl ether–acetic acid (90:10:1, v/v) and recovered by diethyl ether extraction of the appropriate thin-layer band scraped off the chromatoplate. Samples not used immediately were sealed *in vacuo*.

The plasma cholesteryl ester fraction was isolated by TLC using the developing solvent mentioned above. This fraction was converted to fatty acid methyl ester and free sterol by refluxing the scraped-off silica gel band in sulfuric acid–methanol (6:94, v/v, solutions cooled in ice before mixing) at 80° for 6 h. The esters were purified by TLC as above on plates that had been previously washed with a highly polar cleaning solvent to remove contaminating matter[4].

The high quality menhaden oil prepared from the whole fish was generously supplied by Marine Chemurgics, Inc. (Morehead City, N.C.). Lipid standards came from the Hormel Institute, Lipids Preparation Laboratory, Austin, Minn.

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Adduct preparation and chromatography

The methoxy, bromomercuri-adducts prepared in the manner as described previously[1] were dissolved in chloroform and washed with water several times until the solution became clear. Occasionally, when particulate matter could not be completely removed from the chloroform layer by washing, it was necessary to filter the solution through a short column of anhydrous sodium sulfate.

Separation of mercury adducts by TLC and visualization of the unsaturated classes were done as stated earlier[1]. For adduct decomposition 5 ml HCl–methanol (1:4, v/v, constituents cooled separately in ice bath and mixed while cold) was added to scraped-off silica gel bands containing the organomercurials. Recovery of unsaturates was completed in the manner given previously[1].

All solvents were checked for volatile contaminants by evaporation of a 100 ml aliquot and GLC of the residue under the same conditions used for analysis of the fatty acids.

To remove small amounts of one unsaturation class present in another the complete fractionation procedure (derivatization, separation by TLC, and unsaturate regeneration) was repeated twice. The final product was purified by TLC as above prior to analysis by GLC[1].

RESULTS

Isolation of pure trienoic and tetraenoic fatty acid groups

A standard mixture (500 μg) containing approximately equal amounts of methyl-9,12,15-octadecatrienoate ($>$ 99 %) and methyl eicosatetraenoate ($>$ 90 % arachidonate), the latter standard also containing methyl eicosatrienoate and eicosapentaenoate, was submitted to repeated derivatization and resolution into unsaturated classes by TLC. Completeness of the triene, tetraene fractionation was determined by GLC.

In Fig. 1 can be seen that following the first fractionation (A) a small proportion (3.0 %) of tetraenoic acid (peak 3) remained in the trienoic acids (peaks 1 and 2). Two other components (peaks 4 and 5), presumably arising during the experimental handling of the sample, were also present. The tetraenoic acids in turn were contaminated with 3.4 % of trienoic acid.

In the second fractionation (B) the fatty acid impurities in each unsaturation class were lowered to approximately 0.5 %. To remove them entirely required a third fractionation (C). The final purification (D) by TLC removed the non-fatty acid constituents and left the pure trienoic and tetraenoic groups.

Although the recovery of ester in the two unsaturation groups averaged approximately 82% after each derivatization and recovery step, giving a final yield of only about 50 % of the starting material, proportions of the C_{18} and C_{20} acids in the trienoic group varied only slightly (Table I).

The C_{20} tetraenoic component had two positional isomers which were partially resolved on the gas chromatographic column. Measurement of the peak height ratios of the minor to the major isomer gave values of 0.107, 0.122, 0.114 and 0.115 for the successive purification steps (Fig. 1) showing that the experimental procedure did not lead to alteration in isomer proportions.

