A CRITICAL STUDY OF THE FACTORS EFFECTING THE PHYSICAL CHEMICAL DETERMINATION OF VITAMINS D IN OILS

by

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CONTENTS

A Critical Study of the Factors Effecting the Physical Chemical Determination of Vitamins D in Oils.

The results of a study made upon methods for removing substances which interfere in the physical chemical determination of vitamins D in oils are given. Two methods applicable to high-potency irradiated ergosterols in oil are described. A third method which can be applied to oil solutions of irradiated ergosterols, and possibly to multiple vitamin oil solutions, having potencies as low as 2,000 to 3,000 D_2 units per gram is also given.

Various physical chemical methods for determining vitamins D have been proposed in the literature. A great majority of these, however, are limited in application because they can be used only for certain types of solutions or for limited concentration ranges. Others were applied to only a few oils. A critical evaluation of some of these methods, therefore, seems desirable.

Several investigators have attempted to determine vitamins D quantitatively by using the absorption maxima of the untreated solution at 265 mu (22, 27, 33, 44). This method has been used successfully only in cases where the solutions analyzed were those containing vitamins D in the pure state or as the irradiated provitamin. The presence of by-products of irradiation decreases the accuracy of these measurements considerably. Furthermore, if the vitamins are dissolved in a vegetable or fish liver oil, as they usually are, some means for correcting for the absorption of the solvent itself must be provided.

Colorimetric methods have also been proposed in which intensity measurements of a color, produced by direct addition of a reagent to the vitamin solution, serve as a quantitative estimation of the amount of vitamin D present (17, 18, 35, 37, 39, 40, 41, 42). Many of these reagents, however, are not specific for vitamins D and give color reactions with provitamins, other irradiation products, or compounds present in the oil in which the vitamin is dissolved (7, 8, 20, 30, 36). The reagent proposed by Brockmann and Chen (4) has been studied quite extensively and several modifications have been suggested in order to increase its sensitivity and stability (26, 32, 38). Nield, Russell, and Zimmerli (26, 48) found that when acetyl chloride is added to the antimony trichloride reagent of Brockmann and Chen, increased sensitivity resulted and that sterols gave a negligible reaction. Sterols with double bonds in the side chain showed no reaction while those with double bonds in the ring structures gave the following absorption coefficients:

1 double bond	E (1%, 1cm.)	2.2	@ 500 mu
2 double bonds	E (1%, 1cm.)	7	@ 515 mu
D_2 and D_3	E (1%, 1cm.)	1800	@ 500 mu

Vitamin A also has been shown to react with the Brockmann and Chen reagent and must, therefore, first be removed (11).

Several methods have been proposed in which interfering substances such as vitamin A and sterols have been removed from fish liver oils by chemical means or freezing before estimating the amount of vitamins D present from the absorption maxima at 265 mu or colorimetrically. Sterols have frequently been removed by precipitation with digitonin

(32, 46) or by freezing (15). Maleic anhydride has also been suggested for the removal of vitamin A (15, 23).

Chromatographic procedures have been by far the most successful means of separating vitamin A and carotenoids from vitamins D. Several adsorbents such as alumina (3, 25, 34, 45), charcoal (22), Montana earth (46), tri-calcium phosphate (24) and a mixture of magnesia with diatomaceous earth (9) have been suggested.

With few exceptions, all of the proposed methods above were applied to only a small number of oils and in most cases could not be used for solutions of irradiated ergosterols.

In these laboratories, Ewing and Tompkins (13) separated vitamin A from the nonsaponifiable fraction of sixteen (16) fish liver oils by chromatographic adsorption on Superfiltrol from hexane-ether-alcohol solution. After the removal of sterols with digitonin, the modified reagent of Nield, Russell, and Zimmerli was used to determine the vitamins D present. Ewing, Kingsley, Brown, and Emmett (12) modified this procedure by replacing the sterol precipitation step with another chromatographic step in which the sterols were separated from vitamins D from a benzene-Skellysolve solution using Superfiltrol as the adsorbent. Fifty-one fish liver oils with potencies ranging from 250 to 325,000 units/gram were tested by this method and forty-four of these had an average variation of 5.8% from the bioassay value.

Hage (16) further simplified this method eliminating the second chromatographic step by swirling the benzene-Skellysolve solution of vitamins D and sterols with the adsorbent instead of using a packed column.

Several investigators have tried with little success to apply the method of Ewing et al. to solutions of irradiated ergosterols (2, 16, 47). Baker (2) however, observed that the material eluted from the column in the first step of this procedure had an absorption curve similar to that of vitamin D_2 when the original sample was an irradiated ergosterol. A considerable amount of extraneous absorption below 265 mu was observed, and attempts to correct for it were unsuccessful.

Powell (31) found that by using a longer column of the adsorbent, which was prewashed with the solvent, the vitamin D_2 could be separated quantitatively from crude irradiated ergosterols in volatile solvents and the chromatographed material gave an absorption curve similar to that of a standard calciferol. This method was applied to seven irradiated ergosterols produced in the laboratory, as well as eight commercial solutions. Potencies, calculated from the absorption curves of the chromatographed solutions, compared very well with the bioassay values which were available for five different solutions.

Bullard (5) applied this same separation to mixtures of ergosterol and vitamin D_2, and a similar study was made by Pinkerton (29) in which a modification of the solvent, as well as the length of the column, was proposed. Correction for extraneous absorption due to residues contained in the adsorbent was made either by making a blank run or mathematically from the extinction values for the chromatographed solution measured at 230 and 265 mu.

The problem of determining vitamins D in the presence of other products of irradiation is a relatively simple matter, when they are dissolved in volatile solvents. The separation becomes much more com-

plex, however, when solution is made in vegetable or fish liver oils. The nonsaponifiable fraction of both vegetable and fish liver oils have been found to contain squalene type compounds which have been isolated as the hydrochloride by several investigators (14, 19). In addition to this, large amounts of sterols, pigments, and possibly other fat soluble vitamins may be present. A partial separation of these compounds from the nonsaponifiable fraction of vegetable oils such as corn, olive, and wheat germ oil, has been obtained using chromatographic techniques (10, 14, 43).

From this review it becomes evident that any successful spectrophotometric method for determining vitamins D must include one or more of the following: -- (a) A method for separating the vitamins D from interfering substances, such as:

(1) Substances present in the oil or solvent in which the vitamin is dissolved (e.g., sterols, carotenoids, pigments, other fat soluble vitamins, squalene type compounds, and saponifiable material).

(2) Interfering substances introduced by the method itself (e.g., solvent residues, residues eluted from adsorbent of chromatograph column).

(3) Other products of irradiation process by which vitamin D was produced (e.g., lumisterol, toxisterol, suprasterol, and unconverted ergosterol).

(b) A reagent which is very nearly specific for vitamins D in the presence of other substances which were not previously removed.

With this in mind, a study was made to determine what method or methods were most efficient in separating vitamins D from interfering substances with an eye toward developing a method which could be equally adapted to both high- and low-potency fish liver oils, multi-vitamin solutions, and irradiated ergosterols in vegetable oils.

EQUIPMENT AND REAGENTS

Superfiltrol Columns. -- (a) Large Size - A column of Superfiltrol 18 mm. in diameter (unless otherwise specified) and 9 cm. in length is used. For the fish liver oils, the length is 11 cm. It is prepared by the method of Ewing et al. (12). The packed column must be thoroughly washed with the chromatographic developing solution (approximately 60 ml.) before the sample is added.

(b) Small Size - Enough Superfiltrol, packed merely by tapping and applying 10 cm. differential in pressure, is used to prepare a column 8 mm. in diameter and 4 cm. in length. This column is prewashed with 8 ml. of the chromatographic developing solution before the sample is added.

Alumina Columns. -- Four gm. of alumina are packed similar to the small Superfiltrol columns above. This column must be prewashed with 10 ml. ether before addition of the sample.

Spectrophotometers. -- Either a Beckman spectrophotometer (Model DU) equipped with a hydrogen discharge tube and 1 cm. quartz cells or an optical system consisting of a Bausch & Lomb sector photometer and a medium quartz spectrograph is used for absorption measurements in the ultraviolet.

For measurements in the visible range, a Bausch and Lomb visual spectrophotometer equipped with a Martin's polarizing unit and 1 cm. glass cells is employed.

EQUIPMENT AND REAGENTS

Ethyl Alcohol. -- Ethyl Alcohol (95%) is purified by treating with a silver oxide precipitate, decanting, and distilling. Twenty gm. of potassium hydroxide and 10 gm. of silver nitrate per 2 liters of alcohol are added to form the silver oxide.

Alcoholic Potassium Hydroxide. -- Fourteen gm. of high grade potassium hydroxide are dissolved in 500 ml. of purified 95% ethyl alcohol.

Ethyl Ether. -- Anhydrous ether (c.p.) is purified by distilling over crystalline ferrous sulfate. It should be free of peroxides.

Skellysolve. -- Skellysolve B is fractionated and that portion from 65°-68°C is saved and further purified by passing it through a two-foot column of silica gel which has been activated by heating @ 250°C for two hours. Fractions which transmit to 220 mu are collected and used. A commercial grade of hexane may be substituted for the Skellysolve B if of sufficient purity.

Transmission curves of all the purified solvents (Fig. 1) were used as a criterion for their purity.

Developing Solutions for Superfiltrol Columns. -- (a) Large Columns - This solution is made up from the purified reagents described above by taking 50 parts of hexane or Skellysolve, 10 parts of anhydrous ethyl ether, and 1 part of absolute ethyl alcohol.

(b) Small Columns - The solvent mixture, 50 parts hexane or Skellysolve, and 10 parts anhydrous ethyl ether, which was proposed by Pinkerton (29) is used for this size column of adsorbent.

EQUIPMENT AND REAGENTS

Developing Solutions for Alumina Columns.

This developing solution is composed of 1 part of hexane or Skellysolve, and 1 part anhydrous ethyl ether.

Alumina. -- High grade alumina (less than 80 mesh) of chromatographic quality is employed.

Digitonin Solution. -- A one per cent solution of commercial Digitonin in 95% ethyl alcohol is used as a precipitating agent.

HCl. -- A cylinder of commercially prepared anhydrous HCl is equipped with a trap and a small jet for bubbling this gas into solutions.

Chloroform. -- Small amounts of alcohol are removed from c.p. chloroform by washing seven times with an equal volume of distilled water. It is then dried over anhydrous sodium sulfate, decanted, and distilled; the first and last 10% of the distillate are discarded.

Antimony Trichloride Reagent. -- This reagent is prepared fresh for each day's run. Eighteen gm. of c.p. antimony trichloride are dissolved in 100 ml. of purified chloroform. After this solution is filtered, two ml. of redistilled acetyl chloride are added.

PROCEDURES STANDARDIZED FOR THIS STUDY

Saponification and Extraction. — Oil samples are weighed into small glass capsules and then placed in 125 ml. Erlenmeyer flasks containing 10 ml. of alcoholic potassium hydroxide for each gram of sample.

A short-stemmed funnel is then placed in the neck of the flask and the sample is saponified in a water bath at 70°C. from 1/2 to one hour or until saponification is complete. Then 20 ml. of water are added to the saponified solution and the nonsaponifiable fraction is extracted in a separatory funnel, using one 40-ml. followed by three 20-ml. portions of ether. The combined ether extracts are washed with at least four 50-ml. portions of water or until the ether-water interface is clear and the water layer is not alkaline to phenolphthalein. The first two washings are made without shaking to prevent the formation of an emulsion.

The washed ether extract is then filtered through an anhydrous sodium sulfate filter pad into a 125 ml. Erlenmeyer flask. After the separatory funnel is rinsed and the filter pad washed with 10 ml. of ether, the combined ether portions are evaporated to dryness, using gentle suction and a water bath at about 50°C.

Digitonin Treatment. — The nonsaponifiable fraction is taken up in 10 ml. of 95% ethyl alcohol. Twelve ml. of 1% digitonin solution are added and the mixture is heated for two hours at 70°C. The precipitated sterols are then filtered off and the filtrate collected along with two 2-ml. washes of cold ethyl alcohol. An alternative procedure is used, if a chromatographic step follows, in which no filtration is made but the alcohol is evaporated off and the developing solution added directly

PROCEDURES STANDARDIZED FOR THIS STUDY

Digitonin Treatment. -- (continued) - to the residue. In this procedure the column of adsorbent serves as the filter.

HCl Treatment. — The residue from the preceding step is taken up in 10 ml. hexane or purified Skellysolve B. Anhydrous HCl is bubbled slowly through this solution for one hour. Any squalene hydrochloride crystals which form are then removed by a procedure similar to that of the digitonin precipitate above.

Chromatographic Procedure for Large Superfiltrol Columns. -- The residue is taken up in 10 ml. of the chromatographic developing solution. This is allowed to pass through the prepared 9- or 11-cm. column of Superfiltrol which has been previously washed with 40 to 60 ml. of the developer and has not been allowed to become dry. A 10-cm. differential in pressure is maintained throughout the whole procedure.

The flask is rinsed with three to five ml. of the developing solution, which is added at once to the column. By means of a short-stemmed separatory funnel which is fitted to the tube, the developing solution is added to the column drop by drop until the vitamin D_2 has passed through the adsorbent column. In the author's experience this separation is complete when the lowest visible band reaches the bottom of the column.

The filtrate from the column is then evaporated to dryness using suction and a hot water bath (about 50°C.). The residue is taken up in absolute alcohol and the extinction at 265 mu is measured on the Beckman quartz spectrophotometer.

PROCEDURES STANDARDIZED FOR THIS STUDY

Chromatographic Procedures for Small Superfiltrol Columns. -- The residue is taken up in three ml. of the chromatographic developing solution. This is added to the prewashed Superfiltrol column which has not been permitted to become dry.

The flask is rinsed with three ml. of the developing solution which is added to the column. After this has passed through the column, an additional five ml. of the developing solution is added to develop the chromatogram.

The filtrate from the column is then evaporated to dryness, using suction and a hot water bath (about 50°C.).

Alumina Chromatographic Step. -- The residue from the first chromatograph is taken up in three ml. of the developing solution. This is allowed to filter through the prewashed alumina column which has not been permitted to become dry. A 1-cm. pressure differential is maintained throughout the procedure.

A three ml. rinse is then added to the column. The procedure varies then, depending upon the type of sample. -- (a) Irradiated Ergosterols or D_2 in Corn Oil. - An additional five ml. of the developing solution is passed through the column and all the filtrate up to this point is discarded. The D_2 is eluted from the column with 15 ml. ether and its extinction in the developing solution at 265 mu is determined by means of the spectrophotometer.

(b) Multivitamin Preparations or Fish Liver Oils. -- An additional five ml. of the developing solution is passed through the column and all the filtrate up to this point is discarded. The D is eluted from the

PROCEDURES STANDARDIZED FOR THIS STUDY

column with an additional 12 ml. of the developing solution and its extinction at 265 mu is determined directly by means of the spectrophotometer.

EXPERIMENTAL STUDIES

Studies Made on Untreated Oil Solutions of Irradiated Ergosterols. -- From the absorption curve of a typical corn oil dissolved in purified alcohol (Fig. 2), it is obvious that due to the excessively high ratio of oil to vitamin present in a sample (even those having a potency of 1,000,000 units per gram or more) it is impractical to attempt to estimate the vitamin D potency directly from the absorption curve of the sample dissolved in alcohol or similar solvents. For this reason, no study of this type was made.

An attempt, however, was made to correct for the extinction due to the corn oil present in a given sample. This was done by dissolving an equal amount of corn oil in the same volume of the solvent as that of the oil being tested. The extraneous absorption exhibited by the original vitamin solution then, when run with the corn oil solution in the solvent cell, should be automatically corrected for.

Procedure. -- .100 to .120 gm. of the irradiated ergosterol in oil is dissolved in 50 ml. of purified alcohol and placed in the solution cell. An equal weight of corn oil is dissolved in 50 ml. of alcohol and placed in the solvent cell. The extinction at 265 mu is then measured by the spectrophotometer.

The potency of the original oil in vitamin D_2 units per gram is determined by calculating the E(1%, 1Cm.) of the sample @ 265 mu and multiplying by 86,960. This factor is obtained by dividing the number of vitamin D_2 units per gram of the standard calciferol (40,000,000) by the E(1%, 1cm.) at 265 mu, which is 460.

A typical absorption curve is shown in Fig. 3.

EXPERIMENTAL STUDIES

Studies Made on Saponified Oil Solutions of Irradiated Ergosterol.

Assuming that the saponifiable material in corn oil is largely responsible for the extraneous absorption or color reaction produced when an irradiated ergosterol in oil is studied, the following study was made:

Procedure. -- The nonsaponifiable fraction of the oil solution of irradiated ergosterol is obtained by carrying a sample of the original oil through the saponification and extraction procedure described above. The residue is then treated by one of the following methods:

(a) Absorption Curve of Nonsaponifiable Fraction. - The residue is taken up in alcohol and its absorption curve is run directly. The potency of the original oil is then calculated by multiplying the E(1%, 1cm.) at 265 mu by the factor 86,360. A typical curve is shown in Fig. 4.

(b) Antimony Trichloride Colorimetric Method. -- The residue from the ether extract is taken up in 10 ml. of the purified chloroform. To one ml. of this solution, 10 ml. of the antimony trichloride reagent are added. After 30 seconds' swirling, a 1-cm. cell is filled and the extinction at 500 mu is measured in exactly three minutes from the time the reagent was first added, using the Bausch & Lomb visual spectrophotometer.

The potency of the original oil in vitamin D_2 units per gram is then determined by calculating the E(1%, 1cm.) from the extinction at 500 mu and multiplying by the factor 19,300 as determined by Ewing et al (12).

An attempt to correct for the nonsaponifiable part of corn oil, as well as the saponifiable portion, was made by dissolving an equal amount of treated corn oil in the same volume of solvent as that of the treated

irradiated ergosterol, and placing it in the solvent cell. The resulting absorption curve should then be automatically corrected for the extraneous absorption due to corn oil.

Procedure. -- .120 gm. of the irradiated ergosterol in oil is saponified and the nonsaponifiable fraction extracted. An equal amount of corn oil is carried through a similar procedure. The corrected absorption curve is obtained by dissolving each of the above in 50 ml. alcohol, placing the treated D_2 solution in the solution cell, and comparing it with that of the treated corn oil placed in the solvent cell. Potencies are then evaluated by multiplying the E(1%, 1cm.) @ 265 mu by the factor 86,360. See Fig. 5 for a typical curve.

Studies Made on Removal of Interfering Substances by Chemical Means or Chromatography. -- Since it has been shown that the nonsaponifiable fraction of vegetable and fish liver oils contain sterols and squalene type compounds and that they can be removed, at least in part, by treatment with digitonin, anhydrous HCl, or chromatography, a study was made to determine if these preliminary treatments, separately or combined, would increase the accuracy of the potency evaluations.

(a) Chromatographic Separation Attained by Use of a Single Column of Superfiltrol. -- The separation of vitamin D_2 from crude irradiated ergosterols in volatile solvents which was successfully obtained by Powell (31), using a superfiltrol column and solvent mixture, hexane, ether and alcohol, was applied to oil solutions as follows:

EXPERIMENTAL STUDIES

Procedure. -- A one-gm. sample of the vitamin solution is saponified, extracted, and chromatographed using a 9-cm. column of Superfiltrol (large columns) according to the standardized procedures above.

The absorption curve of the treated sample should have a maxima at 265 mu similar to that exhibited by pure calciferol (Fig. 6). The potency of the sample is then determined by multiplying the E(1%, 1cm.) of the sample at 265 mu by the factor 86,360. A typical curve obtained for low-potency oils is shown in Fig. 7.

(b) Separation Achieved by Combined Chemical and Chromatographic Treatment. -- This study was confined to corn oil solutions of irradiated ergosterol or crystalline D_2 having a potency of approximately 10,000 units per gram.

Procedure. -- One gm. samples of the solution are treated separately using standardized procedures in the order described below:

Irradiated Ergosterol in Corn Oil (#73978)

Sample No.	No. of Treatments	Order of Treatments
1	1	Saponification & extraction.
2	2	Saponification & extraction, chromatographed through 9-cm. column of Superfiltrol.
3	2	Saponification & extraction, HCl Treatment.
4	3	Saponification & extraction, chromatographed through 9-cm. column of Superfiltrol, HCl treatment.
5	3	Saponification & extraction, HCl treatment, chromatographed through 9-cm. column of Superfiltrol.

EXPERIMENTAL STUDIES

Crystalline D_2 in Corn Oil

Sample No.	No. of Treatments	Order of Treatments
I	1	Saponification & extraction.
II	2	Saponification & extraction, digitonin treatment.
III	3	Saponification & extraction, digitonin treatment, chromatographed through 9-cm. column of Superfiltrol.

The absorption curve of the residue obtained from the above samples, treated as described, is run. An indication, as to what type of compound is removed by each step, is obtained by subtracting the absorption curves of samples representing two (2) procedures made in the same order but differing by one (1) additional treatment.

Absorption curves of material removed by the various treatments are shown in Figs. 8, 9, and 10.

(c) Separation Obtained Using Separate Superfiltrol and Alumina Columns. -- A partial separation of sterols and squalene type compounds has been obtained by several investigators using alumina columns (10, 14, 43). A chromatographic procedure, therefore, was employed in which the vitamins D are first separated from vitamins A, carotenoids, pigments, and other products of irradiation, by use of the Pinkerton modification of the Superfiltrol column described above (small columns). The vitamins D are contained in the eluate from this column. Any remaining sterols, squalene type compounds, and possibly vitamin E are then separated from the vitamins D by use of an alumina column. In this step the vitamins D are held on the column and then must be eluted to be recovered.

