A CRITICAL STUDY OF THE SPECTROPHOTOMETRIC METHODS FOR THE EVALUATION OF THE POTENCY OF IRRADIATED ERGOSTEROL

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by

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INTRODUCTION

Numerous attempts have been made to quantitatively estimate vitamins D by chemical means, usually colorimetric. One of the most reliable colorimetric reactions is that between vitamins D and antimony trichloride in chloroform solution. This reaction yields at 500 mu an absorption maximum whose extinction (1%, 1 cm) is directly proportional to the amount of vitamins D present. Unfortunately, a number of other compounds react with antimony trichloride, and their absorption at 500 mu is sufficient to prevent an accurate estimation of vitamins D potency. Vitamin A, sterols, carotenoids, and pigments are among these compounds which interfere and which also usually occur with vitamins D in natural oils.

Therefore, after the selection of the antimony trichloride colorimetric reaction for the determination of the vitamins D potency, the most important problem was that of establishing a method of purification whereby the vitamins D might be separated from the interfering compounds mentioned above.

Tomkins (1) used the following procedure: saponification of the oil, cooling and treating with digitonin to remove sterols, chromatographing to separate the vitamin A from vitamins D. The vitamins D were then determined colorimetrically as mentioned earlier. Saponification with N/2 alcoholic potassium hydroxide serves as an important means of separation since the vitamins D are contained in the

non-saponifiable fraction. In the chromatographing vitamin A, carotenoids, and pigments are adsorbed onto activated bentonite clay from a hexane-ether solution.

Kingsley (2 & 3) modified Tomkin's method until the following procedure resulted: saponification; extraction of the non-saponifiable fraction with ether; removal of vitamin A, etc., by chromatographic adsorption. The vitamins D and sterols were then determined colorimetrically with antimony trichloride. The vitamins D were removed from an aliquot of the sample by chromatographic adsorption, and the sterols determined colorimetrically. The difference in the two Log I_o/I measurements was then used to calculate the vitamins D potency. Kingsley calculated that a factor of 19,300, when multiplied by the E (1%, 1 cm) of the antimony trichloride reaction at 500 mu, would give the number of vitamins D units per gram.

Young (4) verified Kingsley's method for natural fish oils containing vitamins D, but he found wide deviations between the physical chemical assay and the bioassay when Kingsley's method was tried on samples containing irradiated ergosterol. Young suggested that some modifications of Kingsley's method might be used on samples of irradiated ergosterols.

Hage (5) modified Kingsley's method for natural oils by substituting a swirling of the sample with the activated bentonite clay for the second chromatograph, or sterol correction. This modification not only shortened the procedure

but also simplified it since it had been difficult to separate out all of the vitamins D by ordinary chromatographing. Naturally incomplete removal of vitamins D would cause a low value for the calculated potency. Kingsley's method with Hage's modification still was not successful when applied to samples of irradiated ergosterol.

EQUIPMENT AND REAGENTS

The adsorption columns must be carefully and uniformly prepared to obtain reproducible results. The tubes for the chromatographic separations are made by sealing a 6 cm. length of 7 mm. Pyrex tubing to the bottom of a 1.6 x 15 cm. (0.625 x 6 inch) Pyrex test tube. These tubes are cleaned before use by soaking in sulfuric acid-dichromic acid solution, rinsing with distilled water and with alcohol, and finally drying in an oven. The suction apparatus is designed so that a bank of 8 columns can be developed simultaneously, controlling the pressure with the aid of an open-tube mercury manometer attached to a suction flask. Unless otherwise specified, the adsorbent used is a finely divided grade of activated bentonite clay (Superfiltrol, obtained from The Filtrol Corp., 315 West 5th St., Los Angeles, California).

The adsorption columns for the first chromatographic separation are prepared by placing a small wad of cotton in the bottom of one of the adsorption tubes, pressing this down firmly, and adding enough of the adsorbent so that, when very firmly pressed down with a piston (a glass rod with a cork on one end) under 6 cm. of suction, the height of the packed

column will be 3 cm. A second and equal portion of the adsorbent is then added and pressed down as before to give a hard, level surface. It is important that there should be no air pockets, as they cause irregularly shaped adsorption bands. To aid in overcoming this, the piston-head cork used in packing the columns is slightly smaller than the inside diameter of the Pyrex tube. This also helps avoid loosening of the adsorbent by suction when the piston is raised.

Unless otherwise stated, all extinction measurements of the antimony trichloride reactions were made with 1 cm. cells on a Bausch and Lomb visual spectrophotometer. Absorption curves in the ultra-violet region were measured with 1 cm. quartz cells on a Beckman quartz spectrophotometer. Too great emphasis cannot be placed on the purification of the solvents used in this work. For example, peroxides in ether, a fairly common occurrence, will cause a large error in measurements since they give a definite color reaction with antimony trichloride.

Alcoholic potassium hydroxide is prepared by dissolving 28 grams of C. P. potassium hydroxide pellets in 95 percent ethyl alcohol to give 1 liter. Usually, however, this solution is made up in 100 or 200 ml. lots as needed.

C. P. ethyl ether is used without further purification for extracting the saponified oils and for elution of adsorption columns.

The anhydrous ethyl ether for the chromatograph is purified by washing C. P. ethyl ether with 1 percent ferrous

sulfate solution to remove peroxides, then 10 times with distilled water to remove alcohol, drying with phosphorus pentoxide, decanting, and storing over sodium. This ether is distilled as needed and kept over ferrous sulfate.

The Skellysolve is purified by washing twice with concentrated sulfuric acid, allowing it to stand for 24 hours each time over the acid, then washing twice with 10 percent sodium carbonate solution, and once with a mixture of 10 percent sodium carbonate and 5 percent potassium permanganate solution. The Skellysolve is allowed to stand over this mixture for 24 hours. Then it is washed 15 times with distilled water, the reagent decanted into a dry flask, and dried over sodium for at least 24 hours. The dried solvent is then distilled (68 to 70 deg. C.), the first 5 percent and the last 10 percent of the distillate being discarded.

The absolute ethyl alcohol is a high-grade commercial product.

C. P. chloroform is washed thoroughly with 7 approximately equal portions of distilled water, dried over anhydrous potassium carbonate, decanted, and distilled, discarding the first and last 10 percent of the distillate. The purified chloroform is kept with activated charcoal and filtered as needed.

C. P. thiophene-free benzene is dried over sodium, distilled, and shaken with Superfiltrol before use.

The antimony trichloride reagent is prepared by dissolving 18 grams of C. P. antimony trichloride in 100 ml. of

the purified chloroform and then adding 2 ml. of redistilled acetyl chloride.

The ether is tested for peroxides by adding a few milliliters to a mixture of potassium iodide and starch solution. The presence of peroxides will be indicated by the appearance of a blue color.

The chloroform is tested for chloride ions by adding a few millliliters to a silver nitrate solution made slightly acidic. A white percipitate indicates the presence of chloride ions.

The chloroform is tested for phosgene by adding a small quantity to a saturated barium chloride solution. An opaque film between the chloroform and water layers indicates the presence of phosgene.

Due to the instability of purified chloroform, it is not advisable to prepare more than a week's supply of chloroform and antimony trichloride reagent at one time.

DESCRIPTION OF PROCEDURES

The following procedures were used in this investigation:

Procedure A (Kingsley's method with Hage's modification)

Step 1. Weigh out sample containing 4,000 to 100,000 U. S. P. Units.

Step 2. Add 10 ml. of alcoholic potassium hydroxide if the sample weighs 1 g. or less, or 10 ml. per gram if more.

Step 3. Place a short stem funnel in neck of flask to serve as condenser.

Step 4. Saponify in water bath for one hour or more at 70 to 75 deg. C. Swirl frequently.

Step 5. Cool and add 20 ml. of water for each 10 ml. of potassium hydroxide.

Step 6. Extract with ethyl ether in separatory funnel using one 40 ml. portion of ether and three 20 ml. portions.

Step 7. Combine ether extracts and wash with six 50 ml. portions of water. Do not agitate during the first three washings.

Step 8. Filter washed extract through anhydrous sodium sulfate to remove water. Rinse separatory funnel with ether.

Step 9. Evaporate to dryness under reduced pressure, using hot water bath.

Step 10. Dissolve residue in 5 ml. of the following mixture (50-10-1): 50 parts Skellysolve, 10 parts anhydrous ether, and 1 part absolute ethyl alcohol (by volume).

Step 11. Prepare 6 cm. adsorption column and wet with 10 ml. of 50-10-1.

Step 12. Add sample, 5 ml. of 50-10-1 for rinsing flask, and 35 ml. for developing the column. Each addition of solvent is made just before the top of the column becomes dry.

Step 13. Use differential pressure of 6 cm. of mercury for chromatographing.

Step 14. Dry by drawing air through column for 5 to 10 minutes.

Step 15. Remove adsorbent down to 2 mm. below the vitamin A ring, which is yellow or orange.

Step 16. Elute the remainder of the column with 10 to 15 ml. of ether.

Step 17. Evaporate combined filtrate and eluate to dryness (see step 9).

Step 18. Dissolve the residue in 10 ml. of chloroform.

Step 19. Add 10 ml. of antimony trichloride reagent to 1 ml. of the chloroform solution, swirl 30 seconds, fill absorption cell, and determine the extinction at 500 mu on the Bausch and Lomb visual spectrophotometer exactly 3 minutes after starting to add the reagent. This reading represents vitamins D plus sterols (D plus S).

Step 20. Evaporate 1 ml. of the chloroform solution (see step 9).

Step 21. Dissolve the residue in 25 ml. of B_2S_1 (2 parts Benzene, 1 part Skellysolve by volume).

Step 22. Add Superfiltrol (the amount which will fill a 5/8 inch test tube to the depth of 1 inch.

Step 23. Allow to stand for 30 minutes with very frequent swirling.

Step 24. Filter and rinse with two 10 ml. portions of B_2S_1.

Step 25. Evaporate filtrate to dryness (see step 9).

Step 26. Dissolve residue in 1 ml. of chloroform.

Step 27. Repeat step 19. This reading represents the sterols (S). To calculate the potency in D units per gram:

1. Subtract sterols from vitamins D plus sterols, (D plus S)-(S).
2. Determine the E(1%, 1 cm.) of the difference.
3. Multiply the E(1%, 1 cm.) by 19,300. The result is the potency in D units/gram.

Procedure B (Direct)

Steps 18 and 19 of Procedure A.

Procedure C (Saponified)

Steps 1 to 9 inclusive, 18, and 19 of Procedure A.

Procedure D (Saponified and First Chromatograph)

Steps 1 to 19 inclusive of Procedure A.

Procedure E (First Chromatograph)

Steps 10 to 19 inclusive of Procedure A.

Procedure F (Second Chromatograph)

Steps 21 to 27 inclusive of Procedure A.

In case, during the abovementioned procedures, it was desired to measure the absorption of the sample in the ultra-violet

region the procedures were modified in the following manner: instead of dissolving the sample in chloroform prior to adding the antimony trichloride reagent the sample was dissolved in absolute ethyl alcohol, and the resulting solution was divided into two or more portions. For the ultra-violet measurements one portion was diluted with more absolute ethyl alcohol until the proper concentration for the most accurate measurements was reached. For the antimony trichloride reaction measurements another portion was evaporated to dryness, taken up in chloroform, and the extinction measured as described previously.

When the samples consisted of irradiated ergosterol, the length of time for saponification was shortened to 30 minutes. Hereafter, it may be assumed that the saponification time for samples of irradiated ergosterol was 30 minutes unless stated otherwise.

STUDY OF THE LENGTH OF TIME REQUIRED FOR SAPONIFICATION

When it was noticed that samples of irradiated ergosterol in corn oil with alcoholic potassium hydroxide during saponification became homogeneous sooner than had been observed for samples of natural oils, it was decided to make a brief investigation to determine the length of time required for complete saponification.

The oils selected for this study were #3772, a solution of pure calciferol in corn oil the bioassay of which is 200,000 D units/gram and Be 103, irradiated ergosterol in corn oil,

which had a bioassay value of 700,000 to 750,000 D units/gram. Table I shows the measurements made on samples of these oils run by Procedure C with varying lengths of time for saponification.

As a result of this investigation, it was decided that 30 minutes would allow sufficient time for complete saponification and that 30 minutes would be adopted as the standard saponification time for samples of irradiated ergosterol.

DISTRIBUTION OF VITAMINS D IN ETHER PORTIONS DURING EXTRACTION

To make sure that all the vitamins D were being extracted after saponification, a brief investigation was made as to the amounts of vitamins D extracted by each of the four portions of ethyl ether used in this part of the procedure.

The same two oils were used in this study as were used in the preceding study of saponification times. Procedure C was followed except that the portions of ether used in extraction were kept separate after extraction and were treated as separate samples thereafter. Table II shows the measurements made on the portions of ether after extraction.

Since the Log I_o/I measurements of the third and fourth ether portions of both oils were so small that they would be attributed to the insensitivity of the eye in reading the instrument at low or zero extinctions, it was decided that extraction with four portions of ether as described in Procedure A was sufficient to extract all of the vitamins D contained in the amounts of sample as were ordinarily used in this investigation.

TABLE I

Oil	Sample Weight	Time of Saponification	Log I_o/I	E(1%, 1 cm.)	Calculated D u/g
3772	0.060g.	3 minutes	0.48	8.80	170,000
"	"	6 "	0.49	8.98	173,000
"	"	10 "	0.48	8.80	170,000
"	"	15 "	0.47	8.62	166,000
"	"	30 "	0.48	8.80	170,000
"	"	45 "	0.47	8.62	166,000
Be 103	0.020g.	1 minute	0.47	25.8	498,000
"	"	6 minutes	0.53	29.2	563,000
"	"	11 "	0.53	29.2	563,000
"	"	15 "	0.48	26.4	510,000
"	"	30 "	0.48	26.4	510,000
"	"	45 "	0.53	29.2	563,000
"	"	60 "	0.52	28.6	552,000

TABLE II

Oil	Sample Weight	Ether Portion	Log I_o/I	E(1%, 1 cm.)	D u/g
3772	0.060g.	1	0.42	7.80	150,500
		2	0.04	0.734	14,150
		3	0.025	0.458	8,850
		4	0.025	0.458	8,850
					182,350
Be 103	0.020g.	1	0.53	29.2	562,000
		2	0.075	4.12	79,500
		3	0.025	1.37	26,400
		4	0.030	1.65	31,900
					699,800

STUDY OF ADSORBENTS

A study was made in which the effectiveness of various adsorbents was compared to that of the adsorbent, Superfiltrol, normally used. The oil B 5684, a standard high D fish oil having a bioassay value of 17,000 D units/gram, was used in this investigation. All samples contained 0.500 g. of this oil. The procedure used was Procedure A, Kingsley's method with Hage's modification. Table III shows the measurements which resulted from the treatment of this oil using the adsorbents listed.

It can be seen from Table III that all the samples of Superfiltrol behaved in a similar manner and that, therefore, any of the types tested might be used for the analysis of a sample of unknown potency without modification of Procedure A.

When Magnesia or Magnasol was used as the adsorbent, both the D plus S and S measurements were high as compared to those obtained when Superfiltrol was used. This would seem to indicate that the first chromatograph did not succeed in removing as much of the interfering substances as did Superfiltrol. However, in the cases of both samples of powdered Magnesia, the difference between the D plus S and S measurements was only slightly less than that difference obtained when Superfiltrol was used as the adsorbent.

In the cases of Alumina and Magnesium Silicate, there seemed to be little or no difference between the action of the two chromatographs. Obviously, these adsorbents could not be used without a drastic modification of the present procedure.

It may be concluded from this study that different adsorbents vary considerably in their effectiveness in removing interfering substances and that a different type of adsorbent might be used only after a modification of the present procedure.

NATURAL OILS

The natural oils listed in Table IV were run by Procedure A, Kingsley's method with Hage's modification. As can be seen from the table there is, for the most part, quite close agreement between the experimentally determined D units/gram and the bioassay values.

SUPERFILTROL CORRECTION FOR ULTRA-VIOLET MEASUREMENTS

Late in the course of this investigation, it was observed by C. W. Carlson, who was carrying on related studies in this laboratory, that when certain solvents were passed through an adsorption column containing Superfiltrol the filtrate contained a substance, apparently eluted from the Superfiltrol, which had an appreciable absorption in the ultra-violet region.

When this observation was made known, it was decided to ascertain whether or not any substance was eluted from the Superfiltrol when the specific mixtures of solvents used in this investigation came in contact with the Superfiltrol.

Therefore, a "blank" was run on each of the two types of chromatographic adsorption. Fifty-five milliliters of the 50-10-1 mixture (the total volume used normally) was passed through a 6 cm. adsorption column, approximately the bottom two cm. of the column eluted with ethyl ether, the filtrates

combined and evaporated to dryness, and the residue taken up in absolute ethyl alcohol to 25 ml. The absorption in the ultra-violet region was measured for this solution on the Beckman quartz spectrophotometer. Then the solution was diluted 1:1 with alcohol and the absorption measured. Finally, a 1:3 dilution of the original alcohol solution was made and the absorption measured. The results of these measurements are shown in Figure I.

The same procedure was carried out with the second chromatograph except that 25 ml. of the B_2S_1 mixture were swirled frequently for one hour with the amount of Superfiltrol normally used in this operation, filtered, rinsed with two 10 ml. portions of the same solvent, evaporated to dryness, and the residue taken up in absolute ethyl alcohol to 25 ml. The same dilutions and measurements were made as in the case of the first chromatograph. The results of those measurements are shown in Figure II.

As a result of this study, it was decided that a correction must be made in ultra-violet measurements of a sample whenever that sample has been treated by any procedure involving the use of Superfiltrol. It is believed that this may best be done by running a "blank" simultaneously with the sample through all the operations involving Superfiltrol. As may be seen from Figures I and II, the absorption of the substance eluted from the Superfiltrol varies inversely with the final volume of the solution, and after absorption has been measured for any one concentration, it may be calculated for any other

TABLE III

Adsorbent	Log I_o/I	E(1%, 1cm.)	D u/g
Superfiltrol Lot 63	(D plus S) 0.53 - (S) -0.14 0.39	0.858	16,600
Superfiltrol Lot 63	0.54 -0.13 0.41	0.902	17,400
Superfiltrol Lot 63	0.55 -0.14 0.41	0.902	17,400
Superfiltrol Lot A	0.50 -0.11 0.39	0.858	16,600
Superfiltrol Lot B	0.48 -0.08 0.40	0.880	17,000
Superfiltrol Lot C	0.48 -0.09 0.39	0.858	16,600
Superfiltrol Lot X-202	0.51 -0.11 0.40	0.880	17,000
Adsorptive Powdered Magnesia, Lot 2641	1.10 -0.73 0.37	0.814	15,700
Adsorptive Powdered Magnesia, Lot 2642	1.08 -0.70 0.38	0.836	16,100
Adsorptive Granular Magnesia, Lot 2652	0.90 -0.23 0.67	1.474	28,400

TABLE III (cont'd.)

Adsorbent	Log I_o/I	E(1%, 1cm.)	D u/g
Magnasol "A"	1.05 -0.42 0.63	1.386	26,750
Alumina, 80 mesh Grade A	(D plus S) 0.15 - (S) -0.15 0.00	0	0
Magnesium Silicate #16	0.57 -0.43 0.14	0.308	5,950
Magnesium Silicate #34, Lot 3	0.58 -0.45 0.13	0.286	5,520

TABLE IV

Oil No.	Type of Oil	Sample Weight	E(1%, 1 cm.)	D u/g	Bioassay
20923	Standard High D Fish Oil	1.000 g.	0.462	8,900*	15,000
		1.000 g.	0.495	9,500*	
		1.000 g.	0.594	11,500*	
		1.000 g.	0.616	11,800*	
		1.000 g.	0.748	14,400	
		1.000 g.	0.737	14,200	
		1.000 g.	0.771	14,900	
		1.000 g.	0.814	15,700	
		0.5000g.	0.771	14,900	
		0.5000g.	0.703	13,600	
		0.5000g.	0.792	15,300	
		0.5000g.	0.771	14,900	
		0.5000g.	0.814	15,700	
		0.5000g.	0.771	14,900	
		0.5000g.	0.771	14,900	
		0.5000g.	0.792	15,300	
		0.5000g.	1.012	19,500	
P 6846	Standard High D Fish Oil	0.800 g.	0.990	19,100	20,000
		0.800 g.	0.990	19,100	
		0.5000g.	1.056	20,400	
		0.500 g.	1.078	20,800	
B 5684	Standard High D Fish Oil	0.5000g.	0.880	17,000	17,000
		0.5000g.	0.902	17,400	
		0.5000g.	0.924	17,800	

* Chloroform probably contaminated

TABLE IV (cont'd.)