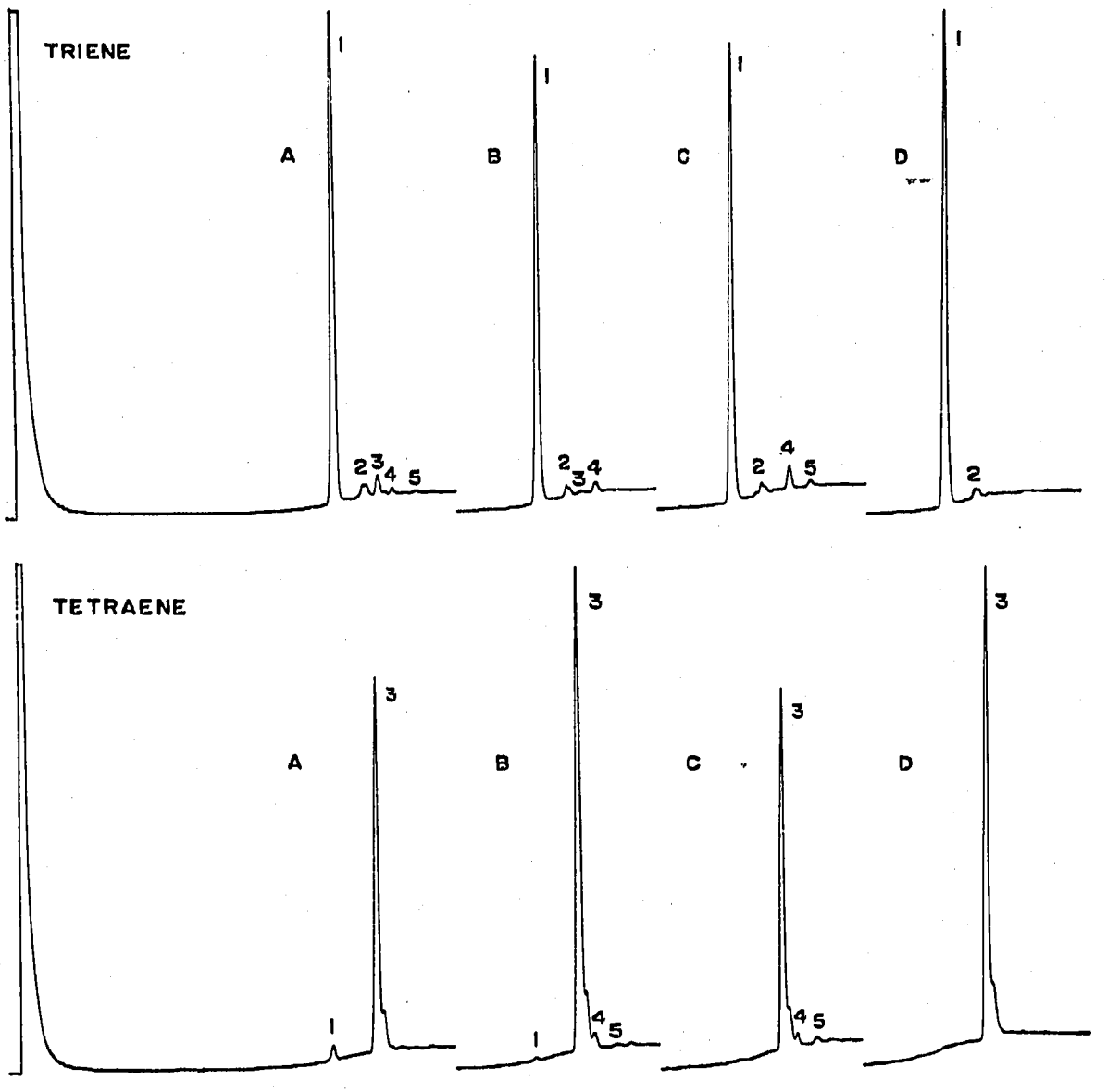

Fig. 1. Photograph of composite gas chromatograms showing progress of the separation of a synthetic mixture of fatty acid methyl esters into trienoic and tetraenoic groups. Octadecatrienoic (1), eicosatrienoic (2), and eicosatetraenoic (3) acids, as the methyl esters, were analyzed following the first (A), second (B), and third (C) fractionation of the mercury adducts by TLC. The final purification step (D) by TLC removed non-fatty acid impurities (4 and 5).

