FATTY ACID COMPOSITION OF ESTERS IN FISH LIVER I. SEPARATION OF FATTY ACID OF VITAMIN A_1 ESTER

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I. SEPARATION OF FATTY ACID OF VITAMIN A_1 ESTER

By

Masahiro HATA and Mitsuo HATA

Department of Fisheries, Faculty of Agriculture, Tohoku University, Sendai, Japan

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It is very difficult to isolate the vitamin A_1 esters from liver oil by the use of ordinary methods such as alumina column chromatography or thin layer chromatography. In these methods, the vitamin A_1 esters fraction obtained contains the cholesterol esters and wax esters (straight chain higher fatty alcohol esters). Table 1 gives the composition of unsaponifiable matters of vitamin A_1 esters fraction obtained by alumina column chromatography.

Table 1. Composition of unsaponifiable matters of vitamin A_1 esters fraction after alumina column chromatography.

Fish liver oil	Cholesterol	Vitain A_1	Straight chain fatty alcohol
Eel (Anguilla japonica)	57.2 %	17.0 %	25.8 %
Carp (Cyprinus carpio)	45.3	0.2	54.5
Rainbow trout (Salmo gairdnerii)	20.0	4.3*	75.7
Bonito (Katsuwonus pelamis)	3.1	24.1	72.8

Cholesterol: Lieberman-Burchard reaction (1)
Vitamin A: Spectrophotometry
Straight chain fatty alcohol: 100%−(Cholesterol%+Vitamin A%)
*: Vitamin A_2 contained.

There are few papers with regard to the ditailed fatty acid composition of natural vitamin A_1 esters. Futterman and Andrews (2) isolated the retinal vitamin A_1 esters of five mammals and trout by alumina column chromatography and silica gel thin layer chromatography, and analyzed the fatty acids by gas-liquid chromatography. Their method, however, is only useful in the presence of small amounts of wax esters because the separation of vitamin A_1 esters from wax esters dose not complete.

Kinumaki et al (3) analyzed the fatty acids of vitamin A_1 esters of fish liver oils by means of dehydration reaction. Vitamin A_1 esters were separated into an-

hydrovitamin A_1 and free fatty acids in benzen solution, in the presence of acethyl chloride as catalyst. Then, fatty acids were analyzed by gas-liquid chromatography. This is very good method, but not available to the samples containing small amounts of vitamin A_1 esters.

The authors investigated the use of dehydration reaction of vitamin A_1 esters and found that p-toluenesulphonic acid was very suitable as catalyst. With the use of this catalyst, it was possible to analyze the fatty acids of small amounts of vitamin A_1 esters quantitatively in the presence of cholesterol and wax esters.

Experimental

Materials and Procedures

Reagents Vitamin A_1 plamitate was obtained from Riken Vitamin Co. Ltd..+ Vitamin A_1 esters mixture was prepared from fatty acid methyl ester mixture and vitamin A_1 acetate, and vitamin A_1 palmitate was added. These vitamin A_1 esters were purified by alumina column chromatography before use. Cholesterol palmitate was obtained from Tokyo Kasei Co. Ltd..++ Wax esters were prepared from sperm whale blubber oil by fractional precipitation in acetone. All solvents used were obtained from commercial sources and purified by distillation.

Separation of fatty acid Vitamin A_1 esters were dissolved in benzen, and small amounts of crystalline p-toluenesulphonic acid were added, and then heated on a water bath while sometimes shaking. After a definite time, added water and cooled the solution to stop the reaction, then washed the solution with water to remove the catalyst until the aqueous layer showed no colour change with methyl red. Fatty acids were prepared by the method of Kinumaki et al (3) or by extracting with a 2% KOH solution from the reaction mixture directly. Fatty acids were esterified to methyl esters with diazomethane, and analyzed by gas-liquid chromatography.

Gas-liquid chromatography Gas-liquid chromatography conditions were as follows: apparatus, Shimadzu GC-2B type (HFD detector); stationary phase, 10% DEGS on Diasolid L prepared by Nihon Chromato Kogyo Co. Ltd.+++; column, 0.4 mm $\times$3 m; column temp., 190°C; carrier gas, N_2 15 ml/min (2 atm).

Results and Discussion

The formation of anhydrovitamin A_1 and free fatty acids were observed by absorption spectrum, thin layer chromatography and fluoressence under U.V. light. Fig. 1 shows the difference spectra (E_{8min}–E_{0min}) of reaction mixtures. The maximum wave length 377 mμ in benzen, and the difference spectra were the same with that of the previous paper (4).