Oil No.	Type of Oil	Sample Weight	E(1%, 1 cm.)	D u/g	Bioassay
21273	High D Fish Oil	1.000 g.	0.583	11,250	12,000
28283	Haliverol D	1.000 g.	0.473	9,100	11,500
29263	High D Fish Oil	1.000 g.	0.561	10,800	13,500
50754	Albacore	0.7000g.	0.628	12,100	12,650
		0.7000g.	0.613	11,800	
50764	Skipjack	0.7000g.	1.385	26,700	24,750
		0.5000g.	1.385	26,700	
50784	Yellow Fin	0.7000g.	0.566	10,900	10,840
		0.7000g.	0.597	11,500	
50814	Blue Fin	0.7000g.	0.707	13,600	11,400
		0.7000g.	0.738	14,200	
50834	Yellow Tail	0.7000g.	0.660	12,700	13,800
		0.7000g.	0.692	13,300	
50854	Bonita	0.7000g.	0.755	14,600	17,150
		0.7000g.	0.738	14,200	

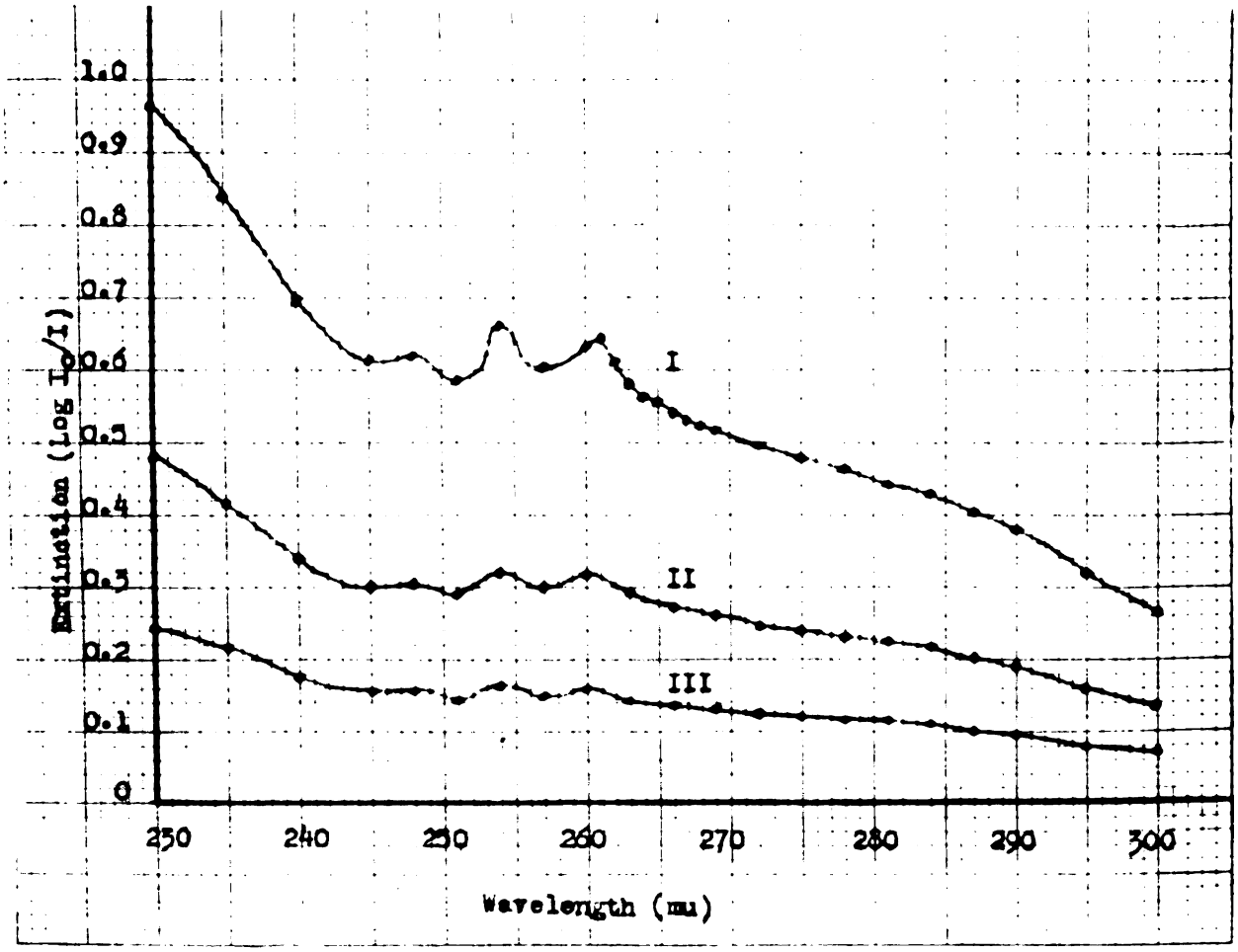


Fig. I- Extinction (Log I_o/ I) of the substance eluted from Super-filtrol, in absolute ethyl alcohol, plotted against wavelength.

I- Procedure E, final volume 25 ml.

II- Procedure E, final volume 50 ml.

III- Procedure E, final volume 100 ml.

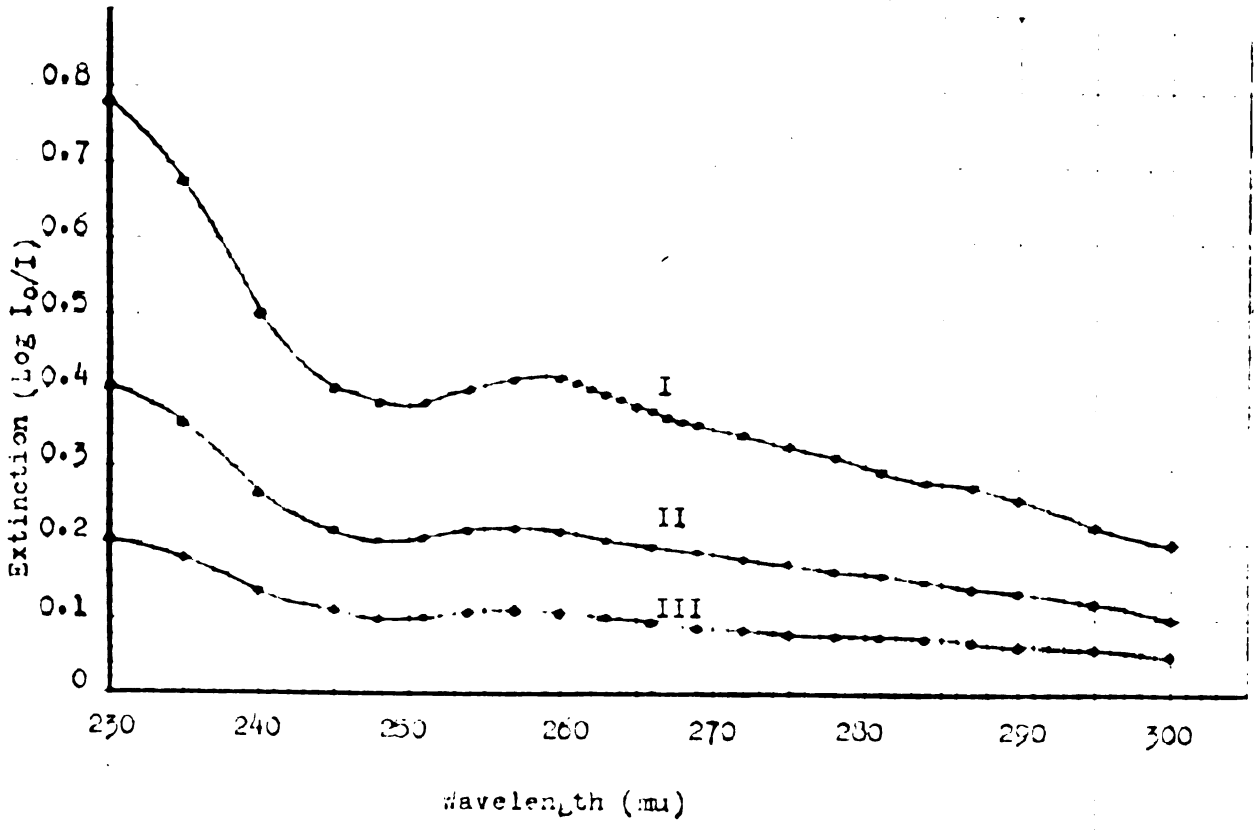


Fig. II- Extinction (Log I_o/ I) of the substance eluted from Super-filtrol, in absolute ethyl alcohol, plotted against wavelength.

I- Procedure F, final volume 25 ml.

II- Procedure F, final volume 50 ml.

III- Procedure F, final volume 100 ml.

concentration. It is obvious, therefore, that the dilution, or final volume, of a sample must be known in order that the proper correction may be applied.

It is believed that this correction is of great value in determining the changes which take place during the procedures, as measured by the ultra-violet absorption.

As far as may be ascertained, there is no interference in the antimony trichloride measurements due to the substance which is eluted from the Superfiltrol.

STUDY OF CALCIFEROL AND ERGOSTEROL

Toward the end of the investigation, it was decided to make a study of calciferol, or vitamin D_2, and ergosterol. In this study, the following solutions were examined:

(1) pure calciferol dissolved in absolute ethyl alcohol,

(2) pure ergosterol dissolved in absolute ethyl alcohol,

(3) a mixture of calciferol and ergosterol, approximately equal parts of each, dissolved in absolute ethyl alcohol. This was made by combining portions of solutions (1) and (2).

(4) Oil No. 3772, a solution of calciferol in corn oil,

(5) a mixture of Oil No. 3772 and the ergosterol solution (2).

Solutions (1), (2), and (3) were run by Procedure B; also the absorption of dilutions of the original solutions was measured in the ultra-violet region. Then all five solutions were run by Procedure A. Portions of the solutions were removed at intervals during the procedure and were measured with antimony trichloride at 500 mu and in the ultra-violet region in alcoholic solution. These intervals corresponded to the ends of

Procedures C and D. Table V shows the results of the antimony trichloride measurements. Figures III to VII inclusive show the absorption in the ultra-violet region.

As Figure III indicates, the calciferol remains unchanged during Procedure A until the second chromatograph; this is verified by the antimony trichloride measurements. The calciferol is apparently removed by the second chromatograph since its characteristic absorption curve in the ultra-violet regions disappears and since there is no measurable extinction with antimony trichloride.

Figure IV shows that the ergosterol is unchanged by saponification but is almost completely removed by the first chromatograph. The adsorption columns through which were passed solutions containing ergosterol showed green bands; after the developer (Step 12, Procedure A) had run through, the green color had spread out below the bands which had turned to a violet color. The adsorption columns of solutions (1) and (4), which did not contain ergosterol, showed no bands except the yellow band of solution (4) due to the corn oil.

According to Figure V solution (3) is changed only slightly by saponification, but as is expected the characteristic absorption curve of the calciferol is evident after the ergosterol is removed by the first chromatograph.

In Figure VI, the calciferol also shows up after the first chromatograph which indicates that the corn oil is removed by the end of chromatographing.

Figure VII indicates that the corn oil and ergosterol are

TABLE V

Procedure	Solution	Sample Weight	E(1%, 1 cm.)	D units/gram	Calculated Potency
B	Calciferol	0.377mg.	1610.	31,000,000	40,000,000
B	Ergosterol	1.275mg.	0.0	0	0
B	Calc-Erg. mixture	0.557mg.	830.	16,000,000	21,700,000
C	Calciferol	0.377mg.	1570.	30,300,000	40,000,000
C	Ergosterol	1.275mg.	0.0	0	0
C	Calc.-Erg. mixture	0.557	850.	16,400,000	21,700,000
C	#3772	0.0600g.	6.97	134,500	200,000
C	#3772-Erg. mixture	61.275 mg.	7.37	142,000	196,000
D	Calciferol	0.226 mg.	1510.	29,100,000	40,000,000
D	Ergosterol	0.815 mg.	0.0	0	0
D	Calc.-Erg. mixture	0.334 mg.	823.	15,900,000	21,700,000
D	#3772	36.0 mg.	6.72	130,000	200,000
D	#3772-Erg. mixture	36.765 mg.	6.65	126,000	196,000
A (Sterol Correction)	Calciferol	0.271 mg.	0.0	0	40,000,000
	Ergosterol	0.392 mg.	0.0	0	0
	Calc.-Erg. mixture	0.347 mg.	0.0	0	21,700,000
	#3772	43.2 mg.	0.0	0	200,000
	#3772-Erg. mixture	37.6 mg.	0.0	0	196,000

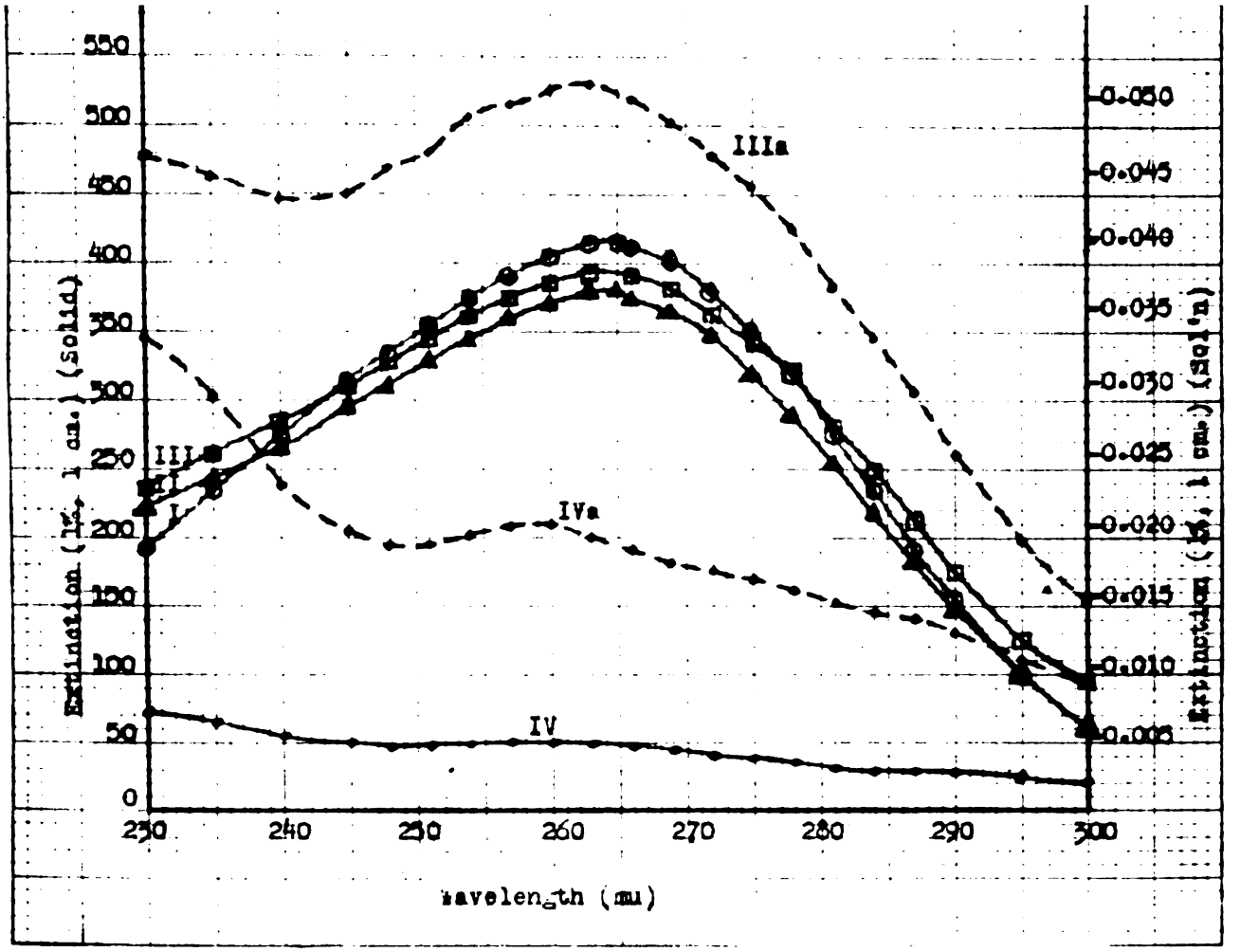


Fig. III- Extinction (1%, 1 cm.) of calciferol in absolute ethyl alcohol plotted against wavelength.

I- Procedure B, Concentration: 0.001508g./100 ml. (Solid)*
15.69g./100 ml. (Sol'n)*

II- Procedure C, Concentration: 0.001508g./100 ml. (Solid)
15.69g./100 ml. (Sol'n)

III- Procedure D, Concentration: 0.000905g./100 ml. (Solid)
9.415g./100 ml. (Sol'n)
Corrected for absorption of Superfiltrol

IIIa- Same as III except uncorrected for Superfiltrol

IV- Procedure A (Sterol Correction)
Corrected for absorption of Superfiltrol
Concentration: 0.002714g./100 ml. (Solid)
28.25g./100 ml. (Sol'n)

IVa- Same as IV except uncorrected for absorption of Superfiltrol

* (Solid) - Grams of pure calciferol/100 ml.
(Sol'n) - Grams of original sol'n/100 ml.

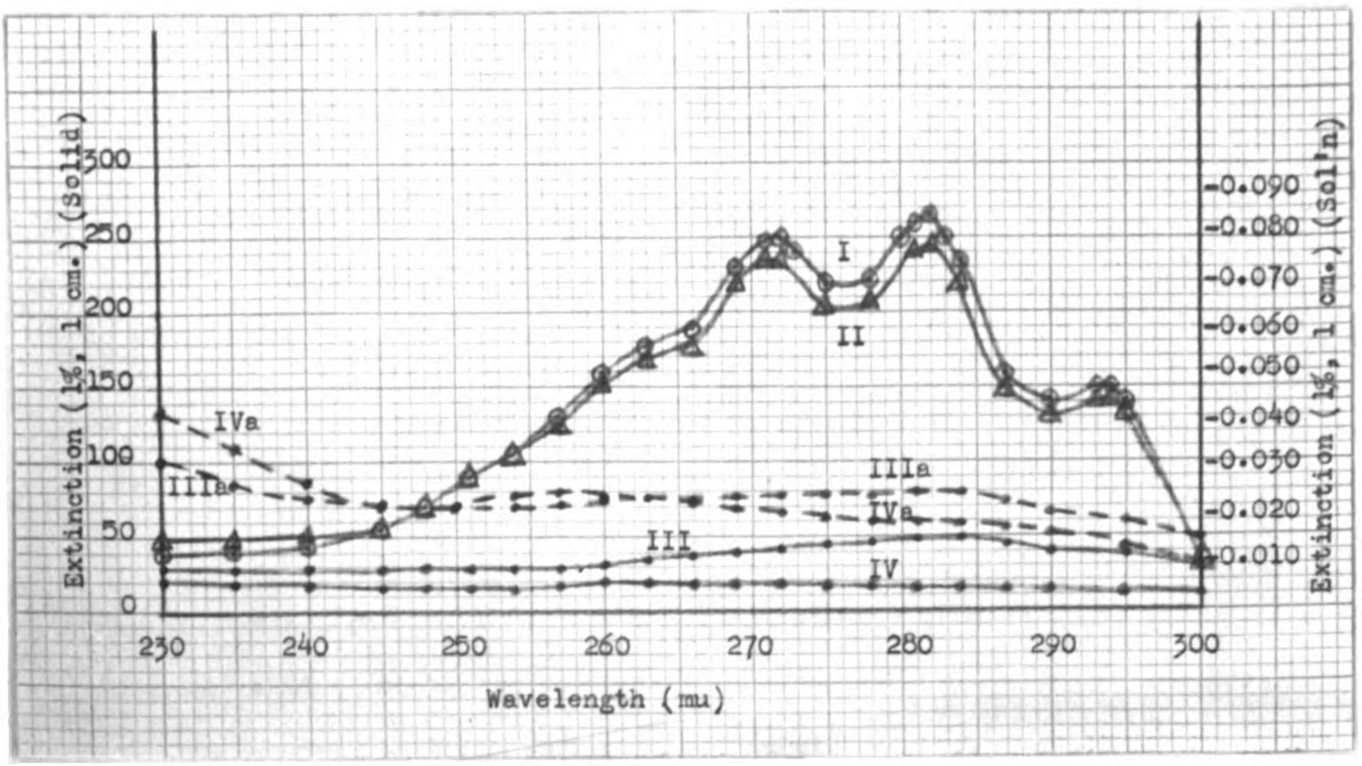


Fig. IV- Extinction (1%, 1 cm.) of ergosterol in absolute ethyl alcohol plotted against wavelength.

I- Procedure B, Concentration: 0.00255g./100 ml. (Solid)*
7.868g./100 ml. (Sol'n)*

II- Procedure C, Concentration: 0.00255g./100 ml. (Solid)
7.868g./100 ml. (Sol'n)

III- Procedure D, Concentration: 0.00765g./100 ml. (Solid)
25.20g./100 ml. (Sol'n)
Corrected for absorption of Superfiltrol

IIIa- Same as III except uncorrected for absorption of Superfiltrol

IV- Procedure A (Sterol Correction)
Corrected for absorption of Superfiltrol
Concentration: 0.00734g./100 ml. (Solid)
24.20 g./100 ml. (Sol'n)

IVa- Same as IV except uncorrected for absorption of Superfiltrol

* (Solid) - Grams of pure ergosterol/100 ml.
(Sol'n) - Grams of original sol'n/100 ml.

