DETERMINATION OF VITAMIN D IN FISH AND FISH PRODUCTS BY CHROMATOGRAPHY WITH JAPANESE ACID CLAY

HIDEO HIGASHI, TAKESHIGE YAMAKAWA, TOYOSUKE KINUMAKI,
KISABURO SUGII AND KIKUKO IWASAKI

*Tokai Regional Fisheries Research Laboratory, Tsukishima,
Kyobashi, Tokyo*

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Methods so far proposed for physicochemical determination of vitamin D are reliable only when a sample contains impurities such as vitamin A in a relatively small amount, *e. g.*, the prescription of the U.S. Pharmacopeia (1). Although it might be applicable to the determination of medicines containing vitamin D, it can not be used for the determination of fish products which usually accompany vitamin A. In order to secure a method for this purpose we used activated Japanese acid clay (hereafter simply called "the clay") as an adsorbent together with activated alumina. As the method seems to be applicable to the determination of vitamin D in various forms of fish products at a high level of accuracy, the technique will be described in detail in this paper.

REAGENTS AND APPARATUS

1. *Alumina*

Merck's preparation was used for an adsorbent.

2. *Petroleum Ether (PE)*

A chemically pure grade preparation of the Nihon Sekiyu Co. was distilled. The main distillate (bp 40—60°) was dried over anhydrous sodium sulfate.

3. *Ether (E)*

A commercial preparation was purified by removing peroxides and dried over anhydrous sodium sulfate, followed by distillation. The main distillate was dried again with the same drying agent.

4. *Chloroform*

A commercial preparation was washed with concentrated H₂SO₄, and then with water and KOH solution. After washing again with water, it was distilled. The main distillate was dried over potassium carbonate overnight.

5. Antimony Trichloride

An extra pure grade preparation of Yotsuhata Kagaku Co. was recrystallized from the purified CHCl_3 .

6. Acetyl Chloride

An extra pure grade preparation of Kanto Kagaku Co. was used.

7. SbCl_3 -Acetyl Chloride Reagent

It was prepared by dissolving 200 mg SbCl_3 per ml CHCl_3 . Acetyl chloride was added before use (0.4 ml to 20 ml of SbCl_3 solution).

8. Vitamin A Alcohol

A preparation of crystalline vitamin A acetate (2,900,000 U. S. P. U./g) of the Riken Vitamin Co. was saponified to extract vitamin A alcohol.

9. Vitamin D_2

A preparation of the Riken Vitamin Co. was recrystallized from wet acetone to a state showing $E_{1\text{cm}}^{1\%}$ 265 $m\mu$ =470, mp 115°, then it was purified by alumina chromatography.

10. Glass Instruments Used for Chromatography

They are shown in Fig. 1. The

FIG. 1 Instruments Used for Chromatography

diameter of each column was corrected.

RESULTS

Studies on the Clay Activity

Attempts to use the clay for vitamin D determination have already been made by Baba (2) and Miwa (3). The methods adopted by the authors have not been described in detail for general use and the conditions for activation do not appear to have been investigated thoroughly. In view of securing a standard clay, therefore, we activated the clay under various conditions.

1. Preparation of the Clay Suitable for Chromatography

Usually commercial clay is so fine as to prevent rapid filtration and results in a loss of vitamin D even if it is mixed with celite for quickening the filtration. For the purpose of preparing the clay with the particles of suitable size, 10 parts of the commercial clay was kneaded with 7 parts of water and was pushed out in a form of noodle. After drying at 95° to 100° until complete evaporation of the water added, the clay was crashed up to the particles of 100 to 150 mesh. The grains were activated at a proper temperature to be mentioned later and kept in a desiccator together with P_2O_5 as a

drying agent.

2. Clay Chromatography

Cotton wool is placed on the bottom of the column. Pour the E-PE mixture (3:7, v/v), and then put a suitable amount¹ of the clay into the column under light agitation with a glass rod up to about 6 cm in height. Then the stopcock is opened to let the solvent flow down. The sample diluted with the E-PE mixture (3:7, v/v) is placed at the top of the column and washed with the same solvent mixture. Use the first 50 ml of the eluate for the determination.