Fractionation of natural mixtures

Analysis of the trienoic and tetraenoic acids in menhaden oil and the cholesteryl ester fraction of human blood plasma can be seen in Fig. 2. The striking difference in complexity of the two samples is readily apparent.

Menhaden oil contained a minimum of 17 trienoic acids ranging in chain length from 12 to 24 carbon atoms. The efficiency of the gas chromatographic column used was not sufficient to resolve completely the C_{18} and C_{20} members. At least four C_{18} trienoic acids were present. Data in Table I show the degree of variation in proportion of several major component acids during the repetitive purification procedure.

TABLE I

COMPARISON OF FATTY ACID PROPORTIONS DURING REPETITIVE PURIFICATION PROCEDURE TO ISOLATE UNSATURATED CLASSES[a]

Component	*Purification steps*[b]		
	1	*2*	*3*
Synthetic mixture			
18:3	1.00	1.00	1.00
20:3	0.046	0.045	0.043
Menhaden oil			
16:3	4.65	4.43	3.86
18:3	1.00	1.00	1.00
18:3	4.23	4.23	4.35
20:3	0.49	0.49	0.43
20:3	0.72	0.60	0.60
18:4	1.00	1.00	1.00
20:4	0.78	0.82	0.83
20:4	1.19	1.25	1.35

[a] Peak area ratios calculated from gas chromatograms are shown.

[b] Each step included derivatization of unsaturated components, separation by TLC, regeneration of unsaturates, and analysis of product by GLC.