+ Riken Vitamin Co. Ltd.. (Wakagi, Itabashi, Tokyo)
++ Tokyo Kasei Co. Ltd.. (Toshima, Kitaku, Tokyo)
+++ Nihon Chromato Kogyo Co. Ltd.. (Toshima, Kitaku, Tokyo)

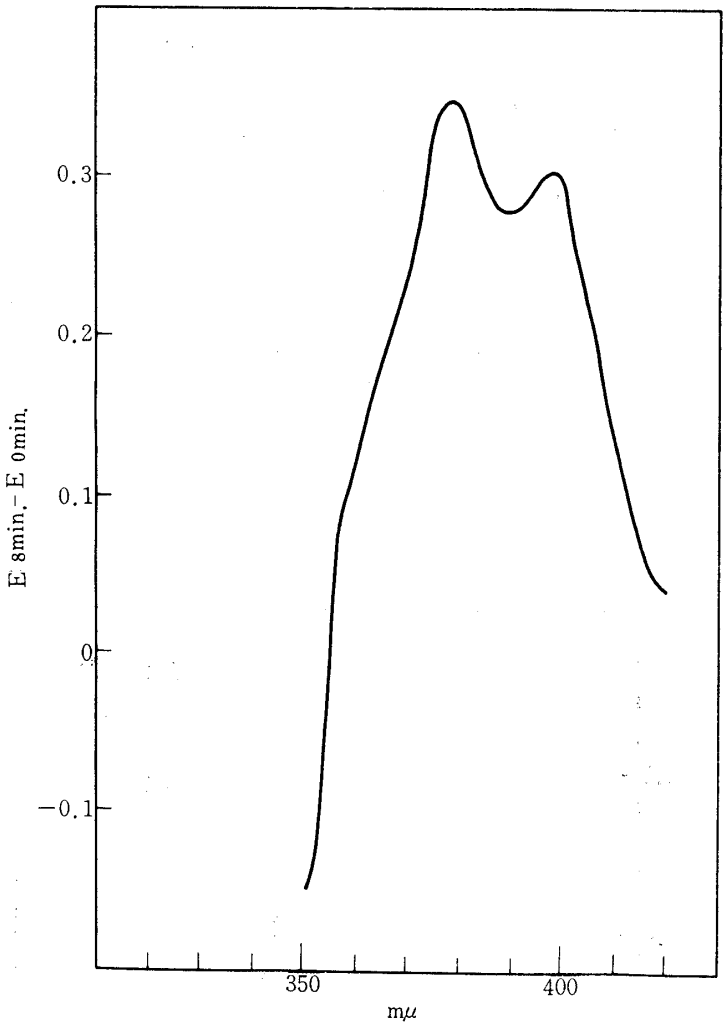

Fig. 1. Difference spectra 60°C, 8 min.

Effect of temperature In this paper, the extinction ratios of E 358 mμ/E 377 mμ (I) and E 399 mμ/E 377 mμ (II) were used to show the progress rate of reaction. (I) and (II) of pure anhydrovitamin A_1 in benzen are 0.692 and 0.868 respectively (4). As the formation of anhydrovitamin A_1 advances, (I) has to decrease and (II) has to increase from that of vitamin A_1. Table 2 shows the changes of (I) and (II) with the passing of times at various reaction temperatures. As given in table 2, the extinction ratio at 60°C, 8 min is more closely than others to that of anhydrovitamin A_1. 60°C is a very suitable reaction temperature.

Fig. 2 shows the changes of absorption spectra at 60°C. The extinction value was maximum at 8 min, and three peaks 358 mμ, 377 mμ and 399 mμ were recognized. The extinction value was decreased with the passing of reaction time, and the maximum peak was recognized at near 350mμ. This suggests the formation of 350mμ substances as the results of subreaction..

Effect of vitamin A_1 ester concentration Table 3 gives the relation of vitamin A_1

Table 2. Changes of extinction ratio at various temperatures.

Time (min)	40°C		50°C		60°C		70°C	
	358/377	399/377	358/377	399/377	358/377	399/377	358/377	399/377
2	3.183	0.133	3.068	0.120	1.603	0.549	1.000	0.716
4	3.141	0.170	2.012	0.545	1.149	0.740	0.867	0.728
6	2.909	0.245	1.586	0.494	0.870	0.735	0.949	0.703
8	2.482	0.244	1.270	0.654	0.822	0.742	1.045	0.702
10	1.895	0.444	0.940	0.725	0.846	0.707	1.043	0.819
12	1.831	0.402	0.953	0.706	0.901	0.663	1.065	0.817
14	1.442	0.594	0.875	0.734	0.980	0.636	1.056	0.847
16	1.333	0.551	0.859	0.960	0.947	0.644	1.045	0.845

Reaction condition: V. A_1 palmitate 4.9 mg, Benzen 2 ml.