FIG. 2 Absorption Spectra of the Colored Solution by $SbCl_3$ of Vitamin A Decomposition Products Produced by Chromatography Using the Clay of Varying Activations

¹ Until better specified later, 4 g of the clay was used in the early stages of the experiments.

3. Alumina Chromatography

When alumina is used as an adsorbent, alumina suspended with the E-PE mixture (1:9) is poured into the tube. Open the stop-cock to let the solvent flow down and the alumina settle down until the column becomes about 11 cm in height. The sample having passed through the clay chromatography is applied to the column. Three fractions are separately caught using 500 ml each of the solvent mixtures, E-PE mixture (1:9, v/v), the same mixture (3:7, v/v) and ether, respectively.

4. Determination of the Color Produced by SbCl_3 Reaction

The residue is dissolved in a certain volume of CHCl_3 ; 0.3 ml of this solution is allowed to react with 3.0 ml of the SbCl_3 reagent. After 1 minute the optical density (500 $\text{m}\mu$, 1 cm) of the solution is determined with a spectrophotometer (4).

5. Relation between the Activity of the Clay and the Decomposition Products Derived from Vitamin A

The clay adsorbs and decomposes vitamin A during chromatography (5, 6). We found that the vitamin A decomposition products are different depending on the activity of the clay.

When 10 mg of pure vitamin A specified above was applied to the clay chromatography mentioned in 1, some of the vitamin A decomposition products appeared in the eluate, whose absorption spectra after adding the SbCl_3 reagent were determined spectrophotometrically as shown in Fig. 2.

The intensity and shape of the absorption spectra of the decomposition products were shown to vary depending on the heating condition of the clay. When the clay activated by heating at 95° to 100° was used, the main absorption maximum of the color exists at 585 $\text{m}\mu$, the intensity being very

FIG. 3 Absorption Spectra of the Fractions Obtained by Alumina Chromatography of Vitamin A Decomposition Products through the Clay Heated at $95-100^\circ$

strong. However, in the case of the clay heated at 190° , the main maximum appeared at $430\text{ m}\mu$, the intensity being very weak. The vitamin A decomposition products, showing the main absorption maximum at 585 and $430\text{ m}\mu$, respectively, under the condition mentioned above are hereafter called "585

FIG. 4 Absorption Spectra of the Fractions Obtained by Alumina Chromatography of Vitamin A Decomposition Products through the Clay Heated at 150°

TABLE I
Alumina Chromatography of Vitamin A Decomposition Products
through the Clay Heated at 95° to 100°

Fraction no.	Solvent used	Weight	Absorption maximum of colored solution	$E_{1\text{cm}}^{1\%}$ 585 $\text{m}\mu$	Absorption maxima in ultraviolet region ^a	Solubility
		%	$\text{m}\mu$		$\text{m}\mu$	
Before chromatography		(100)	585			
I	E-PE (1:9)	30	585	831	335, 370	Sparingly soluble in alcohol
II	E-PE (3:7)	19	585	601	335, 350, 370, 260	Sparingly soluble in alcohol
III	E	4	585	2,060	293.5, 330, 255	Soluble in alcohol

^a Arranged in the order of absorption intensity, with inflections shown in italics throughout Tables I to IV.

and 430 chromogen". From this result, the decomposition products seem to differ each other according to the activity of the clay. To assure this point, these decomposition products were divided into several fractions using the alumina chromatography mentioned in 3 and their properties were compared with the results shown in Tables I to IV as well as Figs. 3 and 4. Comparison of these results with Fig. 2 shows that the 585 chromogen obtained from the clay of weak activity (Fraction I in Tables I and III and Fig. 3) is different from the 430 chromogen separated by the clay of stronger activity (Fr. II in Table II and IV, Fig. 4) in chromatographic behavior and ultraviolet absorption.