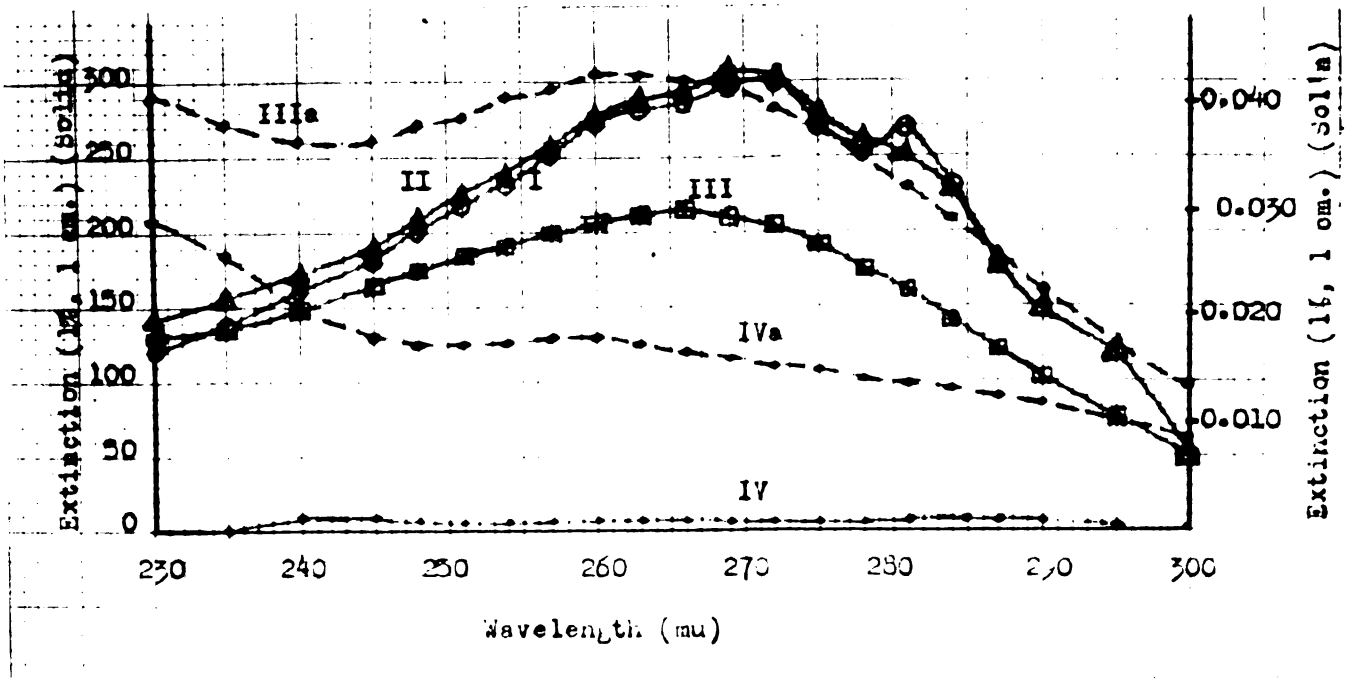


Fig. V- Extinction (1%, 1 cm.) of a calciferol-ergosterol mixture in absolute ethyl alcohol plotted against wavelength.

The original solution contained 0.0003 mg. calciferol/ml. and 0.0510 mg. ergosterol/ml.

I- Procedure B, concentration: 0.00[illegible]g./100 ml. (solid)*
15.73g./100 ml. (sol'n)*

II- Procedure B, concentration: 0.00[illegible]g./100 ml. (solid)
15.73g./100 ml. (sol'n)

III- Procedure B, concentration: 0.00[illegible]g./100 ml. (solid)
14.16g./100 ml. (sol'n)
Corrected for absorption of superfiltrol

IIIa- Same as III except uncorrected for absorption of superfiltrol

IV- Procedure A (sterol correction)
Corrected for absorption of superfiltrol
Concentration: 0.00[illegible]g./100 ml. (solid)
[illegible]g./100 ml. (sol'n)

IVa- [illegible] except uncorrected for absorption of superfiltrol

* (solid) - grams of pure calciferol and ergosterol/100 ml.
(sol'n) - grams of original sol'n/100 ml.

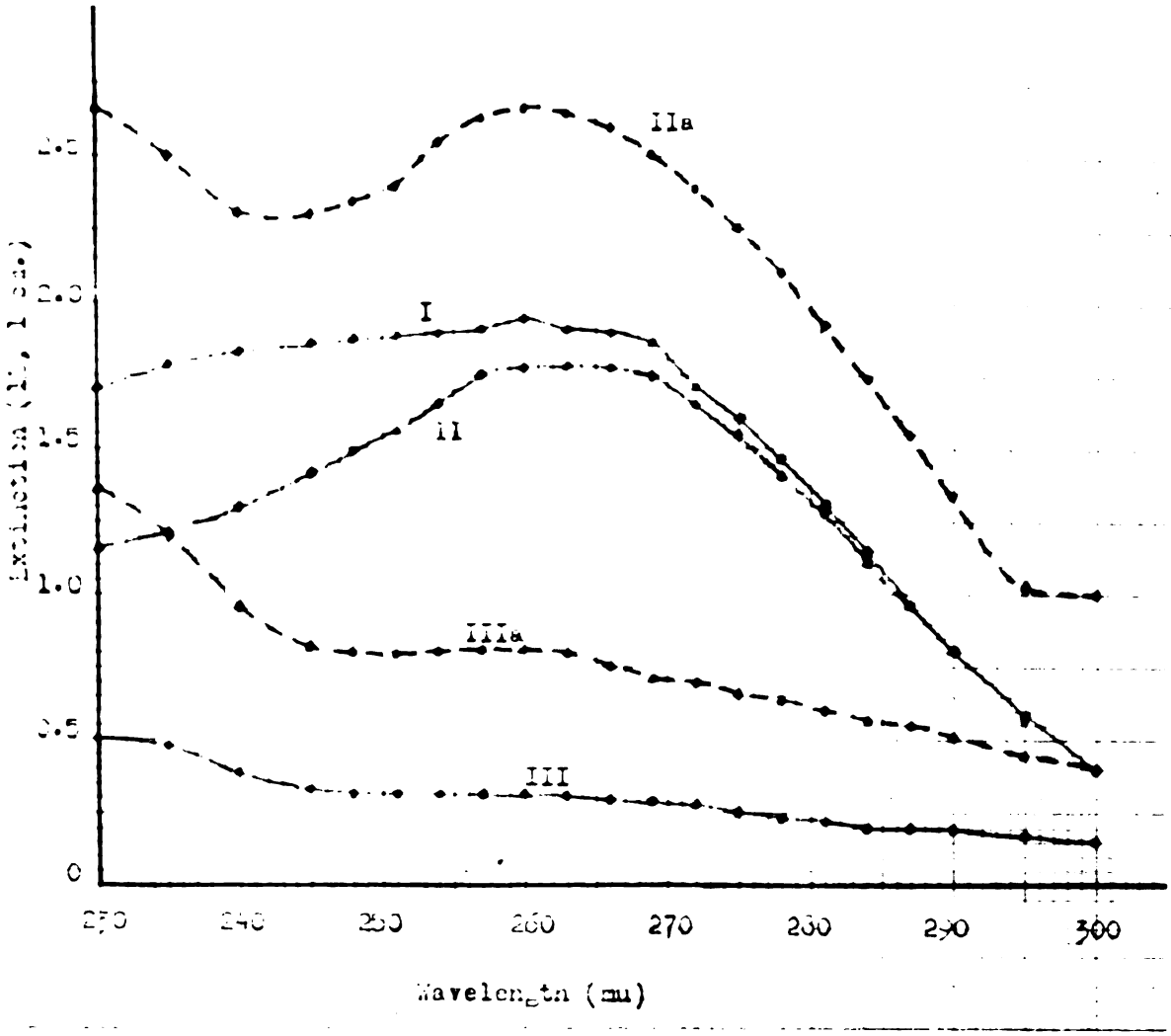


Fig. VI- Extinction (1%, 1 cm.) of Oil No. 3772 in absolute ethyl alcohol plotted against wavelength.

I- Procedure C, Concentration 0.300g./100 ml.

II- Procedure B, Concentration 0.200g./100 ml.
Corrected for absorption of superfiltrol

IIa- Same as II except uncorrected for absorption of Superfiltrol

III- Procedure A (Sterol Correction)
Corrected for absorption of superfiltrol
Concentration 0.432g./100 ml.

IIIa- Same as III except uncorrected for absorption of superfiltrol

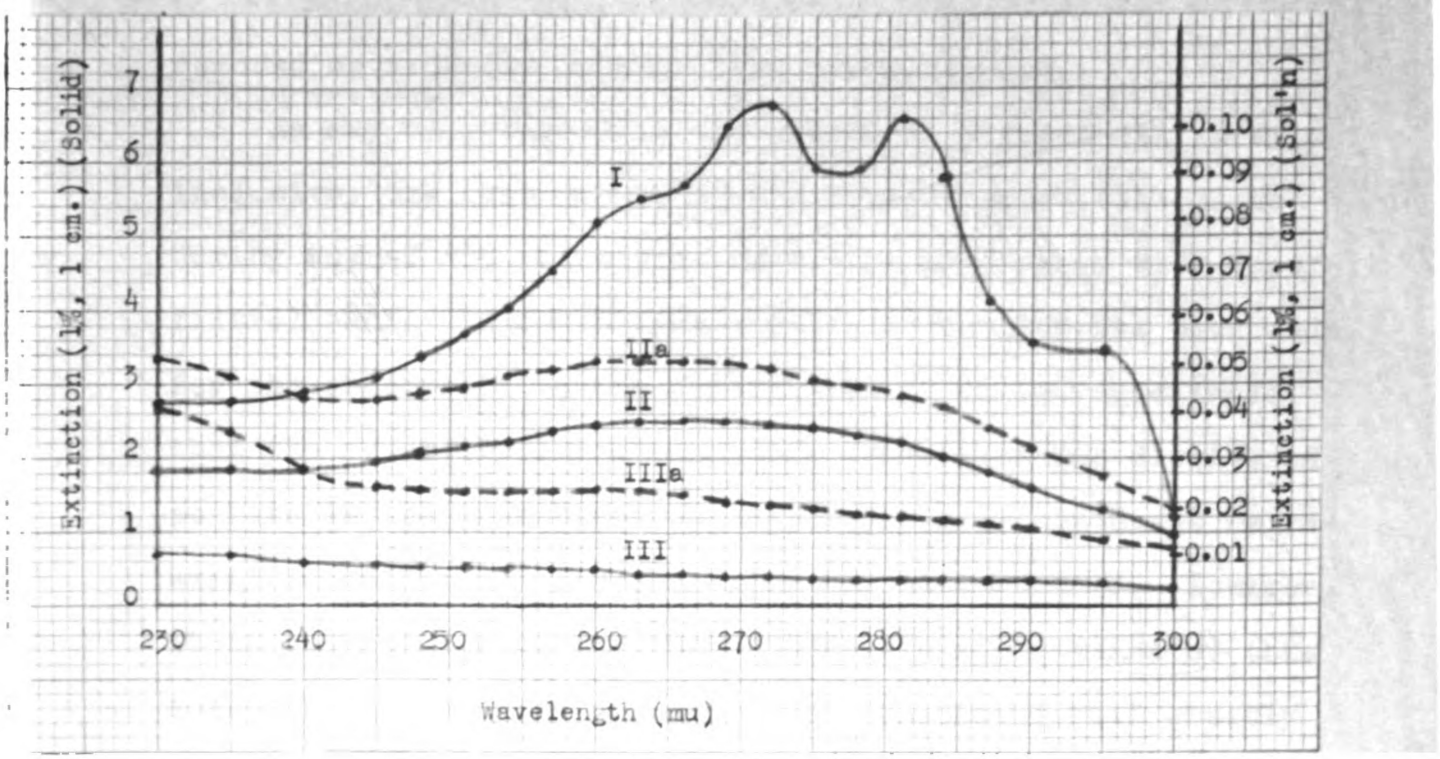


Fig. VII- Extinction (1%, 1 cm.) of a #3772-ergosterol mixture in absolute ethyl alcohol plotted against wavelength.

The original mixture contained 0.3000g. of #3772 and 25 ml. of ergosterol (0.275mg./ml.)

I- Procedure C, Concentration: 0.1021g./100 ml. (Solid)*
6.657g./100 ml. (Sol'n)*

II- Procedure D, Concentration: 0.2206g./100 ml. (Solid)
14.38g./100 ml. (Sol'n)
Corrected for absorption of Superfiltrol

IIa- Same as II except uncorrected for absorption of Superfiltrol

III- Procedure A (Sterol Correction), Concentration: 0.3024g./100 ml. (Solid)
24.92g./100 ml. (Sol'n)
Corrected for absorption of Superfiltrol

IIIa- Same as III except uncorrected for absorption of Superfiltrol

* (Solid) - Grams of #3772 and pure ergosterol/100 ml.
(Sol'n) - Grams of original sol'n/100 ml.

removed as expected by the first chromatograph.

As may be observed in the graphs of Figures III to VII inclusive, the true nature of the changes which take place during any of the procedures involving adsorption with Superfiltrol cannot be determined until a correction has been made for the absorption due to Superfiltrol. It is regrettable that the necessity for making this correction was discovered so late in the investigation that other oils could not be re-examined before the investigation was discontinued. However, it is the opinion of the author that the application of this correction to samples of irradiated ergosterol will greatly decrease the difficulties hitherto encountered in experimentally determining the vitamins D potency of such oils.

It may be concluded from this study that the first chromatograph plays an important part in the separation of calciferol from interfering substances. No correlation between the bioassay value of an oil and its ultra-violet absorption may be made unless the oil has been chromatographed.

SUMMARY OF OIL NO. 3772

Oil No. 3772 is an 160x dilution of calciferol in corn oil, having a potency of 200,000 D units/gram. When samples of irradiated ergosterol were treated, this oil was used as a check in order to verify the purity of the solvents and reagent and the effectiveness of the operations undertaken. Table VI shows the results of measurements of the antimony trichloride extinction for various procedures. Figure VI shows typeical absorption curves in the ultra-violet region for the same procedures.

TABLE VI

Procedure	Sample Weight	E(1%, 1 cm.)	D units/gram	
B	0.060 g.	11.55	223,000	
	0.050 g.	9.23	178,000	
	0.050 g.	9.90	191,000	
	0.050 g.	9.45	183,000	
	0.0600g.	8.80	170,000	
	0.0600g.	9.73	188,000	
	0.0600g.	9.90	191,000	
	0.0600g.	9.73	188,000	
	0.0600g.	9.90	191,000	
	0.0600g.	8.62	167,000	
	0.0500g.	9.45	183,000	
	0.0600g.	8.62	167,000	
	0.0600g.	7.90	152,500	
	0.0500g.	8.36	161,000	
	0.0500g.	9.90	191,000	182,000 av.
C	0.040 g.	9.62	186,000	
	0.060 g.	9.54	184,000	
	0.060 g.	7.61	147,000	
	0.060 g.	8.80	170,000	
	0.060 g.	8.62	167,000	
	0.0600g.	6.97	135,000	
	0.0600g.	8.80	170,000	
	0.0600g.	8.80	170,000	
	0.0600g.	8.25	159,000	

TABLE VI (cont'd.)

Procedure	Sample Weight	E(1%, 1 cm.)	D units/gram	
C	0.0600g.	6.60	127,000	
	0.0500g.	8.36	161,000	
	0.0500g.	8.14	157,000	
	0.0200g.	8.80	170,000	
	0.0500g.	8.80	170,000	
	0.0500g.	9.24	178,000	
	0.0500g.	8.58	166,000	164,000 av.
D	0.060 g.	6.42	124,000	
	0.060 g.	7.89	152,000	
	0.060 g.	7.89	152,000	
	0.0500g.	7.26	140,000	
	0.0500g.	7.26	140,000	142,000 av.

IRRADIATED ERGOSTEROL IN CORN OIL

Perhaps the most extensive phase of this investigation was the study of samples of irradiated ergosterol in corn oil. A large number of samples of this type of oil were run by various procedures, and measurements were made both of the antimony trichloride extinction at 500 mu and of the absorption of the alcoholic solution in the ultra-violet region. Almost all of the samples had been purified to some extent before they reached this laboratory; many were in their final, commercial form. All with but one exception, Ertron, had been irradiated while in solution. Ertron was irradiated while in crystalline form.

As may be observed from Table VII, there is little or no correlation between the bioassay values and experimentally determined D units/gram for Procedure B and only slightly more for Procedure C. In agreement with the conclusions drawn from the study of calciferol and ergosterol, considerable correlation appears to exist between the bioassay values and D units/gram for Procedure D.

Figures VIII to X inclusive show typical ultra-violet absorption curves for samples of irradiated ergosterol in corn oil.

IRRADIATED ERGOSTEROL IN ALCOHOL

A considerable length of time was also devoted to the study of irradiated ergosterol in alcohol. However, in the case of these samples, few, if any, had been purified to any

extent before they reached this laboratory.

Particular attention was paid to oils No. 43573, 43583, 43593, 43603, 45013, 45023, 45033, and 45043. These had been irradiated for different lengths of time; the irradiation times being 60, 45, 33, 15, 15, 33, 45, and 60 minutes respectively. Table VIII shows the results of measurements of the antimony trichloride extinction for the abovementioned oils and those others which fall into the same category. Figures XI and XVIII show the various ultra-violet absorption curves for these oils when run by different procedures. All oils in this group were diluted with absolute ethyl alcohol before treatment. Most were diluted 1 ml. to 100 ml.; oil No. 64174 was diluted 1 ml. to 25 ml.; various dilutions were made of oils No. 96872 and 97182. In Table VIII, the E(1%, 1 cm.) values were calculated for the dilutions, but the D units/gram values are for the original solutions.

IRRADIATED ERGOSTEROL IN FISH OILS

A number of oils of the type irradiated ergosterol in fish oils were treated by various procedures, mainly Procedure A. Table IX shows the results of the measurements made of the antimony trichloride extinctions. As may be seen from the table, Procedure A gives results which are lower than the bioassay values. The Procedure D values are also lower than the bioassay values, but these values divided by the bioassay values and multiplied by 100 fall in very closely with similarly calculated values for samples of irradiated ergosterol in corn

oil (see Table VII). Thus, it would appear that Procedure D, perhaps with some modification, would be the best of those procedures tried so far with which to treat samples of irradiated ergosterol. Obviously, some other factor besides 19,300 would be required; however, once constant results were gained by using this procedure, a different factor would be merely a matter of arithmetic.

A STUDY OF CORN OIL

A brief study was made of corn oil in order to ascertain if it interfered in the antimony trichloride reaction or if it had a significant absorption curve in the ultra-violet region. Table X shows the effect of corn oil and another similar oil on the antimony trichloride extinction; the corn oil used is commercial "Mazola Oil". Figure XIX shows the ultra-violet absorption curves for the corn oil when treated by various procedures.

As Table X indicates, corn oil has a definite effect on the antimony trichloride reaction; however, the effect is quite small when compared to the weight of sample. In agreement with Young (4), it was found by this author that the E(1%, 1 cm.) of corn oil decreased after saponification. This was also true of the other oil tested. In any event, it is not believed that corn oil has an appreciable effect on samples of vitamins D of the potency usually met.

Figure XIX shows that corn oil has a very definite ultra-violet absorption curve. When treated by Procedure B,

measurements cannot usually be taken below 250 mu due to the high absorption of corn oil below that wave length. The absorption of corn oil in the ultra-violet region also decreases after saponification. After the discovery of the necessity for making a correction for Superfiltrol, it was noticed that the ultra-violet absorption curve for corn oil when treated by Procedure D was very similar to that for the correction. Consequently, it may be assumed that corn oil is largely, if not completely, removed by treatment with Procedure D.

STUDY OF SUDAN III

When attention was first focused on ultra-violet absorption measurements, it was decided to determine if the dye Sudan III, used as a marker for the first chromatograph in Kingsley's method, had an appreciable absorption of its own. As can be seen from Figure XX, the dye has an absorption significant enough to prohibit its use in the adsorption column. Consequently, the orange-colored corn oil layer in the adsorption column was used to determine where the column should be cut for elution with ethyl ether. Previous investigation had demonstrated that the corn oil and Sudan III layers occur in the same place in the adsorption column.

TABLE VII

Oil No.	Procedure	Sample Weight	E(1%,1cm.)	D u/g	Bioassay	D u/g x 100 / Bioassay
Hg 2	B	0.040 g.	23.9	462,000	450,000	102.6
Hg 3	B	0.040 g.	23.9	462,000	450,000	102.6
Sample F	B	0.060 g.	27.9	538,000	440,000	122.5
45120	B	0.050 g.	8.24	159,000	250,000	63.6
	B	0.060 g.	8.71	168,000		67.2
61691	B	0.060 g.	6.05	117,000	200,000	58.5
65751	B	0.050 g.	10.32	199,000	250,000 to 275,000	
	B	0.060 g.	10.73	207,000		
13192	B	0.050 g.	9.89	191,000	250,000	76.4
	B	0.060 g.	9.63	185,000		74.0
78272	B	0.060 g.	8.06	157,000	225,000	69.8
	B	0.060 g.	8.53	164,000		73.0
84742	B	0.060 g.	11.57	224,000	275,000	81.5
	B	0.060 g.	11.65	225,000		81.8
15153	B	0.060 g.	10.10	195,000	179,000 to 196,000	
34983	B	0.0700g.	8.33	161,000	275,000	58.7
	B	0.0600g.	8.80	170,000		61.8
35563	B	0.0600g.	10.10	195,000	250,000	78.0
	B	0.0600g.	10.88	210,000		84.0
36323	B	0.0600g.	8.62	166,000	275,000	60.4
	B	0.0600g.	8.43	163,000		59.3
36962	B	0.0700g.	8.48	164,000	200,000	82.0
	B	0.0700g.	8.17	158,000		79.0
38133	B	0.0700g.	7.40	143,000	125,000	114.4

TABLE VII (cont'd.)