EXPERIMENTAL STUDIES

Procedure. -- One gm. samples of the vitamin solution are saponified, extracted, and chromatographed through a small Superfiltrol column as described in the standardized procedures. The residue is then adsorbed upon a column of alumina using the standardized procedure corresponding to the type of vitamin solution being tested. The potency of the original sample is obtained by multiplying the E(1%, 1cm.) at 265 mu of the eluted vitamins D by the factor 100,000. Absorption curves obtained for both types of vitamin solutions at various potency levels are shown in Figs. 11 and 12.

RESULTS

For each method attempted, calculated potency values are compared to bioassay values run by the U.S.P. procedure. All animal tests were run at two or three levels, 15 to 20% apart, and the U.S.P. reference oil was used as the standard. As no attempt was made to interpolate between the bioassay levels, some of the discrepancies between the physical chemical and the biological data may be due to the 15 to 20% range at which the samples were tested.

The values obtained from the animal tests represent the highest biological potency that could be obtained from the levels at which the samples were tested. For example, the data of a sample tested at 1,200,000 and 1,000,000 units per gram might indicate the material to be slightly less than 1,200,000 units per gram but above 1,000,000 units per gram. In this case the assay would be reported at 1,000,000 units per gram, although the material might actually contain 1,100,000 or 1,150,000 units per gram.

There seems to be very little correlation between the true potency values and the absorption curve of the untreated samples. (Table I). Potencies calculated for 13 different samples using the absorption curves of the untreated oil, obtained with corn oil of the same concentration in the solvent cell, showed an average variation of 55.2% from the bioassay value. Only two of these differed from the bioassay value by less than 20%.

Saponification does not seem to increase the accuracy of determining vitamins D present in an oil by use of their absorption curves. For saponified oils having bioassay values, the absorption curves obtained with alcohol in the solvent cell invariably gave potency values which were high (Table II). Eight of the 13 oils tested had bioassay values and showed an

Table I. POTENCIES OF IRRADIATED ERGOSTEROLS IN OIL AS DETERMINED BY THEIR ABSORPTION CURVES IN ALCOHOL WHEN COMPARED TO A SOLUTION OF CORN OIL IN THE SOLVENT CELL.

Sample No.	E (1%, 1cm.) @ 265 mu	Calculated D_2 Units/G.	Bioassay U.S.P. D_2 Units/G.
64184	2.14	186,073	250,000
64824	1.77	153,901	250,000
65104	3.75	326,062	250,000
65464	2.22	148,129	200,000
67784	1.83	158,249	250,000
68564	1.36	118,252	250,000
69934	3.10	269,545	250,000
70514	2.66	230,417	200,000
72114	2.76	239,982	300,000
73554	2.70	234,765	180,000
77424	1.19	103,470	500,000
80434	2.49	215,505	300,000
87114	24.29	2,112,015	1,200,000
88875	8.19	712,120	1,200,000
91095	5.18	520,301	400,000

Table II. POTENCIES OF IRRADIATED ERGOSTEROLS IN OIL AS DETERMINED FROM THE ABSORPTION CURVE OF THEIR NONSAPONIFIABLE FRACTION IN ALCOHOL.

Sample No.	E (1%, 1cm.) @ 265 mu	Calculated D_2 Units/G.	Bioassay U.S.P. D_2 Units/G.
Bal03	22.0	1,912,900	
	24.8	2,156,360	
CP3	20.7	1,799,865	
	22.5	1,956,375	
DP3	13.8	1,199,910	
	14.6	1,269,470	
FP3	12.7	1,104,265	
3772	4.7	408,665	200,000
	4.9	426,055	
64824	4.20	365,190	250,000
65104	4.65	404,317	250,000
65464	5.12	445,184	200,000
M9023	7.50	652,125	
B11299	19.8	1,721,610	525,000
65624	1.15	99,992	48,000
64184	3.75	326,062	250,000
Syn 2	8.9	773,855	

RESULTS

average variation of 127%. The results are high, probably, due to presence of solvent residues obtained from the extraction procedure.

Placing an equal amount of the nonsaponifiable fraction of corn oil in the solvent cell, as that of the vitamin solution in the solution cell, tends to correct for residues obtained from the extraction procedure, but the calculated potencies still vary considerably from the bioassay values. The results from five different samples listed in Table III show an average variation of 24.5% and a sixth sample which was not averaged with the group varied 234% from the bioassay.

Table IV gives the potencies of various irradiated ergosterols in corn oil as determined by the antimony trichloride colorimetric method applied to the nonsaponifiable fraction of the sample. The first part of Table IV is made up of values obtained for high-potency irradiated ergosterols in corn oil, and the second part consists of values for low-potency samples in corn oil containing around 10,000 units per gram. These were obtained on the open market.

Out of the 51 high-potency oils assayed by the antimony trichloride colorimetric method, 10 oils showed a difference from the bioassay of more than 25%. The maximum difference shown by this method was 57.0%, while the average variation of all the oils tested was 15.8%. The maximum variation shown when testing six low-potency oils of about 10,000 vitamin D_2 units per gram by this method was 21.4%. The average difference was 16.4%.

The separation obtained when a single column of Superfiltrol is employed is readily shown by Tables V, VI, and VII. Comparison of the

Table III. POTENCIES OF IRRADIATED ERGOSTEROLS AS DETERMINED FROM THE ABSORPTION CURVE OF THEIR NONSAPONIFIABLE FRACTION WHEN COMPARED TO AN ALCOHOL SOLUTION OF THE NONSAPONIFIABLE FRACTION OF CORN OIL.

Sample No.	E (1%, 1cm.) @ 265 mu	Calculated D_2 Units/G.	Bioassay U.S.P. D_2 Units/G.
65104	2.879	250,329	250,000
Syn 2	1.553	135,033	
65464	3.171	275,718	200,000
67784	3.458	300,673	250,000
69934	4.058	352,843	250,000
70514	7.713	670,645	200,000

Table IV. POTENCIES OF IRRADIATED ERGOSTEROLS AS DETERMINED BY ANTIMONY TRICHLORIDE COLORIMETRIC METHOD.

Sample No.	E (1%, 1cm.) @ 500 mu	Calculated D_2 Units/G.	Bioassay U.S.P. D_2 Units/G.
	<u>High-Potency Samples in Corn Oil</u>		
87114	72.16	1,592,688	1,200,000
	70.40	1,358,720	
	Av. 71.28	Av. 1,375,704	
88875	57.20	1,103,960	1,200,000
	58.96	1,137,928	
	Av. 58.08	Av. 1,120,944	
97195	70.40	1,358,720	1,200,000
	70.40	1,358,720	
	Av. 70.40	Av. 1,358,720	
1015	56.76	1,095,468	1,000,000
1735	52.80	1,019,040	1,000,000
7305	48.84	942,612	1,200,000
	52.14	1,006,202	
	Av. 50.49	Av. 974,407	
89025	15.84	305,712	600,000
	17.16	331,188	
	17.16	331,188	
	Av. 16.72	Av. 322,696	
94435	23.76	458,568	600,000
25446	25.52	492,536	525,000
	25.96	501,028	
	25.08	484,022	
	25.08	484,022	
	Av. 25.41	Av. 490,402	
6105	24.42	471,306	500,000
	21.78	420,354	
	23.10	446,000	
	Av. 23.10	Av. 445,887	
4985	22.22	428,846	600,000

Table IV (Continued).

Sample No.	E (1%, 1cm.) @ 500 mu	Calculated D_2 Units/G.	Bioassay U.S.P. D_2 Units/G.
7705	20.46	394,878	600,000
	20.68	399,124	
	Av. 20.57	Av. 397,001	
19656	22.60	436,000	525,000
92345	18.58	377,894	525,000
	18.48	356,664	
	Av. 18.53	Av. 367,279	
25476	25.08	484,044	515,000
	24.64	475,552	
	Av. 24.86	Av. 479,798	
25456	25.52	492,536	500,000
	25.30	488,290	
	24.42	471,506	
	25.96	501,028	
	25.96	501,028	
	26.18	505,274	
	26.40	509,520	
	25.74	496,792	
	25.96	501,028	
	26.40	509,520	
	Av. 25.77	Av. 497,652	
21226	23.75	459,000	525,000
21976	23.76	458,568	525,000
	22.44	433,092	
	Av. 23.10	Av. 445,830	
21986	26.10	504,000	525,000
25196	25.10	484,000	525,000
91095	23.76	458,568	400,000
	23.76	458,568	
	Av. 23.76	Av. 458,568	
25496	21.34	412,000	450,000
8645	15.40	297,500	325,000

Table IV. (Continued).

Sample No.	E (1%, 1cm.) @ 500 mu	Calculated D_2 Units/G.	Bioassay U.S.P. D_2 Units/G.
78174	14.08	271,744	330,000
	14.08	271,744	
	14.30	275,990	
	14.08	271,744	
	14.52	280,236	
	14.08	271,744	
Av.	14.11	Av. 274,867	
84614	11.22	216,546	330,000
	11.22	216,546	
Av.	11.22	Av. 216,546	
77424	9.46	182,578	300,000
	9.46	182,578	
Av.	9.46	Av. 182,578	
80434	12.98	250,514	300,000
	12.98	250,514	
Av.	12.98	Av. 250,514	
79404	14.08	271,744	275,000
	13.86	267,498	
Av.	13.97	Av. 269,621	
85024	13.42	259,006	275,000
	13.42	259,006	
Av.	13.42	Av. 259,006	
64184	13.86	267,498	250,000
	13.64	263,252	
	13.64	263,252	
Av.	13.71	Av. 264,667	
64824	10.12	195,316	250,000
	10.12	195,316	
Av.	10.12	Av. 195,316	
65104	12.10	233,550	250,000
	12.54	242,022	
Av.	12.32	Av. 237,776	
67784	9.46	182,578	250,000
	9.46	182,578	
Av.	9.46	Av. 182,578	

Table IV. (Continued).

Sample No.	E (1%, 1cm.) @ 500 mu	Calculated D_2 Units/G.	Bioassay U.S.P. D_2 Units/G.
68564	9.68	186,824	250,000
	9.68	186,824	
	Av. 9.68	Av. 186,824	
69934	9.90	191,070	250,000
	10.12	195,316	
	Av. 10.01	Av. 193,193	
84944	11.00	212,300	250,000
	11.00	212,300	
	Av. 11.00	Av. 212,300	
5305	10.23	197,439	250,000
80904	9.35	180,453	204,500
84174	10.34	199,562	204,500
65464	10.23	197,439	200,000
	10.12	199,562	
	Av. 10.18	Av. 198,500	
70514	10.56	203,808	200,000
	10.56	203,808	
	10.56	203,808	
	Av. 10.56	Av. 203,808	
74294	10.85	209,500	200,000
	10.85	209,500	
	Av. 10.85	Av. 209,500	
73534	10.56	203,808	180,000
	10.56	203,808	
	10.56	203,808	
	Av. 10.56	Av. 203,808	
42943	6.16	118,888	160,000
	6.38	123,134	
	5.83	112,519	
	5.94	114,642	
	6.05	116,765	
	6.16	118,888	
	Av. 6.09	Av. 117,473	

Table IV. (Continued).

Sample No.	E (1%, 1cm.) @ 500 mu	Calculated D_2 Units/G.	Bioassay U.S.P. D_2 Units/G.
38133	5.39	104,027	125,000
	4.84	93,412	
	5.06	97,658	
	5.06	97,658	
	5.39	104,027	
	5.39	104,027	
	Av. 5.19	Av. 100,135	
75584	8.14	157,102	100,000
	8.14	157,102	
	Av. 8.14	Av. 157,102	
18546	22.70	438,000	400,000
19196	20.45	395,000	450,000
20826	8.03	154,800	250,000
24646	23.10	446,000	450,000
24876	22.45	433,000	450,000
	Low-Potency Samples in Corn Oil		
0855	0.594	11,464	14,000
0865	0.550	11,000	14,000
2985	0.605	12,000	14,000
0905	0.418	8,000	10,000
74334	0.450	8,775	10,000
89545	0.594	11,500	10,000
89535	0.489	9,500	10,000

Table V. EXTINCTION RATIOS OF CRYSTALLINE CALCIFEROL AND CHROMATOGRAPHED SAMPLE IN ETHANOL.

Wave Length, Mu	Extinction Ratios (265 mu) Test Material	Calciferol
240	0.76	0.67
250	0.89	0.85
260	0.98	0.98
270	0.95	0.98
280	0.75	0.73
290	0.47	0.43
300	0.20	0.23

Table VI. POTENCIES OF IRRADIATED ERGOSTEROLS AS DETERMINED BY CHROMATOGRAPHIC ULTRAVIOLET ABSORPTION CURVE AT 265 MU.

Sample No.	E (1%, 1cm.) 265 mu	Calculated D_2 Units/G.	Bioassay, U.S.P. D_2 Units/G.
	High-Potency Samples in Corn Oil		
97195	16.77	1,458,000	1,200,000
87114	17.89	1,555,000	1,200,000
3155	15.18	1,318,000	1,200,000
7305	13.39	1,164,000	1,200,000
1015	14.07	1,221,000	1,000,000
1735	11.89	1,033,000	1,000,000
4985	5.43	472,000	600,000
25446	5.51	479,000	525,000
2915	4.68	407,000	525,000
3265	4.95	430,000	525,000
0145	5.04	437,500	525,000
89025	4.27	371,000	525,000
91285	4.26	370,000	525,000
92345	4.11	357,000	525,000
94435	5.16	448,000	525,000
97025	6.09	529,000	525,000
98655	5.70	495,000	525,000
21226	5.52	479,000	525,000
21976	4.47	389,000	525,000
21986	5.86	509,000	525,000
24646	5.04	438,000	525,000
23196	4.64	404,000	525,000
6105	5.44	473,500	500,000
7705	5.13	446,500	600,000
0555	4.68	407,000	475,000
18546	5.05	439,000	400,000
99775	5.48	476,000	450,000
97775	5.11	444,000	450,000
3005	5.91	513,000	400,000
19196	4.58	398,000	450,000
24876	4.54	395,000	450,000
19196	5.26	456,000	450,000
78174	3.22	280,000	330,000
84163	3.09	268,500	330,000
80434	2.75	239,000	300,000
79404	3.35	281,000	275,000
83024	2.96	257,000	275,000
64184	2.69	234,000	250,000
64824	2.60	226,000	250,000
65104	2.74	238,000	250,000
67784	2.28	198,000	250,000
68564	2.25	195,500	250,000
69934	2.60	226,000	250,000

Table VI. (Continued).

Sample No.	E (1%, 1cm.) 265 mu	Calculated D_2 Units/G.	Bioassay, U.S.P. D_2 Units/G.
	High-Potency Samples in Corn Oil		
5305	2.43	211,000	250,000
20826	1.81	157,500	250,000
80904	2.52	219,000	204,500
84174	2.60	226,000	204,500
65464	2.48	216,000	200,000
70514	2.46	214,000	200,000
	High-Potency Irradiated Ergosterols in Fish Liver Oil		
74254	2.57	225,000	250,000
66844	2.42	210,500	200,000
71064	2.32	202,000	200,000
20206	2.97	258,300	300,000
25646	2.02	175,800	200,000

Table VII. POTENCIES OF IRRADIATED ERGOSTEROLS AS DETERMINED BY CHROMATOGRAPHIC ULTRAVIOLET ABSORPTION CURVE AT 265 MU

Sample No.	E (1%, 1cm.) 265 mu	Calculated D_2 Units/G.	Bioassay, U.S.P. D_2 Units/G.
	Low-Potency Irradiated Ergosterols in Oil		
0855	0.296	25,800	14,000
0865	0.236	20,520	14,000
0905	0.196	17,070	10,000
2985	0.262	22,800	14,000
39597	0.234	20,370	10,000
89535	0.153	13,300	10,000
White Lab. D_2 in Corn Oil	0.511	44,400	40,000
ADMA D_2 in Corn Oil	0.365	26,650	15,000
Viosterol in Sesame Oil	0.272	23,600	10,000

RESULTS

absorption curves of ethanol solutions of crystalline D_2 and of the chromatographed sample is well shown in Table V. The extinction ratios for given wavelengths are tabulated according to the method of Oser, Melnick, and Pader (28).

Of 49 irradiated ergosterols in corn oil assayed by the chromatographic ultraviolet absorption curve method, only 8 differed from the bioassay values by more than 25%. (Table VI). The maximum % difference from the bioassay figures was 36.9, and the average variation of all the oils run by this method was 14.5%.

The results obtained when using the chromatographic method for five high-potency irradiated ergosterols, in fish liver oil containing vitamin A, are tabulated in the second part of Table VI. The close agreement of these results with the bioassay values indicated that the chromatographic method might also be applicable to fish liver oils fortified with irradiated ergosterol. However, not enough oils of this type were tested to recommend using this method for them.

When the chromatographic ultraviolet absorption curve method is applied to low-potency oils, invariably the potencies calculated will be high. This is shown in Table VII. The average deviation from the bioassay value of nine different oils tested was 65.5%. This method, therefore, can be applied only to high-potency oils.

The most successful method for determining the potencies of low valued oils was the two chromatographic step ultraviolet absorption curve method. Potencies evaluated for both irradiated ergosterols and multiple vitamin solutions are given in Table VIII. Out of six irradiated ergosterols

Table VIII. POTENCIES OF IRRADIATED ERGOSTEROLS AND MULTIPLE VITAMIN SOLUTIONS AS DETERMINED BY TWO CHROMATOGRAPHIC STEP ULTRA-VIOLET ABSORPTION CURVE METHOD.

Sample No.	Type	E (1%, 1cm.) 265 mu	Calculated D_2 Units/G.	Bioassay U.S.P. D_2 Units/G.	Bioassay U.S.P. Vit. A Units/G.
0339	Irradiated Ergosterol in Corn Oil	8.81	881,000	1,000,000	.....
97359	Irradiated Ergosterol in Corn Oil	8.46	846,000	1,000,000	.....
98449	Irradiated Ergosterol in Corn Oil	4.14	414,000	464,000	.....
3299	Irradiated Ergosterol in Corn Oil	4.44	444,000	404,000	.....
96399	Irradiated Ergosterol in Corn Oil	1.40	140,000	151,000	.....
2319	Irradiated Ergosterol in Corn Oil	0.0807 0.0797	8,070 7,970 Av. 8,020	11,250	.....
Syn III	Crystalline D_2 in Corn Oil	0.1023 0.0900	10,230 9,000 Av. 9,615	9,680*	.....
0899	Irradiated Ergosterol in fish liver oil	1.75	175,000	250,000	33,800
37786	Natola	0.115	11,500	11,000	55,000

* Theoretical potency calculated on basis of weight of crystalline D_2 present in sample.

Table VIII. (Continued).

Sample No.	Type	E (1%, 1cm.) 265 mu	Calculated D_2 Units/G.	Bioassay U.S.P. D_2 Units/G.	Bioassay U.S.P. Vit. A Units/G.
0399	High D Oil (Mostly D_3)	0.102 0.098	10,220 9,800 Av. 10,010	10,630	12,200
37756	Haliver Oil	0.170	17,000	10,000	60,000
4969	Natola	0.222	22,200	11,000	59,800
6299	Oil Mix for Natola (75% D_2, 25% D_3)	0.0492 0.0588 0.0582	4,920 5,880 5,820 Av. 5,540	4,000	19,860
ABDEC	Multiple Vitamin Solution in Polyethylene glycol.	0.0246 0.0276 0.0294	2,460 2,760 2,940 Av. 2,720	1,590 (Label claim)	7,950

RESULTS

and one crystalline D_2 solution in corn oil, only one showed a variation from the bioassay value above 25% and that was 28.7%. The average deviation from the bioassay of all seven samples was 12.11%. These oils ranged from potencies of 1,000,000 down to 9,680 units per gram and, as shown in Table IX, the method is reliable for oils having a potency as low as 2,000 - 3,000 units/gram. The absorption curves obtained in all cases resemble that of pure calciferol and have very little extraneous absorption in the low ultraviolet regions.

This method was also applied to various types of multivitamin solutions. Results for these varied somewhat, but the greatest differences seemed to be exhibited by those samples containing high amounts of vitamin A. The absorption curves in most cases, however, were very similar to that exhibited by pure calciferol: Of the seven different multivitamin solutions tested, ranging from 250,000 to approximately 1,590 vitamin D_2 or D_3 units per gram, an average deviation of 41.7% was obtained. In most cases the calculated values were higher than the bioassay value.

Experiments were made to determine the reproducibility of both the single chromatographic step ultraviolet absorption curve method and the antimony trichloride colorimetric method. These results are shown in Table X. Five separate samples of oil #65464 were run by the chromatographic ultraviolet absorption curve method and the maximum deviation from the average value was 8.7%. Ten different determinations for oil #25456 by the antimony trichloride colorimetric method showed a maximum deviation from the average of 1.6%.

Table IX. DILUTION SERIES OF OIL #6105 SHOWING LIMIT OF ACCURACY OF TWO CHROMATOGRAPHIC STEP ULTRAVIOLET ABSORPTION CURVE METHOD.