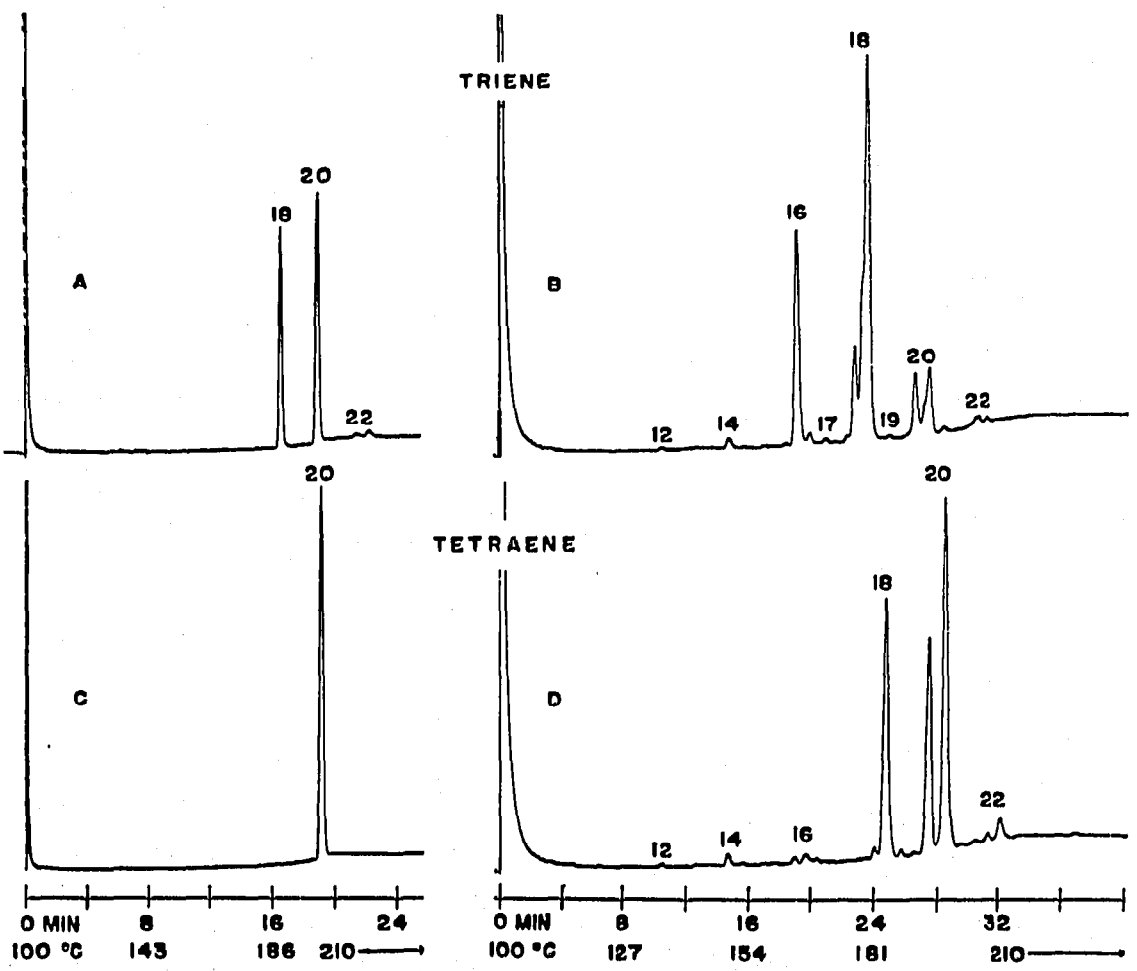

Fig. 2. Photograph of composite gas chromatograms showing trienoic and tetraenoic members in two mixtures: fatty acid methyl esters derived from blood plasma cholesteryl ester (A and C) and menhaden oil (B and D). Chain length denoted by number at peak apex.

Hydrogenation of the trienoic mixture and rechromatography revealed the following chain length distribution: C_{12}, trace; C_{14}, 2.2 %; C_{15}, 0.5 %, C_{16}, 27.3 %; C_{17}, 1.0 %; C_{18}, 50.7 %; C_{19}, 0.6 %; C_{20}, 15.6 %; C_{22}, 1.5 %; C_{24}, 0.6 % (Fig. 3). No branched chain acids were present.

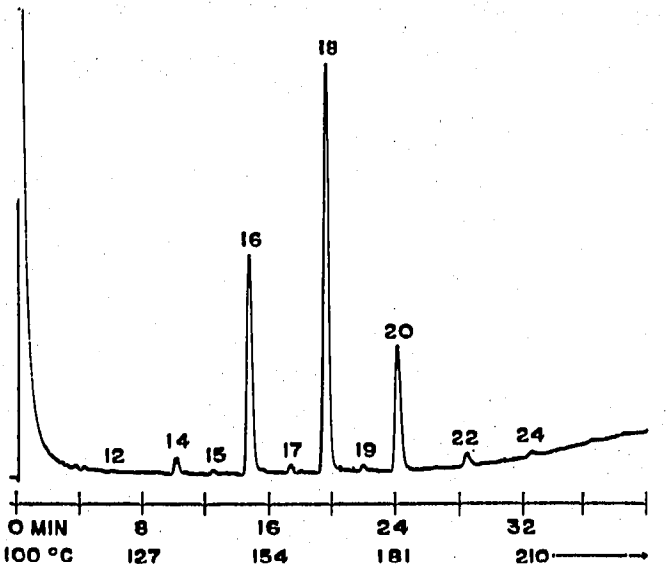

Fig. 3. Gas chromatogram of the hydrogenated trienoic fatty acids isolated from menhaden oil. Chain length denoted by number at peak apex.

There were at least 15 tetraenoic acids in menhaden oil containing 12 to 24 carbon atoms (Fig. 2). One C_{18} and two C_{20} acids constituted 29.1 % and 63.1 % of the mixture, respectively.

Assuming that double bonds in the chain occurred only in divinyl methane rhythm[5], the C_{12} tetraene would necessarily have a double bond in conjugation with the carboxyl group. Reaction yields in adduct synthesis had previously been determined only for acids with non-conjugated double bonds[1]. Accordingly, a test was needed of the ability to derivatize an unsaturate with a structure in the carboxyl group region corresponding to that suggested for the C_{12} menhaden acid. Methyl crotonate was used and found to react with mercuric acetate in 81.5 % yield.

In the cholesteryl ester sample trienoic and tetraenoic acids smaller than C_{18} were absent (Fig. 2). Much in contrast to the results with menhaden oil, only single C_{18} and C_{20} peaks were observed in the plasma fraction triene. A C_{20} component, probably arachidonic acid, was the lone tetraene present.