TABLE II
*Alumina Chromatography of Vitamin A Decomposition Products
through the Clay Heated at 150°*

Fraction no.	Solvent used	Weight	Absorption maxima of colored solution	$E_{1cm}^{1\%}$	Absorption maxima in ultraviolet region	Solubility
		%	$m\mu$			
Before chromatography		(100)	430, 585 494			
I	E-PE (1:9)	58	430, 585 494	111 (430 $m\mu$)	290, 245, 322, 338	Sparingly soluble in alcohol
II	E-PE (3:7)	28	430, 585	175 (430 $m\mu$)	290, 278	Soluble in alcohol
III	E	2	585, 430	126 (585 $m\mu$)	290, 249	Soluble in alcohol

TABLE III
*Alumina Rechromatography of the Substance Purified from
Fr. I in Table II*

Fraction no.	Solvent used	Weight	Absorption maxima of colored solution	$E_{1cm}^{1\%}$	Solubility
		%	$m\mu$		
Before chromatography		(100)	430, 495, 590	136	White powder, sparingly soluble in alcohol
I	E-PE (1:9)	74	430, 495, 590	175	"
II	E-PE (3:7)	24	430, 500, 585	27	"

6. Vitamin D Recovery in the Clay Chromatography

The complicated feature of vitamin A decomposition products was shown in the foregoing observations. The clay to be used for vitamin D determination should adsorb vitamin A decomposition products as much as possible without destroying vitamin D.

The recovery test of 20 μ g vitamin D in the clay chromatography has

TABLE IV
*Alumina Rechromatography of the Substances Purified from
 Fr. II in Table II*

Fraction no.	Solvent used	Weight	Absorption maxima of colored solution	$E_{1\text{cm}}^{1\% 430\text{m}\mu}$	Solubility
		%	$\text{m}\mu$		
Before chromatography		(100)	430, 585	403	Soluble in alcohol
I	E-PE (1:9)	28	430, 585, 500	378	Practically soluble in alcohol
II	E-PE (3:7)	64	430, 585	307	Soluble in alcohol

proved that only the clay heated at 95° to 100° can be used for vitamin D determination, whereas those heated at higher temperatures (130° to 190°) promote 430 chromogen formation, lowering the recovery rate unsuitably as shown in Table V.

TABLE V
*Recovery Test of Vitamin D Using the Clay Heated under
 Various Conditions*

Heating conditions for activating acid clay	Recovery
	%
One hour, 95°	100
30 minutes, 95°; 15 minutes, 130°	50
30 minutes, 95°; 15 minutes, 150°	34
30 minutes, 95°; 15 minutes, 190°	0

7. Conditions for Activating the Clay

If the heating time of the clay is too long even at the temperature below 100°, the recovery sometimes becomes low. Furthermore, the heating time has an effect on the quantity of 585 chromogen appearing in the vitamin D fraction, which has to be kept at a minimum. With this point in mind, the conditions for preparing the clay suitable for chromatography were investigated.

Optimal Amount of Water for Preparing Granular Clay — In recovery tests to determine an optimal quantity of water needed for preparing granular clay, the clay mixed with 50 or 70 parts of water was heated until it gained its initial weight. Then heating was continued for either 2 or 4 hours more. From Table VI showing the vitamin D recovery through each of these clays, it can be seen that with 50 parts of water the same duration of heating was necessary for about the same recoveries of vitamin D as well as appearance of 585 chromogen.