Wt. of Samples, Grams	Theoretical Potency U.S.P. D_2 Units/Gram	E (1%, 1cm.) 265 mu	Calculated Potency D_2 Units/Gram
1.0027	13,400	0.1272	12,720
1.0087	13,400	0.1207	12,070
1.0100	13,400	0.0945	9,450
1.0175	6,660	0.0606	6,060
1.0099	3,380	0.0354	3,540
1.0170	1,652	0.0232	2,320

Table X. REPRODUCIBILITY FOR IRRADIATED ERGOSTEROLS IN CORN OIL.

E (1%, 1cm.)	Calculated D_2 Units/G.	Bioassay D_2 Units/G.
Chromatographic Ultraviolet Absorption Curve Method, Oil #65464.		
2.28	187,000	200,000
2.09	182,000	
2.12	184,500	
2.02	176,000	
2.10	183,000	
	Av. 182,500	
Antimony Trichloride Colorimetric Method, Oil #25456		
25.52	492,500	500,000
25.30	488,500	
24.42	471,500	
25.96	501,000	
25.96	501,000	
26.18	505,500	
26.40	509,500	
25.74	497,000	
25.96	501,000	
26.40	509,500	
	Av. 497,700	

RESULTS

Reliable determinations were obtained by the antimony trichloride colorimetric method with oils containing as low as 10,000 vitamin D_2 units per gram and the indications are that oils of much lower potency can be evaluated successfully.

In order to determine how small an amount of sample may be employed and still an accurate potency determination can be obtained by the antimony trichloride colorimetric method, various amounts of an oil containing about 10,000 vitamin D_2 units per gram were put through the procedure. The results, as shown in Table XI indicate that samples containing as low as 500 vitamin D_2 units can be assayed with a fair degree of reliability. However, when working with very small amounts of the sample, it is necessary to add the reagent directly to the ether extract residue. Ordinarily, in a large sample, the residue is taken up in chloroform and a 1-ml. aliquot of this solution is mixed with 10 ml. of reagent. Thus, the concentration of the reagent in the solution cell is slightly more dilute than that in the solvent cell. The difference cannot be detected on the visual spectrophotometer at 500 mu, however, and no appreciable error is introduced by adding the reagent directly to the dry residue.

Although very small amounts of vitamin D_2 may be measured as indicated by Table XI, the antimony trichloride colorimetric method is not recommended for oils containing below 10,000 vitamin D_2 units per gram. Corn oil alone also exhibits, to a slight extent, the same color reaction with the antimony trichloride reagent as vitamin D_2, thus introducing another error in the determination.

Table XI. EFFECT OF AMOUNT OF SAMPLE UPON ACCURACY OF ANTIMONY TRICHLORIDE COLORIMETRIC METHOD.

Wt. of Sample, Grams	Calculated D_2 Units in Sample (Based on Bioassay)	E (1%, 1cm.) 500 mu	Calculated Potency D_2 Units/Gram
1.000	14,000	0.606	11,690
0.4992	7,000	0.473	9,130
0.2514	3,520	0.542	10,480
0.1262	1,769	0.634	12,220
0.0640	897	0.676	13,030
0.0372	521	0.537	10,370
0.0144	202	0.389	7,510

RESULTS

Experiments were also made to determine how low valued an oil can be determined accurately by means of the two chromatographic step ultraviolet absorption curve method. Irradiated ergosterol #6105, having a potency of approximately 500,000 units per gram, was diluted with corn oil to give samples ranging from 13,400 units to 1,652 units per gram. Potency values of the diluted samples were then determined by this method and the results are shown in Table IX. The absorption curves of the treated samples are shown in Fig. 13. These results indicate that oils containing as low as 2,000-3,000 units per gram can be run with a fair degree of accuracy.

DISCUSSION

Since various procedures in this study have been standardized, it seems fitting that a discussion as to how they were developed, their effectiveness, and the affect of various factors upon each of them should be made.

Saponification and Extraction.

The saponification and extraction procedure used is essentially the same as that of Ewing et al (12) with the exception that a single 40 ml. followed by three 20 ml. portions of ether were used for extraction instead of five 20 ml. portions of ether. This change helped to prevent emulsions and facilitated faster and cleaner separations of the ether and aqueous layers.

In spite of all attempts to obtain optically pure solvents, considerable extraneous absorption is introduced from residues in the solvents used in the saponification and extraction procedure. This was readily shown by running 10 ml. alcohol samples as a blank through the saponification and extraction procedure and obtaining absorption curves for a different one at successive steps of the procedure. The absorption curves are all similar to that exhibited by the residue contained in 100 ml. of anhydrous ethyl ether (Fig. 14) and accounts in part for the extraordinarily high results obtained for potencies calculated on the basis of the absorption curves of saponified oils.

DISCUSSION

Precipitation of Sterols.

The digitonin procedure was developed by determining experimentally what volume of 1% digitonin in ethanol is required to precipitate completely .015 gm. of ergosterol in the presence of 5,000 units of crystalline D_2 dissolved in 5 ml. alcohol. These proportions were used to approximate the ratio of sterols to D_2 present in .5 gm. of a 10,000 unit oil, assuming corn oil contains approximately 3% sterols. From the curves shown in Fig. 15, seven ml. of 1% digitonin solution is sufficient to precipitate the sterols present or approximately 14 ml. per gram of corn oil. In order to have a slight excess of digitonin present, 20 ml. of digitonin solution per gram of oil was finally decided upon.

Very little decrease in extraneous absorption is actually made by the digitonin treatment and substances which are precipitated exhibit only a general absorption as shown in Fig. 10. These curves were obtained using a crystalline D_2 solution in corn oil having a theoretical potency of 9,930 units/gram.

HCl Treatment.

The HCl treatment is essentially the same as that used by Heilbron, Kamm, & Owen (19) with the exception that Skellysolve B is used as the solvent. The material removed by this treatment, when testing an irradiated ergosterol in corn oil, has maxima at 260, 270, and 280 mu and appears to be partly vitamin D_2 (Fig. 8). For this reason, the procedure was discontinued.

DISCUSSION

Large Superfiltrol Column Characteristics.

The material removed by a single Superfiltrol chromatographic step is readily shown in Fig. 9. Absorption maxima at 250 and 280 mu strongly indicate that toxisterol and lumisterol are adsorbed upon the column and the vitamin D_2 passes on through into the eluate. The procedure used in this experiment was that described by Powell (31) using a 9-cm. column of Superfiltrol and a solvent mixture of 50 parts Skellysolve, 10 parts ether, and 1 part alcohol.

Since digitonin treatment appeared to make little difference in the absorption curve of the treated sample and the HCl treatment seemed to remove part of the vitamin D_2 as well as impurities both were discarded and chromatographic methods were resorted to completely.

For low-potency oils a two step chromatographic procedure was decided upon. A Superfiltrol column was employed for removal of vitamins A, pigments, carotenoids and products of irradiation other than vitamin D_2. This was followed by an alumina column to separate vitamins D from squalene type compounds and possibly vitamin E. An extensive study was made to determine the effect of changing such factors as column length, solvents, and corn oil concentration upon the degree of separation and potency evaluation of samples added to each of the two chromatographic columns. All these studies were made using solutions of crystalline D_2 in alcohol or corn oil with the samples ranging in potency values from 10,000 - 20,000 units unless otherwise indicated. The adsorption columns, in every case, were of 8-mm. inside diameter.

DISCUSSION

Alumina Column Characteristics. -- (a) With Skellysolve as the solvent.

Separation achieved. -- When a hexane solution of the nonsaponifiable fraction of crystalline D_2 in corn oil, having a potency of approximately 20,000 units per gram, is added to a six gm. column of alumina previously wet with Skellysolve, the vitamin D_2 can be separated to some extent from the interfering materials in corn oil. This is shown in Figs. 16 and 17. Attention must be called, however, to the fact that the Skellysolve developer does not carry through all the interfering substances of corn oil without using excessively large amounts of solvent. This is evidenced by the shape of the absorption curves for the first and last fractions of the eluate in Fig. 17.

Effect of changing length of column. -- Very little change in the absorption curve of the eluate is obtained when the length of the alumina column is varied. If the nonsaponifiable fraction of crystalline D_2 in corn oil is treated in the same manner as in part (1) using 30-ml. Skellysolve as the developer and discarding the filtrate up to that point, the eluate obtained, by passing 30 ml. ether through various lengths of alumina columns, gave the following extinctions:

Wt. in grams of Alumina in Column	Wave Length in mu									
	235	245	255	262	264	266	270	280	290	300
1	.347	.384	.428	.452	.456	.453	.439	.344	.212	.109
2	.367	.393	.432	.456	.458	.455	.439	.347	.214	.109
3	.358	.383	.428	.448	.450	.447	.429	.334	.204	.099
4	.316	.359	.411	.448	.450	.448	.431	.338	.207	.100
6	.351	.373	.422	.442	.442	.439	.423	.334	.202	.096

DISCUSSION

Results in this table indicate that the D_2 is very tightly held by the adsorbent. The fact that Sudan III, having adsorption characteristics similar to D_2, remains near the top of the column throughout the development of the chromatogram also tends to confirm this. Addition of amounts as high as 100 ml. of Skellysolve to the column failed to carry through any of the vitamin D_2 into the eluate. For this reason no threshold volumes using this solvent were determined for the column.

If the Superfiltrol chromatographic step, using the long 9-cm. column, precedes the alumina chromatographic procedure, very little difference is observed in the results, except that slightly larger absorption is exhibited in the 250 mu region when longer alumina columns are used. This is shown in the following table:

Wt./grams of Alumina in Column	Wave Length in mu									
	235	245	255	262	264	266	270	280	290	300
1	.348	.393	.442	.458	.453	.448	.428	.322	.197	.103
2	.402	.423	.459	.473	.471	.463	.441	.328	.194	.098
4	.398	.423	.461	.472	.470	.462	.438	.314	.179	.086

These results were also confirmed when solutions of crystalline D_2 in alcohol were substituted in place of the saponified corn oil solutions. Since complete separation is not achieved using Skellysolve as the solvent, various solvent mixtures were tried.

(b) With Hexane - Alcohol Mixtures As the Developing Solution. - The effect of adding small amounts of alcohol to Skellysolve and using the mixture as a developing solution for the alumina chromatographic step is graphically shown in Fig. 18. Approximately 10,000 units of D_2 in one ml. Skellysolve were added to the wet alumina column, and five ml. portions of

DISCUSSION

the developing solution were used to develop the chromatogram, saving the corresponding fractions of the filtrate. The approximate threshold volumes for each solvent mixture were determined by plotting the extinction of the filtrate fraction @ 265 mu against the number of the fraction. Any great increase in extinction at 265 mu indicated the presence of D_2 in the filtrate and in all cases if a large increase occurred, the complete absorption curve resembled that of pure D_2.

No further work was done using Skellysolve-alcohol mixtures as a solvent because it was found that interfering substances of corn oil were also carried into the filtrate along with the D_2.

(c) With Skellysolve-Ether Mixtures As the Developing Solution.— The approximate threshold volume for the alumina column, using Skellysolve-ether mixtures as the solvent, was determined in the same manner as for the Skellysolve-alcohol mixtures in part (b). This is shown in Fig. 19. The small increase in extinction in the first fraction indicates that a residue is eluted from the alumina. Absorption curves of the residue eluted from a four gm. alumina column by successive five-ml. portions of ether is shown in Fig. 20. Approximately 10 ml. of ether is required to reduce this to a minimum. The characteristics of a prewashed column of alumina were then determined.

A special apparatus with which fractions of the percolate could be taken without disturbing the equilibrium of the column was constructed (Fig. 21). Approximately 10,000 units of crystalline D_2 in three ml. of 1:1 Skellysolve-ether solution was added to a four gm. column of alumina

DISCUSSION

which had been prewashed with 10 ml. of ether. The absorption curves of the various fractions taken are shown in Fig. 22.

From these curves it appears that the filtrate obtained up to the point where eight ml. of developing solution has passed through the column, may be discarded without loss of vitamin D_2. The D_2 can be quantitatively recovered from the alumina column at this point by eluting with 15 ml. ether. The recovery of the D_2 (10,000 units) from the column is shown by comparison of the absorption curves in Fig. 23 of an untreated sample and a sample carried through the procedure using eight ml. of the 1:1 developing solution followed by elution with 15 ml. of ether.

In order to determine if this same separation and recovery could be obtained for crystalline D_2 in corn oil, a solution was prepared to contain 9,680 units D_2 per gram. The nonsaponifiable portion of a 1.000 gram sample of this solution was chromatographed through Superfiltrol and then added to a four gm. alumina column as above. Absorption curves of various fractions of the filtrate from the alumina column are shown in Fig. 24. Here again, it appears that the filtrate may be discarded up to the point where eight ml. of developing solution has passed through the column.

Two additional samples were run discarding all the filtrate up to this point, and eluting the D_2 from the column with 15 ml. of ether. The recovery of the D_2 is quantitative as shown by the following table:

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Sample	E (1%, 1cm.)	Calculated D_2 Units/gm.	Theoretical D_2 Units/gm.
1	.1023	10,230	9,680
2	.0900	9,000	
		Av. 9,615	

A similar study was made with a Natola oil and absorption curves of various fractions of the filtrate from the alumina column are shown in Fig. 25. From this set of curves it appears also that eight ml. of developer may be passed through the column and the filtrate discarded without loss of vitamins D. Elution with ether is not practical, however, since extraneous material is eluted after 9 - 12 ml. of additional developer has passed through.

To check the results from these curves, 1/2 ml. of each of these fractions was treated with antimony trichloride reagent and its extinction at 500 mu was measured on the spectrophotometer. Results were as follows:

Filtrate Fraction	Log I_o/I @ 500 mu	Remarks
1	.27	Cherry-red color produced
2	.27	Cherry-red color produced
3	.25	Cherry-red color produced
4	.38	Typical D_2 orange color produced
5	.40	Typical D_2 orange color produced
6	.37	Typical D_2 orange color produced
7	.22	Typical D_2 orange color produced
8	.22	Typical D_2 orange color produced

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Thus, both the ultraviolet absorption curves and the color reaction with antimony trichloride reagent of these fractions indicate complete separation of the vitamin D_3 from other interfering compounds.

Small Superfiltrol Column Characteristics:

The separation achieved by a six-cm. Superfiltrol column for both fish liver oils and irradiated ergosterols using a Skellysolve-ether-alcohol mixture as a solvent has been discussed by various authors (3, 12, 13, 16, 47). Powell (31) found that by increasing the length to 9-cm. and prewashing the column with the solvent mixture a quantitative separation of vitamin D_2 could be obtained from irradiated ergosterols in volatile solvents. The longer column was then applied to high-potency irradiated ergosterols in corn oil for this study. This column, however, requires large amounts of solvents and adsorbent so a study was made to determine if a smaller diameter column (8 mm.) could be used.

Pinkerton (29) has quantitatively separated calciferol, from simple synthetic mixtures of the vitamin with the provitamin ergosterol, by means of a short four-cm. column of Superfiltrol. A mixture of 50 parts Skellysolve and 10 parts ether was used as a solvent. Since no visible bands appear on this column, a fixed amount of developing solution (8 ml.) must be added after the sample which is taken up in three ml. of the solution is added to the column. A mathematical means of correcting for residues from Superfiltrol and solvents was employed.

In order to compare the 8-mm. diameter, long and short Superfiltrol columns, the nonsaponifiable fraction of one gm. of an irradiated

DISCUSSION

ergosterol in corn oil containing approximately 500,000 units per gram (#6105) was carried through their specific procedures. This was followed in each case by the regular alumina chromatographic step using 1:1 Skellysolve-ether mixture as the solvent. The absorption curves of the filtrate from each column are shown in Fig. 26. It is quite evident from these curves that while the 9-cm. column retains almost 50% of the D_2, little or no D_2 is retained by the short column and a good separation is achieved. The short four-cm. column was, therefore, adopted for the Two Chromatographic Step Ultraviolet Absorption Curve Method.

Some discussion of the various conversion factors used in this study seems desirable.

For the antimony trichloride colorimetric method the factor 19,500 proposed by Ewing et al (12) was used and a thorough discussion as to how it was obtained is given by these authors. This factor was also used successfully by Powell (31) when applied to irradiated ergosterols in volatile solvents.

The factor 86,960, which has been applied to almost all methods in this study in which the E(1%, 1cm.) @ 265 mu is used as a measure of the amount of vitamins D present, is obtained by dividing the number of vitamin D_2 units per gram of the standard calciferol (40,000,000) by the E(1%, 1cm.) @ 265 mu, which is 460. Arnold (1) has indicated that calciferol contains 49,000,000 units per gram. Livingood (21), in a statistical study carried on simultaneously in these laboratories using the single chromatographic step ultraviolet absorption curve method for high-potency oils proposed in this investigation, found that the conversion factor should

be more near 100,000. For this reason the 100,000 factor is used tentatively for the Two Chrometographic Step Ultraviolet Absorption Curve Method.

SUMMARY

1. Very little or no correlation can be made between the ultraviolet absorption curves of an untreated or saponified corn oil solution of an irradiated ergosterol and its vitamin D potency value.

2. Chemical treatment of the nonsaponifiable fraction of an irradiated ergosterol with digitonin or anhydrous HCl does not increase appreciably the accuracy of the potency evaluation of the oil from its ultraviolet absorption curve. The anhydrous HCl probably removes the vitamin D_2 along with squalene type compounds.

3. Interfering substances can be successfully removed by chromatographic adsorption upon a Superfiltrol column and an alumina column.

(a) Vitamin A, carotenoids, pigments, and possibly toxisterol and lumisterol are removed by means of a prewashed Superfiltrol column using either a mixture of Skellysolve B and ether or a Skellysolve-ether-alcohol mixture as the solvent. The vitamins D are collected in the filtrate.

(b) Solvent and Superfiltrol residues, and squalene type compounds can be removed by use of an alumina column and a 1:1 Skellysolve-ether mixture as the solvent. The vitamins D are retained on the column and must be eluted with ether or additional developing solution.

4. Two physical chemical methods for determining the vitamin D_2 content of high potency samples of irradiated ergosterol in corn oil have been developed.

SUMMARY

One method, using the ultraviolet absorption curve of the nonsaponifiable fraction of the oil sample which had been chromatographed with Superfiltrol to separate the impurities, gave an average variation from the bioassay value of 14.5% when 49 different oils were tested. This method is recommended for oils containing 50,000 or more vitamin D_2 units per gram, when one-gram samples are used.

A colorimetric method, using the color reaction of vitamin D_2 obtained by adding an antimony trichloride reagent to the nonsaponifiable fraction of the oil sample, gave an average variation of 15.8% from the bioassay values of 51 high-potency oils and an average difference of 16.4% from the bioassay values of six low-potency oils. This method is recommended for oil solutions of irradiated ergosterol, having a potency of 10,000 vitamin D_2 units or more per gram.

5. A two chromatographic step method for determining the vitamin D content of both high or low potency irradiated ergosterols in corn oil and possibly multivitamin oil solutions has been developed.

The nonsaponifiable fraction of the oil is first chromatographed using a four-cm. Superfiltrol column. The vitamins D collected in the filtrate are then adsorbed upon an alumina column for further purification. The ultraviolet absorption curve of the eluted vitamins D is then used to evaluate the potency of the original oil.

The average deviation from the bioassay value for six irradiated ergosterols and one crystalline D_2 solution in corn oil was 12.11%. These

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ranged from 1,000,000 to 9,680 units/gram. The method was shown to be accurate for oils having potencies as low as 2,000 - 3,000 units/gram by diluting a higher valued oil to this range.

Seven multiple vitamin solutions tested by this method ranging from 250,000 to 1,590 units/gram showed an average variation of 41.7%.