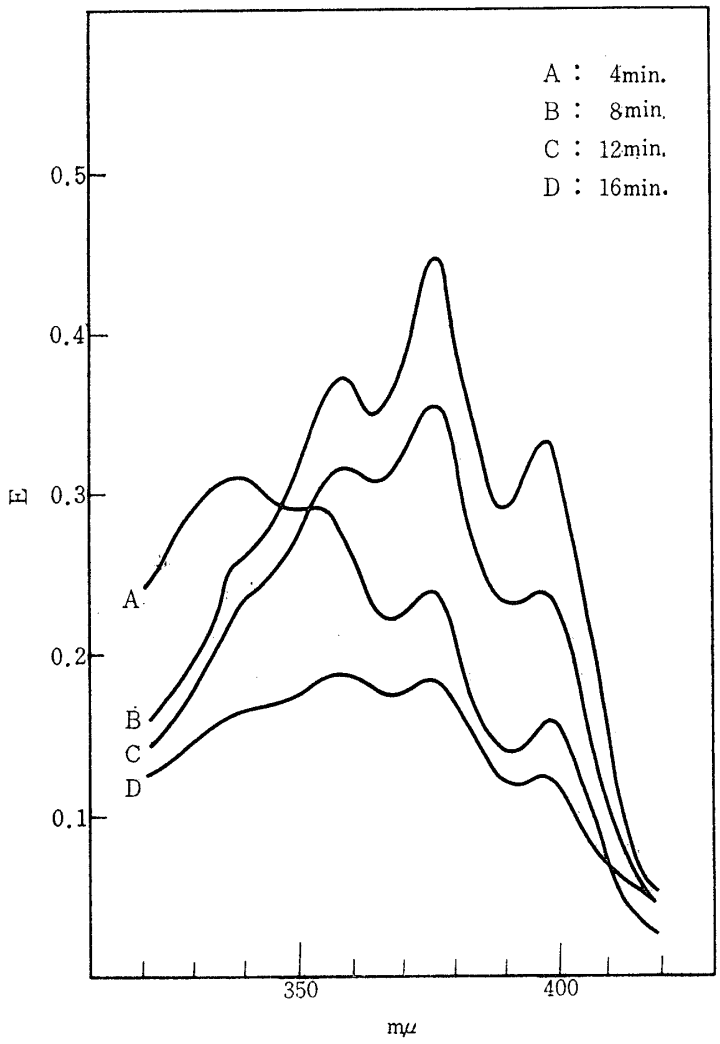

Fig. 2. Changes of absorption spectra at 60°C.

Table 3. Changes of extinction ratio in various V. A_1 palmitate concentration.

Time (min)	4.9mg		9.8mg		19.6mg		29.4mg	
	358/377	399/377	358/377	399/377	358/377	399/377	358/377	399/377
2	2.153	0.834	1.667	0.566	1.293	0.645	1.704	0.494
4	0.892	0.654	1.000	0.754	0.919	0.747	1.169	0.172
6	0.841	0.726	0.758	0.761	0.804	0.754	0.906	0.742
8	0.769	0.740	0.806	0.733	0.847	0.731	0.818	0.754
10	0.821	0.701	0.844	0.710	0.897	0.667	0.826	0.742
12	0.868	0.664	0.906	0.681	0.906	0.607	0.868	0.727
14	0.900	0.640	0.962	0.643	1.005	0.626	0.939	0.678
16	0.993	0.637	0.965	0.605	1.078	0.614	0.975	0.633

Reaction condition: Temp. 60°C, Benzen 2 ml.

Table 4. Fatty acid yield and composition.

Time (min)	Yield (mg)	Fatty acid composition as C 16:0=100			
		12:0	14:0	16:0	18:0
4	2.4	9.7	10.0	100	13.8
8	6.8	9.9	9.5	100	12.8
12	6.7	10.1	9.6	100	10.0
—	7.8+	9.6	9.6	100	13.7

+ saponification in KOH-EtOH, 70°C, 90 min.
Reaction condition: V. A_1 esters mixture 16.6 mg, Benzen 5 ml, 60°C.

Table 5. Changes in free fatty acid composition.