TABLE VI
Conditions for Producing Granular Acid Clay

Volume of water added	Heating time	Vitamin D recovery	Extinction value of 585 chromogen
%	hr	%	
50	8	100	71.5
50	10	82	31.9
50	14	85	22.5
70	9	100	40.7
70	11	100	44.0
70	15	100	22.0

Optimal Length of Heating Time for Activation—Granular clay 25g, prepared from the clay mixed with 70 parts of water was placed in a Petri dish, 6 cm in diameter. In the present test, the layer of the clay was made comparatively thin. Prior to desiccation, one of the clay samples thus prepared was not heated; other samples were heated for 2, 4, 6 and 8 hours, respectively; all of them were then desiccated over P_2O_5 for 16 hours. Using these clay samples, vitamins A and D were subjected to chromatography to determine the recovery of vitamin D and the quantity of 585 chromogen (Fig. 5). The results indicate:

(a) The quantity of 585 chromogen is roughly inversely proportional to the weight loss of the clay by heating, probably representing the quantity of

FIG. 5 Relationships among Weight Loss of Granular Acid Clay, Quantity of 585 Chromogen and Recovery of Vitamin D, When the Clay Activated in a Thin Layer was Used

×, weight loss; ○, recovery of vitamin D; ●, 585 chromogen.

FIG. 6 Relationships among Weight Loss of Granular Acid Clay, Quantity of 585 Chromogen and Recovery of Vitamin D, When the Clay Activated in a Thick Layer was Used

×, weight loss; ○, recovery of vitamin D; ●, 585 chromogen.

water in the caly; (b) the vitamin D recovery, independent of the weight loss on heating, lowers with the progress of heating after the first 2 hours; (c) Heating the clay for more than 2 hours should be avoided. The use of a thin layer of the clay as in these experiments is not practical. Therefore in the following experiments, the clay layer, about 3 cm in thickness, was used (Fig. 6). Although the weight loss in these experiments occurred later than in the thin layer, other behaviors were roughly the same as in the experiments shown in Fig. 5.

Relation between the Desiccating Period and Formation of 585 Chromogen

The fact that the quantity of 585 chromogen formed was roughly inversely proportional to the weight loss of the clay suggests a possibility of reducing the quantity of 585 chromogen without affecting the vitamin D recovery using the clay desiccated for a long time. The experiments on chromatography along this line proved the idea to be valid as shown in Table VII.

TABLE VII
Correlation between Desiccating Periods and Formation of 585 Chromogen

Desiccating periods	Extinction value of 585 chromogen	Vitamin D recovery
<i>day</i>		<i>%</i>
1	33.0	100
4	9.3	100

Relation between the Quantity of Clay and Formation of 585 Chromogen

Now that the method to prepare the clay suitable for chromatography has been secured, the second step was tried to reduce the formation of 585 chromogen by increasing the quantity of the clay used. It was found that an increase of the clay from 4 to 6 g resulted in the decrease in chromogen formation to some extent without affecting the vitamin D recovery (Table VIII). For this reason it is expected that a sample which is supposed to contain much greater amounts of vitamin A and impurities other than vitamin D can be subjected to vitamin D determination by increasing the quantity of the clay.

TABLE VIII
Correlation between Weight of Acid Clay and Formation of 585 Chromogen

Weight of acid clay	Extinction value of 585 chromogen	Vitamin D recovery	Volume of solvent
<i>g</i>		<i>%</i>	<i>ml</i>
4	46.0	100	50
6	22.0	97	50
8	14.4	92	60

Various Brands of the Clay — In all the experiments described above, the

clay supplied by the Mizusawa Kagaku Kogyo Co., Ltd. was used. We examined, therefore, whether or not other products from different localities or batches would give the same results. Of the three samples of the clay examined for this purpose, the batches *a* and *b* from the Mizusawa Kogyo and another from a second company were all found useful (Table IX).

TABLE IX
The Chromatography of Vitamins A and D Using Various Brands of the Clay

Producer	Place of production (Prefecture)	Extinction value of 585 chromogen	Vitamin D recovery %
Mizusawa Kagaku Kogyo Co., Ltd. (<i>a</i>)	Yamagata	50.0	97
Mizusawa Kagaku Kogyo Co., Ltd. (<i>b</i>)	Yamagata	63.5	100
Nihon Kassei Hakudo Co., Ltd.	Niigata	62.4	100

Separation of Vitamin D and Vitamin A Decomposition Products with Gradient Alumina Chromatography—Since a little amount of 585 chromogen still appeared in the vitamin D fraction even after clay chromatography, it seemed necessary to separate the chromogen and vitamin D before measurement of the color of SbCl_3 reaction.