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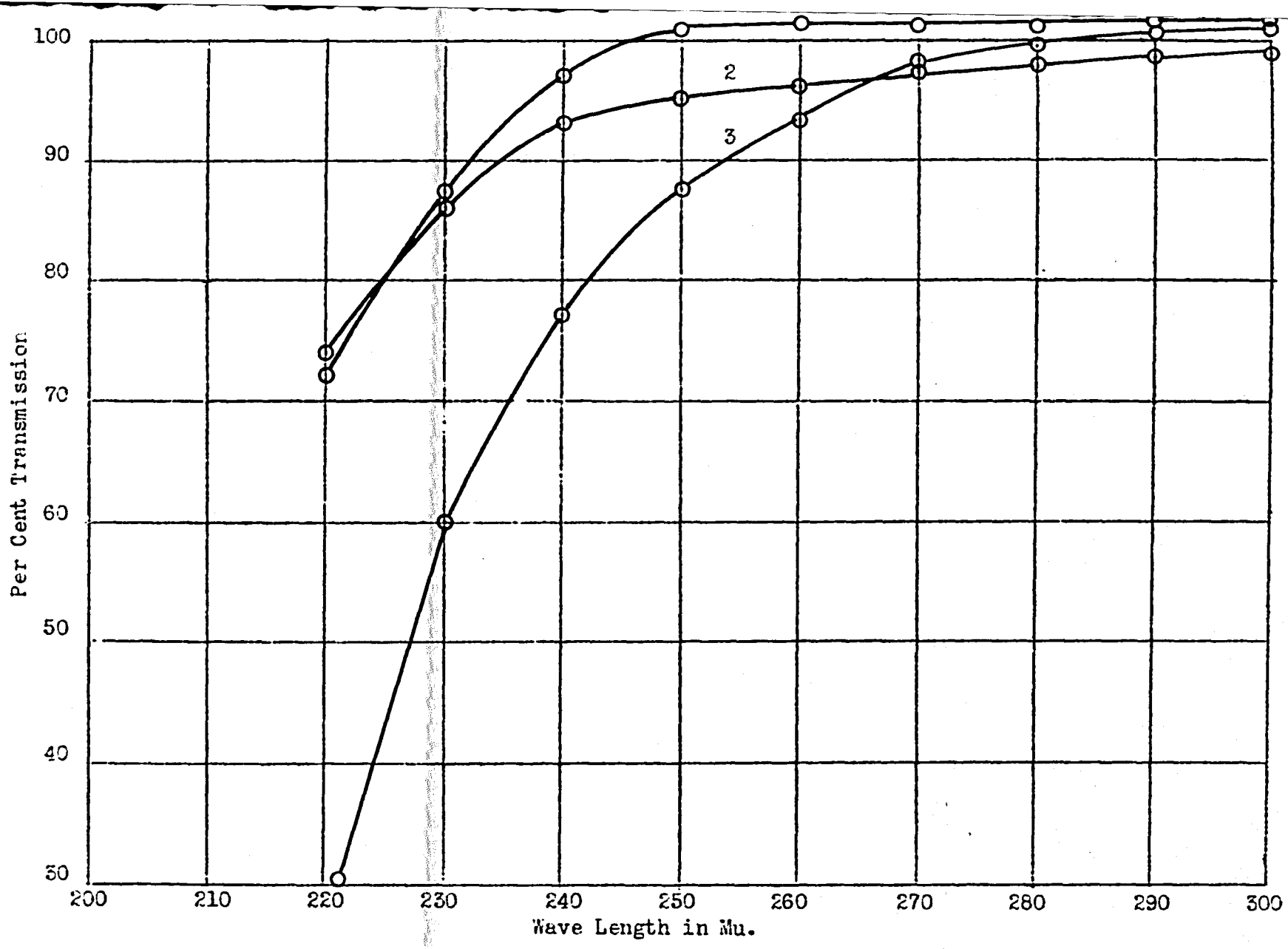

Figure 1. - Transmission curves of purified solvents as compared to water: 1, 95% ethanol; 2, Skellysolve B; 3, anhydrous ethyl ether.

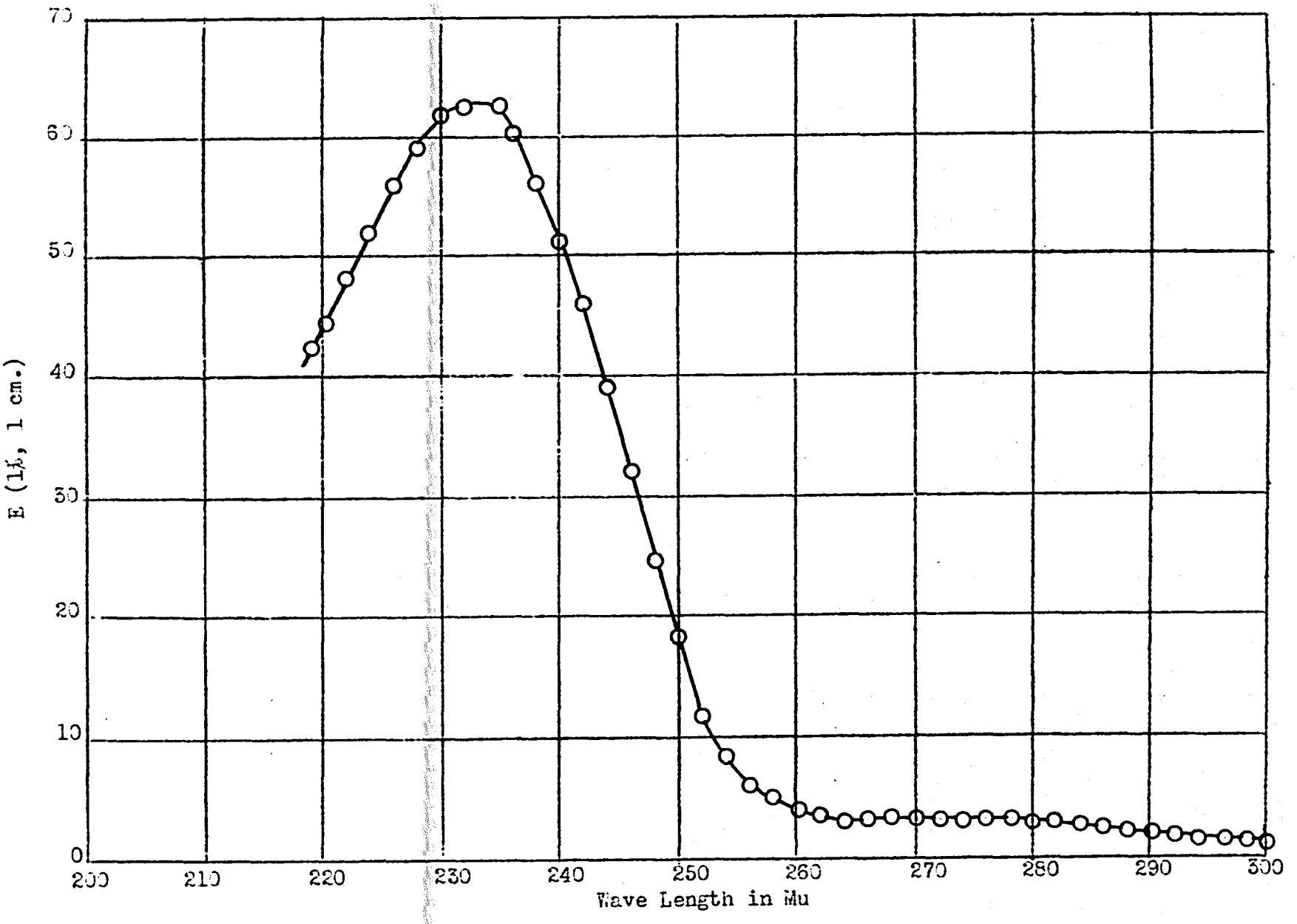

Figure 2. Absorption Curve of Corn Oil in Ethanol.

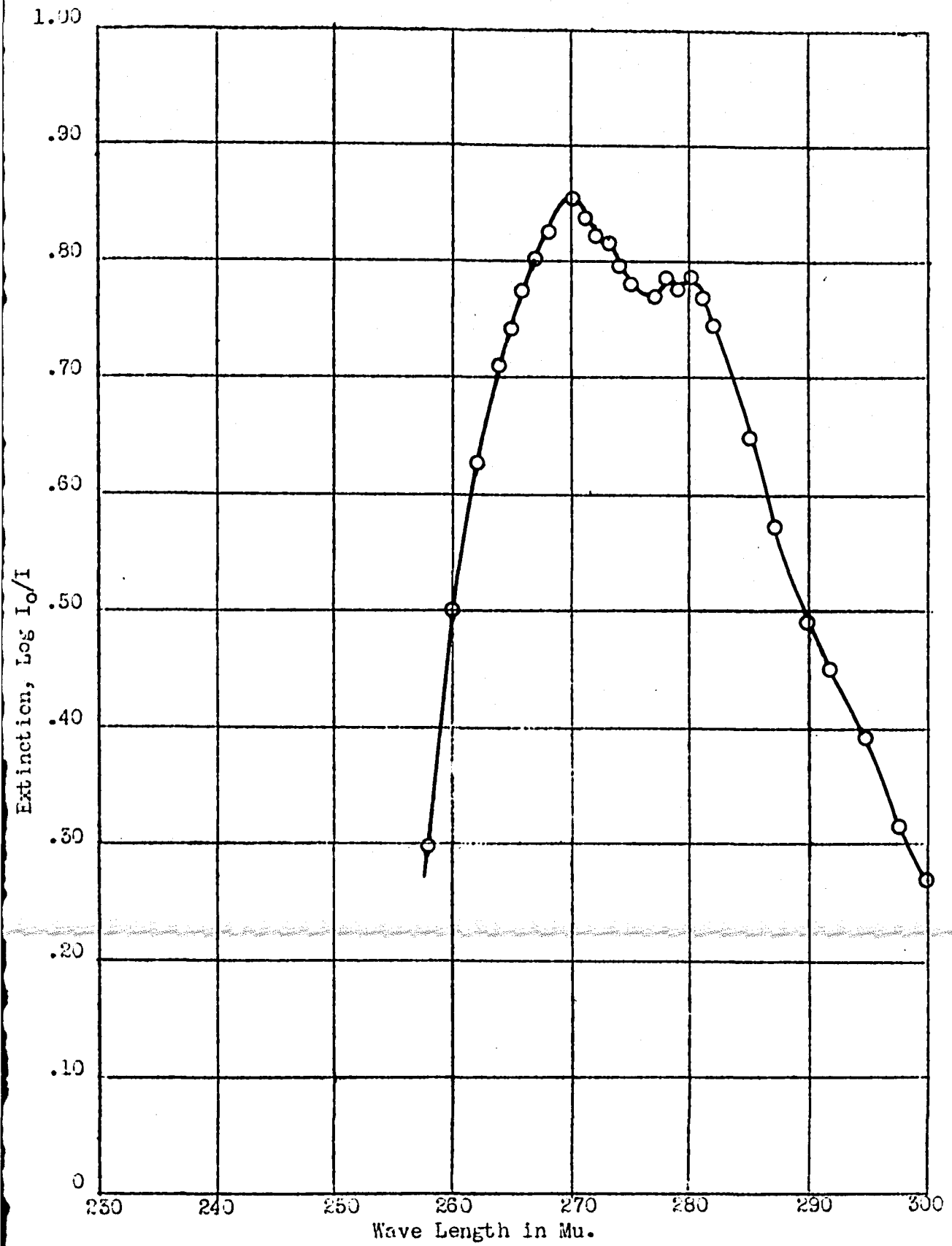

Figure 3. Absorption Curve of Irradiated Ergosterol #69934 in Ethanol Ran With an Ethanol Solution of Corn Oil in the Solvent Cell.

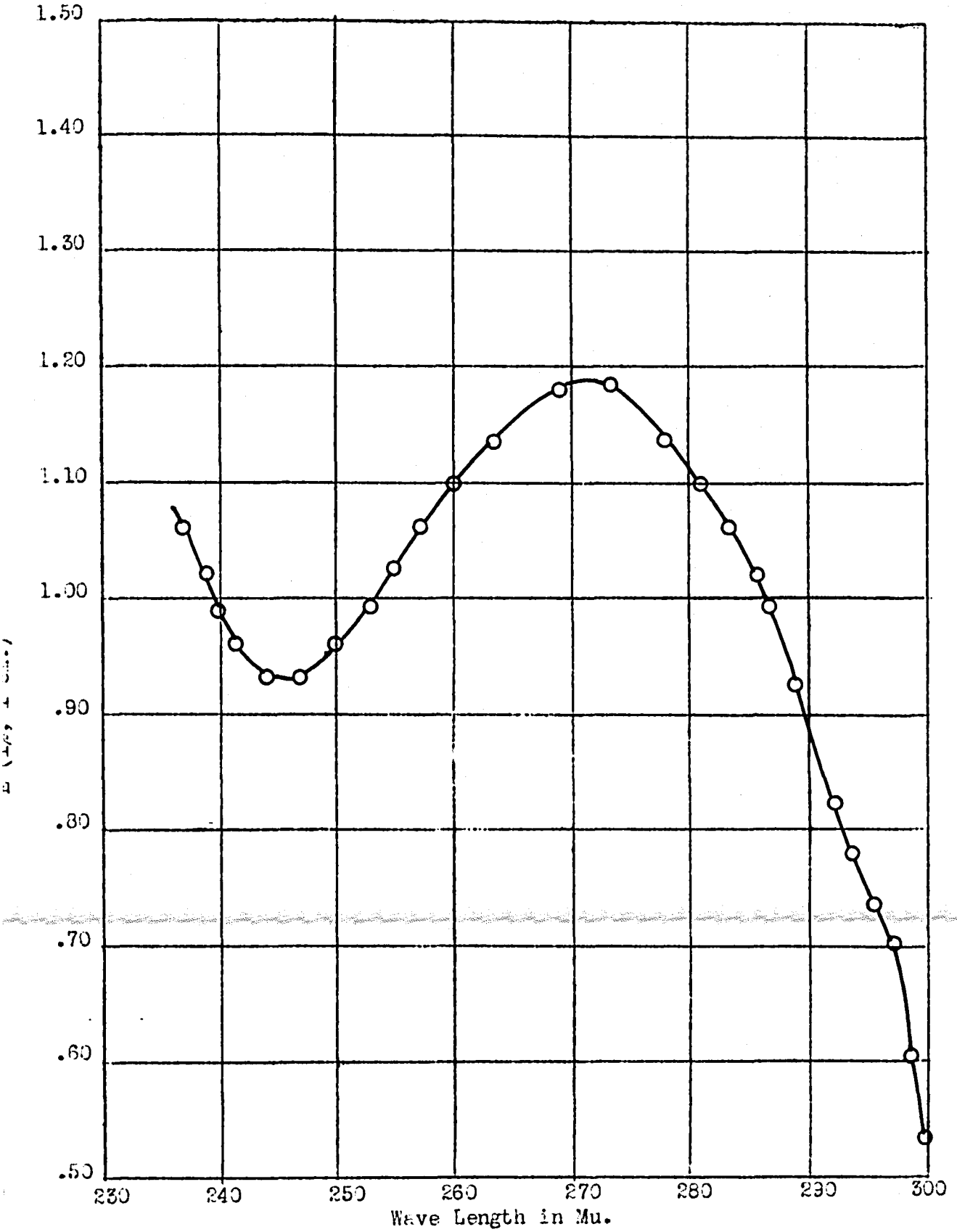

Figure 4. Absorption Curve of Irradiated Ergosterol #65624 in Ethanol After Saponification.

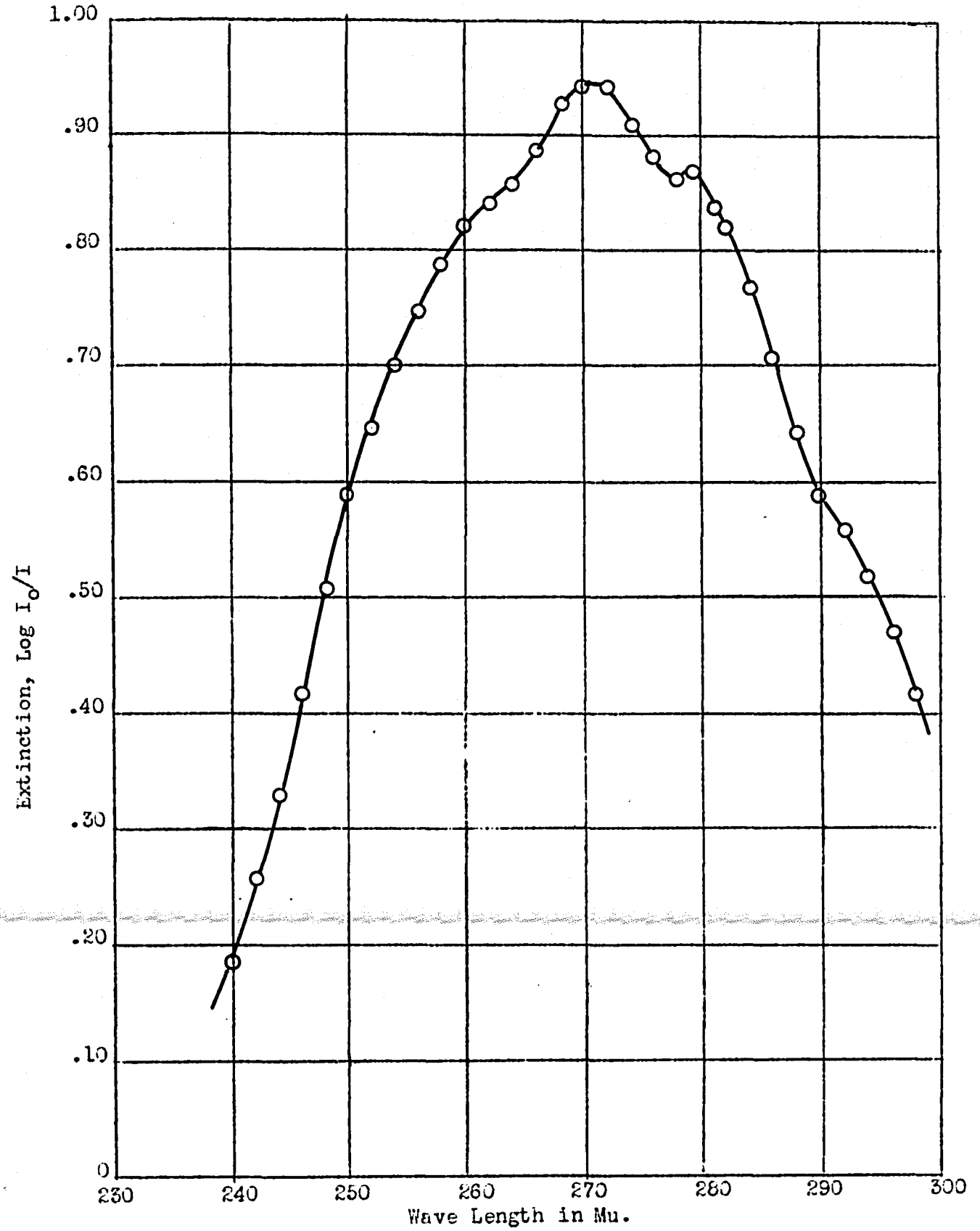

Figure 5. Absorption Curve of Saponified Irradiated Ergosterol #69934 in Ethanol Ran With an Ethanol Solution of Saponified Corn Oil in the Solvent Cell.

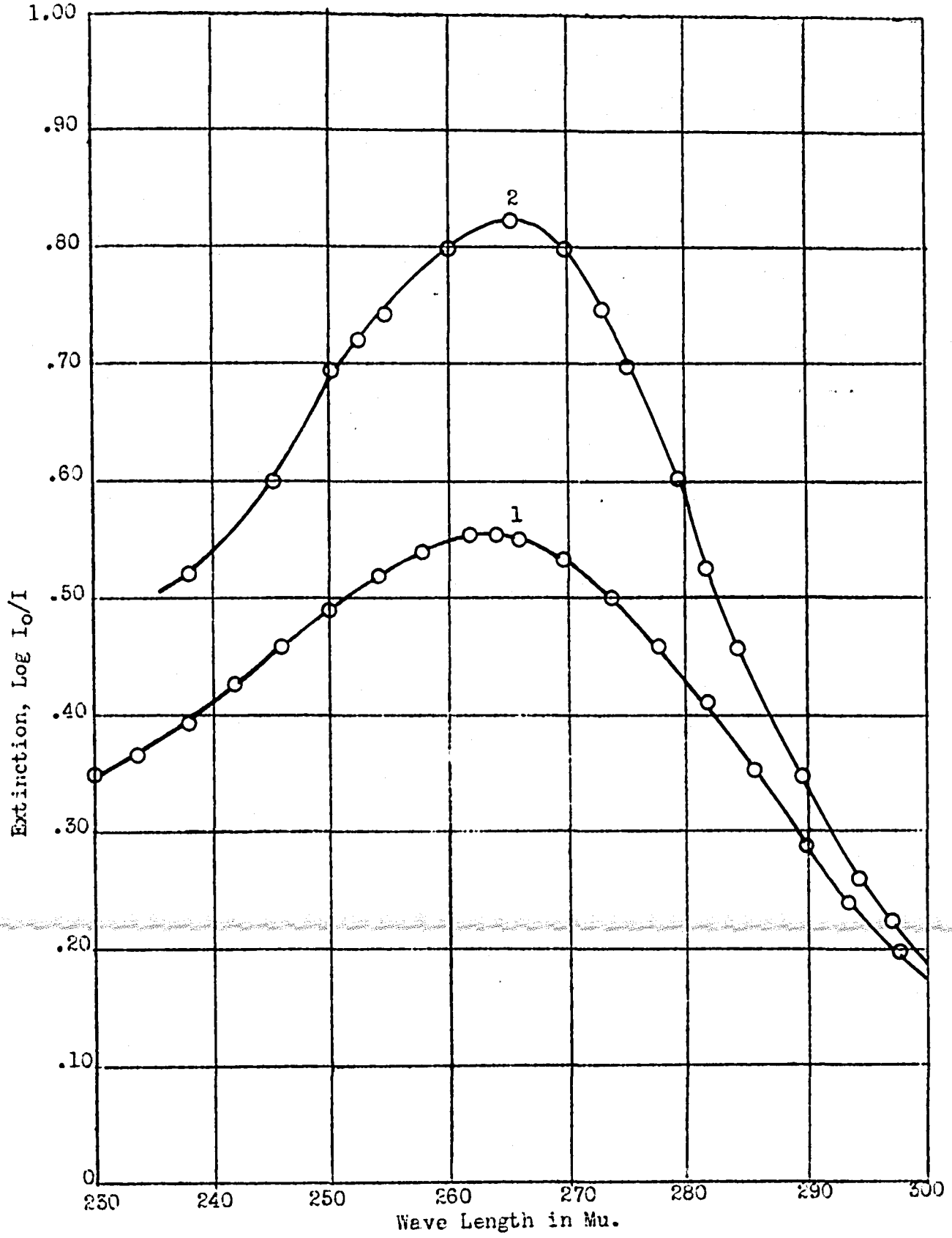

Figure 6. - Absorption curve of oil #B-11299 in ethanol after saponification and chromatographing through column of Superfiltrol: 1, treated oil B-11299; 2, standard calciferol.