Oil No.	Procedure	Sample Weight	E(1%,1cm.)	D u/g	Bioassay	D u/g x 100 / Bioassay
	B	0.0600g.	8.25	159,000		127.2
42943	B	0.0700g.	8.96	173,000	160,000	108.0
	B	0.0600g.	8.80	170,000		106.3
42953	B	0.0600g.	9.17	177,000	175,000	101.0
	B	0.0600g.	8.80	170,000		97.1
42963	B	0.0700g.	6.60	128,000	150,000	84.6
	B	0.0700g.	6.77	131,000		87.3
Ba 103	B	0.015 g.	48.1	929,000	920,000	101.0
	B	0.030 g.	46.9	905,000		98.4
	B	0.010 g.	45.5	880,000		95.7
Bb 103	B	0.040 g.	28.7	554,000	500,000	110.8
	B	0.030 g.	28.9	558,000		111.6
	B	0.020 g.	28.9	558,000		111.6
Bc 103	B	0.035 g.	24.4	471,000	480,000	96.0
	B	0.025 g.	25.3	488,000		101.5
Bd 103	B	0.010 g.	75.8	1,460,000	1,000,000	146.0
	B	0.010 g.	80.2	1,550,000		155.0
	B	0.0100g.	74.8	1,440,000		144.0
Be 103	B	0.030 g.	41.9	810,000	775,000	104.5
	B	0.015 g.	42.8	827,000		106.7
	B	0.015 g.	45.8	884,000		114.0
Bf 103	B	0.020 g.	47.5	917,000	920,000	99.5
	B	0.010 g.	47.3	915,000		99.3
Bg 103	B	0.020 g.	35.5	685,000	625,000	109.5
	B	0.020 g.	36.6	706,000		113.0

TABLE VII (cont'd.)

Oil No.	Procedure	Sample Weight	E(1%,1 cm.)	D u/g	Bioassay	D u/g x 100 / Bioassay
9031	B	0.0200g.	34.7	670,000		
	B	0.0200g.	33.0	637,000		
	B	0.0200g.	30.3	585,000		
B 6727	B	0.0200g.	35.2	678,000		
	B	0.0200g.	34.7	670,000		
	B	0.0200g.	30.8	595,000		
B 6851	B	0.0200g.	28.6	552,000		
	B	0.0200g.	28.6	552,000		
	B	0.0200g.	25.9	500,000		
B 6975	B	0.0200g.	28.1	542,000		
	B	0.0200g.	27.5	530,000		
	B	0.0200g.	25.3	488,000		
A 14290	B	0.0100g.	79.2	1,530,000		
Ertron	B	0.0128g.	43.2	834,000		
	B	0.0106g.	44.4	857,000		
50254	B	0.0500g.	10.56	204,000		
	B	0.0500g.	11.12	217,000		
59824	B	0.0250g.	29.1	562,000		
	B	0.0200g.	31.3	605,000		
Hg 2	C	0.040 g.	19.8	381,000	450,000	84.6
Hg 3	C	0.040 g.	18.4	356,000	450,000	79.1
Sample F	C	0.060 g.	23.3	450,000	440,000	102.3
45120	C	0.050 g.	7.47	144,000	250,000	57.6
	C	0.060 g.	6.05	117,000		46.8
61691	C	0.060 g.	5.04	97,000	200,000	48.5

TABLE VII (cont'd.)

Oil No.	Procedure	Sample Weight	E(1%,1 cm.)	D u/g	Bioassay	D u/g x 100 / Bioassay
65751	C	0.050 g.	10.65	205,000	250,000 to 275,000	
	C	0.060 g.	8.98	174,000		
13192	C	0.050 g.	9.24	178,000	250,000	71.2
	C	0.060 g.	7.88	152,000		60.8
78272	C	0.060 g.	7.96	154,000	225,000	68.5
	C	0.060 g.	7.06	136,000		60.5
84742	C	0.060 g.	11.57	224,000	275,000	81.5
	C	0.060 g.	9.52	184,000		67.0
15153	C	0.060 g.	8.45	163,000	179,000 to 196,000	
	C	0.060 g.	8.65	167,000		
34983	C	0.0600g.	5.87	113,000	275,000	41.2
	C	0.0600g.	6.42	124,000		45.1
35563	C	0.0600g.	7.80	151,000	250,000	60.4
	C	0.0600g.	8.06	155,000		62.0
36323	C	0.0600g.	6.23	120,000	275,000	43.7
	C	0.0600g.	7.15	138,000		50.2
36963	C	0.0600g.	5.50	106,000	200,000	53.0
	C	0.0600g.	6.28	121,000		79.0
38133	C	0.0600g.	5.50	106,000	125,000	84.8
	C	0.0600g.	6.05	117,000		93.6
42943	C	0.0600g.	6.43	124,000	160,000	77.5
	C	0.0600g.	6.97	134,000		83.8
	C	0.0500g.	7.92	153,000		95.6
	C	0.0500g.	7.26	140,000		87.5

TABLE VII (cont'd.)

Oil No.	Procedure	Sample Weight	E(1%,1 cm.)	D u/g	Bioassay	D u/g x 100 / Bioassay
42953	C	0.0600g.	6.60	128,000	175,000	73.2
	C	0.0600g.	6.97	134,000		76.5
	C	0.0500g.	7.04	135,000		77.1
	C	0.0500g.	7.04	135,000		77.1
42963	C	0.0500g.	5.06	98,000	150,000	65.3
	C	0.0700g.	5.82	112,000		74.7
	C	0.0500g.	6.60	127,000		85.3
Ba 103	C	0.020 g.	34.6	687,000	920,000	74.7
	C	0.020 g.	38.5	743,000		80.8
	C	0.020 g.	36.9	712,000		77.3
Bb 103	C	0.030 g.	21.3	410,000	500,000	82.0
	C	0.030 g.	26.1	504,000		100.8
Bc 103	C	0.035 g.	18.9	364,000	480,000	75.8
	C	0.040 g.	20.1	388,000		80.8
	C	0.040 g.	20.1	388,000		80.8
Bd 103	C	0.010 g.	51.7	997,000	1,000,000	99.7
	C	0.010 g.	52.8	1,020,000		102.0
	C	0.010 g.	52.8	1,020,000		102.0
Be 103	C	0.020 g.	30.2	584,000	775,000	75.4
	C	0.020 g.	33.0	637,000		82.2
	C	0.020 g.	38.0	733,000		94.6
Bf 103	C	0.020 g.	31.3	605,000	920,000	65.7
	C	0.020 g.	38.5	743,000		79.7
Bg 103	C	0.020 g.	26.9	520,000	625,000	83.2
	C	0.020 g.	31.4	607,000		97.0

TABLE VII (cont'd.)

Oil No.	Procedure	Sample Weight	E(1%,1 cm.)	D u/g	Bioassay	D u/g x 100 / Bioassay
	C	0.020 g.	28.6	552,000		88.3
9031	C	0.0200g.	19.3	373,000		
B 6727	C	0.0200g.	21.4	413,000		
B 6851	C	0.0300g.	22.0	424,000		
B 6975	C	0.0300g.	17.6	339,000		
A 14290	C	0.0100g.	62.7	1,210,000		
Ertron	C	0.0213g.	35.2	680,000		
	C	0.0213g.	35.7	688,000		
52054	C	0.0600g.	10.10	195,000		
	C	0.0600g.	9.90	191,000		
59824	C	0.0200g.	28.0	542,000		
	C	0.0200g.	28.0	542,000		
63824	C	0.0500g.	10.78	208,000		
	C	0.0500g.	11.22	214,000		
63834	C	0.0500g.	3.30	63,500		
	C	0.1000g.	2.42	46,500		
Hg 2	D	0.040 g.	13.75	265,000	450,000	58.9
Hg 3	D	0.040 g.	11.55	223,000	450,000	49.5
Sample F	D	0.060 g.	17.8	344,000	440,000	78.2
Ba 103	D	0.020 g.	29.2	563,000	920,000	61.2
	D	0.020 g.	29.7	573,000		62.3
Bb 103	D	0.030 g.	16.5	318,000	500,000	63.6
Bc 103	D	0.040 g.	13.6	262,000	480,000	54.5
	D	0.040 g.	14.3	276,000		57.5
Bd 103	D	0.010 g.	42.9	830,000	1,000,000	83.0

TABLE VII (cont'd.)

Oil No.	Procedure	Sample Weight	E(1%,1 cm.)	D u/g	Bioassay	D u/g x 100 / Bioassay
Be 103	D	0.020 g.	23.1	446,000	775,000	57.5
	D	0.020 g.	27.5	530,000		68.4
Bf 103	D	0.020 g.	31.4	606,000	920,000	65.8
Bg 103	D	0.020 g.	18.7	366,000	625,000	57.7
	D	0.020 g.	20.9	403,000		64.6
Ertron	D	0.0213g.	29.5	570,000		
	D	0.0213g.	30.0	578,000		

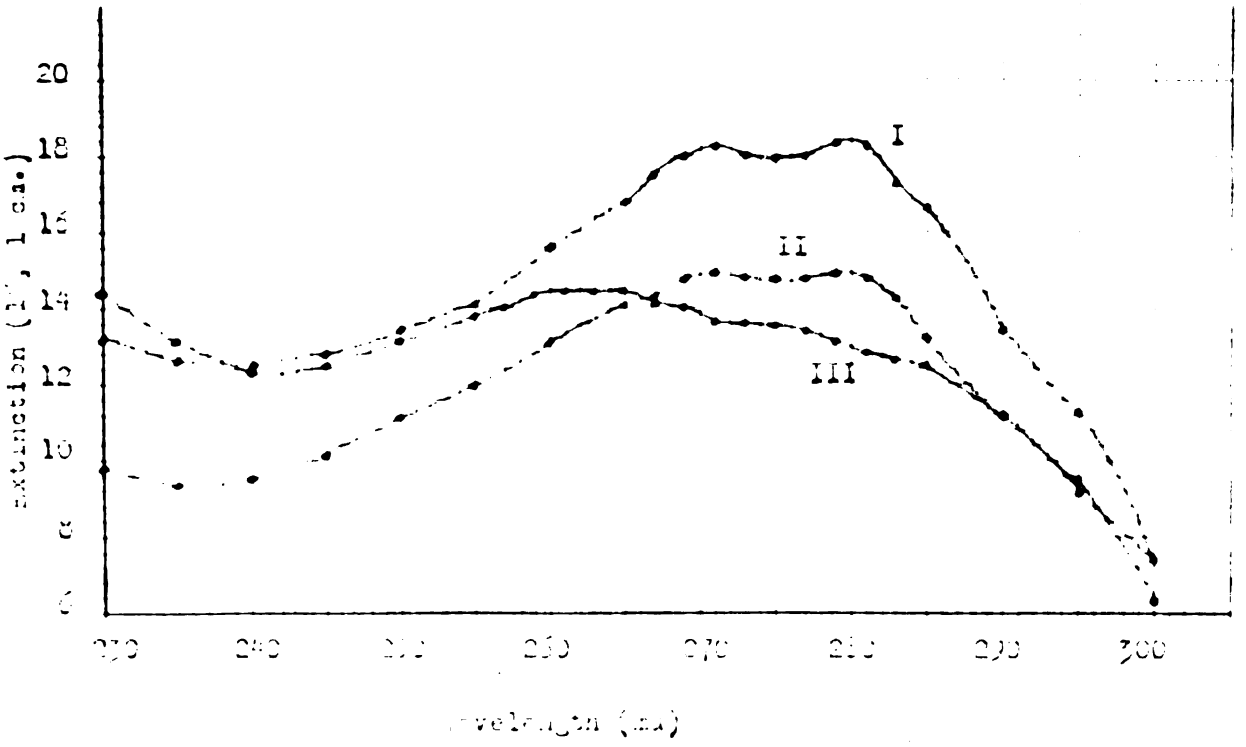


Fig. XIII- Extinction (E', 1 cm.) of iron in absolute ethyl alcohol plotted against wavelength.

I- [illegible], Concentration 0.[illegible] γ/1 ml.

II- [illegible], Concentration 0.[illegible] γ/1 ml.

III- [illegible], Concentration 0.[illegible] γ/1 ml.

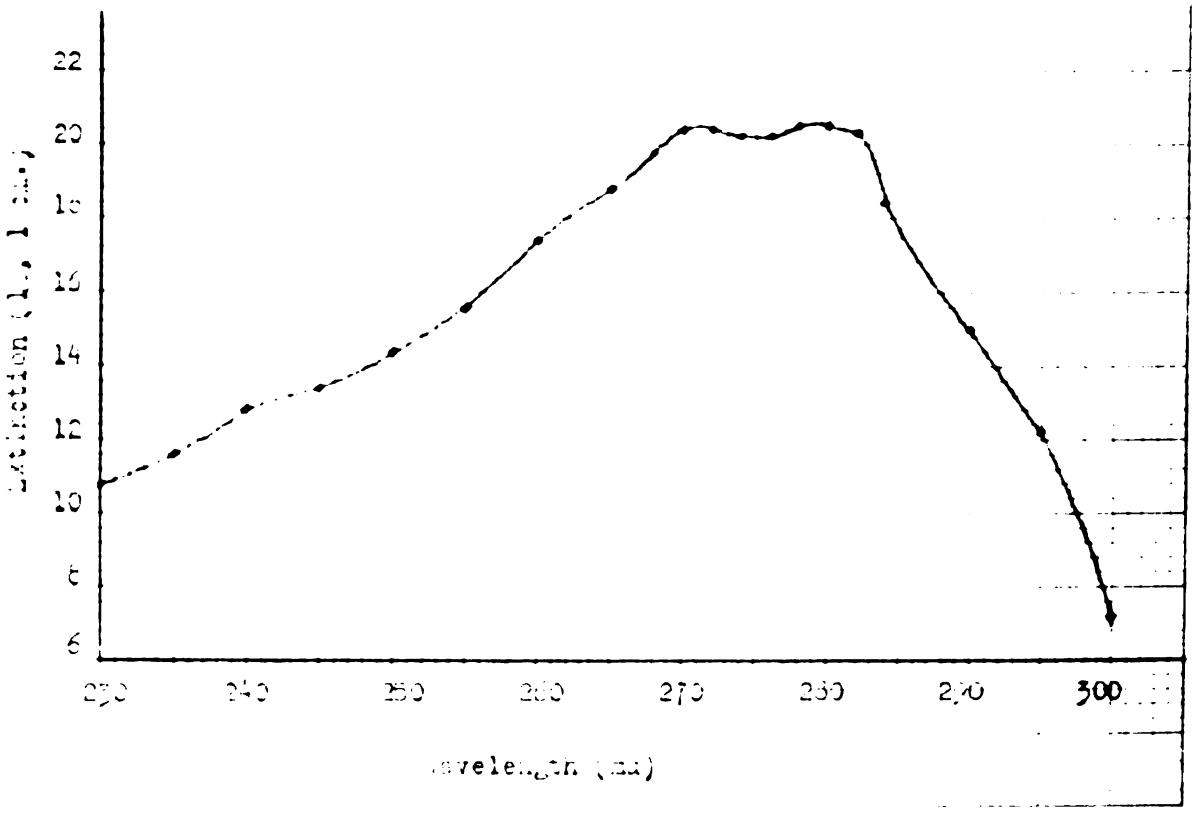


Fig. IA- Extinction (1%, 1 cm.) of Oil No. A 14290 in absolute ethyl alcohol plotted against wavelength. Treated by Procedure C. Concentration 0.0799 g./100 ml.

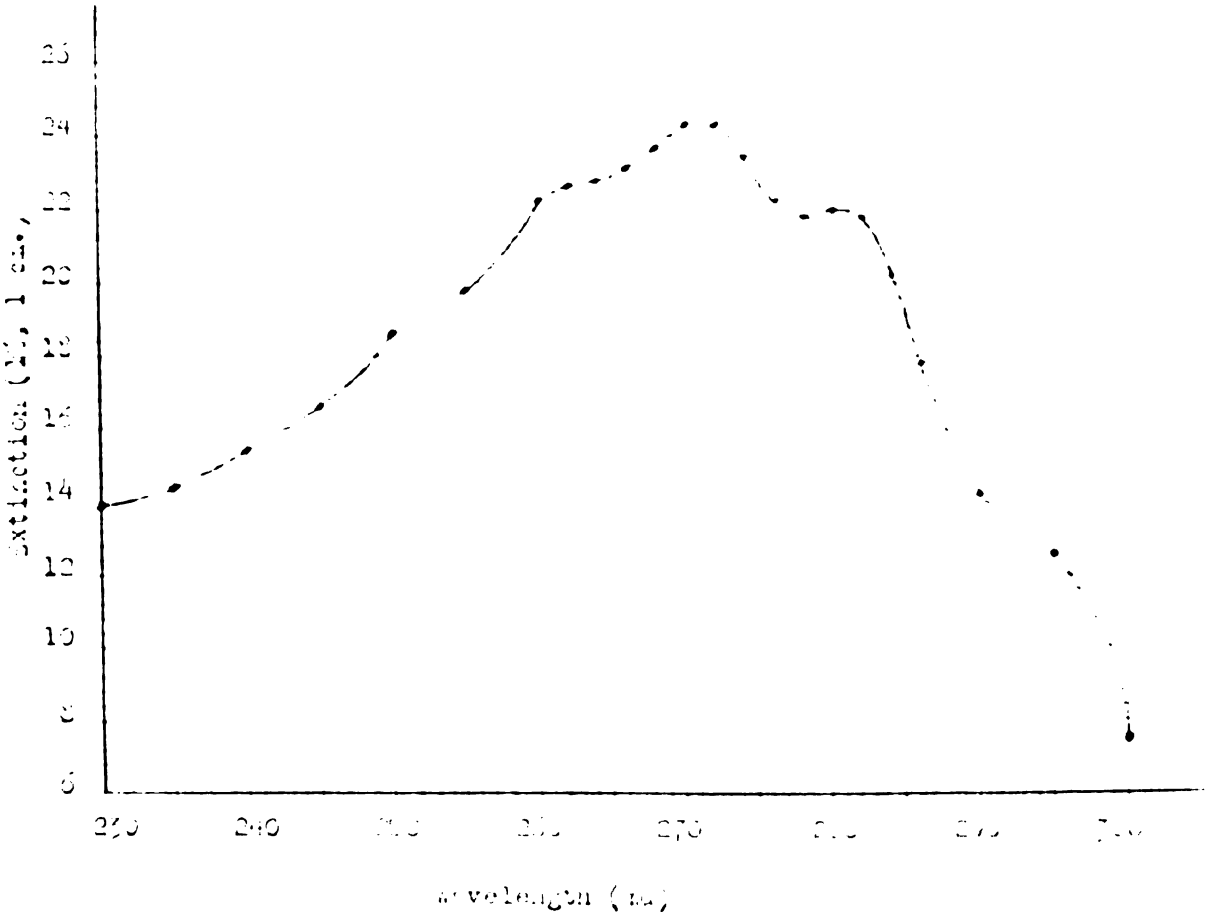


Fig. X- Extinction (1%, 1 cm., of Oil No. 8a 10% in absolute ethyl alcohol plotted against wavelength. Treated by procedure B. Concentration 0.0333 g./100 ml.

TABLE VIII

Oil No.	Procedure	Sample Amount	E(1%, 1 cm.)	D units/gram	Bioassay
96872	B	1 ml.	0.977	1,430,000	1,000,000 to 1,250,000
	B	1 ml.	0.985	1,440,000	
	B	1 ml.	0.861	1,230,000	
	B	1 ml.	1.065	1,560,000	
	C	1 ml.	0.574	840,000	
	C	1 ml.	0.910	1,340,000	
	C	1 ml.	0.836	1,226,000	
	C	1 ml.	0.863	1,265,000	
	C	1 ml.	0.917	1,340,000	
	C	1 ml.	0.714	1,280,000	
	C	0.4 ml.	2.17	1,300,000	
	C	0.4 ml.	2.105	1,260,000	
	E	1 ml.	0.985	1,440,000	
	E	1 ml.	0.920	1,350,000	
	F	1 ml.	0.310	371,000	
	F	1 ml.	0.310	371,000	
97182	B	1 ml.	0.593	860,000	1,000,000
	B	1 ml.	0.566	830,000	
	B	1 ml.	0.675	810,000	
	C	1 ml.	0.546	801,000	
	C	1 ml.	0.492	722,000	
	C	1 ml.	0.468	690,000	
	C	1 ml.	0.478	701,000	
	F	2 ml.	0.1347	161,000	
	F	2 ml.	0.1550	185,000	

TABLE VIII (cont'd.)