FIG. 7 Schematic Outline of Vitamin D Determination

In accordance with Plack's method (7), 585 chromogen derived from vitamin A in the clay chromatography and 50 μg of vitamin D_2 were separated using gradient alumina chromatography. The method of elution is to obtain the first fraction with 25 ml of E-PE mixture (1:9) and each of the further fractions with 10 ml of the same mixture, whereby the polarity was gradually strengthened one after another (Fig. 7). The elution curve of gradient alumina chromatography reveals that 585 chromogen produced from vitamin A is divided into three groups (Fig. 8).

Then, using the sample containing 10 mg vitamin A and 20 μg vitamin D, it was tested to see if the separation of the vitamins was possible by combining the clay and gradient alumina chromatography. A satisfactory result was thus obtained, the recovery of vitamin D during the procedure being practically perfect.

In our experiment vitamin D appears in the fractions 7 to 9 which can be applied to the determination, though the fraction may occasionally slip according to the activity of alumina.

Separation of Unsaponifiable Components Other than Vitamins A and D—The separation of vitamins A and D was described above. However, samples to be treated may contain various unsaponifiable components other than vitamin A and D. The color produced by SbCl_3 reaction of these components and the methods of separation were therefore investigated. Fig. 9 shows the absorption spectra of the SbCl_3 -color of fairly pure specimens of oleyl, sela-chyl, and batyl alcohol and cholesterol.

Comparing with the intensity of SbCl_3 -color of vitamin D, those of the former three alcohols are so weak that we can neglect their effects, even when they appear in the vitamin D fraction. Though cholesterol produces a color of significantly stronger intensity, it can be easily removed from the samples by low temperature crystallization or digitonin treatment.

Besides these alcohols was found in the course of experiments a steroid which increased SbCl_3 -color with the progress of time, thus disturbing vitamin D determination. This steroid appears in the vitamin D fraction even after the combined clay and gradient alumina chromatography. Moreover, it cannot be completely separated by low temperature precipitation. It forms,

FIG. 8 Separation of Vitamin D_2 and 585 Chromogen with Gradient Alumina Chromatography

FIG. 9 Absorption Spectra of Colored Solutions of Oleyl Selachyl, Batyl Alcohol and Cholesterol by SbCl_3 Reagent

however, digitonide and after digitonin treatment the intensity of SbCl_3 -color becomes zero within the whole range of wavelengths from 340 to 600 mμ. Other unsaponifiable components, occurring often in the course of vitamin D determination of fish and fish products can be removed by the combined clay and alumina chromatography.

Proposed Method of Determination

1. Preparation of Samples

Saponification and Extraction—Saponify 1 to 10 g¹ of the oil extracted from fish or fish products with methanolic KOH solution and extract the unsaponifiable matter several times with ether specified above.

Methanol Treatment—Cool the methanol solution of the unsaponifiable matter in a refrigerator, and remove the precipitates by filtration.

Digitonin Treatment—Dissolve in 2 ml of 80% ethanol the residue obtained by evaporating the filtrate. Add 1 or 2 ml of digitonin solution (30 mg per 1 ml of 80% ethanol), warm the mixture with stirring and allow to stand for an hour. Filter the mixture into a test tube to separate the digitonide. Wash the digitonide with 1 ml of 80% ethanol and then with 4 ml

¹ Although the weight of the oil is to be determined according to the potency of vitamin A, the total amount of vitamin A in the oil must not exceed 33,000 I.U.

of PE. Shake and let the filtrate settle to transfer the upper layer to another test tube. Repeat the extraction of the lower layer 4 times using 4 ml each of PE. Wash the combined extract with 3 ml of water, place the extract in a flask and remove the solvent completely under reduced pressure.