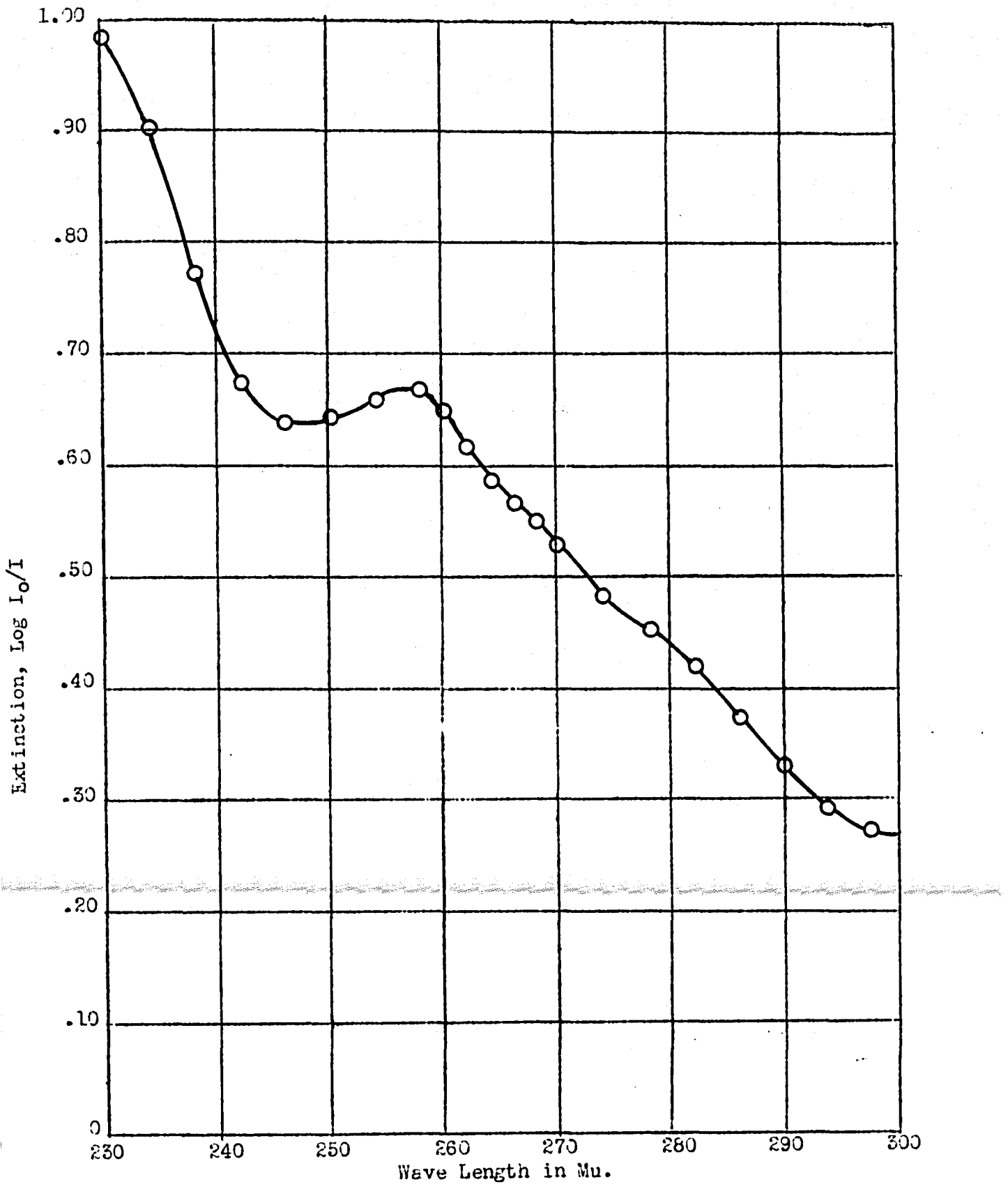

Figure 7. Absorption Curve of Low Potency Irradiated Ergosterol #0905 After Carrying It Through the Single Chromatographic Step Ultraviolet Absorption Curve Method. (Superfiltrol Column).

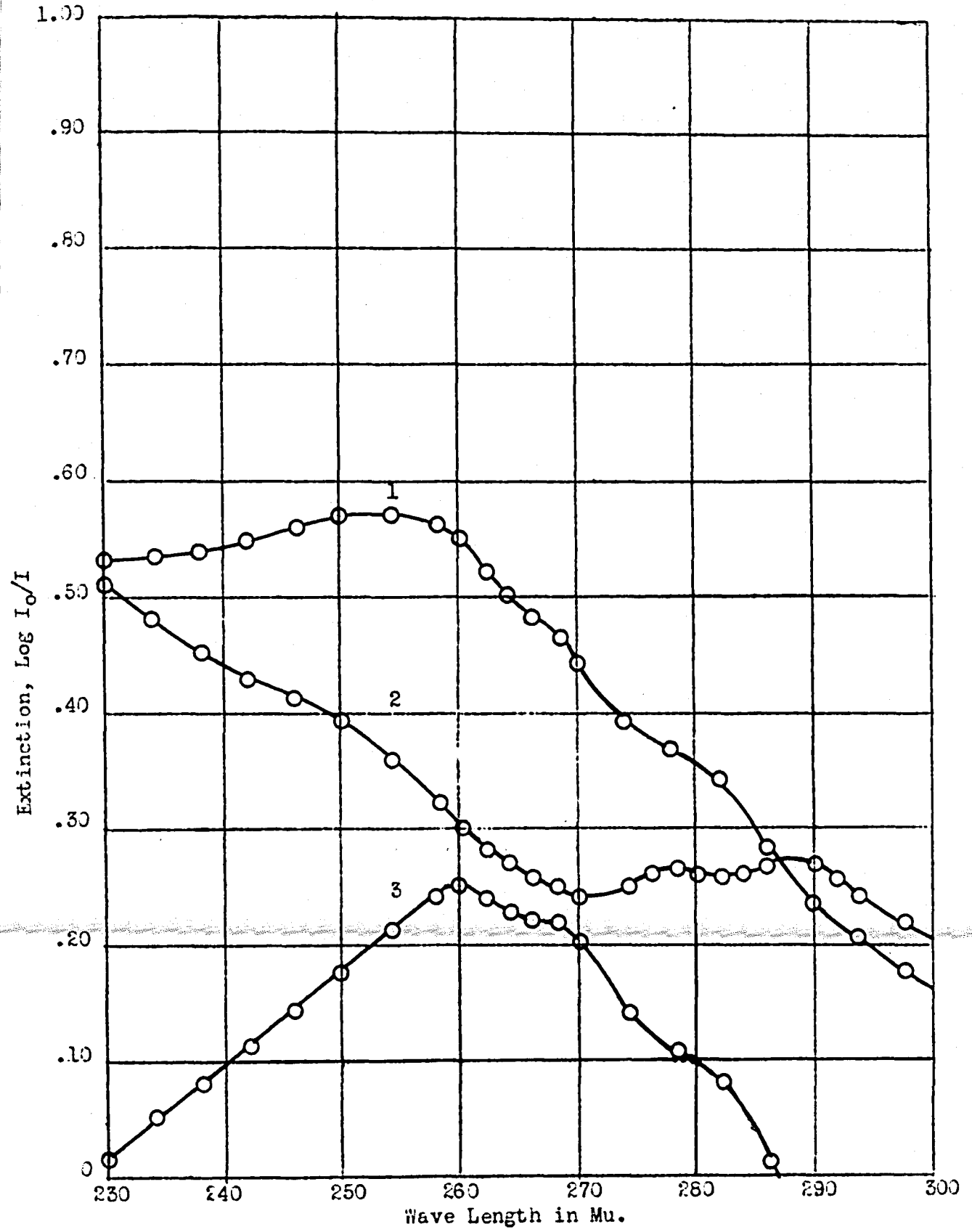

Figure 8. - Calculated absorption curve of material removed from corn oil solution of irradiated ergosterol #73978 by HCl treatment: 1, Oil #73978 after saponification; 2, Oil #73978 after saponification and HCl treatment; 3, curve 1 minus curve 2 (material removed by HCl treatment).

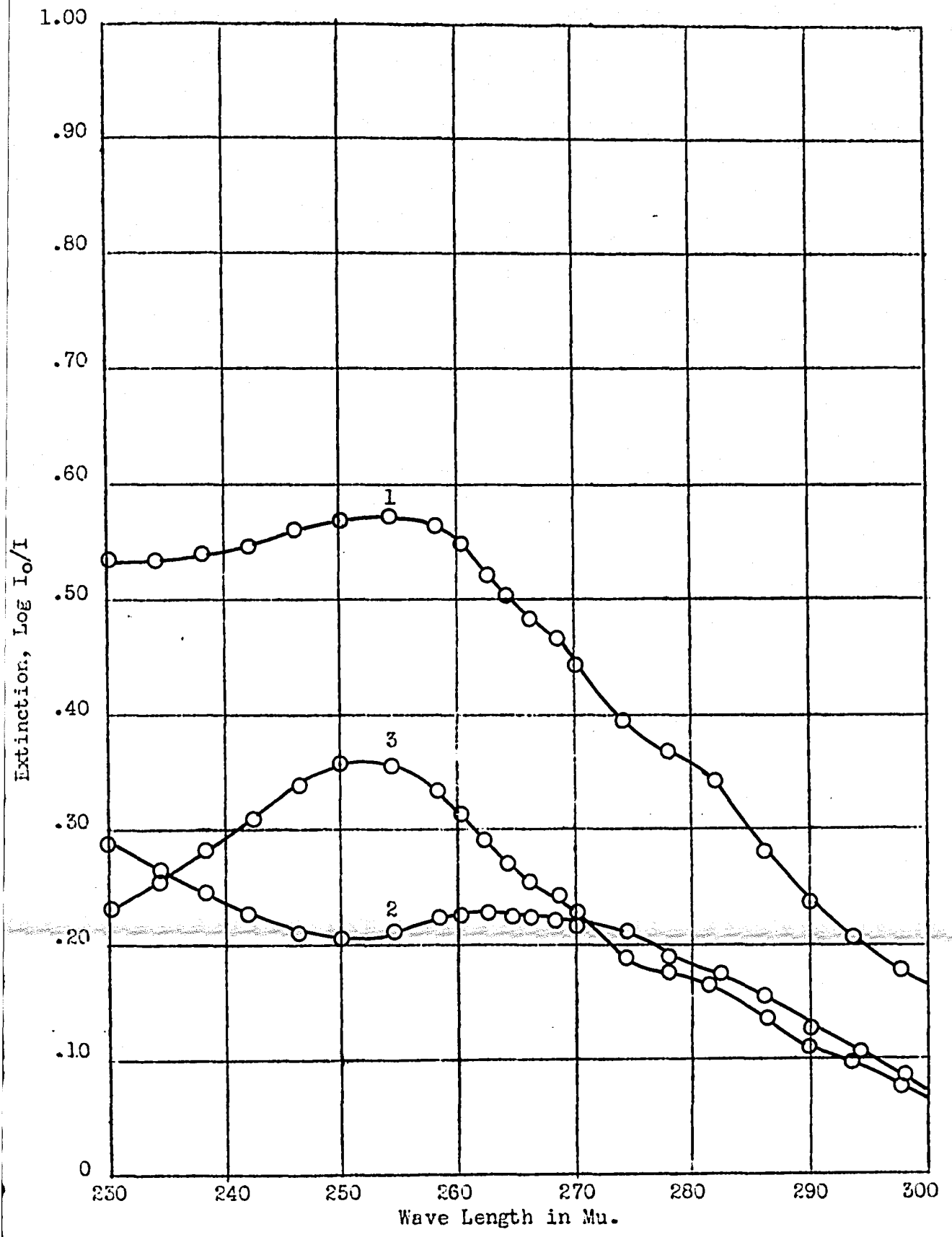

Figure 9. - Calculated absorption curve of material removed from corn oil solution of irradiated ergosterol #73978 by a column of Superfiltrol: 1, Oil #73978 after saponification; 2, Oil #73978 after saponification and chromatographing with a column of Superfiltrol; 3, curve 1 minus curve 2. (material removed by Superfiltrol).

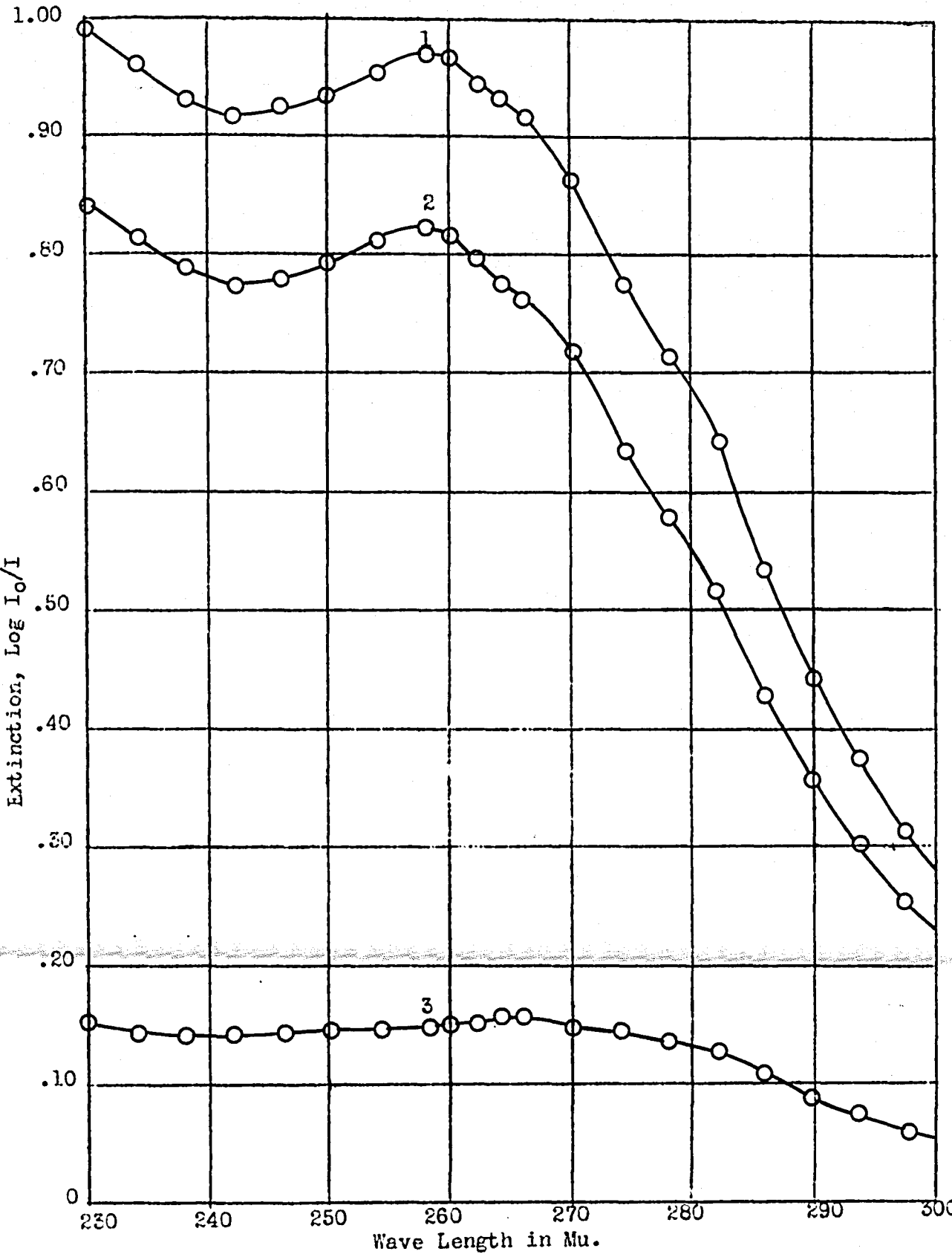

Figure 10. - Absorption curve of material removed from corn oil solution of crystalline vitamin D_2 by digitonin treatment: 1, D_2 solution after saponification; 2, D_2 solution after saponification and digitonin treatment; 3, curve 1 minus curve 2. (material removed by digitonin).

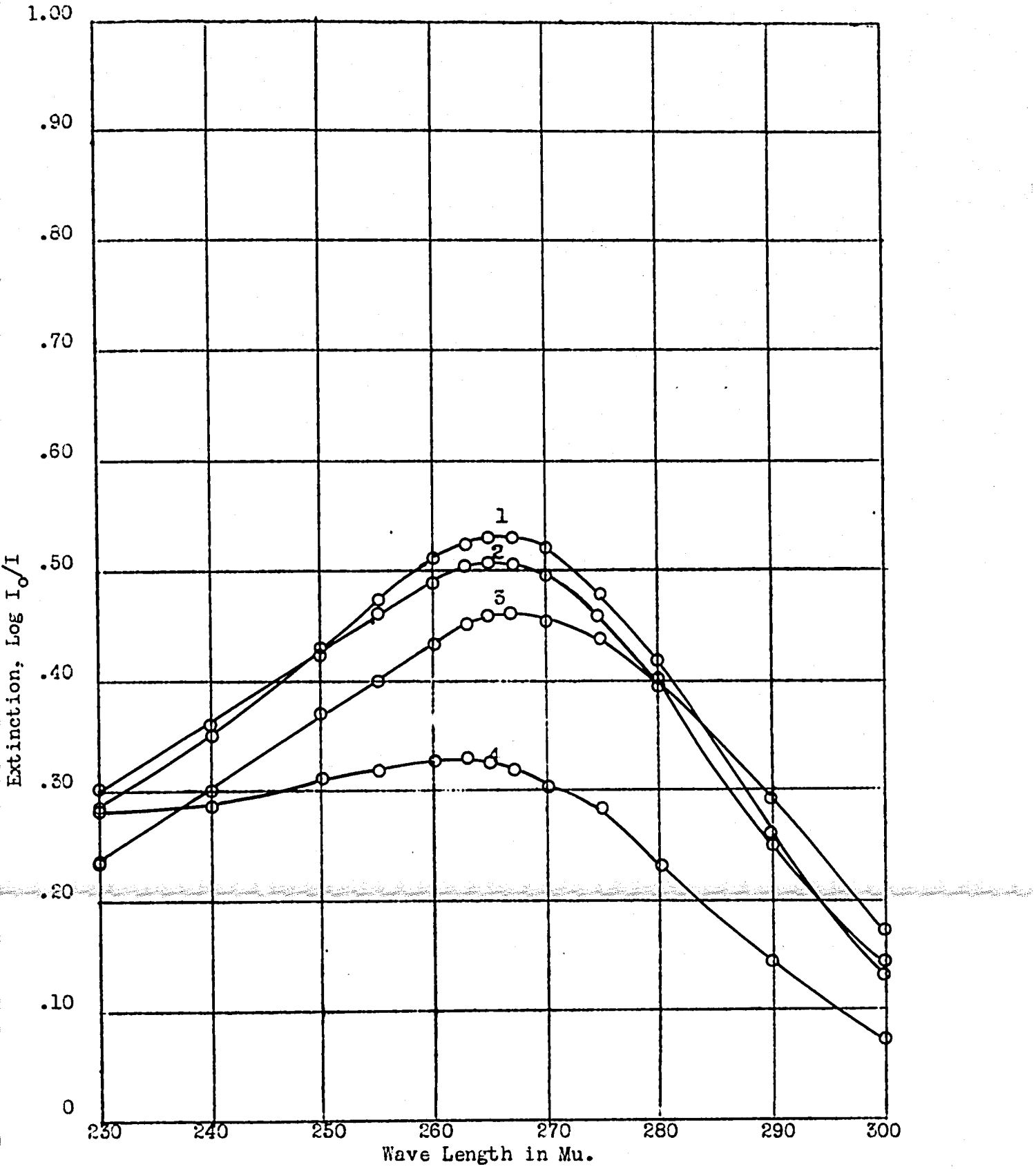

Figure 11. - Absorption curves of irradiated ergosterols in oil after treatment by the Two Chromatographic Step Ultraviolet Absorption Curve method: 1, Oil #0339, 1,000,000 u/gm.; 2, Oil #96339, 151,000 u/gm.; 3, Oil #98449, 464,000 u/gm.; 4, Oil #2319, 11,250 u/gm.