Oil No.	Procedure	Sample Amount	E(1%, 1 cm.)	D units/gram	Bioassay
64174	B-F	0.0314g.	23.5	455,000	
43573	B	1 ml.	0.392	757,000	
	B	2 ml.	0.364	703,000	
	B	2 ml.	0.378	730,000	
	C	3 ml.	0.126	243,000	
	C	3 ml.	0.160	308,000	
	C	3 ml.	0.160	308,000	
	E	2 ml.	0.245	473,000	
	E	2 ml.	0.189	365,000	
43583	B	1 ml.	0.770	1,485,000	
	B	1 ml.	0.770	1,485,000	
	C	1 ml.	0.588	1,135,000	
	C	1 ml.	0.588	1,135,000	
	C	1 ml.	0.588	1,135,000	
	E	1 ml.	0.532	1,025,000	
	E	1 ml.	0.448	865,000	
43593	B	1 ml.	0.518	1,000,000	
	B	1 ml.	0.742	1,435,000	
	B	1 ml.	0.728	1,405,000	
	C	1 ml.	0.588	1,135,000	
	C	1 ml.	0.560	1,080,000	
	C	1 ml.	0.546	1,055,000	
	E	1 ml.	0.532	1,025,000	
	E	1 ml.	0.407	785,000	

TABLE VIII (cont'd.)

Oil No.	Procedure	Sample Amount	E(1%, 1 cm.)	D units/gram	Bioassay
43603	B	1 ml.	0.504	973,000	
	B	1 ml.	0.532	1,025,000	
43603	C	2 ml.	0.392	757,000	
	C	2 ml.	0.364	703,000	
	C	2 ml.	0.371	716,000	
	E	2 ml.	0.280	540,000	
	E	2 ml.	0.273	527,000	
45013	B	2 ml.	0.385	743,000	
	B	1 ml.	0.392	756,000	
	C	2 ml.	0.301	581,000	
	C	2 ml.	0.287	553,000	
	E	2 ml.	0.308	595,000	
	E	2 ml.	0.315	608,000	
45023	B	1 ml.	0.407	785,000	
	B	1 ml.	0.434	838,000	
	C	2 ml.	0.294	567,000	
	C	2 ml.	0.294	567,000	
	E	2 ml.	0.336	648,000	
	E	2 ml.	0.301	582,000	
45033	B	1 ml.	0.770	1,485,000	
	B	1 ml.	0.743	1,435,000	
	C	1 ml.	0.602	1,162,000	
	C	1 ml.	0.588	1,134,000	
	E	1 ml.	0.532	1,025,000	
	E	1 ml.	0.490	945,000	

TABLE VIII (cont'd.)

Oil No.	Procedure	Sample Amount	E(1%, 1 cm.)	D units/gram	Bioassay
45043	B	1 ml.	0.925	1,785,000	
	B	1 ml.	0.910	1,755,000	
	C	1 ml.	0.770	1,485,000	
	C	1 ml.	0.742	1,430,000	
	E	1 ml.	0.617	1,190,000	
	E	1 ml.	0.562	1,085,000	

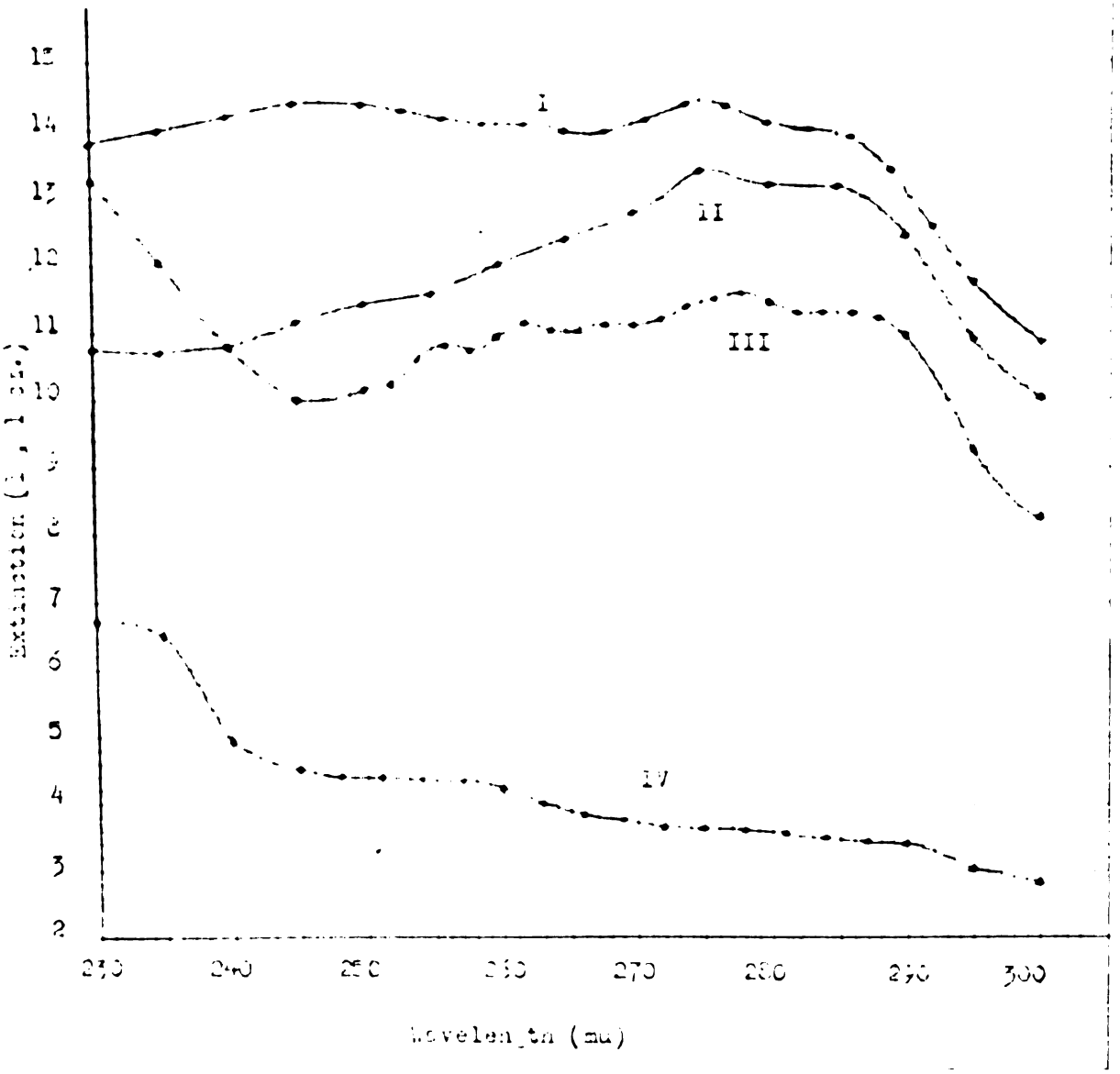


Fig. XI- Extinction (1%, 1 cm.) of Oil No. 43573 plotted against wavelength.

I- Procedure B, Concentration 0.0306g./100 ml. in absolute ethyl alcohol

II- Procedure C, Concentration 0.04575g./100 ml. in absolute ethyl alcohol

III- Procedure E, Concentration 0.0540g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.162g./100 ml. in absolute ethyl alcohol

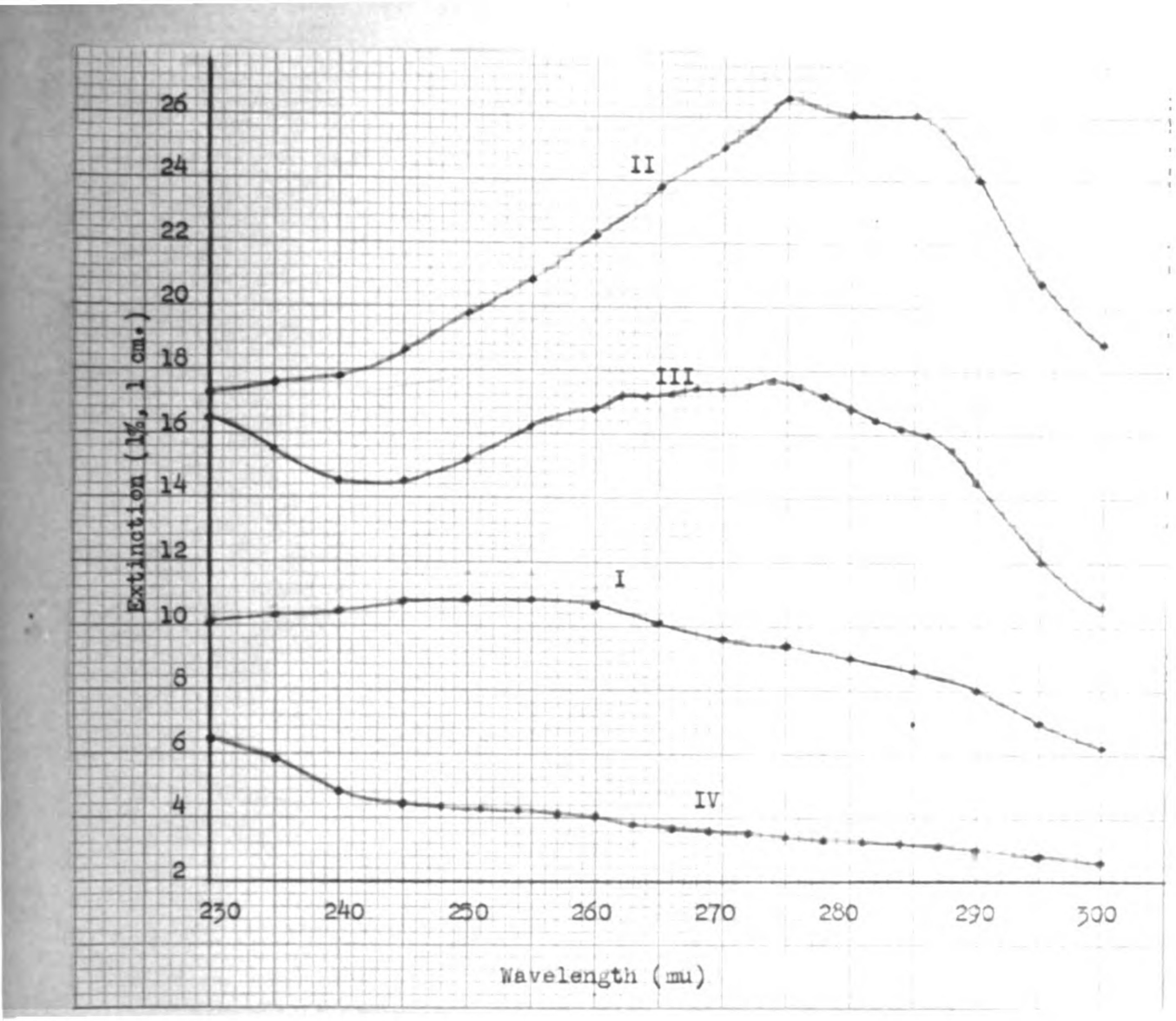


Fig. XII- Extinction (1%, 1 cm.) of Oil No. 43583 plotted against wavelength.

I- Procedure B, Concentration 0.0735g./100 ml. in 95% ethyl alcohol

II- Procedure C, Concentration 0.0277g./100 ml. in absolute ethyl alcohol

III- Procedure E, Concentration 0.0416g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.1664g./100 ml. in absolute ethyl alcohol

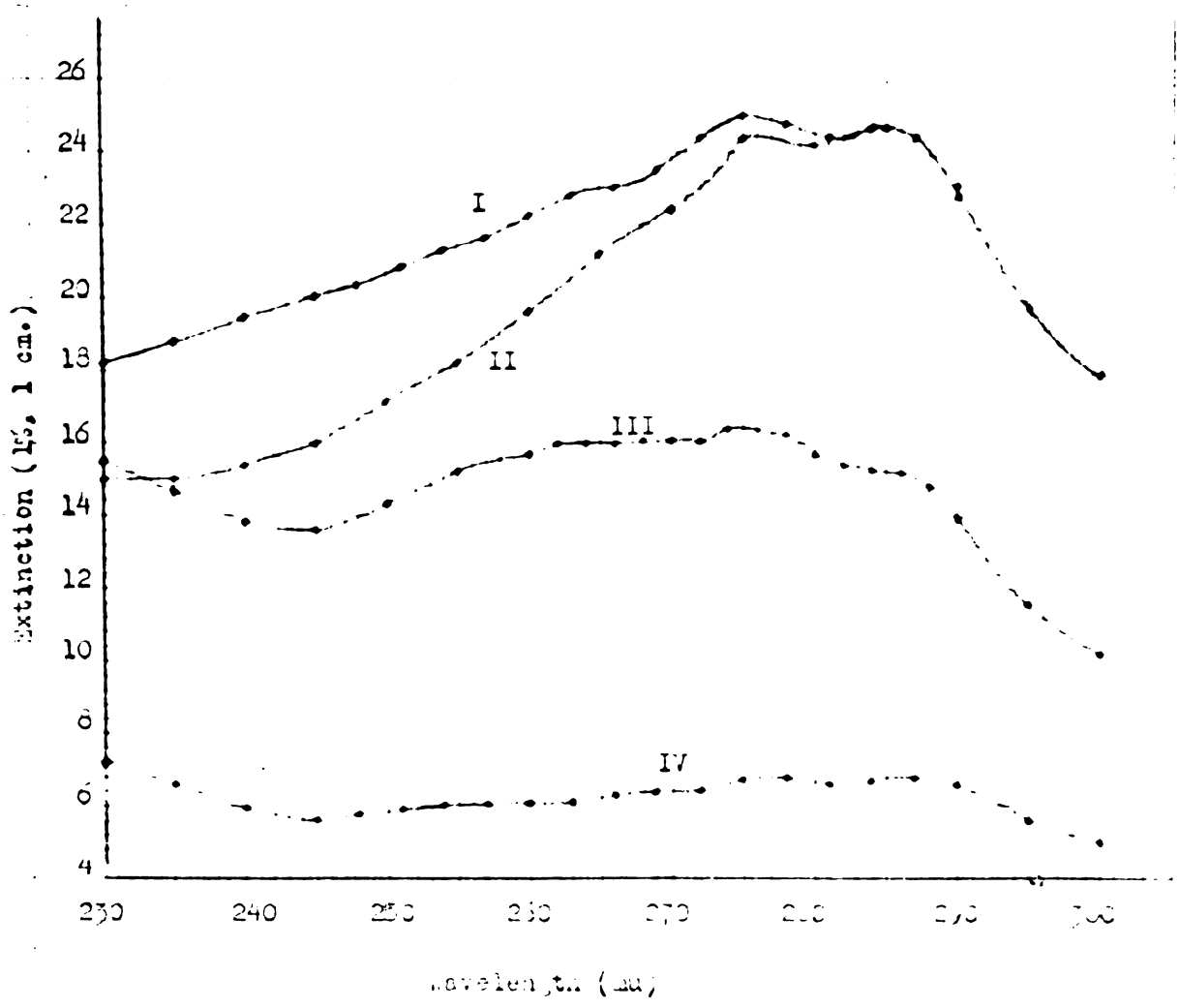


Fig. XIII- Extinction (E, 1 cm.) of Oil No. 4[illegible] plotted against wavelength.

I- Procedure B, Concentration 0.[illegible]g./100 ml. in absolute ethyl alcohol

II- Procedure C, Concentration 0.0[illegible]g./100 ml. in absolute ethyl alcohol

III- Procedure E, Concentration 0.0[illegible]g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.0[illegible]g./100 ml. in absolute ethyl alcohol

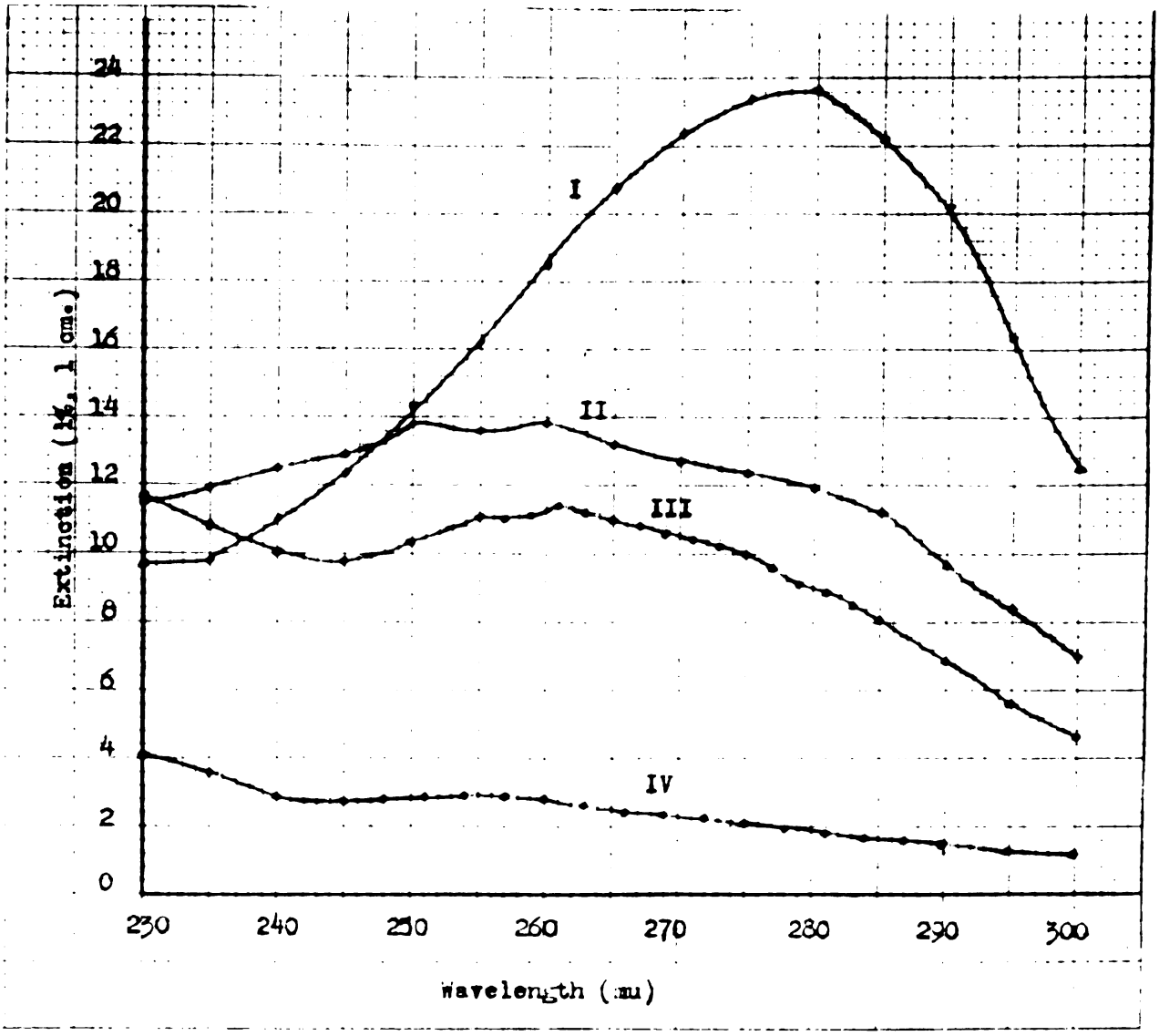


Fig. XIV- Extinction (1%, 1 cm.) of Oil No. 43603 plotted against wavelength.