	Untreated	Treated
C 14:0	7.2 %	7.1 %
C 16:0	26.8	25.9
C 16:1	10.4	9.4
C 18:0	5.7	5.9
C 18:1	20.9	21.9
C 18:2	3.4	4.0
C 20:1(18:3)	5.3	4.2
C 20:3	5.2	4.0
C 22:2	4.9	4.2
C 22:6	13.6	12.7

Free fatty acids mixture was prepared from triglyceride of bonito body oil.
Reaction condition: Fatty acid 47.4 mg, Benzen 5 ml, 60°C, 8 min.

palmitate concentration to reaction time. In the concentration 4.9–29.4 mg/2 ml, the formation of anhydrovitamin A_1 was maximum apparently for 6–8 min at 60°C.

Gas-liquid chromatography of fatty acid The vitamin A_1 esters mixture was treated at 60°C and fatty acids were analyzed by gas-liquid chromatography. Table 4 gives the fatty acid composition. The standard was prepared by saponification in 1/2 N KOH-EtOH, 70°C, 90 min. To confirm the possibility of the denatura-

tion of separated fatty acid by a catalyst, fatty acids mixture was treated as mentioned above, and fatty acid composition was analyzed (Table 5).

These results in table 4 and table 5 suggest that this reaction proceeds uniformly and the separated free fatty acids do not change by the catalyst.

Reaction of wax esters and cholesterol palmitate To analyze only the fatty acids of vitamin A_1 esters, it is necessary that wax esters and cholesterol esters do not split any free fatty acids by this reaction, since vitamin A_1 esters are not separable from other esters by means of alumina column chromatography. Cholesterol palmitate 4.8 mg, 20.0 mg and wax esters 5.5 mg, 30.3 mg were dissolved in benzen 2 ml respectively, a catalyst added and then treated at 60°C for 8 min. Silica gel thin layer chromatography of reaction mixtures showed that these esters did not change.

From these results, it is clear that this reaction is useful to obtain only fatty acids of vitamin A_1 esters from other existing esters.

Fatty acid composition of vitamin A_1 esters of bonito liver oil The liver oil of bonito (Katsuwonus pelamis) was chosen as an example. The liver oil 17 g was dissolved in petroleum ether and subjected to chromatography on an alumina column. The vitamin A_1 esters were eluted with diethyl ether-petroleum ether 5:95, concentrated to dryness under a stream of nitrogen. The residue was 236. 7 mg. It was dissolved in benzen 20 ml, allowed to react at 60°C for 8 min. The separated free fatty acids were 20.4 mg. The methyl ester of fatty acid was analyzed by gas-liquid chromatography (Table 6).

Table 6. Fatty acid composition of V.A_1 esters and triglyceride in liver oil of bonito.

	V.A_1 esters	Triglyceride
C 14:0	1.6 %	1.5 %
C 15:0	1.2	0.4
C 16:0	56.8	30.3
C 16:1	2.1	0.6
C 17:0	3.8	0.5
C 18:0	4.0	8.7
C 18:1	15.4	21.7
C 18:2	2.8	1.3
C 18:4	—	0.6
C 20:1 (18:3)	2.1	1.6
C 20:3	0.9	2.6
C 20:4	—	0.4
C 22:2	1.4	8.3
C 22:5	tr	1.3
C 22:6	tr	8.8

The saturated fatty acids present, in the largest percentage, was about 64%. The major fatty acids were palmitic 57%, oleic 15% and palmitoleic 8%. Polyunsaturated fatty acids were minimal. In general, these results are similar to that of Kinumaki et al (3).

In Kinumaki's method, at least 1 g of oil and about 70 mg of vitamin A_1 esters calculated as palmitate are necessary. In this method, however, it is possible to analyze small amounts of vitamin A_1 esters. The minimum quantity of vitamin A_1 esters required in this method depends wholly on the minimum quantity of fatty acids required by gas-liquid chromatographic analysis, and at least about 4 mg of vitamin A_1 esters are necessary when the hydrogen flame ionozation gas-liquid chromatograph is applied.

Summary

Among many esters contained in liver oil, only vitamin A_1 ester was separated into anhydrovitamin A_1 and free fatty acid in benzen solution, in the presence of p-toluenesulphonic acid as catalyst.

The followings were found suitable as reaction conditions: temperature 60°C, reaction time 8–10 min, vitamin A_1 esters concentration 3–15 mg/ml benzen, catalyst 15–20 mg.

In this reaction, wax esters and cholesterol esters did not split any free fatty acids.

Micro analysis of fatty acids of vitamin A_1 esters is possible with use of this method.

The liver oil of bonito was analyzed as an example. The major fatty acids were saturated ones and among which palmitic acid was most. There was little sign of polyunsaturated fatty acids.

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