Effect of α -Tocopherol Deficiency on Carp—III. Analytical Method for Triglyceride Composition of Fish Lipid

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Quantitative and qualitative gas chromatography of triglycerides has been extended to fish lipids containing both odd and even carbon number fatty acids. A 50 cm short glass column containing 2% OV-17 on 80/100 mesh Shimalite W gave good resolution of triglyceride peaks at a higher temperature, 280°–340°C. Qualitative analysis was found to be possible by using calibration factors determined by the internal normalization technique. The triglycerides of two fish oils (carp and rainbow trout), soybean oil and cod liver oil were analysed by this method. This method was also useful for determining the triglyceride compositions of other fish oils.

Although recently the triglycerides of animal lipids have been studied extensively by direct gas chromatographic analysis¹⁻⁵⁾, only a few reports are available for the composition of fish oil^{6,7)}. We, at first, tried to establish an analytical method for fish lipids in order to determine the triglyceride composition of α -tocopherol deficient fish. This paper describes a method for the quantitative and qualitative analyses of fish triglycerides by gas chromatography.

Experimental

Materials Tricaprin, trilaurin, trimyristin, tripalmitin, trimargarin, tristearin, and triarachidin calibration standards of greater than 99% purity were purchased from Applied Science Laboratories, Inc. Sampling origins of carp and rainbow trout will be described in the following paper (–IV). Soybean oil and cod liver oil were obtained from a commercial source. Extraction of fats from fish was carried out using a chloroform-methanol (2:1) mixture as described by FOLCH *et al.*⁸⁾

Preparation of Triglycerides In the case of plant oil, the triglycerides can be analysed by direct injection of the oils, but since fish oils usually contain various kinds of lipids such as hydrocarbons, sterol esters, free fatty acids, sterols, and polar lipids other than triglycerides, a preliminary separation by means of preparative thin-layer chromatography is necessary prior to gas chromatographic analysis. The triglycerides were isolated from

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each of the fats with preparative thin-layer chromatography on 0.3 mm thick plates coated with silica gel (Wako Gel B-5) which had been activated for 1 hr at 120°C. The plates were run using petroleum ether: diethyl ether: acetic acid (75: 15: 1)⁹⁾ and developed by charring with sulfuric acid. The triglyceride fraction was the major neutral lipid component in all the crude fat samples. The triglyceride band was extracted with chloroform-methanol (2: 1), and the extract analysed by gas chromatography.

Gas Chromatography A Shimadzu Seisakusho Model GC-5APF gas chromatograph equipped with a hydrogen flame ionization detector was used. A glass column, 50 cm in length $\times$ 3 mm i.d., was packed with 2% OV-17 (phenyl methyl silicone) supported on 80/100 mesh silanized Shimalite W. The flow rate of nitrogen carrier gas was 80 ml/min. For triglyceride analyses, the column temperature was programmed from 200° to 340°C at 4°C/min. Detector temperature and injection port temperature were 350°C. Optimum operating conditions are listed in Fig. 1. Triglyceride peaks were identified by co-chromatography with known standards. Peak areas were determined by triangulation.

Fig. 1. Gas chromatogram of synthetic mixture of standard triglycerides.

Operating conditions: Shimadzu Seisakusho Model GC-5APF gas chromatograph with a hydrogen flame ionization detector; 50 cm $\times$ 3 mm i.d. glass column packed with 2% OV-17 (phenyl methyl silicone) on 80/100 mesh silanized Shimalite W; column programmed 200–340°C at 4°C/min with 80 ml/min nitrogen carrier gas; detector and injection port temperatures at 350°C.

Results and Discussion

The chromatogram of authentic standards of triglycerides is shown in Fig. 1. The resulting chromatogram showed good peak resolution eluting in regular order of low molecular weight components in such a short column irrespective of the degree of unsaturation of fatty acids. The figures on the peaks show the total carbon numbers of triglyceride composing fatty acids. This means that in the case of tristearin the total carbon number become 54. The first step in the qualitative analysis of triglycerides was to determine the linearity of retention time vs. carbon number to variations in sample

size. As shown in Fig. 2, the retention times were found to be proportional to the amount of sample injected, and correlation curves were linear in the 1–10 μg range with respect to sample size. This phenomenon usually occurs when a short column is used along with low concentration liquid phase such as 2% OV-17. However, it was found that the

Fig. 2. Relationship between retention time and carbon number vs. amount of triglyceride injected.

retention times become constant and irrespective of the amount of sample injected when they were determined based on the distances from starting point of solvent peak to standing points of each sample peak on the chromatogram. Thus, total carbon number of unknown triglycerides can be easily found by their respective retention times using this fact, however, triglycerides composed of unsaturated and saturated fatty acid mixture cannot be separated by the type of short column used in this experiment.