I- Procedure B, Concentration 0.0201g./100 ml. in 95% ethyl alcohol

II- Procedure C, Concentration 0.04125g./100 ml. in absolute ethyl alcohol

III- Procedure E, Concentration 0.0550g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.1650g./100 ml. in absolute ethyl alcohol

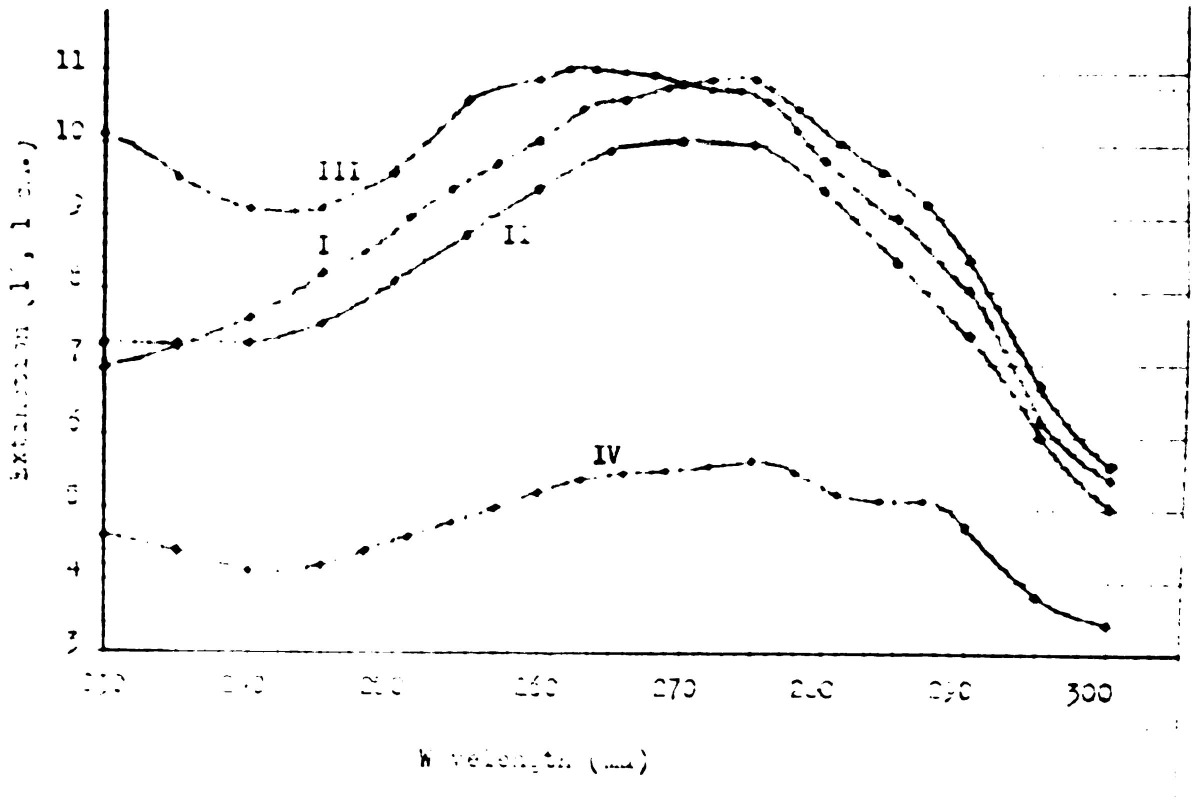


Fig. [illegible] Extinction (1 [illegible], 1 cm.) of oil No. [illegible] plotted against [illegible]

I- [illegible], [illegible] 0.0710[illegible] 100 ml. in absolute [illegible]

II- [illegible] 100 ml. in absolute [illegible]

III- [illegible] 100 ml. in absolute [illegible]

IV- [illegible] 100 ml. in absolute [illegible]

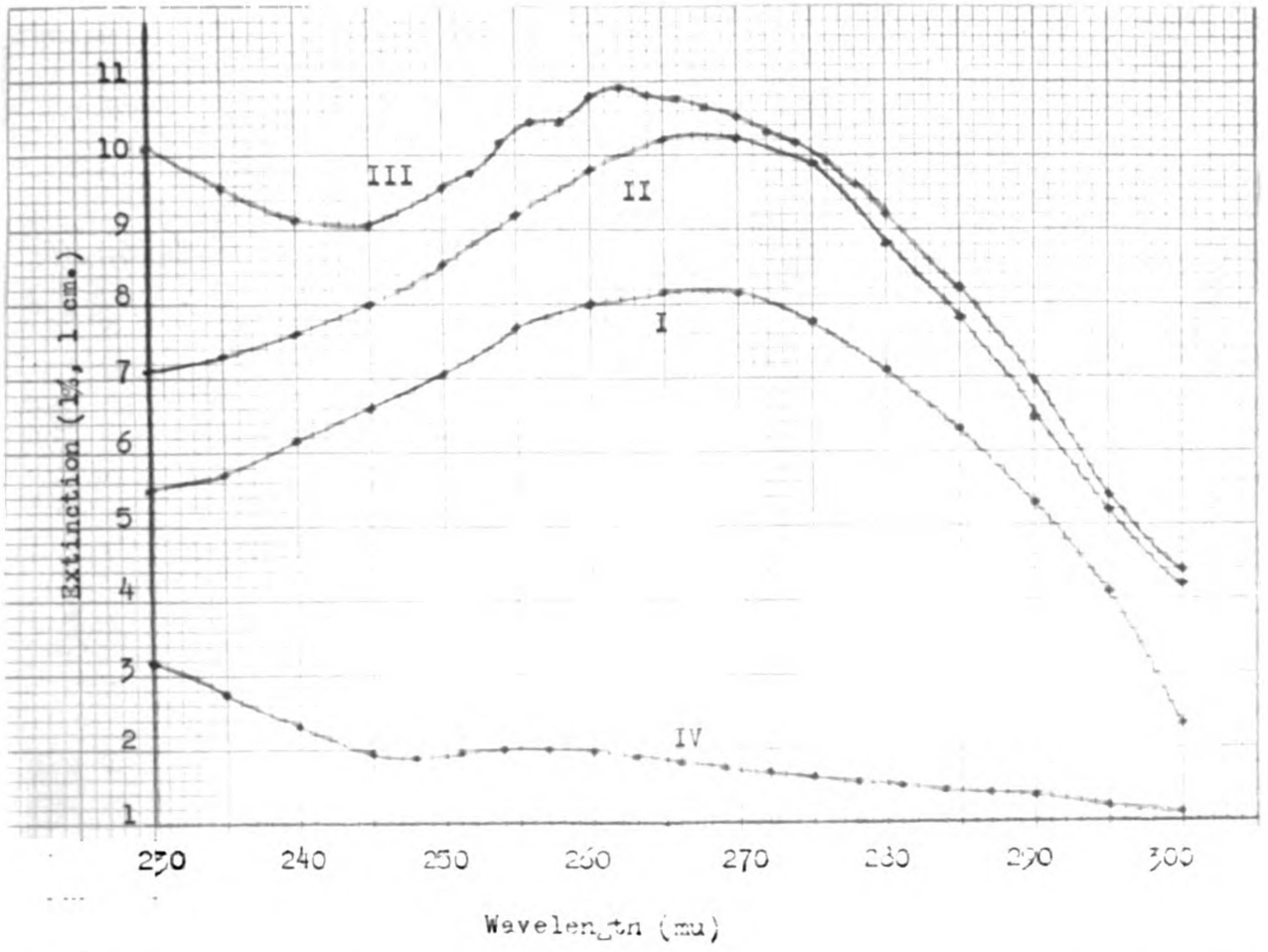


Fig. XVI- Extinction (1%, 1 cm.) of Oil No. 45023 plotted against wavelength.

I- Procedure B, Concentration 0.0739g./100 ml. in 95% ethyl alcohol

II- Procedure C, Concentration 0.0[illegible]25g./100 ml. in absolute ethyl alcohol

III- Procedure E, Concentration 0.0[illegible]3g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.1044g./100 ml. in absolute ethyl alcohol

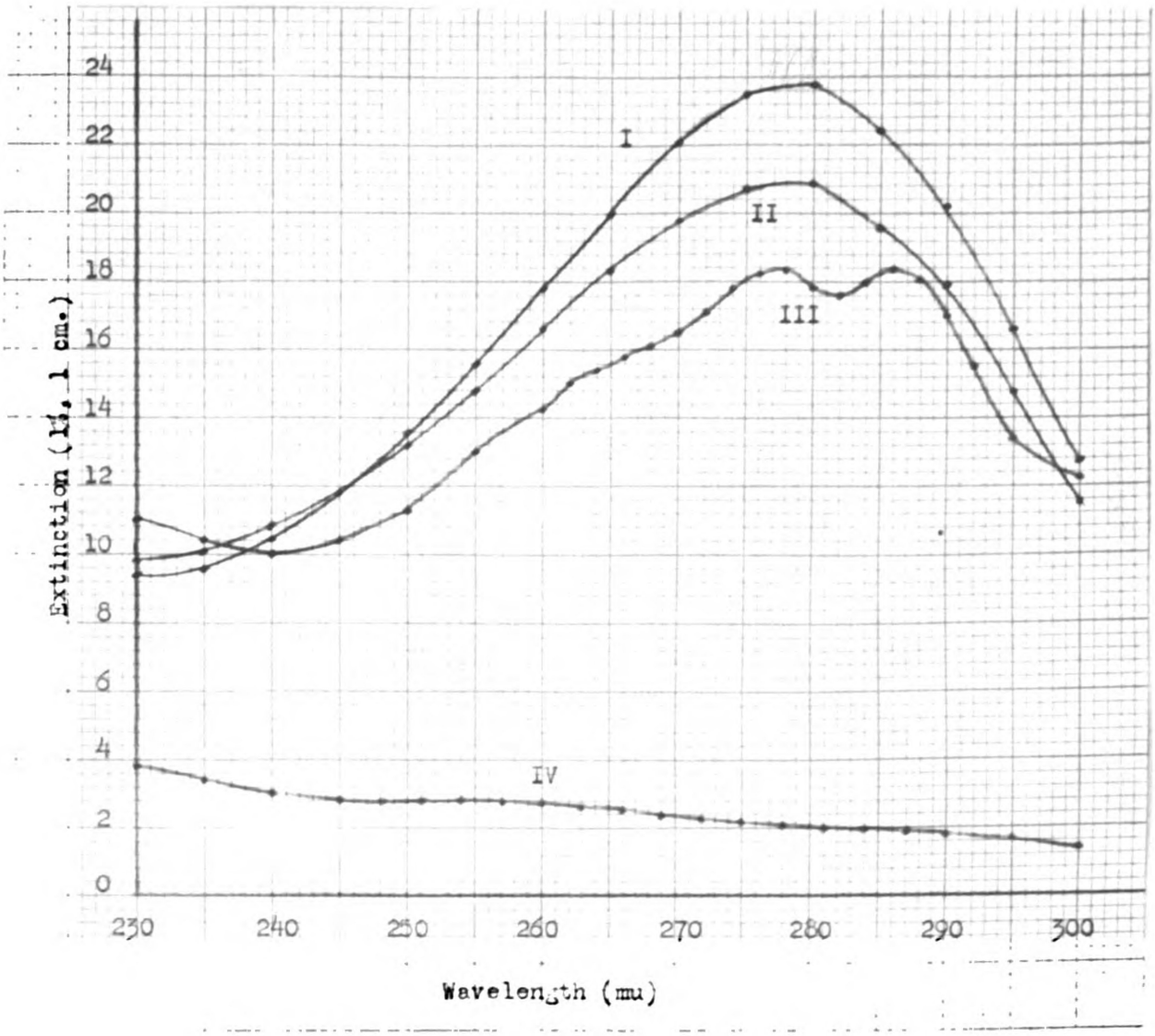


Fig. XVII- Extinction (1%, 1 cm.) of Oil No. 45033 plotted against wavelength.

I- Procedure B, Concentration 0.0247g./100 ml. in 95% ethyl alcohol

II- Procedure C, Concentration 0.0271g./100 ml. in absolute ethyl alcohol

III- Procedure E, Concentration 0.04065g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.1626g./100 ml. in absolute ethyl alcohol

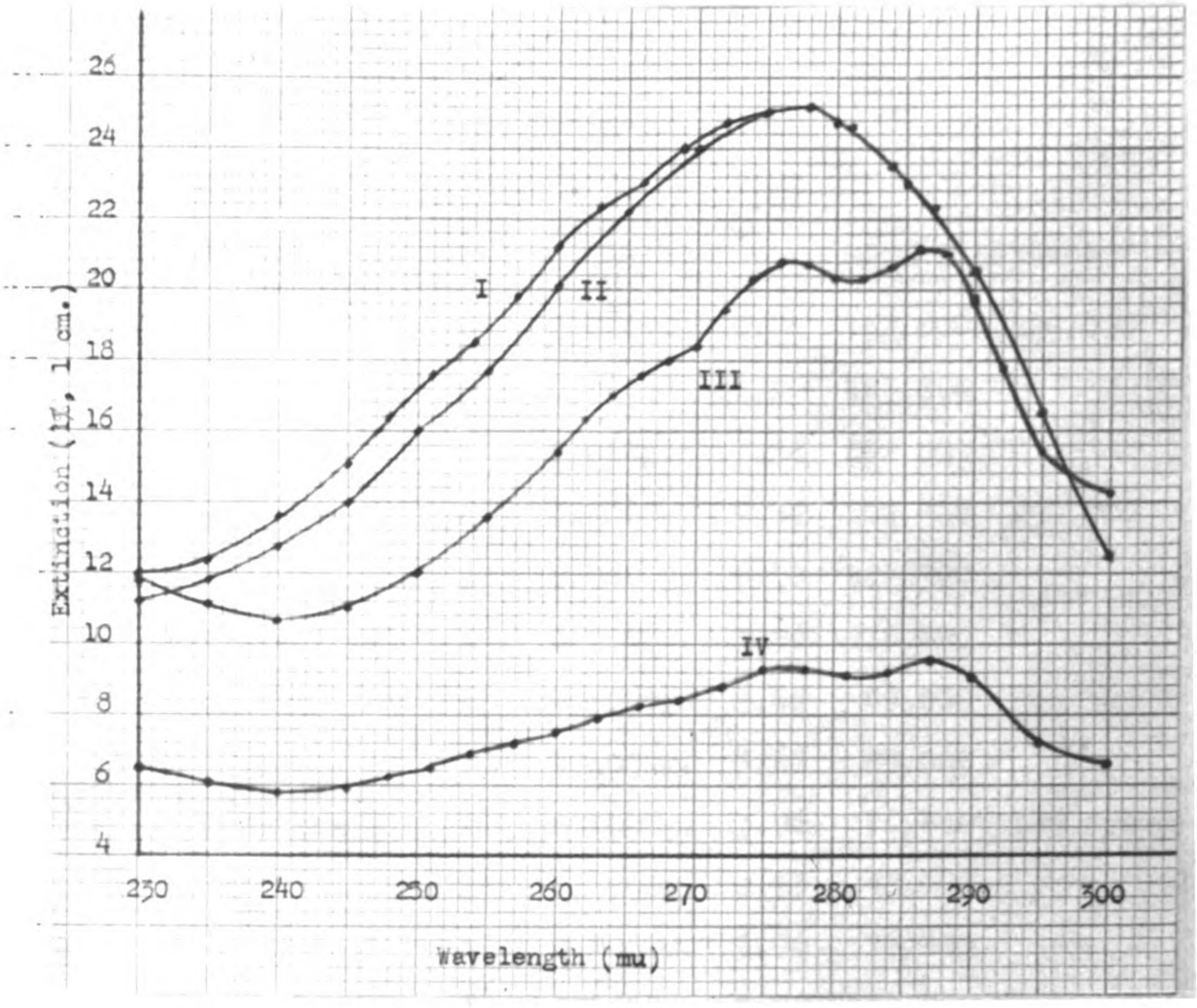


Fig. XVIII- Extinction (1%, 1 cm.) of Oil No. 45043 plotted against wavelength.

I- Procedure B, Concentration 0.0330g./100 ml. in absolute ethyl alcohol

II- Procedure C, Concentration 0.0275g./100 ml. in absolute ethyl alcohol

III- Procedure E, Concentration 0.0330g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.0661g./100 ml. in absolute ethyl alcohol

11 50

3391

4523

3783

3215

4071

4302

4318

600

TABLE IX

Oil No.	Type of Fish Oil	Procedure	Sample Weight	$E^{1\%}_{1cm}$	D u/g	Bioassay	D u/gx100 / Bioassay
56391	Halibut Liver Oil	B	0.060g.	23.5	453,000	225,000	201.5
		C	0.060g.	21.1	407,000		181.0
		D	0.060g.	7.88	152,000		67.5
34523	Cod Liver Oil	A	0.5000g.	5.32	102,500	250,000	41.0
		D	0.5000g.	6.60	127,500		51.0
35783	Halibut Liver Oil	A	0.1000g.	3.96	76,400		
		A	0.1000g.	4.62	89,000		
		A	0.1000g.	4.62	89,000		
39215	Fish Liver Oil	A	0.1000g.	3.96	76,400	125,000	61.2
		A	0.1000g.	2.53	48,000		38.4
		A	0.1000g.	3.96	76,400		61.2
40713	Fish Liver Oil	A	0.1000g.	5.72	110,000	150,000	73.4
		A	0.1000g.	4.62	89,000		59.4
		A	0.1000g.	5.72	110,000		73.4
43023	Cod Liver Oil	A	0.0500g.	5.68	109,500	250,000	43.8
		D	0.0500g.	7.15	138,000		55.3
43125	Halibut Liver Oil	A	0.1000g.	5.17	100,000	160,000	66.2
		A	0.1000g.	4.07	78,500		49.1
		A	0.1000g.	4.95	95,500		59.7
60074	Fish Liver Oil	A	0.100g.	3.74	72,200		
		A	0.200g.	3.74	72,200		

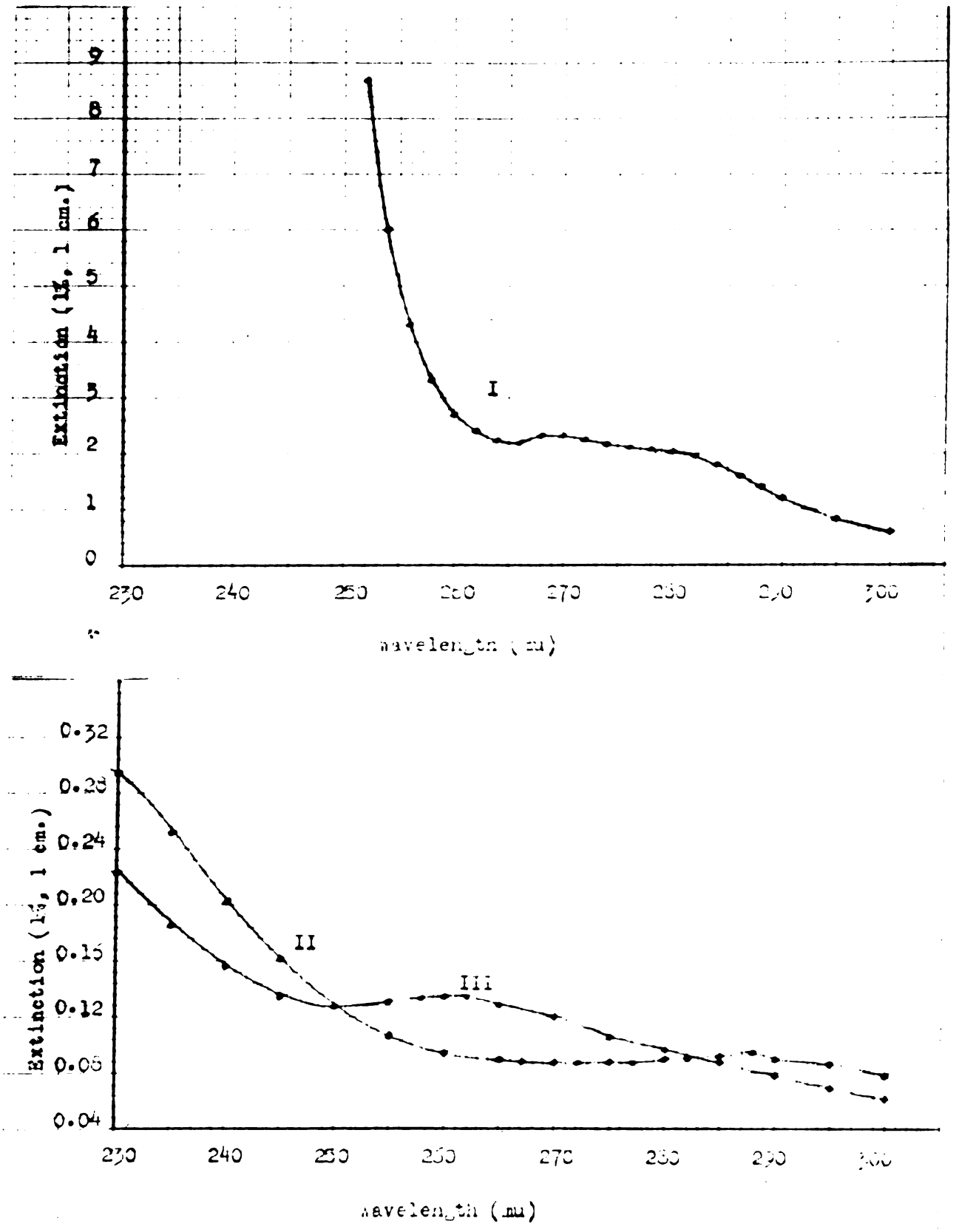


Fig. XIX- Extinction (1%, 1 cm.) of Corn Oil in absolute ethyl alcohol plotted against wavelength.

I- Procedure B, Concentration 0.1875g./100 ml.

II- Procedure C, Concentration 3.333g./100 ml.

III- Procedure D, Concentration 5.000g./100 ml.