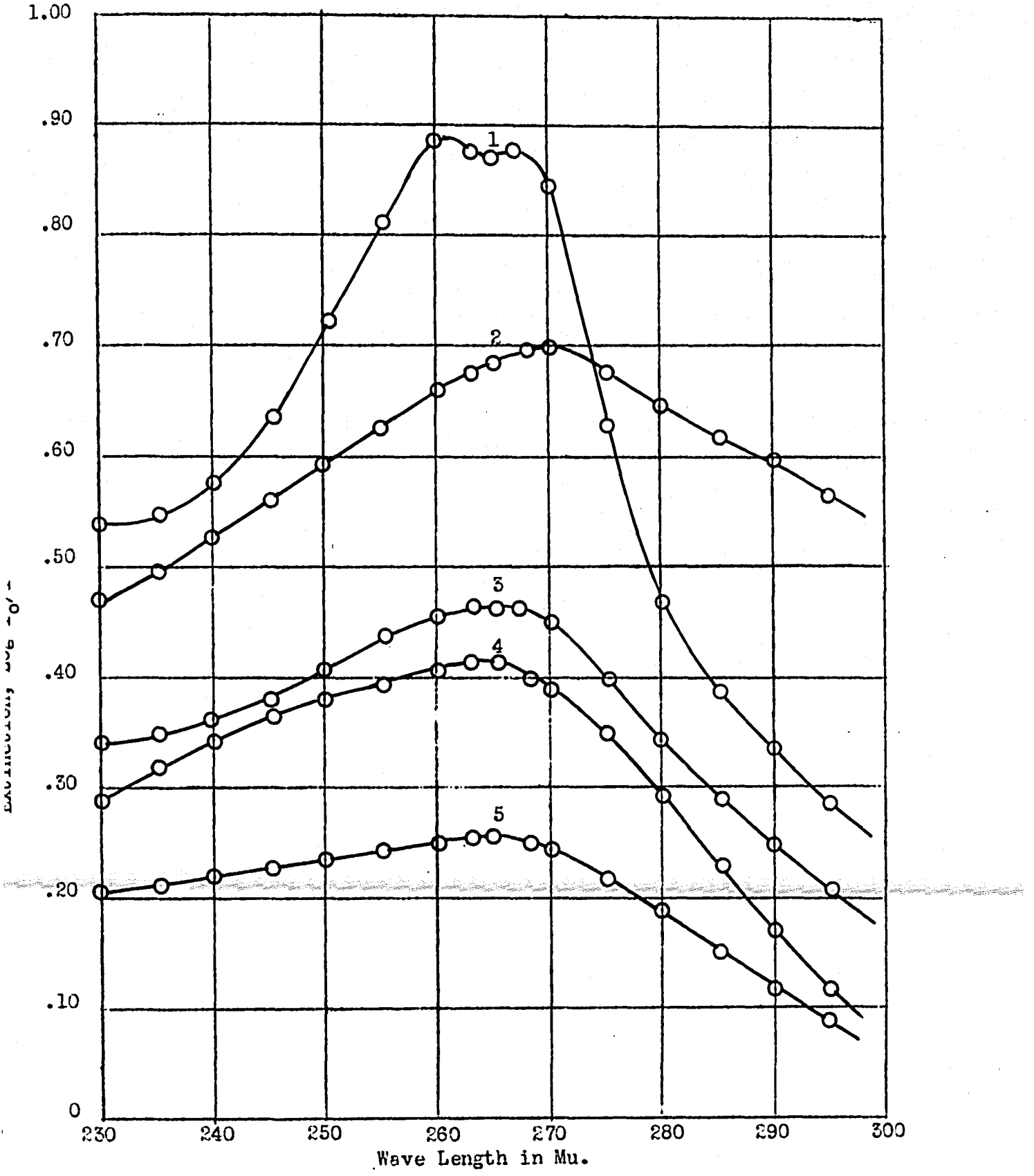

Figure 12. - Absorption curves of various multiple vitamin solutions after treatment by the Two Chromatographic Step Ultraviolet Absorption Curve method: 1, ABDEC, multiple vitamin solution in ethylene glycol; 2, Haliver Oil #37756; 3, Natola #37786; 4, High D oil (mostly D_3) #0399; 5, Oil mix for Natola (75% D_2, 25% D_3) #6299.

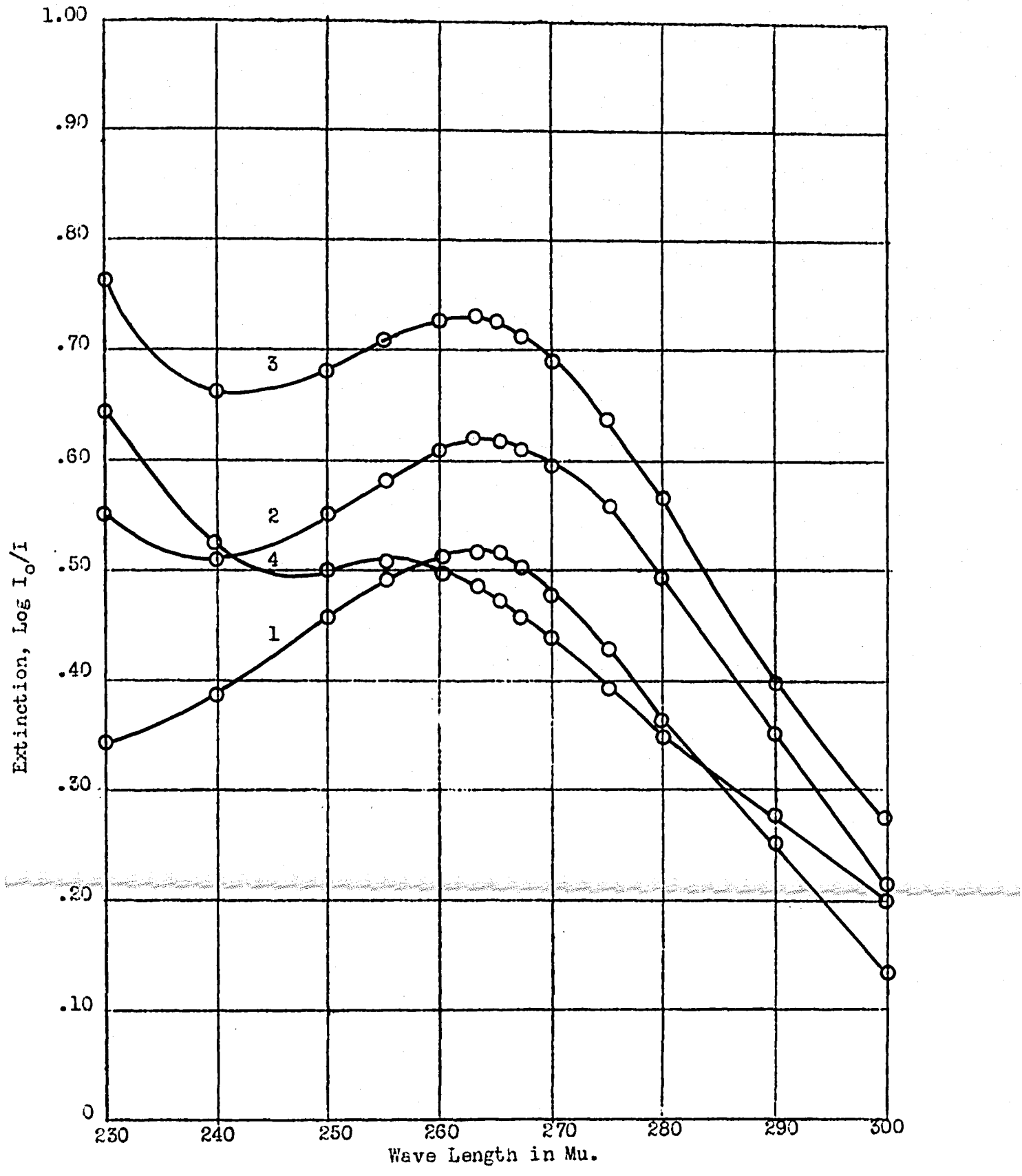

Figure 13. - Absorption curves of various dilutions of irradiated ergosterol #6105 with corn oil which were treated by the Two Chromatographic Step Ultraviolet Absorption Curve method. Dilutions ran were: (1) 13,400 D_2 u/gm.; (2) 6,660 D_2 u/gm.; (3) 3,380 D_2 u/gm.; (4) 1,652 D_2 u/gm.

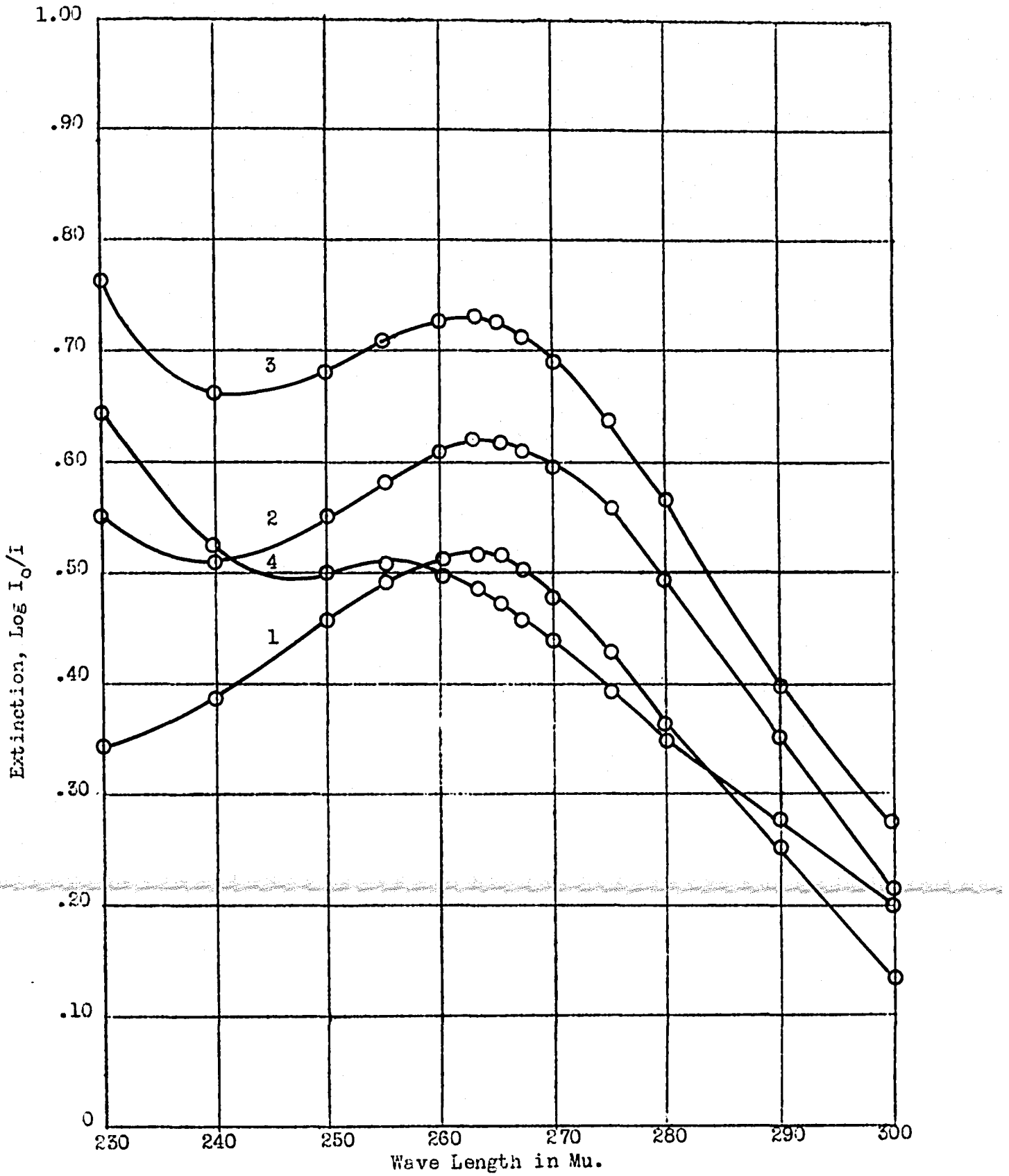

Figure 13. - Absorption curves of various dilutions of irradiated ergosterol #6105 with corn oil which were treated by the Two Chromatographic Step Ultraviolet Absorption Curve method. Dilutions ran were: (1) 13,400 D_2 u/gm.; (2) 6,660 D_2 u/gm.; (3) 3,380 D_2 u/gm.; (4) 1,652 D_2 u/gm.

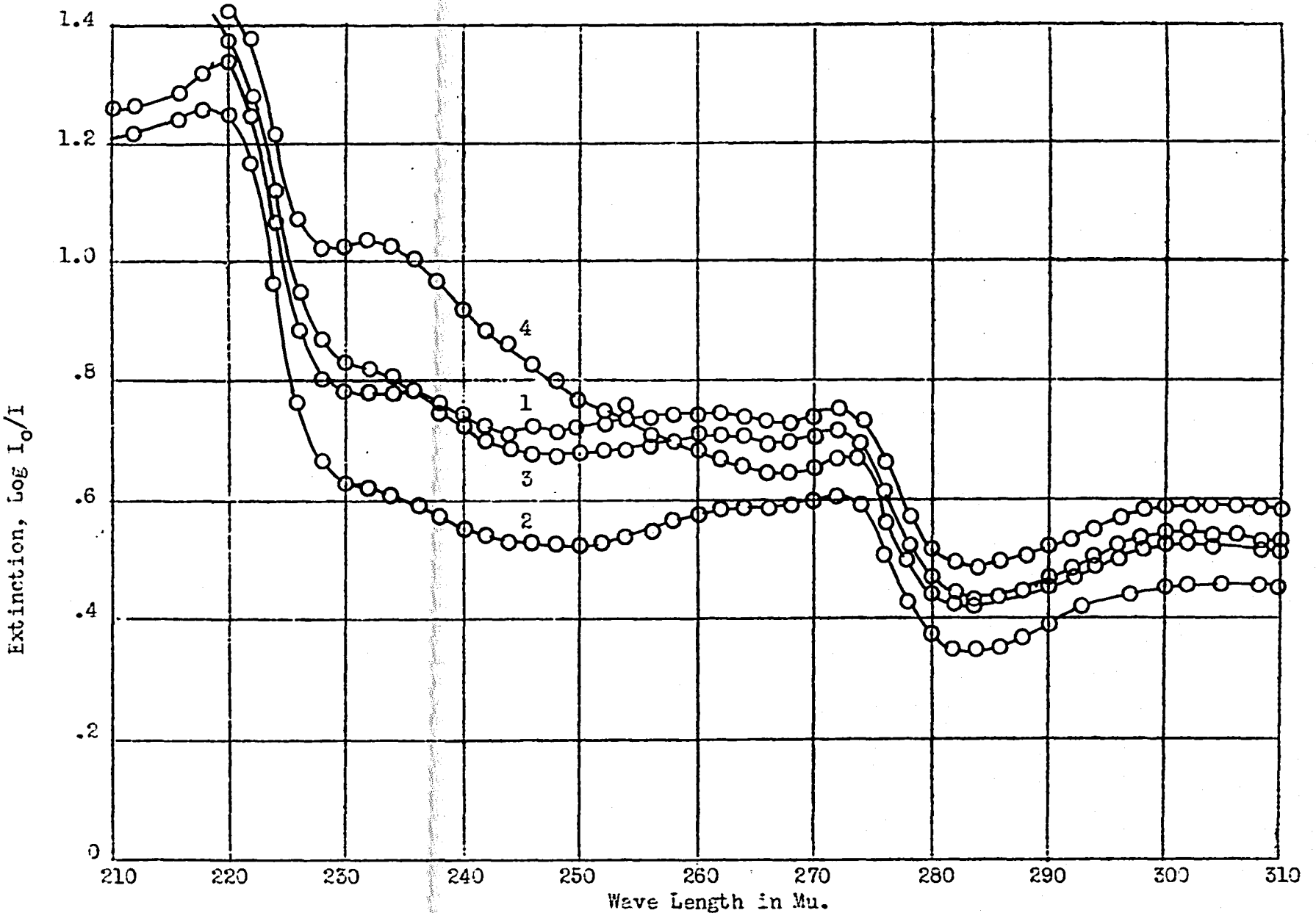

Figure 14. - Absorption curve of blank sample (10 ml. ethanol) at the end of various steps in the saponification procedure.: (1) after saponification and extraction; (2) after saponification, extraction, and washing; (3) after saponification, extraction, washing, and drying through Na_2SO_4; (4) residue from 100 ml. ethyl ether (anhyd.).

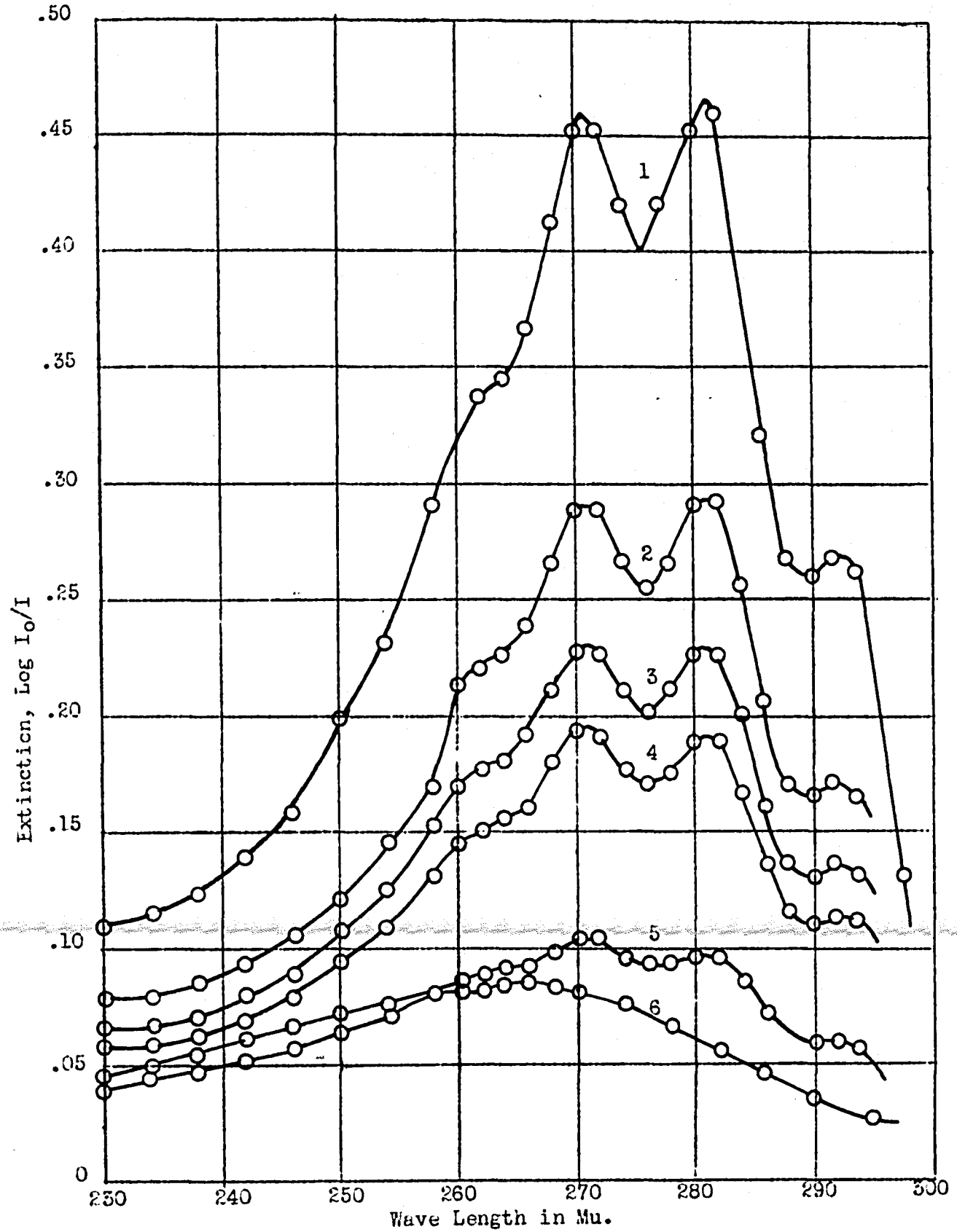

Figure 15. - Absorption curves of an ergosterol-calciferol mixture after treating with various amounts of 1% digitonin. Amounts are: (1) untreated solution; (2) 2 ml.; (3) 3 ml.; (4) 4 ml.; (5) 6 ml.; (6) calculated curve of calciferol present.