2. Clay and Alumina Chromatography

Apply the sample thus prepared to the clay and gradient alumina chromatography. In the case of the sample containing very little impurities, alumina chromatography can be simplified as follows. After washing the alumina column with 100 ml of E-PE mixture (1:9), 50 ml of the eluate with E-PE mixture (3:7) is taken as vitamin D fraction.

3. Determination and Calculation

Vitamin D determination is carried out as described above. The content (I. U. per g) is calculated by the following formula:

$$\text{Vitamin D content} = \frac{R \times k \times d \times 40}{w}$$

where R =optical density, k =conversion factor, which is determined from the standard solution of vitamin D_2 , d =volume of CHCl_3 for dilution, w =weight of the sample used (g).

Examples of Vitamin D Determination by the Proposed Method — Vitamin D contents in fish and fish products determined by the present method are shown in Tables X to XII. Though scarcity of the available data makes it difficult to generalize the results, the following might be noticed. (a) Vitamin D contents vary depending on the part of the body as well as species. In one group of fish such as mackerel, bluefin tuna, yellowtail, and Japanese flounder, vitamin D exists mainly in the liver but little in other organs. On the other hand, in the sardine vitamin D occurs both in the flesh and the wastes including the head and bones at a fairly high level, comparable with the level of the viscera. Another group, such as horse mackerel and flatfish, contains little or no detectable amount of vitamin D in the flesh and viscera (Table X). (b) Among the commercial products tested for the applicability of the method, it is interesting to note that vitamin D, which is un-

FIG. 10 Absorption Spectra of Colored Solution of Sterol Fraction Separated from Great Blue Shark by SbCl_3 Reagent

TABLE X
Vitamin D Contents in Various Fishes

Common and scientific name	Part of fish	Oil content %	Vitamin A, i.u. per		Vitamin D, i.u. per		A/D (i.u.)
			1 g of oil	100g of tissue	1 g of oil	100g of tissue	
Sardine <i>Sardinops melanosticta</i>	Flesh	6.4	13	83	83	532	0.2
	Viscera	8.4	114	946	71	590	1.6
	Waste	7.2	10	72	137	990	0.1
Horse mackerel <i>Trachurus trachurus</i>	Flesh	2.8	4	11	...	...	
	Viscera	12.6	—	—	134	1,695	
	Waste	5.7	—	—	...	...	
Mackerel <i>Scomber japonicus</i>	Flesh (with skin)	23.8	3	72	6	143	0.5
	Skin	17.2	9	16	32	55	0.3
	Liver	13.3	28,600	380,000	435	5,780	66
Bluefin tuna <i>Thunnus thynnus</i>	Liver	6.7	8,480	56,700	7,800	52,200	1.1
Yellowtail <i>Seriola quinqueradiata</i>	Flesh	11.8	—	—	...	...	
	Liver	18.3	15,200	278,000	7,450	136,300	20
	Pyloric caeca	51.7	395	20,400	16	830	25
	Viscera	12.2	38	460	22	270	1.7
	Roe	3.2	1,710	5,430	...	...	
	Eye	23.7	122	2,890	...	...	
	Head (without eye)	11.8	...	...	57	668	
Young yellowtail	Dorsal flesh	6.6	...	...	...	...	
	Ventral flesh	8.1	...	...	7	53	
	Liver	9.3	1,470	13,600	636	5,880	2.3
	Pyloric caeca	12.5	4,230	52,900	12	150	350
	Kidney	4.2	684	2,570	...	...	
	Viscera	3.8	624	2,600	...	...	
	Waste	13.3	...	...	47	630	
Flatfish <i>Pleuronichthys cornutus</i>	Flesh	2.5	29	73	24	60	1.2
	Viscera	1.7	—	—	68	116	
	Waste (without milt and liver)	3.5	38	133	...	...	
Japanese flounder <i>Paralichthys olivaceus</i>	Flesh	0.2	...	...	222	43	
	Liver	1.4	569	787	3,100	4,200	0.2
	Roe	2.1	...	...	90	193	
	Viscera	1.2	3,080	3,700	600	720	5.1

—, no data; ..., no vitamin detected.