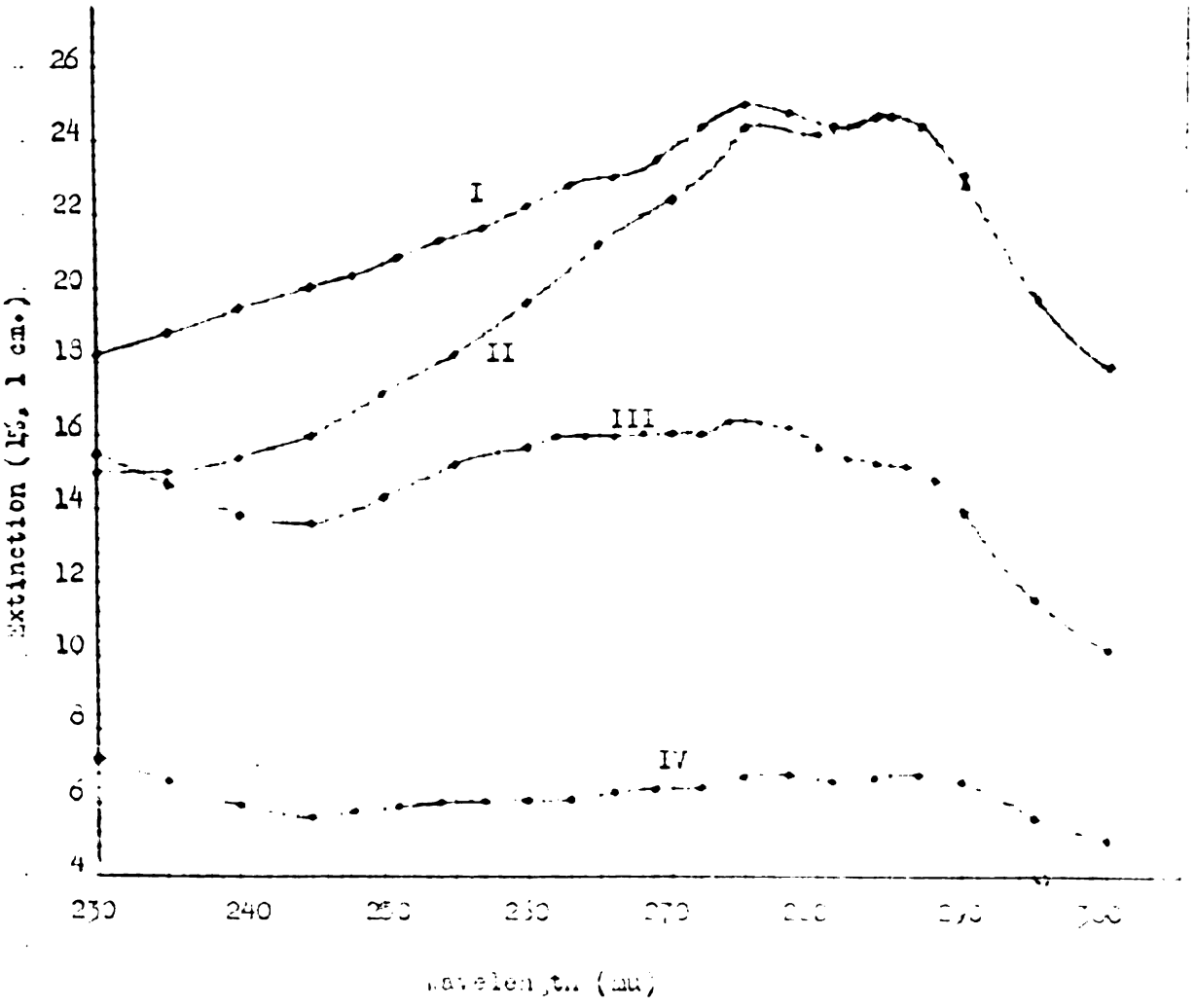


Fig. XIII- Extinction (1%, 1 cm.) of oil No. 4[illegible] plotted against wavelength.

I- Procedure D, Concentration 0.[illegible]g./100 ml. in absolute ethyl alcohol

II- Procedure C, Concentration 0.02[illegible] g./100 ml. in absolute ethyl alcohol

III- Procedure E, Concentration 0.04[illegible] g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.0[illegible]g./100 ml. in absolute ethyl alcohol

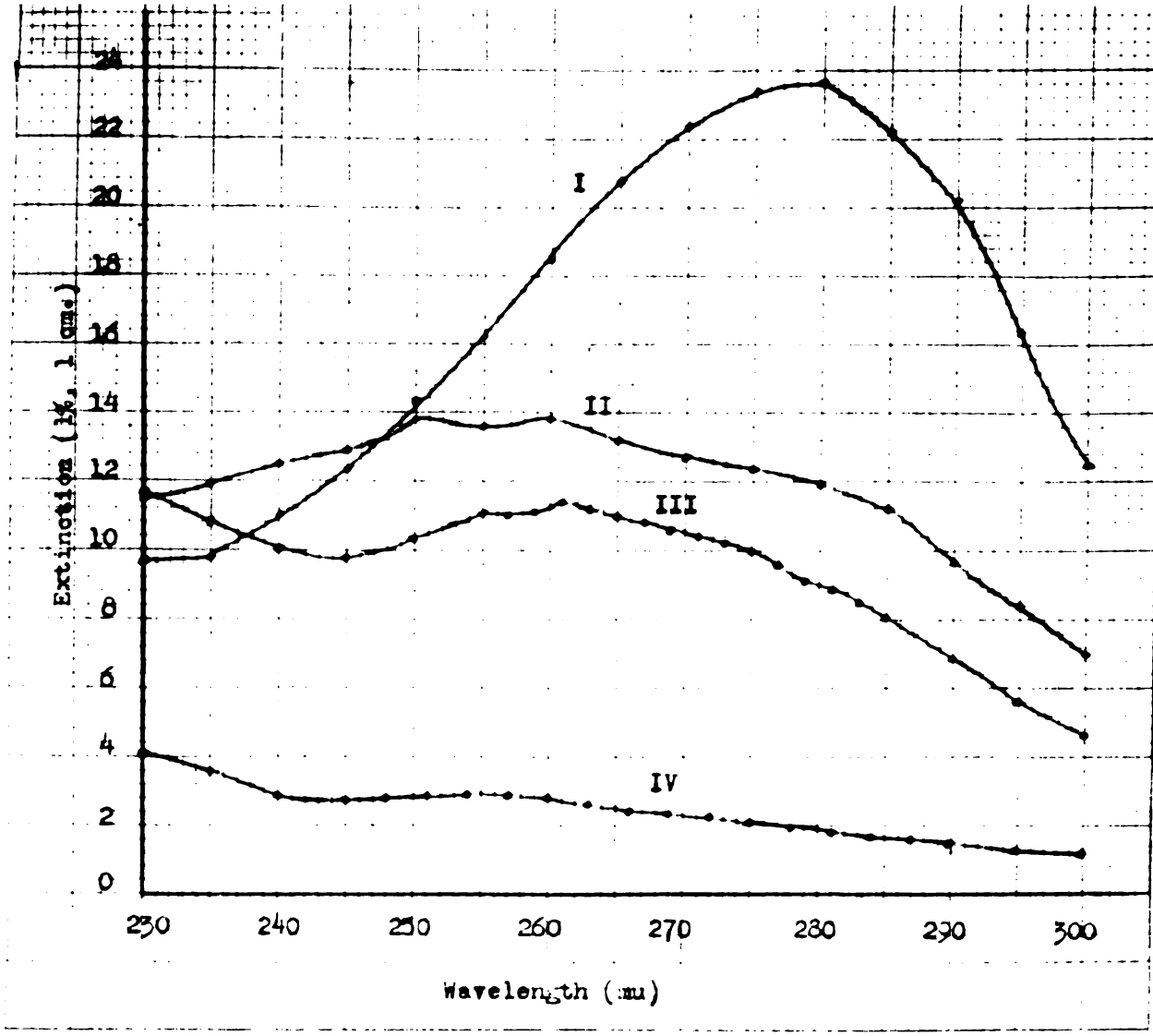


Fig. XIV- Extinction (1%, 1 cm.) of Oil No. 43603 plotted against wavelength.

I- Procedure B, Concentration 0.0201g./100 ml. in 95% ethyl alcohol

II- Procedure C, Concentration 0.04125g./100 ml. in absolute ethyl alcohol

III- Procedure E, Concentration 0.0550g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.1650g./100 ml. in absolute ethyl alcohol

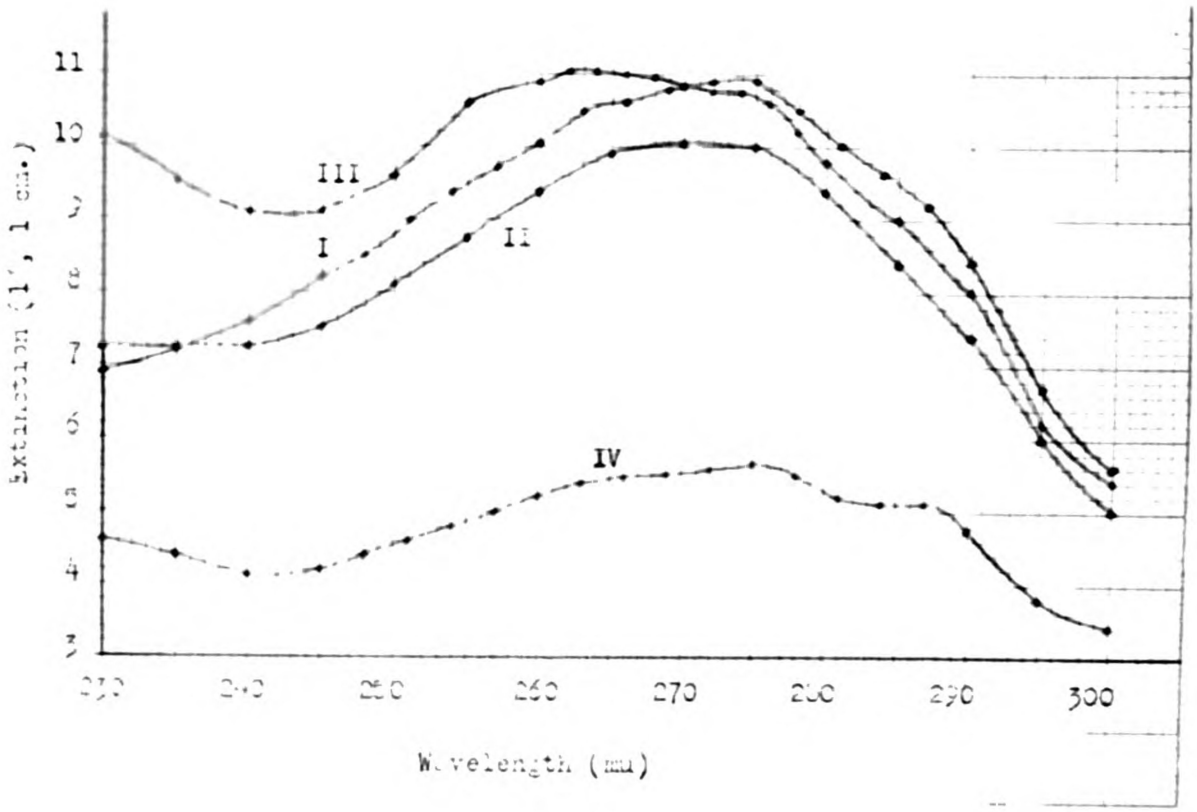


Fig. XV- Extinction (1%, 1 cm.) of oil No. 45613 plotted against wavelength.

I- Procedure B, Concentration 0.0710g./100 ml. in absolute ethyl alcohol

II- Procedure B, Concentration 0.0750g./100 ml. in absolute ethyl alcohol

III- Procedure B, Concentration 0.0750g./100 ml. in absolute ethyl alcohol

IV- Procedure B, Concentration 0.1160g./100 ml. in absolute ethyl alcohol

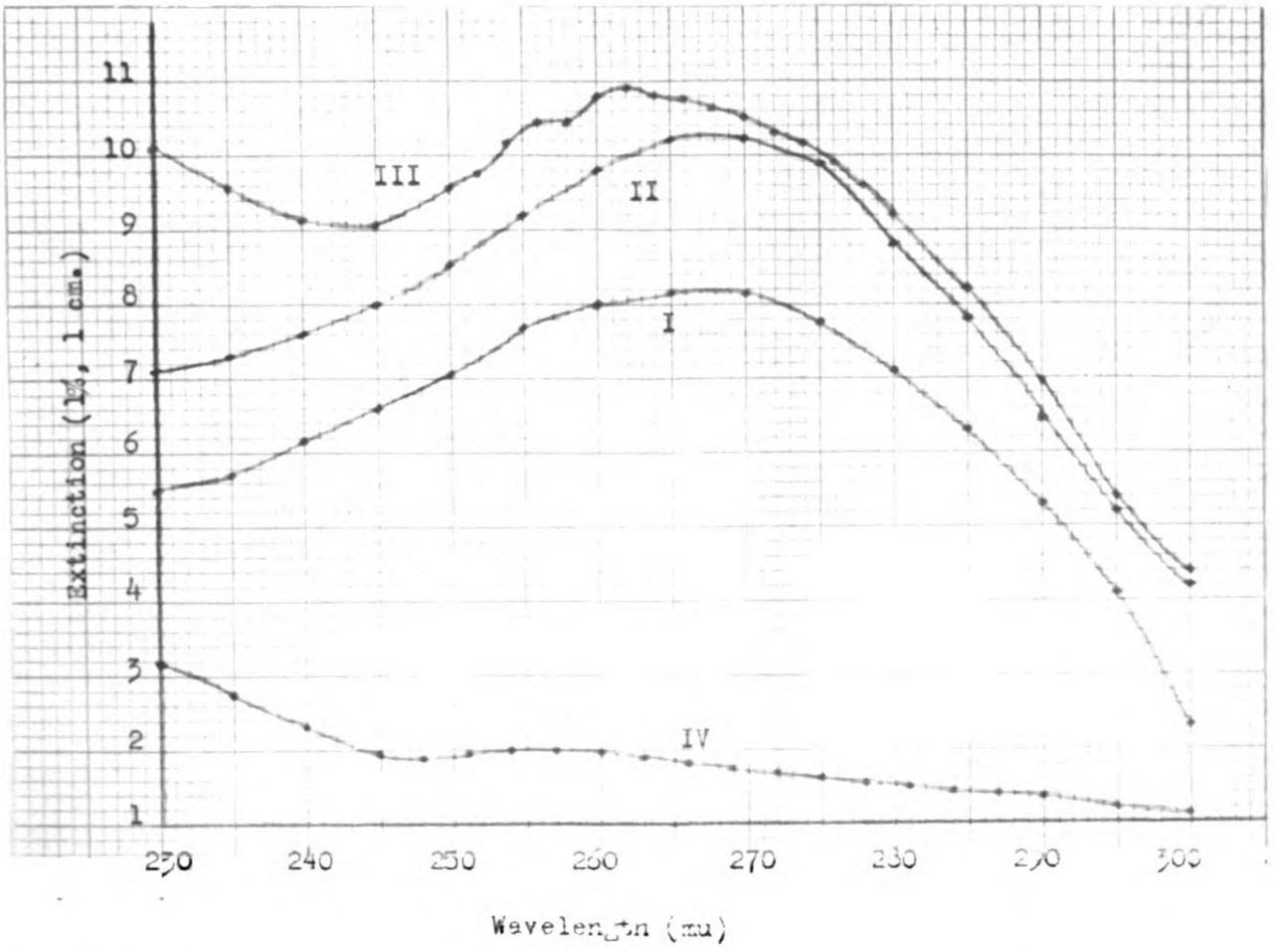


Fig. XVI- Extinction (1%, 1 cm.) of Oil No. 45023 plotted against wavelength.

I- Procedure B, Concentration 0.0739g./100 ml. in 95% ethyl alcohol

II- Procedure C, Concentration 0.05625g./100 ml. in absolute ethyl alcohol

III- Procedure E, Concentration 0.0533g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.1644g./100 ml. in absolute ethyl alcohol

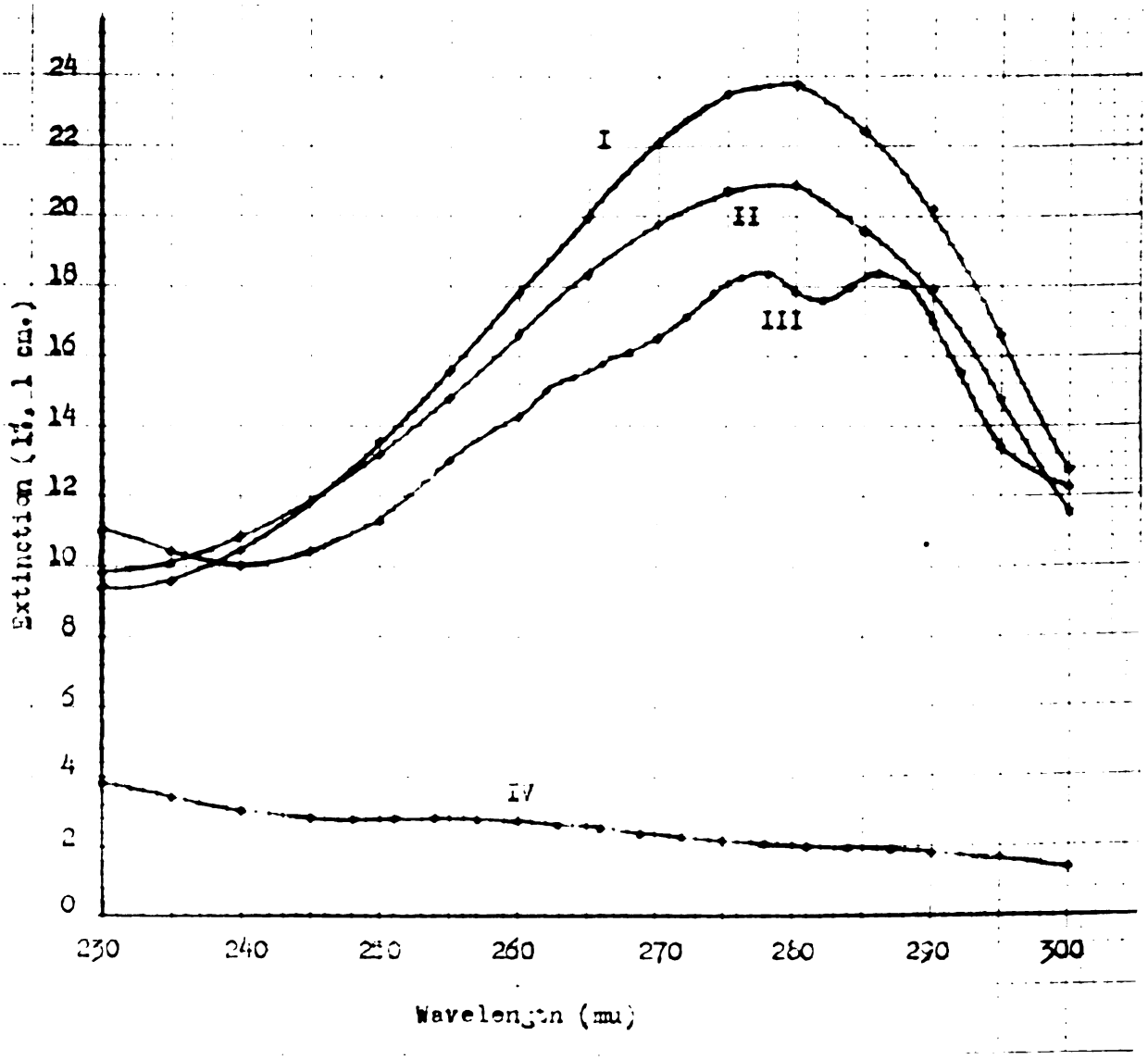


Fig. XVII- Extinction (1%, 1 cm.) of Oil No. 45033 plotted against wavelength.