The chromatograms of carp and rainbow trout triglycerides analysed by this method are shown in Figs. 3 and 4, respectively. Carp oil contained C-even triglycerides from

Fig. 3. Gas chromatogram of triglycerides in carp oil. Operating conditions as for Fig. 1.

Fig. 4. Gas chromatogram of triglycerides in rainbow trout oil. Operating conditions as for Fig. 1.

C-42 to C-58, while rainbow trout oil contained the C-43 to C-60 series with small amounts of C-odd triglycerides. In both species the dominant components are C-50 to C-56 triglycerides. The distribution patterns of triglyceride peaks of soybean oil and cod liver oil are presented in Figs. 5 and 6. Soybean oil consisted of C-50, C-52 and C-54 triglycerides. On the other hand, the triglyceride pattern of cod liver oil was largely different from that of soybean oil as shown in Fig. 6. Cod liver oil contained various kinds of triglycerides, and furthermore, unknown peaks a-f were detected on the chromatogram. Each retention time of these unidentified peaks a-f obtained between C-even triglycerides were found to be 0.5 units longer than those of the corresponding C-even triglycerides, respectively. This probably indicates the occurrence of C-odd triglycerides⁷⁾

Fig. 5. Gas chromatogram of triglycerides in soybean oil.
Operating conditions as for Fig. 1.

Fig. 6. Gas chromatogram of triglycerides in cod liver oil.
Operating conditions as for Fig. 1.

or some kinds of mixed triglycerides judging from the gas chromatographic fact that given two kinds of triglycerides (A) $\text{CH}_2\text{-OCOR}_n$ and (B) $\text{CH}_2\text{-OCOR}_x$,

and if $R_n + R_n + R_n = R_x + R_y + R_z$ in total carbon numbers, the retention time of triglyceride (B) becomes longer than that of triglyceride (A). For instance, the retention time of a simple triglyceride such as tristearin is shorter than that of a mixed triglyceride composed of three different fatty acids, palmitic, stearic, and arachidic acid.

In the quantitative analysis of high molecular weight triglycerides the calibration factors, f_w values (weight calibration factors determined by the internal normalization

technique²⁾) of the standard triglycerides were calculated and listed in Fig. 7. The calibration data in Fig. 7 are in agreement with those reported by KUKSIS¹⁾ and LITCHFIELD *et al.*⁷⁾ and indicate that significant amounts of C-54 and higher triglycerides are lost during analysis. This has been accounted for due to the occurrence of loss of some

Fig. 7. Calibration factors determined by internal normalization technique.

● C 30—0.91	C 40—1.03	C 50—0.87
C 32—0.95	● C 42—1.03	● C 51—0.85
C 34—0.98	C 44—1.01	C 52—0.83
● C 36—1.00	C 46—0.97	● C 54—0.76
C 38—1.02	● C 48—0.93	C 56—0.70

triglycerides having molecular weight above C-42, even under optimum operating conditions.⁷⁾ Sample losses should probably occur in the flash heater or on the column. Flash heater losses could be caused by incomplete vaporization or pyrolysis⁷⁾. Losses on the column could be attributable to irreversible adsorption, condensation, or pyrolysis at the high operating temperatures.

The quantitative data for carp and rainbow trout triglycerides determined by using these calibration factors are listed in Table 1.

In this experiment it was found that to separate triglycerides by gas chromatography, a short column, from 35 to 75 cm in length should be used and operated at a higher temperature, 280°–340°C. Triglycerides having the same number of carbon atoms show the same retention times in such a short column irrespective of the degree of unsaturation of the fatty acids. Identification of triglyceride peaks on the chromatogram was found to be effected through correlation curves between the numbers of carbon atoms and retention times. The quantitative analysis was also found to be possible by using calibration

Table 1. Triglyceride composition of carp and rainbow trout.

Carbon number	Carp (%)	Rainbow trout (%)
C 42	0.45	—
C 43	—	0.47
C 44	0.31	—
C 45	0.31	0.49
C 46	0.16	0.64
C 47	0.16	—
C 48	2.03	3.45
C 49	0.65	—
C 50	12.07	24.91
C 52	35.65	28.26
C 54	39.44	25.64
C 56	7.13	10.68
C 58	1.56	4.16
C 60	—	1.31

factors. Thus, the gas chromatographic method examined in this experiment is found to be available for both the qualitative and quantitative analysis of fish triglycerides.

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