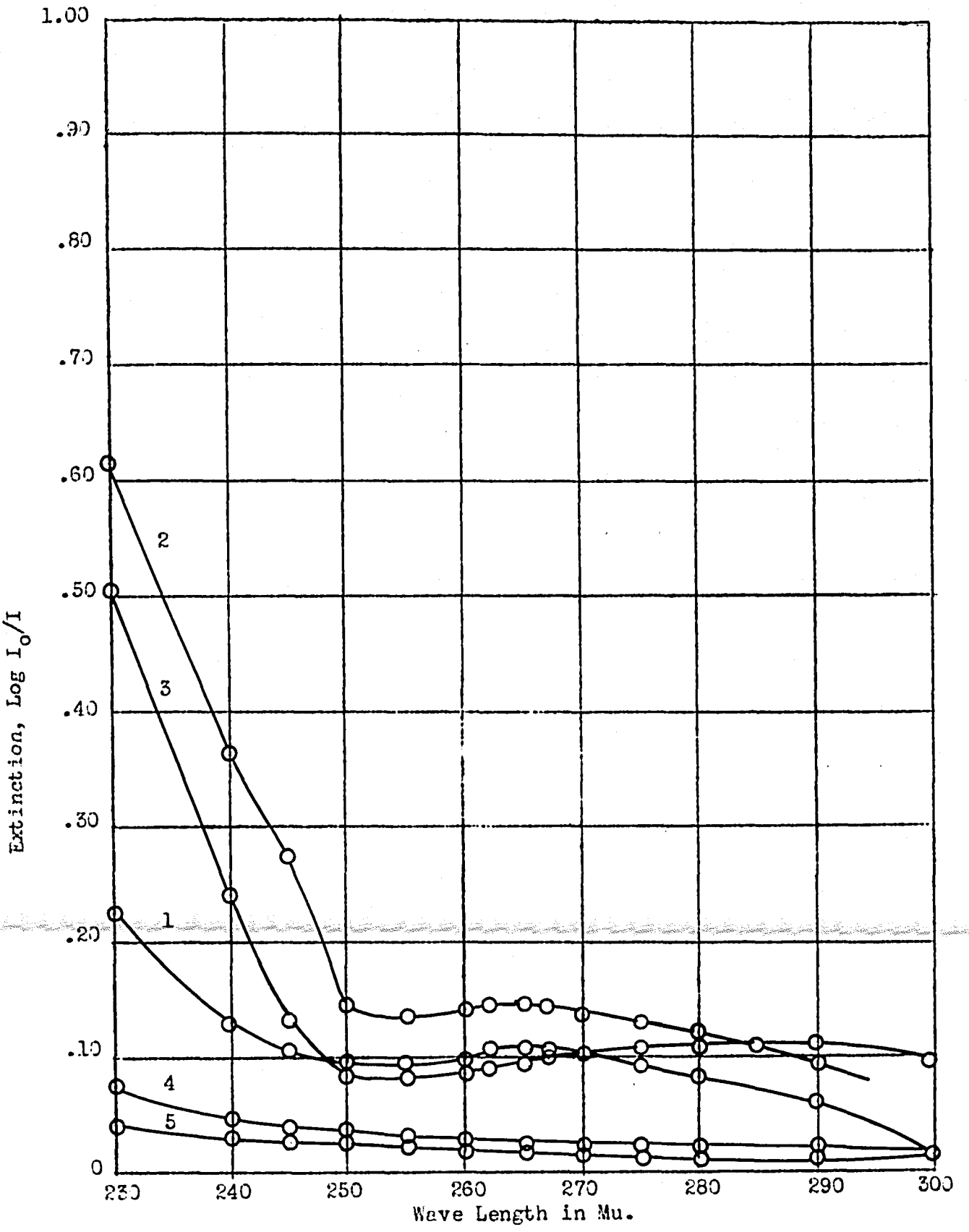

Figure 16. - Absorption curves of various fractions of the nonsaponifiable portion of crystalline D_2 in corn oil taken as filtrate from 6 gram alumina column using Skellysolve as the solvent. Volumes for each fraction are listed in order taken: (1) 4 ml.; (2) 4 ml.; (3) 4 ml.; (4) 5 ml.; (5) 5 ml.

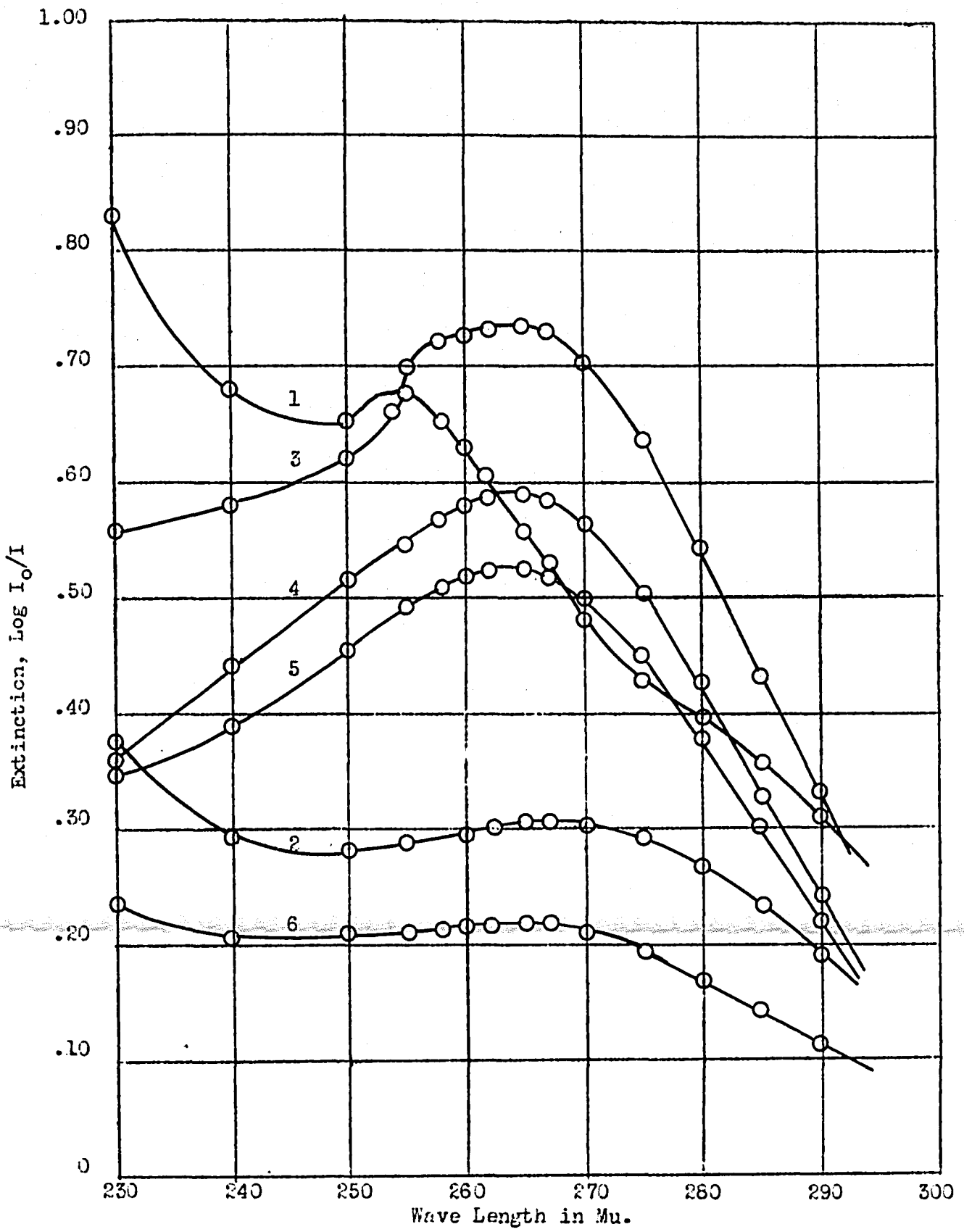

Figure 17. - Absorption curves of various fractions of the nonsaponifiable portion of crystalline D_2 in corn oil taken as eluate from 6 grams alumina column using Skellysolve as the solvent. Volumes for each fraction are listed in order taken: (1) 5 ml.; (2) 5 ml.; (3) 5 ml.; (4) 5 ml.; (5) 5 ml.; (6) 5 ml.

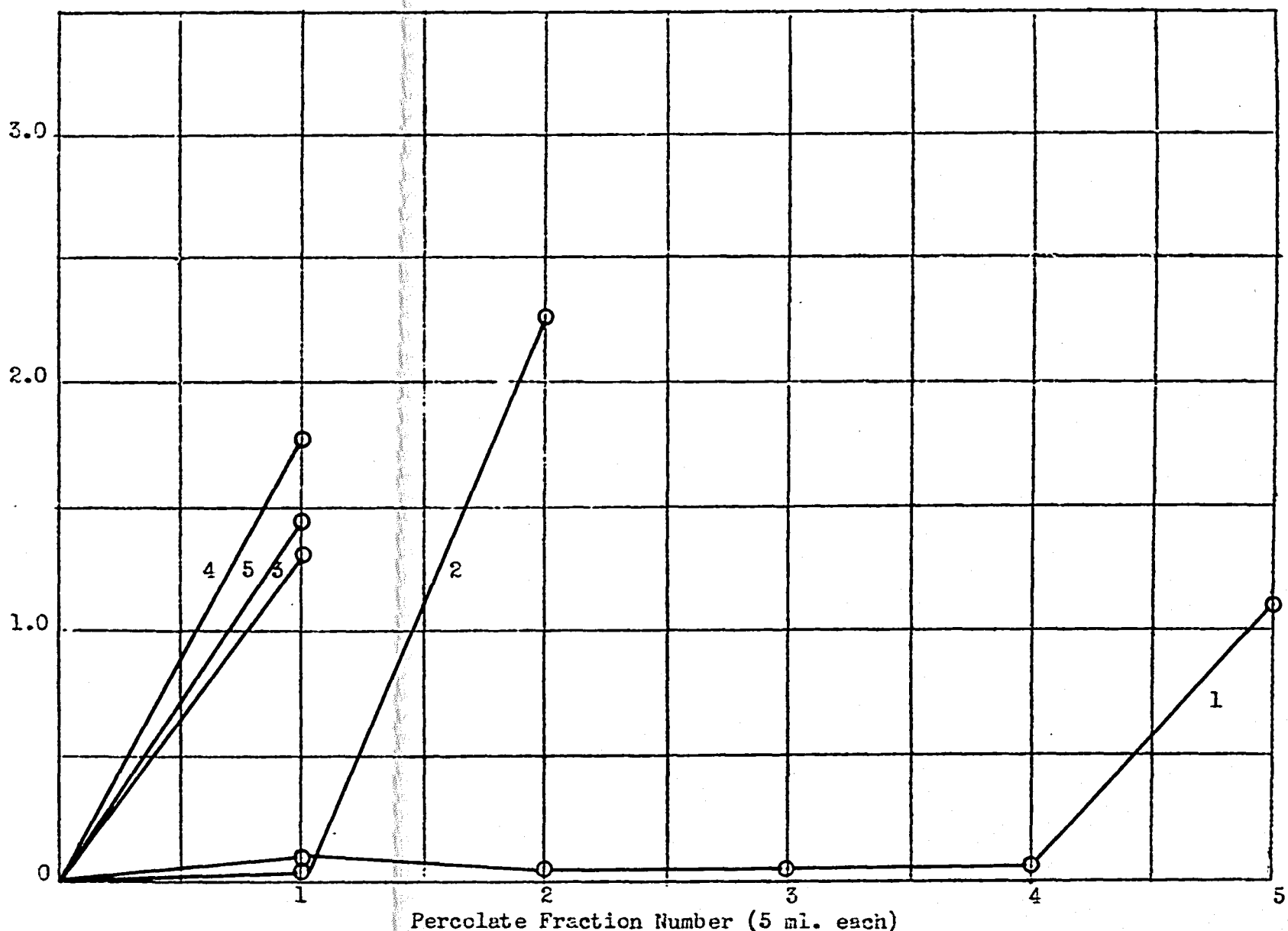

Figure 18. - Approximate threshold volume of 4 gram alumina column for vitamin D_2 using various Skellysolve-alcohol mixtures as the solvent. Skellysolve-alcohol ratios used were: (1) 100:1; (2) 50:1; (3) 50:3; (4) 50:5; (5) 50:8.

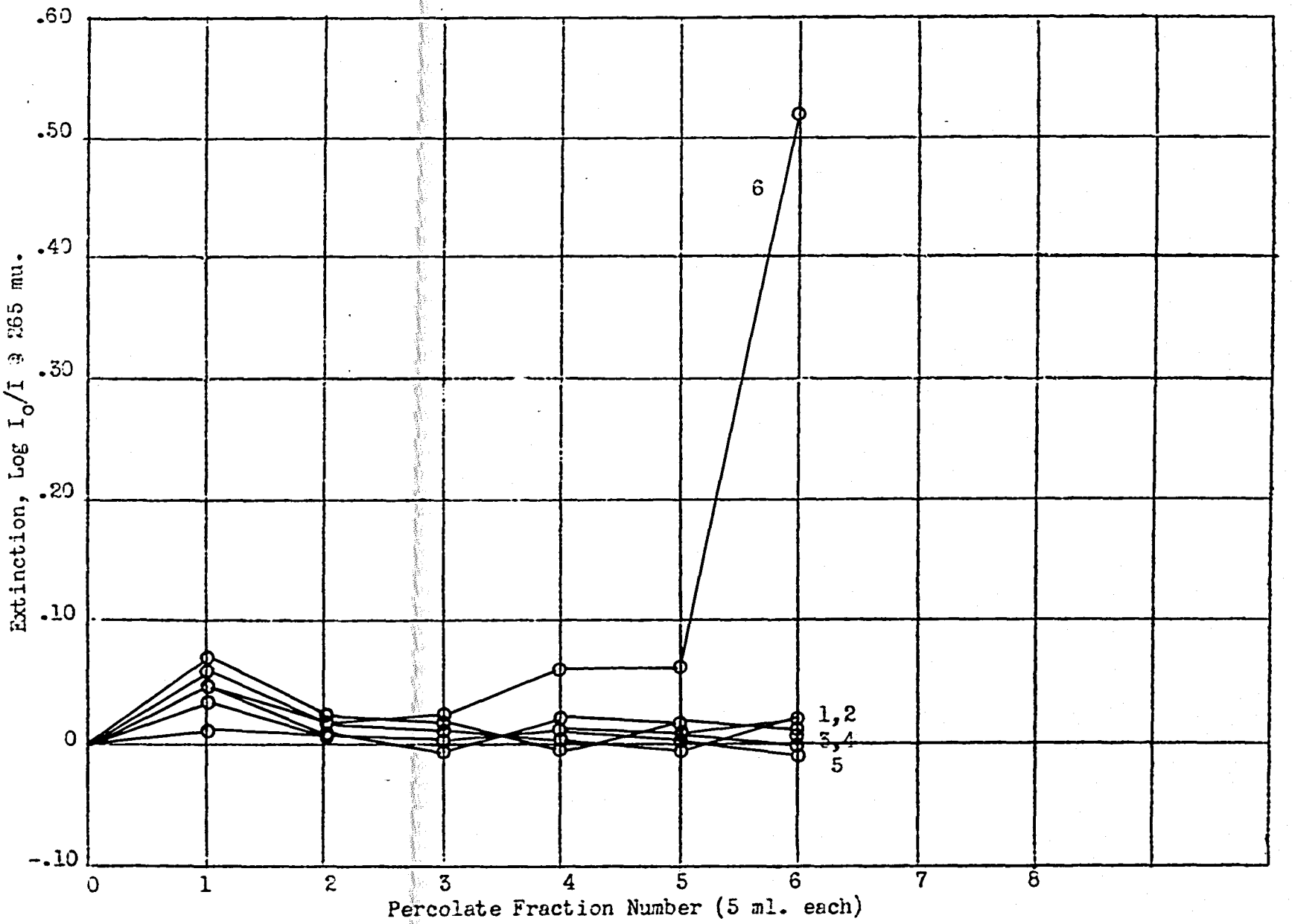

Figure 19. - Approximate threshold volume of 4 gram alumina column for vitamin D_2 using various Skellysolve-ether mixtures as the solvent. Skellysolve-ether ratios used were: (1) 50:1; (2) 50:3; (3) 50:5; (4) 50:8; (5) 40:15; (6) 50:50.

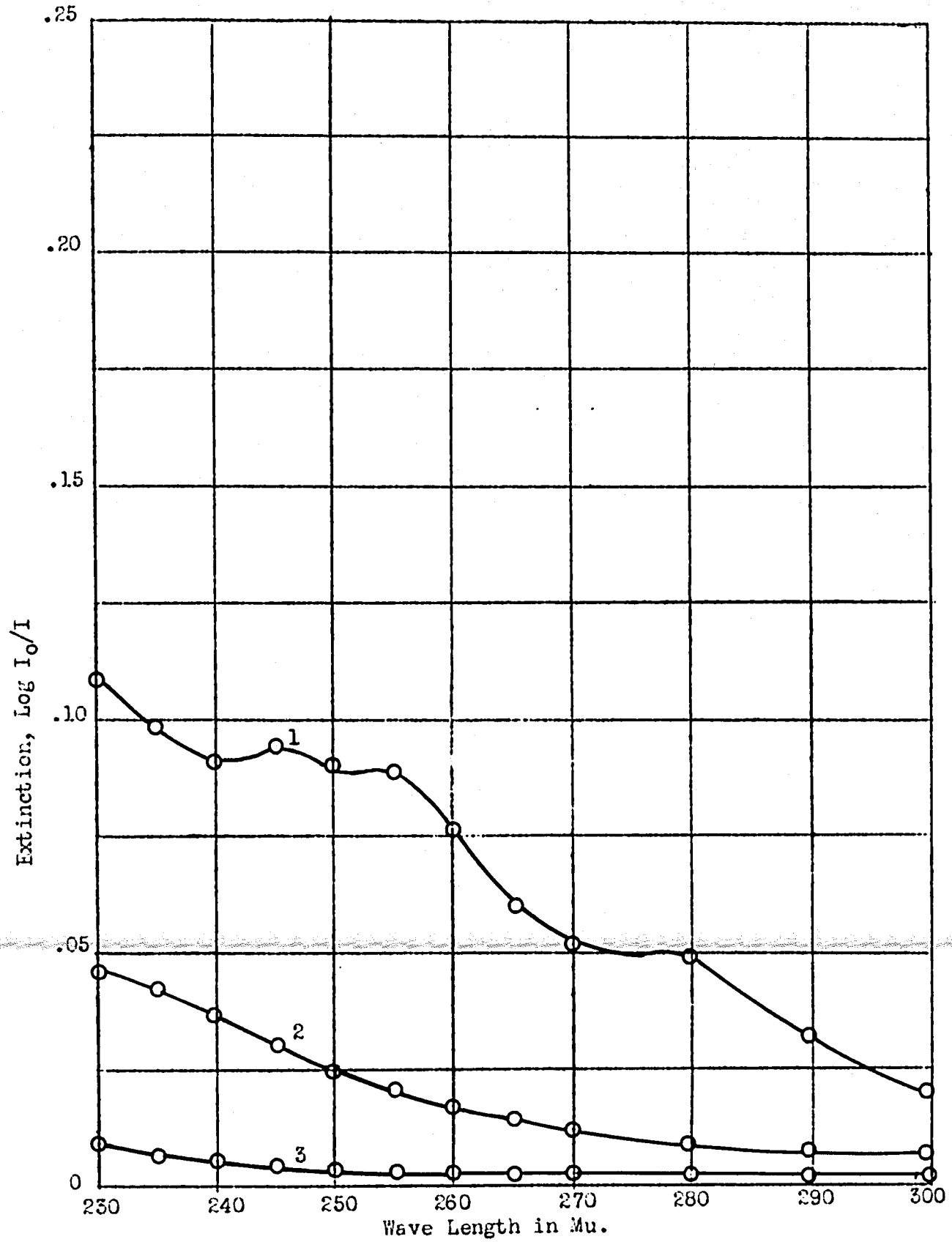

Figure 20. - Absorption curve of residue eluted from 4 gram alumina column by successive 5 ml. portions of ether: 1, first portion; 2, second portion; 3, third portion.

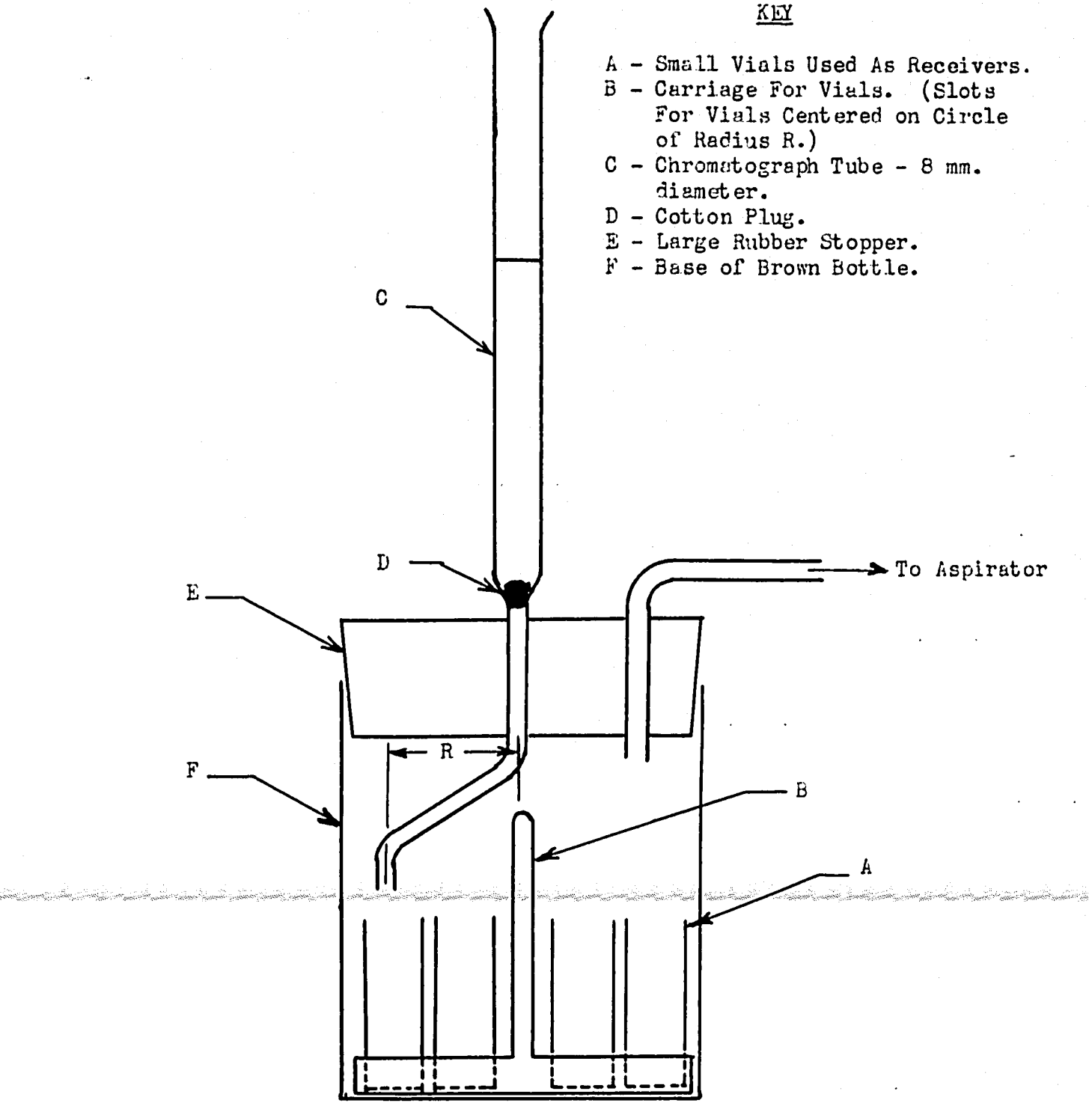

Figure 21. Special Apparatus Designed For Collecting Fractions From Chromatograph Column Without Disturbing Equilibrium.