I- Procedure B, Concentration 0.0247g./100 ml. in 95% ethyl alcohol

II- Procedure C, Concentration 0.0271g./100 ml. in absolute ethyl alcohol

III- Procedure E, Concentration 0.04065g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.1626g./100 ml. in absolute ethyl alcohol

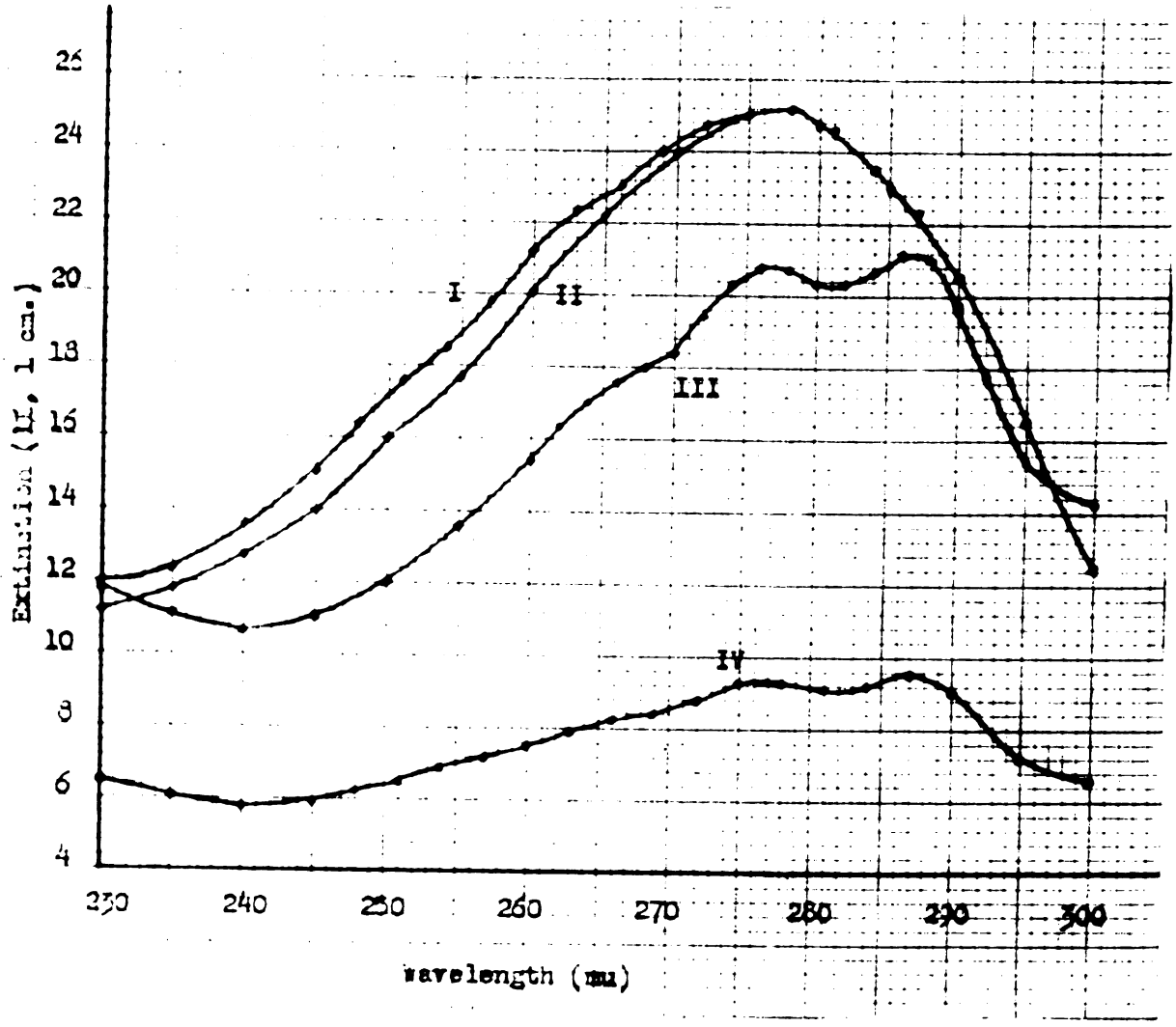


Fig. XVIII- Extinction (1%, 1 cm.) of Oil No. 45043 plotted against wavelength.

I- Procedure B, Concentration 0.0330g./100 ml. in absolute ethyl alcohol

II- Procedure C, Concentration 0.0275g./100 ml. in absolute ethyl alcohol

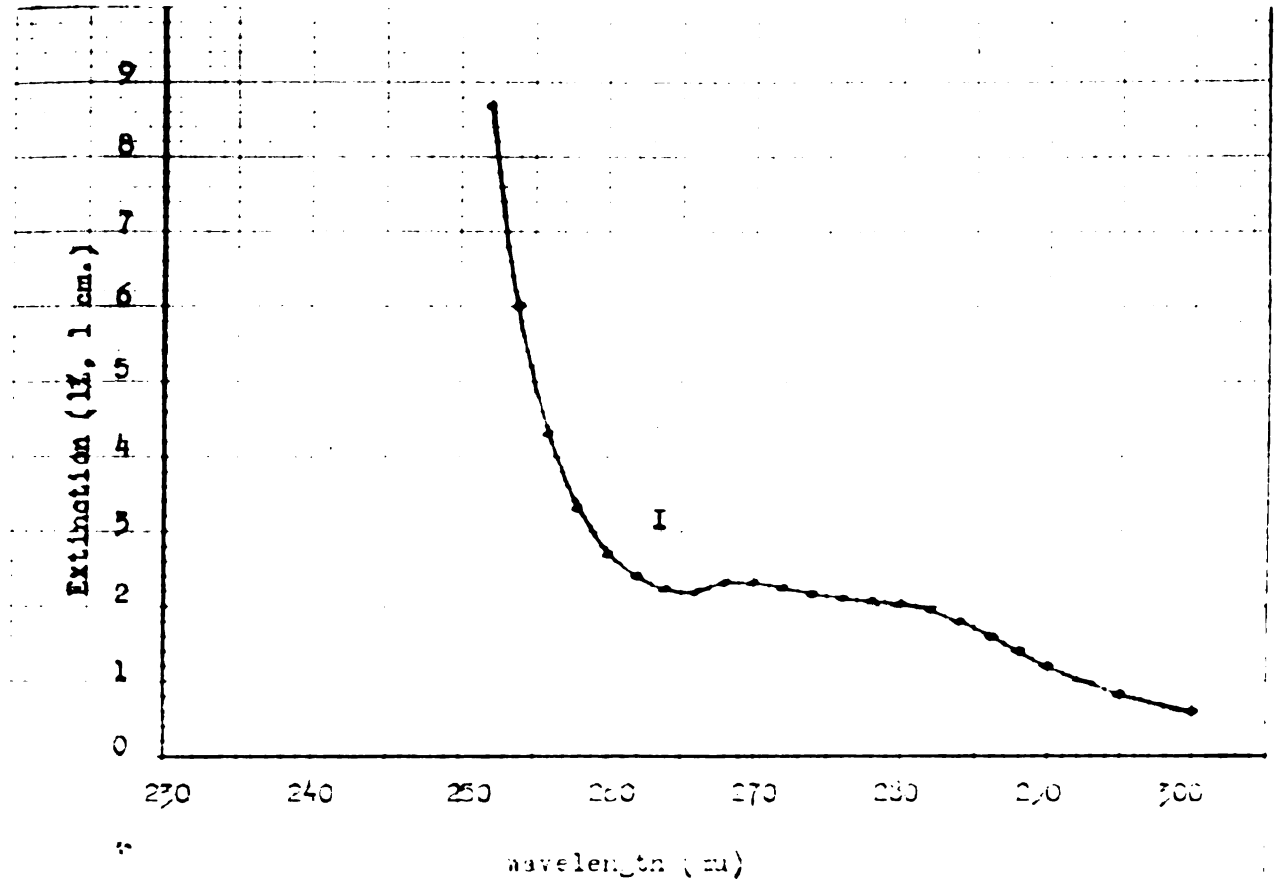
III- Procedure E, Concentration 0.0330g./100 ml. in absolute ethyl alcohol

IV- Procedure F, Concentration 0.0661g./100 ml. in absolute ethyl alcohol

ll No.
5391
5523
5783
9215
4071
4302
4312
6007

TABLE IX

Oil No.	Type of Fish Oil	Procedure	Sample Weight	$E^{1\%}_{1cm}$	D u/g	Bioassay	D u/gx100 / Bioassay
56391	Halibut Liver Oil	B	0.060g.	23.5	453,000	225,000	201.5
		C	0.060g.	21.1	407,000		181.0
		D	0.060g.	7.88	152,000		67.5
34523	Cod Liver Oil	A	0.5000g.	5.32	102,500	250,000	41.0
		D	0.5000g.	6.60	127,500		51.0
35783	Halibut Liver Oil	A	0.1000g.	3.96	76,400		
		A	0.1000g.	4.62	89,000		
		A	0.1000g.	4.62	89,000		
39215	Fish Liver Oil	A	0.1000g.	3.96	76,400	125,000	61.2
		A	0.1000g.	2.53	48,000		38.4
		A	0.1000g.	3.96	76,400		61.2
40713	Fish Liver Oil	A	0.1000g.	5.72	110,000	150,000	73.4
		A	0.1000g.	4.62	89,000		59.4
		A	0.1000g.	5.72	110,000		73.4
43023	Cod Liver Oil	A	0.0500g.	5.68	109,500	250,000	43.8
		D	0.0500g.	7.15	138,000		55.3
43125	Halibut Liver Oil	A	0.1000g.	5.17	100,000	160,000	66.2
		A	0.1000g.	4.07	78,500		49.1
		A	0.1000g.	4.95	95,500		59.7
60074	Fish Liver Oil	A	0.100g.	3.74	72,200		
		A	0.200g.	3.74	72,200		



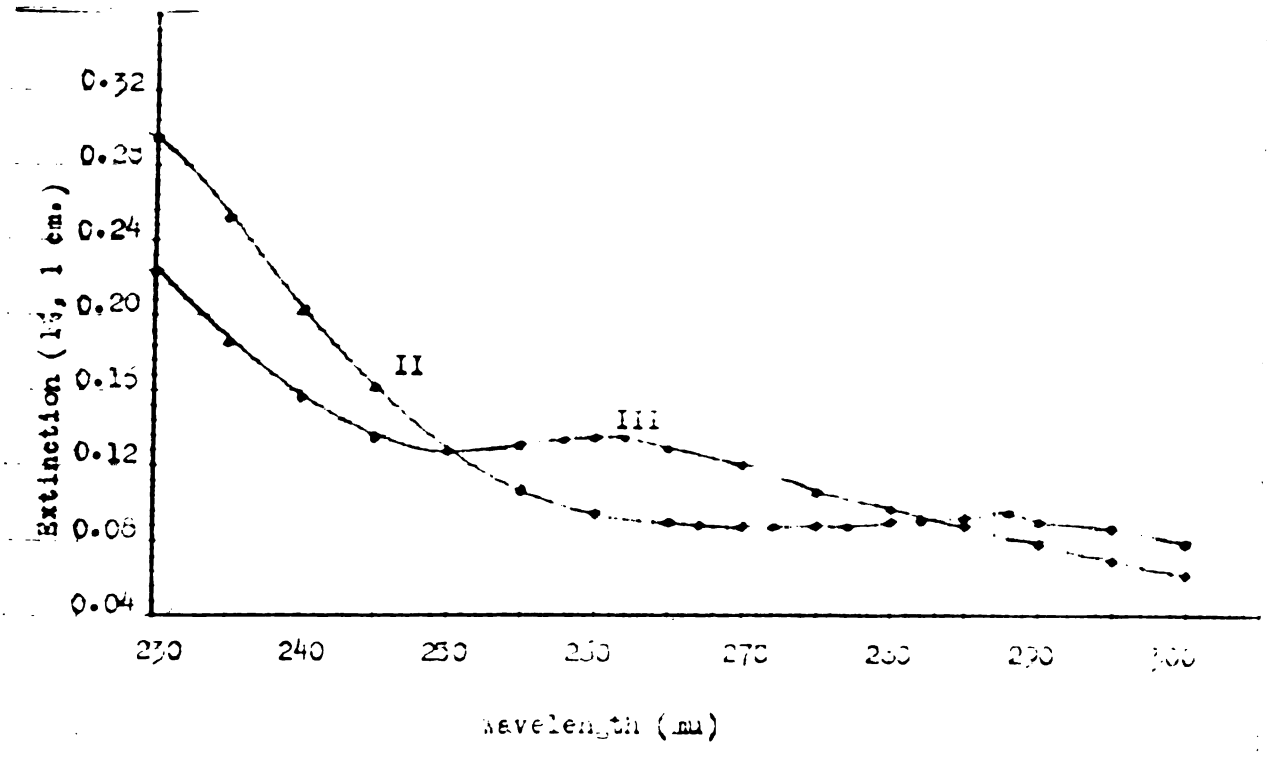


Fig. XIX- Extinction (1%, 1 cm.) of Corn Oil in absolute ethyl alconol plotted against wavelength.

I- Procedure B, Concentration 0.1875g./100 ml.

II- Procedure C, Concentration 3.333g./100 ml.

III- Procedure D, Concentration 5.000g./100 ml.

TABLE X

Oil	Procedure	Sample Weight	E(1%, 1 cm.)	D units/gram
Corn Oil	B	3.000g.	0.382	7,370
	B	2.000g.	0.423	8,180
	C	3.000g.	0.147	2,830
	C	3.000g.	0.147	2,830
Special #1	B	2.000g.	0.490	9,450
	C	3.000g.	0.323	6,230
	C	2.000g.	0.330	6,370

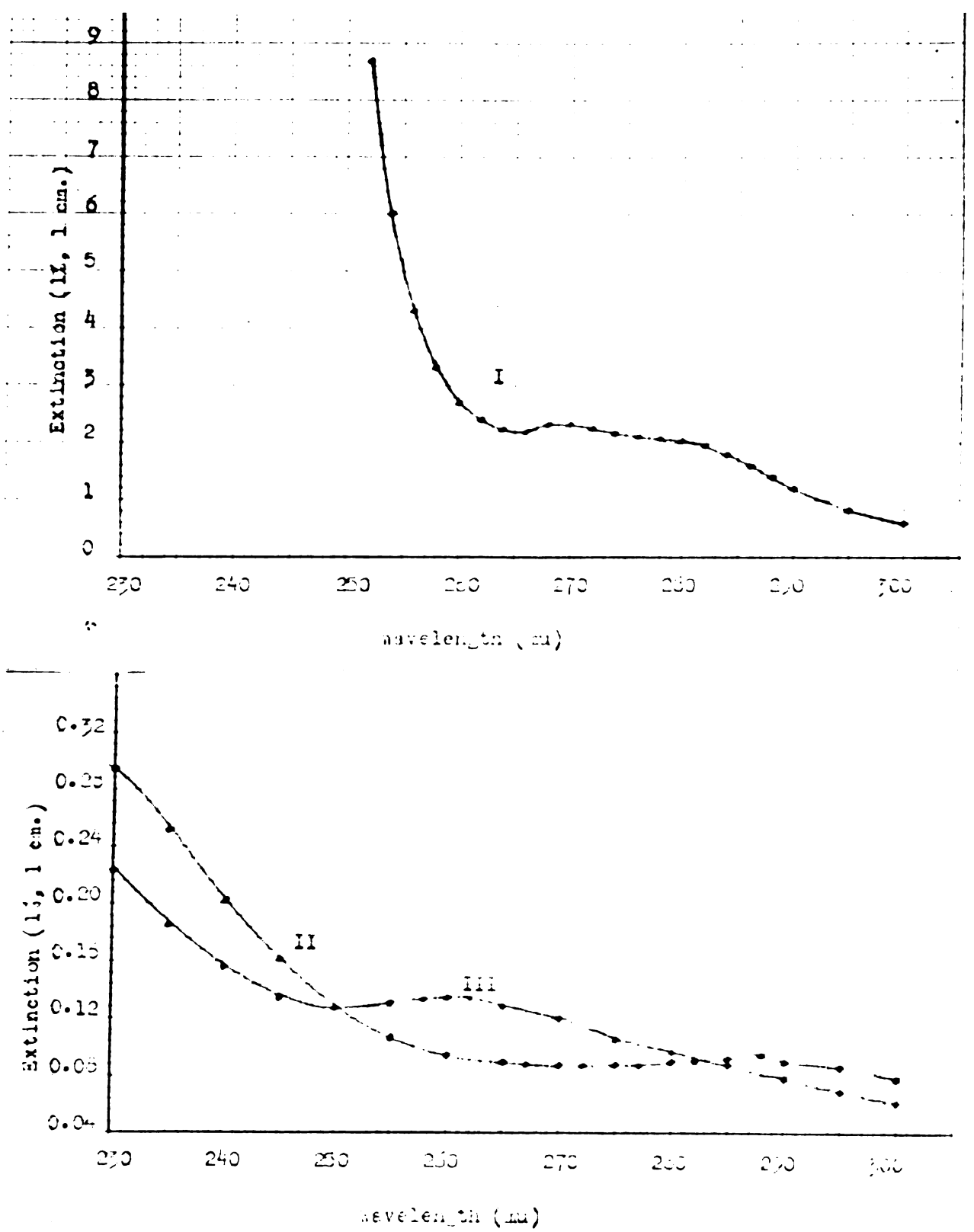


Fig. XIX- Extinction (1%, 1 cm.) of corn oil in absolute ethyl alcohol plotted against wavelength.

I- Procedure B, Concentration 0.1875 g./100 ml.

II- Procedure C, Concentration 3.333 g./100 ml.

III- Procedure D, Concentration 5.000 g./100 ml.

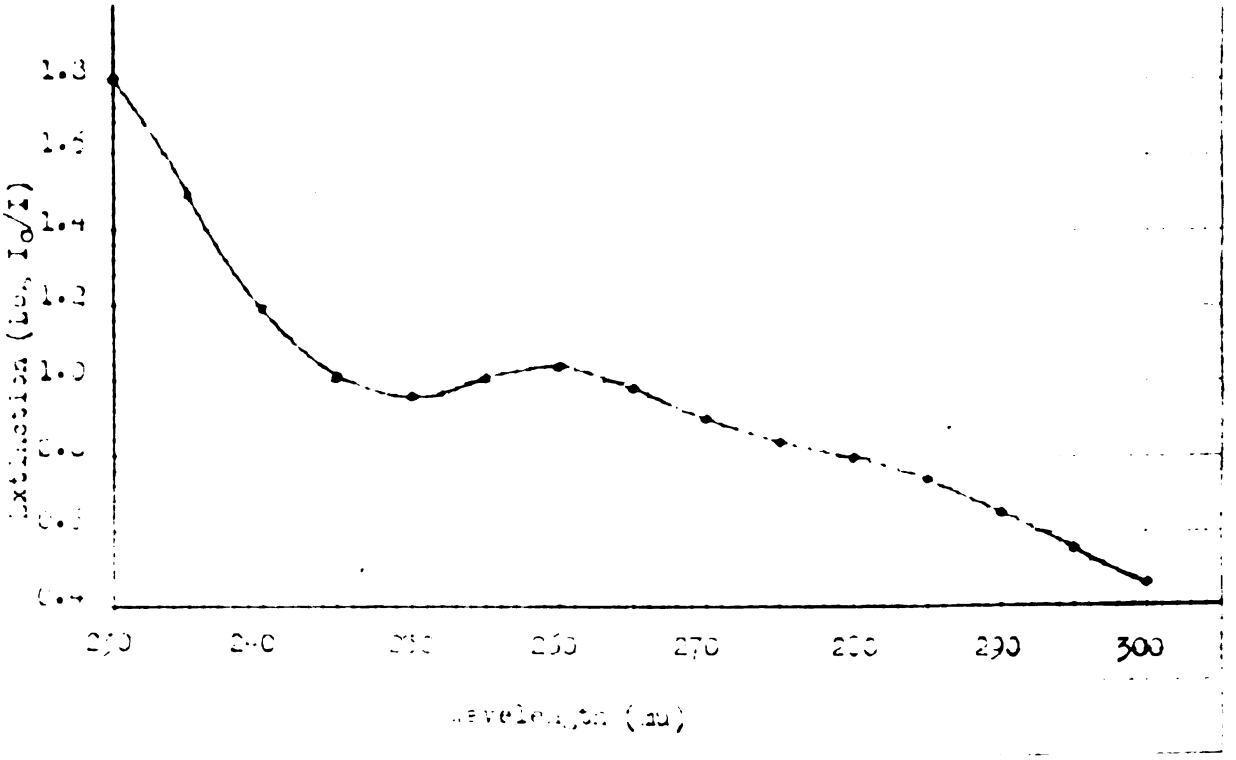


Fig. XX- Extinction ($\log I_0/I$) of Dilan III in absolute ethyl alcohol plotted against wavelength. Treated by procedure E.

SUMMARY

Thirty minutes was shown to be ample time for complete saponification of samples of irradiated ergosterol.

Four portions of ethyl ether, for the extraction of saponified samples as described in Procedure A, were shown to be sufficient for the extraction of samples of irradiated ergosterol.

In the study of adsorbents, considerable difference was shown to exist in the behavior of various adsorbents. Different samples of Superfiltrol acted alike, but, aside from magnesia, none of the other adsorbents examined could be used in this investigation without some modification of the present procedures.

Twelve natural oils were run by Kingsley's method with Hage's modification. For the most part, there was close agreement between the experimentally determined potency and the bioassay value. It is not felt that further modification of the procedure is necessary for natural oils.

After investigating the ultra-violet absorption of a substance eluted from Superfiltrol during chromatographing, it was shown that a correction must be made for the absorption of this substance in the measurements of the ultra-violet absorption of oils run by certain procedures. This substance has a definite ultra-violet absorption curve the intensity of which varies inversely with the concentration of the solution.

In the study of calciferol and ergosterol, it was demonstrated that calciferol and ergosterol are unchanged by

saponification. However, ergosterol is held back during chromatographing, while calciferol goes through the chromatograph column. Corn oil was also shown to be removed by saponification and chromatographing. It is believed that any method for determining the potency of irradiated ergosterol must have saponification and chromatographing incorporated in it.

Numerous samples of irradiated ergosterol in corn oil were treated by various procedures. Practically no correlation was found to exist between the experimentally determined potency and the bioassay values unless the samples were saponified and chromatographed (Procedure D). This result is in agreement with the conclusion reached after the study of calciferol and ergosterol.

Eight samples of irradiated ergosterol in fish oils were treated by various procedures. Most values obtained were lower than the bioassay values. However, results obtained by using Procedure D compared favorably with those obtained by using the same procedure on samples of irradiated ergosterol in corn oil.

Corn oil was found to have considerable effect in the measurement of the ultra-violet absorption of irradiated ergosterol and a slight effect on antimony trichloride extinction measurements. The effect in both cases was less after saponification.

It was shown that the dye Sudan III could not be used as a marker for the adsorbent column since enough of the dye goes through the column to interfere with ultra-violet measurements.

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