DISCUSSION

The methods described in this report have been shown to be capable of isolating in high purity trienoic and tetraenoic acids at the μg level from a synthetic mixture and several natural mixtures. These methods are also applicable to the isolation of fatty acids with other degrees of unsaturation.

Purity of the isolated fractions when tested by GLC was distinctly improved by repetition of the derivatization and resolution by TLC procedure. In earlier work[1] a difficulty in interpretation of results was the trace contamination of one unsaturation

class in another. As demonstrated in Fig. 1 after the third fractionation of the mercury adducts by TLC the separation into trienoic and tetraenoic groups was complete. At this point one was sure that all peaks on the gas chromatogram, no matter how small, represented fatty acids with the same number of double bonds.

The agreement in percentage of individual components within a particular unsaturation group following the various stages of purification can be seen in Table I. In general no preferential loss within the longer chain fatty acids (C_{18} and above) was observed. However, there was a tendency for shorter chain members to decrease in proportion during the purification steps because of the difficulty in quantitatively retaining these acids when solvents were removed in the experimental manipulations.

These experiments are yet another example of how greatly simplified and accelerated the analysis of complex fatty acid mixtures has become through the use of TLC[6]. In earlier work, before the rapid development of TLC, procedures often employed in determining fatty acids according to chain length and degree of unsaturation were much more complicated and time-consuming; for example, fractional crystallization, repeated fractional distillation, column chromatography, and counter current distribution. Moreover, these older methods required the use of larger amounts of material than does TLC; for example, g rather than μg samples.

Detection of the complicated array of trienoic and tetraenoic fatty acids in menhaden fish oil (Fig. 2) demonstrates the value of prefractionation in unmasking compound peaks on gas–liquid chromatograms. Although difficulties caused by chain length overlap may be reduced by using liquid phases other than DEGS[7] and gas chromatographic conditions of greater resolution ability, for detecting all components in complex oils like menhaden the necessity of subfractionating the mixture prior to the final analysis by GLC is clear. A total of 11 trienoic and tetraenoic fatty acids, all even-numbered with chain lengths C_{16}–C_{20}, have previously been described in menhaden body oil[5,8–10]. Polyunsaturated components less than 0.1% of the aggregate were not specified. On using the complementary TLC, GLC procedure described here, we found an extended chain length range and the presence of polyunsaturated odd-numbered acids (Fig. 2). Many minor component acids became evident. Calculations revealed that the level of 17:3, which is used as an example of a component near the lowest level measured, was 0.01% of the total fatty acids in menhaden oil.

Another procedure for isolating into groups fatty acids with a common number of double bonds is separation on thin layers of silicic acid impregnated with silver nitrate[11]. This approach, particularly useful in triglyceride analysis[12,13], does not require unsaturate derivatization. A consideration of the two TLC systems reveals that double bond position and geometrical configuration as well as chain length of the molecule influence R_F values more in silver nitrate-TLC[13] than in TLC of mercury adducts using the heptane–dioxane developing solvent[1]. In the limited amount of data available for comparison the purity of unsaturate class fractions separated and repurified by silver nitrate-TLC[14] is less than that demonstrated with the mercury adduct procedure described here.

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SUMMARY

A recently described procedure for the analysis of fatty acid methyl esters through the complementary use of thin layer and gas–liquid chromatography has been further investigated. Conditions necessary for the complete removal of one unsaturation class from another by TLC of the mercury adducts have been worked out.

The improved methodology has been applied to a study of the trienoic and tetraenoic acids of menhaden oil which were shown to be considerably more complex than was heretofore realized. At least 17 trienoic acids were found, including odd-numbered members, containing from 12 to 24 carbon atoms. A minimum of 15 tetraenoic acids was detected, extending over a chain length range similar to that of the trienoic acids, with C_{18} and C_{20} acids prominent.

Analysis of the cholesteryl ester fraction from human blood plasma revealed a much less complicated group of acids.

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