The same are applicable to the following Tables.

stable against exposition to sunlight, still remained in the dried mackerel. It was detected even in fish solubles as well (Table XI). (c) The amount of vitamin D in shark liver oil is so small that it will possibly be decomposed and become undetectable when the oil is subjected to distillation (Table XII). (d) No correlation exists between the contents of vitamins A and D in most cases regardless of the species or anatomical part of fish, suggesting that

TABLE XI
Vitamin D Contents in Various Fish Products

Sample	Part of fish product	Oil content %	Vitamin A, I.U. per		Vitamin D, I.U. per		A/D (I.U.)
			1 g of oil	100 g of sample	1 g of oil	100 g of sample	
Dried mackerel	Flesh	27.4	2	55	9	247	0.2
	Waste	14.4	8	110	19	270	0.4
Fish soluble (N. Co.)		8.4	965	8,100	431	3,620	2.2
" (T. Co.)		10.0	7,180	71,800	750	7,500	9.6

TABLE XII
Vitamin D Contents in Various Vitamin Oils

Sample	Vitamin A	Vitamin D	Vitamin A/Vitamin D
	I.U./g	I.U./g	I.U.
Mixed shark liver oil distilled	89,700	...	...
Rockfish liver oil	83,000	978	85
Swordfish liver oil distilled	79,100	6,930	11
Dogfish liver oil distilled	94,700	...	...
Great blue shark liver oil	5,600	67	84

the mechanisms of accumulating these two vitamins in the body of fish are different from each other.

DISCUSSION

Many methods of physicochemical determination of vitamin D proposed up to the present seem to be successful only when they are applied to pharmaceutical preparations (8, 9, 10, 11, 12, 13, 14). In cases of vitamin D contained in food, they do not secure a reliable result. A few methods reported as applicable to fish products can be applied only to liver oils containing a fairly large amount of vitamin D (3, 15, 16, 17). By the use of the present method, however, vitamin D seems to be successfully determined in dried products and fish solubles as well as in fish body as given above. Nevertheless, the present method can not be said free from any defects. For instance, when the method is applied to fish roes, it sometimes happens that a small amount of the chromogen having a main absorption maximum at $480\text{ m}\mu$ appears in the vitamin D fraction, thus making the correct determination impossible unless the amount of vitamin D in a sample is large enough. In some samples obtained from the liver of fish such as young yellowtail, another chromogen having an absorption maximum of SbCl_3 color at $500\text{ m}\mu$ appeared in the fractions prior to the vitamin D fraction. This chromogen differed from vitamin D in chromatographic behaviors and color: the color produced from this chromogen was pink, while that from vitamin D orange. Resemblance of the absorption spectrum of $500\text{ m}\mu$ chromogen to that of vitamin D may suggest a possibility that the former is a decomposition product of vitamin D,

though further investigation is necessary to clarify the point. These problems in the application of the method are the subjects for future research, since various unsaponifiable components contained in fish have not yet been clearly identified at the present stage of investigation.

In addition to these problems, it should be recalled that a minimum amount detectable of vitamin D, which is about 200 i.u. for 10 g of a sample in the present method, depends on such factors as the capacity of a spectrophotometer, the concentration of vitamin D in the solution to be assayed, and the thickness of the solution in the cell.

SUMMARY

Methods hitherto proposed for vitamin D determination are not applicable to separating vitamin D from the impurities contained in fish and fish products. However, a series of experiments carried out by the authors demonstrated that a combined use of clay and gradient alumina chromatography is highly efficient for the purpose.

Based on the studies on the nature of Japanese acid clay to be used for chromatography, suitable conditions and methods for activation of the clay were secured.

Satisfactory results were obtained in the experiments performed by the use of sample oils prepared from various parts of fish, dried fish and fish solubles.

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