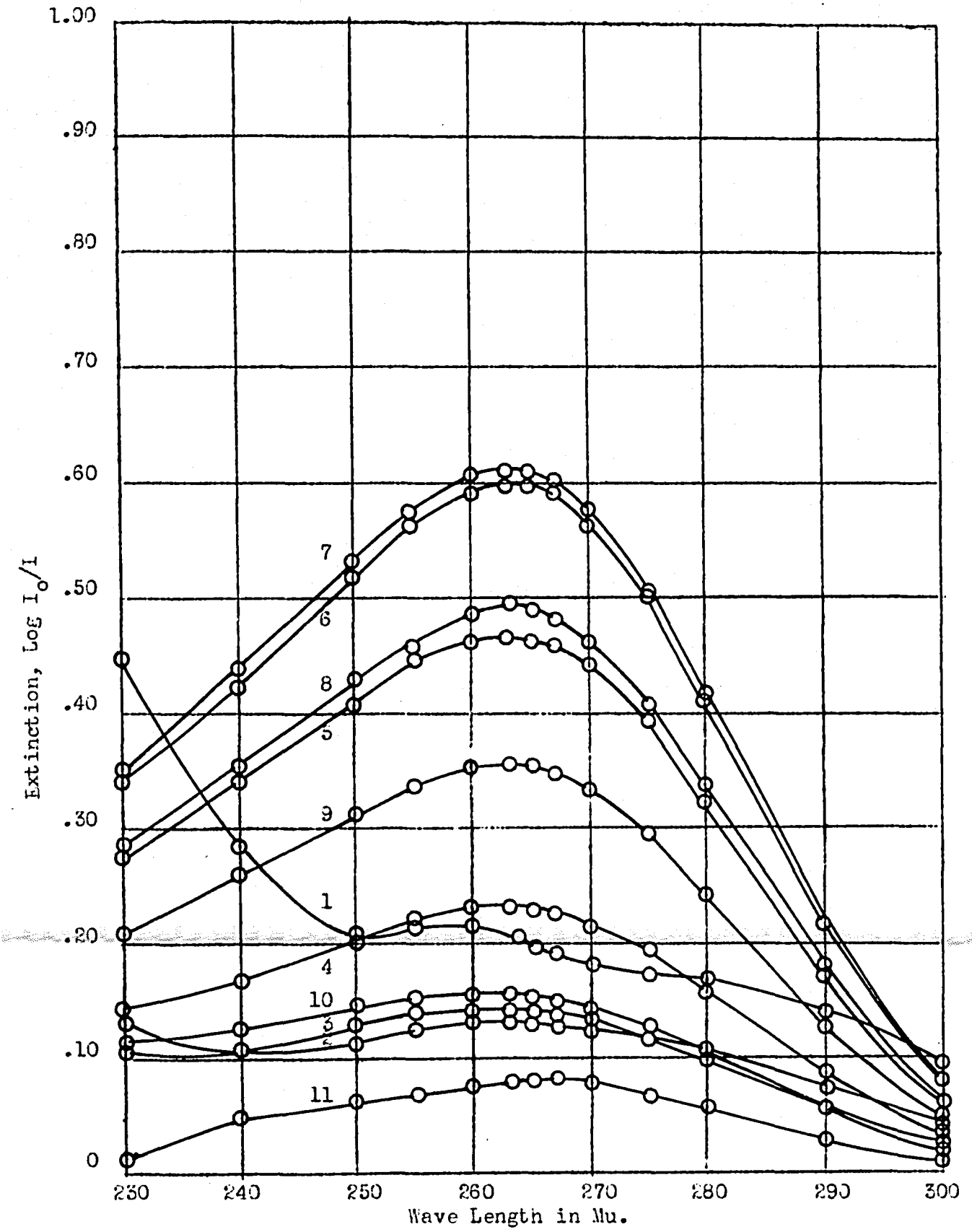

Figure 22. - Absorption curves of various fractions of 10,000 units crystalline D taken from alumina column. Volumes for each fraction are listed in order taken: (1) 3 ml.; (2) 5 ml.; (3) 3 ml.; (4) 2 ml.; (5) 2 ml.; (6) 2 ml.; (7) 2 ml.; (8) 2 ml.; (9) 2 ml.; (10) 2 ml.; (11) 2 ml.

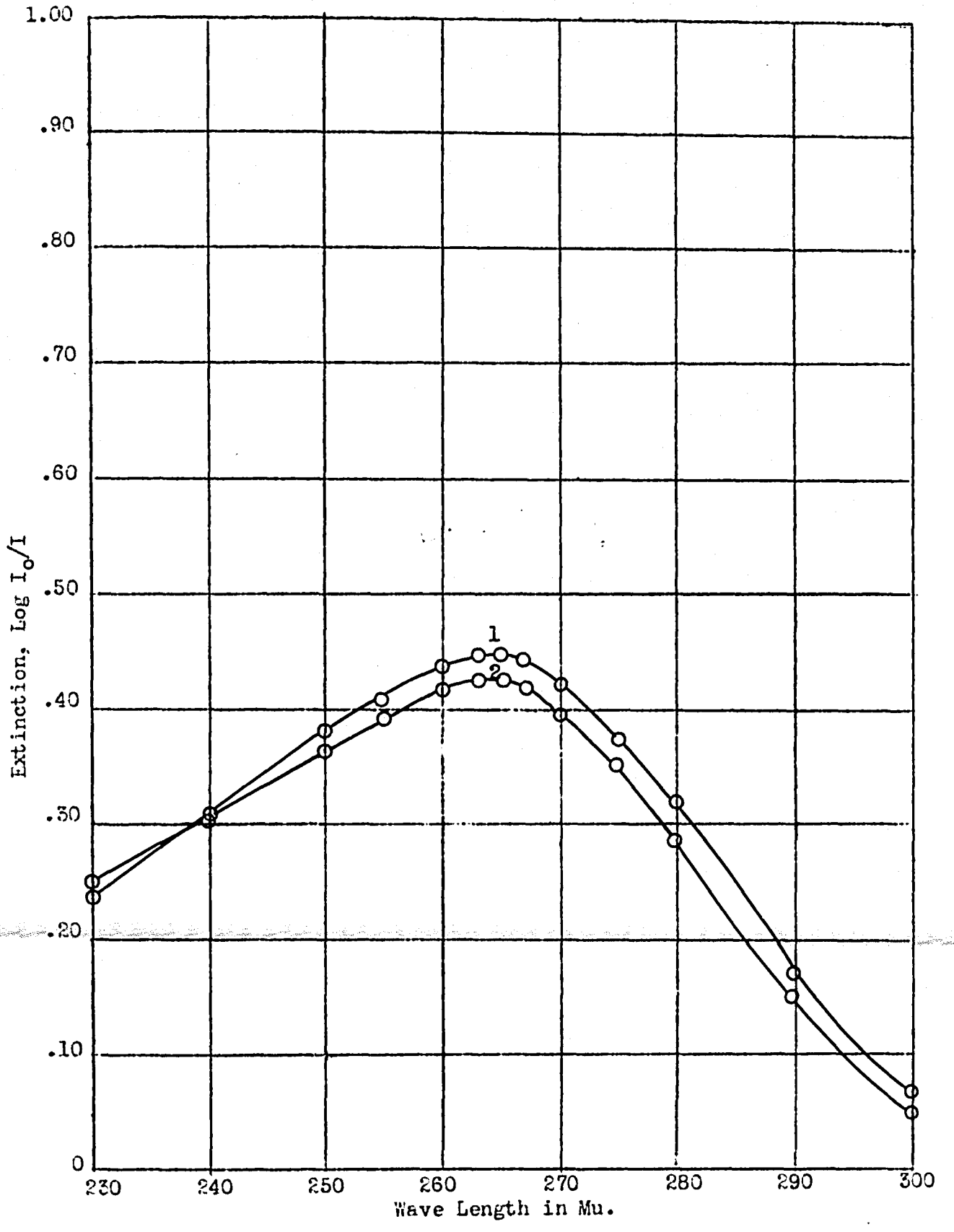

Figure 23. - Recovery of crystalline D_2 from alumina column: 1, untreated D_2 solution in 1:1 Skellysolve-ether; 2, same D_2 solution carried through alumina chromatographic procedure.

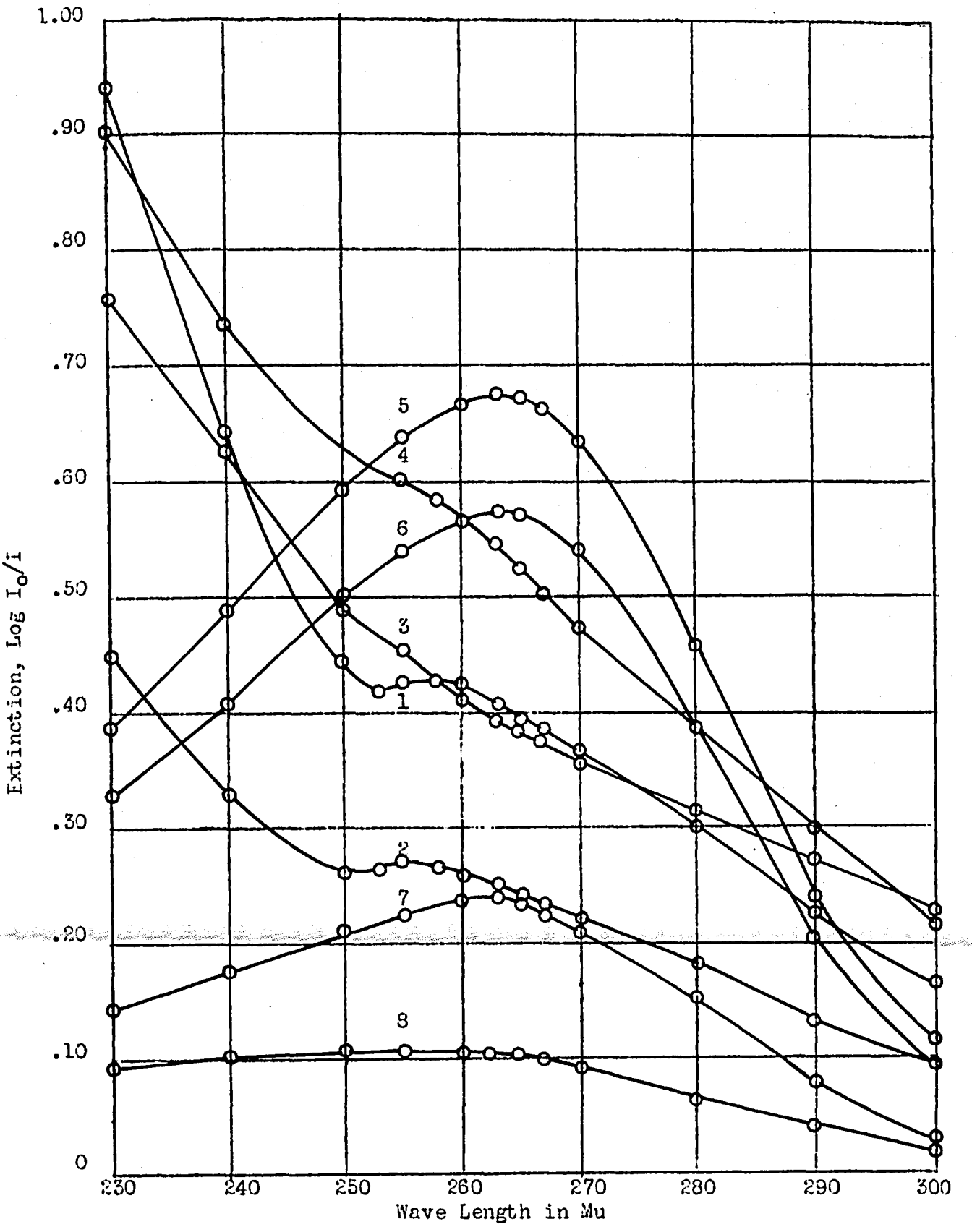

Figure 24. - Absorption curves of various fractions of the nonsaponifiable portion of crystalline D_2 in corn oil (9,680 u/gm.) taken from the alumina column. (Superfiltrol step preceding). Volumes for each fraction are listed in order taken: (1) 2 ml.; (2) 2 ml.; (3) 2 ml.; (4) 2 ml.; (5) 3 ml.; (6) 3 ml.; (7) 3 ml.; (8) 3 ml.

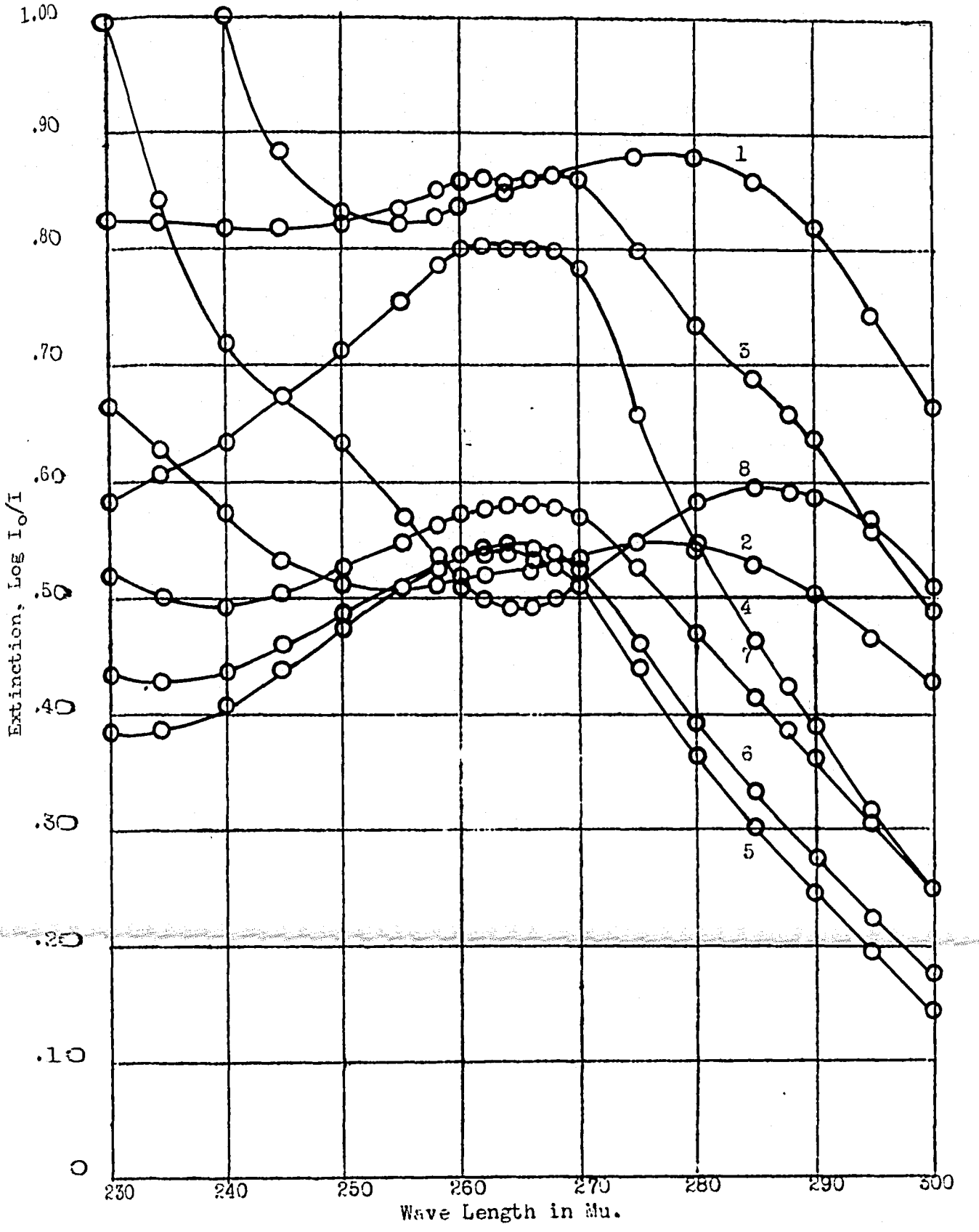

Figure 25. - Absorption curves of various fractions of the nonsaponifiable portion of Natola oil taken from the alumina column. (Super-filtrol step preceding). Volumes for each fraction are listed in order taken: (1) 2 ml.; (2) 3 ml.; (3) 3 ml.; (4) 2 ml.; (5) 2 ml.; (6) 2 ml.; (7) 3 ml.; (8) 3 ml.

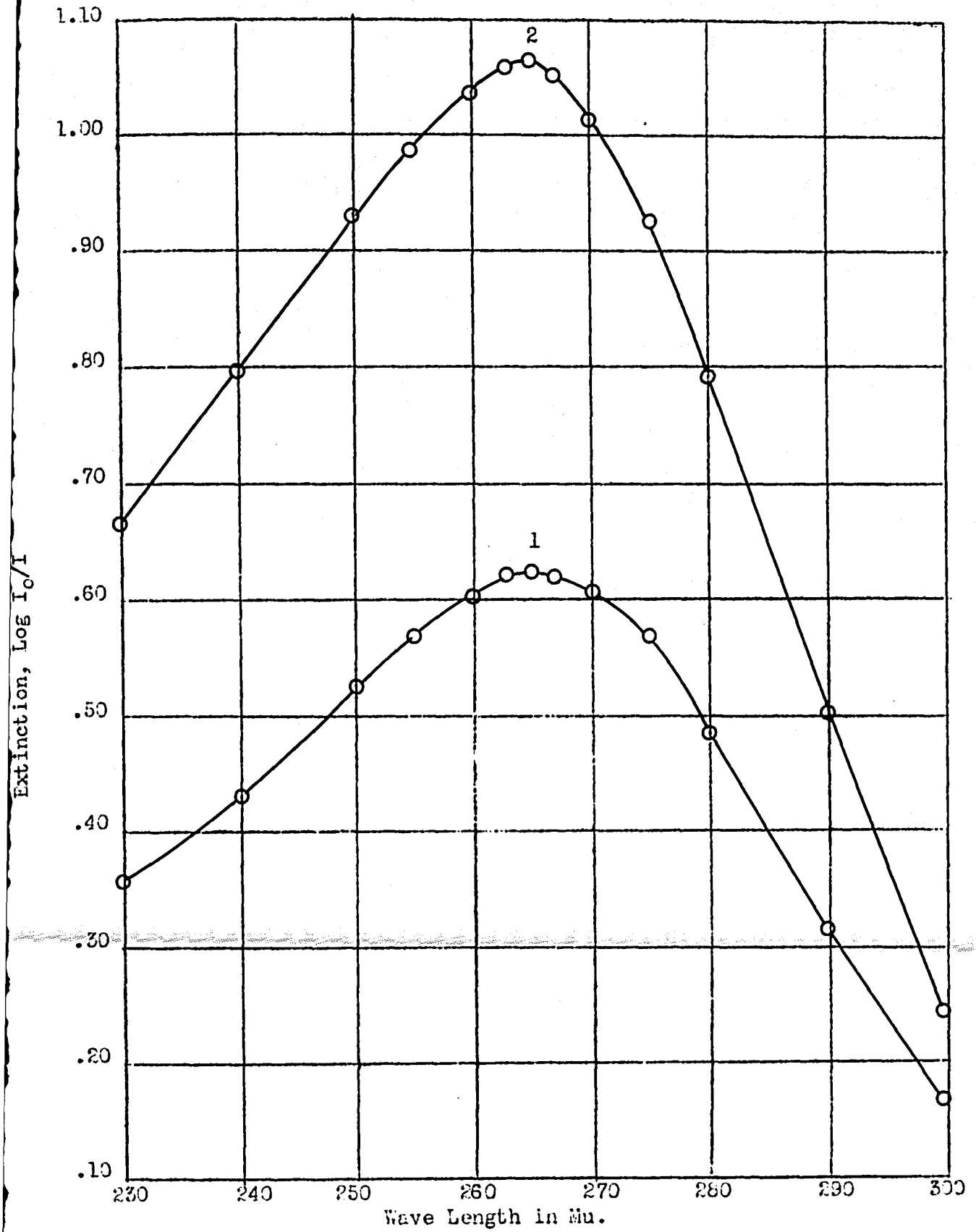

Figure 26. - Comparison of filtrate from 4 cm. and 9 cm. Superfiltrol Columns using the nonsaponifiable fraction of 1 gram of Irradiated ergosterol #6105 as the solute: 1, filtrate from 9 cm. column; 2, filtrate from 4 cm. column.